Phosphatidylinositol 3-kinase/Akt Stimulates Androgen Pathway Through GSK3β Inhibition And Nuclear β-catenin Accumulation

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Running title: β-catenin mediates the interaction between PI3K/Akt and AR.

Key words: androgen receptor, Akt, β-catenin, GSK3β, PI3 kinase, PTEN, prostate cancer.

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SUMMARY

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 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 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 between the PI3K, wnt, and androgen pathways, and provide fresh insights into the mechanisms of prostate tumor development and progression.
INTRODUCTION

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). 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 which participate in multiple cellular processes, including cell growth, transformation, differentiation, and survival (8). An oncprotein, 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). Activation of Akt results in the phosphorylation of a number of downstream substrates, such as glycogen synthase kinase (GSK3), Bad, caspase9, 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 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 (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 (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, 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 have 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 mechanisms have been suggested for this crosstalk, 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 crosstalk between the androgen and PI3K/Akt pathways is mediated through 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 (GIBCO/BRL, Gaithersburg, MD) with 5% fetal calf serum. Transient transfections were carried out using LipofectAMINE 2000 (GIBCO/BRL) as described previously (23). In the experiments with the PI3K inhibitor, LY294002 (Alexis, San Diego, CA), cells were usually cultured in T-medium for 16 hr, and then were treated with different concentrations of the inhibitor in Me2SO or vehicle only for 20 min to 2 hr. For androgen induction experiments, cells were grown in T-medium with charcoal-stripped fetal calf serum (HyClone, Denver, CO) for 14 hr and treated with 10 nM DHT in ethanol and different concentrations of LY294002 for 4 hr.

**Northern Blot analysis:** Total RNAs were isolated from LNCaP cells treated with LY294002 for 4 hr 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, Arlington Heights, IL) by capillary blotting in 20X SSC, and hybridized with a DNA fragment (amino acids: 1-261) derived from the human PSA gene. 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 PBS and were resuspended in RIPA buffer (1% NP-40, 0.1% SDS, 50mM NaF, 0.2mM Na3VO4, 0.5mM DTT, 150mM NaCl, 2mM EDTA, 10mM sodium phosphate buffer pH 7.2). Nuclear extracts were prepared from LNCaP cells, essentially according to the method of...
Dignam et al with minor modifications (31). Briefly, the cells were washed with PBS and mechanically disrupted by scraping into homogenization buffer A (10mM Hepes pH 7.9, 10mM KCl, 1.5mM MgCl2, 0.5mM DTT, and 0.5mM PMSF) 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 20mM Hepes pH 7.9, 420mM NaCl, 1.5mM MgCl2, 0.2mM EDTA, 0.5mM DTT, 0.5mM PMSF, and 25% glycerol, and then homogenized with 10 strokes. The lysate was incubated on ice for 30min 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, 150mM NaCl, 50mM Tris-HCl pH 7.5, 10mM MgCl2). The lysates were centrifuged at 13,000 rpm for 10 min, and 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, CA), Phospho Akt (Ser 473) (Cat # 9271, Cell Signaling Tech., Beverly, MA), Phospho GSK3α/β (Ser21/9) (Cat # 9331, Cell Signaling Tech.), AR ( Cat # sc-816, Santa Cruz Inc., Santa Cruz, CA), Sin3A (Cat # sc-996, Santa Cruz Inc.), tubulin (Cat # MS-581-P, Neomarker, Fremont, CA), β-catenin (Cat # C19220, Transduction Labs, Lexington, KY), and GSK-3β (Cat # G22320, Transduction Labs). Proteins were detected using the ECL kit (Amersham, Arlington Heights, IL). The nuclear fractions were analyzed by SDS-PAGE. Equal loading of the nuclear proteins was ascertained by reversible staining with the Ponceau S solution (Cat #: P7767, Sigma, St. Louis MO).
**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). 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 2 or 3 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 pcDNA 3 vector (Invitrogen, San Diego, CA). All constructs were sequenced from both ends of the inserts to confirm that no extraneous mutations were introduced by PCR.

**Luciferase and β-gal assay:** Luciferase activity was measured in relative light units (RLU) as previously described (30). 50 µl of cell lysate was used for luciferase assays. The light output is measured after a 5 sec delay following injection of 50 µl luciferase buffer and 50 µl luciferin by the dual injector luminometer, according to manufacturer’s instruction (Analytical Luminesence Lab., San Diego, CA). The RLU from individual transfections were normalized by 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 mean luciferase/β-galactosidase (+SD) 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 expression of the endogenous prostate specific antigen (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 approximately four-fold in LNCaP cells over that found in cells not treated with DHT (Fig. 1A). At concentrations of LY294002 from 25-100 μM, the expression of PSA was significantly reduced. An approximately 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 (Figure 1A). Low concentrations (5 μM) of LY294002 induced only a slight reduction of PSA expression during a 4 hour treatment, but showed a significant reduction of PSA expression after 16 hours (data not shown). To ensure that this repression was not due to 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 (Figure 1B). 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 due to inhibition of Akt, we evaluated Akt activation using a phosphorylation-specific antibody for Ser473. As shown in Figure 2A, 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.

Since 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. 2B). 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 hours (data not shown). Taken together, the results demonstrate that 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 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 due to decreased degradation (17,35). To evaluate 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 Figure 3A, there was no significant change in the amount of total β-catenin protein in the treated compared to the untreated cells. However, there was a 2- to 3-fold reduction in nuclear β-catenin in the cells treated with LY294002 (Figures 3A & 3B). In contrast, the controls, total nuclear protein and the transcriptional repressor, Sin3A, showed no change (Figure 3A). To confirm these findings, we examined the level of free cytosolic β-catenin protein in LNCaP cells
treated with LY294002 (36). As shown in Figure 3C, after LY294002 treatment, free β-catenin in the cytosolic compartment (Digi) was significantly reduced while 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, overexpression of AR induces about a 10-fold induction of the PSA promoters. Co-transfection with the wild type β-catenin expression vector augments AR activity to nearly 20-fold above baseline (Fig. 4A). Addition of LY294002 to the cells results in a large reduction in AR activity. At 5 μM LY294002, AR activity was reduced by approximately 60%. Co-expression 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 (Figure 4A). These data directly demonstrate that repression of AR activity by LY294002 is mediated through down-regulation of PI3K and the subsequent inactivation of Akt activity.

Next, we performed the transient transfection experiments using either wild-type or β-catenin mutants containing a point mutation within the NH2 terminal GSK3β binding site. Since 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 Figure 4B, an approximately 40% reduction in expression was induced by 5 μM LY294002 in the cells that were co-transfected with wild type β-catenin, but not in the cells co-transfected with the β-catenin mutants. As mentioned above, since the β-catenin mutants used in these experiments are impervious to the effects of the destructive complex due 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 activation of the downstream target, Akt (24). Mutations in the PTEN gene were found in prostate cancer tissues and cell lines (25). In a previous report, Li and his colleagues (28) showed that 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 inhibition of PI3K/Akt signaling represses 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 Figure 5A, in the absence of PTEN vector, both the wild type and β-catenin mutants augment AR-mediated transcription about 1.5-fold using a 7Kb 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 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, 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 Figure 5B, both the phosphorylation of Akt and GSK3β proteins were 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 samples (Figure 5C).

Next, we examined whether 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 Figure 5D, in the presence of PTEN, 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, inhibition of GSK3β by LiCl treatment increases AR activity in the presence of wild-type β-catenin (Black bar 3), while 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 crosstalk 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).

Dysregulation of β-catenin expression and Wnt-mediated signaling are 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 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, 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). 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 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. Since the β-catenin mutants used in our experiments are impervious to degradation by the destruction complex, we conclude that 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 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 due 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 reported recently (23,46), β-catenin was 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 or/and other tumor cells.

In this study, we demonstrate that β-catenin mediates the crosstalk between PI3K/Akt and androgen pathways. Based on these results and previous studies by others, we summarize our
findings in Figure 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. Loss of expression or mutational inactivation of PTEN has been frequently observed in human tumors, which induces the suppression of apoptosis and accelerates cell cycle progression (24, 25). Additionally, mutation or aberrant expression of the destruction complex and 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 downregulating androgen induced cell growth and survival. Further study of the regulation of the interaction between 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.
Footnotes:

1 This work was supported by National Institutes of Health Grants CA70297 and CA87767, and Department of Army Prostate Cancer Grant (PC01-0690).

2 Abbreviations used in this paper are: PI3K, phosphatidylinositol 3,4,5-trisphosphate; GSK3β, glycogen synthase kinase 3β; PTEN, phosphatase and tensin homolog deleted on chromosome ten; AR, Androgen receptor; TAD, transcription activation domain; LBD, ligand binding domain; DHT, dihydrotestosterone; PSA, prostate specific antigen; ARE, androgen responsive element; β-gal, β-galactosidase.

3 We are especially grateful for the various reagents received from Drs. Jan Trapman, Richard Roth, and William Sellers. We thank Homer Abaya for administrative assistance and help in preparing this manuscript.
REFERENCES


**FIGURE LEGENDS**

**Figure 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 hr 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 OD units PSA/OD units 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.

**Figure 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 Figure 1 and the "EXPERIMENTAL PROCEDURES" section, 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.

**Figure 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 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 (OD units nuclear proteins/OD units total proteins). (C) Both cytosolic fraction (Digi) and cytoskeletal fraction (RIPA) were prepared from LNCaP cells as described in the "EXPERIMENTAL PROCEDURES" section, and were analyzed by Western-blotting. Both β-catenin and tubulin were detected using specific antibodies.
**Figure 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-gal, and 50 ng of wild type pcDNA3-Flag-β-catenin, in the presence or absence of 50 ng of an inactive mutant or a dominantly active mutant of Akt. The cells were incubated in T-medium with 5% charcoal-stripped FCS for 12 hours, and then were treated with different concentrations of LY294002 in the presence or absence of 10 nM DHT for 18 hours. Cell lysates were measured for luciferase and β-gal activities. The data represent the mean ± S.D. of three independent samples. (B) LNCaP cells were co-transfected 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.

**Figure 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-gal (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 were cotransfected with the above plasmids. Ten hours after transfection, cells were treated with 10 nM DHT or with vehicle only for 18 hours. Cell lysates were measured for luciferase and β-gal activities. The data represent the mean ± S.D. of three independent samples. (B) The PTEN expression constructs were transfected into LNCaP cells. Nuclear extracts and whole cell lysates were prepared from the cells 30 hr after transfection, and were analyzed by Western-blotting. (C) Transient transfections were performed with the plasmids as labeled in the figure. After 10 hr transfection, 10 nM DHT and 50 mM LiCl were added to the cells. Whole cell lysates were prepared after another 18 hr of incubation and were used to measure luciferase and β-gal activities.
**Figure 6.** β-catenin acts as a mediator in the crosstalk between PI3K and androgen signaling. A model summarizing PI3K/Akt signaling in prostate cells and the pathways for PTEN and the PI3K inhibitor, LY294002, in regulation of AR activity.
Figure 1

A. 10nM DHT - + + + +
LY294002 - - 25 50 100 μM

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<th>PSA</th>
<th>β-actin</th>
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Relative PSA Density (PSA/β-actin)

B. 10nM DHT - + + + +
LY294002 - - 25 50 100 μM

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**A.**
- Phosphorylated AKT (Ser 473)
- Total AKT Proteins

**B.**
- Phosphorylated GSK3β
- Total GSK3β Proteins
Figure 3

A. 

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<th>Condition</th>
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<th>Total cellular β-catenin</th>
<th>Total nuclear proteins</th>
<th>Nuclear Sin3A</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 nM DHT</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LY294002</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

B. 

Relative (Nuclear/Total) β-catenin density

10 nM DHT  | - | + | + | + |
LY294002   | - | - | 50 μM | 20' 120' |

C. 

10 nM DHT  | + | + | + | + |
LY294002   | + | + | 50 μM | 20' 120' |

Digi, RIPA, β-catenin, Tubulin
Figure 4

A.

Relative Luciferase Units (Luc/βgal)

Without DHT
With DHT

PSA 7Kb-Luc + + + + + + +
peDNA3-AR + + + + + + +
β-catenin-Wt - + + + + + +
LY294002 - 5 μM - 5 μM 5 μM 5 μM

B.

Relative Luciferase Units (Luc/βgal)

10 nM DHT - + + + + + + +
PSA 7Kb-Luc + + + + + + + +
peDNA3-AR + + + + + + + +
LY294002 - - - 1 5 - 1 5 - 1 5 μM
β-Cat Wt
β- Cat S33F
β- Cat S37A
Figure 5

A. Relative Luciferase Units (Luc/gal) with DHT

B. Phosphorylated AKT (Ser 473)

C. Phosphorylated GSK3

D. Relative Luciferase Units (Luc/gal) without DHT
PI3K

PTEN
LY294002

Akt/PKB-

GSK3β-
(Inactivation)

β-catenin

AR

Proliferation
Survival

Figure 6
Phosphatidylinositol 3-kinase/Akt stimulates androgen pathway through GSK3β inhibition and nuclear β-catenin accumulation
Manju Sharma, William W. Chuang and Zijie Sun

J. Biol. Chem. published online June 12, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M201919200

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