Linking β-catenin to Androgen Signaling Pathway

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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 co-factors. 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 PRβ, ERα, and GR. The ligand binding domain of AR and the N-terminus combined with the first 6 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.
INTRODUCTION

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). There is increasing evidence that the nuclear hormone receptors, including AR, interact with other signal transduction pathways (2). The regulation by co-factors 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 N-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 (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. 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. These data identify a new role for β-catenin in nuclear hormone receptor-mediated transcription. 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, Palo Alto, CA). 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 (OD600). pGBT9 constructs with three different AR fragments, including the partial TAD (1-333 amino acids), DBD (505-676 amino acid), 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 C-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 down-stream of GAL4 TAD in the pGAD10 vector (Clontech, Palo Alto, CA). 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 N-terminal 513 bp was generated by PCR and
cloned into the pcDNA3 vector at EcoR I 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, NY, NY). 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 DMEM supplemented with 5% fetal calf serum (HyClone, Denver, CO). An AR-positive prostate cancer cell line, LNCaP, was maintained in T-medium (GIBCO/BRL, Gaithersburg, MD) 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 (GIBCO/BRL, Gaithersburg, MD) for CV1, and LipofectAMINE 2000 (GIBCO/BRL) for TSU.pr-1 and LNCaP cells. Approximately 1.5-2x10^4 cells were plated in a 48-well plate 16 hr before transfection. 12-16 hr after transfection, the cells were washed and fed medium containing 5% charcoal-stripped fetal calf serum (HyClone, Denver, CO) in the presence or absence of steroid
hormones. Cells were incubated for another 24 hr and luciferase activity was measured as relative light units (RLU) (21). The RLU 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 RLU/β-galactosidase (±SD).

**In vitro binding assay:** GST-β-catenin fusion proteins were constructed in the pGEX-4T-1 vector (Amersham, Arlington Heights, IL). Expression and purification of GST fusion proteins were performed according to the manufacturer's instructions. Full-length human AR proteins were generated and 35S-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 35S-labeled proteins at 4°C for 2 hr in the lysis buffer as described above. Beads were carefully washed 3 times with washing buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40). GST fusion proteins were then eluted by incubating with buffer containing 10 mM glutathione and 50mM Tris-HCl, pH 8.0 for 10 min at room temperature. The bound proteins were analyzed by SDS-PAGE followed by autoradiography.

**Immunofluorescence:** 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 (Gibco/BRL, Rockville, MD). After 2 hr, transfected cells were fed with fresh medium plus/minus 10 nM DHT, incubated for 4 hr and then fixed for 10 minutes with 3% paraformaldehyde in PBS and washed with 0.1% NP-40/PBS buffer. Non-specific sites were blocked with 5% skim-milk powder in PBS for 30 minutes. The cells were then incubated with either anti-Flag monoclonal or anti-AR polyclonal antibody for 1 hr at room temperature. Cells were washed three times followed by incubation with FITC-conjugated anti-mouse or Rhodamine-conjugated anti-rabbit secondary antibody (Santa Cruz Biotech). 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, St. Louis, MO) 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 2x10^7 transformants, 73 grew under selective conditions and showed increased adenine and β-gal production in medium containing 100 nM dihydrotestosterone (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 co-transformed 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. 1A). 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 approximately 97-fold induction with pGBT9-AR-LBD in the presence of DHT (Fig. 1B). 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 unique NH2 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. 2A). As shown in Figure
2B, deletion of the C-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 N-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 N-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. 3A). The deletion constructs containing the N-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. 3A). 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 is 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. 3B). 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 & 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 Figure 4A, 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, co-immunoprecipitation 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 truncated 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). Overexpression of β-
catenin plasmids 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. 4D, 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 co-transfected with AR compared to 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 co-activator 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 co-transformed 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, SRC1, only 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 (HREs) (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.

**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 mediated 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. Co-transfection of the wild type of β-catenin expression construct increased AR activity to nearly 10-fold above baseline (Fig. 6A). Expression of the β-catenin mutant lacking the armadillo repeat 12 still showed 6 to 7-fold induction, whereas the mutants lacking repeat 6 showed no enhancement on AR-mediated transcription (Fig. 6A). The data indicates 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 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. 6B). 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 co-activator 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, were 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. 6C, 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 polyclonal 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. 7A). 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. 7B). 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. 7E). 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 Figure 7C, enhancement of ligand dependent AR activation in TSU.pr-1/Neo cells was specifically repressed by co-transfection with the β-catenin antisense construct. This was correlated with a decreased level of β-catenin protein in the cells (Fig. 7D). 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 Figure 7F, unlike the results that we observed in Figure 7E, 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 nuclear 
hormone receptors can be modulated by co-activators and co-repressors (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 suggests 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 alpha 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 N-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 alpha-helix which binds to a hydrophobic cleft in the AF2 domain 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 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 (RAR) 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.
FOOTNOTES:

1. Abbreviations used in this paper 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; AF2, activation domain 2; 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.

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FIGURE LEGENDS:

**Figure 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 co-transformed 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.

**Figure 2.** Delineation of the domain in β-catenin that mediates the interaction with AR-LBD in the yeast-two-hybrid system. (A) Both the N- and C-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 co-transformed into yeast strain PJ69 with AR-LBD. Specific interactions between the two fusion proteins were measured by appearance of colonies on SD-Ade-Leu-Trp plates and a liquid β-gal assay in the presence or absence of 100 nM DHT. Liquid β-gal assay is presented as the mean ± S.D. of three independent colonies.

**Figure 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 C-terminal truncations (A) and internal deletions (B) in pGAD10 were made and co-transformed 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 Figure 2.

**Figure 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 Figure 3B were
constructed. The conjugated GST fusion proteins were incubated with in vitro expressed and 35S-labeled full-length AR in the presence or absence of 10 nM DHT for 2 hours at 4°C, and then eluted by 10mM GSH in 50mM 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 FITC-conjugated secondary antibody (green).

**Figure 5.** β-catenin specifically interacts with AR. (A) The full-length β-catenin or SRC-1 in pGAD10 was co-transformed 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 (E2), progesterone, or Vitamin D3, respectively. The plates were incubated at 30°C for five 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 60ng of pcDNA3 vector or pcDNA3-Flag-β-catenin. Cells were incubated with or without 10 nM DHT, dexamethasone (DEX) or progesterone, respectively. Cell lysates were measured for luciferase and β-gal activities.

**Figure 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 hours. Cell lysates were prepared for
assessment of luciferase and β-gal activities (as controls of transfection efficiency). (B) Similar to A, except that a 2xARE-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 hours. Cell lysates were measured for luciferase and β-gal activities. The data represent the mean ± S.D. of three independent samples.

Figure 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+SD. (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 reporter 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 pSV-β-gal, 100 ng 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.
### A.

<table>
<thead>
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<tr>
<td>559-624</td>
<td>DBD</td>
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<td>624-676</td>
<td>HR</td>
</tr>
<tr>
<td>676-919</td>
<td>LBD</td>
</tr>
</tbody>
</table>

- **AR**
- **AR-pTAD (1-330)**
- **AR-DBD (505-676)**
- **AR-LBD (629-919)**

### B.

<table>
<thead>
<tr>
<th>Bait</th>
<th>Prey</th>
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<tr>
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<tr>
<td>pGBT9/ARLBD</td>
<td>pGAD10</td>
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</table>
Fig. 2

A. 

\( \beta \)-Catenin

\( \beta \)-cat-F

\( \beta \)-cat-t671

\( \beta \)-cat-t423

\( \beta \)-cat-t134-671

\( \beta \)-cat-t671-781

B. 

Relative Gal Activity

- With DHT
- Without DHT

pGAD10-

pGBT9/AR-LBD
A. β-Catenin

<table>
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B. Relative β-Gal Activity

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<td>β-cat-R12</td>
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Fig. 3
Fig. 4

A. 

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<td>Δ</td>
<td>Δ</td>
</tr>
<tr>
<td>R12</td>
<td>Δ</td>
<td>Δ</td>
</tr>
</tbody>
</table>

DHT-       DHT +

35S-AR

B. 

<table>
<thead>
<tr>
<th>WT</th>
<th>R6</th>
<th>R7</th>
<th>R12</th>
</tr>
</thead>
</table>


C.

<table>
<thead>
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<th>DHT</th>
<th>AR+β-catenin (F)</th>
<th>AR+β-catenin (△R6)</th>
<th>AR+β-catenin (△R12)</th>
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</thead>
<tbody>
<tr>
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<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Flag Ab

AR Ab

Flag Ab + AR Ab

1 2 3 4 5 6
Fig. 5

A.

Relative \(\beta\)-Gal Activities

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<th>Construct</th>
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<th>With ligand</th>
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<tr>
<td>pGAD10-(\beta)-Cat SRC1</td>
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<td>pGBT9-(\beta)-Cat SRC1 AR-LBD</td>
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<td>pGBT9-(\beta)-Cat SRC1 ER-LBD</td>
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<td>pGBT9-(\beta)-Cat SRC1 PR-LBD</td>
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<td>pGBT9-(\beta)-Cat SRC1 VDR-LBD</td>
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</table>

B.

Relative Luciferase Activity (luc/\(-\)gal)

<table>
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<tr>
<th>Constructs</th>
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<th>With ligand</th>
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<tr>
<td>MMTV-Luc (\beta)-cat-F</td>
<td></td>
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<tr>
<td>hSV-hAR</td>
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<td>hSV-hGR</td>
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<tr>
<td>hSV-hPR</td>
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</tr>
</tbody>
</table>
A. CV1 cells

- MMTV-LUC
- pSV-hAR
- pcDNA3-Flag
- Without DHT
- With DHT

B. CV1 cells

- 2XARE-LUC
- pSV-hAR
- Without DHT
- With DHT

C. LNCaP cells

- PSA7Kb-LUC
- 50 ng
- 100 ng
- 100 ng
- Without DHT
- With DHT

Fold Induction

Relative Luciferase Activity (Luc/gal)
Fig. 7

A.

TSU.pr-1/ E-cad  TSU.pr-1/ Neo

E-cadherin Antibody

β-catenin Antibody
Fig. 7

B. Relative Luciferase Activity (Luc/gal) without DHT

C. Relative Luciferase Activity (Luc/gal) with DHT

D. β-catenin

E. Relative Luciferase Activity (Luc/gal) without DHT

F. Relative Luciferase Activity (Luc/gal) with DHT