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PRINCIPAL INVESTIGATOR: Zijie Sun, Ph.D.

CONTRACTING ORGANIZATION: Stanford University
Stanford, California 94305-5401

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Zijie Sun, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Stanford University
Stanford, California 94305-5401

EMail: zsun@stanford.edu

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The androgen-signaling pathway is important for the growth and progression of prostate cancer. The cell cycle signaling regulated by mitogen activated protein/extracellular-signal-regulated kinase (MAPK/ERK) have been linked to tumorigenesis. 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 be targeted for the development of a drug in the treatment of prostate cancer.

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INTRODUCTION:

The majority of primary prostate cancers are dependent upon the AR and blocking the AR is an extremely important method for treating prostate cancer. Most advanced, androgen independent prostate cancers also express AR and the mechanisms that lead to the androgen independent prostate cancer are currently unclear. Experiments with AR and other steroid receptors have identified several associated proteins that can modulate the receptor-mediated transcriptions. 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. A novel AR-interacting protein, PAK6, was identified in our group previously. PAK6 is a 75 kDa protein that contains a putative amino-terminal Cdc42/Rac Interactive Binding (CRIB) motif and a carboxy-terminal kinase domain. The goal of this study is to determine the biological roles of PAK6 in the tumorigenesis of prostate cancer. Two specific aims, including assessing the role 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 significant efforts within this funding in order to achieve our goals.

BODY:

The majority of primary prostate cancers are dependent upon the AR and blocking the AR is an extremely important method for treating prostate cancer. PAK proteins are the direct effectors of the Rho family of GTPases, Rac1 and Cdc42. The GTPases bind to a conserved p21-binding domain and stimulate their serine/threonine kinase activities (1, 3). Although the biological functions of PAKs remain unclear, PAKs are implicated in the regulation of a number of cellular processes, including rearrangement of the cytoskeleton, apoptosis, MAPK signaling pathway. Interactions between the AR and PAK6 pathways may be an important mechanism both for the development and progression of prostate cancer. In our original grant application, we proposed four specific tasks to investigate the biological roles of PAK6 and the significance of its interaction with AR, including (1) To characterize the biological roles of PAK6 in prostate cancer cells, (2) To detect expression of PAK6 protein in prostate cancer samples by immunohistochemistry, (3) To elucidate the interaction between PAK6 and AR in prostate cancer cells, and (4) To study the mechanisms by which PAK6 represses AR-mediated transcription. However, both the scientific and programmatic reviewers recommended to fund only tasks 1 and 2. The panel also made a significant reduction of the budget. To reflect these changes, we have revised our specific aims. Within this year, we have made some significant progresses in both the approved tasks and we described them in detail as followed:

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

Based on the protein sequence and structure of PAK6, it suggests that the protein may possess the biological functions comparable to other PAK members. The selective expression of PAK6 in testis and prostate tissues is consistent with the interaction between AR and PAK6 that was identified in our previous study. PAK6 was also identified to bind to AR and to inhibit AR mediated transcription. We have further study biological roles of PAK6 in androgen signaling. We first investigated the role of the kinase activity of PAK6 in this inhibition. We compared the kinase activity of PAK6 with two other members of the PAK family PAK1 and PAK4. Like PAK4, PAK6 possesses a constitutively kinase activity. However, the kinase activity of PAK6 is

not modulated by GTPases, such as Rac/Cdc42, which is different from PAK1. To study the involvement of PAK6 kinase activity in AR-mediated transcription, we generated the kinase dead (KD) and kinase active (KA) mutants of PAK6. Interestingly, transient transfection experiments showed that PAK6 kinase activity is implicated in PAK6 repressed AR-mediated transcription. The wild type (WT) form of PAK6 showed a strong inhibitory effect on AR-mediated transcription as we observed previously (6), while the KD and the KA mutants of PAK6 inhibited AR activity by 20% and 90%, respectively. Importantly, either kinase dead or constitutively active mutants of PAK1 and PAK4 did not show a significant effect on AR-mediated transcription. These results shown that PAK6 repression of AR-mediated transcription is specific for and dependent on the kinase activity of PAK6. Modulation of this activity may be responsible for regulation of AR signaling in prostate cancer cells.

In this period, we also studied the molecular basis of PAK6 inhibitory effect in AR-mediated transcription. One possible mechanism for repression by PAK6 could be due to disrupting recruitment of other co-activators to the AR-complex. Several AR co-activators that specifically interact with AR and enhance AR transactivation have been identified by us (5) and others (2). Cotransfection of AR and cofactor including b-catenin, SRC1 and ARA55 showed augmentations of AR-mediated transactivation in PC3 and LNCaP cells. In the presence of PAK6 WT and PAK6 KA, enhancements of AR activity by these cofactors were significantly reduced. However, augmentations by these cofactors were not significantly affected. Although it not clear how PAK6 affects the cofactors in augmenting AR activity, our data suggests that inhibitions of AR and its cofactors by modulation of these proteins may be a potential mechanism by which PAK6 affects AR activity.

To further search the molecular mechanism by which PAK6 represses AR function, we examined whether PAK affects the phosphorylation status of AR. Expression vectors of PAK6 WT, KA, and KD were co-transfected with different AR truncated mutants into CV1 cells. The above transfected cells were incubated with medium with ^{32}P-orthophosphate in the presence or absence of the appropriate ligands. After 12-24 hrs, different fragments of AR proteins were immunoprecipitated with the Flag antibody from the CV1 cells and analyzed in SDS-PAGE. Through these analyses, we demonstrated that PAK6 WT and KA were able to phosphorylate the DBD domain of AR.

As proposed previously, we will continue to analyze biological roles of PAK6 in prostate cancer cells. We are in the process of making different adenoviral vectors of PAK6, which will be used to infect either primary prostate cells or prostate cancer cell lines. In addition, we are also generating the LNCaP cell lines that are stabily transfected with PAK6. With these tools, we will be able to use a more biologically relevant approach to study the crosstalk between the PAK6 and androgen pathways.

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

In this aim, we will search for a direct link between PAK6 and prostate cancer and assess whether PAK6 expression is altered at different stages of prostate cancers and in particular

whether this alteration occurs in the late stage of prostate cancer, and therefore may be used as a marker for progression.

The samples used in this study are from a prostate tissue bank in the Department of Urology at Stanford Medical Center. So far, we have collected approximately 10 samples from normal, BPH, and tumor tissues. A rabbit polyclonal antibody against human PAK6, was generated in the PI's lab. We have been used it in this study. In our preliminary experiments, we used the standard three-step immunoperoxidase-based method as described previously (4). The PAK6 antibody, at a 1:100 dilution, was be added to the slides and incubated overnight at 4°C. After three washes, slides were be incubated with biotinylated secondary antibody and washed again. Color was developed with the ImmunoCruz staining system (Santa Cruz, sc 2053). Interestingly, we did not observed strong staining in tissues, and instead, there are only a few of cells that seem to be stained with the antibody in the luminal epithelial areas. We are in the process of repeating the above experiments in different experimental conditions.

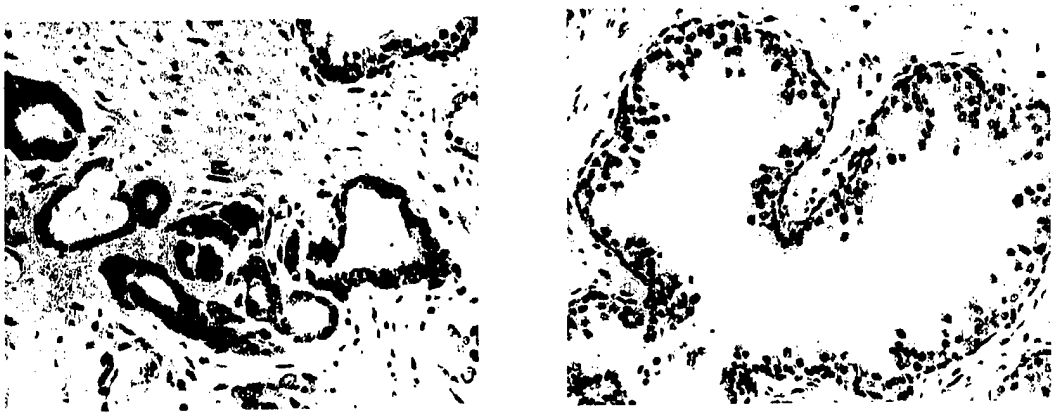

Figure 1: Immunohistochemistry of human prostate cancer tissues with the anti-PAK6 antibody. Two postate tissues were stained with the rabbit polyclonal antibody against human PAK6 protein. Color was developed with the ImmunCruz Staning System (Santa Cruz, sc-2023). Sections were lightly counterstained with 5% (w/v) hematoxylin.

Although the above anti-PAK6 antibody that has been successfully used to detect the overexpressed protein, it is possible that it may not work in our tumor samples. Currently, we are in the process of generating new PAK6 antibodies. The peptides from the N-terminal or C-terminal region of human PAK6 have be chosen based upon their predicted immunogenicity and minimal homology to other family members. The peptides will be synthesized and injected into rabbits by Sierra BioSource Inc (Gilroy, CA), which has successfully made several antibodies for the PI's lab. In the unlikely event that a specific antibody to PAK6 protein is not successful to carry out the above study, in situ hybridization will be used to assess message expression. In this regards, we started to make the construct that will be used for making either the sense or antisense RNA probe of PAK6. Our effort will be adjusted based on our progresses.

KEY RESEARCH ACCOMPLISHNETS:

1) Identify that the kinase domain of PAK6 is involved in repression of AR activity.
2) Demonstrate that PAK6 phosphorylates the DBD of AR.
3) Assess the expression of PAK6 in prostate cancer tissues with the PAK6 antibody.

REPORTABLE OUTCOMES:

Publications:

1. Yang F, Li X, Sharma M, Sasaki CY, Longo DL, Lim B, **Sun ZJ.** (2002). Linking beta-catenin to androgen signaling. *J. Biol. Chem.* 277:11336-11344.

2. Sharma M, Chuang WW, **Sun ZJ.** (2002). Regulation of androgen signaling by the phosphatidylinosital 3-kinase/Akt is mediated through GSK3beta/beta-catenin pathway. *J. Biol. Chem.* 277:30935-30941.

3. Sharma M, Li X, Wang Y-Z, Zarnegar M, Huang C-Y, Palvimo JJ, Lim B, **Sun ZJ.** Novel PIAS-like protein, hZimp10, acts as the androgen receptor coactivator and forms a complex at replication foci. Submitted, 2003.

Abstracts:

N. M. Schrantz, Z. Sun & G. M. Bokoch. Repression of AR-mediated transcription by PAK6 is dependent on PAK6 kinase activity. ASCB, San Francisco, Dec, 2002.

Patent application:

Zijie Sun & Fajun Yang. "Androgen Receptor Specifically Interacts with a Novel p21-Activated Kinase, PAK6" (Stanford University S01-034).

Funding applied for based on work supported by this award:

1. 2003-2008 NIH/2RO1CA70297, Principal Investigator: Zijie Sun
   Androgen receptor associated proteins in prostate cancer

2. 2003-2008 NIH/RO1DK61002, Principal Investigator: Zijie Sun
   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:

In this funding year, we have further characterized biological roles of PAK6 in prostate cancer cells and to access the stages of prostate cancer where PAK6 expression is altered. We

demonstrated that PAK6 is a true regulator of the androgen receptor in the more biological relevant experiments. As proposed, we will continue to investigate the crosstalk between the PAK6 and androgen pathways. We will also make a new antibody of PAK6 to study expression of PAK6 in prostate tissues. If aberrant expressions of PAK6 are found to correlate with a particular stage of the disease, our efforts will be adjusted to carry out more experiments to further characterize this abnormality. We expect that the results from our experiments will provide more definitive evidence for a central role of PAK6 in the regulation of prostate cell growth.

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5. Yang, F., X. Li, M. Sharma, C. Y. Sasaki, D. L. Longo, B. Lim, and Z. Sun. 2002. Linking beta-catenin to androgen signaling pathway. J Biol Chem 277:11336-11344.

6. Yang, F., X. Li, M. Sharma, M. Zarnegar, B. Lim, and Z. Sun. 2001. Androgen receptor specifically interacts with a novel p21-activated kinase, PAK6. J Biol Chem 276:15345-353.

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Linking β-Catenin to Androgen-signaling Pathway*

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Fajun Yang‡, Xiaoyu Li§, Manju Sharma‡, Carl Y. Sasaki¶, Dan L. Longo¶, Bing Lim§, and Zijie Sun‡‖

From the ‡Department of Surgery and Department of Genetics, Stanford University School of Medicine, Stanford, California 94305-5328, the §Division of Hematology/Oncology, Department of Medicine, Beth Israel Deaconess Medical Center, Boston, Massachusetts 02115, and the ¶Laboratory of Immunology, NIA, National Institutes of Health, Baltimore, Maryland 21224

The androgen-signaling pathway is important for the growth and progression of prostate cancer cells. The growth-promoting effects of androgen on prostate cells are mediated mostly through the androgen receptor (AR). There is increasing evidence that transcription activation by AR is mediated through interaction with other cofactors. β-Catenin plays a critical role in embryonic development and tumorigenesis through its effects on E-cadherin-mediated cell adhesion and Wnt-dependent signal transduction. Here, we demonstrate that a specific protein-protein interaction occurs between β-catenin and AR. Unlike the steroid hormone receptor coactivator 1 (SRC1), β-catenin showed a strong interaction with AR but not with other steroid hormone receptors such as estrogen receptor α, progesterone receptor β, and glucocorticoid receptor. The ligand binding domain of AR and the NH_2 terminus combined with the first six armadillo repeats of β-catenin were shown to be necessary for the interaction. Through this specific interaction, β-catenin augments the ligand-dependent activity of AR in prostate cancer cells. Moreover, expression of E-cadherin in E-cadherin-negative prostate cancer cells results in redistribution of the cytoplasmic β-catenin to the cell membrane and reduction of AR-mediated transcription. These data suggest that loss of E-cadherin can elevate the cellular levels of β-catenin in prostate cancer cells, which may directly contribute to invasiveness and a more malignant tumor phenotype by augmenting AR activity during prostate cancer progression.

Prostate cancer is the most commonly diagnosed malignancy among males-in western countries (1). However, in contrast to some other tumors, the molecular events involved in the development and progression of prostate cancer remain largely unknown. Androgen ablation, used as an effective treatment for the majority of advanced prostate cancers, indicates that androgen plays an essential role in regulating the growth of prostate cancer cells. The growth-promoting effects of androgen in prostate cells are mediated mostly through the androgen receptor (AR).[1] There is increasing evidence that the nuclear hormone receptors, including AR, interact with other signal transduction pathways (2). The regulation by cofactors can modulate AR activities, which may contribute to the development and progression of prostate cancer.

β-Catenin plays a pivotal role in cadherin-based cell adhesion and in the Wnt-signaling pathway (3, 4). Corresponding to its dual functions in the cells, β-catenin is localized to two cellular pools. Most of the β-catenin is located in the cell membrane where it is associated with the cytoplasmic region of E-cadherin, a transmembrane protein involved in homotypic cell-cell contacts (5). A smaller pool of β-catenin is located in the nucleus and cytoplasm and mediates Wnt signaling. In the absence of a Wnt signal, β-catenin is constitutively down-regulated by a multicomponent destruction complex containing GSK3β, axin, and a tumor suppressor, adenomatous polyposis coli (APC). These proteins promote the phosphorylation of serine and threonine residues in the NH_2-terminal region of β-catenin and thereby target it for degradation by the ubiquitin proteasome pathway (6). Wnt signaling inhibits this process, which leads to an accumulation of β-catenin in the nucleus and promotes the formation of transcriptionally active complexes with members of the Tcf/LEF family (7). Activation of Tcf/LEF and β-catenin targets has been shown to induce neoplastic transformation in cells, suggesting a potential role of β-catenin in tumorigenesis (8).

The link between stabilized β-catenin and tumor development and progression was considerably strengthened by discoveries of mutations in both β-catenin and components of the destruction complex in a wide variety of human cancers, which cause increased cellular levels of β-catenin (3, 9). About 85% of all sporadic and hereditary colorectal tumors show loss of APC function, which correlates with the increased levels of free β-catenin found in these cancer cells (10–12). It appears that inappropriate high cellular levels of β-catenin play a fundamentally important role in tumorigenesis.

In normal epithelial tissues, E-cadherin complexes with actin cytoskeleton via cytoplasmic catenins to maintain the functional characteristics of epithelia. Disruption of this complex, due primarily to the loss or decreased expression of E-cadherin, is frequently observed in many advanced, poorly differentiated carcinomas (13, 14). There is a strong correlation between decreased expression of E-cadherin and an invasive and metastatic phenotype of human prostate cancers

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‖ To whom correspondence should be addressed: Depts. of Surgery and Genetics, R135, Edwards Bldg., Stanford University School of Medicine, Stanford, CA 94305-5328. E-mail: zsun@stanford.edu.

[1] The abbreviations used are: AR, androgen receptor; GR, glucocorticoid receptor; ERα, estrogen receptor α; PRβ, progesterone receptor β; VDR, vitamin D receptor; TAD, transcription activation domain; DBD, DNA binding domain; LBD, ligand binding domain; DHT, dihydrotestosterone; PSA, prostate specific antigen; ARE, androgen responsive element; MMTV, mouse mammary tumor virus; GST, glutathione S-transferase; ARA70, androgen receptor-associated protein 70; β-gal, β-galactosidase; APC, adenomatous polyposis coli.

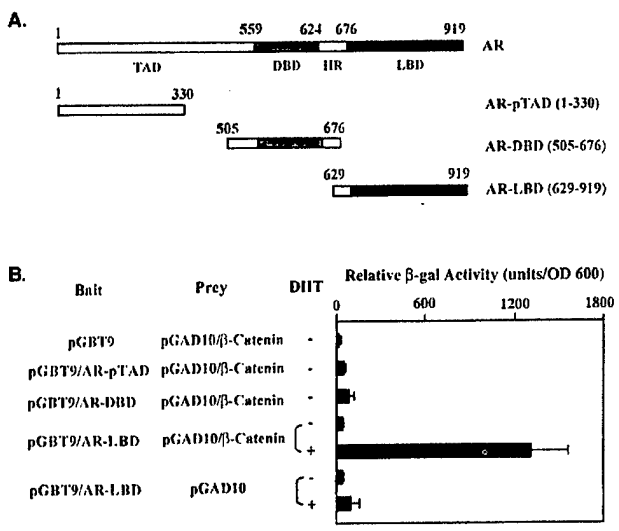

FIG. 1. **Specific interaction of β-catenin with the LBD of AR.** *A*, schematic representation of different portions of the human AR that were used in the yeast experiments. Numbers correspond to amino acid residues. *B*, full-length β-catenin clone or an empty library vector (pGAD10) was cotransformed into yeast strain PJ69 with either the bait vector (pGBT9), AR-pTAD, AR-DBD, or AR-LBD. Transformed cells were plated on SD-Ade-Leu-Trp plates with or without 100 nM DHT and SD-Leu-Trp plates for monitoring the transformation efficiency. Three independent colonies were inoculated from each transformation experiment for a β-gal assay. The data for the liquid β-gal assay is shown as the mean ± S.D.

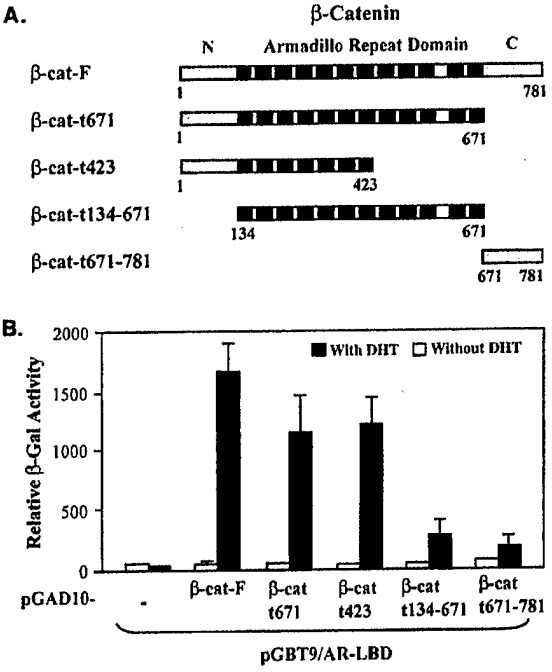

FIG. 2. **Delineation of the domain in β-catenin that mediates the interaction with AR-LBD in the yeast two-hybrid system.** *A*, both the NH_2- and COOH-terminal regions and a central armadillo domain of β-catenin are shown schematically. Numbers correspond to amino acid residues. Full-length (*F*) and truncated (*t*) β-catenin constructs were generated. *B*, the various truncated β-catenin genes in pGAD10 were cotransformed into yeast strain PJ69 with AR-LBD. Specific interactions between the two fusion proteins were measured by the appearance of colonies on SD-Ade-Leu-Trp plates and a liquid β-gal assay in the presence or absence of 100 nM DHT. A liquid β-gal assay is presented as the mean ± S.D. of three independent colonies.

(15). Besides playing a role in retaining normal cell-cell contact, E-cadherin can also modulate the cytoplasmic pools of β-catenin for signaling (16).

Here, we demonstrated a specific protein-protein interaction between β-catenin and AR. Importantly, unlike the steroid receptor cofactor 1 (SRC1), β-catenin selectively binds to AR in a ligand-dependent manner but not to other steroid hormone receptors such as the estrogen receptor α (ERα), the progesterone receptor β (PRβ), and glucocorticoid receptor (GR). The ligand binding domain (LBD) of AR and the central region spanning the armadillo repeats 1–6 of β-catenin were found to be responsible for the interaction. Using transient transfection experiments, we further demonstrated that β-catenin augments the ligand-dependent activity of AR in prostate cancer cells through this specific interaction. These data identify a new role for β-catenin in nuclear hormone receptor-mediated transcription. Moreover, transfection of an E-cadherin expression construct into an E-cadherin-negative prostate cancer cell line, TSU.pr-1, resulted in redistribution of β-catenin to the cell membrane and reduction of AR-dependent transcriptional activity. They suggest that reduced expression of E-cadherin can elevate the cellular levels of β-catenin in prostate cancer cells, which may directly contribute to the invasiveness and more malignant tumor phenotype by augmenting AR activity during the progression of prostate cancer.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid System—Yeast two-hybrid experiments were basically performed as described previously (17). The LBD of human AR (amino acids 629–919) was fused in frame to the GAL4 DBD in the pGBT9 vector (CLONTECH, Palo Alto, CA). The construct was transformed into a modified yeast strain PJ69-4A (18). A cDNA library from human brain tissue was used in this screening (CLONTECH). Transformants were selected on Sabouraud dextrose medium lacking adenine, leucine, and tryptophan in the presence of 100 nM dihydrotestosterone (DHT). The specificity of interaction with AR was determined by a liquid β-galactosidase (β-gal) assay as described previously (17). β-Gal activities were measured using the Galacto-light Plus kit (Tropix Inc., Bedford, MA) and normalized by cell density (A_{600}). pGBT9 constructs with three different AR fragments, including the partial TAD (amino acids 1–333), DBD (amino acids 505–676), and LBD were used to confirm the interaction.

Plasmid Construction—A yeast clone containing the full-length cDNA of human β-catenin was isolated in the screen. Using it as a template, the COOH-terminal and internal deletions of β-catenin clones were generated by PCR with specific primers containing the appropriate restriction enzyme sites. After cleavage, the fragments containing different portions of the β-catenin were cloned downstream of GAL4 TAD in the pGAD10 vector (CLONTECH). The LBD fragments of ERα (amino acids 250–602), PRβ (amino acids 633–952), VDR (amino acids 90–427) were generated by PCR with specific primers and subcloned in-frame to the GAL4 DBD in pGBT9. An antisense construct of β-catenin containing the NH_2-terminal 513 bp was generated by PCR and cloned into the pcDNA3 vector at *Eco*RI site. All constructs were sequenced to confirm that there were no mutations introduced by PCR.

The AR expression vector, pSV-hAR, was provided by Dr. Albert Brinkmann (Erasmus University, Rotterdam, The Netherlands). The expression constructs for human ERα and pERE-luc plasmid were generously given by Dr. Myles Brown (Dana-Farber Cancer Institute, Boston, MA). A human PRβ and PRE-luc reporter were provided by Dr. Kathryn B. Horwitz (University of Colorado). The expression constructs of human GR and VDR, and the pVDRE-luc reporter plasmid, were the kind gifts of Dr. David Feldman (Stanford University, Stanford, CA). pSV-β-gal, an SV40-driven β-galactosidase reporter plasmid (Promega, Madison, WI) was used in this study as an internal control. The pSG5-ARA70 plasmid and the reporter plasmid pARE-luc were the kind gifts of Dr. Chawnshang Chang (19). pMMTV-pA3-luc was provided by Dr. Richard Pestell (Albert Einstein College of Medicine, New York). The reporter plasmids, pPSA7kb-luc, with the luciferase gene under the control of promoter fragments of the human prostate-specific antigen was obtained from Dr. Jan Trapman (20).

Cell Cultures and Transfections—The monkey kidney cell line, CV-1, was maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (HyClone, Denver, CO). An AR-positive pros-

required for the interactions, and a minimum of 6–7 repeats are sufficient for detectable binding (29, 30). Those data are

β-catenin and its mutants lacking either repeat 7 or 12. The interaction is more pronounced in the presence of DHT than in

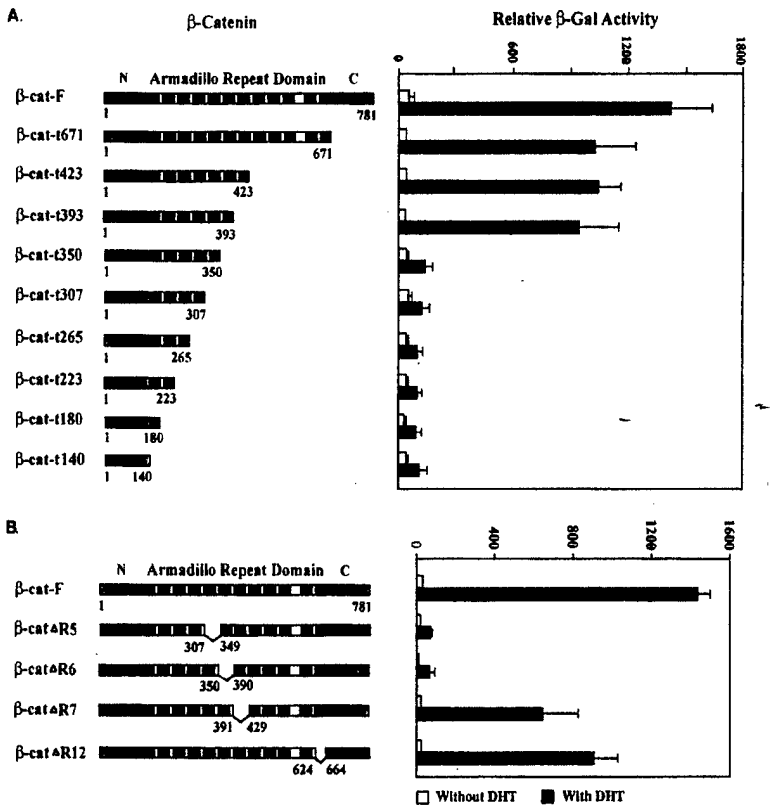

FIG. 3. **Mapping the armadillo repeats in β-catenin that mediate the interaction with AR-LBD in yeast two-hybrid system.** Mutants of β-catenin containing COOH-terminal truncations (*A*) and internal deletions (*B*) in pGAD10 were made and cotransformed into yeast strain PJ69 with pGBT9/AR-LBD in the presence or absence of 100 nM DHT. Numbers corresponding to amino acid residues are indicated for each construct. Liquid β-gal was measured as described in the legend to Fig. 2.

tate cancer cell line, LNCaP, was maintained in T-medium (Invitrogen) with 5% fetal calf serum. The two sublines derived from TSU.pr-1 prostate cancer cells (16) were maintained in RPMI 1640 medium with 10% fetal calf serum and G418 (500 μg/ml).

Transient transfections were carried out using a LipofectAMINE transfection kit (Invitrogen) for CV1 and LipofectAMINE 2000 (Invitrogen) for TSU.pr-1 and LNCaP cells. Approximately 1.5–2 × 10^4 cells were plated in a 48-well plate 16 h before transfection. 12–16 h after transfection, the cells were washed and fed medium containing 5% charcoal-stripped fetal calf serum (HyClone) in the presence or absence of steroid hormones. Cells were incubated for another 24 h, and luciferase activity was measured as relative light units (21). The relative light units from individual transfections were normalized by β-galactosidase activity in the same samples. Individual transfection experiments were done in triplicate and the results are reported as mean relative light units/β-galactosidase (+S.D.).

In Vitro Binding Assay—GST-β-catenin fusion proteins were constructed in the pGEX-4T-1 vector (Amersham Biosciences, Inc.). Expression and purification of GST fusion proteins were performed according to the manufacturer's instructions. Full-length human AR proteins were generated and ^{35}S-labeled *in vitro* by the TNT-coupled reticulocyte lysate system (Promega, Madison, WI). Equal amounts of GST fusion proteins coupled to glutathione-Sepharose beads were incubated with ^{35}S-labeled proteins at 4 °C for 2 h in the lysis buffer as described above. Beads were carefully washed three times with washing buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40). GST fusion proteins were then eluted by incubating with buffer containing 10 mM glutathione and 50 mM Tris-HCl, pH 8.0, for 10 min at room temperature. The bound proteins were analyzed by SDS-PAGE followed by autoradiography.

Immunofluoresence—CV-1 cells were plated onto gelatin-coated (2%) coverslips the day before transfection. The pcDNA3-AR and the wild type or mutants of β-catenin plasmids were cotransfected into cells with the LipofectAMINE-PLUS reagent (Invitrogen). After 2 h, transfected cells were fed with fresh medium plus/minus 10 nM DHT, incubated for 4 h, and then fixed for 10 min with 3% paraformaldehyde in phosphate-buffered saline and washed with 0.1% Nonidet P-40/phosphate-buffered saline buffer. Nonspecific sites were blocked with 5% skim milk powder in phosphate-buffered saline for 30 min. The cells were then incubated with either anti-FLAG monoclonal or anti-AR polyclonal antibody for 1 h at room temperature. Cells were washed three times followed by incubation with fluorescein isothiocyanate-conjugated anti-mouse or rhodamine-conjugated anti-rabbit secondary antibody (Santa Cruz Biotechnology). Indirect immunofluorescence staining was performed according to the procedure described previously (16). In TSU cells, E-cadherin was stained with the rat monoclonal antibody against uvomorulin (6 μg/ml; Sigma) and donkey anti-rat immunoglobulin conjugated to Alexa-488 (20 μg/ml; Molecular Probes, Eugene, OR). β-Catenin was stained with a mouse monoclonal anti-β-catenin antibody conjugated to TRITC (10 μg/ml; Transduction Laboratories).

RESULTS

Androgen Receptor Interacts with β-Catenin in a Ligand-dependent Manner—Using a bait construct containing the LBD and hinge region of the AR, we employed a modified yeast two-hybrid system to identify proteins that interact with AR in an androgen-dependent manner (17, 18). Of 2 × 10^7 transformants, 73 grew under selective conditions and showed increased adenine and β-gal productions in medium containing 100 nM DHT. Rescue of the plasmids and sequencing of the inserts revealed several different cDNAs, including the previously identified SRC1 (22), an AR-associated protein (ARA70) (19), and several other AR-interacting proteins identified recently by others or us (17, 21, 23). Importantly, 23 of these clones perfectly matched the sequence of the full-length coding region of β-catenin. To confirm the interaction, we cotransformed one of these β-catenin clones with various constructs containing either GAL4DBD alone or the AR fusion proteins with a partial transactivation domain (pTAD), the DBD, and the LBD (Fig. 1*A*). pGAD10-β-catenin showed a specific interaction with GAL4DBD-AR-LBD by producing adenine in the presence of 100 nM DHT (data not shown). In the liquid β-gal assays, pGAD10-β-catenin showed an ~97-fold induction with pGBT9-AR-LBD in the presence of DHT (Fig. 1*B*). This result demonstrated that the LBD of AR specifically interacts with β-catenin in a ligand-dependent manner.

Armadillo Domain of β-Catenin Is Responsible for Binding to AR—β-Catenin and its *Drosophila* homolog, armadillo, contain a central core domain of 12 armadillo repeats flanked by

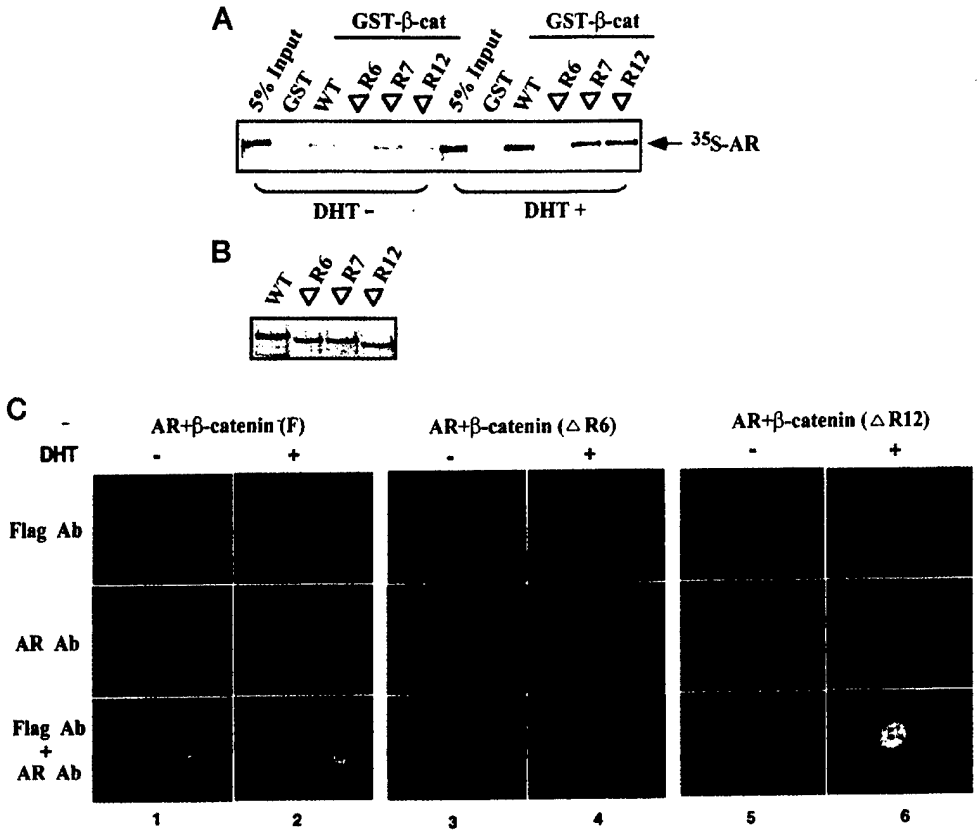

FIG. 4. **β-Catenin interacts with AR *in vitro* and *in vivo*.** *A*, GST fusion proteins containing full-length (*WT*), and different internal deletion mutants of β-catenin as indicated in the legend to Fig. 3*B* were constructed. The conjugated GST fusion proteins were incubated with *in vitro* expressed and ^{35}S-labeled full-length AR in the presence or absence of 10 nM DHT for 2 h at 4 °C and then eluted by 10 mM GSH in 50 mM Tris-HCl, pH 8.0, and resolved by 10% SDS-PAGE. *B*, GST fusion proteins were resolved in SDS-PAGE and stained with Coomassie Blue for measuring expression. *C*, CV-1 cells were transfected with an AR expression vector and FLAG-tagged wild type or mutants of β-catenin plasmids as indicated in the figure. The cells were cultured in the presence or absence of 10 nM DHT. AR proteins were detected with a polyclonal AR antibody and revealed by rhodamine-conjugated secondary antibody (*red*). FLAG-tagged β-catenin proteins were detected with a monoclonal anti-FLAG antibody and revealed with fluorescein isothiocyanate-conjugated secondary antibody (*green*).

unique NH_2 and COOH termini (7). To identify the region of β-catenin that interacts with AR, we generated several truncated mutants of β-catenin and assessed their ability to interact with AR using the yeast two-hybrid system (Fig. 2*A*). As shown in Fig. 2*B*, deletion of the COOH-terminal activation domain of β-catenin (β-cat-t671) alone, or in combination with the last five armadillo repeats (β-cat-t423), did not significantly affect the binding. However, a mutant in which the NH_2-terminal activation domain alone (β-cat-t134–671), or in combination with the central armadillo domain (β-cat-t671–781) was deleted, showed no interaction. This result suggests that the primary binding region for AR spans the NH_2 terminus and the first seven armadillo repeats of β-catenin.

To precisely map the interacting region, a series of truncated mutants were made in which each single armadillo repeat was subsequently deleted (Fig. 3*A*). The deletion constructs containing the NH_2-terminal region and the first six repeats (β-cat-t393) showed about two-thirds the activity of the full-length protein (β-cat-F) (Fig. 3*A*). However by deleting repeat 6 (β-cat-t350), the interaction was essentially abolished, indicating that armadillo repeat 6 is crucial for binding to AR. Deletion of repeats 1–5 obviously had little further effect.

It has been shown that most β-catenin-binding proteins such as Tcf/LEF family members (24), axin (25), APC (26, 27), and cadherins (26, 28) bind β-catenin mainly through the central armadillo repeats. In most of cases, the first 10 repeats are required for the interactions, and a minimum of 6–7 repeats are sufficient for detectable binding (29, 30). Those data are consistent with our finding that deletion of repeat 6 fully abolished the interaction with AR. To more precisely map the interaction region within the first six armadillo repeats, we used a PCR-based, site-directed mutagenesis techniques to generate several internal deletion mutants. As shown in the figure, the wild type β-catenin and the mutant with deletion of repeat 7 (ΔR7) or 12 (ΔR12) all reacted avidly with the AR-LBD. In contrast, mutants lacking repeat 6 (ΔR6) showed no interaction with the AR-LBD (Fig. 3*B*). Moreover, deletion of repeat 5 alone also fully abolished the interaction, indicating that the armadillo repeats 1–5 may be also involved in the interaction. To confirm this result, an additional internal deletion mutant lacking repeat 3 (ΔR3) was generated and tested. As we expected, the mutant also showed no interaction with AR (data not shown). Taken together, the results allow us to conclude that the region spanning armadillo repeats 1–6 is mainly responsible for binding to AR.

β-Catenin Interacts with AR in Vitro and in Vivo—Physical interaction between AR and β-catenin was further assessed by GST pull-down experiments. A series of GST fusion proteins with the full-length β-catenin and internal deletion mutants were generated and immobilized onto a glutathione-Sepharose matrix. The binding of [^{35}S]methionine-labeled AR protein to GST-β-catenin fusion proteins was analyzed by SDS-PAGE and detected by autoradiography. As shown in Fig. 4*A*, AR protein bound to the GST fusion protein containing wild type β-catenin and its mutants lacking either repeat 7 or 12. The interaction is more pronounced in the presence of DHT than in

the absence of DHT, and as much as 5% of the input protein was recovered (Fig. 4A). However, a significant reduction of binding was observed between the AR protein and the β-catenin mutants lacking repeat 6 when equal amounts of the GST fusion proteins were used in the experiments (Fig. 4B). These results are consistent with our observations from the yeast two-hybrid system and show a domain-dependent interaction *in vitro*.

To confirm that endogenous AR and β-catenin are physically associated in intact cells, coimmunoprecipitation assays were carried out to detect a possible protein complex in a prostate cancer cell line, LNCaP. Using specific antibodies, we further confirmed that AR and β-catenin proteins form a protein complex in LNCaP cells and the formation of AR and β-catenin complexes in these cells was also enhanced by DHT (data not shown). These results are consistent with a recent report by Truica and colleagues (31).

Next, we examined whether a dynamic interaction between β-catenin and AR existed in cells. FLAG-tagged vectors containing either full-length or mutants of β-catenin were transfected into CV-1 cells, and the expressed protein showed a cytoplasmic and nuclear distribution, which was not altered by treatment with DHT (data not shown). Overexpressed β-catenin protein with AR vector, in the absence of DHT, showed the same cellular distribution as transfection of β-catenin plasmid alone, while transfected AR protein is localized mainly in the cytoplasm (Fig. 4C, *panels 1, 3,* and *5*). In the presence of DHT, AR proteins are fully translocated into the nuclei (*panels 2, 4,* and *6*). Importantly, both the wild type (*panel 2*) and the ΔR12 mutant of β-catenin (*panel 6*) showed increased levels of nuclear translocation when cotransfected with AR compared with cells transfected with the ΔR6 mutant of β-catenin in which cytoplasmic staining of β-catenin persisted (*panel 4*). These results provide the first evidence that β-catenin can translocate into the nucleus as part of a complex with AR by an interaction through armadillo repeat 6.

β-Catenin Binds Selectively to the AR—To assess the possibility that β-catenin functions as a general coactivator of nuclear receptors, we examined the interaction of β-catenin with other members of the nuclear receptor family in yeast. The LBD of ERα, PRβ, and VDR were generated and fused to GAL-DBD in the pGBT9 vector. These plasmids were cotransformed with either pGAD10-β-catenin or pGAD10-SRC1 as a positive control in the presence of corresponding ligands. The yeast transformants were grown on the selective media, and a liquid β-gal assay was performed to quantify the interactions. All receptors were shown to have a ligand-dependent interaction with the SRC1 (Fig. 5A), which is consistent with the previous reports (22, 32). However, β-catenin showed a strong interaction with AR but not with ERα and PRβ. VDR showed a weaker interaction with β-catenin in comparison to SRC1. These results indicate that β-catenin selectively interacts with AR.

The specificity of interaction between β-catenin and AR proteins was further tested in CV1 cells. Since AR, GR, and PRβ all can activate the MMTV promoter, we examined whether β-catenin is able to enhance GR and PRβ activity under identical experimental condition. Transfection experiments were repeated with β-catenin and AR, GR, and PRβ expression plasmids, along with a luciferase reporter plasmid regulated by an MMTV promoter containing the steroid hormone-responsive elements (33, 34). As shown in Fig. 5B, all receptors showed a ligand-dependent transactivation with the MMTV promoter. However, β-catenin specifically augmented only AR-mediated transcription but not GR and PRβ (Fig. 5B). Taken together, our results suggest that β-catenin differs from SRC1 and selectively affects AR.

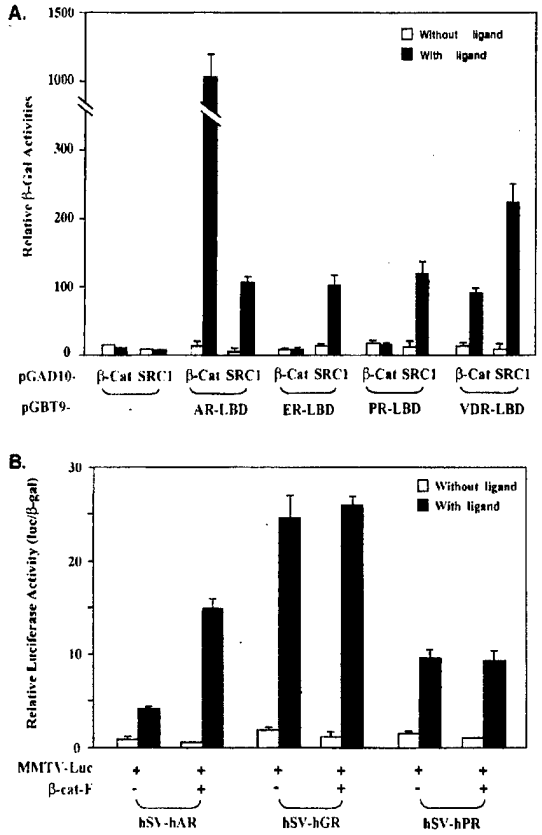

FIG. 5. **β-Catenin specifically interacts with AR.** *A*, the full-length β-catenin or SRC-1 in pGAD10 was cotransformed into yeast strain PJ69 with either the bait vector (pGBT9) or the fusion proteins containing the LBD of AR, ERα, PRβ, or VDR. Transformed cells were plated on SD-Ade-Leu-Trp plates with or without 100 nM of DHT, 17β-estradiol (E_2), progesterone, or vitamin D_3, respectively. The plates were incubated at 30 °C for 5 days. Specific interactions were measured by the appearance of yeast colonies on SD-Ade-Leu-Trp plates and a liquid β-gal assay. The data represent the mean ± S.D. of three independent colonies. *B*, CV-1 cells were transiently transfected with 100 ng of pMMTV-Luc, 50 ng of pSV40-β-gal, 10 ng of pSV-AR, pSV-GR, or pSV-PRβ as indicated, and 60 ng of pcDNA3 vector or pcDNA3-FLAG-β-catenin. Cells were incubated with or without 10 nM DHT, dexamethasone, or progesterone, respectively. Cell lysates were measured for luciferase and β-gal activities.

β-Catenin Augments AR-mediated Transcription through Specific Protein-Protein Interaction—Transient transfection assays were performed to further investigate the possible effect of β-catenin on AR-ediated transcription. Plasmids capable of expressing AR, wild type or mutants of β-catenin, and a luciferase reporter plasmid regulated by the MMTV-LTR (MMTVpA3-Luc), were transfected into CV-1 cells (35). A nearly 3-fold ligand-dependent transactivation was observed in the cells transfected with AR plasmid alone. Cotransfection of the wild type of β-catenin expression construct increased AR activity to nearly 10-fold above base line (Fig. 6A). Expression of the β-catenin mutant lacking the armadillo repeat 12 still showed 6–7-fold induction, whereas the mutants lacking repeat 6 showed no enhancement on AR-mediated transcription (Fig. 6A). These data indicate that β-catenin augments AR-mediated transcription, and this enhancement is mediated through the physical interaction between these proteins.

β-Catenin can form a transcriptional complex with members of the Tcf/LEF family to activate target genes (7, 25). To ensure that augmentation of the MMTV promoter by β-catenin is mediated solely through the AR, rather than through other transcription factors, we examined the effect of β-catenin on

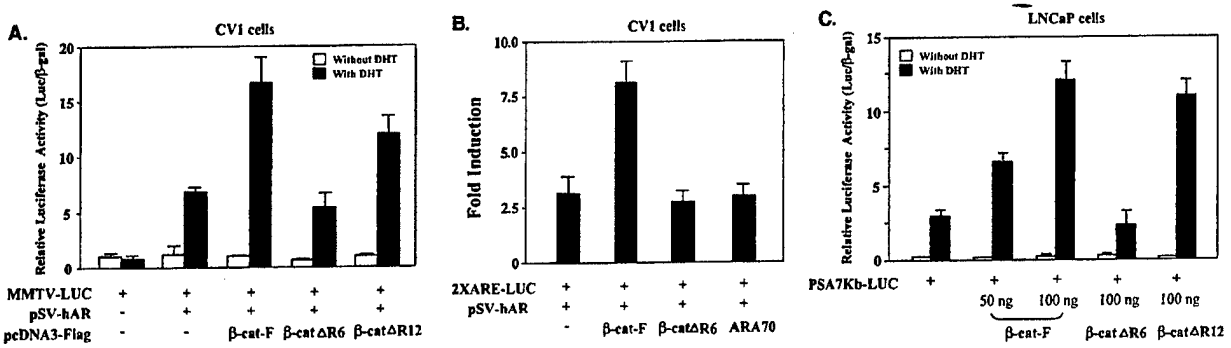

FIG. 6. **β-Catenin enhances AR-mediated transcription.** *A*, CV-1 cells were transfected with MMTV-luc reporter (100 ng), pcDNA3-β-gal (25 ng), pSV-hAR (5 ng), and the wild type and mutants of pcDNA3-FLAG-β-catenin (100 ng) as indicated. Twenty-four hours after transfection, cells were incubated with or without 10 nM DHT for 24 h. Cell lysates were prepared for assessment of luciferase and β-gal activities (as controls of transfection efficiency). *B*, similar to *A*, except that a 2×ARE-luc reporter (100 ng) was used. *C*, LNCaP cells were transfected with PSA7kb-luc reporter (100 ng), pcDNA3-β-gal (25 ng), and the wild type and mutants of pcDNA3-FLAG-β-catenin 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. The data represent the mean ± S.D. of three independent samples.

the transcription from a luciferase reporter driven by a minimum promoter with two androgen response elements (AREs). A similar ligand-dependent enhancement of AR-mediated transcription was observed with the full-length β-catenin construct (Fig. 6*B*). As we observed above, the mutant lacking repeat 6 showed no enhancement. Interestingly, ARA70, an AR coactivator, did not affect the AR-mediated transcription of this minimum promoter. Nevertheless, these results further confirm that β-catenin is truly a coactivator of AR and can enhance AR-mediated transcription on a minimum promoter with AREs.

To evaluate the enhancement by β-catenin of AR-mediated transcription in a physiologically relevant cellular context, an AR-positive prostate cancer cell line, LNCaP, was transfected with β-catenin expression constructs and a luciferase reporter driven by the 7-kb prostate-specific antigen (PSA) gene promoter, which is an AR-regulated target gene and has been widely used as a prostate-specific tumor marker (36). As seen in Fig. 6*C*, the wild type and repeat 12 deletion mutants of β-catenin enhance endogenous AR-mediated transcription from the PSA promoter, and the wild type β-catenin showed a dose-dependent effect. However, as we observed previously, the mutant with a deletion of repeat 6 showed no effect. These data further support the transfection data with MMTV and ARE-minimum promoters and demonstrate that the augmentation of endogenous AR activity by β-catenin in prostate cancer cells is mediated through the AR/β-catenin interaction.

E-cadherin Modulates the Level of Cytoplasmic Pools of β-Catenin to Enhance AR-mediated Transcription—The observation that β-catenin can function as an oncogene when inappropriately expressed highlights the importance of regulating β-catenin level in the cells. Recent studies show that tumor cells can bypass this regulation by acquiring loss-of-function mutations in components of the destruction complex or by altering regulatory sequences in β-catenin itself, which makes it impervious to the effects of the destruction complex. Moreover, in normal epithelial tissues, E-cadherin complexes with the actin cytoskeleton via catenins to maintain the functional characteristics of epithelia (37, 38). Disruption of this complex, due primarily to loss or decreased expression E-cadherin, is frequently observed in many advanced, poorly differentiated prostate cancer patient samples (39, 40). It has been reported that β-catenin is mainly accumulated in both the cytoplasm and the nucleus of some prostate cancer cell lines in which there is a reduction or loss of E-cadherin expression (41, 42).

To test whether loss of E-cadherin can augment AR activity by increasing cytoplasmic and nuclear levels of β-catenin, we stably transfected E-cadherin expression vectors into an E-cadherin-negative prostate cancer cell line, TSU.pr-1. In TSU.pr-1 cells, E-cadherin expression is silenced by hypermethylation of the promoter region (41). Immunostaining of the polycolonal subline transfected with the E-cadherin expression vector (TSU.pr-1/E-CAD) showed that β-catenin is partially redistributed into the cell membrane, resulting in reduction of its cytoplasmic and nuclear levels compared the pool transfected with a control vector, TSU.pr-1/Neo (Fig. 7*A*). Transfection of an AR expression vector and the PSA-luciferase reporter into these two TSU.pr-1 sublines showed a ligand-dependent AR activity (Fig. 7*B*). However, a stronger AR activity was observed in TSU.pr-1/Neo cells than TSU.pr-1/E-CAD cells with equal amounts of AR plasmid, and a dose-dependent induction was only shown in TSU.pr-1/Neo cells. Using an ARE-luciferase reporter, we also showed a similar dose-dependent augmentation of AR activity by the cytoplasmic pool of endogenous β-catenin in TSU.pr-1/Neo cells (Fig. 7*E*). The results from the above experiments suggest that endogenous β-catenin in the cytoplasmic pool can augment AR-mediated transcription and that reducing its level can decrease this enhancement.

To further confirm that the enhancement of AR activity in TSU.pr-1/Neo cells was directly mediated by β-catenin, we repeated the above experiments with an antisense construct of β-catenin. As shown in Fig. 7*C*, enhancement of ligand-dependent AR activation in TSU.pr-1/Neo cells was specifically repressed by cotransfection with the β-catenin antisense construct. This was correlated with a decreased level of β-catenin protein in the cells (Fig. 7*D*). To ensure that the enhancement of AR activity in TSU.pr-1/Neo cells was specifically mediated by endogenous β-catenin, rather than a general effect on the basal transcriptional machinery or other nonspecific effects from this subline, we examined the transcriptional activities of other nuclear hormone receptors in the cells. As shown in Fig. 7*F*, unlike the results that we observed in Fig. 7*E*, ERα, PRβ, and VDR showed no significant differences in ligand-dependent activities between TSU.pr-1/Neo and TSU.pr-1/E-CAD cells. These results are consistent with our yeast data showing that β-catenin selectively interacts with AR. Taken together, we conclude that overexpression of E-cadherin in TSU.pr-1 induces a redistribution of the cellular localization of β-catenin protein, which can directly affect AR-mediated transcription.

DISCUSSION

The nuclear receptor superfamily coordinates the complex events involved in development, differentiation, and physiological response to diverse stimuli. Transcriptional activity of the

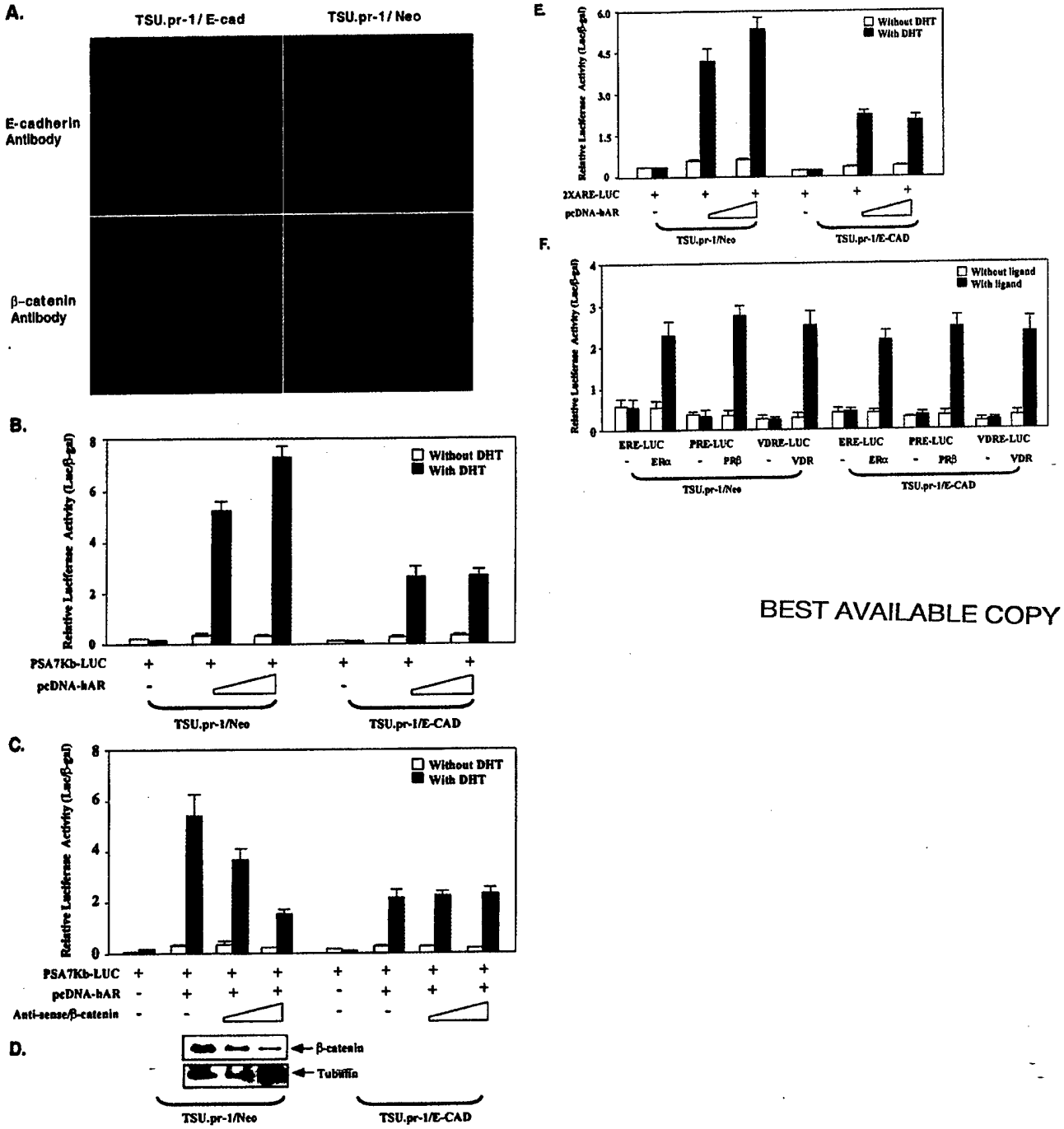

FIG. 7. **β-Catenin augments AR-mediated transcription in human prostate cancer cells.** *A*, two TSU.pr-1 sublines stably transfected with an E-cadherin expression plasmid (TSU.pr-1/E-CAD) or a control vector, pcDNA3 (TSU.pr-1/Neo), were stained with E-cadherin (*green*) and β-catenin (*red*) antibodies. *B*, 100 ng of PSA7Kb-Luc alone or with 10 or 30 ng of pcDNA-hAR were transfected into the TSU.pr-1 cells. *White bars* represent the absence of DHT; *black bars* represent the addition of 10 nM DHT. Luciferase activity is reported as relative light units and is represented as mean ± S.D. *C*, an antisense construct of β-catenin (20 and 60 ng) was cotransfected with the PSA-luc reporter and AR expression plasmid. Relative luciferase activities were measured. *D*, total cell lysates isolated from the above experiment were analyzed by Western blotting to determine the cellular levels of β-catenin proteins. *E*, a luciferase reported driven by two AREs (100 ng) and an AR expression vector (10 or 30 ng) were transfected into TSU.pr-1/NEO and TSU.pr-1/E-CAD cells. *F*, for each sample, 25 ng of pSV-β-gal, 100 ng of luciferase reporter vectors containing the different hormone response elements such as pERE-luc, pPRE-luc, and pVDRE-luc, and 10 ng of the corresponding receptor expression constructs were transfected. The specific ligands for each receptor were added for induction, and these included 10 nM β-estradiol, progesterone, and 1α,25-dihydroxyvitamin.

nuclear hormone receptors can be modulated by coactivators and corepressors (43). Aberrations of these cofactors may lead to enhancement of receptor activity to provide an adaptive advantage for cell growth. Changes in the transcriptional programs of nuclear receptors such as the AR are important but poorly understood events in tumor development and progression. The experiments reported here demonstrate a specific protein-protein interaction between AR and β-catenin. Further characterization of the interaction in the yeast two-hybrid assays and in *in vitro* GST-pull down experiments showed that the LBD of AR is necessary and sufficient for the interaction with β-catenin in an androgen-dependent manner. This sug-

gests that conformational changes in the LBD of AR, following binding to ligand, are necessary for the interaction of β-catenin with AR protein. Using immunofluorescence studies, we first demonstrated that β-catenin can translocate into the nucleus as part of a complex with AR. These multiple lines of evidence clearly indicated that AR and β-catenin proteins can specifically interact and given the consequences of the interaction, probably represents a biologically relevant interaction.

In this study, we also demonstrated a functional interaction between AR and β-catenin in prostate cancer cells, which is consistent with a recent report by Truica *et al.* (31). Importantly, using internal deletion mutants of β-catenin, we showed that enhancement of intact, natural AR-dependent promoters from MMTV and the PSA gene by β-catenin can be completely abolished by deleting armadillo repeat 6 of the protein. Similar results were obtained with a mini-promoter containing only two androgen response elements. These results provide the first line of evidence demonstrating that augmentation of AR activity by β-catenin is mediated solely through a specific protein-protein interaction. These results further support the possibility that the AR/β-catenin interaction characterized in this study is biologically relevant.

As a key player in both cell-cell adhesion and Wnt signaling, β-catenin is regulated by multiple signaling pathways through binding to other protein partners, including Tcf/LEF family members, axin, APC, and cadherins (44, 45). The crystal structure of the armadillo domain of β-catenin revealed that each of the armadillo repeats consists of three α-helices, and together the 12 repeats form a superhelix (29). This unique structure provides a long positively charged groove for binding. It has been shown that most of the β-catenin-binding proteins bind β-catenin mainly through the central armadillo domain (29, 30). The binding regions overlap, but in general, a minimum of 6–7 repeats is sufficient for detectable binding. Using a series of deletion mutants, we demonstrated that the NH_2 terminus and first six armadillo repeats of β-catenin are primarily responsible for binding to AR. Significantly, multiple lines of evidence from this study showed that a deletion lacking repeat 6 can completely abolish the binding, which suggests this region may directly form an interface with AR. Although most of β-catenin-interacting proteins bind β-catenin in the central armadillo domain, each of these proteins may bind to β-catenin differently depending on divergent binding regions. Further structural studies of AR/β-catenin interaction should lead to important information that may provide the basis for designing compounds to block this interaction.

A number of cofactors have been identified that interact with the LBD of nuclear hormone receptors (22, 46–48). Among them, the best characterized coactivators are the p160 family and p300/CBP, which appear to bind to most of the nuclear receptors in a ligand-dependent manner through the conserved protein motif, LXXLL (49, 50). The motif forms a two-turn amphipathic α-helix, which binds to a hydrophobic cleft in the activation domain 2 of nuclear receptors. β-Catenin contains five LXXLL motifs, all of which are located in a central armadillo domain (29). Among them, four are localized in the second helix of the armadillo repeats 1, 7, 10, and 12. Based on the structure of β-catenin protein, the Leu residues in these motifs are buried in the hydrophobic core of the armadillo repeats, and it seems unlikely that they would interact with AR or other nuclear hormone receptors (29). Using a series of deletion mutants, we have shown that constructs lacking the repeats 7, 10, and 12 retained the capacity to bind AR, whereas the construct lacking only repeat 6 fully abolished the interaction. Under identical experimental conditions, other steroid hormone receptors such as ERα, PRβ, and GR did not show an interaction with β-catenin. These data are consistent with an earlier structural study of β-catenin and suggest that the LXXLL motifs in β-catenin may not directly contribute to the interaction that we have identified between AR and β-catenin. An earlier report showed that β-catenin associates with a retinoic acid receptor and enhance activation of a retinoic acid-responsive promoter (51). In the yeast two-hybrid system, we also observed a weak interaction between β-catenin and VDR. In this regard, it will be important to determine the protein motifs involved in the interaction with these receptors, which will expand our understanding of the cross-talk between β-catenin and nuclear hormone receptors.

The cellular levels of β-catenin are tightly regulated in normal cells. Mutations affecting the degradation of β-catenin can increase the cellular levels of the proteins to induce neoplastic transformation (52). A tumor suppressor, APC, which is an important component of the degradation machinery, was frequently mutated in both sporadic and hereditary colorectal tumors (12). Mutations of β-catenin within the GSK binding region were also found in prostate cancer samples (53), suggesting a potential role of β-catenin in prostate cancer cells. Our results showing a detailed molecular basis of the interaction of β-catenin with AR provide a direct link between β-catenin and androgen signaling. Due to an abnormal cadherin-catenin interaction in the cell membrane, increasing the cytoplasmic and nuclear levels of β-catenin as a consequence of loss of E-cadherin is frequently observed in late stages of prostate cancer cells (15). Using an E-cadherin-negative prostate cancer cell line, TSU.pr-1, we further showed that the endogenous β-catenin that accumulated in cytoplasm and nucleus are capable of augmenting AR-mediated transcription, and the effect of β-catenin on AR can be enhanced by loss of E-cadherin expression. These results suggest that loss of E-cadherin expression may promote AR-mediated cell growth in late stages of prostate cancer. In addition, as observed previously (16), β-catenin was shown to have no effect with a TCF reporter gene in TSU.pr-1 cells (data not shown). A similar observation was also reported recently in breast cancer cells containing transcriptional silencing of the E-cadherin gene (54). This raises the question as to whether the growth-promoting effect of β-catenin is mediated through other partners rather than through the TCF/LEF pathway in prostate cancer or/and other tumor cells.

Our results suggest a new role for E-cadherin and β-catenin in prostate cancer cells. During prostate cancer progression, loss of expression of E-cadherin frequently occurs, which leads to an increase in the cytoplasmic levels of β-catenin. Under normal conditions, the cellular β-catenin is tightly regulated by the destruction complex which includes APC, GSK3β, and axin. When the functional activities of these components are changed, such as by mutation or aberrant expression of the proteins, the excessive free β-catenins overload the system and are translocated into the nucleus, where they specifically interact with the AR to augment AR-mediated transcription. In addition, enhancement by β-catenin may also be able to maintain or increase AR activity in the setting of decreased androgen levels during androgen ablation therapy, which can adapt prostate cancer cells to become androgen insensitive. Therefore, studying the interaction of β-catenin with AR in prostate cancer should provide fresh insight into the progression of prostate cancer that may help us to identify new steps that can be targeted for prostate cancer treatment.

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Phosphatidylinositol 3-Kinase/Akt Stimulates Androgen Pathway through GSK3β Inhibition and Nuclear β-Catenin Accumulation*

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Manju Sharma, William W. Chuang, and Zijie Sun‡

From the Departments of Surgery and Genetics, Stanford University School of Medicine, Stanford, California 94305-5328

PI3K/Akt plays a critical role in prostate cancer cell growth and survival. Recent studies have shown that the effect of PI3K/Akt in prostate cells is mediated through androgen signaling. The PI3K inhibitor, LY294002, and a tumor suppressor, PTEN, negatively regulate the PI3K/Akt pathway and repress AR activity. However, the molecular mechanisms whereby PI3K/Akt and PTEN regulate the androgen pathway are currently unclear. Here, we demonstrate that blocking the PI3K/Akt pathway reduces the expression of an endogenous AR target gene. Moreover, we show that the repression of AR activity by LY294002 is mediated through phosphorylation and inactivation of GSK3β, a downstream substrate of PI3K/Akt, which results in the nuclear accumulation of β-catenin. Given the recent evidence that β-catenin acts as a coactivator of AR, our findings suggest a novel mechanism by which PI3K/Akt modulates androgen signaling. In a PTEN-null prostate cancer cell line, we show that PTEN expression reduces β-catenin-mediated augmentation of AR transactivation. Using the mutants of β-catenin, we further demonstrate that the repressive effect of PTEN is mediated by a GSK3β-regulated degradation of β-catenin. Our results delineate a novel link among the PI3K, wnt, and androgen pathways and provide fresh insights into the mechanisms of prostate tumor development and progression.

Prostate cancer is the most common malignancy in men and the second leading cause of cancer death in the United States (1). The fact that androgen ablation is an effective treatment for the majority of prostate cancers indicates that androgen plays an essential role in regulating the growth of prostate cancer cells (2, 3). The growth-promoting effects of androgen in prostate cells are mediated mostly through the androgen receptor (AR).[1] The AR belongs to the nuclear receptor superfamily and acts as a ligand-dependent transcription factor (4, 5). Recent studies suggest that other signal transduction pathways can modulate AR activity and that they may also contribute to the development and progression of prostate cancer (6, 7).

The phosphatidylinositol 3-kinase (PI3K) consists of regulatory (p85) and catalytic (p110) subunits that participate in multiple cellular processes including cell growth, transformation, differentiation, and survival (8). An oncoprotein, Akt/PKB, has been identified as a key effector of the PI3K signaling pathway (9, 10). The binding of PI3K-generated phospholipids to Akt results in the translocation of Akt from the cytoplasm to the inner surface of the plasma membrane where Akt is phosphorylated by the upstream kinases, PDK-1, PDK-2, and ILK (11, 12). The activation of Akt results in the phosphorylation of a number of downstream substrates such as glycogen synthase kinase (GSK3), Bad, and caspase9 and the forkhead transcription factors, Raf, Iκb kinase, and phosphodiesterase 3B (13). As one of the principal physiological substrates of Akt, GSK3 is a ubiquitously expressed protein serine/threonine kinase that was initially identified as an enzyme that regulates glycogen synthesis in response to insulin (14, 15). It has been shown that GSK3β plays an important role in the Wnt pathway by regulating the degradation of β-catenin (16, 17).

β-catenin plays a pivotal role in cadherin-based cell adhesion and in the Wnt signaling pathway (18). Corresponding to its dual functions in cells, β-catenin is localized to two cellular pools. Most of the β-catenin is located in the cell membrane where it is associated with the cytoplasmic region of E-cadherin, a transmembrane protein involved in homotypic cell-cell contacts (19). A smaller pool of β-catenin is located in both the nucleus and cytoplasm where it mediates Wnt signaling. In the absence of a Wnt signal, β-catenin is constitutively down-regulated by a multicomponent destruction complex containing GSK3β, axin, and the tumor suppressor adenomatous polyposis coli. These proteins promote the phosphorylation of serine and threonine residues in the amino-terminal region of β-catenin and thereby target it for degradation by the ubiquitin proteasome pathway (20). Wnt signaling inhibits this process, which leads to an accumulation of β-catenin in the nucleus and promotes the formation of transcriptionally active complexes with members of the Tcf/LEF family (21) and other transcription factors (22, 23).

The tumor suppressor PTEN is a phosphoprotein/phospholipid dual specificity phosphatase (24). Early studies indicated that somatic mutation of *PTEN* is a common event in a variety of human tumors including prostate cancer (25). *PTEN* was found to be mutated in primary prostate tumors, metastatic prostate cancers, and in prostate cancer cell lines (25, 26). In addition, the reduced expression of PTEN protein as well as increased Akt activity has been observed in xenograft models (27). Recently, it has been shown that PTEN inhibits PI3K/Akt-stimulated androgen-promoted cell growth and AR-mediated transcription in prostate cancer cells (28).

PI3K/Akt has been shown to promote prostate cancer cell survival and growth via enhancing AR-mediated transcription. Both PTEN and the PI3K inhibitor LY294002 negatively regulate this process (28, 29). Although several potential mecha-

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‡ To whom correspondence should be addressed: Departments of Surgery and Genetics, R135, Edwards Bldg., Stanford University School of Medicine, Stanford, CA 94305-5328. E-mail: zsun@stanford.edu.

[1] The abbreviations used are: AR, androgen receptor; PI3K, phosphatidylinositol 3,4,5-trisphosphate; GSK3β, glycogen synthase kinase 3β; PTEN, phosphatase and tensin homolog deleted on chromosome 10; DHT, dihydrotestosterone; PSA, prostate-specific antigen.

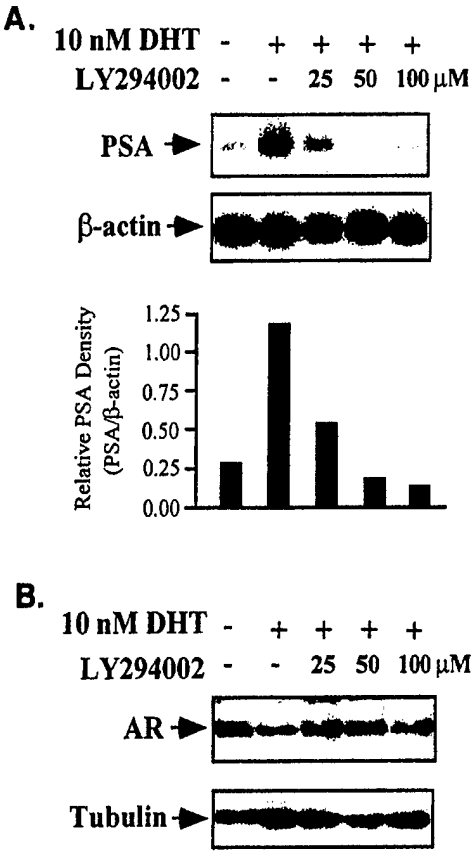

FIG. 1. **The PI3K inhibitor represses AR-mediated transcription.** *A*, total RNAs were isolated from LNCaP cells cultured in T-medium with or without 10 nM DHT, treated for 4 h with the PI3K inhibitor LY294002 or vehicle, and analyzed by Northern blotting. Expression of the endogenous *PSA* gene was detected by a cDNA probe derived from the human PSA gene. A β-actin probe was used to confirm equal RNA loading. Densitometry of the membrane blot was performed, and the relative numbers were reported as optical density units of β-actin (*PSA/β-actin*). *B*, whole cell lysates were isolated from LNCaP cells treated as described above and analyzed by Western blotting to detect the expression of AR and tubulin proteins.

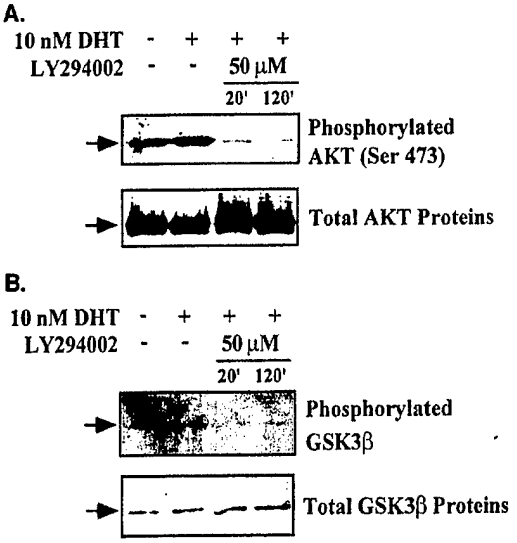

FIG. 2. **Inhibition of Akt and GSK3β phosphorylation by LY294002 in prostate cancer cells.** Whole cell lysates were isolated from LNCaP cells that were treated as indicated in Fig. 1 and "Experimental Procedures," and were analyzed by Western blotting. Both total and phosphorylated Akt (*A*) and GSK3β (*B*) were detected by specific antibodies as indicated in the figure.

nisms have been suggested for this cross-talk, the precise molecular basis by which PI3K/AKT and PTEN regulate AR-mediated transcription is currently unclear. Recently, a specific protein-protein interaction between β-catenin and AR was identified by us and others (22, 23). Through this interaction, β-catenin augments the ligand-dependent activity of AR in prostate cancer cells. Here, we provide multiple lines of evidence showing that the cross-talk between the androgen and PI3K/Akt pathways is mediated through the modulation of the PI3K/Akt downstream effector GSK3β. Its inactivation by phosphorylation results in increased nuclear levels of β-catenin, which augment AR activity. These findings delineate a novel mechanism by which PI3K/Akt and PTEN regulate the androgen pathway during prostate cell growth and survival.

EXPERIMENTAL PROCEDURES

Cell Cultures and Transfections—An AR-positive prostate cancer cell line LNCaP was maintained in T-medium (Invitrogen) with 5% fetal calf serum. Transient transfections were carried out in RPMI 1640 medium using LipofectAMINE 2000 (Invitrogen) as described previously (23). In the experiments with the PI3K inhibitor LY294002 (Alexis, San Diego, CA), cells were usually cultured for 16 h and then were treated with different concentrations of the inhibitor in Me_2SO or vehicle only for 20 min to 2 h. For androgen induction experiments, cells were grown in T-medium with charcoal-stripped fetal calf serum (HyClone, Denver, CO) for 14 h and treated with 10 nM DHT in ethanol and different concentrations of LY294002 for 4 h.

Northern Blot Analysis— Total RNAs were isolated from LNCaP cells treated with LY294002 for 4 h in the presence of 10 nM DHT in ethanol or vehicle alone using an RNAwiz kit (Ambion, Austin, TX). For Northern blotting, 5 μg of total RNA were electrophoresed on a 1% agarose-formaldehyde gel, transferred to Hybond-N nylon membranes (Amersham Biosciences) by capillary blotting in 20× SSC, and hybridized with a DNA fragment (amino acids 1–261) derived from the human prostate-specific antigen (*PSA*) gene. The blots were stripped and rehybridized with a β-actin probe (30).

Preparation of Whole Cell and Nuclear Extracts—LNCaP cells were cultured in duplicate flasks to collect both whole cell lysates and nuclear extracts. To make the whole cell lysates, cells were washed with phosphate-buffered saline and were resuspended in RIPA buffer (1% Nonidet P-40, 0.1% SDS, 50 mM NaF, 0.2 mM Na_3VO_4, 0.5 mM dithiothreitol, 150 mM NaCl, 2 mM EDTA, 10 mM sodium phosphate buffer, pH 7.2). Nuclear extracts were prepared from LNCaP cells essentially according to the method of Dignam *et al.* (31) with minor modifications. The cells were washed with phosphate-buffered saline and mechanically disrupted by scraping into homogenization buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and incubated on ice for 10 min. Cells were further disrupted by 10 strokes of a homogenizer and centrifuged at 15,000 rpm for 20 min. The pellet was resuspended in buffer containing 20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol and then homogenized with 10 strokes. The lysate was incubated on ice for 30 min and centrifuged for 10 min at 15,000 rpm. The supernatant was saved and analyzed as the nuclear fraction.

To prepare the cytosolic fraction, LNCaP cells treated with LY294002 were lysed in digitonin lysis buffer (1% digitonin, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$). The lysates were centrifuged at 13,000 rpm for 10 min, and the supernatants were saved as cytosolic components. The pellets representing cytoskeletal and nuclear components were lysed in RIPA buffer.

SDS-PAGE and Immunoblotting—Protein fractions for immunoblotting were boiled in SDS sample buffer and then resolved on a 10% SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane and probed with appropriate antibodies including an anti-human Akt (provided by Dr. Richard Roth, Stanford University, Stanford, CA), phospho-Akt-(Ser-473) (catalog number 9271, Cell Signaling Technology, Beverly, MA), phospho-GSK3α/β-(Ser-21/Ser-9) (catalog number 9331, Cell Signaling Technology), AR (catalog number sc-816, Santa Cruz Biotechnology, Santa Cruz, CA), Sin3A (catalog number sc-996, Santa Cruz Biotechnology), tubulin (catalog number MS-581-P, Neomarker, Fremont, CA), β-catenin (catalog number C19220, Transduction Laboratories, Lexington, KY), and GSK3β (catalog number G22320, Transduction Laboratories). Proteins were detected using the ECL kit (Amersham Biosciences). The nuclear fractions were analyzed by SDS-PAGE. Equal loading of the nuclear proteins was ascertained

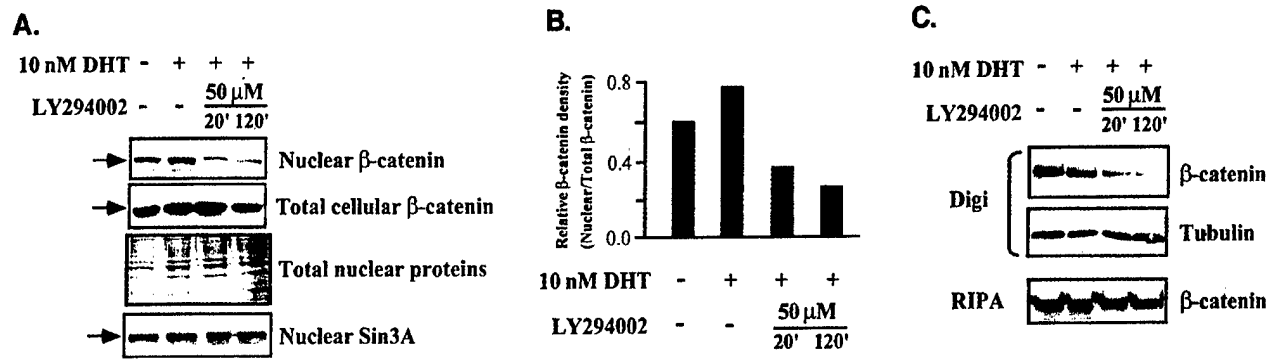

FIG. 3. **Inhibition of PI3K signaling results in decreased nuclear accumulation of β-catenin in prostate cancer cells.** *A*, both nuclear extracts and whole cell lysates were isolated from LNCaP cells treated with LY294002 and DHT and resolved by SDS-PAGE. The β-catenin and Sin3A antibodies were used for the detection of protein expression. The same membrane used for the Western blotting was also stained with Ponceau S stain solution for measuring equal protein loading. *B*, densitometry of nuclear β-catenin proteins is shown as relative β-catenin density (optical density units of nuclear proteins/optical density units of total proteins). *C*, both cytosolic fraction (*Digi*) and cytoskeletal fraction (*RIPA*) were prepared from LNCaP cells as described under "Experimental Procedures" and were analyzed by Western blotting. Both β-catenin and tubulin were detected using specific antibodies.

by reversible staining with the Ponceau S solution (Catalog number P7767, Sigma).

Plasmid Construction—The pcDNA3-AR expression vector was generated in the laboratory and used for the transient transfection experiments. Expression constructs of human PTEN were generously provided by Dr. William Sellers (Dana-Farber Cancer Institute, Boston, MA) and used for subcloning into the pCMV5 vector. PLNCX-HA-myr-AKT and PLNCX-HA-myr-AKT179M were also kindly provided by Dr. Sellers (32). The reporter plasmid pPSA7kb-luc with the luciferase gene under the control of promoter fragments of the human prostate-specific antigen was provided by Dr. Jan Trapman (33). The mutants of β-catenin with a single point mutation in the GSK3β phosphorylation sites were generated by a PCR-based mutagenesis scheme. The key serine amino acid residues were mutagenized by using sets of primers containing two or three nucleotide changes in conjunction with upstream and downstream primers. The appropriate fragments with in-frame restriction enzyme sites were generated by PCR, cleaved with restriction enzymes, and cloned into the pcDNA3 vector (Invitrogen). All of the constructs were sequenced from both ends of the inserts to confirm that no extraneous mutations were introduced by PCR.

Luciferase and β-Galactosidase Assay—Luciferase activity was measured in relative light units as described previously (30). 50 μl of cell lysate was used for luciferase assays. The light output is measured after a 5-s delay following injection of 50 μl of luciferase buffer and 50 μl of Luciferin by the dual injector luminometer according to manufacturer's instruction (Analytical Luminescence Laboratories, San Diego, CA). The relative light units from individual transfections were normalized by the measurement of β-galactosidase activity expressed from a cotransfected plasmid in the same samples. Individual transfection experiments were done in triplicate, and the results are reported as the luciferase/β-galactosidase mean ± S.D. from representative experiments.

RESULTS

Inhibition of the PI3K/AKT Pathway Represses AR-mediated Transcription—PI3K/Akt enhances the activity of AR-regulated reporter genes in transient transfection experiments (28, 29). To evaluate the effect of PI3K/Akt on AR-mediated transcription in a physiologically relevant cellular context, we examined the expression of the endogenous *PSA* gene in an AR-positive prostate cancer cell line LNCaP treated with the PI3K inhibitor LY294002. In the presence of 10 nM DHT, PSA expression was increased ~4-fold in LNCaP cells over that found in cells not treated with DHT (Fig. 1*A*). At concentrations of LY294002 from 25–100 μM, the expression of PSA was significantly reduced. An ~4-fold reduction of PSA transcripts was found in the cells treated with 100 μM LY294002 using the level of β-actin transcripts as an internal control (Fig. 1*A*). Low concentrations (5 μM) of LY294002 induced only a slight reduction of PSA expression during a 4-h treatment but showed a significant reduction of PSA expression after 16 h (data not shown). To ensure that this repression was not the result of LY294002-induced changes in the intracellular steady-state levels of AR protein, we examined both the AR and tubulin protein levels in the cell samples used for the Northern blotting. We found that there was no significant change in protein expression (Fig. 1*B*). This result provided the first line of evidence that inhibition of PI3K/Akt could suppress endogenous AR-mediated transcription in prostate cancer cells.

Repression of the PI3K/AKT Pathway Inhibits Phosphorylation of GSK3β and Nuclear Accumulation of β-catenin in Prostate Cancer Cells—To further elucidate the mechanism by which LY294002 inhibits endogenous AR transactivation in LNCaP cells, we first assessed the phosphorylation state of Akt. It has been reported that PDK-1 phosphorylation of threonine 308 in the activation loop of the catalytic domain of Akt allows autophosphorylation of serine 473 (a hydrophobic phosphorylation site) in the carboxyl terminus (34). To demonstrate that the effect of LY294002 on PSA transcription was attributed to inhibition of Akt, we evaluated Akt activation using a phosphorylation-specific antibody for Ser-473. As shown in Fig. 2*A*, the phosphorylation of Akt proteins was significantly inhibited by LY294002 in LNCaP cells, even after a very short pulse (20 min). In contrast, the total amount of Akt protein showed no differences in the presence or absence of LY294002.

Because GSK3β is one of the major downstream targets of Akt, we next assessed whether LY294002 also affected the phosphorylation of GSK3β. Using specific antibodies, we examined both the total and phosphorylated GSK3β proteins in the same cell samples used for detecting Akt. As expected, the phosphorylation of GSK3β proteins was also significantly impaired by treatment with LY294002, whereas almost equal amounts of total GSK3β proteins were found in both treated and untreated cells (Fig. 2*B*). At either 5 or 20 μM LY294002, we observed a similar inhibitory effect on the phosphorylation of both Akt and GSK3β in cells treated for 12 h (data not shown). Taken together, the results demonstrate that the suppression of the PI3K pathway by the PI3K inhibitor LY294002 blocks the phosphorylation of both Akt and GSK3β proteins in LNCaP cells.

The above data demonstrate that the treatment of LNCaP cells with LY294002 results in a decreased level of expression of the endogenous *PSA* gene and an inhibition of the phosphorylation of Akt and GSK3β. It has been shown that GSK3β regulates the cellular levels of β-catenin by targeting it to the ubiquitin proteasome pathway via the destruction complex (20). Previous studies have shown that inactivation of GSK3β by phosphorylation can induce the nuclear accumulation of β-catenin because of decreased degradation (17, 35). To evalu-

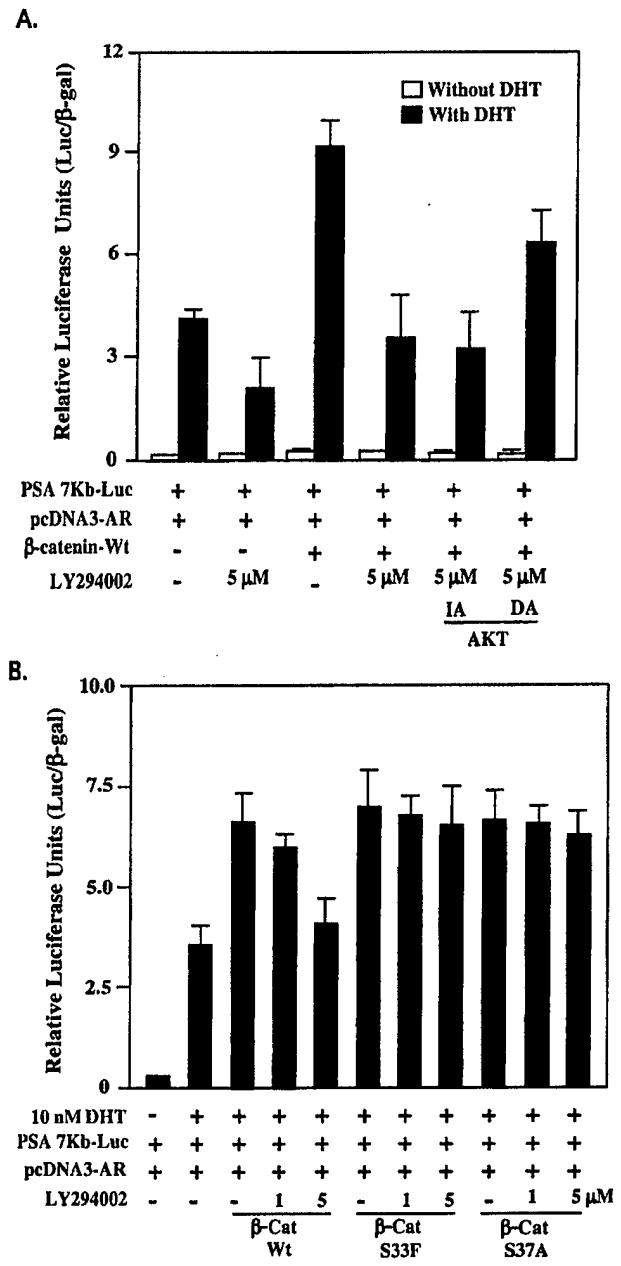

FIG. 4. **Inhibition of AR activity by LY294002 is mediated through Akt-and GSK3β.** *A*, transient transfections were performed in LNCaP cells with 100 ng of PSA7kb-luc reporter, 5 ng of pcDNA3-AR, 25 ng of pcDNA3-β-galactosidase, and 50 ng of wild type pcDNA3-FLAG-β-catenin in the presence or absence of 50 ng of an inactive mutant (*IA*) or a dominantly active mutant (*DA*) of Akt. The cells were incubated in RPMI 1640 medium with 5% charcoal-stripped fetal calf serum for 12 h and then were treated with different concentrations of LY294002 in the presence or absence of 10 nM DHT for 18 h. Cell lysates were measured for luciferase and β-galactosidase activities. The data represent the mean ± S.D. of three independent samples. *B*, LNCaP cells were cotransfected with 50 ng of wild type β-catenin or the mutants of β-catenin containing a point mutation within the GSK3β binding site as well as with the other plasmids indicated in the figure. The cells were treated with DHT and LY294002 as described above.

ate the downstream effect of GSK3β in LNCaP cells, we next examined the nuclear levels of β-catenin. Nuclear extracts and whole cell lysates were prepared from cells that were treated with LY294002 or with vehicle only. As shown in Fig. 3*A*, there was no significant change in the amount of total β-catenin protein in the treated compared with the untreated cells. However, there was a 2–3-fold reduction in nuclear β-catenin in the cells treated with LY294002 (Fig. 3, *A* and *B*). In contrast, the controls, total nuclear protein, and the transcriptional repressor Sin3A showed no change (Fig. 3*A*). To confirm these findings, we examined the level of free cytosolic β-catenin protein in LNCaP cells treated with LY294002 (36). As shown in Fig. 3*C*, after LY294002 treatment, free β-catenin in the cytosolic compartment (Digi) was significantly reduced, whereas β-catenin in the cytoskeletal compartment (RIPA) remained unchanged. Taken together, these results demonstrate that blocking PI3K signaling results in a decrease in both the free cytosolic and nuclear β-catenin in prostate cells.

Repression of AR Activity by LY294002 Is Mediated through the Downstream Effectors of PI3K, Akt, and GSK3β—To further study the repressive effect of LY294002 on AR-mediated transcription, we next used an inactive and a dominantly active mutant of Akt to directly examine the involvement of Akt in LY294002-induced AR repression. Transient transfection assays were performed in LNCaP cells. In the presence of 10 nM DHT, the overexpression of AR induces approximately a 10-fold induction of the PSA promoters. Cotransfection with the wild type β-catenin expression vector augments AR activity to nearly 20-fold above base line (Fig. 4*A*). The addition of LY294002 to the cells results in a large reduction in AR activity. At 5 μM LY294002, AR activity was reduced by ~60%. Coexpression of the dominantly active Akt reversed the inhibition of AR activity by LY294002, whereas an inactive mutant of Akt used as a control showed no effect (Fig. 4*A*). These data directly demonstrate that repression of AR activity by LY294002 is mediated through the down-regulation of PI3K and the subsequent inactivation of Akt activity.

We next performed the transient transfection experiments using either wild type or β-catenin mutants containing a point mutation within the NH_2-terminal GSK3β binding site. Because these mutants are resistant to GSK3β-mediated degradation, we further assessed whether the repression of AR by LY294002 is mediated through GSK3β. As shown in Fig. 4*B*, an ~40% reduction in expression was induced by 5 μM LY294002 in the cells that were cotransfected with wild type β-catenin but not in the cells cotransfected with the β-catenin mutants. As mentioned above, because the β-catenin mutants used in these experiments are impervious to the effects of the destructive complex attributed to point mutations within the GSK3β phosphorylation sites (20), the results from these experiments suggest that GSK3β is involved in the regulation of β-catenin-mediated augmentation of AR activity.

Expression of PTEN in LNCaP Cells Represses β-Catenin-mediated Augmentation of AR Activity—Recent data have shown that the tumor suppressor PTEN appears to negatively control the PI3K signaling pathway by blocking the activation of the downstream target Akt (24). The mutations in the *PTEN* gene were found in prostate cancer tissues and cell lines (25). In a previous report, Li *et al.* (28) showed that the transfection of the wild type PTEN repressed an AR-regulated reporter gene in PTEN-null prostate cancer cells. The results from our experiments indicate that the inhibition of PI3K/Akt signaling represses the expression of an endogenous AR target gene and reduces the levels of nuclear β-catenin. To further examine whether repression of AR activity by PTEN is also mediated by PI3K/Akt modulation of nuclear β-catenin, we performed transient transfections using either the wild type β-catenin or the β-catenin mutants described above. As shown in Fig. 5*A*, in the absence of PTEN vector, both the wild type and β-catenin mutants augment AR-mediated transcription ~1.5-fold using a 7-kilobase PSA promoter in the PTEN-null cells, LNCaP. However, when a wild type PTEN vector was cotransfected into the cells, the wild type β-catenin showed less enhancement of AR

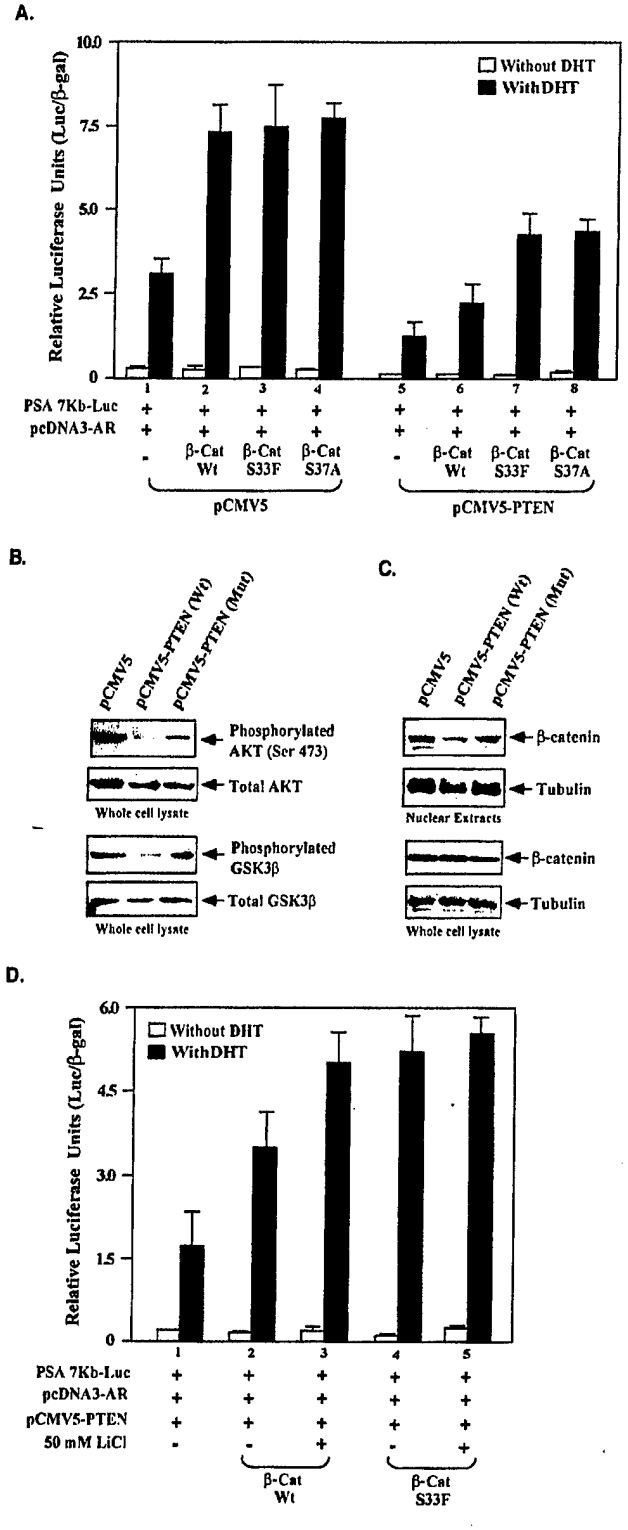

FIG. 5. **PTEN represses β-catenin-mediated augmentation of AR activity by reducing nuclear β-catenin protein.** *A*, LNCaP cells were transfected with a PSA7kb-luc reporter (100 ng), pcDNA3-β-galactosidase (25 ng), pcDNA3-AR (5 ng), and the wild type or mutants of pcDNA3-FLAG-β-catenin (50 ng) as indicated. Either an empty pCMV5 vector or pCMV5-PTEN was cotransfected with the above plasmids. Ten hours after transfection, the cells were treated with 10 nM DHT or with vehicle only for 18 h. Cell lysates were measured for luciferase and β-galactosidase activities. The data represent the mean ± S.D. of three independent samples. (*B* and *C*) The PTEN expression constructs were transfected into LNCaP cells. Nuclear extracts and whole cell lysates were prepared from the cells 30 h after transfection and analyzed by Western-blotting. *D*, transient transfections were performed with the plasmids as labeled in the figure. After a 10 h transfection, 10 nM DHT and 50 mM LiCl were added to the cells. Whole cell lysates were prepared after another 18 h of incubation and were used to measure luciferase and β-galactosidase activities.

activity than the mutants, indicating a repressive effect of PTEN on wild type β-catenin ($p < 0.05$). The results with the mutants of β-catenin demonstrate that the effect of PTEN on AR-mediated transcription is regulated through GSK3β via degradation of nuclear β-catenin. To further confirm this finding, we examined the phosphorylation status of Akt and GSK3β proteins as well as the levels of nuclear β-catenin protein in LNCaP cells, which were transfected with either wild type or the loss-of-function PTEN expression vector. As shown in Fig. 5*B*, both the phosphorylation of Akt and GSK3β proteins was significantly reduced in the cells transfected with wild type PTEN vector. Moreover, a reduction of nuclear β-catenin protein was observed only in the nuclear extracts isolated from cells transfected with the wild type PTEN vector, although the total β-catenin protein detected was almost equal in all of the samples (Fig. 5*C*).

We next examined whether the inhibition of GSK3β can directly affect β-catenin-mediated augmentation of AR activity. As lithium chloride has been shown to inhibit GSK3β through a mechanism independent of serine 9 phosphorylation (37), we examined whether β-catenin-mediated AR augmentation is affected in cells treated with LiCl. As shown in Fig. 5*D*, in the presence of PTEN, the transfection of wild type β-catenin showed less stimulation of AR-mediated PSA promoter activity than that of the mutant β-catenin (*black bars 2* and *4*). However, the inhibition of GSK3β by LiCl treatment increases AR activity in the presence of wild type β-catenin (*black bar 3*), whereas there is little change in the PSA promoter activity in the mutant β-catenin-transfected cells treated with LiCl (*black bar 5*). These data are consistent with previous reports on other human cell lines (36, 38). Taken together, our results demonstrate that PTEN negatively regulates the augmentation of AR activity by β-catenin through targeting of the β-catenin degradation pathway mediated by GSK3β.

DISCUSSION

The PI3/Akt pathway plays a critical role in prostate cell proliferation and survival (24). PTEN, which is frequently mutated in prostate cancer cells, negatively regulates this process by blocking the PI3K/Akt pathway. Recently, several lines of evidence showed that PI3K/Akt and PTEN can modulate androgen-induced cell growth and AR-mediated transcription in prostate cancer cells (28, 29), suggesting a potential link between the PI3K/Akt and androgen pathways. In this study, we demonstrated that β-catenin acts as the point of convergence for the cross-talk between the PI3K/Akt and androgen signaling pathways. The data presented here are consistent with what is known regarding the degradation of β-catenin by GSK3β, a downstream effector of PI3K/Akt, and fit very well with our recent finding that β-catenin interacts with AR and augments its ligand-dependent transcription (23).

The dysregulation of β-catenin expression and Wnt-mediated signaling is now recognized as important events in the pathogenesis of variety of human malignancies including prostate cancer (18, 39). Tumor cells contain high levels of free cellular β-catenin by acquiring loss-of-function mutations in the components of the destruction complex or by altering regulatory sequences in β-catenin itself. Besides Wnt signaling, other signaling pathways are also involved in regulating cellular β-catenin levels (36, 38, 40). In this study, we showed that PI3K/Akt increases the stability of nuclear β-catenin by phosphorylation and inactivation of the downstream substrate

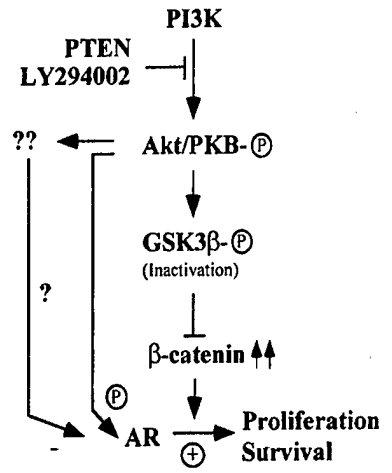

FIG. 6. **β-catenin acts as a mediator in the cross-talk between PI3K and androgen signaling.** A model summarizes PI3K/Akt signaling in prostate cells and the pathways for PTEN and the PI3K inhibitor LY294002 in the regulation of AR activity.

GSK3β in prostate cancer cells. Given that β-catenin acts as a transcriptional coactivator of AR, these data provide evidence to suggest a new mechanism whereby PI3K/Akt can affect prostate cell proliferation and survival through androgen signaling.

Earlier studies showed that PTEN negatively regulates the PI3K/Akt pathway in prostate cancer cells (28). The expression of PTEN in LNCaP, a PTEN-null prostate cancer cell line, blocks androgen-induced cell growth and AR-mediated transcription. In this study, we demonstrated that the overexpression of PTEN in LNCaP reduces β-catenin-mediated augmentation of AR activity; however, PTEN showed no effect in cells transfected with β-catenin mutants containing a single point mutation within the GSK3β phosphorylation sites. The results from our biochemical experiments further demonstrated that PTEN reduces the nuclear accumulation of β-catenin proteins in prostate cells. Because the β-catenin mutants used in our experiments are impervious to degradation by the destruction complex, we conclude that the regulation of β-catenin by PTEN is mediated through GSK3β. Our results are consistent with a recent study showing that nuclear β-catenin protein is constitutively elevated in PTEN null cells, and this elevated expression can be reduced upon the reexpression of PTEN (41). The data presented here also confirm that PTEN negatively regulates the PI3K pathway by inhibiting phosphorylation of Akt. In addition, the experiments using PTEN as a natural PI3K inhibitor are consistent with our data showing the important effects mediated by the synthetic PI3K inhibitor LY294002.

Modification of the AR protein such as by phosphorylation or acetylation has been suggested to be an important mechanism for modulating AR activity in prostate cancer cells (42–44). The putative consensus sequences for Akt phosphorylation were identified in both the transactivation and the ligand binding domains of AR (29). Those authors showed that Akt can directly bind to and phosphorylate AR (29). However, using both biochemical and functional approaches, we were not able to show a physical protein-protein interaction between Akt and AR or the phosphorylation of AR by Akt *in vitro* (data not shown). Results similar to ours were also reported by Li *et al.* (28). These conflicting results may be attributed to the use of different reagents and experimental conditions, but they also suggest that other alternative pathways may be involved in this regulation (Fig. 6). As presented in this study, we propose a novel molecular mechanism for PI3K/Akt and PTEN regulation of androgen signaling in prostate cancer cells.

The major role of β-catenin in tumorigenesis has been implicated via its interaction with the Tcf/LEF transcription factors (45). Interestingly, as we and others have reported recently (23, 46), β-catenin is shown to have no effect on the activation of Tcf/LEF-mediated transcription in prostate cancer cells despite the expression of Tcf/LEF. A similar observation was also reported recently in breast cancer cells (47). In this study, using Tcf/LEF reporters, we were also not able to demonstrate an effect of PTEN on the regulation by β-catenin of Tcf/LEF-mediated transcription in LNCaP cells (data not shown). This raises the question as to whether the growth-promoting effect of β-catenin is mediated through partners outside of the Tcf/LEF pathway in prostate cancer and/or other tumor cells.

In this study, we demonstrate that β-catenin mediates the cross-talk between PI3K/Akt and androgen pathways. Based on these results and previous studies by others, we summarize our findings in Fig. 6. The PI3K/Akt signal induces phosphorylation and inactivation of GSK3β, resulting in increased nuclear levels of β-catenin. Consequently, increased β-catenin elevates AR activity to stimulate prostate cell growth and survival. Both the PI3K inhibitor LY294002 and PTEN negatively regulate these processes. A loss-of-expression or mutational inactivation of PTEN has been frequently observed in human tumors, which induce the suppression of apoptosis and accelerates cell cycle progression (24, 25). Additionally, the mutation or aberrant expression of the destruction complex and the reduction of E-cadherin, which results in increased nuclear β-catenin, also occurs during prostate cancer progression (39). Our data showing that PTEN reduces nuclear β-catenin in prostate cancer cells suggest a novel role of PTEN in down-regulating androgen-induced cell growth and survival. A further study of the regulation of the interaction among PI3K, Wnt, and the androgen signaling pathways in prostate cancer cells should provide fresh insight into the pathogenesis of prostate cancer that may help us to identify new pathways that can be targeted for prostate cancer treatment.

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