APPL Suppresses Androgen Receptor Transactivation via Potentiating Akt Activity*

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The androgen receptor (AR) is a member of the nuclear receptor superfamily that cooperates with multiple proteins to exert its biological function (1–4). Upon binding to ligand testosterone/5α-dihydrotestosterone (DHT), the AR can bind to the androgen-response-element (ARE) on the 5’ promoter of the target gene, which results in the modulation of cell growth (5, 6). Prostate cancer is the most common form of cancer in men and the second leading cause of cancer deaths in men in the United States (7). Androgen ablation is the mainstream of therapy for progressive prostate cancer; however, most of prostate cancer patients eventually fail with androgen ablation therapy and die of recurrent androgen-independent prostate cancer. The failure of androgen ablation therapy may be due to an alteration of the normal androgen axis through mutation of AR, an alteration in the expression of AR coregulators, or a dysregulation of AR activity through signal transduction cascades. A substantial body of literature suggests that the AR can be regulated directly or indirectly by growth factor signal transduction pathways, which may contribute to the development and progression of prostate cancer (8).

The phosphatidylinositol 3-kinase (PI3K) consists of regulatory (p85) and catalytic (p110) subunits that participate in multiple cellular processes, including cell survival, growth, transformation, and differentiation (9). Akt, a serine/threonine kinase can prevent cell apoptosis by phosphorylation and inactivation of several pro-apoptotic proteins such as Bad, caspase-9, and forkhead transcription factors (10–12) and is the key effector of the PI3K pathway (12, 13). The binding of PI3K-generated phospholipids to Akt results in the translocation of Akt from the cytoplasm to the inner surface of the plasma membrane, where Akt is phosphorylated by the upstream kinases phosphoinositide-dependent protein kinase (PDK)-1, PDK-2, and integrin-linked kinase (14, 15). Three Akt homologs, Akt1, Akt2, and Akt3, have been identified and characterized (16–18). Tissue distribution studies suggest that these Akt homologs are expressed ubiquitously in human tissue and may share similar mechanisms in exerting their biological functions (19).

APPL (adapter protein containing PH domain, PTB domain, and leucine zipper motif) was identified as an Akt-interacting protein (20) and detected in many human tissues including the prostate. The ability of APPL to interact with Akt1 and P110α, the catalytic domain of PI3K, suggests that APPL may function as an adapter to tether Akt and PI3K (20). The detailed mechanisms by which APPL influences the PI3K/Akt signal pathway, however, remains unclear. Our early reports show that Akt can suppress AR transactivation (21). In this study, we demonstrate that various AR functions can be suppressed by a new signal pathway of APPL → Akt → AR, which may influence AR-mediated prostate cancer growth.

EXPERIMENTAL PROCEDURES

Materials—pCDNA3-cAkt (a constitutively active Akt with a deletion at amino acids 4–129 replaced with a consensus myristoylation domain) and pCDNA3-dAkt (a kinase-deficient mutant, K179A) were obtained from Dr. Robert Freeman (22). Δp85 was kindly provided by M. Kasuga, Kobe University, Kobe, Japan (23), and p110α1 was from L. T. Williams, Chiron Corp., Emeryville, CA (24). The promoter region of the human p27Kip1 gene (p27PF) was provided by Dr. Toshiyuki Sakai (25). LY294002 was purchased from Calbiochem, and DHT was...
from Sigma. The plasmids pCMV-AR, pSG5-AR, mouse mammary tumor virus promoter-luciferase (MMTV-luc), prostate-specific antigen promoter-luciferase (PSA-luc), promoter-luciferase reporters containing four copies of androgen responsive elements (ARE-4-luc), pRL-SV40, and pRL-TK have been previously described (2, 26). The anti-AR polyclonal antibody, NH27, was produced as previously described (27). The anti-AR monoclonal antibody (G122–77) was purchased from Pharmingen. The anti-FLAG monoclonal antibody was from Sigma, and the AP-1 (c-jun and c-fos) secondary anti-mouse, and anti-rabbit antibodies were from Santa Cruz.

**APPL Constructs**—APPL cDNA was PCR-amplified from the human testis Marathon-Ready cdNA library (Clontech) using primers APPL-5’ (5’ CCT TCC GAG ATG CCG GGG ATC GAC AAG 3’) and APPL-3’ (5’-CGG GCC TTA TGC TTA TCA TGC TCT TCT-3’). The underlined nucleotides represent BamHI in APPL-5’ and ApaI in APPL-3’. The PCR products were cut as a BamHI to ApaI fragment and inserted between the BamHI and ApaI sites in pCDNA3-FLAG and in-frame to the down-stream of the FLAG tag. APPL N- and C-terminal DNA fragments (1–1284 and 1329–2130 bp, respectively) were produced by PCR from pCDNA3-FLAG-APPL and inserted between the BamHI and XhoI sites in pCDNA3-FLAG. The APPL N-terminal DNA fragment was inversely inserted into pFLAG-CMV vector (Sigma) with XbaI and BamHI sites as antisense APPL.

**Reverse Transcription (RT)-PCR Analysis**—Reverse transcription was performed at 42 °C for 50 min in a total volume of 20 µl of first-strand buffer (Invitrogen) containing 0.5 µM of oligo(dT)12-18 primers (Invitrogen), 0.5 mM each dNTP (Invitrogen), 10 mM dithiothreitol, 200 units of Moloney murine leukemia virus reverse transcriptase (U. S. Biochemical Corp.), 200 units of SuperScript II reverse transcriptase, and 1 µg of total RNA. The absence of contaminating DNA from each RNA sample was checked by omitting the reverse transcriptase from the RT reaction (RT control). When the reaction was complete, 2 µl of the cDNA solutions were amplified in a final volume of 50 µl of PCR buffer (20 mM Tris/HCl, 50 mM KCl, 1.5 mM MgCl2) containing 0.2 µM each primer, 0.2 mM each dNTP (Invitrogen), and 1 unit of Taq DNA polymerase (Invitrogen). The PCR primers used were: PTB-N 5’-GCATCCAATTTCTCTCTCTTGG-3’; PTB-C 5’-CGGAGATGTGAGCATCTGAGCC-3’; PTB-X 5’-GTCTGATATGACACTGACCTTCTG-3’ (250-bp product).

**Luciferase Reporter Assays**—The cells were transfected with plasmids in the 10% CD-FBS media for 16 h and then treated with ethanol or 10 nM DHT for 24 h. After the cells were washed with phosphate-buffered saline and harvested, cell lysates were prepared and used for luciferase assay according to the manufacturer’s instructions (Promega). The results were obtained from at least three sets of transfection and are presented as the mean ± S.D.

**Glutathione S-Transferase (GST) Pull-down Assay**—APPL was subcloned into pGEX-KG vector (Amersham Biosciences). GST fusion proteins were generated from BL21 (DE3) strain and purified as described by the manufacturer (Amersham Biosciences). The purified GST proteins were resuspended with 100 µl of interaction buffer (20 mM Tris-HCl/pH 8.0, 60 mM NaCl, 1 mM EDTA, 6 mM MgCl2, 1 mM dithiothreitol, 8% glycerol, 0.05% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and proteinase inhibitors) and incubated with 800 µg of LN CaP cell lysates or 35S-labeled TNT-expressed AR in the presence or absence of 10 nM DHT on a rotating disk at 4 °C for 2 h. Translation reactions were performed in the TNT-coupled rabbit reticulocyte lysate (Promega) system according to the manufacturer’s instructions. After extensive washes with NENT (20 mM Tris; pH 8.0, 100 mM NaCl, 6 mM MgCl2, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, 8% glycerol, 1 mM phenylmethylsulfonyl fluoride) buffer, the bound proteins were solubilized in 5% SDS-polyacrylamide gel and analyzed by Western blot with anti-AR NH27 antibody.

**Immunoprecipitation and Western Blot Analysis**—A standard protocol was performed for immunoprecipitation. For the immunoprecipitation of APPL and AR, COS-1 cells (at a cell density of 1 x 10^6 cells/10-cm dish) were maintained in 10% CD-FBS medium and transfected with APPL, AR, Akt, or combinations of these genes. After 16 h, cells were treated with or without DHT for 24 h. For the immunoprecipitation of APPL and PI3K, COS-1 cells (at a cell density of 1 x 10^6 cells/10-cm dish) were maintained in 10% FBS medium and transfected with APPL and wild-type p85, a regulatory subunit. After 24 h, cells were lysed by radioimmunoprecipitation assay buffer containing 1 × phosphate-buffered saline, 1% IGE-PAI CA-630 (Sigma), 0.5% sodium deoxycholate, and 0.1% SDS and supplemented with 10 µg/ml protease inhibitors (PIB, 30 mg/ml). Lysates were cleared by centrifugation at 12,000 × g for 15 min at 4 °C, and 800 µg supernatants were incubated with 1 µg of individual antibodies and 30 µl of packed protein A/G PLUS--Sepharose beads at 4 °C for 1 h. After incubation, the beads were washed five times with radioimmuno precipitation assay buffer. The resulting immunoprecipitated immunocomplexes were solubilized in 60 µl of protein sample buffer, resolved by 8% SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. The protein complex was detected by Western blot analysis and developed by the AP conjugate substrate kit (Bio-Rad).

**Northern Blot Analysis**—LN CaP cells were cultured following the methods previously described (28) and treated with or without DHT for 24 h. Total RNA was isolated from each plate using the TRIZOL reagent (Invitrogen), and 25 µg of each RNA was loaded onto denaturing agarose gels. The RNA samples were separated by electrophoresis and blotted onto a nylon membrane using a vacuum blotter. PSA cdNA was used as the hybridization probe, and β-actin RNA was used as a control for equivalent RNA loading.

**RESULTS**

**APPL Expression in Different Cell Lines**—We first applied RT-PCR analysis to test the expression of endogenous APPL mRNA in COS-1 and prostate cancer lines, which will be used in the current studies. As shown in Fig. 1, the amplified APPL transcript of 250 bp was detected in the androgen-dependent LNCaP cells, androgen-independent PC-3 cells, and the COS-1 cells.

**APPL Suppresses AR Transactivation**—APPL has been identified as an Akt-interacting protein that contains phosphotyrosine binding, pleckstrin homology (PH), and leucine zipper domains (20). Our earlier study showed that Akt could suppress AR transactivation in prostate cancer cells (21). To investigate the potential influence of APPL on the AR transactivation, we first co-transfected APPL with AR in the presence of 10 nM DHT to see if APPL could influence the activity of the luciferase reporter linked to three different promoters containing AREs, MMTV 5′ promoter, PSA 5′ promoter, and four copies of a synthetic ARE (ARE4) in PC-3 cells. As shown in Fig. 2A, 10 nM DHT induces AR transactivation up to 25-fold, and the addition of APPL can then repress AR transactivation in a dose-dependent manner in all three different ARE promoters in PC-3 cells. To reduce the potential artificial effect of exogenous overexpression of AR, we replaced PC-3 cells with LNCaP cells that express endogenous, mutated but functional AR. As shown in Fig. 2B, the
addition of APPL also represses endogenous AR-induced (ARE)4-luc activity in a dose-dependent manner. In contrast, APPL showed only marginal effects on the estrogen receptor (ER)/H9251-mediated transactivation, even with high concentrations of APPL in PC-3 cells (Fig. 2C). These findings suggest that the APPL effect on AR transactivation is selective and may not be due to general transcriptional squenching. To further prove the APPL repression effect on AR transactivation, we tested antisense APPL effect on AR activity in LNCaP cells with natural promoter PSA-luc. As shown in Fig. 2D, APPL also suppressed endogenous AR-induced PSA-luc activity in a dose-dependent manner, and antisense APPL enhanced AR activity and could reverse the inhibitory effect of transfected APPL. Overall, the results from Fig. 2 suggest that APPL can repress AR transactivation in prostate cancer cells, such as LNCaP and PC-3 cells.

**APPL Enhances Akt Effect on Suppression of AR Transactivation**—Because we previously showed that Akt could repress AR transactivation (21), we were interested in seeing if APPL, an Akt-interacting protein, had any influence on the Akt-repressed AR transactivation. As shown in Fig. 3A, in PC-3 cells cAkt can repress AR transactivation in all reporters with three different AREs, and the addition of APPL can then further suppress AR transactivation. In contrast, the addition of cAkt enhances the ERα-mediated transactivation (Fig. 3B), which is consistent with a recent publication (29).
from Fig. 3 clearly indicate that both APPL and Akt can repress AR transactivation and that APPL can further enhance the Akt effect on AR transactivation.

Suppression of AR Transactivation by APPL Is Dependent on the PI3K/Akt Pathway—An earlier report suggested that APPL could interact with Akt and PI3K and might function as an adapter affecting the PI3K/Akt pathway (20). We were interested in seeing if APPL-mediated suppression of AR transactivation relied on the PI3K/Akt pathway. Three components, Δp85 (a dominant negative form of PI3K), LY294002 (a selective PI3K inhibitor), and dAkt (a dominant negative form of Akt), were applied to examine potential effects on APPL-mediated AR transactivation. As shown in Fig. 4, in PC-3 cells the addition of APPL (lane 3) or cAkt (lane 4) alone suppresses AR transactivation (lane 2). Simultaneous addition of APPL and cAkt further suppresses AR transactivation (lane 5). As expected, the addition of dAkt (lane 6), LY294002 (lane 8), and Δp85 (lane 10) alone enhance AR transactivation, which is consistent with a previous report (21). Furthermore, the suppression effect of APPL on AR transactivation can be reversed after adding either dAkt (lanes 3 versus 7), LY294002 (lanes 3 versus 9), or Δp85 (lanes 3 versus 11). These results suggest that the PI3K/Akt pathway plays a major role in the APPL-mediated inhibition of AR transactivation. Taken together, the results from Fig. 4 clearly demonstrate that the suppression of AR transactivation by APPL is dependent on the PI3K/Akt pathway.

APPL Enhances Akt Phosphorylation Dependent on PI3K Activity—APPL was first identified as an Akt interaction protein (20). However, the potential physiological roles of APPL as well as how APPL influences Akt function remain largely unknown. As our data show, because APPL can enhance the Akt-mediated suppression of AR transactivation and the suppression of AR transactivation by APPL is dependent on PI3K/Akt pathway, we would like to see if it is possible that APPL may also be able to influence PI3K/Akt activity. Using a co-immunoprecipitation assay, we found that APPL could interact with p85, a subunit of PI3K (Fig. 5A). Because the PI3K/Akt pathway can down-regulate the gene expression of p27Kip1 (30), we used p27Kip1 as a surrogate gene to monitor the Akt activity in the presence and absence of APPL. As shown in Fig. 5B, p27Kip1 promoter activity (lane 1) was suppressed by both APPL (lane 2) and cAkt (lane 3). The simultaneous addition of cAkt and APPL (lane 4) could further suppress p27Kip1 promoter activity (lane 4). In contrast, the addition of dAkt (lane 5), LY240092 (lane 6), or Δp85 (lane 7) could reverse the APPL-repressed p27Kip1 promoter activity, implying that APPL may influence p27Kip1 promoter activity via activating the PI3K/Akt pathway.

To further confirm this notion, we directly assayed the Akt activity by monitoring the Akt phosphorylation status in the presence and absence of APPL. As shown in Fig. 5C, IGF-1 can activate the Akt activity (lane 2), and this effect can be blocked by Δp85 (lane 8) or LY294002 (lane 12), suggesting that PI3K serves as an upstream activator for IGF-1 mediated Akt acti-

### Figure 4

Suppression of AR transactivation by APPL is via PI3K/Akt pathway. Dominant-negative Akt (dAkt) and LY294002 blocked the effect of APPL on AR activity in PC-3 cells. Cells were transfected with 50 ng of pSG5-AR, 150 ng of MMTV-luc or (ARE)4-luc, 5 ng of pRL-TK, 150 ng of APPL, 150 ng of cAkt, 150 ng of dominant-negative Akt, and 20 μg of LY294002. Transfected cells were treated for 24 h with 10⁻⁸ M DHT or ethanol as vehicle control. Duplicate samples were analyzed for each data point.

### Figure 5

APPL enhances Akt activity. A, APPL associates with PI3K in vivo. The COS-1 cell were transfected with FLAG-APPL and wild-type p85, a regulatory subunit of PI3K for 24 h, followed by harvesting for co-immunoprecipitation (IP) as described under “Experimental Procedures.” B, suppression effect of APPL via Akt on the human p27Kip1 promoter activity in PC-3 cells. Cells were transfected with 300 ng of APPL in the presence or absence of 300 ng of cAkt, 300 ng of dominant-negative Akt (dAkt), or 300 ng of Δp85 in combination with 50 ng of pRL-TK and 150 ng of promoter p27PF-luc. Luciferase activity was analyzed after 36 h. C, the COS-1 cells were seeded in 100-mm dishes and transfected with 10 μg of empty vector, 5 μg of APPL, 5 μg Δp85, or combinations as indicated. Empty vector was used to balance the DNA amount to 10 μg. After 24 h, the cells were serum-starved for another 24 h then treated with 20 μg of LY294002 10 min before treatment with vehicle or 50 ng/ml IGF-1 for 20 min, and the cells were harvested by radioimmune precipitation assay buffer. The Akt activity was determined by the Western blot using the phospho-(Ser-473) Akt (pAkt) antibody. D, the COS-1 cells were seeded in 100-mm dishes and transfected with 10 μg of empty vector, 5 μg of pCDNA3-FLAG-APPL, 5 μg of antisense APPL or co-transfected with 5 μg of pCDNA3-FLAG-APPL and 5 μg of antisense APPL followed by serum starvation for another 24 h. Empty vector was used to balance the DNA amount to 10 μg. The cells were treated with or without 50 ng/ml IGF-1 for 20 min before harvesting cells. Cell lysates were immunoblotted with anti-phosphorylation Akt antibody (Ser-473), anti-FLAG M2 antibody, or anti-β-actin antibody.
Fig. 6. APPL and Akt suppressed endogenous PSA mRNA expression and inhibition of PI3K/Akt signaling enhanced PSA protein expression level. A, APPL cooperated with Akt to further reduce the PSA mRNA expression level in LNCaP cells. The LNCaP cells were transfected with 10 μg of empty vector (lane 1), 5 μg of APPL plus 5 μg of empty vector (lanes 2 and 3), 5 μg of APPL plus 5 μg of cAkt (lane 4), and 5 μg of cAkt plus 5 μg of empty vector (lane 5) for 24 h followed by treatment with ethanol or 10 nM DHT for another 24 h. The cells were harvested and lysed by TRIZOL. 20 μg of total RNA was loaded for the Northern blot analysis. Quantification of the relative PSA mRNA level was done by ImageQuant software (upper panel). B, LY294002 influenced PSA mRNA expression. The LNCaP cells were cultured in 10% (CD-FBS)-containing medium for 24 h and then treated with vehicle, DHT, 20 μM LY294002, or combinations. After 4 h (lanes 1–4) and 24 h (lanes 5–8), cells were harvested for Northern blot analysis. Quantification of the relative PSA mRNA level was done by ImageQuant software (upper panel). C, LY294002 influenced PSA protein expression level. The LNCaP cells were cultured in 10% (CD-FBS)-containing medium for 24 h and then treated with vehicle, DHT, or DHT plus 20 μM LY294002. After 4 h and 24 h, cells were harvested for Western blot analysis with the indicated antibodies, and the relative fold change in PSA signal was analyzed by QuantityOne software (Bio-Rad) (upper panel). D, androgen ablation of LNCaP cells increases Akt activation. LNCaP cells were cultured under serum-starvation conditions with or without 1 nM DHT for 4 days. Cell lysates were analyzed by immunoblotting with anti-phospho-Ser-473 Akt (pAkt) and whole Akt protein antibodies, and the relative fold change in phospho-Ser-473-Akt signal was analyzed by QuantityOne software (Bio-Rad) (upper panel).

As expected, APPL significantly enhanced the IGF-1 mediated Akt activation (lane 4). The enhancement effect of APPL on Akt activation was abrogated by Δp85 (lane 6) or LY294002 (lane 10), indicating that APPL may affect Akt activation by modulation of PI3K activity or PI3K upstream activators. Furthermore, we used the antisense APPL construct to block endogenous APPL expression to see if APPL was required for the mitogenic signal activating Akt. As shown in Fig. 5D, overexpression of APPL significantly enhanced Akt activity (lane 3), but the phosphorylated Akt became undetectable after co-transfection with antisense APPL, even under the treatment of IGF-1 (lane 6 versus lane 2). These findings suggest that APPL is an important adapter protein that may be necessary for Akt activation upon mitogenic stimulation.

Inhibition of the PI3K/Akt Pathway Increases the PSA Expression Level—Data from Figs. 2–5 suggest that APPL may suppress AR transactivation via interaction with PI3K to mediate the growth factor signal for the activation of Akt. To avoid potential artificial effects from the reporter gene assay on the results in Figs. 2–4, we replaced PC-3 cells with LNCaP cells and tested the influence of Akt and APPL on the mRNA expression of PSA, an androgen-induced target gene that has been used as a marker to monitor the progress of prostate cancer (31). As shown in Fig. 6A, androgen-induced PSA mRNA expression (middle panel, lane 2) can be repressed by the addition of cAkt or APPL (middle panel, lanes 3 and 5, respectively). As expected, the simultaneous addition of cAkt and APPL can further suppress the PSA mRNA expression (middle panel, lane 4). Quantification of the Northern blot in Fig. 6A is presented (upper panel). To further prove the PI3K/Akt signaling repression on AR transactivation, we treated LNCaP cells with LY294002 to determine the effect of the PI3K/Akt pathway on PSA mRNA and protein expression. As shown in Fig. 6B, 4 h of treatment of 20 μM LY294002 decreased the PSA mRNA level (middle panel, lane 4 versus lane 2) but increased PSA mRNA expression at 24 h (middle panel, lane 8 versus lane 6). Quantification of the PSA mRNA expression level in Fig. 6B is shown in the upper panel. Western blot results indicated that blockade of PI3K/Akt pathway with LY294002 (24 h) causes a significant increase of protein expression level of PSA (Fig. 6C, lane 3 versus lane 2), but 4 h of treatment of LY294002 only marginally influenced PSA protein level (data not shown). We also tested the effect of androgen ablation on Akt activity by growing LNCaP cells under serum-starvation conditions for 4 days in the presence and absence of DHT. Removal of androgens resulted in increased levels of active phosphorylated Akt (Fig. 6D, lane 2 versus lane 1), but no changes were observed in Akt protein levels. These data indicate that acute androgen deprivation of LNCaP cells triggered an increase in PI3K and Akt activity.

APPL C-terminal Domain Suppresses AR Transactivation—The APPL C terminus is responsible for binding to Akt (20),
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**Fig. 7.** The APPL C terminus is responsible for the suppression of AR transactivation. A. full-length APPL and truncated mutants of APPL encompassing various functional domains were cloned into pCDNA3 vector with a FLAG tag. B. APPL C terminus suppressed AR activity in PC-3 cells. Cells were transfected with 50 ng of pCMV-AR, 150 ng of ARE4-luc, 2.5 ng of pRL-SV40, and 300 ng of APPL or APPL N or APPL C termini. Transfected cells were treated for 24 h with 10^-8 M DHT or ethanol as vehicle control. Duplicate samples were analyzed for each data point. The membranes were then probed individually with the anti-AR (G122), anti-APPL (FLAG M2), and anti-HA antibodies.

**DISCUSSION**

APPL contains multiple important regulatory motifs, including a PH domain, a phosphotyrosine binding domain, and a leucine zipped coiled-coil domain. The PH domain exists in diverse signaling molecules and permits anchorage of proteins to the cell membrane via phospholipid interactions (20). The leucine zipped coiled-coil domain is found in many transcription factors and has been proposed to play a role in the dimerization/polymerization of proteins (32). The phosphotyrosine binding domain was originally identified from mammalian signaling proteins such as Shc and has been shown to mediate protein-protein interaction as well as protein-phospholipid interactions (33). Although APPL is identified as an Akt-associated protein from the yeast two-hybrid screening using Akt2 as bait (20), the detailed mechanism of how APPL may affect the Akt-signaling pathway and its biological function remains largely unknown. In this study, we demonstrate that APPL suppresses the AR transactivation in a dose-dependent manner in the androgen-dependent and androgen-independent prostate cancer cells and that this suppressive effect is largely dependent
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First, suppression of AR transactivation by APPL is blocked by LY294002, Δp85, or dAkt. Second, APPL down-regulates gene expression of p27Kip1, which is blocked by LY294002, Δp85, or dAkt. Finally, APPL markedly enhances the IGF-1-mediated Akt activation and the destruction of endogenous APPL by antisense APPL, leading to the blockade of Akt activation upon mitogenic stimulation. How APPL affects the Akt phosphorylation is currently unclear. The fact that the enhancement of Akt activation by APPL is blocked by LY294002, Δp85, or antisense APPL suggests that APPL may affect PI3K. Because APPL contains the PH domain and interacts with PI3K (Fig. 5A), it is likely that APPL serves as an adapter protein that recruits the PI3K to the cell membrane, where it can be activated by growth factor receptors like the IGF-1 receptor, followed by subsequent Akt activation.

AR is a well known critical proliferation factor in prostate cancer. The study on the role of PI3K/Akt in the progression of prostate cancer suggests that PI3K/Akt may function as a dominant growth factor-activated cell survival pathway in LNCaP cells (34). Thus, PI3K/Akt and AR may cooperate with each other to promote the prostate cancer cell survival and growth. Based on our data and other reports, PI3K/Akt and AR may also negatively regulate each other to maintain homeostasis of prostate cancer cells. Supporting this hypothesis, Lin et al. (34) find that in LNCaP cells inhibiting the PI3K/Akt pathway will induce apoptosis, but DHT pretreatment can prevent this apoptosis. Our data further indicate that inhibition of PI3K/Akt strongly induces AR functioning, suggesting that LNCaP cells are able to enhance AR activity to rescue themselves from apoptosis caused by the blockade of PI3K/Akt. Thompson et al. (35) demonstrate that Akt can suppress AR transactivation in PC-3 cells, which is also consistent with our previous report (21). In addition, Murillo et al. (36) show that androgen ablation can increase PI3K-Akt activity, as our data suggest (Fig. 6D), implying that enhanced Akt activity may rescue LNCaP cells from impairment of androgen withdrawal. Taken together, these findings suggest that the survival factor Akt and the proliferation factor AR may mutually influence prostate cancer cell survival and growth in a balanced way.

Interestingly, using reporter gene assays, Thompson et al. (35) and Lin et al. (21) indicate that Akt can suppress AR transactivation in PC-3 cells, whereas Wen et al. (37) find that Her2/Neu was able to go through Akt to enhance PSA promoter activity in LNCaP cells in the absence of androgen. Manin et al. (38) show that LY294002 treatment could decrease AR protein and PSA mRNA levels but was unable to influence AR mRNA expression in LNCaP cells, whereas Sharma et al. (39) find that LY294002 treatment did not suppress the AR protein level in LNCaP cells, but 4 h of LY294002 treatment decreased the PSA mRNA level, which is consistent with our report (Fig. 6B). The detailed mechanisms for these discrepancies remain unclear. This may be due to the use of different reagents and different cell lines with various passage numbers. Furthermore, different experimental conditions, such as varied concentrations and treatment times of LY294002, applied by individual investigators, may also influence the results. We used parental LNCaP cells with passage numbers 23–30 under the ATCC-medium culture condition in our studies. According to our data and the results from a recent report which show that LNCaP cells with androgen withdrawal treatment show increased Akt activity sustained throughout the progression process (36) combined with the evidence that PI3K/Akt is the dominant factor for the LNCaP cells survival (34), it is possible that AR activity can be induced by LY294002 to play a dominant proliferation role to compensate for the loss of PI3K/Akt signaling. Overall, AR and PI3K/Akt signaling both appear to be important proliferation

on the PI3K/Akt pathway. We also observed that APPL could further enhance the Akt-mediated suppression of AR transactivation. Furthermore, we also found that APPL can interact with PI3K and Akt (Fig. 5A and data not shown). These data suggest that APPL may regulate PI3K/Akt activity by controlling AR transactivation. In support of this notion, we found that APPL suppresses the reporter gene expression of p27Kip1, an Akt downstream target, and that this effect is blocked by LY294002, Δp85, or dAkt. Thus, APPL may affect the activity of PI3K or the PI3K upstream activator to control p27Kip1 expression. In addition, APPL markedly enhances the IGF-1-mediated Akt activation. This effect is abrogated by LY294002, Δp85, and antisense APPL. These data indicate that APPL modulates the Akt activity via a PI3K-dependent manner. Several lines of evidence suggest that APPL regulates the AR transactivation via the PI3K/Akt-dependent pathway. First, suppression of AR transactivation by APPL is blocked by

Fig. 8. APPL was detected in the AR immunocomplexes but did not directly interact with AR. A, GST-APPL can pull down endogenous AR in LNCaP cells. GST-APPL was used as bait to incubate with LNCaP cell lysate with or without DHT treatment. The incubation and washing were performed as described under “Experimental Procedures,” and then the lysates were analyzed with anti-AR (NH27) antibody. B, APPL does not interact with AR directly. GST-APPL or GST-ARA70 was incubated with S35-labeled AR for 2 h in the presence or absence of 10 μM DHT. C, Akt markedly enhances the APPL/AR complex formation. COS-1 cells were co-transfected with FLAG-APPL, AR, and HA-Akt. The cell lysates were immunoprecipitated (IP) with anti-FLAG M2. The immunoprecipitated complexes were immunoblotted (WB) with anti-AR antibody (G122–77), anti-FLAG M2 antibody, or anti-HA antibody.
and survival factors in prostate cancer cells and also seem to antagonize each other to maintain cell homeostasis.

In conclusion, our finding that APPL may suppress AR function via the PI3K-Akt pathway may represent a unique pathway that further expands the importance of the PI3K-Akt-AR pathway in prostate cancer. Further development of blockers to interrupt this pathway may help us to battle the prostate cancer.

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