

Suppression of Androgen Receptor-mediated Transactivation and Cell Growth by the Glycogen Synthase Kinase 3 β in Prostate Cells*

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Androgens play important roles in the growth of normal prostate and prostate cancer via binding to the androgen receptor (AR). In addition to androgens, AR activity can also be modulated by selective growth factors and/or kinases. Here we report a new kinase signaling pathway by showing that AR transactivation was repressed by wild type glycogen synthase kinase 3 β (GSK3 β) or constitutively active S9A-GSK3 β in a dose-dependent manner. In contrast, the catalytically inactive kinase mutant GSK3 β showed little effect on the AR transactivation. The suppression of AR transactivation by GSK3 β was abolished by the GSK3 β inhibitor lithium chloride. The *in vitro* kinase assay showed that GSK3 β prefers to phosphorylate the amino terminus of AR that may lead to the suppression of activation function 1 activity located in the NH₂-terminal region of AR. GSK3 β interrupted the interaction between the NH₂ and COOH termini of AR, and overexpression of the constitutively active form of GSK3 β , S9A-GSK3 β , reduced the androgen-induced prostate cancer cell growth in stably transfected CWR22R cells. Together, our data demonstrated that GSK3 β may function as a repressor to suppress AR-mediated transactivation and cell growth, which may provide a new strategy to modulate the AR-mediated prostate cancer growth.

Androgen exerts its effects via the intracellular androgen receptor (AR),¹ a member of the superfamily of nuclear receptors (1, 2). Like other nuclear receptors, the AR protein is comprised of a nonconserved amino-terminal domain, a highly conserved DNA-binding domain (DBD), a hinge region, and a ligand-binding domain (LBD). Upon androgen binding, AR dissociates from the heat-shock proteins and binds to androgen response elements, resulting in up-regulation or down-regulation of the transcription of AR target genes. Mutations in the AR gene cause a range of androgen insensitivity abnormalities

in male sexual development and play a key role in human prostatic carcinogenesis, which is the most common invasive malignancy and second leading cause of cancer deaths in males in the United States (3, 4). In the early stage of this disease, most patients respond favorably to androgen ablation and antiandrogen therapy. However, the effects of androgen ablation are usually transient as cancer cells eventually progress into the androgen-independent phenotype. Several different molecular mechanisms might be responsible for the transition to androgen independence. Many of these involve the mutations in the AR and the altered AR coregulator signaling (5, 6), but they might also include enhanced expression of growth factor receptors and their associated ligands (7).

In addition to responding to ligands, the AR is affected by kinase signaling pathways, which directly or indirectly alter the biological responses to androgens. This phenomenon is mediated by the AR as antiandrogens have been shown to block kinase-induced transcriptional activation (8). Growth factors, cytokines, and neuropeptides have been implicated in various *in vitro* and *in vivo* models of human malignancies, including prostate cancers (9). In the absence of androgens, insulin-like growth factor-1, keratinocyte growth factor, and epidermal growth factor are able to activate transcription of AR-regulated genes in prostate cancer cells (10). Mitogen-activated protein kinase and Akt kinase cascades have been shown to be involved in growth factor-mediated AR activation (7, 11–13). Some neuropeptides, such as bombesin and neurotensin, can stimulate AR activation and cancer cell growth in the absence of androgen by activation of tyrosine kinase signaling pathways (14). To date, it is well established that the AR is a phosphoprotein, and its activity is correlated with its phosphorylation status. Prostate cancer cells may progress from androgen dependence to a refractory state resulting from activation of AR by various kinases, thus circumventing the normal growth inhibition caused by androgen ablation.

Glycogen synthase kinase 3 β (GSK3 β) is a serine/threonine protein kinase that was first described in a metabolic pathway for glycogen synthase regulation (15). It is now clear that GSK3 β is a multifunctional kinase that regulates a wide range of cellular processes, ranging from intermediate metabolism and gene expression to cell fate determination, proliferation, and survival (16–19). GSK3 β phosphorylates a broad range of substrates, including several transcription factors such as c-Myc, c-Jun, rat glucocorticoid receptor, heat-shock factor-1, nuclear factor of activated T-cells c, and β -catenin (20–25). In contrast to other kinases, GSK3 β is highly active in unstimulated cells and becomes inactivated in response to mitogenic stimulation (26). Growth factors down-regulate GSK3 β activity through the phosphatidylinositol 3-kinase/Akt signaling cascade and the mitogen-activated protein kinase/p90RSK pathway (27, 28). Consistent with its position downstream of the phosphatidylinositol 3-kinase/Akt and mitogen-activated pro-

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¹ The abbreviations used are: AR, androgen receptor; GSK3 β , glycogen synthase kinase 3 β ; DBD, DNA-binding domain; GST, glutathione S-transferase; LBD, ligand-binding domain; GR, glucocorticoid receptor; N-C interaction, interaction between the NH₂ and COOH termini of AR; Luc, luciferase; DHT, 5 α -dihydrotestosterone; WT, wild type; MMTV, mouse mammary tumor virus; PSA, prostate-specific antigen; ARE, androgen-responsive element; AF, activation function; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; KM, kinase mutant; HA, hemagglutinin; ARN, AR NH₂ terminus; DL, AR-DBD-LBD.

tein kinase/p90RSK pathways, GSK3 β activity suppresses cell proliferation and induces apoptosis (29, 30). Phosphorylation of serine 9 of GSK3 β inhibits its activity by creating an inhibitory pseudosubstrate for the enzyme. Conversely, a mutation that prevents this phosphorylation results in activation of the kinase. GSK3 β is also inhibited by Wnt signaling, which may contribute to progression of the prostate cancer (31).

In the present study, the role of GSK3 β in AR signaling was assessed. In transfected cell lines, GSK3 β inhibited AR-dependent transactivation of several reporter genes as well as endogenous 5 α -dihydrotestosterone (DHT)-mediated prostate-specific antigen (PSA) expression. Additionally our data indicated that the effect of GSK3 β was mediated through the NH₂-terminal activation function 1 (AF-1) of the AR and through suppressing the interaction between the NH₂ and COOH termini of AR. Moreover our results suggested that GSK3 β can interact directly with the AR and inhibit androgen-stimulated cell growth. These findings suggest that GSK3 β might modulate AR signaling and, therefore, may play important roles in the control of the proliferation of normal and malignant androgen-regulated tissues.

EXPERIMENTAL PROCEDURES

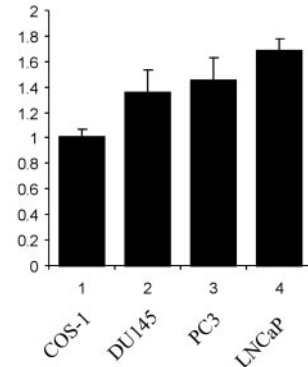
Materials and Plasmids—DHT and LiCl were obtained from Sigma. Antibodies to GSK3 β and phospho-GSK3 β were purchased from New England Biolabs. Purified GSK3 β was purchased from Upstate Biotechnology, Lake Placid, NY. The anti-AR polyclonal antibody, NH27, was produced as described previously (32). The GSK3 β plasmids, including wild type (WT), constitutively active, and dominant negative forms, were kindly provided by J. Sadoshima, Pennsylvania State University. Plasmids pCMX-GAL4-AR-DE, pCMX-VP16-AR-N, pCDNA3-AR-N, and pCDNA3-AR-C were constructed as described previously (33). The plasmid pBIG-S9A-GSK3 β was constructed by inserting full-length S9A-GSK3 β cDNA into pBIG2i expression vector.

Cell Culture and Transfection Assay—COS-1 and PC-3 cells were maintained in early to midlog phase in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μ g/ml streptomycin in incubators with humidified air and 5% carbon dioxide at 37 °C. LNCaP cells were maintained in RPMI 1640 medium (Invitrogen). Twenty-four hours prior to transfection, cells were washed with Hanks' buffered saline solution, trypsinized, and seeded to be at a density of 40–60% confluence for transfection. Cells in 24-well plates were refed with fresh medium 2 h before transfection and transfected according to the SuperFect transfection instructions (Qiagen). After 2–3 h of incubation, cells were treated with medium supplemented with charcoal-dextran-treated fetal bovine serum containing either ethanol or ligands. Cells were further incubated at 37 °C for 24 h, washed with phosphate-buffered saline, and harvested. The luciferase results were normalized to *Renilla* luciferase activity, and the data represent means \pm S.D. from duplicate sets of three independent experiments.

In Vitro Kinase Assay—Purified recombinant murine GSK3 β (New England Biolabs) was assayed as described previously (7). The kinase buffer contained 25 mM HEPES/pH 7.4, 10 mM MgCl₂, and 1 mM dithiothreitol. The kinase reactions were performed for 30 min at 30 °C in the presence of 10 μ Ci of [γ -³²P]ATP, 10 μ M ATP, and 0.05 pmol of GSK3 β . The same amounts of various AR fragments were purified from *Escherichia coli* and used as substrates, whereas GST protein was utilized as negative control. The reactions were terminated by addition of 4 \times SDS sample buffer. The samples were boiled and loaded on 12% SDS-polyacrylamide gels.

Real Time Reverse Transcription PCR—Total RNA was isolated using the TRIzol (Invitrogen) reagent according to the manufacturer's instructions, and 1 μ g of RNA was subjected to reverse transcription using Superscript II (Invitrogen). Specific primers for GSK3 β , 5'-CTA AGG ATT CGT CAG GAA CAG-3' (forward) and 5'-TTG AGT GGT GAA GTT GAA GAG-3' (reverse), were designed according to Bacon Designer2 software. 36B4 primers, 5'-TAC ACC TTC CCA CTT ACT G-3' (forward) and 5'-GAT TCC TCC GAC TCT TCC-3' (reverse), were used as controls (34). The real time PCR was performed with 1 μ l of reverse transcription product, 12.5 μ l of 2 \times SYBR Green PCR Master Mix (Bio-Rad), and 0.5 μ l of each primer (10 μ M) in a total volume of 25 μ l. PCR was performed as follows: 94 °C for 3 min and 40 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s on an iCycler iQ multicolor real

A



B

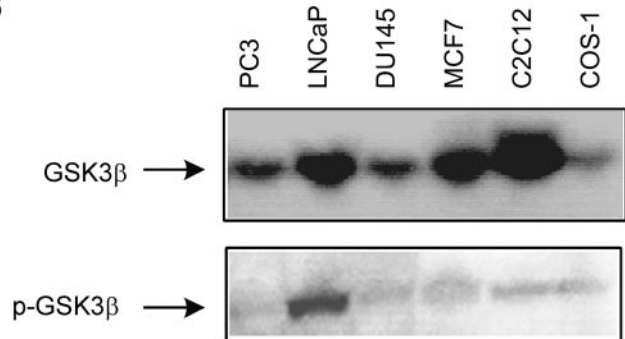


FIG. 1. The expression and activity of GSK3 β . A, total RNA was extracted from COS-1 (lane 1), DU145 (lane 2), PC-3 (lane 3), and LNCaP cells (lane 4), and 1 μ g of RNA from each cell line was subjected to reverse transcription using Superscript II. GSK3 β mRNA expression was analyzed by real time PCR as shown under "Experimental Procedures." B, expression of GSK3 β protein in cell lines. Several cell lines were incubated with 5% fetal bovine serum for 24 h. For the total amount of GSK3 β , 50 μ g of cell lysate was subjected to immunoblot analysis using anti-GSK3 β antibody (top panel). The inactive form of GSK3 β was detected by specific anti-phospho-GSK3 β (p-GSK3 β) antibody (bottom panel).

time PCR detection system (Bio-Rad). Each sample was run in triplicate. Data were analyzed by iCycler iQ software (Bio-Rad).

Stable S9A-GSK3 β Transfection in CWR22R Cells—The constitutively active form of GSK3 β , S9A-GSK3 β , was inserted into pBIG vector with hygromycin resistance. The S9A-GSK3 β -transfected CWR22R cells were selected and maintained in RPMI 1640 medium containing 50 μ g/ml hygromycin (Invitrogen).

Thiazolyl Blue (MTT) Assay—The MTT assay is a quantitative colorimetric assay for mammalian cell survival and proliferation. 5 \times 10³ CWR22R cells were seeded in 24-well plates and incubated in RPMI 1640 medium with 5% charcoal-dextran-treated fetal calf serum for 48 h. Cells were then treated with ethanol, 10 nM DHT, and/or 2 μ g/ml doxycycline for another 5 days. Then 200 μ l of MTT (5 mg/ml, Sigma) was added into each well with 1 ml of medium for 3 h at 37 °C. After incubation, 2 ml of 0.04 M HCl in isopropyl alcohol was added into each well. After 5 min of incubation at room temperature, the absorbance was read at a test wavelength of 570 nm.

RESULTS

GSK3 β Is Ubiquitously Expressed in Prostate Cancer Cells—Early studies showed that GSK3 β mRNA was prominently expressed in testis, thymus, prostate, and ovary (35). We first applied real time PCR to determine the expression of endogenous GSK3 β in COS-1 and several prostate cancer cells, including PC-3, LNCaP, and DU145 cells. As shown in Fig. 1A, GSK3 β mRNA was detected in all the cell lines tested with the highest level of GSK3 β mRNA in LNCaP and the lowest level in COS-1. To further examine the protein expression and activity of endogenous GSK3 β in cancer cells, several prostate cancer cell lines, including PC-3, LNCaP, and DU145, were

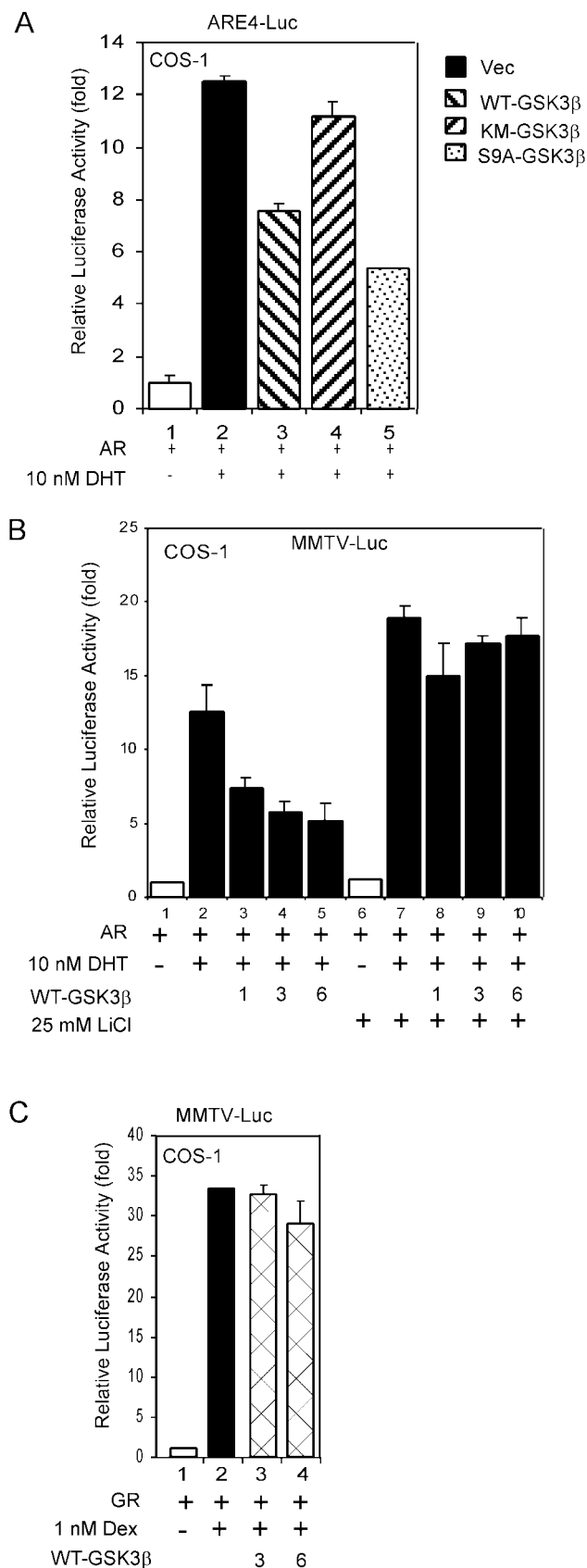


FIG. 2. Effect of GSK3 β on androgen receptor transcriptional activity. A, expression of WT GSK3 β , but not the kinase mutant GSK3 β , suppressed AR transactivation in COS-1 cells. AR-negative COS-1 cells were transiently transfected using SuperFect transfection reagent (Qiagen) with 3 μ g of ARE4-Luc reporter plasmid, 100 ng of pRLtk-Luc as an internal control, 1 μ g of pSG5-AR expression plasmid, and 6 μ g of WT, S9A, or kinase mutant GSK3 β expression plasmids as

subjected to Western blotting analysis along with some non-prostate cancer cell lines, including MCF7, C2C12, and COS-1. GSK3 β protein was ubiquitously expressed in all cell lines analyzed. GSK3 β can be phosphorylated at Ser-9, and phosphorylation at this site has been shown to inhibit kinase activities of GSK3 β (36). Therefore, the inactive form of GSK3 β with phosphorylation at serine 9 was determined by Western blotting with phosphospecific antibodies. LNCaP cells showed strongly phosphorylated GSK3 β compared with PC-3 and DU145 cells (Fig. 1B), suggesting that LNCaP cells may have relatively lower endogenous activity of GSK3 β .

Suppression of AR Transactivation by GSK3 β —Since growth factors, neuropeptides, and protein kinase A inhibit GSK3 β and enhance AR activity concurrently (8, 14, 37–39), we were interested to see whether co-expression of GSK3 β might alter AR-dependent transcriptional activity. We took advantage of a dual luciferase assay system (Promega) using reporter and internal control plasmids together. The ARE4-Luc reporter is driven by four androgen-responsive elements (AREs) in the promoter region and functions as a monitor of AR transcriptional activity. We transiently co-transfected GSK3 β , AR, and the two reporter plasmids in COS-1 cells, which lack endogenous AR. As shown in Fig. 2A, WT GSK3 β reduced the AR-mediated transcription of the luciferase reporter by about 40% (lane 3 versus lane 2). While the KM-GSK3 β had only a marginal effect on AR (lane 4), the constitutively active form of the GSK3 β (S9A-GSK3 β), which prevents serine 9 phosphorylation and inactivation of GSK3 β , strongly inhibited AR activity (lane 5), suggesting that the kinase activity of GSK3 β is necessary to suppress AR activity.

Since the context of upstream promoter elements may influence transcriptional efficiency, we tested another reporter plasmid, MMTV-Luc, to confirm the suppression effect of GSK3 β on AR transcriptional activity. MMTV-Luc is driven by the natural MMTV long terminal repeat promoter that contains several AR response elements. Fig. 2B demonstrates that GSK3 β inhibits DHT-mediated AR transactivation in a dose-dependent manner (lanes 2–5). LiCl, a specific inhibitor of GSK3 β , not only abolished the inhibitory effect of GSK3 β on AR (lanes 8–10) but also slightly enhanced AR transcriptional activity (lane 7 versus lane 2). This result indicates that LiCl may block both exogenously transfected GSK3 β as well as the endogenous GSK3 β activity in COS-1 cells. Moreover LiCl did not alter luciferase expression in the absence of AR, ensuring that LiCl has no nonspecific effect on the MMTV-Luc reporter (data not shown). To rule out the possibility that GSK3 β may have nonspecific effects on the general transcription machinery, we also tested its effect on the human glucocorticoid receptor (GR) since early studies reported that GSK3 β has little effect on the phosphorylation of human GR. As shown in Fig. 2C (lanes 3 and 4), addition of GSK3 β failed to inhibit human GR transactivation.

indicated. The total amount of plasmids was adjusted to 10 μ g with vector plasmids. Transfected cells were induced with 10 nM DHT for 18 h before the luciferase activities were measured. Luciferase activity was analyzed following the manufacturer's instructions (Promega). The results are shown as mean \pm S.D. of three independent experiments. B, overexpression of GSK3 β inhibits AR transcriptional activity in a dose-dependent manner. COS-1 cells were transfected with increasing amounts of WT GSK3 β expression plasmids as indicated. Experiments were performed and analyzed as described in A using MMTV-Luc instead of ARE4-Luc reporter. LiCl, a specific inhibitor of GSK3 β , was added into cell medium as indicated 1 h prior to DHT treatment. The results are shown as mean \pm S.D. of three independent experiments. C, overexpression of GSK3 β has no effect on human GR transcriptional activity. Experiments were performed and analyzed as described in B using GR instead of AR. The results are shown as mean \pm S.D. of three independent experiments. *Vec*, vector.

Inhibition of AR Transactivation and PSA Expression by GSK3 β in LNCaP Cells—To examine whether the inhibitory effect of GSK3 β on AR transactivation extends to cells that express endogenous AR, LNCaP cells, which have mutated yet functional AR, were co-transfected with the androgen-responsive reporter MMTV-Luc and GSK3 β . As shown in Fig. 3A, addition of GSK3 β reduced the activity of AR in a dose-dependent manner (lanes 3–5). Moreover, addition of LiCl abrogated the GSK3 β -mediated inhibition of AR activity (lane 6). A similar suppression effect also occurred when we replaced MMTV-Luc reporter with the ARE4-Luc reporter system (data not shown).

PSA is a clinically significant androgen-stimulated gene that is used to monitor the response to treatment, the prognosis, and the progression of prostate cancer. The DHT-mediated induction of transcription from the PSA promoter by DHT was repressed by overexpression of WT GSK3 β in a dose-dependent manner in LNCaP cells (Fig. 3B, lanes 4, 6, and 8 versus lane 2). The results from Northern blot assays further demonstrated that the expression of PSA mRNA was reduced about 40% by the ectopic expression of GSK3 β (Fig. 3C). Together, both the reporter assay and the Northern blot assay suggest that GSK3 β inhibits AR transactivation and influences expression of the PSA target gene downstream of the AR.

GSK3 β Phosphorylates the NH₂ Terminus of AR in Vitro and Inhibits the Function of the Ligand-independent Activation Domain (AF-1)—Since our data suggest that GSK3 β kinase activity is necessary for inhibiting AR transactivation, we wanted to determine whether AR is a substrate for GSK3 β . We purified three proteins, GST-ARN, GST-AR-DBD-LBD, and His₆-AR-LBD, that cover most of the N terminus (amino acids 38–560), DBD-LBD (amino acids 551–918), and LBD (amino acids 666–918) of AR, respectively (Fig. 4A). Fig. 4B demonstrates that GSK3 β significantly phosphorylated the GST-AR-(38–560) (lane 2), while GST protein alone could not be phosphorylated (lane 1). We also observed the phosphorylation of some degraded fragments of GST-AR-(38–560) by GSK3 β (lane 1). In contrast, under the same experimental conditions GSK3 β only slightly phosphorylated GST-AR-DBD-LBD (lane 3) or His₆-AR-LBD (lane 4). Thus, it appears that the NH₂ terminus of AR serves as a substrate for GSK3 β *in vitro*.

As AF-1 is located in the NH₂ terminus of AR and Fig. 4B shows GSK3 β can phosphorylate AR at the NH₂ terminus, we wanted to determine the potential effect of GSK3 β on AF-1 function. COS-1 cells were transfected with a fusion construct linking the GAL4 DNA-binding domain to the NH₂ terminus of AR (GAL4-AR-N). The transcriptional response of this construct was assessed using an upstream activating sequence-Luc reporter (pG5-Luc). Fig. 4C (lower panel) shows that the addition of GSK3 β inhibited the constitutive transcriptional activity of GAL4-AR-N (lanes 3 and 4 versus lane 2). In contrast, in the presence of DHT, GSK3 β did not influence the weak activity of GAL4-AR-LBD, which contains the AF-2 domain (lanes 7 and 8 versus lane 6). These results suggest that GSK3 β may suppress AR transactivation via the AF-1 functional domain that is located in the AR NH₂ terminus.

AR Interacts with GSK3 β —To test whether GSK3 β can associate with AR *in vitro*, we used the GST pull-down assay to examine the interaction between GSK3 β and AR. Full-length WT GSK3 β was constructed in a GST fusion vector. As shown in Fig. 5A, *in vitro* translated [³⁵S]methionine-labeled AR was found to bind specifically to purified GST-GSK3 β in the presence or absence of DHT (lanes 3 and 4). In contrast, there was no AR detected in the complex pulled down by GST protein alone (lane 2). To dissect the GSK3 β -interacting domain in AR, we found that both AR NH₂-terminal and COOH-terminal domains could bind to GSK3 β as shown in Fig. 5B.

To further demonstrate that GSK3 β interacts with AR in mammalian cells, we next used co-immunoprecipitation to examine their interaction by co-transfecting AR and HA-tagged GSK3 β into COS-1 cells. The COS-1 cell extracts were immunoprecipitated with an anti-HA antibody. As shown in Fig. 5C, the HA-GSK3 β immunocomplexes contained the AR (lane 3), suggesting that AR interacts with GSK3 β in the COS-1 cells. HA-tagged GSK3 β was also observed in the immunocomplexes pulled down with an anti-AR antibody (data not shown). Next we used LNCaP cells, which express endogenous AR and GSK3 β , to examine whether GSK3 β interacts with AR physiologically. As demonstrated in Fig. 5D, GSK3 β forms a stable complex with AR, suggesting that GSK3 β can interact with AR in the same cell and that AR could be a substrate for GSK3 β *in vivo*.

GSK3 β Suppresses Androgen/AR-induced Cell Growth—As previous reports revealed that androgen/AR might play important roles in the initiation and progression of prostate cancer, we wanted to know whether the suppression of AR by GSK3 β could modulate prostate cancer cell growth. We introduced the inducible constitutively active form of GSK3 β (S9A-GSK3 β) into the androgen-dependent CWR22R cell line by stable transfection. To distinguish exogenously transfected GSK3 β from endogenous GSK3 β in CWR22R cells, a Myc-tagged S9A-GSK3 β was constructed in the pBIG vector. Doxycycline stimulated the constitutively active S9A-GSK3 β expression in CWR22R-S9A-GSK3 β cells but not in the vector-transfected CWR22R-pBIG cells (Fig. 6A). Using a luciferase reporter assay, we found that induction of the constitutively active S9A-GSK3 β reduced AR transactivation by 30%, while doxycycline had a marginal effect on CWR22R-pBIG cells in the presence of DHT (Fig. 6B). This effect likely represents an underestimate of the total impact of GSK3 β on AR activity since CWR22R cells express endogenous GSK3 β . To correlate the inhibitory effect of GSK3 β on AR with prostate cancer cell growth, the growth of stably transfected CWR22R cells was tested in an MTT assay. The MTT assay (Fig. 6C) shows that addition of DHT induced cell growth in both CWR22R-pBIG and CWR22R-S9A-GSK3 β cells. As expected, the doxycycline treatment caused obvious growth arrest in the CWR22R-S9A-GSK3 β cells but not in the CWR22R-pBIG cells, suggesting that GSK3 β may repress AR-mediated cell proliferation. Taken together, these data indicate that activation of GSK3 β inhibits AR transcriptional activity and correlates with the reduced cell growth.

Reduction of the Interaction between the NH₂ and COOH Termini of AR—One potential mechanism through which GSK3 β may inhibit AR transactivation is by altering the level of AR expression. To address this issue, AR expression was measured by immunoblot in LNCaP cells transfected with the pCMV vector or with pCMV-GSK3 β . As shown in Fig. 7A, little change was seen in the endogenous expression of AR in LNCaP cells. In addition, AR localization was not altered by expressing S9A-GSK3 β in LNCaP cells (data not shown). Similar data were observed in transiently transfected COS-1 and in stably transfected CWR22 cells. These data therefore suggest that GSK3 β suppression of AR transactivation is not through alteration of endogenous androgen receptor stability or its nucleus-cytosol distribution.

We then studied the potential GSK3 β effect on the interaction between the NH₂- and COOH termini of AR (AR N-C interaction) as early studies indicated that AR N-C interaction plays important roles for the AR transactivation. We used a mammalian two-hybrid system to study the effects of GSK3 β on AR N-C interaction. GAL4-AR-LBD and VP16-AR-N plasmids were transfected into COS-1 cells. As shown in Fig. 7B, addition of GSK3 β inhibited the interaction of AR NH₂ termi-

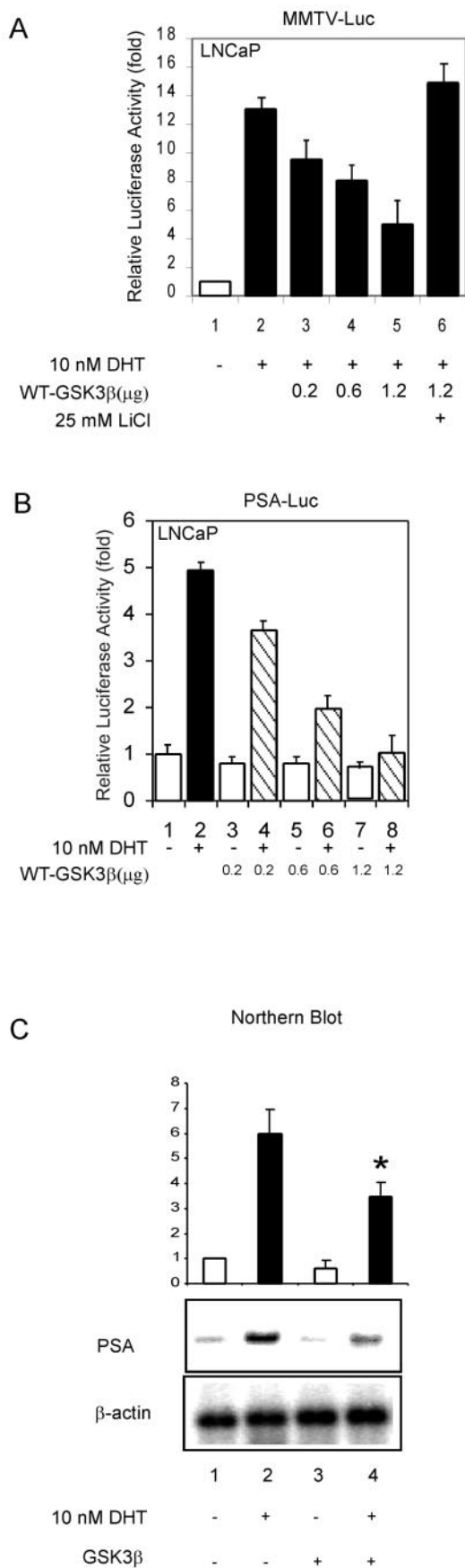


FIG. 3. Suppression of AR transactivation and PSA expression by GSK3 β in LNCaP cells. *A*, the LNCaP cells were transfected with WT GSK3 β for 3 h followed by DHT treatment for 18 h. Transactivation was measured by luciferase activity using MMTV-Luc as a reporter.

nus with AR-LBD (*lanes 4, 6, and 8 versus lane 2*), whereas this inhibitory effect was blocked by the presence of 25 mM LiCl, suggesting that the inhibition of AR transactivation by GSK3 β may involve reduced AR N-C interaction. In addition, we used a reconstituted AR transcription assay to further explore the interruption of the AR N-C interaction. The addition of the NH₂ terminus of AR dramatically increased the reporter induction by the AR-DBD-LBD (*Fig. 7C, lane 4 versus lane 2*). The reduced AR transactivation was observed by the co-expression of GSK3 β (*lanes 6 and 8 versus lane 4*), while LiCl could eliminate the inhibition of GSK3 β on AR activity. Thus, these results suggest GSK3 β may play an inhibitory role in the AR N-C interaction.

DISCUSSION

A principal clinical problem in prostate cancer treatment is the progression of androgen-dependent tumors to a hormone-refractory state after antiandrogen or androgen ablation therapy. Although the molecular basis for androgen independence is largely unknown, studies of patient specimens indicate that the AR signaling pathway can still be functional in hormone-refractory cancers. The AR is a phosphorylated protein, and its phosphorylation status is associated with its transcriptional activation. The NH₂ terminus of AR contains the majority of the sites phosphorylated *in vivo* (40). The alteration of AR phosphorylation by factors with elevated expressions in some prostate cancers may provide one possible mechanism involved in stimulating the progression of prostate cancer. These factors include cytokines, growth factors, and G-protein-coupled receptors, and their activity often leads to the inactivation of GSK3 β .

In this report, we have demonstrated that GSK3 β modulates AR transcriptional activity by measuring the expression of several androgen-regulated reporters. Previous studies indicate that protein kinase A can activate the AR through modification of its NH₂-terminal domain in the absence of androgen (8). Because it is known that protein kinase A reduces AR phosphorylation (41) and that the NH₂ terminus of AR mediates the effect of both protein kinase A and GSK3 β and can be phosphorylated by GSK3 β , our results suggest that GSK3 β may, in part, regulate the effects of protein kinase A on AR. Future studies are needed to confirm this hypothesis. Since GSK3 β is highly active in normal prostate cells, the kinase may inhibit AR transactivation under normal physiological conditions. This hypothesis fits well with our data showing that the inhibition of GSK3 β activity by LiCl enhances AR activity (*Fig. 2*). Our data demonstrate that GSK3 β suppresses AR activity (*Figs. 2 and 3*), phosphorylates AR (*Fig. 4*), and interacts with AR *in vivo* (*Fig. 5*), suggesting the AR may be a novel target of the GSK3 β signaling pathway. Addition of the constitutively active S9A-GSK3 β leads to the growth arrest of prostate cancer cells (*Fig. 6*); thus, the inhibition of GSK3 β may contribute to the development and progression of androgen-independent prostate cancers. Considering that protein kinase A, Akt, and mitogen-activated protein kinase inhibit GSK3 β (27, 42, 43), the data presented here are consistent with what is known

The data are means \pm S.D. from three independent experiments. *B*, overexpression of GSK3 β represses PSA promoter activity. Experiments were performed and analyzed as described in *A* using PSA-Luc instead of MMTV-Luc reporter. *C*, inhibition of AR target gene PSA expression by GSK3 β . LNCaP cells were transfected with WT GSK3 β (*lanes 3 and 4*) or vector (*lanes 1 and 2*). The cells were treated with ethanol or 10 nM DHT for 18 h as indicated. Total RNA was isolated, and the levels of PSA and β -actin were monitored by Northern blot assay (*lower panel*). The relative mRNA levels are presented as the ratio of PSA mRNA: β -actin mRNA, and values of bars represent the mean \pm S.D. of three similar experiments (*upper panel*). (*, $p = 0.019$ versus lane 2, two-tailed Student's *t* test).

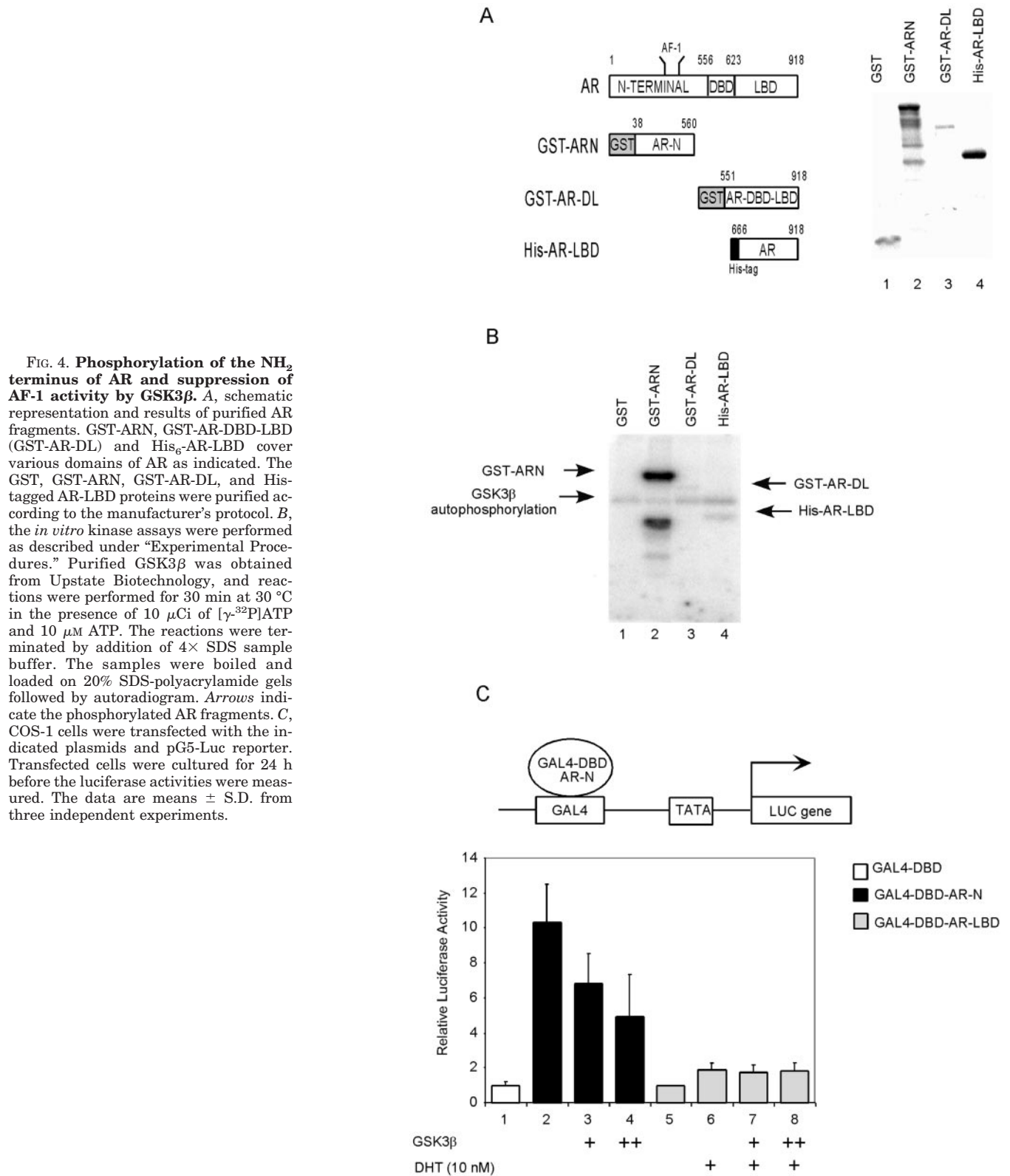


FIG. 4. Phosphorylation of the NH₂ terminus of AR and suppression of AF-1 activity by GSK3 β . *A*, schematic representation and results of purified AR fragments. GST-ARN, GST-AR-DBD-LBD (GST-AR-DL) and His₆-AR-LBD cover various domains of AR as indicated. The GST, GST-ARN, GST-AR-DL, and His-tagged AR-LBD proteins were purified according to the manufacturer's protocol. *B*, the *in vitro* kinase assays were performed as described under "Experimental Procedures." Purified GSK3 β was obtained from Upstate Biotechnology, and reactions were performed for 30 min at 30 °C in the presence of 10 μ Ci of [γ -³²P]ATP and 10 μ M ATP. The reactions were terminated by addition of 4 \times SDS sample buffer. The samples were boiled and loaded on 20% SDS-polyacrylamide gels followed by autoradiogram. Arrows indicate the phosphorylated AR fragments. *C*, COS-1 cells were transfected with the indicated plasmids and pG5-Luc reporter. Transfected cells were cultured for 24 h before the luciferase activities were measured. The data are means \pm S.D. from three independent experiments.

regarding the stimulation of prostate cancer cell growth by growth factors and cytokines and fit very well with the proapoptotic roles of GSK3 β in other tissues (16, 44, 45).

Numerous studies have suggested potential links between the androgen/AR and GSK3 β signaling pathways. First, testosterone, but not estrogen, prevents the heat shock-induced overactivation of GSK3 β , suggesting that androgen may display a neuroprotective effect against Alzheimer's disease (46). Second,

GSK3 β plays a pivotal role in the degradation of the free cytoplasmic β -catenin, an AR coregulator, through the ubiquitin proteasome pathway (25). Recent studies indicate that dysregulation of β -catenin expression is found in a variety of human malignancies, including prostate cancer in which β -catenin may act as a coactivator of AR (47). Third, GSK3 β also phosphorylates c-Myc and cyclin D1, resulting in ubiquitin-mediated degradation (20, 48). This is relevant in that

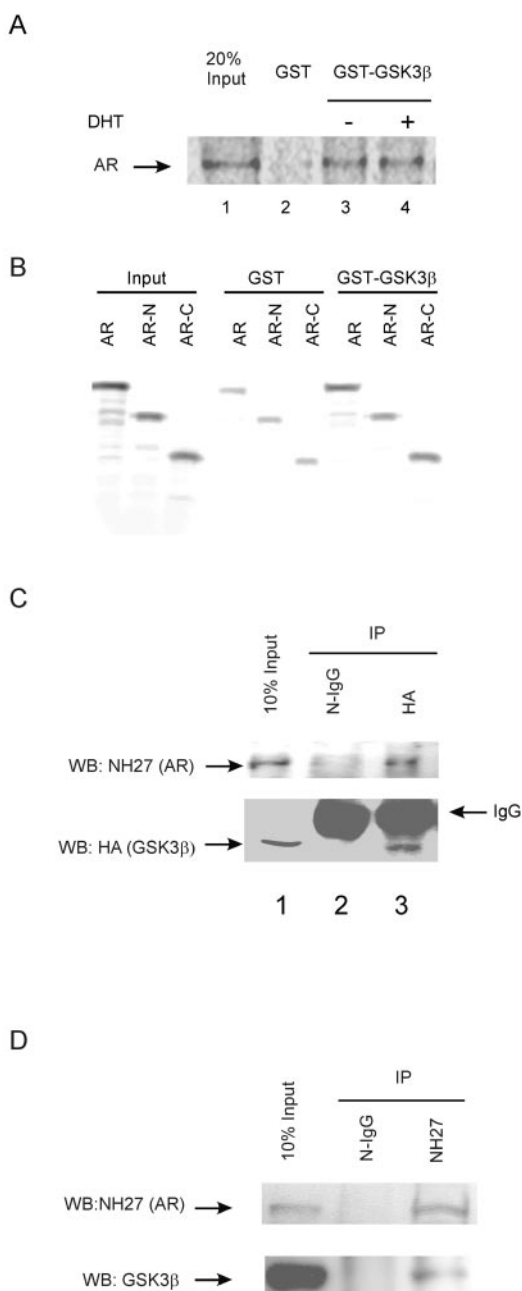


FIG. 5. GSK3 β interacts with AR *in vitro* and *in vivo*. *A*, GST and GST-fused GSK3 β were expressed in *E. coli* and purified by glutathione-Sepharose 4B beads as instructed by the manufacturer (Amersham Biosciences). 5 μ l of *in vitro* translated 35 S-labeled AR was incubated with the GST or GST-GSK3 β bound to glutathione-Sepharose beads in a pull-down assay. After extensive washing, bead-bound protein complexes were resolved by 8% SDS-PAGE and analyzed by PhosphorImager. The input represents 20% of 35 S-labeled proteins used in each pull-down assay. *B*, the AR fragments, the AR NH₂ terminus containing amino acids 1–556, and the AR COOH terminus containing amino acids 507–919 were *in vitro* translated and labeled with 35 S as indicated. GST pull-down assay was carried out as described in *A*. *C*, COS-1 cells plated on 100-mm dishes were transfected with pSG5-AR and pCMV-GSK3 β -HA for 24 h. COS-1 cells were solubilized in radioimmune precipitation assay buffer containing 0.5% Nonidet P-40 and protease inhibitors. Immunoprecipitation was performed using mouse HA antibody (1:1000) or normal mouse IgG (*N-IgG*), then analyzed by Western blot with anti-AR NH27 (1:1000) or anti-GSK3 β (1:1000) antibodies followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit or rabbit anti-mouse IgM antibodies, and visualized with an alkaline phosphatase conjugate kit (Bio-Rad). *D*, 500 μ g of total proteins from LNCaP cells was immunoprecipitated with normal rabbit IgG or rabbit anti-AR NH27, and the immunoprecipitates were subjected to a Western blot analysis using the antibody for GSK3 β and the NH27 for AR. *WB*, Western blot; *IP*, immunoprecipitation.

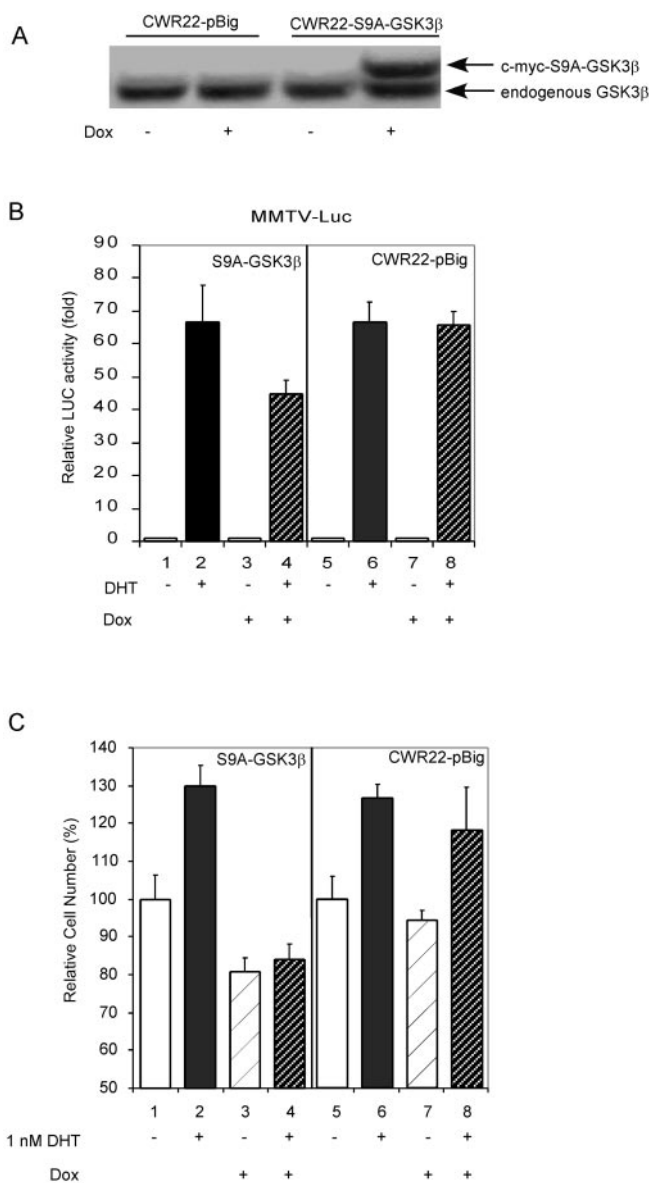


FIG. 6. Stably transfected GSK3 β inhibits prostate cancer CWR22R cell growth. *A*, Myc-tagged S9A-GSK3 β or the inducible pBIG vector were stably transfected into prostate cancer CWR22R cells. CWR22R-S9A-GSK3 β and CWR22R-pBIG cells were cultured in 5% fetal bovine serum for 24 h followed by doxycycline (Dox) treatment for 16 h. Whole cell lysates were subjected to immunoblot analysis using anti-GSK3 β antibody. *B*, CWR22R-pBIG or CWR22R-S9A-GSK3 β cells were transfected with MMTV-Luc and pRLtk-Luc for 3 h followed by DHT treatment for 18 h. Transactivation was measured by luciferase activity as described under "Experimental Procedures." *C*, growth assays were performed by the MTT method as instructed by the manufacturer (Sigma). 5 \times 10³ CWR22-S9A-GSK3 β and CWR22-pBIG cells were seeded in 24-well plates and incubated in RPMI 1640 medium with 5% charcoal-dextran-treated fetal bovine serum for 48 h. Cells were then treated with ethanol, 10 nM DHT, and/or 2 μ g/ml Dox as indicated. After 5 days of treatments, cells were harvested for an MTT assay. Values are the means \pm S.D. of A₅₇₀ from three independent wells of cells.

elevated cyclin D1 and c-Myc levels may be associated with prostate cancer progression (49–51).

Recent studies also demonstrate that GSK3 β may regulate AR activity through an AR coactivator, β -catenin. Our study raises the possibility that GSK3 β directly influences AR activity independently of the β -catenin-mediated pathway. The interaction between AR and β -catenin is DHT-dependent, and our data demonstrate that the inhibition of GSK3 β by LiCl

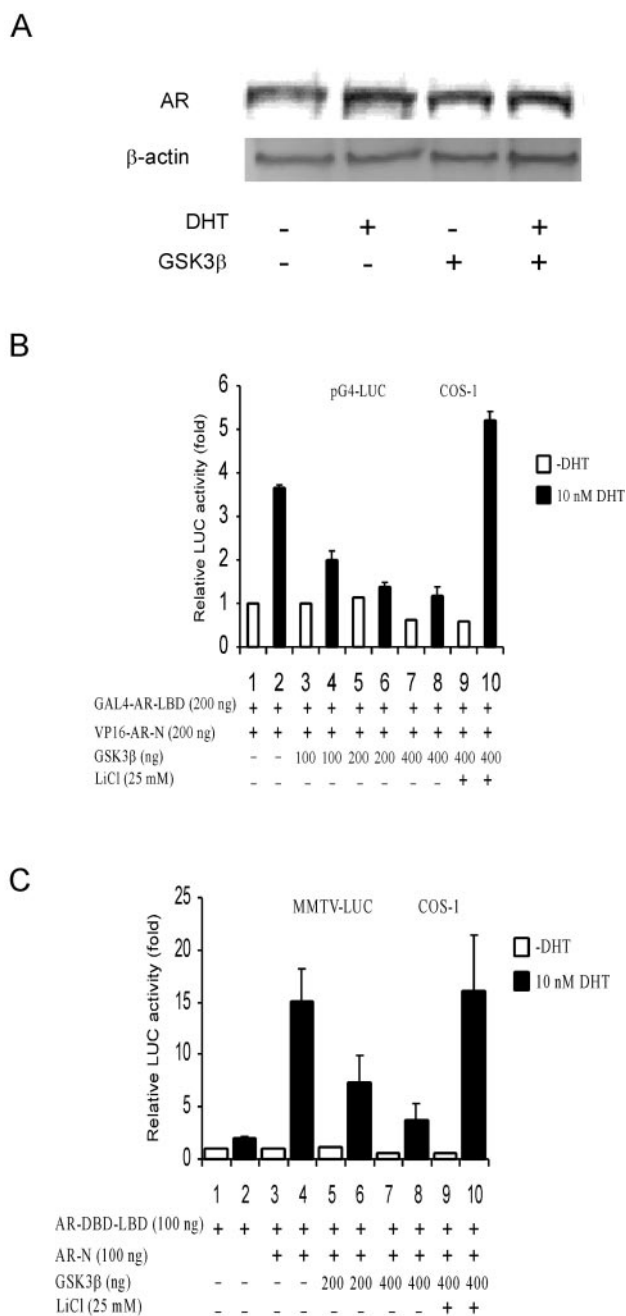


FIG. 7. Effect of GSK3 β on the AR N-C interaction. *A*, GSK3 β does not change AR protein amount. COS-1 cells were transfected with WT GSK3 β or mock vector as indicated. After a 24-h transfection, 50 μ g of whole cell extract was immunoblotted with AR antibody (NH27). *B*, inhibition of the interaction between the NH₂ and COOH termini of AR by GSK3 β . The COS-1 cells were transfected with GAL4-AR-LBD (amino acids 624–918) and VP16-AR-N (amino acids 1–556). The interactions between the NH₂ and COOH termini of AR were determined by luciferase assay by using pG5-Luc as a reporter. *C*, the reconstituted AR transcription assays were performed to determine the association of the AR NH₂ terminus with the AR-DBD-LBD. COS-1 cells were transfected with MMTV-Luc, pCDNA3-AR-N (amino acids 1–556), pCDNA3-AR-DBD-LBD (amino acids 556–919), and GSK3 β as indicated. After transfection, cells were treated with 10 nM DHT or 25 mM LiCl for 24 h before harvesting. The LUC activity relative to lane 1 was calculated, and results are the mean \pm S.D. of three independent experiments.

increases AR transcriptional activity in the absence of DHT. Also, several factors that inhibit GSK3 β , such as insulin-like growth factor-1 and insulin, do not stabilize β -catenin or stimulate β -catenin-dependent gene transcription (52). This obser-

vation argues for the direct effect of GSK3 β on AR. Moreover, β -catenin enhances AR activity through interaction with the AR-LBD, which contains the AF-2 domain. Our data suggest that AF-1 activity, but not that of AF-2, is reduced by GSK3 β (Fig. 4). Furthermore GSK3 β directly phosphorylates the NH₂-terminal region of AR. Our GST pull-down assay and co-immunoprecipitation assay indicate the interaction between GSK3 β and AR (Fig. 5A). Together, these lines of evidence indicate that GSK3 β and β -catenin may affect the AR at different levels and that the inhibition of GSK3 β followed by elevated β -catenin levels may cooperate to enhance AR activity and lower the requirement for androgen in prostate cancer cells.

Although we favor the hypothesis that AR phosphorylation and the resulting inhibition of AR activity contribute to the blockage of DHT-induced cell growth imposed by activated GSK3 β (Fig. 6), we cannot rule out the possibility that the phosphorylation of a variety of other substrates by GSK3 β might influence cell growth. For example, by inhibiting GSK3 β , growth factors might promote the dephosphorylation and stabilization of cyclin D1 and c-Myc (20, 48, 53). Elevated cyclin D1 might enhance the activities of cyclin-dependent protein kinases CDK4 and CDK6, resulting in the inactivation of the retinoblastoma gene and entry into the S phase of the cell cycle. c-Myc is known to stimulate prostate cancer cell proliferation and survival as shown in many reports (54, 55). GSK3 β is also known to phosphorylate c-Jun, resulting in inhibition of the DNA binding of this transcription factor that has been implicated in cell growth, differentiation, and development (56, 57). Active GSK3 β , therefore, is implicated as a key factor in maintenance of the basal states of several important signaling pathways, and dysregulation of GSK3 β may lead to transformation to malignancy.

In summary, our data demonstrate that AR might be a substrate for GSK3 β and that GSK3 β negatively regulates AR-mediated gene transcription to modulate androgen/AR-mediated cell growth. Our study raises the possibility that this pathway may function in the progression of prostate cancer to androgen independence. These findings suggest potential approaches for the development of pharmacological inhibitors designed to increase GSK3 β activity, an effect that may be useful for prostate cancer therapy.

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