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13. ABSTRACT (Maximum 200 Words)

Data suggest androgen/androgen receptor (AR) may be involved in proliferation of prostate cancer, but opposite roles of in inhibition of cell growth and apoptosis are documented. The detailed mechanism of how androgen/AR functions in apoptosis, remains unclear. Serine/threonine kinase (Akt) plays a role in promoting cell survival through anti-apoptotic effects. Akt was found active in prostate cancer LNCaP cells and plays an essential role for survival. Our preliminary data demonstrated that Akt phosphorylates AR at Ser210 and Ser790. Mutation at Ser210 results in reversion of Akt-mediated suppression of AR transactivation. Activation of phosphatidylinositol-3-OH kinase/Akt pathway results in the suppression of AR target genes. Our hypothesis is that Akt may control androgen/AR-induced apoptosis by phosphorylating and inhibiting AR. In this past year our Akt studies have led into study of the PTEN pathway. We have shown 1) via PI3K/Akt-dependent pathway, PTEN regulates AR activity in high passage LNCaP cells and suppresses AR activity in the early passage LNCP cells, 2) PI3K/Akt pathway promoted AR ubiquitylation, leading to AR degradation, and 3) restoration of AR function or PI3K/Akt pathway rescues cells from PTEN-induced apoptosis.

Our project's success may enhance our understanding of cross-talk between Akt and androgen/AR pathway on prostate cancer progression.

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INTRODUCTION:

Most data suggest androgen/AR may be involved in proliferation of prostate cancer, however opposite roles of androgen/AR in inhibition of cell growth and apoptosis are also documented. The detailed mechanism of how androgen/AR functions in apoptosis, however, remains unclear. A serine/threonine kinase (Akt) was demonstrated to play a role in promoting cell survival with anti-apoptotic effects. Akt was also found to be constitutively active in prostate cancer LNCaP cells and play an essential role for LNCaP survival. Our hypothesis is that Akt may control androgen/AR-induced apoptosis by phosphorylating and inhibiting AR. Our aims are 1) to prove that Akt can promote AR degradation via phosphorylation of AR *in vivo*, 2) to dissect the molecular mechanism by which Akt promotes AR protein degradation, 3) to determine whether Akt can suppress androgen/AR-induced cell growth inhibition and apoptosis, and 4) to generate site-specific phospho-AR antibodies and use these Abs to monitor the AR phosphorylation status and their relationship to the progression of prostate cancer in archival human tissues. Our project's success may enhance our understanding of cross-talk between Akt and androgen/AR pathway on prostate cancer progression.

BODY:

Much of our progress is summarized in the manuscript published in the *J. Biol. Chem.* "Suppression vs induction of androgen receptor functions by the phosphatidylinositol 3-Kinase/Akt pathway in prostate cancer LNCaP cells with different passage numbers, by Hui-Kuan Lin, Yueh-Chiang Hu, Lin Yang, Saleh Altuwaijri, Yen-Ta Chen, Hong-Yo Kang and Chawnshang Chang. *J. Biol. Chem.* 278, 50902-50907. The shortened abstract is below, and the entire manuscript is attached as Appendix B.

J. Biol. Chem Abstract: The phosphatidylinositol 3-kinase (PI3K)/Akt pathway controls several important biological functions, such as cell growth regulation, apoptosis, and migration. However, how 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, can suppress AR activity in androgen-dependent LNCaP cells with low passage numbers and also enhance AR activity in LNCaP cells with high passage numbers. We also demonstrate that insulin-like growth factor-1 (IGF-1) can activate the PI3K/Akt pathway that results in the phosphorylation of AR at S210 and S790 to 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 a combined therapy of antiandrogens and anti-PI3K/Akt inhibitors may be worth consideration as future therapeutic approaches to battle the prostate cancer.

Narrative on Specific progress in the Aims.

Aim 1: To prove that Akt can promote AR degradation via phosphorylation of AR *in vivo*. Our preliminary studies indicated that Akt phosphorylates AR *in vitro* and could suppress AR transactivation in prostate cancer cells. Because phospho-inositol 3 kinase (PI3K). is an upstream activator of Akt, we applied the PI3K inhibitor LY294002 in LNCaP cells to block the PI3K/Akt pathway, to see whether AR expression and activity can be really influenced by this signaling pathway. Our studies in year 1 indicated that the blockade of this PI3K/Akt pathway causes increased AR expression and activity, proving the *in vivo* phosphorylation of AR by Akt through the PI3K activation pathway. See attached *J. Biol. Chem* manuscript. Our additional studies in 2003 led us into studies involving the PTEN pathway and its relationship to our Akt studies. See Figures in Appendix A. The most relevant studies are those below which are being written up for submission to *Cancer Research.*

Aim 2: To dissect the molecular mechanism by which Akt promotes AR protein degradation. The same *in vivo* studies described in Aim 1 above are also mechanistic studies. See studies in *J. Biol. Chem.* manuscript. Here is a somewhat abbreviated abstract for submission to *Cancer Research.* "Regulation of androgen receptor signaling by PTEN tumor suppressor through distinct mechanisms in prostate cancer

cells by Hui-Kuan Lin, Yueh-Chiang Hu, Hong-Yo Kang, Dong Kun Lee, and Chawnshang Chang, followed by some results/conclusions also being submitted. Also in Results/conclusions find specific Figure references (the numbers shown refer to the figure numbers in the manuscript being submitted).

Cancer Research. Abstract: Here we show that PTEN suppresses androgen receptor (AR) activity via a PI3K/Akt-independent pathway in the early passage number of prostate cancer LNCP cells. As androgen/AR plays important roles in prostate cancer progression, understanding the factors involved in the regulation of androgen/AR action may provide molecular targets for prostate cancer treatment. Here we demonstrate that PTEN regulates AR activity in low-passage number LNCaP cells via a PI3K/Akt-independent pathway and interacts directly with AR to suppress androgen-induced AR nuclear translocation. The interaction between AR and PTEN may expose the active site of the AR for the recognition of caspase-3, leading to AR degradation. In contrast, PTEN regulates AR activity in high passage number LNCaP cells via a PI3K/Akt-dependent pathway.

Results/Conclusions: **(See Appendix A for all figures)** We also studied AR protein stability by pulse-chase labeling. As shown in Fig. 1A, PTEN clearly reduced the half-life of newly synthesized [^{35}S]-AR 4- to 5-fold and accelerated AR degradation. Interestingly, when we replaced PTEN with either the dominant negative form of Akt (dAkt) or PI3K inhibitor LY294002, the results (Fig. 1B) indicated that dAkt and LY294002 did not promote AR degradation, ruling out the possibility that PTEN promotes AR degradation via regulation of the PI3/Akt pathway. These data strongly suggest that direct PTEN-AR protein-protein interaction may play major roles for the PTEN-promoted AR degradation. In contrast, in high passage number LNCaP cells (passage 65) where the PI3/Akt pathway becomes dominant (Fig. 2A and B), PTEN-induced AR degradation was reversed by cAkt (Fig. 1C and 2B), suggesting that the suppressive effect of PTEN on AR involves Akt pathway and Akt might not promote AR ubiquitylation and degradation in high passage LNCaP cells.

We reported recently that the PI3K/Akt pathway promoted AR ubiquitylation, leading to AR degradation by the 26 S proteasome (See attached manuscript). These data clearly suggest that both PTEN and the PI3K/Akt pathway can promote AR degradation via distinct mechanisms. How can PTEN negatively regulate the PI3K/Akt pathway and at same time promote AR degradation? Since PI3K/Akt signaling promotes AR degradation, PTEN inhibition of this pathway would be expected to result in increased AR protein levels. It is possible that PTEN can go through both pathways by inhibition of PI3K/Akt-mediated AR degradation by the 26 S proteasome and caspase-3-mediated AR degradation. Yet the overall balance may favor the caspase-3-mediated AR degradation. These figures will be included in manuscript being submitted.

Aim 3: To determine whether Akt can suppress androgen/AR-induced cell growth inhibition and apoptosis. Studies for this aim are planned for the coming year.

Data for manuscript submission: The loss of PTEN expression in prostate LNCaP cells leads to constitutive activation of Akt (1). Akt is an important survival factor in a variety of cell types including LNCaP cells. Several lines of evidence have indicated that PI3K/Akt is able to suppress cell apoptosis induced by growth factor deprivation (2, 3, 4). Abrogation of PI3K/Akt activity by PI3K inhibitors causes LNCaP cell apoptosis (5, 6). On the other hand, the androgen/AR signal is thought to play important roles in the prostate cancer cell growth and survival, and this signal can protect cells from apoptosis in response to treatment of PI3K inhibitors (5, 7). Thus, the PI3K/Akt and the androgen/AR signaling pathways represent two major survival pathways in the LNCaP prostate cancer cells. As PTEN could repress the androgen/AR signal and PI3K/Akt pathway in LNCaP cells, we propose that inhibition of these two pathways by PTEN might contribute to PTEN-induced cell apoptosis in the LNCaP prostate cancer cells. This assertion was further supported by the observation that restoration of AR function or the PI3K/Akt pathway rescues cells from PTEN-induced apoptosis (Fig. 3).

Aim 4: To generate site-specific phospho-AR antibodies and use these Abs to monitor the AR phosphorylation status and their relationship to the progression of prostate cancer.

Several site-specific phospho-AR antibodies have been recently tested for their specificity and to determine appropriate and efficient testing concentrations and procedures. However these prepared antibodies proved to be ineffective and/or inefficient. We are hoping to have additional antibodies available soon for testing. The studies on archival human tissue samples will proceed after the evaluations of the antibodies are completed.

KEY RESEARCH ACCOMPLISHMENTS:

- Via a PI3K/Akt-dependent pathway PTEN regulates AR activity in high passage number LNCaP cells.
- PI3K/Akt pathway promoted AR ubiquitylation, leading to AR degradation by the 26 S proteasome.
- Via a PI3K/Akt-independent pathway PTEN suppresses androgen receptor (AR) activity in the early passage number of prostate cancer LNCP cells.
- Restoration of AR function or the PI3K/Akt pathway rescues cells from PTEN-induced apoptosis
- Site-specific antibodies evaluated with poor results, however others are in process of being developed.

REPORTABLE OUTCOMES:

- One manuscript associated with the proposal published by *J. Biol. Chem.*

CONCLUSIONS:

As a summary, we ask how to interpret these findings and what is the physiological role of increased AR function after PI3K/Akt is blocked? We found that removal of androgens in LNCaP cells resulted in increased levels of active phosphorylated Akt. Thus, we believe that the AR and PI3K/Akt signaling both appear to be important proliferation and survival factors in prostate cancer cells, and seem to antagonize each other to maintain the cell homeostasis. The AR activity can be induced by LY294002 to play a dominant proliferation role to compensate for the loss of PI3K/Akt signaling. Our additional studies in 2003 led us into studies involving the PTEN pathway and its relationship to our Akt studies. Although we had expected to have tissue studies with antibodies by now, we hope to be able to do these studies within the next several months.

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APPENDIX A: Figures 1, 2, and 3

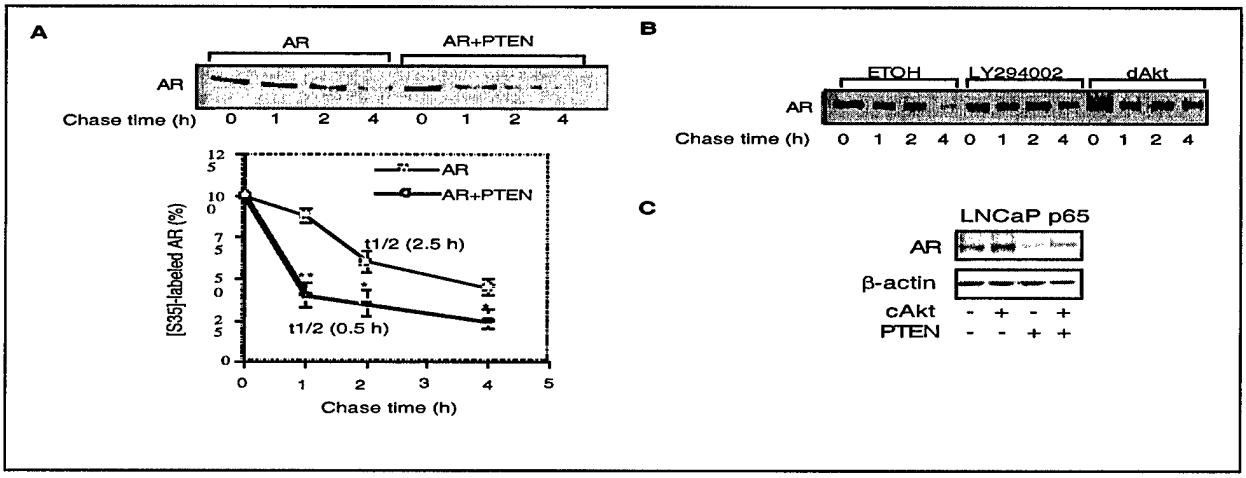
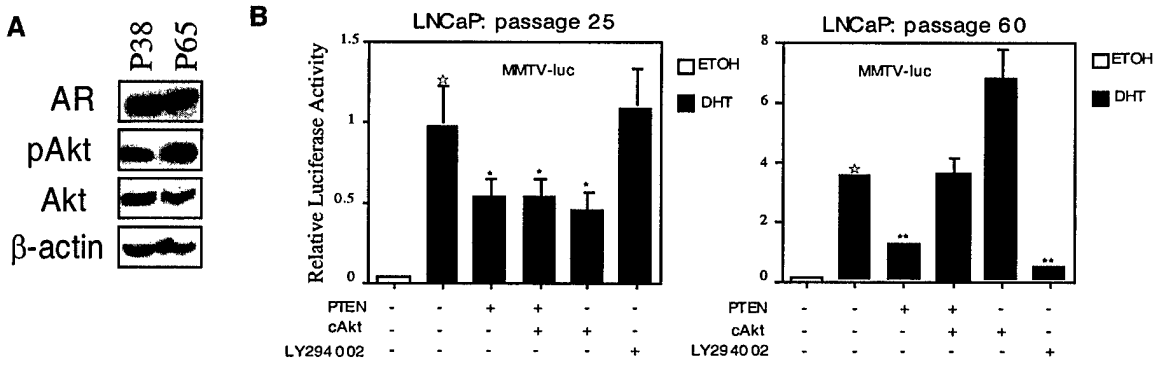


Fig. 1 PTEN decreases AR protein levels via PI3K/Akt-independent or –dependent pathways. (A) COS-1 cells were transfected with AR along with pCDNA3 or PTEN in 10% CDS media for 16 h. The cells were then pulsed with [^{35}S]-methionine for 45 min in the presence of 10 nM DHT and harvested at different chase times as indicated. The cell extracts were immunoprecipitated with AR antibody and subjected to SDS-PAGE followed by autoradiography. The intensity of the bands was quantitated using ImageQuant (Molecular Dynamic). Data were from three identical results. (B) COS-1 cells were transfected with AR along with pCDNA3 or dAkt in 10% CDS media for 16 h, pulsed with [^{35}S]-methionine for 45 min, and then harvested at different chase times as indicated. LY294002 (20 μM) was added 2 h before pulsing with [^{35}S]-methionine. (C) PTEN regulates AR degradation is inhibited by Akt in high passage number LNCaP cells. LNCaP cells at passage 65 (p65) were transfected with plasmids, as indicated, for 24 h, treated with 10 nM DHT for another 24 h, and harvested for Western blot assay. (*, $p<0.05$; **, $p<0.001$ vs. AR alone, Student's two-tailed *t*-test)



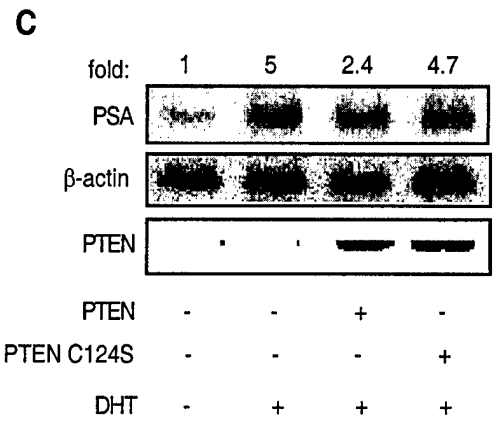


Fig. 2. PTEN suppresses AR transactivation involving PI3K/Akt pathways. (A) Akt activity is higher in high passage number of LNCaP cells. Different passages (passage 38 vs. 65) of LNCaP cells were cultured in 10% FBS media and harvested for Western blot analysis. (B) LNCaP cells (passage 25) or LNCaP cells (passage 60) 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 LY294002 for 16 h. The results were normalized by pRL-SV40 activity and the data are represented as means ± s.d of three independent experiments. (*, $p<0.05$; **, $p<0.001$ vs. control (indicated as ☆), Student's two-tailed *t*-test)

(C) LNCaP cells were transfected with plasmids, as indicated, in 10% CDS media for 24 h and then treated with DHT for 24 h. The cells were harvested for Northern blot analysis.

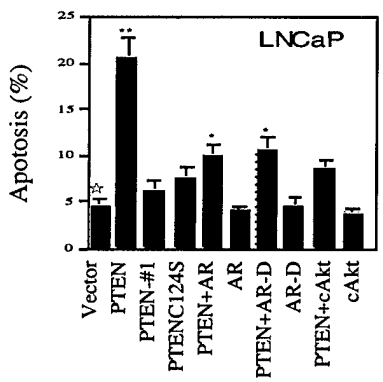


Fig. 3. Interaction between PTEN and AR contributes to PTEN-mediated apoptosis and suppression of AR functions. The LNCaP cells were transfected with plasmids, as indicated, for 16 h, and the medium was changed to 0.1% CDS media for 2 days. The cell apoptosis was determined by TUNEL assay. PTEN#1, PTEN N-terminus (aa 1~107) serves as control for AR-D. AR (aa 486-651) serves as PTEN-AR interaction inhibitor. Data for apoptosis are means ± s.d. from three independent experiments. (*, $p<0.05$; **, $p<0.001$ vs. control (indicated as ☆), Student's two-tailed *t*-test).

APPENDIX B: "Suppression vs induction of androgen receptor functions by the phosphatidylinositol 3-Kinase/Akt pathway in prostate cancer LNCaP cells with different passage numbers, by Hui-Kuan Lin, Yueh-Chiang Hu, Lin Yang, Saleh Altuwaijri, Yen-Ta Chen, Hong-Yo Kang and Chawnshang Chang. *J. Biol. Chem.* 278, 50902-50907.

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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 Ser^{210} and Ser^{790}. 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. 5α-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 (Ser^{473}) 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 μg/ml), and 10% fetal calf serum (FCS). LNCaP cells were maintained in RPMI 1640 with 10% FCS. Transfections were performed using SuperFect™ 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 ± 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 μg/ml hygromycin. Individual colonies were picked and grown until 70% confluent followed by 4 μg/ml Dox treatment. The positive clones were confirmed by Western blot analysis.

Generation of an Anti-phospho-AR Antibody (Ser^{210})—The phospho-AR peptide (SGRAREADGAPTSSKD) was generated and used for generation of anti-phospho-AR (Ser^{210}) antibody (clone 156C135.2) according to the manufacture'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, 5α-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 ± 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. 1*A*, *P25*) but enhanced AR activity in high passage number LNCaP cells (Fig. 1*B*, *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 μM 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. 1*A*), it apparently increased androgen-induced PSA expression, an AR target gene, in low passage number LNCaP cells (Fig. 1*C*). Similar to the reporter gene assay, LY294002 suppressed PSA expression in high passage number LNCaP cells (Fig. 1*C*). 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. 1*D*). 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. 1*A*) 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 antiandrogen 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. 1*E*). In contrast, high passage LNCaP cells show a strong basal Akt activity (Fig. 1*E*). Our data show that Akt negatively modulates AR activity in low passage LNCaP cells (Fig. 1*A*), 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. 1*F*), 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. 1*F*), 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. 1*F*). 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 Ser^{210} and Ser^{790} residues, which are the Akt consensus phosphorylation sites (3). Overexpression of cAkt, but not the kinase-dead Akt

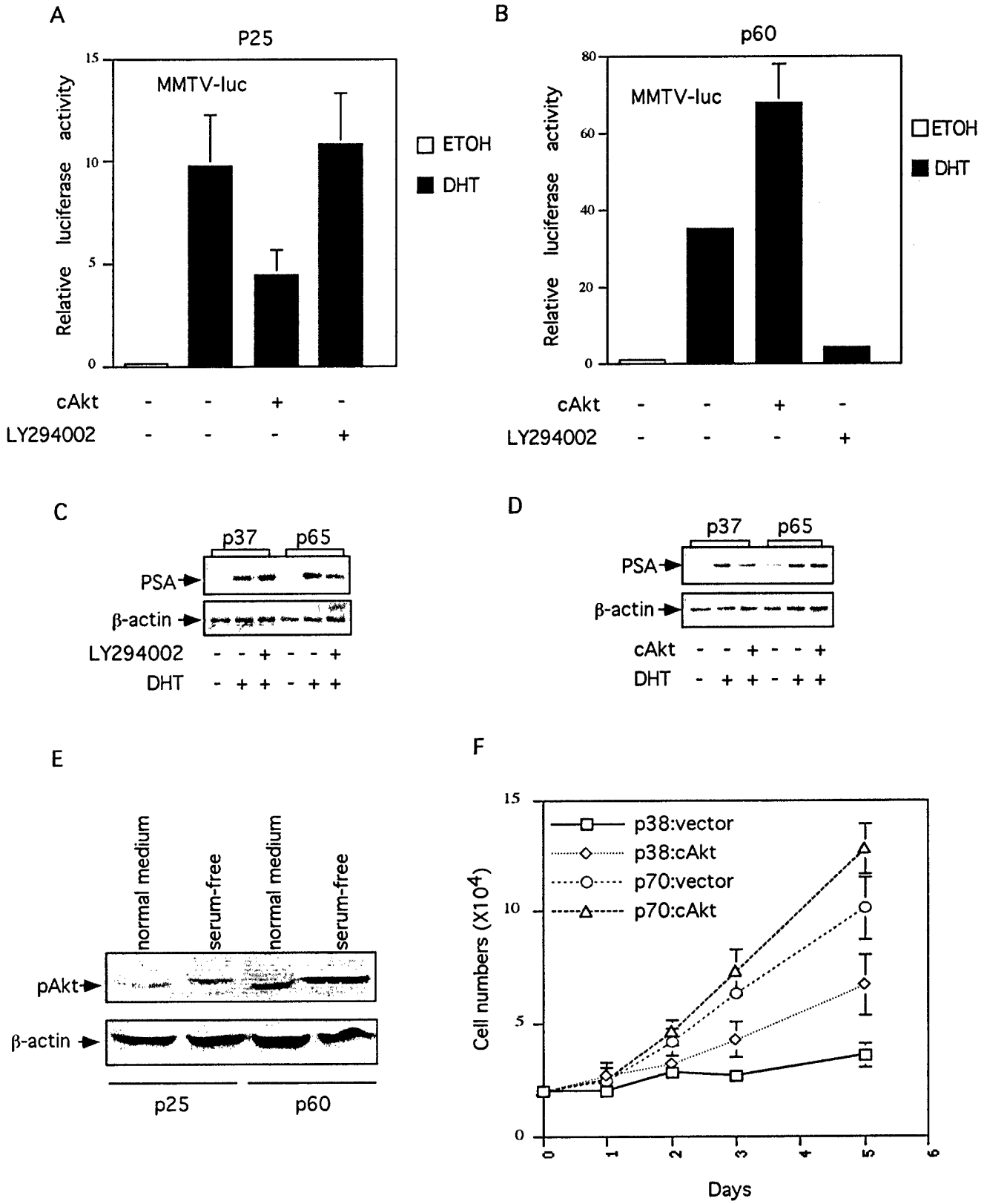

FIG. 1. **Passage-dependent effect of the PI3K/Akt pathway on AR transactivation in LNCaP cells.** *A*, LNCaP cells (passage 25 (*P25*)) were transfected with MMTV-luc along with plasmids, as indicated, for 16 h, and cells were then treated with EtOH or 10 nM DHT in the presence or absence of 20 μM LY294002 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 μM 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 (Ser^{473}) 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."

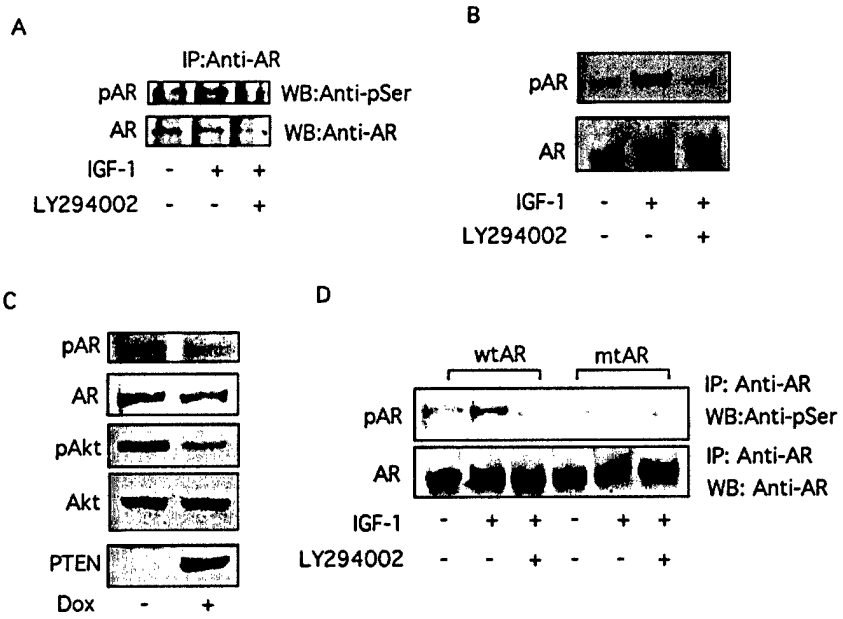

FIG. 2. **Activation of the PI3K/Akt pathway induces AR phosphorylation *in vivo*.** *A*, LNCaP cells at passage 38 were serum-starved for 2 days, incubated with 20 μM LY294002 for 30 min prior to treatment with 100 μg/ml IGF-1 for 4 h, and then harvested for immunoprecipitation (*IP*) with AR antibody. *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 (Ser^{210}) antibody (*pAR*). *C*, PTEN-inducible LNCaP cells at passage 40 were cultured in 10% FCS, treated with 4 μg/ml Dox for 24 h, treated with 100 μg/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*, Ser^{210}Ala/Ser^{790}Ala) for 16 h, serum-starved for 24 h, and then incubated with 20 μM LY294002 for 30 min prior to treatment with 100 μg/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 Ser^{210} and Ser^{790} *in vitro*.

We and others (3, 7) and have found that Akt can phosphorylate AR at Ser^{210} and Ser^{790}. However, Gioeli *et al.* (13) found that Akt fails to phosphorylate AR at Ser^{210} and Ser^{790} 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 Ser^{210} 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 Ser^{210} (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 phosphorylation. 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 Ser^{210} and Ser^{790} (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 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. 3*A*). In contrast, LY294002 enhanced AR protein levels in low passage LNCaP cells but slightly reduced AR protein levels in high passage LNCaP cells (Fig. 3*B*). 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. 3*C, left panel*). cAkt did not promote AR degradation in high passage LNCaP cells but slightly enhanced AR stability (Fig. 3*C, 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.

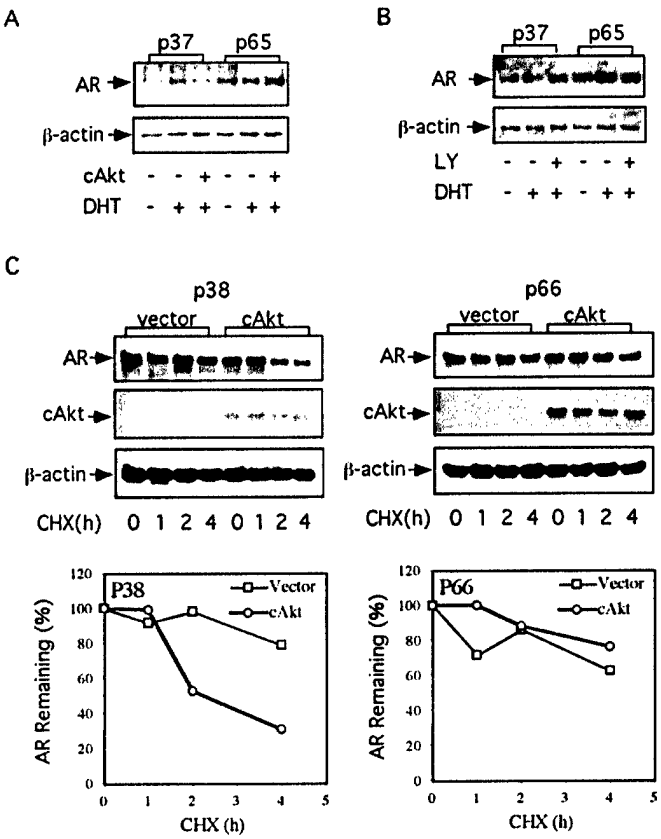

FIG. 3. **Distinct regulation of AR protein degradation by the PI3K/Akt pathway at various passage numbers of LNCaP cells.** *A*, LNCaP cells at different passage numbers were transfected with vector or cAkt for 24 h, and cells were treated with EtOH or 10 nM DHT for another 24 h followed by harvesting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

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

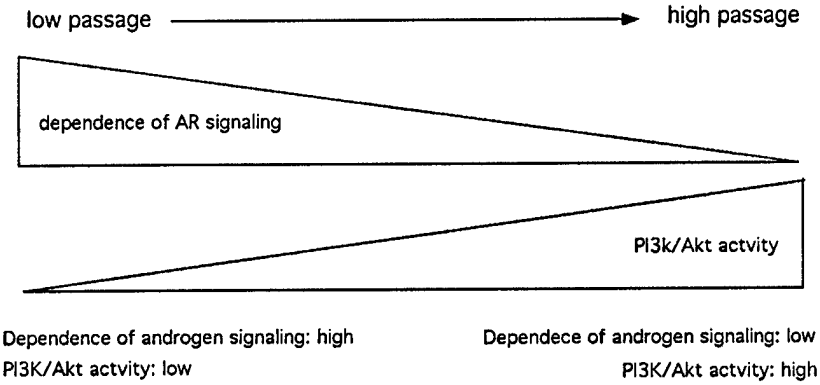

FIG. 4. **Model for PI3K/Akt pathway on AR signaling in prostate LNCaP cells.** In low passage LNCaP cells, the basal activity of PI3K/Akt signaling is low, and cells are strongly dependent on androgen signaling for growth and survival. In contrast, in high passage LNCaP cells, the basal activity of 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.

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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