

Suppression *Versus* Induction of Androgen Receptor Functions by the Phosphatidylinositol 3-Kinase/Akt Pathway in Prostate Cancer LNCaP Cells with Different Passage Numbers*

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The phosphatidylinositol 3-kinase (PI3K)/Akt pathway controls several important biological functions, such as cell growth regulation, apoptosis, and migration. However, the way in which PI3K/Akt controls androgen receptor (AR)-mediated prostate cancer cell growth remains unclear and controversial. Here, we demonstrate that the PI3K/Akt pathway regulates AR activity in a cell passage number-dependent manner. Specifically, PI3K/Akt pathway can suppress AR activity in androgen-dependent LNCaP cells with low passage numbers. In contrast, it can also enhance AR activity in LNCaP cells with high passage numbers. Furthermore, we also demonstrate that insulin-like growth factor-1 can activate the PI3K/Akt pathway that results in the phosphorylation of AR at Ser²¹⁰ and Ser⁷⁹⁰. The consequence of these events may then change the stability of AR protein. Together, our results demonstrate that the PI3K/Akt pathway may have distinct mechanisms to modulate AR functions in various stages of prostate cancer cells and that a combined therapy of antiandrogens and anti-PI3K/Akt inhibitors may be worth considering as a future therapeutic approach to battle prostate cancer.

Prostate cancer is the second leading cause of cancer-related death among men in the United States. The normal prostate and prostate cancers at early stages require androgen for growth and survival. In addition to androgen signaling, which plays an essential role in survival of prostate cancer, the phosphatidylinositol 3-kinase (PI3K)¹/Akt pathway represents another important survival signal for prostate cancer cells. It appears that these two pathways can compensate for each other in growth regulation of prostate cancer LNCaP cells, because androgen treatment can rescue cells from apoptosis induced by application of PI3K inhibitors (1). Furthermore,

activation of the PI3K/Akt pathway protects cells from apoptosis induced by serum starvation and androgen deprivation (2).

Recent rapid progress of the PI3K/Akt signal pathway studies, as well as its influence on the androgen receptor (AR)-mediated prostate cancer growth, has resulted in many exciting yet controversial results. Here we address these controversial results by summarizing Akt-AR-related results and provide new data, as well as possible explanations for the distinct roles of the PI3K/Akt pathway in AR-mediated prostate cancer growth. Particular emphases will be: 1) Akt suppresses *versus* induces AR activity, 2) Akt phosphorylation sites on AR protein, and 3) promotion of AR degradation by the PI3K/Akt pathway.

EXPERIMENTAL PROCEDURES

Reagents—pCDNA3 cAkt (3) and mutant AR S210A/S790A were described previously (4). pCDNA3-PTEN was a gift from Dr. Charles L. Sawyers, and pGEX-KG-PTEN was from Dr. Frank B. Furnari. Insulin-like growth factor-1 (IGF-1) and LY294002 was from Calbiochem. 5 α -Dihydrotestosterone (DHT), doxycycline (Dox), and cycloheximide were from Sigma. The anti-AR polyclonal antibody, NH27, was produced as described previously (3). The mouse monoclonal PTEN and prostate-specific antigen (PSA) antibodies and the goat polyclonal β -actin antibody were from Santa Cruz Biotechnology. The mouse monoclonal Akt and phospho-Akt (Ser⁴⁷³) antibodies were purchased from Cell Signaling.

Cell Culture and Transfections—DU145, PC-3, and COS-1 cell lines were maintained in Dulbecco's minimum essential medium containing penicillin (25 units/ml), streptomycin (25 μ g/ml), and 10% fetal calf serum (FCS). LNCaP cells were maintained in RPMI 1640 with 10% FCS. Transfections were performed using SuperFectTM according to standard procedures (Qiagen).

Luciferase Reporter Assays—Luciferase reporter assay was as described previously with some modifications (5). The cells were transfected with plasmids in 10% charcoal-stripped serum (CSS) medium for 16 h and then treated with ethanol or 10 nM DHT for 16 h. The cells were lysed, and luciferase activity was detected by the dual luciferase assay according to standard procedures (Promega). Mouse mammary tumor virus-luciferase (MMTV-luc), which contains the AR response elements, was used as an AR transactivation reporter. The results were normalized by *Renilla* luciferase activity (pRL-SV40-luc), and the data represent means \pm S.D. from triplicate sets of three independent experiments.

LNCaP Stable Transfectants—For the Dox-inducible system, PTEN was released from pGEX-KG-PTEN using EcoRI digestion and inserted into pBIG2i vector. The LNCaP cells were transfected with pPIB2i PTEN for 24 h. The cells were selected using 100 μ g/ml hygromycin. Individual colonies were picked and grown until 70% confluent followed by 4 μ g/ml Dox treatment. The positive clones were confirmed by Western blot analysis.

Generation of an Anti-phospho-AR Antibody (Ser²¹⁰)—The phospho-AR peptide (SGRAREADGAPTSSKD) was generated and used for generation of anti-phospho-AR (Ser²¹⁰) antibody (clone 156C135.2) according to the manufacturer's procedures (AndroScience, San Diego, CA).

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¹ The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; AR, androgen receptor; cAkt, constitutively active form of Akt; CSS, charcoal-stripped serum; DHT, 5 α -dihydrotestosterone; Dox, doxycycline; E3 ligase, ubiquitin-protein isopeptide ligase; FCS, fetal calf serum; IGF, insulin-like growth factor; MMTV-luc, mouse mammary tumor virus-luciferase; PSA, prostate-specific antigen; PTEN, phosphatase and tensin homolog deleted on chromosome ten.

Immunoprecipitation and Western Blot Analysis—Immunoprecipitation and Western blotting were performed as previously described (3). Cell extracts (1 mg) were immunoprecipitated with the indicated antibody. The immunocomplexes were subjected to 8% SDS-PAGE and immunoblotted with the indicated antibody.

Cell Growth Assay—LNCaP cells (2×10^4) with different passage numbers were grown in 12-well plates, transfected with parent vector or the constitutively active form of Akt (cAkt), and cultured in 10% CSS medium after 3 h of transfection. Cells were stained by trypan blue on different days, as indicated, and cell numbers were determined by direct counting on hemacytometers. The data represent means \pm S.D. from triplicate sets of three independent experiments.

RESULTS AND DISCUSSION

Cell-specific and Passage-dependent Effect of PI3K/Akt Signaling on AR Activity—The PI3K/Akt pathway plays an important role in cell growth, survival, adhesion, and migration in a variety of cell types. In prostate cancer LNCaP cells, the PI3K/Akt pathway is a dominant survival signal pathway for cells, and inhibition of this pathway by PI3K inhibitors leads to cell growth arrest and apoptosis (6). Recently, it has been demonstrated that the PI3K/Akt pathway regulates AR activity and phosphorylation (3, 7). Although activation of the PI3K/Akt pathway suppresses AR activity in androgen-independent prostate cancer DU145 cells (3), other reports also demonstrated that the PI3K/Akt pathway enhances AR activity in androgen-dependent prostate cancer LNCaP cells (7, 8). Although the detailed mechanisms of these differential effects remain unclear, it is possible that different cell types may have differential PI3K/Akt effects on AR activity, which led to our examination of various prostate cancer cells.

Interestingly, we found that the PI3K/Akt pathway could regulate AR activity in a passage-dependent manner in LNCaP cells. cAkt suppressed AR activity in low passage number LNCaP cells (passage number 25) (Fig. 1A, P25) but enhanced AR activity in high passage number LNCaP cells (Fig. 1B, P60), in reporter gene assays. It should be noted that the reporter gene activation by androgen was much higher in higher passage LNCaP cells (Fig. 1, compare panel B with A). The reason for this phenomenon is currently unknown. This may suggest that some factors that preferentially exist or are over-expressed in higher passage LNCaP cells may contribute to the enhancement of this androgen response. Blockage of the PI3K/Akt pathway by LY294002 slightly enhanced AR activity in low passage number LNCaP cells but suppressed AR activity in high passage number LNCaP cells (Fig. 1, A and B, 4th lanes on right). Although LY294002 has been widely used as a PI3K inhibitor, we cannot rule out the possibility that at 20 μ M this reagent may affect other kinases that influence AR activity. We performed a Western blot assay to examine the role of the PI3K/Akt pathway in regulating AR target gene expression. Even though LY294002 only marginally enhanced AR activity in low passage LNCaP cells in the reporter gene assays (Fig. 1A), it apparently increased androgen-induced PSA expression, an AR target gene, in low passage number LNCaP cells (Fig. 1C). Similar to the reporter gene assay, LY294002 suppressed PSA expression in high passage number LNCaP cells (Fig. 1C). Moreover, cAkt reduced androgen-induced PSA expression in low passage number LNCaP cells but slightly enhanced PSA expression in high passage number LNCaP cells (Fig. 1D). These results suggest that distinct passage numbers of LNCaP cells might influence the effects of the PI3K/Akt effect on AR activity. Using PC-3 cells, Thompson *et al.* (9) also demonstrated that the PI3K/Akt pathway could suppress AR activity, which is consistent with our data (Fig. 1A) and with early reports using DU145 cells as the cell model (3). Together, these results demonstrate that the effects of the PI3K/Akt signaling pathway on AR activity may change with different prostate

cancer cell lines and within the same cell line at different passage numbers.

At early stages, prostate cancer cells may need androgen signaling for growth and survival. Androgen ablation or anti-androgen treatment may lead to cell growth arrest and apoptosis of these androgen-sensitive cancer cells (1). The basal activity of the PI3K/Akt pathway in the early stage prostate tumors is lower and may not be adequate to play a major role in the maintenance of prostate cancer cell growth and survival in the absence of concurrent androgen signaling. However, androgens may become less important factors for tumor cell growth and survival in late stage prostate cancer. In contrast, tumor cells at this later stage have higher basal activity of the PI3K/Akt pathway, which may contribute to the development of prostate cancer progression by preventing cells from apoptosis (10).

To support the above hypothesis, we found that the low passage LNCaP cells possess a low basal level of Akt activity (Fig. 1E). In contrast, high passage LNCaP cells show a strong basal Akt activity (Fig. 1E). Our data show that Akt negatively modulates AR activity in low passage LNCaP cells (Fig. 1A), suggesting that LNCaP cells at this early stage require more androgen to compensate for the suppressive effect of the low basal Akt activity and that the low basal Akt activity may not be sufficient to provide the survival signal necessary for maintenance of cell growth and survival.

To determine whether Akt is a determining factor for the androgen reliance of LNCaP cell growth, we cultured LNCaP cells in CSS medium lacking androgen to compare the growth pattern of LNCaP cells at different passage numbers in the presence or absence of cAkt. As expected, early passage LNCaP cells, with low basal activity of Akt, showed little cell growth in the CSS medium (Fig. 1F), suggesting that the androgens are important for cell growth. In contrast, high passage LNCaP cells, with higher basal Akt activity, grew much faster than early passage LNCaP cells (Fig. 1F), suggesting less dependence on the androgens. Elevation of the basal Akt activity by transfection of cAkt significantly increased the LNCaP cell growth at both cell passages, although the effect of cAkt was more profound in the early passage LNCaP cells (Fig. 1F). Thus, the Akt signal may be a key factor in driving LNCaP cell growth and survival at this late stage with weaker androgen reliance.

Considering the biphasic effect of PI3K/Akt and androgen signaling on the progression of prostate cancer, we found that androgen ablation therapy, which removes most of the androgens available for prostate tumors, may result in increased activation of the PI3K/Akt pathway, promoting tumor cell growth and survival. This hypothesis is further supported by a recent report (11) showing that the PI3K/Akt pathway is elevated in LNCaP cells cultured in androgen-depleted medium. It is possible that increased PI3K/Akt signaling upon loss of androgen signaling may contribute to the failure of androgen ablation therapy at later stages of prostate cancer. For this reason, using a combination therapy that includes androgen ablation at early stages and suppression of the PI3K/Akt pathway at later stages may provide a better strategy for battling prostate cancer.

The Effect of PI3K/Akt Signaling on AR Phosphorylation—AR is a phosphoprotein, and its activity can be modulated by phosphorylation (12). We demonstrated that activation of PI3K/Akt pathways by IGF-1 in COS-1 cells induces AR phosphorylation *in vivo* (3). The *in vitro* kinase assay further revealed that Akt, but not PI3K, phosphorylates AR at Ser²¹⁰ and Ser⁷⁹⁰ residues, which are the Akt consensus phosphorylation sites (3). Overexpression of cAkt, but not the kinase-dead Akt

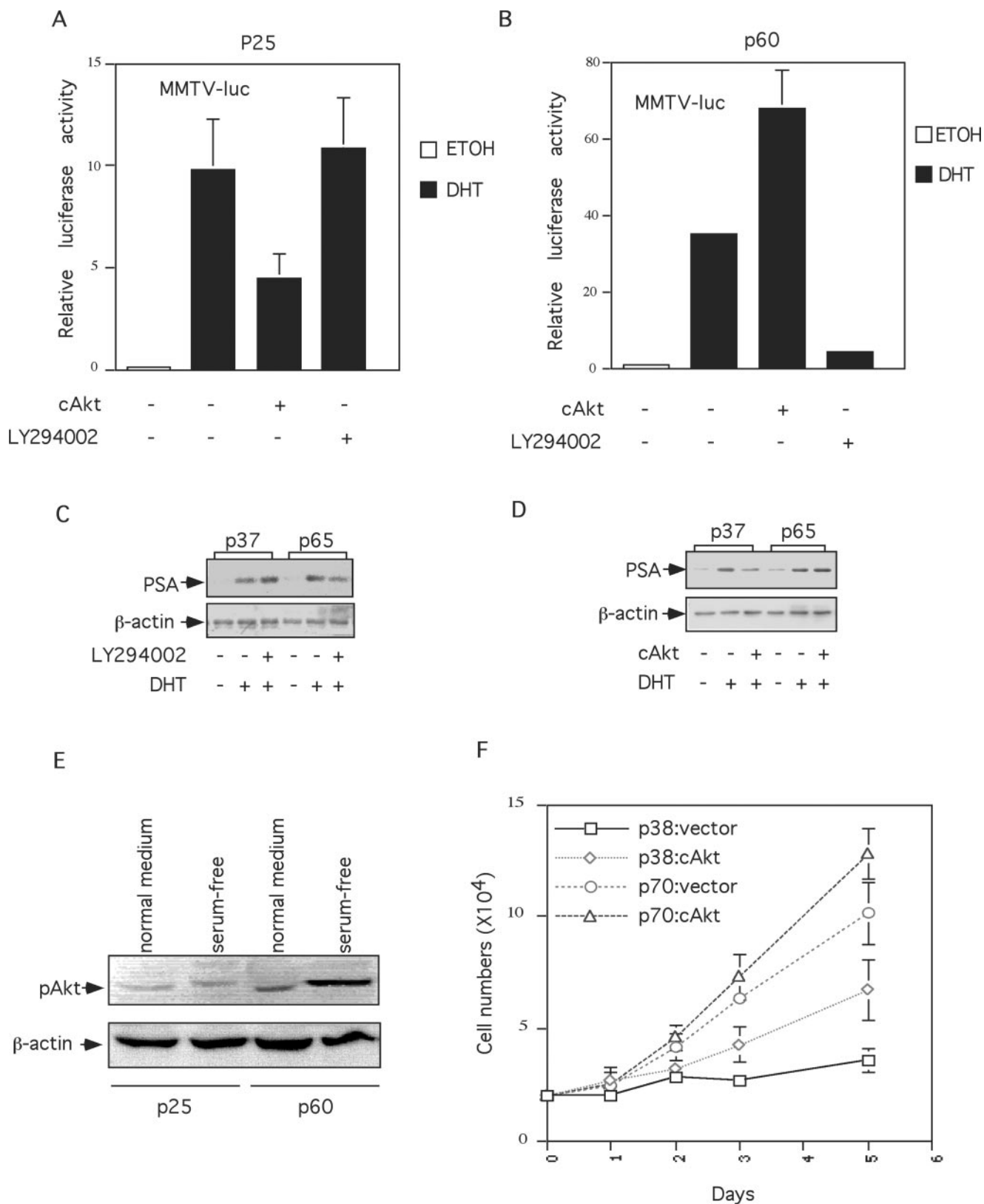


FIG. 1. Passage-dependent effect of the PI3K/Akt pathway on AR transactivation in LNCaP cells. *A*, LNCaP cells (passage 25 (P25)) were transfected with MMTV-luc along with plasmids, as indicated, for 16 h, and cells were then treated with EtOH or 10 nM DHT in the presence or absence of 20 μ M LY294002 for 24 h. The cells were harvested for luciferase assay. *B*, the same experiment as described in *A* was carried out with LNCaP cells at passage 60 (P60). *C*, LNCaP cells at different passage numbers were cultured in 10% CSS for 24 h, treated with 20 μ M LY294002 10 min prior to 10 nM DHT treatment for another 24 h, and harvested for Western blot assay. *D*, LNCaP cells at different passage numbers were transfected with vector or cAkt for 24 h, and cells were treated with EtOH or 10 nM DHT for another 24 h, followed by harvesting cells for Western blot assay. *E*, different passage numbers of LNCaP cells were cultured in the 10% FCS medium or serum-free medium for 2 days, and the cells were harvested for Western blot analysis. Akt activity is determined by the levels of Akt phosphorylation (pAkt) using anti-phospho-Akt (Ser⁴⁷³) antibody. *F*, LNCaP cells at different passages were transfected with vector or cAkt and cultured in CSS medium. Cells were stained by trypan blue on different days, and cell numbers were determined as described under "Experimental Procedures."

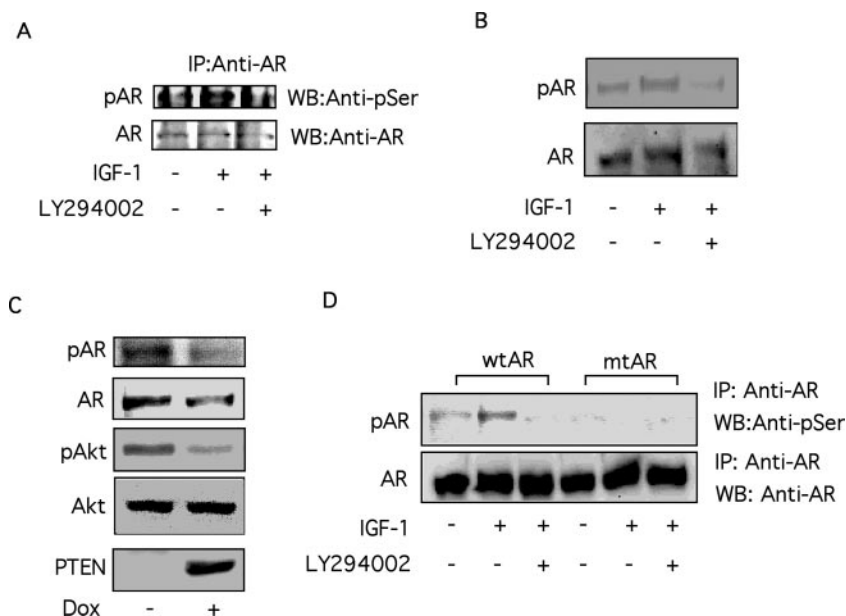


FIG. 2. Activation of the PI3K/Akt pathway induces AR phosphorylation *in vivo*. *A*, LNCaP cells at passage 38 were serum-starved for 2 days, incubated with 20 μ M LY294002 for 30 min prior to treatment with 100 μ g/ml IGF-1 for 4 h, and then harvested for immunoprecipitation (IP) with AR antibody. *WB*, Western blot; *Anti-pSer*, anti-phosphoserine antibody. *B*, LNCaP cells at passage 38 were treated as described in *A* and harvested for Western blot analysis. Total AR protein was blotted using an anti-AR antibody (*AR*), and AR phosphorylation was detected using an anti-phospho-AR (Ser²¹⁰) antibody (*pAR*). *C*, PTEN-inducible LNCaP cells at passage 40 were cultured in 10% FCS, treated with 4 μ g/ml Dox for 24 h, treated with 100 μ g/ml IGF-1 for 4 h, and then harvested for Western blot analysis. *D*, COS-1 cells were transfected with wild-type (*wtAR*) or mutant AR (*mtAR*, Ser²¹⁰Ala/Ser⁷⁹⁰Ala) for 16 h, serum-starved for 24 h, and then incubated with 20 μ M LY294002 for 30 min prior to treatment with 100 μ g/ml IGF-1 for 4 h. The cells were then harvested for immunoprecipitation with anti-AR antibody and Western blot analysis. *Anti-pSer*, anti-phosphoserine antibody.

mutant (dAkt), induced AR phosphorylation *in vivo*, and mutations at the consensus serine residues reduced Akt-mediated AR phosphorylation (3). Consistent with our results, Wen *et al.* (7) also found that Akt associated with AR and phosphorylated AR at Ser²¹⁰ and Ser⁷⁹⁰ *in vitro*.

We and others (3, 7) and have found that Akt can phosphorylate AR at Ser²¹⁰ and Ser⁷⁹⁰. However, Gioeli *et al.* (13) found that Akt fails to phosphorylate AR at Ser²¹⁰ and Ser⁷⁹⁰ in LNCaP cells. They also found that PI3K inhibitor LY294002 did not change the levels of AR phosphorylation in a two-dimensional gel electrophoresis assay (13). These contrasting results may be because of the use of different cell lines (COS-1 versus LNCaP cells) to test AR phosphorylation under various transfection and treatment conditions. Alternatively, another explanation for the discrepancy may be that the overexpression of Akt via transient transfection may produce protein levels that are far higher than that seen under physiological conditions.

To determine whether gene overexpression was a confounding factor in the interpretation of our AR phosphorylation assays, we used IGF-1 to activate endogenous PI3K/Akt and therefore mimic physiological conditions. As shown in Fig. 2*A*, we demonstrated that IGF-1 treatment induced AR phosphorylation in LNCaP cells (passage number 38), and adding the PI3K inhibitor LY294002 blocked IGF-1-mediated AR phosphorylation, suggesting that the PI3K/Akt pathway is involved in the phosphorylation of AR. Using a site-specific anti-phosphoserine AR antibody, AR phosphorylation at Ser²¹⁰ was detected when LNCaP cells were treated with IGF-1 (Fig. 2*B*). Moreover, using the Dox-inducible system we generated the inducible PTEN clone, a tumor suppressor that antagonizes the PI3K/Akt pathway (14), in LNCaP cells at passage number 40. PTEN expression induced by Dox treatment inhibited Akt activation and AR phosphorylation at Ser²¹⁰ (Fig. 2*C*). IGF-1 also induced wild-type AR phosphorylation in COS-1 cells (Fig. 2*D*, *wtAR*), and LY294002 blocked the IGF-1-mediated phosphoryl-

ation. In contrast, IGF-1 did not induce phosphorylation of the mutant AR (S210A/S790A), in which two Akt consensus sites were mutated from Ser to Ala (Fig. 2*D*, *mtAR*). These data therefore strongly support our early findings that the PI3K/Akt pathway activated by IGF-1 mediates AR phosphorylation at Ser²¹⁰ and Ser⁷⁹⁰ (3). In contrast, Gioeli *et al.* (13) did not add growth factors such as IGF-1 to activate the PI3K/Akt pathway. It is therefore possible that the level of the Akt activity in LNCaP cells may not be sufficient to induce AR activity, given that the basal level of Akt activity is low in early passage LNCaP cells (Fig. 1*E*) in which AR phosphorylation by Akt may not occur and may require the addition of growth factors to amplify the PI3K/Akt signal.

Regulation of AR Protein Turnover by the PI3K/Akt Pathway—AR controls several biological functions, including prostate cell growth and apoptosis (12). However, the mechanism by which AR maintains its stability for proper function remains largely unknown. Growing evidence implies that AR may be degraded by the ubiquitin-proteasome pathway (15–17). In support of this notion, we have recently demonstrated that activation of the PI3K/Akt pathway induces AR ubiquitylation and subsequent degradation by the 26 S proteasome (4). The effect of Akt on AR ubiquitylation and degradation seems to be dependent on AR phosphorylation, because activation of Akt did not induce ubiquitylation or degradation of mutant AR, which lacks Akt-mediated phosphorylation. Interestingly, the AR mutant was remarkably stable compared with wild-type AR, suggesting that phosphorylation of AR by Akt reduces AR stability (4).

Mdm2, a Ring Finger protein, consists of an E3 ligase and suppresses p53 activity by regulation of ubiquitylation and degradation of p53 (18, 19). In addition to regulation of p53 function, Mdm2 can also regulate AR activity via regulation of ubiquitylation and degradation of the AR (4). We further identified Mdm2 as an E3 ligase for AR and a mediator for Akt-induced AR ubiquitylation and degradation (4). AR protein

normally undergoes degradation several hours after its synthesis in cells. However, the signals responsible for AR turnover remain unclear. Based on our data, we propose that the PI3K/Akt/Mdm2 pathway represents an important mechanism to control AR turnover rate. When LNCaP cells are cultured in normal medium, growth factors such as IGF-1 can activate the PI3K/Akt pathway, which may then be responsible for the turnover of AR protein. In support of this hypothesis, blockage of the PI3K/Akt pathway by LY294002 in LNCaP cells leads to increased AR protein levels (4).

Because the PI3K/Akt pathway differentially regulates AR

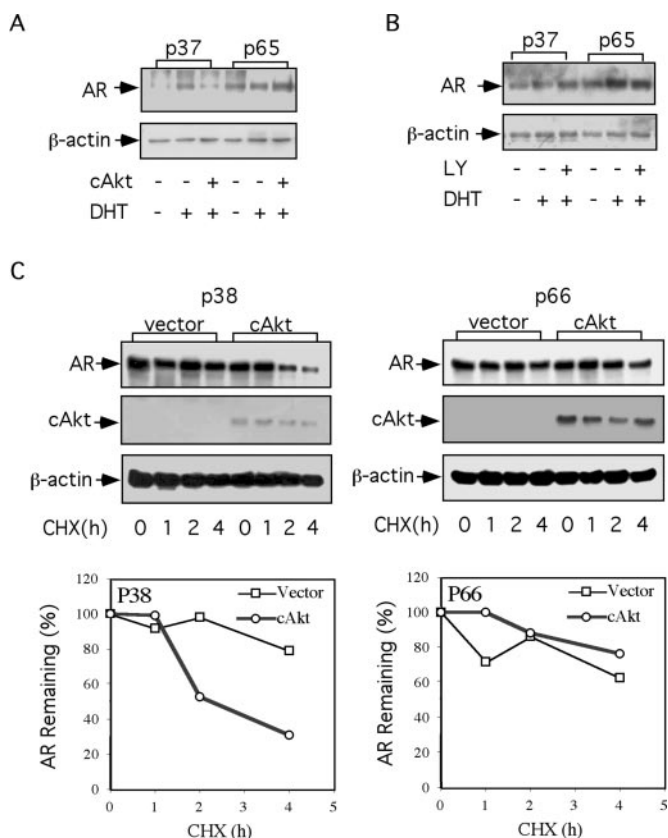


FIG. 3. Distinct regulation of AR protein degradation by the PI3K/Akt pathway at various passage numbers of LNCaP cells. A, LNCaP cells at different passage numbers were transfected with vector or cAkt for 24 h, and cells were treated with EtOH or 10 nM DHT for another 24 h followed by harvesting for Western blot assay. B, LNCaP cells at different passage numbers were cultured in 10% CSS medium for 24 h, treated with 20 μ M LY294002 10 min prior to 10 nM DHT treatment for another 24 h, and harvested for Western blot assay. C, LNCaP cells at different passage numbers were transfected with vector or cAkt for 24 h, and cells were treated with 20 μ g/ml cycloheximide (CHX) for different times, as indicated, in 10% FCS medium followed by harvesting for Western blot assay.

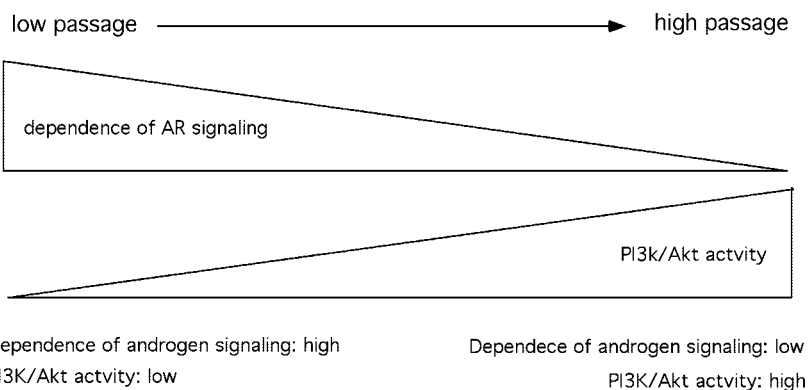
FIG. 4. Model for PI3K/Akt pathway on AR signaling in prostate LNCaP cells. In low passage LNCaP cells, the basal activity of PI3K/Akt signaling is low, and cells are strongly dependent on androgen signaling for growth and survival. In contrast, in high passage LNCaP cells, the basal activity of the PI3K/Akt pathway is high, and cells are less dependent on androgen signaling. The PI3K/Akt pathway not only provides the growth and survival signals for prostate cancer cells but also enhances AR activity in high passage LNCaP cells via an unknown mechanism.

activity in different passage numbers of LNCaP cells (Fig. 1, A–D), we next determined whether the PI3K/Akt pathway has a distinct effect on AR degradation in these cells. cAkt down-regulated AR protein levels in low passage LNCaP but slightly enhanced AR protein levels in high passage LNCaP cells (Fig. 3A). In contrast, LY294002 enhanced AR protein levels in low passage LNCaP cells but slightly reduced AR protein levels in high passage LNCaP cells (Fig. 3B). To prove the role of Akt in regulation of AR degradation directly, we examined the effect of Akt on AR protein stability. Overexpression of cAkt in low passage LNCaP cells led to accelerated AR degradation (Fig. 3C, left panel). cAkt did not promote AR degradation in high passage LNCaP cells but slightly enhanced AR stability (Fig. 3C, right panel), which indeed correlated with the effect of PI3K/Akt on AR transcriptional activity in Fig. 1, A–D, and AR protein levels in Fig. 3, A and B. These results suggest that the PI3K/Akt pathway induces AR degradation in low passage LNCaP cells but not in high passage LNCaP cells.

Exactly how the cell passage number affects PI3K/Akt modulation of AR activity remains unclear. However, it is possible that the variant basal Akt activity levels among cells of different passages may be a key factor contributing to this phenomenon. Alternatively, different cell contexts may exist in LNCaP cells of different passage numbers contributing to the modulating effect of the PI3K/Akt pathway on AR activity. Because Mdm2 is a downstream effector of the PI3K/Akt pathway, it would be useful to determine whether the levels of Mdm2 in various passage numbers of LNCaP cells are significantly different. A more global assay, such as proteomics, may be required to elucidate the factors that may contribute to this phenomenon.

Summary—On the basis of this study and our previous reports (3, 4) we propose a model for the PI3K/Akt pathway action on the regulation of AR activity in prostate cancer LNCaP cells (Fig. 4). The PI3K/Akt pathway exhibits a cell passage-dependent regulation of AR activity. In low passage LNCaP cells, the basal activity of PI3K/Akt signaling is low and cells are strongly dependent on androgen signaling for growth and survival. However, in high passage LNCaP cells, the basal activity of the PI3K/Akt pathway is high and cells are less dependent on androgen signaling. The PI3K/Akt pathway not only provides the growth and survival signals for prostate cancer cells, but it also enhances AR activity in high passage LNCaP cells via an unknown mechanism.

Several important questions have been raised throughout this study. First, what are the factors that determine the differential effects of the PI3K/Akt pathway on AR activity in different passage numbers of LNCaP cells? Second, what is the molecular mechanism by which the PI3K/Akt pathway enhances AR activity in the high passage LNCaP cells? Future studies should focus on these issues, and systematic analysis is required to solve these puzzles. Finally, the PI3K/Akt pathway



provides a survival and growth signal for prostate cancer cells and induces AR activation in the presence or absence of androgen. Given its activation during prostate cancer progression, PI3K/Akt signaling may represent a new chemotherapeutic target with the potential to be particularly effective. A therapy that suppresses the PI3K/Akt pathway combined with classic androgen ablation therapy could reach the maximal effect in the battle against prostate cancer.

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