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Phosphorylation/Dephosphorylation of Androgen Receptor as a Determinant of Androgen Agonistic or Antagonistic Activity[1]

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Protein phosphorylation/dephosphorylation is an important posttranslational modification that plays a critical role in signal transduction. The androgen receptor (AR) is under such control. We demonstrate that androgen receptor phosphorylation determines whether or not AR ligands perform as agonists or antagonists in LNCaP cells. Androgen receptor ligands (such as dihydrotestosterone and β-estradiol) stimulate receptor expression and phosphorylation and, as a result, they act as agonists or partial agonists. In contrast, agents such as bicalutamide and estramustine inhibit the receptor phosphorylation and act as antagonists. This model is supported by gene expression and transactivation assays. Significant increases in levels of both mRNA and protein of prostate-specific antigen (PSA), a natural AR target gene, occur following the treatment of LNCaP cells with DHT, β-estradiol, or hydroxyflutamide. In contrast, exposure of LNCaP cells to bicalutamide or estramustine results in a sharp decrease of PSA expression. Agonistic or antagonistic effect of these compounds on PSA expression parallels the level of phosphorylated, but not dephosphorylated androgen receptors. These agonistic or antagonistic effects are also observed in HeLa cells transfected with wild-type AR expression plasmid (pAR0) and AR-driven luciferase expression plasmid GRE-tk-LUC in the presence of different groups of AR blockers. Our data indicate that the functional status of androgen receptors is strongly correlated with the phosphorylation status of the receptors, and that the phosphorylated androgen receptor is the form of the receptor transcriptionally active in regulation. Thus the androgen receptor phosphorylation/dephosphorylation may serve as a new molecular target for screening androgen antagonists for the treatment of prostate cancer.

Key Words: **androgen receptors; phosphorylation/dephosphorylation; agonists; antagonists; gene expression.**

Hormone related therapy of prostate cancer includes treatment with antiandrogens, such as estrone and β-estradiol, and androgen receptor (AR) blockers, such as flutamide, and bicalutamide [1–2]. These agents exhibit their androgen antagonistic effect by binding to wild-type androgen receptors, leading to interruption of AR mediated signal transduction. A high frequency of androgen receptor mutations occurs in advanced prostate cancers [3–5], and recent studies indicate that such mutation(s) may change the characteristics of androgen receptor ligands from antagonists to agonists [6–11]. Hydroxyflutamide, anandron, cyproterone acetate, estradiol, and progesterone can compete with endogenous androgen to bind to a mutated androgen receptor. Under these conditions, they exhibit androgen agonistic- instead of antagonistic- effects [6–11].

The androgen receptor, a member of the nuclear receptor superfamily of ligand-regulated transcription factors [12–16], is a phosphorylated protein [17–20], Several phosphorylation sites in the AR molecule have been identified, including Ser 81, 94, 503, 641, 650 and 653 [21–23]. The phosphorylation of the receptor protein has been found to be required for hormone binding, suggesting that there is a link between the phosphorylation status and the activation state of the receptor [24]. In addition, stimulation of androgen-regulated transactivation is modulated by protein phosphorylation [25], and inhibition of AR phosphorylation by the protein kinase A (PKA) stimulator, fors-

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Abbreviations used are: AR, androgen receptor; wt-AR, wild type androgen receptor; m-AR, mutated androgen receptor; PSA, prostate-specific antigen; SRBC, steroid receptor binding consensus; DHT, dihydrotestosterone; FBS, fetal bovine serum.

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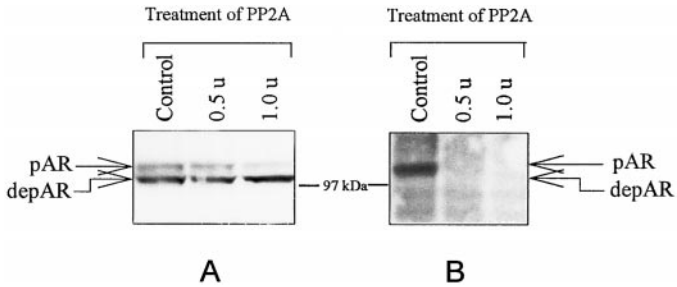

FIG. 1. Androgen receptors as phosphorylated- and dephosphorylated-proteins in LNCaP cells. LNCaP cells grown exponentially were metabolitically labeled for 6 h with 500 μCi/ml of [^{35}S]-methionine or [^{32}P] orthophosphate in methionine- or phosphate-free RPMI 1640 medium. The cells were harvested, washed, and total proteins extracted. One hundred μg of the protein extracts were inmmunoprecipitated with AR specific antibody for 1 h in the presence of proteinase inhibitors (1 mM PSMF, 5 μg/ml leupeptin, 10 μg/ml aprotinin, and 25 u/ml bacitracin) followed by addition of 15 μl of Protein agarose A + G, and incubated at 4°C overnight in a Speci-Mix shaker. The immunoprecipiates were washed, and separated by 4%/7% stack SDS–PAGE. The AR dephosphorylation was performed by addition of indicated amounts of protein phosphatase-2A to immunoprecipitates from ^{35}S (panel A) or ^{32}P (panel B) proteins, and incubated for 30 min at 37°C followed by SDS–PAGE. The protein bands were obtained by exposure of the dried gel to ECL films at −70°C overnight.

kolin, results in a decrease in AR mediated transcriptional regulation of two endogenous genes (PAS and β1-subunit of Na/K-ATPase) [23]. In this report, we demonstrate that androgen receptor phosphorylation/dephosphorylation plays a dominant role in the action of androgen receptor ligands. This molecular switch is a potential new molecular target to allow development of pure androgen antagonists for the treatment of prostate cancer, and to distinguish androgen antagonists from agonists for the widely used anti-androgen therapeutic drugs.

MATERIALS AND METHODS

Reagents. Antibodies against AR, corresponding to amino acids 900-919 (mapping at the carboxy terminus of the androgen receptor of human origin), and anti-rabbit IgG-HRP were purchased from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA). A Western blotting detection kit was purchased from Amersham (Arlington Heights, Illinois). Reagents for SDS–PAGE and protein determination were obtained from Bio-Rad, (Richmond, CA). RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Gibco BRL Life Technologies, Inc., (Gaithersburg, MD), and [^{35}S]-methionine and [^{32}P] orthophosphate from ICN Pharmaceuticals, Inc. (Costa Mesa, CA) and Amersham Life Science Inc., (Arlington Heights, Illinois), respecitively. Other chemicals including dihydrotestosterone (DHT) were purchased from Sigma Chemical Company, (St.

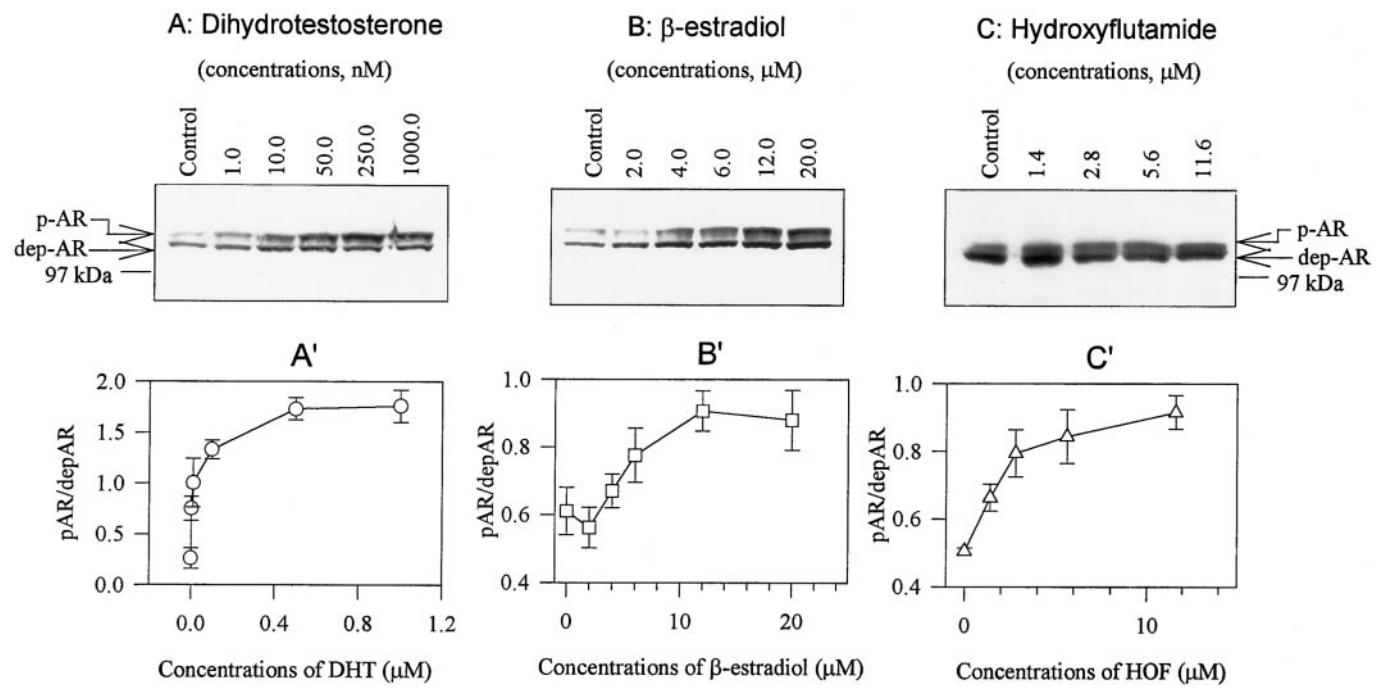

FIG. 2. Stimulation of androgen receptor phosphorylation by androgen agonists in LNCaP cells. Synchronized LNCaP cells at G1 in serum free RPMI 1640 medium were exposed to indicated concentrations of dihydrotestosterone (A, A′), or LNCaP cells at 65–75% confluence in RPMI 1640 medium containing 10% fetal bovine serum were treated with various concentrations of β-estradiol (B, B′), and hydroxyflutamide (C, C′) for 24 hours. The cells were harvested, washed once with cold PBS, and proteins extracted as described in the method section. One hundred μg of the proteins were subjected to electrophoresis on 4%/7.5% stack SDS-ploy arylamide for 4 hours. After electrophoresis, the proteins on the gel were electro-transferred onto nitrocellulose membrane and the phosphorylated- (105 kDa) and dephosphorylated- (99 kDa) androgen receptor proteins were immuno-detected by AR-specific antibody. The pAR/depAR (lower panels A′, B′ and C′) was calculated from the density of the plots of AR determined from ECL films by an Imaging Densitometer Model GS-700. Data are the means ± SD of three separate experiments scored by the densitometer and normalized to Control.

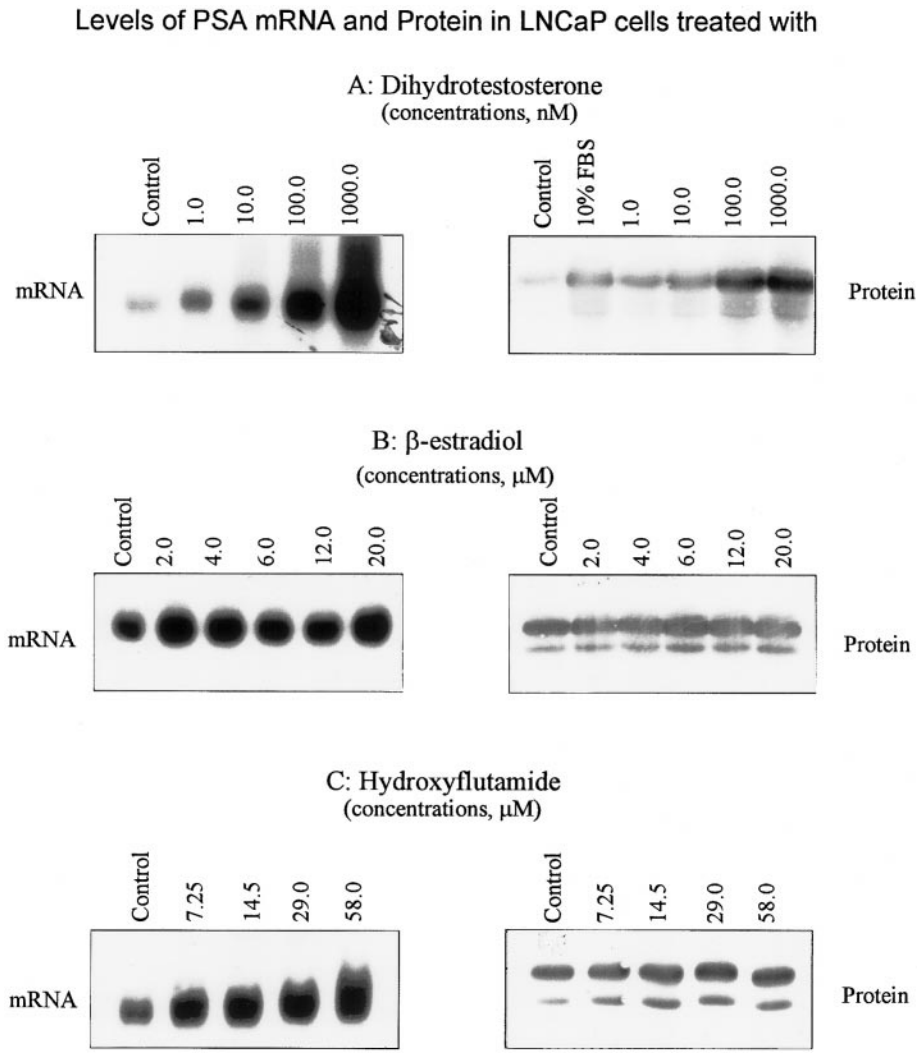

FIG. 3. Up-regulation of the expression of prostate specific antigen (PSA), a natural AR- target gene, by androgen agonists. Synchronized LNCaP cell at G1 in serum free RPMI 1640 medium were exposed to indicated concentrations of dihydrotestosterone (panel A), or LNCaP cells at 65–75% confluence in RPMI 1640 medium containing 10% fetal bovine serum were treated with various concentrations of β-estradiol (panel B), or hydroxyflutamide (panel C) for 24 hours. The cells were harvested, washed with cold PBS and total RNA, and proteins extracted. For Northern blotting assay, 20 μg of total RNA were separated on 1.2% agarose-formaldehyde gels and transferred to Zeta-Probe Blotting Membranes (Bio-Rad, Richmond CA). The double-stranded PSA specific oligonucleotide probe, (5′-AGCCTAGAGAAGGCTGTGAGCCAAG-GAGGGAGGGTCTTCCTTTGGCATGGGATGGGGATGAAGTAAGGAGAGGGACT-3′), 5′-end-labeled was used for hybridization. For Western blotting assay, 100 μg cellular extracts cells were separated by 4%/7.5% stack SDS–PAGE, electro-transferred to nitrocellulose membrane, and immunoblotted with antibodies against PSA.

Louis, MO). Hydroxyflutamide, bicalutamide, β-estradiol, estramustine, estromustine were provided by Schering, Zeneca Pharmaceuticals, and Pharmacia Corporations, respectively.

Cell culture. The prostatic carcinoma cell lines LNCaP, PC-3, and DU 145 were purchased from the American Type Culture Collection (Rockville, Maryland). Cells were maintained under conditions as described previously [26]. The cell lines used for the experiments were transferred through less than 30 passages.

Cell treatment. LNCaP cells at 65–75% confluence in RPMI 1640 medium containing 10% FBS were treated with indicated androgen agonists or antagonists for 24 h. The cells were harvested and washed with cold PBS. Total RNA and cellular proteins were extracted as described previously [27]. For stimulation studies with dihydrotestosterone (DHT), the LNCaP cells at 65–75% confluence were washed twice with pre-warmed serum free RPMI 1640 medium and reincubated in serum free medium for 48 hr to synchronize the cells at G_1 phase (over 80% of cells were in G_1 as examined by flow cytometer). Upon synchronization, the cells were exposed for 24 hr to the serum- free RPMI 1640 medium containing indicated concentrations of DHT. The total proteins were then extracted as described above.

Determination of androgen receptor phosphorylation. Androgen receptor phosphorylation were determined by Western blotting and immuno-precipitation using AR specific antibody. For the Western blotting assay, 100 μg cellular extracts were separated by stack 4%/7.5% SDS–PAGE, electro-transferred to nitrocellulose filters, and probed with AR specific antibody using standard techniques [28]. Quantitation by densitometry of the ECL films

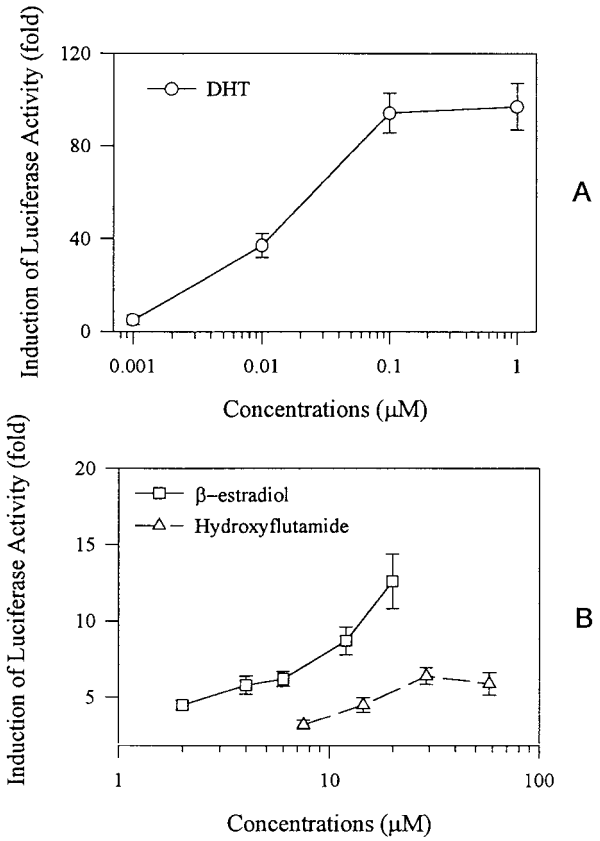

FIG. 4. Transactivation of androgen agonists on the wild-type AR mediated luciferase reporter gene expression. 3×10^5 HeLa cells in RPMI 1640 medium containing 10% FBS were sown in 10 cm dishes and incubated at 37°C for 24 hrs. Ten μg w-pAR0 cDNA and 2 μg GRE-tk-LUC cDNA in 125 mM $CaCl_2$–HEPES buffer (0.14 M NaCl, 0.05 M, HEPES acid, 1.5 mM Na_2HPO_4 pH 7.05) were added to the culture and incubated for 16 hrs. The precipitates were washed out with pre-warmed PBS and refed with fresh RPMI 1640 medium containing 10% FBS. After incubation of the transfected cells for additional 32 hrs at 37°C in the presence of various concentrations of DHT, β-estradiol, hydroxyflutamide, the cells were harvested and the cell extracts used for luciferase activity assay as described under Materials and Methods.

was done using an Imaging Densitometer Model GS-700 (Bio-Rad Lab. Hercules, CA).

For immunoprecipitation, LNCaP cells grown exponentially were metabolitically labeled for 6 h with 500 μCi/ml [^{35}S]-methionine [^{35}S]-methionine or [^{32}P] orthophosphate in methionine- or phosphate-free RPMI 1640 medium. The cells were harvested, washed, and total proteins extracted. One hundred μg of the protein extracts were incubated with AR specific antibody for 1 h in the presence of proteinase inhibitors (1 mM PSMF, 5 μg/ml leupeptin, 10 μg/ml aprotinin, and 25 u/ml bacitracin) followed by addition of 15 μl of Protein agarose A + G and incubated at 4°C overnight in a Speci-Mix shaker. The immunoprecipiates were washed four times with PBSTDS (0.58 M $NaHPO_4$, 0.17 M $NaHPO_4$, 0.68 M NaCl, 1% Trition X-100, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate), and separated by 4%/7% stack SDS–PAGE. For the protein dephosphorylation assay, the indicated amount of protein phosphatase-2A was added to immunoprecipitates from proteins labeled with ^{35}S or ^{32}P after the washes, and incubated for 30 min at 37°C followed by SDS–PAGE. The protein bands were obtained by exposure of the dried gel to ECL films at −70°C overnight.

Northern blotting assay. For the Northern blotting assay, 20 μg aliquots of the total RNA were fractionated on 1.2% agarose-formaldehyde gels and transferred to Zeta-Probe Blotting Membranes (Bio-Rad, Richmond CA). The double-strand PSA specific oligonucleotide probe, (5′-AGCCTAGAGAAGGCTGTGAGCCAAGG-AGGGAGGGTCTTCCTTTGGCATGGGATGGGGATGAAGTAAGG-AGAGGGACT-3′) described by Young *et al.* [29], with 5′-end-label was used for hybridization as described previously [28].

Gene transactivation assay. HeLa cells were co-transfected with wild-type AR expression plasmid pAR0 and reporter-plasmid GRE-tk-LUC using standard calcium phosphate precipitation methods [30–31], and the efficiency of the transfection was monitored by the luciferase activity (Promega Assay Systems) in the presence of 10 nM DHT. Briefly, 3×10^5 HeLa cells in RPMI 1640 medium containing 10% FBS were sown in 10-cm dishes and incubated at 37°C for 24 hrs. Ten μg w-pAR0 cDNA and 2 μg GRE-tk-LUC cDNA in 125 mM $CaCl_2$–HEPES buffer (0.14 M NaCl, 0.05 M, HEPES acid, 1.5 mM Na_2HPO_4 pH 7.05) were added to the culture and incubated for 16 hrs. The precipitates were washed out with pre-warmed PBS and refed with fresh RPMI 1640 medium containing 10% FBS. After incubation of the transfected cells for additional 32 hrs at 37°C in the presence of various concentrations of DHT, β-estradiol, hydroxyflutamide, estramustine, or bicalutamide or other test compounds, the cells were harvested. The luciferase activity in cell extracts was evaluated as described above.

RESULTS

Androgen Receptor Is a Phosphorylated Protein

Western blotting or immunoprecipitation of proteins extracted from [^{35}S] labeled LNCaP cells with AR specific antibody produced two major protein bands with molecular weight approximately 99 and 105 kDa (Fig. 1, A, lane 1, and Fig. 2, upper panel). These protein bands represent dephosphorylated (dep-AR, 99 kDa) and phosphorylated (pAR, 105 kDa) forms of the androgen receptors as indicated by the antibody supplier. To confirm this observation, immunoprecipitation assay was performed using ^{32}P orthophosphate labeled protein from LNCaP cells that produced only one protein band with a molecular weight of approximately 105 kDa (Fig. 1, B, lane 1). Treatment of the proteins labeled with ^{35}S-methionine or ^{32}P orthophosphate with protein phosphatase-2A resulted in the disapperance of the 105 kDa protein band. These data demonstrate the presence of the phosphorylated- and dephosphorylated-forms of androgen receptors (Fig. 1, A and B, lanes 2–3).

Androgen Agonists

To determine the changes of phosphorylation status of androgen receptors in response to dihydrotestosterone (DHT) stimulation, synchronized LNCaP cells were exposed for 24 h or different periods of time to varying concentrations of DHT (1.0 to 1000.0 nM), and the phosphorylation status examined by Western blotting. As shown in Fig. 2A, two major AR antibody-reactive proteins with molecular weights of approximately 99 and 105 kDa were observed. The dephosphorylated androgen receptor in the arrested LNCaP cells was found to be the major form of the

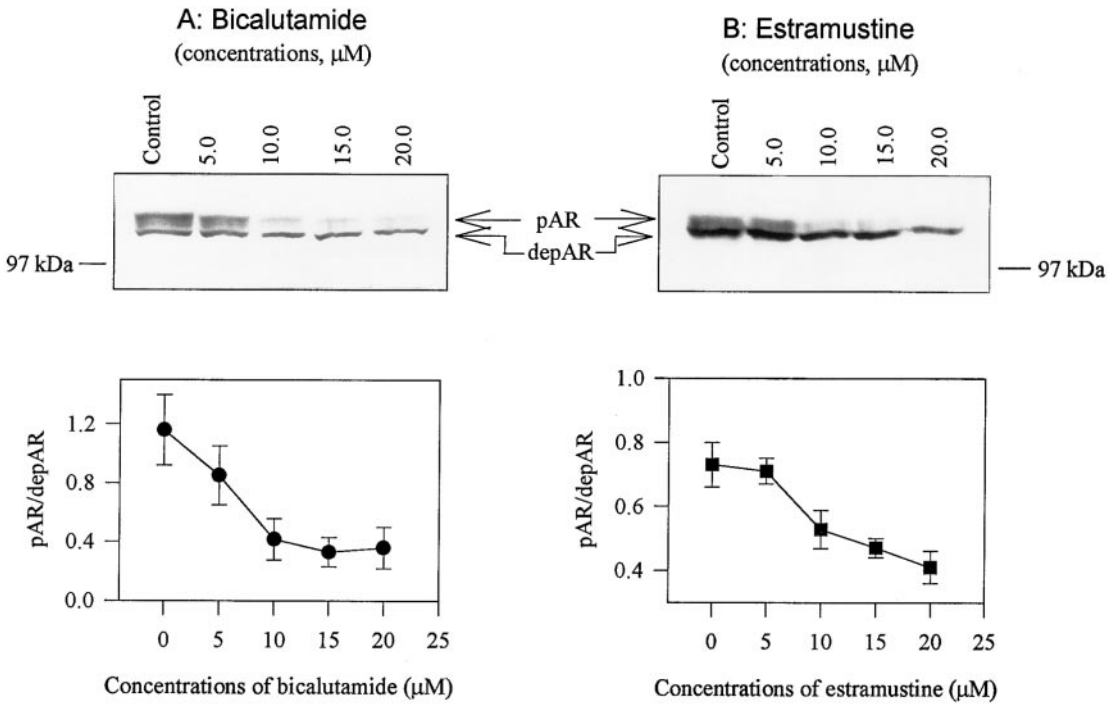

FIG. 5. Inhibition of androgen receptor phosphorylation by androgen antagonists in LNCaP cells. LNCaP cells at 65–75% confluence in RPMI 1640 medium containing 10% fetal bovine serum were treated with various concentrations of bicalutamide (A), and estramustine (B) for 24 hours. The cells were harvested, washed once with cold PBS, and proteins extracted as described under Materials and Methods. One hundred μg proteins were subjected for electrophoresis on 4%/7.5% stack SDS-polyarylamide for 4 hours. After electrophoresis, the proteins on the gel were electro-transferred onto nitrocellulose membrane and the phosphorylated- (105 kDa) and dephosphorylated- (99 kDa) androgen receptor proteins were immuno-detected by AR-specific antibody. The phosphorylated AR were significantly inhibited by both bicalutamide (A) and estramustine (B) in a concentration-dependent manner. The pAR/depAR (lower panels) was calculated from the density of the plots of AR determined from ECL films by an Imaging Densitometer Model GS-700. Data are the means ± SD of three separate experiments scored by the densitometer and normalized to Control.

receptor (lane 1 in Fig. 2A). The ratio of phosphorylated to dephosphorylated androgen receptors were approximately 1:4 in arrested LNCaP cells. The levels of AR phosphorylation were found to be a function of the androgen concentrations present in the medium and to the time of that exposure. DHT was an efficient stimulius for AR phosphorylation. A 1.5-fold increase in the level of the phosphorylated protein resulted from exposure to 1 nM of DHT and an 8.9-fold increase with 1.0 μM of the androgen. Interestingly, no significant increase of dephosphorylated AR was observed as illustrated in Fig. 2, whereas total AR proteins were moderately increased (1.5-fold). Thus, the pAR/depAR ratio went up markedly with the increase of the androgen concentrations (Fig. 2, panel A′). A dynamic study showed that a significant increase of receptor phosphorylation in synchronized LNCaP cells occured at 4 h and reached maximal levels at 6 h. The maximal level was maintained up to 48 h after exposure of the cells to 50 nM of the DHT (data not shown).

β-Estradiol also showed a significant stimulation of both AR expression and phosphorylation when LNCaP cells grown exponentially (non-synchronized) were treated with β-estradiol in normal culture conditions. This effect is different from DHT stimulation in that the increase of phosphorylated AR induced by β-estradiol parallels the increase of dephosphorylated form and the amount of total receptor proteins, even though the increase of pAR/depAR ratio is a function of the drug concentrations (Fig. 2, panel B′). The agonistic effect of β-estradiol is less than that of DHT.

Hydroxyflutamide is generally considered as androgen antagonist. However, as demonstrated in Fig. 2, panel C, this agent showed the same pattern as β-estradiol, moderately affecting both androgen receptor expression and phosphorylation in LNCaP cells.

As shown in Fig. 3, strongly stimulated the expression of prostate specific antigen (PSA)—a native AR target gene [32–34]. The levels of both mRNA and protein were significantly increased in a concentration-dependent manner when synchronized LNCaP cells were exposed to DHT ($P < 0.01$). Both mRNA and protein levels in arrested LNCaP cells were much lower than regular grown cells (Fig. 3, lane 1 in A compared to lane 1 in B or C). Moderate increases in the amount of mRNA and protein of PSA were obtained after treatment of LNCaP cells with different concentrations of β-estradiol or hydroxyflutamide. This find-

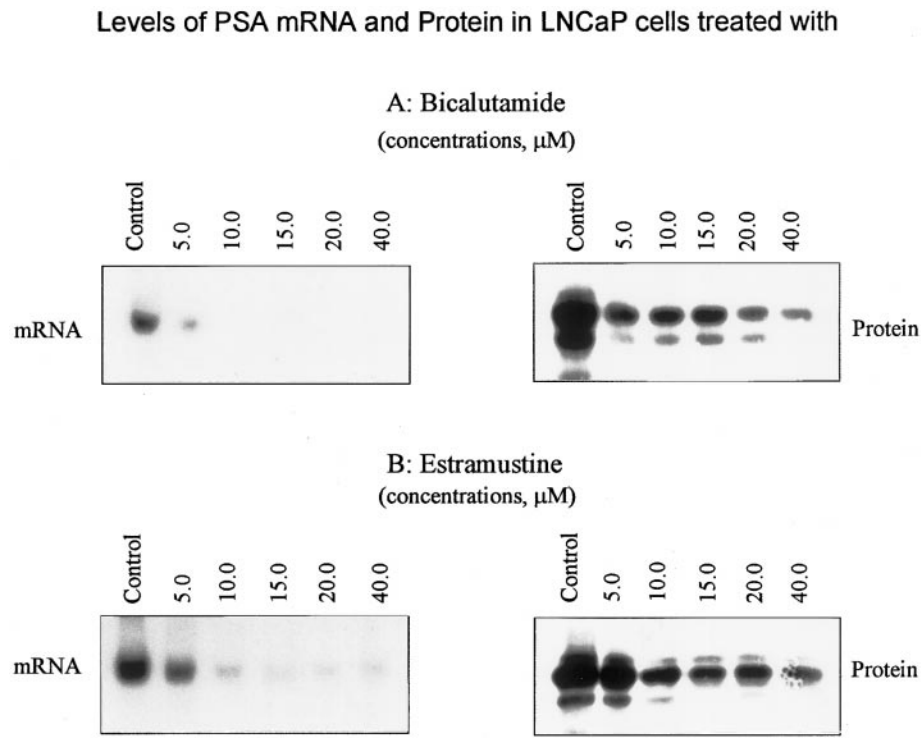

FIG. 6. Down-regulation of the expression of prostate specific antigen (PSA), a natural AR- target gene, by androgen antagonists. LNCaP cells at 65–75% confluence in RPMI 1640 medium containing 10% fetal bovine serum were treated with various concentrations of bicalutamide (panel A), and estramustine (panel B) for 24 hours. The cells were harvested, washed with cold PBS and total RNA or proteins extracted. For Northern assay, 20 μg of total RNA were separated on 1.2% agarose-formaldehyde gels and transferred to Zeta-Prpbe Blotting Membranes (Bio-Rad, Richmond CA). The double-strand PSA specific oligonucleotide probe, (5′-AGCCTAGAGAAGGCTGTGAGCCAA-GGAGGGAGGGTCTTCCTTTGGCATGGGATGGGGATGAAGTAAGGAGAGGGACT-3′), 5′-end-labeled was used for hybridization. For Western blotting assay, 100 μg cellular extracts cells were separated by 4%/7.5% stack SDS–PAGE, electro-transferred to nitrocellulose membrane, and immunoblotted with antibodies against PSA.

ing is also valid for wild type androgen receptors as demonstrated by a gene transactivation assay system. As shown in Fig. 4, a strong concentration-dependent induction of luciferase activity was obtained in the presence of various concentrations (1 nM to 1,000 nM) of DHT. As partial agonists, both β-estradiol and hydroxyflutamide exhibited a moderate and significant induction of the enzyme activities, respectively.

Androgen Antagonists

In contrast to androgen agonists, exposure of LNCaP cells to androgen antagonists in RPMI 1640 medium containing 10% FBS, would be expected to cause a significant concentration-dependent decrease in levels of phosphorylated androgen receptor. Figure 5 shows that this prediction is borne out. Bicalutamide and estramustine are typical androgen antagonists. After treatment of LNCaP cells with bicalutamide at a concentration as low as 5.0 μM, the level of phosphorylated AR decreased by approximately 3.5-fold ($P < 0.01$). The decrease of the pAR/depAR ratio was found to be a function of the concentration of bicalutamide. A similar result was also obtained when cells were treated with estramustine. Inhibition of AR phosphorylation resulting from the exposure to bicalutamide or to estramustine was paralleled down-regulation of PSA expression (Fig. 6). Substantial decreases in the levels of both PSA mRNA and protein in a concentration-dependent manner were obtained when the cells treated with either bicalutamide or estramustine. The levels of PSA mRNA decreased over 90% when LNCaP cells were treated with 5 μM or 10 μM of bicalutamide or estramustine ($P < 0.001$). Consistant with this finding, when HeLa cells were transfected with the wild type expression plasmid pAR0 and the AR-driven expression reporter-plasmid GRE-tk-LUC in the presence of various concentrations of either bicalutamide or estramustine (Fig. 7), no induction of luciferase activities were obtained. In contrast, a 98-fold increase of the enzyme activity was obtained in the presence of 100 nM of DHT ($P < 0.001$).

DISCUSSION

In this study, we demonstrated that androgen receptor phosphorylation/dephosphorylation plays a domi-

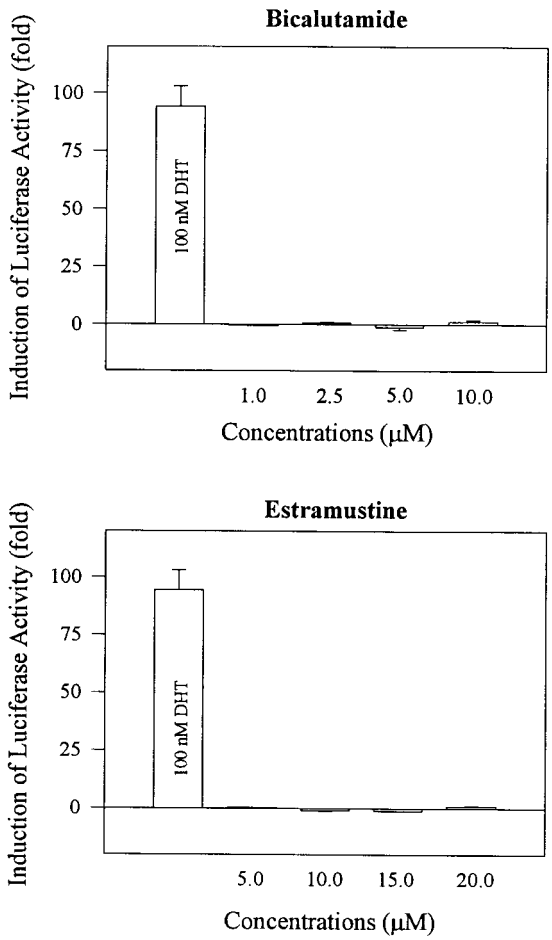

FIG. 7. Transactivation of androgen antagonists on the wild-type AR mediated luciferase reporter gene expression. 3×10^5 HeLa cells in RPMI 1640 medium containing 10% FBS were sown in 10 cm dishes and incubated at 37°C for 24 hrs. Ten μg w-pAR0 cDNA and 2 μg GRE-tk-LUC cDNA in 125 mM $CaCl_2$–HEPES buffer (0.14 M NaCl, 0.05 M, HEPES acid, 1.5 mM Na_2HPO_4 pH 7.05) were added to the culture and incubated for 16 hrs. The precipitates were washed out with pre-warmed PBS and refed with fresh RPMI 1640 medium containing 10% FBS. After incubation of the transfected cells for additional 32 hrs at 37°C in the presence of various concentrations of bicalutamide and estramustine, the cells were harvested and the cell extracts used for luciferase activity assay as described under Materials and Methods.

nant role in determination of activity of androgen receptor ligands as agonists or antagonists. If an agent can significantly stimulate AR phosphorylation, it will act as an androgen agonist. If an agent stimulates AR phosphorylation moderately or not at all, the drug will act as a partial agonist or have minimal effect on androgen receptors. In contrast, if an agent significantly inhibits AR phosphorylation, it will exhibit pure antagonistic effect. Thus the androgen receptor phosphorylation/dephosphorylation may serve as new molecular target for screening androgen antagonists for the treatment of prostate cancer. Of importance, although the LNCaP cell contained a mutated androgen receptor in codon 868 as assay system [35–36], this model also appears to be valid for the wild type androgen receptor as demonstrated in a gene transactivation assay using wild type AR expression plasmid and AR driven reporter GRE-tk-LUC.

Phosphorylation of proteins is a critical post-translational modification in signal transduction. Many steroid receptors have been shown to be phosphorylated upon hormone binding, suggesting that there is a link between the phosphorylation status and the activation state of the receptor [24]. The androgen receptor is a transcription factor whose function is androgen dependent. Hormone binding initiates the process of receptor transformation that eventually results in specific binding of the steroid-receptor complex to hormone-responsive elements of steroid-regulated genes and in transcriptional regulation [37–39]. We and others recently demonstrated that the binding of AR to the steroid receptor binding consensus (SRBC) is essential for the activation of AR mediated gene expression. [28, 34]. In this study, we have shown that DHT, a typical agonist, stimulated receptor protein phosphorylation (Fig. 2). Stimulation of androgen receptor phosphorylation by androgen agonists results in up-regulation of AR-mediated gene expression in both LNCaP cells and wt-pAR cDNA transfected HeLa cells (Figs. 2–4). In contrast, inhibition of AR phosphorylation by androgen antagonists caused down-regulation of AR target gene expression (Figs. 5–7). These data indicate that the function of androgen receptors is strongly correlated with the phosphorylation status of the receptors, rather than the level of total AR proteins (Fig. 2, panels B and C). Furthermore, the data suggests that the phosphorylated androgen receptor is the form of the receptor transcriptionally active in regulation.

Previous reports suggested a role of receptor phosphorylation in receptor cycling between nucleus and cytoplasm [40–42]. The antiandrogens, cyproterone acetate, estrogen and progesterone have been reported not only to promote nuclear transport, but enhance transcriptional activation by AR [34, 40]. Our studies indicate that these antiandrogens, including β-estradiol, can stimulate AR phosphorylation. Since we have shown that DHT facilitates the phosphorylated androgen receptor transport from cytoplasm to nucleus, indicating that the phosphorylation of the androgen receptor is necessary for the protein transport [42], this phenomenon may explain why only the agents that inhibit AR phosphorylation show true androgen antagonistic effects.

The mechanisms by which regulation of androgen receptor phosphorylation by androgen agonists and antagonists occurs are not well established. The effect of estradiol on the androgen receptor and prostate specific antigen secretion have been reported recently and parallels our findings [43]. The authors indicated that this activation involves sex hormone-binding globulin (SHBG) [43]. Because estradiol-SHBG stimulates SHBG receptor to increase intracellular cAMP [44–45],

and both cAMP and a cell- permeable analog of cAMP, 8-(4-chlorophenylthio)-cAMP can stimulate PSA secretion, the protein kinase A (PKA) signal pathway may be involved in the regulation. Our model is also consistent with the recent report that down-regulation of two endogenous PSA and β1-subunit of Na/K-ATPase were followed by dephosphorylation of the androgen receptor induced by forskolin, a PKA stimulator [23]. Whether or not there is a link between estradiol-SHBG-cAMP-PKA and AR phosphorylation need to be defined. The effects of androgen agonists and antagonists on the PKA pathway are currently under investigation.

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