

Glycogen Synthase Kinase-3 β Activity Is Required for Androgen-Stimulated Gene Expression in Prostate Cancer

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Despite the specificity inferred by its name, glycogen synthase kinase (GSK)-3 β is an important kinase with a plethora of significant cellular targets, including cytoskeletal proteins and transcription factors, and its activity is regulated by phosphorylation on tyrosine/serine residues. As part of our efforts to dissect the molecular basis responsible for androgen-independent progression of prostate cancer, we investigated the role of GSK-3 β in androgen-stimulated gene expression in human prostate cancer cells. Pretreatment of prostate cancer cells harboring wild-type or mutant androgen receptor with the GSK-3 β inhibitors, lithium chloride (LiCl), RO318220, or GF109203X, inhibited R1881-stimulated androgen-responsive reporter activity in a dose- and time-dependent manner. In addition, the expression of two endogenous androgen-stimulated gene products, prostate-specific antigen and matrix metalloproteinase-2, was suppressed by the GSK-3 β inhibitors in those cells. Most importantly, knocking down GSK-3 β ex-

pression via a small interference RNA-mediated gene silencing approach also reduced R1881-stimulated gene expression, demonstrating the specificity of GSK-3 β involvement. Moreover, R1881 treatment of the cells increased phosphorylation status of GSK-3 β on tyrosine residue Y²¹⁶ but not on serine residue S⁹. Pretreatment of the cells with phosphatidylinositol 3-kinase inhibitor LY294002 or wortmannin, which blocks androgen action in cells, abolished R1881-induced GSK-3 β Y²¹⁶ phosphorylation. However, the phosphatidylinositol 3-kinase or GSK-3 β inhibitors did not block R1881-induced nuclear translocation of androgen receptor. Finally, knocking down the expression of Akt or β -catenin, the two GSK-3 β -related signaling molecules, via siRNA-mediated gene silencing did not significantly affect R1881-stimulated gene expression. These findings suggest that GSK-3 β activity is required for androgen-stimulated gene expression in prostate cancer cells. (*Endocrinology* 145: 2941–2949, 2004)

PROSTATE CANCER IS the second leading cause of cancer death among American men (1). Whereas digital rectal exams and early prostate-specific antigen (PSA) screening have led to earlier detection and diagnosis, the number of new cases continues to rise and presents a major worldwide health threat (1). Medical treatment for advanced prostate cancer has mainly relied on androgen ablation. However, there has been a growing appreciation that most patients treated by androgen ablation ultimately relapse to more aggressive androgen-independent prostate cancer (reviewed in Ref. 2). The mechanism(s) involved in androgen-independent progression of prostate cancer is (are) not fully known.

Originally, glycogen synthase kinase (GSK)-3 was named as a kinase for glycogen synthase (3). Currently studies have demonstrated that GSK-3 is a multifunctional kinase involved in cellular metabolism, signaling transduction, growth, differentiation, and cell fate determination (reviewed in Ref. 4, 5). Two GSK-3 isoforms (α and β) were

cloned in mammals (6). GSK-3 β has been shown to phosphorylate numerous substrates, including several transcription factors such as c-jun, c-myc, cAMP response element binding protein, and heat shock factor-1, cytoskeletal proteins such as the microtubule-associated protein τ , and the multifunctional protein β -catenin (reviewed in Ref. 5) as well as the glucocorticoid receptor (7). GSK-3 β can be both activated and inhibited, in which activation is usually associated with phosphorylation of Y²¹⁶ (8), or inhibited by phosphorylation of S⁹ (9). Transient increases in intracellular calcium result in GSK-3 β activation (10). Activation of the Wnt (11) or PI3K-Akt (12, 13) pathways induces GSK-3 β inhibition (also see Refs. 4, 5 for detailed review).

It is believed that the androgen receptor (AR) resides primarily in the cytoplasm and associates with heat shock proteins in an inactive state before androgen binding (14, 15). Binding of androgen to AR triggers a conformational change, subsequent nuclear translocation, and eventually binding to specific promoter response elements, leading to transcription initiation or repression of its target genes (16–18). However, the exact mechanisms involved in this cascade are not fully understood. Previous reports have shown that the phosphatidylinositol 3-kinase (PI3K) pathway is involved in androgen-stimulated gene expression (19–22). To understand the mechanisms involved in androgen-independent progression of prostate cancer, it is important first to determine the regulatory kinases involved in androgen-stimulated gene expression. In this report, we screened several kinase-specific

Abbreviations: AR, Androgen receptor; cFBS, charcoal-stripped fetal bovine serum; CMV, cytomegalovirus; GSK, glycogen synthase kinase; LiCl, lithium chloride; MMP, matrix metalloproteinase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; pro-MMP-2, latent form of MMP-2; PSA, prostate-specific antigen; PTEN, phosphatase and tensin homolog deleted from chromosome 10; Pyk2, protein tyrosine kinase 2; SEAP, secreted alkaline phosphatase; si, small interference.

Endocrinology is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

inhibitors for their effect on androgen action in human prostate cancer cells. We determined that GSK-3 β activity is required for androgen-stimulated gene expression but not for AR nuclear translocation.

Materials and Methods

Cell culture and reagents

The human prostate cancer LNCaP, LAPC-4, PC-3, and DU145 cells were grown as described previously (22, 23). The androgen-insensitive cell line C4-2 was obtained from UroCor Inc. (Oklahoma City, OK), and 22Rv1 cell line was purchased from American Type Culture Collection (Manassas, VA). The inhibitors LY294002, rapamycin, PD98059, RO318220, GF109203X, GO6976, SB203580, H89, and PP2 were purchased from Calbiochem (San Diego, CA). R1881 was obtained from Perkin-Elmer (Wellesley, MA). Bicalutamide was a gift from AstraZeneca (London, UK). Where indicated, the inhibitor was added from a 1000-fold concentrated stock in the solvent, dimethylsulfoxide, or ethanol. Control cultures received similar amounts of the solvent only. Final concentrations of the solvent did not exceed 0.1%. Type-1 rat-tail collagen and wortmannin were purchased from Sigma (St. Louis, MO). The antibodies against phospho-specific GSK-3 β S⁹ and Y²¹⁶ and phospho-specific Akt S⁴⁷³ were purchased from Cell Signaling (Beverly, MA). The antibodies against GSK-3 α/β , AR, PSA, β -catenin, actin, and secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Charcoal-stripped fetal bovine serum (cFBS) was obtained from Atlanta Biologicals (Norcross, GA). Fluorescein isothiocyanate- or Alexa Fluor 568-conjugated goat antimouse IgG was purchased from Molecular Probes (Eugene, OR).

Immunoblot analysis

Cell lysates were prepared and Western blot was conducted as described previously (22, 23). Briefly, proteins were resolved in sodium dodecyl sulfate-polyacrylamide gels, transferred to polyvinylidene difluoride membrane, and incubated with antibodies at 4 C overnight with gentle agitation. Immunoblots were developed using horseradish peroxidase-conjugated goat antimouse or goat antirabbit IgG, followed by chemiluminescent detection (SuperSignal West Dura Substrate kit, Pierce Biotechnology Inc., Rockford, IL). The density of protein bands were quantitatively measured with a densitometer.

Immunofluorescence

Cells were cultured on poly-D-lysine-coated glass coverslips and subjected to treatments as indicated. The cells were washed twice with PBS and then fixed and permeabilized in ice-cold methanol-acetone (1:1) for 10 min at -20 C. The coverslips were washed twice with PBS and incubated overnight at 4 C with 10 μ g/ml of mouse monoclonal anti-AR antibody diluted in PBS containing 2% BSA. The coverslips were washed three times with PBS and incubated for 1 h at room temperature with 10 μ g/ml fluorescein isothiocyanate- or Alexa Fluor 568-conjugated antimouse antibody diluted in PBS containing 2% BSA. The coverslips were then washed with deionized water and mounted onto glass slides using VECTASHIELD mounting medium with 4',6'-diamino-2-phenylindole (Vector Laboratories, Burlingame, CA), and examined by a fluorescence microscope (Nikon, Tokyo, Japan) set at \times 400 magnification.

Small interference (si)RNA synthesis and transfection

Sequence information regarding human GSK-3 β gene (GenBank accession no. NM_002093) was extracted from the NCBI Entrez nucleotide database. Several siRNAs with different targeting sequences were selected, and each targeting segment was searched with NCBI Blast to confirm specificity only to the targeted gene. The siRNAs were synthesized as described previously (23) using a transcription-based method with the *Silencer* siRNA construction kit (Ambion, Austin, TX) according to the manufacturer's instructions. The 29-mer sense and antisense DNA oligonucleotide templates (21 nucleotides specific to the targets and eight nucleotides specific to T7 promoter primer sequence 5'-CCT-GTCTC-3') were synthesized by Integrated DNA Technologies (Coral-

ville, IA). The quality of the synthesized siRNA was estimated by agarose gel analysis and found to be very clean (data not shown). In addition, a pooled siRNA mixture containing four siRNA duplexes against human GSK-3 β , *akt*, β -catenin, or AR gene was purchased from Dharmacon (Lafayette, CO). The siRNA transfection was carried out with Oligofectamine transfection agent as described previously (23) obtained from Invitrogen (Carlsbad, CA) according to the manufacturer's protocol. Three days after transfection with the siRNA duplexes, cells were subjected to a reporter gene assay as described below.

Reporter gene assay and gelatinolytic zymography

The luciferase reporter controlled by the human matrix metalloproteinase (MMP)-2 promoter (pMMP2-LUC) and the pCMV-secreted alkaline phosphatase (SEAP) reporter were described previously (22). Two androgen-responsive SEAP reporter constructs, pSH1.PSA-SEAP (PSA-SEAP) and pSH1.ARR2PB-SEAP (PB-SEAP) were described elsewhere (24). Luciferase and SEAP assays were performed as described (22–24). Briefly, cells were plated in six-well tissue culture plates and transfected the following day with the reporter constructs by using the Cytofectene reagent (Bio-Rad Laboratories, Hercules, CA) in serum-free media overnight. Cells were then treated as indicated for 24 h and culture supernatants were collected for SEAP activity. Cell lysates were assayed for luciferase activity. Unconcentrated media from the cell cultures were analyzed for MMP-2 gelatinolytic activities by gelatin zymography as described previously (22).

Statistical analysis

All experiments were repeated two or three times. Western blot results are presented from a representative experiment. The mean and SD from two experiments for reporter gene assay are shown. The significant differences between groups were analyzed using the SPSS computer software (SPSS Inc., Chicago, IL).

Results

GSK-3 inhibitor blocks androgen-stimulated gene expression

To gain insights into the signaling molecules involved in androgen-stimulated gene expression, we screened several commonly used kinase-specific inhibitors in a cell-based androgen-responsive gene reporter assay. These inhibitors include LiCl for GSK-3 β , PD98059 for MAPK kinase-1, LY294002 for PI3K, rapamycin for mammalian target of rapamycin, H89 for protein kinase A, SB203580 for p38 MAPK, and PP2 for Src kinase. A previous described reporter construct PSA-SEAP (24) was used in this study. After transfection with the reporter constructs and serum starvation for 24 h, LNCaP cells were pretreated for 45 min with the inhibitors at the indicated concentrations in culture media containing 2% charcoal-stripped serum. After the addition of synthetic androgen R1881, SEAP activity was measured 24 h later. As shown in Fig. 1A, LiCl completely abolished androgen-stimulated PSA-SEAP reporter activity. The same is true for the PI3K inhibitor LY294002, which is consistent with previous reports in terms of androgen-responsive gene expression (19–22). In contrast, LiCl or LY294002 had no effect on a universal promoter CMV-mediated gene expression (CMV-SEAP) in LNCaP cells (data not shown), ruling out an interference of the inhibitors with the basic transcription/translation machinery. Other inhibitors for MAPK kinase-1 (PD98059), mammalian target of rapamycin (rapamycin), protein kinase A (H89), p38/MAPK (SB203580), and Src kinase (PP2) had no effect on androgen-stimulated PSA-SEAP activity.

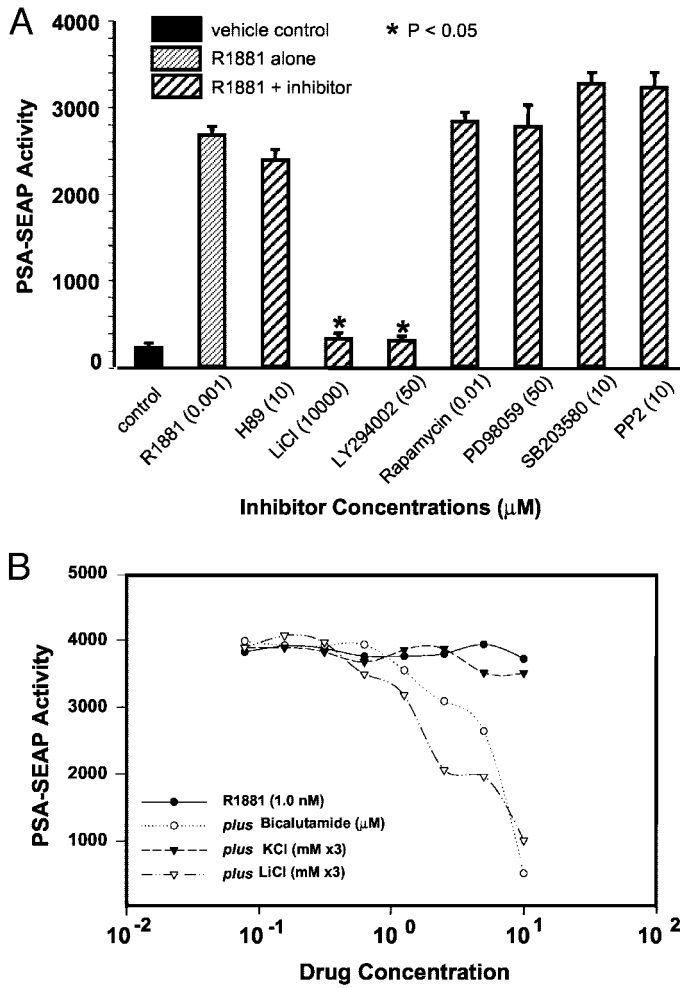


FIG. 1. Involvement of GSK-3 β activity in androgen-stimulated reporter activity. A, After transfection with PSA-SEAP reporter, LNCaP cells were serum starved for 24 h and then pretreated with various kinase inhibitors for 45 min. Thereafter, cells were treated with R1881 (1.0 nM) or vehicle control in 5% cFBS-containing medium and SEAP activity was measured 24 h later. Data represent three experiments. B, After pretreatment with half-log increasing doses of the drugs indicated for 45 min, LAPC-4 cells were treated with R1881 in 5% cFBS-containing medium and SEAP activity was measured 24 h later. The concentration of potassium and lithium chloride (millimoles) was three times higher than the value as shown on the x-axes.

LNCaP cell harbors a mutant AR (25) and a mutant phosphatase and tensin homolog deleted from chromosome 10 (PTEN) (26), and a mutant AR may have an altered transactivation capacity. PTEN is also involved in androgen-stimulated gene expression (20). Therefore, we examined LiCl's effect on a newly developed human prostate cancer LAPC-4 cell line, which has a wild-type AR and PTEN, and expresses PSA (27). Cells were serum starved for 24 h after transfection with PSA-SEAP constructs. After pretreatment with different doses of the androgen antagonist bicalutamide, LiCl, and a salt control KCl for 45 min, cells were treated with R1881 in 2% cFBS-containing medium, and SEAP activity was measured 24 h later. As shown in Fig. 1B, LiCl inhibited androgen-stimulated PSA promoter activity in a dose-dependent manner, which is similar to the effect of the androgen antagonist bicalutamide. As expected, the control salt KCl had

not effect on androgen-stimulated gene expression at similar concentrations, compared with LiCl pretreatment, indicating the Li⁺ specificity.

To understand the prevalence of the involvement of GSK-3 β in androgen-responsive gene expression, we next investigated whether GSK-3 β inhibitor blocks androgen-stimulated endogenous gene expression. Recently we demonstrated that MMP-2 is regulated by androgen in prostate cancer cells (22). In addition to PSA protein expression, we also examined the secretion of the latent form of MMP-2 (pro-MMP-2) protein. Besides LiCl, we used two other GSK-3 β inhibitors, RO318220 and GF109203X (28, 29), which were originally developed as protein kinase C (PKC) inhibitors (30, 31). As a control, another PKC inhibitor GO6976 (32) was included in this experiment. Serum-starved LAPC-4 cells were pretreated for 45 min with various inhibitors before addition of R1881. PSA protein level was determined 24 h later by Western blot, and pro-MMP-2 secretion was assessed by gelatin zymography as described previously (22). As shown in Fig. 2, R1881-induced PSA expression or pro-MMP-2 secretion was dramatically suppressed by LiCl, RO318220, and GF109203X but not by GO6976. These data demonstrate that inhibition of GSK-3 β activity suppresses androgen-stimulated gene expression.

siRNA-mediated GSK-3 gene silencing suppresses androgen-stimulated gene expression

It has been shown that LiCl has multiple noncompetitive targets in addition to GSK-3 β (29), and RO318220 and GF109203X can inhibit nonclassical PKC isoforms in addition to classical isoforms (30–32). To confirm that GSK-3 β inhibition leads to disruption of androgen-stimulated gene ex-

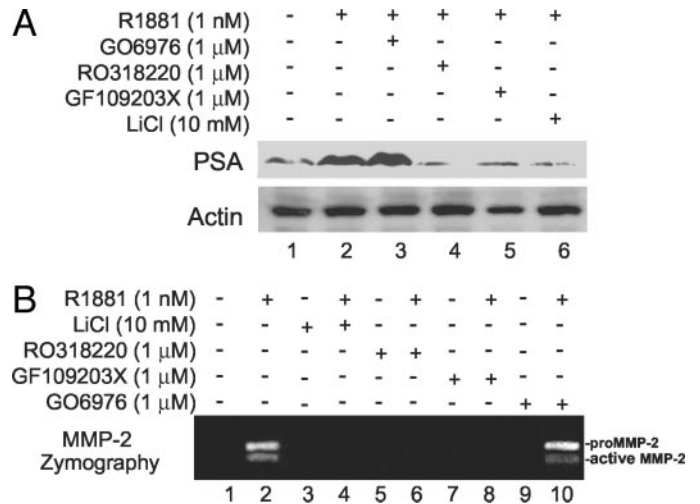


FIG. 2. Suppression of endogenous androgen target gene expression by GSK-3 β inhibitors. A, LAPC-4 cells were serum starved for 24 h and then pretreated with various kinase inhibitors for 45 min in serum-free medium. Thereafter, R1881 (1.0 nM) or vehicle control were added to the media, and PSA expression was measured 24 h later by Western blot. Actin blot served as loading control. B, After serum starvation for 24 h, LAPC-4 cells were pretreated with the kinase inhibitors at the concentration as indicated for 45 min followed by addition of R1881. Twenty-four hours later, MMP-2 secretion was measured by a gelatinolytic zymography assay as described in the text. The results were reproduced in two different experiments.

pression, we next evaluated the effect of GSK-3 β gene silencing mediated by a siRNA duplex on androgen-stimulated probasin promoter activity (pSH1.ARR2.PB-SEAP, described in Ref. 24). Two siRNA duplexes against human GSK-3 β mRNA at different regions were previously described (23). A nonspecific siRNA duplex with scrambled sequence (Ambion) was used as a negative control. After 48 h of transfection with the siRNA duplexes, LAPC-4 cells were cotransfected again with the PB-SEAP reporter for another 24 h. After R1881 addition, SEAP activity was determined 24 h later and the cellular levels of targeted GSK-3 β gene products were determined by Western blot. As shown in Fig. 3A, transfection with the no. 4 or no. 16 siRNA duplexes significantly knocked down the protein level of GSK-3 β , whereas the negative control siRNA had no effect. In parallel, the GSK-3 β siRNA duplexes caused a significant decrease of R1881-stimulated probasin promoter activity. Similar results were also obtained when LNCaP cells were used (data not shown). In addition, a pooled siRNA mixture against human GSK-3 β or AR gene was used in a separate experiment. As shown in Fig. 3B, transfection of the pooled siRNA mixture significantly reduced the expression of the targeted protein, which was similar to the effect of the no. 16 siRNA duplex. In parallel, androgen-stimulated MMP2-LUC

reporter activity was significantly reduced by the siRNAs for either GSK-3 β or AR gene. As expected, the control siRNA had no effect. These results further demonstrate that GSK-3 β is required for androgen-stimulated gene expression.

Androgen treatment increases GSK-3 tyrosine phosphorylation

Although GSK-3 β is a constitutively active enzyme, its activity is associated with the phosphorylation status of the tyrosine residue Y²¹⁶ (8) and serine residue S⁹ (9). Increased Y²¹⁶ phosphorylation usually correlates with GSK-3 β enzymatic activity, in contrast, induction of S⁹ phosphorylation will result in inactivation of the kinase (reviewed in Refs. 4, 5, and 33–36). Because GSK-3 β activity is required for androgen-stimulated gene expression, we asked whether there were any changes with regard to GSK-3 β phosphorylation status after androgen treatment. To address this issue, LNCaP cells were serum starved for 24 h and then treated with R1881 in serum-free media. GSK-3 β tyrosine phosphorylation status was examined using a phospho-specific antibody against GSK-3 β Y²¹⁶. In addition, a phospho-specific antibody against Akt S⁴⁷³ was used to determine Akt phos-

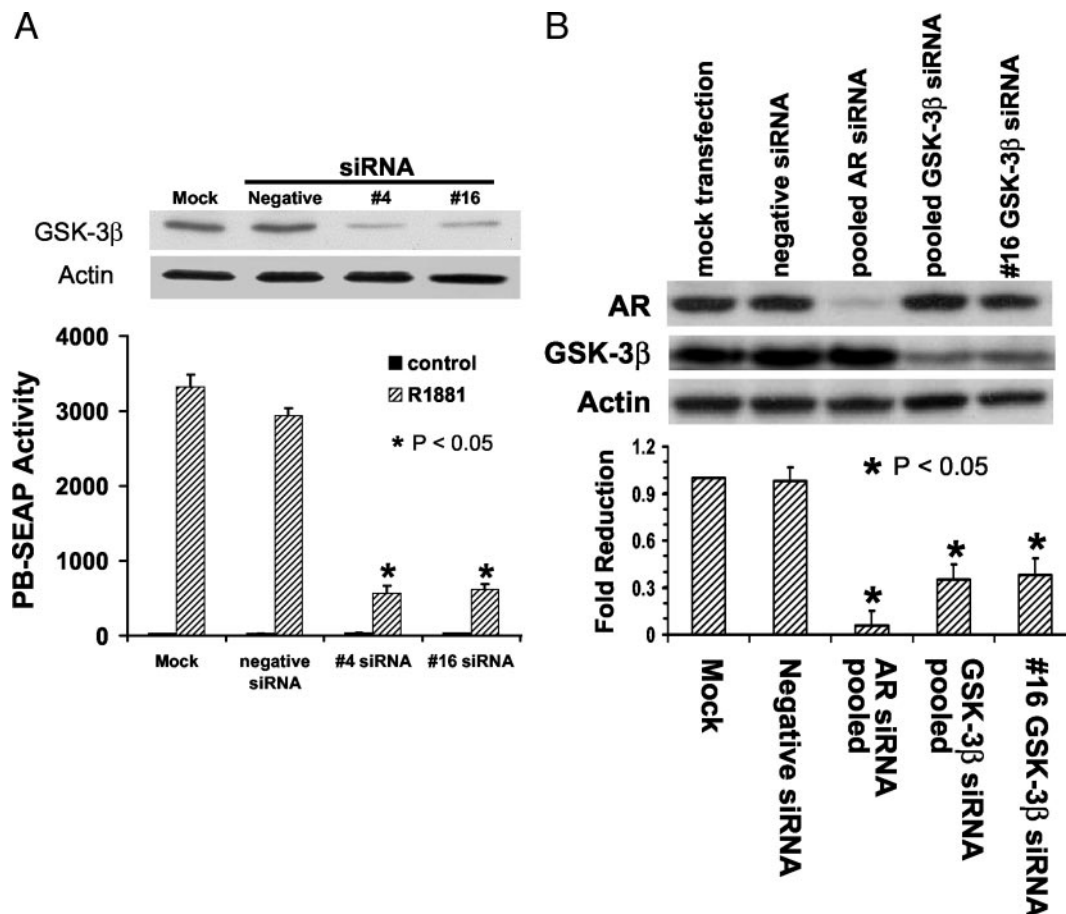


FIG. 3. Knocking down GSK-3 β expression suppresses androgen-stimulated gene expression. After transfection with a single siRNA preparation (10 nM) or a pooled siRNA mixture (100 nM) as indicated for 48 h, LAPC-4 cells were transfected again with PB-SEAP (A) or MMP2-LUC (B) reporters for 24 h and then treated with R1881 (1.0 nM) or vehicle control in serum-free medium. SEAP or luciferase activity was measured 24 h later as described in the text. The protein levels of targeted genes were determined by Western blot. Actin blot served as loading control. Data represent three experiments.

phorylation status. As shown in Fig. 4A, R1881 treatment increased GSK-3 β Y²¹⁶ phosphorylation level as early as 15 min. However, Akt S⁴⁷³ phosphorylation was not altered by R1881 stimulation, which is consistent with a previous report (21) but not with a recent report (37). To address this issue again in a longer period time, LAPC-4 cells were serum starved for 24 h and then treated with R1881 in serum-free media for 24 h after pretreatment (45 min) with various inhibitors as indicated. As shown in Fig. 4B, R1881 treatment considerably augmented Y²¹⁶ phosphorylation of GSK-3 β , but it was totally blocked by pretreatment with bicalutamide or LY294002, which is consistent with the observation of those inhibitors on R1881-stimulated gene expression. In addition, pretreatment with LY294002 resulted in a decline of the basal level of Y²¹⁶ phosphorylation, indicating that a PI3K-dependent pathway is required for androgen-induced GSK-3 β Y²¹⁶ phosphorylation. On the other hand, R1881 treatment did not cause significant alteration on the S⁹ phosphorylation level, compared with the control. Consistent with a previous report (21), LY294002 eliminated the S⁹ phosphorylation in both the control and R1881-treated cells. The relative induction of GSK-3 β Y²¹⁶ and S⁹ phosphorylation is quantitatively presented in Fig. 4C. These data suggest that androgen increases GSK-3 β Y²¹⁶ phosphorylation, which is associated with androgen-stimulated gene expression. How-

ever, LY294002-mediated alternation of GSK-3 β S⁹ phosphorylation might be a nonrelated event to androgen-stimulated gene expression.

Because androgen-independent progression is the major concern in prostate cancer management and we found that GSK-3 β activity is involved in androgen-stimulated gene expression, it is of interest to know whether there is any correlation between androgen sensitivity and GSK-3 β status. Thus, we compared the status of GSK-3 protein expression in a total of six commonly used prostate cancer cell lines including androgen-insensitive cells C4-2 and 22RV1 as well as AR-null cell lines DU145 and PC-3 in addition to the LAPC-4 and LNCaP cells mentioned above. Exponentially growing cells were harvested, and equal amount of protein from each cell preparation was used for Western blot as described above. For most of the cell lines tested, there is no significant variation with regard to GSK-3 α protein expression and phosphorylation status (Fig. 4D). However, GSK-3 β protein was found higher in two androgen-insensitive cells C4-2 and 22RV1 than that in other cell lines. Most interestingly, GSK-3 β Y²¹⁶ is highly phosphorylated in C4-2 cells, compared with others. These data suggest that GSK-3 β or its Y²¹⁶ phosphorylation might be involved in prostate cancer progression in some extent, which needs to be defined in the future.

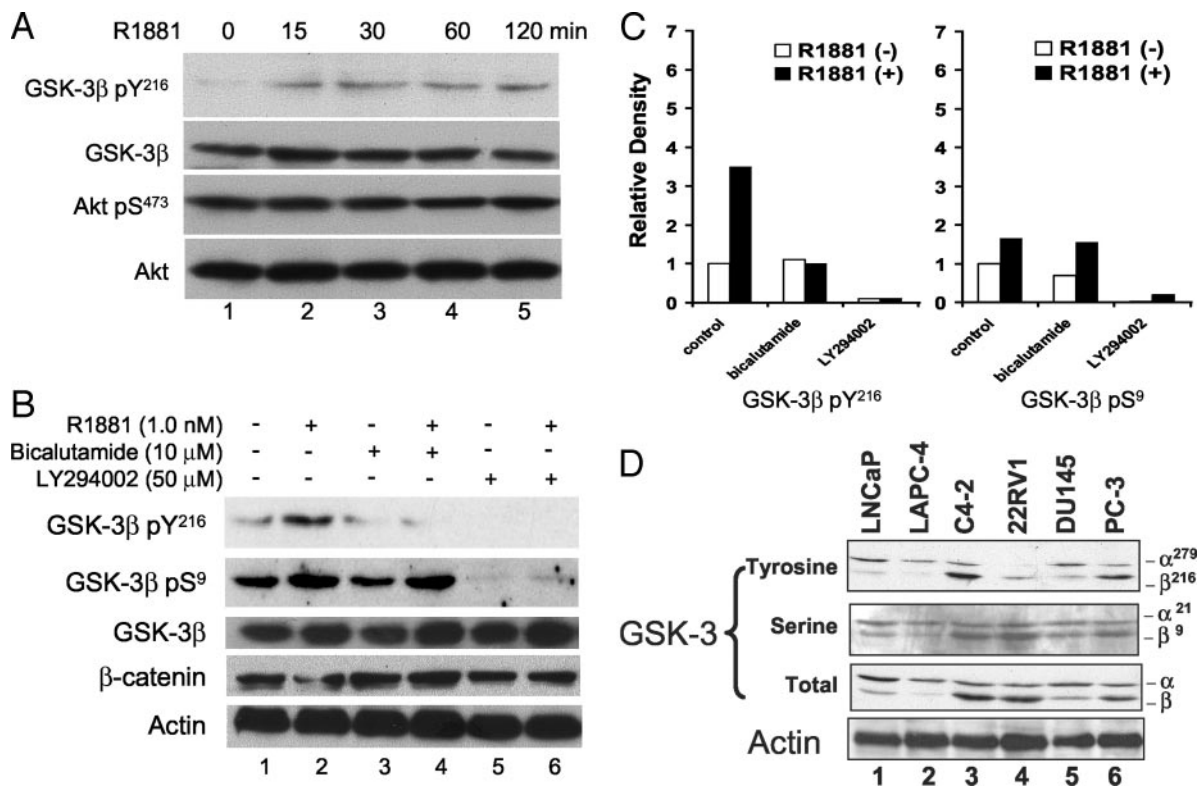


FIG. 4. A–C, R1881 treatment increases GSK-3 β phosphorylation at Y216. A, After serum starvation, LNCaP cells were left untreated or treated with R1881 (1.0 nM). At each time point as indicated, cells were harvested, and phosphorylation status of GSK-3 β or Akt was determined by Western blot using phospho-specific antibody as indicated. Immunoblot for plain GSK-3 β or Akt served as loading control. B, After serum starvation, LAPC-4 cells were pretreated with LY294002 or bicalutamide for 45 min followed by addition of R1881 for another 24 h. Phosphorylation status of GSK-3 β was determined by Western blot using phospho-specific antibodies. The expression of GSK-3 β and β -catenin were also evaluated. Actin immunoblot served as loading control. C, The quantitative data of the band density for GSK-3 β phosphorylation at Y²¹⁶ or S⁹ are shown. D, GSK-3 status in multiple prostate cancer cell lines. Exponentially growing cells were harvested, and an equal amount of cellular protein was used for Western blot as described above.

GSK-3 inhibitor does not block androgen-induced AR nuclear translocation

The AR is a member of the steroid hormone receptor super family and is translocated into the nucleus after androgen binding to mediate androgenic effects of male secondary sexual differentiation and prostate development (14–18). Because we observed that the inhibitors of GSK-3 β or PI3K blocked androgen-stimulated gene expression, we were interested in whether these inhibitors blocked androgen-induced AR nuclear translocation. To answer this question, we examined AR distribution by immunofluorescence staining after androgen stimulation in the presence or absence of the inhibitors. LAPC-4 (Fig. 5, A–D) and LNCaP (Fig. 5, E–I) cells were serum starved for 24 h, pretreated with the inhibitors as indicated for 30 min, and then stimulated with androgen for 6 h (Fig. 5). In both cell lines, AR is mainly localized in the cytoplasmic compartment before R1881 stimulation (Fig. 5E), and AR nuclear translocation was accomplished after androgen addition (Fig. 5, B and F). Interestingly, none of the inhibitors blocked androgen-induced AR nuclear translocation in both cell lines, indicating that the PI3K or GSK-3 β activity is not required for AR nuclear translocation.

The role of β -catenin or Akt in androgen-stimulated gene expression

Recently inconsistent results were reported from different groups regarding the relationship between TCF4/ β -catenin pathway and AR transactivation (21, 38–45). Because the abundance of β -catenin level is regulated by GSK-3 β -related pathways after cellular stimulation (reviewed in Ref. 46) and we found that GSK-3 β activity is required for androgen-stimulated gene expression, we were interested in whether

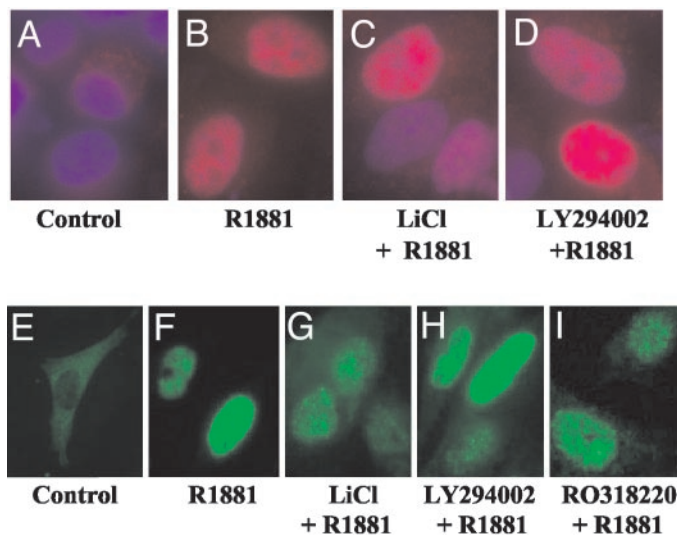


FIG. 5. Inhibition of GSK-3 β or PI3K did not block R1881-induced AR nuclear translocation. After 24 h serum starvation, LAPC-4 (A–D) or LNCaP (E–I) cells were left untreated as control or pretreated with inhibitors (LiCl 10 mM, RO318220 1.0 μ M, or LY294002 50 μ M) as indicated for 45 min. Thereafter, vehicle control or R1881 (1.0 nM) was added for an additional 16 h. Localization of androgen receptor (red in B–D, green in E–I) was carried out by immunofluorescent staining, and the nuclei were counterstained with 4',6'-diamino-2-phenylindole (blue in A–D) as described in the text.

there is any alteration regarding the protein level of β -catenin after R1881 treatment or whether the change is related to androgen-stimulated gene expression. As shown in Fig. 4B, R1881 treatment did not result in any significant alteration of β -catenin protein level, compared with the control, indicating that β -catenin might not participate in androgen-stimulated gene expression. To clarify that β -catenin is not involved in androgen-stimulated gene expression, we knocked down β -catenin expression by using a pooled siRNA duplex preparation against human β -catenin in LAPC-4 cells and tested the effect of β -catenin knockdown on R1881-stimulated PB-SEAP reporter activity. Cells were transfected with the β -catenin siRNA duplexes for 3 d in full culture media followed by a PB-SEAP reporter transfection in 2% cFBS overnight. After 24 h stimulation with R1881, SEAP activity was measured as mentioned earlier and cellular protein levels of β -catenin were determined by Western blot. As shown in Fig. 6A, transfection of the cells with the pooled siRNAs largely reduced the protein level of β -catenin in a dose-dependent manner. However, R1881-stimulated PB-SEAP reporter activity was not significantly affected in β -catenin knockdown cells, compared with the control (Fig. 6B). These data are in contrast to previous reports showing β -catenin's enhancement on AR transactivation (21, 39, 42, 44) but is in agreement with others (41, 45) under the condition of β -catenin overexpression.

As a downstream target of PI3K, Akt is rapidly activated via phosphorylation on two key sites of T³⁰⁸ and S⁴⁷³. Akt was previously reported to phosphorylate AR and subsequently

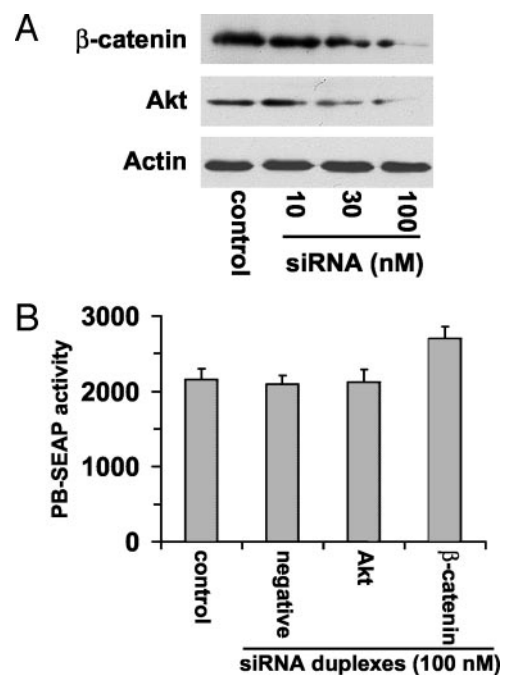


FIG. 6. Akt or β -catenin is not involved in androgen-stimulated gene expression. After transfection with a pooled siRNA mixture as indicated for 48 h, LAPC-4 cells were transfected again with the PB-SEAP reporter for 24 h and then treated with R1881 (1.0 nM) or vehicle control in serum-free medium. SEAP or luciferase activity was measured 24 h later as described in the text. The expression of targeted genes was determined by Western blot. Actin blot served as loading control. Data represent two experiments.

enhance transactivation once overexpressed in cells (47), but other groups (48) later did not confirm it using a proteomic approach. Because PI3K inhibitor LY29402 suppressed androgen-stimulated gene expression, we asked whether Akt is participating in PI3K-dependent androgen-stimulated gene expression. We knocked down Akt protein by the siRNA-mediated gene silencing approach and examined the effect of Akt knockdown on R1881-induced PB-SEAP activity. As mentioned earlier, a pooled siRNA preparation against human *akt* gene was transfected into LAPC-4 cells for 3 d followed by transfection of PB-SEAP reporter. As shown in Fig. 6A, the protein level of endogenous Akt gene was knocked down dramatically by the pooled siRNA duplexes in a dose-dependent manner. However, R1881-stimulated PB-SEAP reporter activity remained unchanged (Fig. 6B), indicating that Akt is not involved in androgen-induced gene expression, which is consistent with a previous report (20).

Discussion

The nature of androgen-stimulated AR transactivation is not clear. Our data demonstrated for the first time that GSK-3 β activity is required for androgen-stimulated gene expression. We showed that: 1) inhibition of GSK-3 β activity by its inhibitors blocks androgen-stimulated gene expression; 2) siRNA-mediated GSK-3 β gene silencing results in reduction of androgen-stimulated gene expression, confirming the specificity of GSK-3 β inhibition; and 3) androgen increases GSK-3 β Y²¹⁶ phosphorylation that is suppressed by PI3K inhibitors. These results suggest a pathway involving PI3K-dependent GSK-3 β activation in androgen-stimulated gene expression.

To determine the kinases and/or their related pathways involved in androgen-stimulated gene expression, we surveyed a group of kinase-specific inhibitors for multiple pathways in an androgen-responsive gene reporter assay. Among those inhibitors, we found that LiCl blocked androgen-stimulated PSA promoter activity. Because LiCl can inhibit

multiple enzymes in addition to GSK-3 β , we verified the involvement of GSK-3 β by two approaches. One was to use different type of GSK-3 β inhibitors, RO318220 and GF109203X; the other was to use siRNA-mediated gene silencing against GSK-3 β . Both approaches demonstrated the requirement of GSK-3 β for androgen-stimulated gene expression. Because RO318220 and GF109203X also inhibit PKC isoforms (classic and novel groups), we used another inhibitor G06976 (for classic PKCs) as control and found that G06976 had no effect on androgen-stimulated gene expression. Most interestingly, we found that androgen treatment enhanced GSK-3 β Y²¹⁶ phosphorylation, which is dependent on PI3K activity. These data provide a direct evidence of GSK-3 β involvement in androgen-stimulated gene expression.

In this study, we observed a PI3K-dependent GSK-3 β Y²¹⁶ phosphorylation after androgen stimulation; however, the mechanism is not clear. Although it was reported that GSK-3 β kinase activity is associated with Y²¹⁶ phosphorylation, the responsive tyrosine kinase is still unknown in mammals. It has been reported that the tyrosine kinase Pyk2 and the Src family member Fyn are able to phosphorylate GSK-3 β , in which Fyn might be not responsible for androgen-induced GSK-3 β Y²¹⁶ phosphorylation because Src kinase inhibitor PP2 did not suppress androgen-stimulated PSA promoter activity in this study. Further investigation is being undertaken by our group to determine whether Pyk2 or other tyrosine kinases are responsive for androgen-induced GSK-3 β activation (Fig. 7).

PI3K has been implicated in androgen signaling in prostate cancer cells. Previous studies including ours (20–22) showed that PI3K inhibitors LY294002 and wortmannin block androgen-stimulated gene expression and overexpression of PTEN, an inhibitory protein phosphatase for PI3K pathway, suppresses androgen-stimulated AR transactivation. In androgen-independent prostate cancer cells, increased PI3K activity was observed, compared with androgen-dependent ones, and consistently LY294002 blocked progression to the

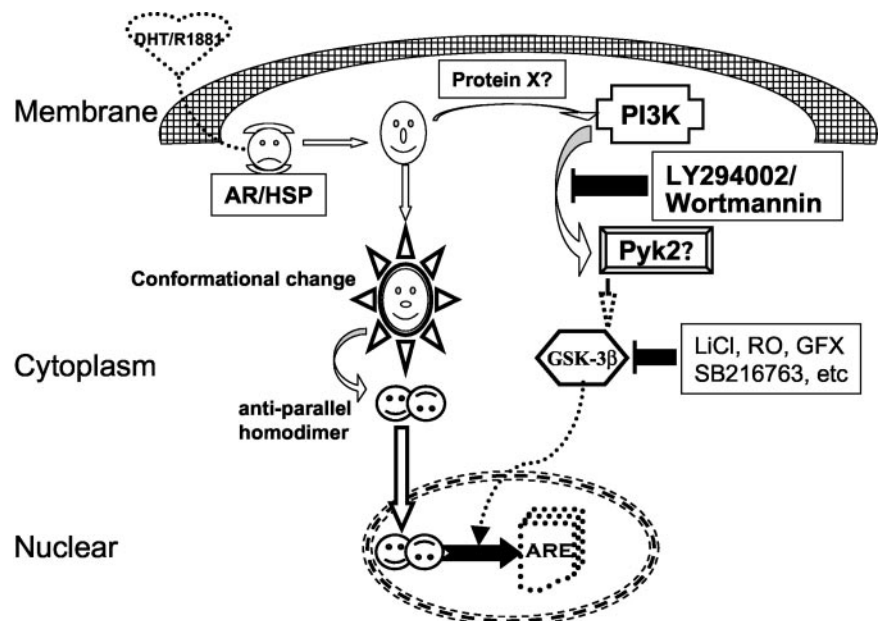


FIG. 7. Schematic illustration of the proposed mechanism of PI3K/GSK-3 β involvement in androgen-stimulated gene expression. Upon androgen binding, the AR is dissociated from the chaperon heat shock proteins (HSP) and then translocated into the nuclear as an antiparallel homodimer after a conformational change. In addition, androgen treatment also results in PI3K activation, which in turn leads to GSK-3 β tyrosine phosphorylation through unknown mechanism (Pyk2?). Finally, activated GSK-3 β regulates the assembly of AR-mediated transcriptional complex in the nuclear.

androgen-independent state of androgen-dependent prostate cancer LNCaP cells (49, 50). Furthermore, a functional genomic analysis revealed that increased expression of genes that converge on the PI3K pathway is observed in androgen-independent prostate cancers (51). Consistent with previous reports, we found in this study that PI3K inhibitor LY294002 suppressed androgen-stimulated gene expression, both endogenous targets (PSA and MMP-2), and exogenous androgen-responding reporters. In contrast to PI3K, some controversy exists regarding whether Akt is playing a role in AR transactivation. Akt is one of the downstream targets in PI3K signaling cascade. Overexpression of active Akt enhances androgen-stimulated gene expression in some systems (47) but not in others, although it can protect cell death induced by PTEN overexpression (20). Recently a proteomic analysis showed that previously proposed Akt phosphorylation sites on AR are not phosphorylated after androgen stimulation (48). Consistent with previous reports (20, 21) but not with a recent report (37), we did not observe an androgen-induced Akt activation (S⁴⁷³ phosphorylation) in all prostate cancer cells tested. Most interestingly, knocking down Akt protein by siRNA-mediated gene silencing did not change androgen-stimulated PSA promoter activity. These data suggest that the possibility of Akt's involvement in androgen-stimulated gene expression is very low. The differences regarding Akt in AR transactivation may come from different experimental conditions and strategies, *i.e.* overexpression *vs.* gene silencing, as well as different promoter-driven reporters.

A recently report proposed that PI3K/Akt activates AR transactivation by inhibiting GSK-3 β -mediated β -catenin degradation (21). However, a direct cause-effect correlation among androgen stimulation, Akt phosphorylation, GSK-3 β activity, and β -catenin degradation was not established in their study. In addition, LiCl-enhanced PSA promoter activity was seen only after β -catenin is overexpressed. Furthermore, whether β -catenin can interact directly with AR or enhance AR transactivation ubiquitously remains controversial (38–45). In contrast to previous studies using ectopic overexpression approach to evaluate β -catenin's role in androgen-stimulated gene expression, we took the advantage of the newest technique of siRNA-mediated gene silencing approach in which the artificial effect due to an aberrant protein presence is avoided in cells and the result is more relevant to biological condition. We found that knocking down endogenous β -catenin protein did not reduce but slightly enhance androgen-stimulated PSA promoter activity, although the difference is not significant. Consistent with this finding, a β -catenin-induced reduction of AR transactivation or *vice versa* was seen in neuronal cells (40), suggesting that a dynamic modulation between these two transcription factors exists the same as that reported between AR and activator protein-1 (52).

In conclusion, we demonstrated for the first time that GSK-3 β activity is required for androgen-stimulated gene expression. The phosphorylation of the regulatory site tyrosine 216 on GSK-3 β is increased after androgen stimulation, which is dependent on PI3K activity. However, neither GSK-3 β nor PI3K is involved in androgen-stimulated AR nuclear translocation. The mechanism of GSK-3 β regulation on androgen action needs further investigation.

Acknowledgments

We thank Dr. Michael Soares for valuable discussion and Mrs. Donna Barnes and Shontell Banks for excellent secretarial assistance.

Received November 10, 2003. Accepted February 18, 2004.

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This work was supported by the William L. Valk Endowment and grants from Mason's Foundation and Lied Foundation (to B.L.).

Part of this study was presented at the 2003 Annual Meeting of the American Urological Association, Chicago, Illinois.

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