The PTEN tumor suppressor is a negative modulator of androgen receptor transcriptional activity

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Abstract

To investigate whether the tumor suppressor gene PTEN affects the activity of the androgen receptor (AR), we monitored the expression of the apoptotic gene HA-Bax (inserted in an adenovirus where it is driven by the AR-responsive promoter ARR2PB) in the presence or absence of dihydrotestosterone, in PTEN (+) or (−) prostate cancer cell lines, infected with an adenovirus containing wild-type PTEN (Av-CMV-PTEN) or a control LacZ-expressing construct. Our results showed that AR transcriptional activity was antagonized by PTEN expression. This antagonism was not cell line dependent, as it was observed in both LNCaP and LAPC-4 cells, or promoter dependent, as it was observed for a reporter gene (HA-Bax) driven by an exogenous androgen-responsive promoter (the ARR2PB promoter), and for a native gene (prostate-specific antigen; PSA) driven by an endogenous AR-responsive promoter. Additional experiments performed with viruses containing constitutively active (Adeno-myrAkt) or dominant negative (Adeno-dnAkt) forms of Akt demonstrated that Akt, a protein kinase whose activation is known to be inhibited by PTEN, mediated the observed antagonism between PTEN and AR transcriptional activity. Recently, two putative Akt phosphorylation sites have been identified in the AR sequence. Site-directed mutagenesis was utilized to convert these two serine into alanine residues. The resulting construct, named CMV-AR S213A&S791A was transfected in AR (−) and PTEN (−) PC-3 cells in the presence or absence of Av-CMV-PTEN and of two reporter plasmids (GRE2E1b-Luc and PSA P/E-luc) containing the luciferase gene driven by well-characterized androgen responsive promoters. These experiments demonstrated that, similarly to the wild-type molecule, AR S213A&S791A was transcriptionally inhibited by PTEN, suggesting that Akt does not have an effect on AR transcription by direct phosphorylation, but probably by affecting the availability of a downstream molecule whose main mechanism of action is that of modulating AR transcription. The data presented here suggest that loss of PTEN function may facilitate activation of AR signaling and progression to androgen independence in prostate cancer.

Introduction

The widespread use of prostate-specific antigen (PSA) has significantly increased our ability to correctly identify patients affected by prostate cancer (CaP). This powerful diagnostic tool has changed the epidemiology of CaP and an increasing number of patients are now diagnosed with organ-confined disease (Hankey et al. 1999). In addition, overall death rates are falling in many industrialized countries due to early diagnosis (Oliver et al. 2001). Despite these encouraging statistics, CaP is still the most frequently diagnosed visceral cancer in American men, and there will have been an estimated 189 000 new cases and 30 200 deaths from it in 2002 (Schroder 1999, Jemal et al. 2002).

CaP can be eradicated when organ confined, but systemic disease is incurable. Systemic CaP is usually treated with hormonal ablative therapy,
but virtually all patients receiving this treatment relapse and develop androgen-independent tumors for which only experimental treatments exist (Schroder 1999). Urgently needed is a better understanding of CaP progression to androgen independence at the molecular level, in order to identify new targets for novel therapy design.

Centrally located in the pathway activated by circulating androgens is the androgen receptor (AR), a member of the nuclear receptor family. After binding ligand, this molecule becomes activated with an associated change in conformation, translocates to the nucleus and binds DNA, ultimately regulating the transcription of androgen-responsive target genes (Balk 2002). In the prostate, AR is believed to work by stimulating activities which antagonize apoptosis and induce cell proliferation (Denmeade et al. 1996). AR is expressed in a normal or amplified way in patients with androgen-independent disease, and mutations of its ligand-binding domain have been described which expand binding specificities (Van-der-Kwast et al. 1991, Visakorpi et al. 1995, Taplin et al. 1999) and are associated with disease progression. Nevertheless, the large majority of AR analyzed at the molecular level does not contain mutations (Marcelli et al. 2000), and so other mechanisms must be involved with progression to androgen-independent disease.

According to a recent paper, the presence of AR is essential for androgen-independent CaP cell proliferation (Zegarra-Moro et al. 2002). In addition, AR expression level increases in androgen-independent CaP (Balk 2002). Therefore, to reconcile the apparent contradiction that AR is essential for proliferation of androgen-independent CaP cells (Zegarra-Moro et al. 2002) but use of AR antagonists in association with inhibitors of testosterone synthesis is ineffective in patients with androgen-independent disease (Eisenberger et al. 1998), many authors have hypothesized that AR can function in a ligand-independent way. In support of this hypothesis, a wide body of literature has been published demonstrating that AR can be activated by mechanisms involving protein kinase A, or tyrosine kinase receptors pathways (Culig et al. 1994, Nazareth & Weigel 1996, Craft et al. 1999, Yeh et al. 1999, Ueda et al. 2002a,b).

A frequent molecular abnormality detected in advanced CaP consists of the loss of the tumor suppressor gene PTEN (Suzuki et al. 1998, McMenamin et al. 1999, Dong et al. 2001). This molecule works by antagonizing the phosphatidylinositol 3-kinase (PI3K) pathway to induce apoptosis and growth arrest (Cantley 2002). The predominant enzymatic activity of PTEN consists in dephosphorylating the glycerophospholipid phosphatidylinositol 3,4,5-triphosphate (PI3,4,5,P3) at the D3 position to form phosphatidylinositol 4,5-biphosphate (Vivanco & Sawyers 2002). PI3,4,5,P3, the main substrate formed after activation of the PI3K pathway, is essential to achieve activation of the serine/threonine kinase Akt by anchoring it to the inner surface of the cell membrane through its pleckstrin homology (PH) domain (Andjelkovic et al. 1997). Once anchored to the plasma membrane, Akt achieves its final active state through phosphorylation at threonine 308 by 3-phosphoinositide-dependent protein kinase (PDK) 1 (Vanhaesebroeck & Alessi 2000), and at Ser473 by PDK2 (Vanhaesebroeck & Alessi 2000). Thus, the main mechanism through which PTEN exerts tumor suppression consists in antagonizing PI3,4,5,P3 formation, and thus preventing Akt activation (Stocker et al. 2002), which signals survival and mitogenesis to the cell.

PTEN and AR play opposing roles in the prostate (AR induces proliferation and antiapoptosis [Denmeade et al. 1996], while PTEN induces apoptosis and growth arrest [Yuan & Whang 2002]). Previous studies have linked PTEN (and Akt signaling) and AR activity, but the conclusions are controversial, as Li et al. (2001a) have shown that PTEN (through down-regulation of Akt) works as an antagonist of AR activity, while Lin et al. (2001) have provided evidence in support of the fact that Akt signaling inhibits AR activity. To further characterize the modality of AR-PTEN (Akt) interaction, we took advantage of adenoviral constructs developed in our laboratories to perform a number of experiments using PTEN positive (+) or negative (−) CaP cell lines. Our data suggest that PTEN antagonizes AR transcriptional activity through inhibition of Akt activation and that this effect is not cell line or promoter dependent. In addition, our data suggest that PTEN inhibition of AR transcription does not depend on prevention of Akt-mediated AR phosphorylation. This suggests that the effect of PTEN on AR transcription is probably mediated by one of the downstream post-translational/transcriptional effects mediated by Akt. As PTEN is frequently inactivated in androgen-independent CaP, these
results suggest that loss of PTEN function may facilitate activation of AR signaling and progression to androgen independence, and identify the PTEN-Akt pathway as an additional therapeutic target for the treatment of androgen-independent CaP.

Materials and Methods

Materials

Fetal bovine serum (FBS), tissue culture media and antibiotics were from Invitrogen Corporation (Carlsbad, CA, USA). Chemicals were from Sigma (St Louis, MO, USA) unless stated otherwise. Restriction endonucleases were from New England Biolabs (Beverly, MA, USA). Hybond ECL nitrocellulose membranes and ECL+ Western blotting detection system were from Pharmacia Biotech (Piscataway, NJ, USA). Antibodies were: PTEN (Cascade BioScience, Winchester, MA, USA) (working dilution: 1000:1), Akt (total) (Cell Signaling, Beverly, MA, USA) (working dilution: 1000:1), (phospho)-Akt (Ser473) (Cell Signaling) (working dilution: 1000:1), PSA (Dako, Carpinteria, CA, USA) (working dilution: 1000:1), /αfii9826-actin (Sigma) (working dilution: 5000:1), Bax (BD-Biosciences, Franklin Lakes, NJ, USA) (working dilution: 1000:1), AR (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (working dilution 300:1), and HA (BD-Biosciences). Secondary antibodies were: anti-mouse IgG, peroxidase-linked (Amersham-Biotech, Piscataway, NJ, USA) (working dilution: 1000:1) and anti-rabbit IgG, peroxidase-linked (Amersham-Biotech) (working dilution: 1000:1). Dihydrotestosterone (DHT) was from Steraloids (Newport, RI, USA). R1881 was from NEN (Boston, MA, USA). The PI3K inhibitor LY294002 was from Cell Signaling.

Plasmids

pCMV-AR contains the wild-type (wt) AR cDNA under the control of the CMV promoter (Tilley et al. 1989). pCMV-AR S213A&S791A contains an AR cDNA in which the putative Akt phosphorylation sites S213 and S791 have been mutated from serine to alanine residues. GRE2E1b-Luc is a luciferase reporter plasmid driven by two androgen response elements from the tyrosine amino transferase promoter, followed by the adenovirus E1b TATA box (Allgood et al. 1993). PSA P/E-luc is a reporter plasmid driven by the AR-dependent 2·4 kb PSA enhancer and 564 bp PSA promoter (Craft et al. 1999). pRL-CMV-TK contains Renilla luciferase cDNA (Promega, Madison, WI, USA) under the control of the constitutively active CMV promoter.

Adenoviral constructs

The following adenoviral constructs were used: Av-ARR2PB-HA-Bax, Av-CMV-PTEN, Av-CMV-PTEN(mut), Av-CMV, Adeno-myrAkt, Adeno-dnAkt, Av-CMV-GFP and Av-CMV-LacZ.

Preparation of adenovirus Av-ARR2PB-HA-Bax has already been described (Andriani et al. 2001). This adenovirus contains an hemagglutinin (HA)-tagged cDNA of the pro-apoptic protein Bax under the control of the ARR2PB (Zhang et al. 2000) promoter. The ARR2PB promoter is inducible by AR only in AR (+) cell lines deriving from prostatic epithelium, after addition to the medium of DHT or non-metabolizable androgens such as mibolerone or R1881. The HA-Bax protein induced from this system after addition of androgens to the medium is recognizable from the wt form because it is slightly larger by immunoblot analysis. Its induction is a direct function of the amount of agonist used in the experiment (Andriani et al. 2001).

Adenoviruses Av-CMV-PTEN and Av-CMV-PTEN(mut) and Av-CMV have been previously described (Yuan & Whang 2002). Av-CMV-PTEN contains the wt PTEN cDNA under the control of the CMV promoter. Av-CMV-PTEN(mut) expresses a mutant form of PTEN (G129E), which has lost its lipid phosphatase activity and the ability to inhibit Akt activation (Yuan & Whang 2002). Adenovirus Av-CMV contains the CMV promoter and no cDNAs subcloned downstream to it. Adenovirus Av-CMV-LacZ has already been described, and contains the LacZ cDNA subcloned downstream of the CMV promoter (Marcelli et al. 1999). Both Av-CMV and Av-CMV-LacZ were used as a control to Av-CMV-PTEN. The dominant negative Akt mutant (Adeno-dnAkt) has alanine residues substituted for threonine at position 308 and serine at position 473 (Suhara et al. 2001). The constitutively active Akt (Adeno-myrAkt) has the c-src myristoylation sequence fused in frame to the N-terminus of the wt Akt coding sequence that targets the fusion protein to the membrane. Membrane-bound Akt is constitutively active (Suhara et al. 2001). The cDNAs of these Akt
mutants were subcloned under the control of the CMV promoter, and inserted in the context of a replication-defective first generation adenovirus. Both these adenoviral constructs were gifts from Dr K Walsh, Tuft University. Adenovirus Av-CMV-GFP contains the cDNA of the green fluorescent protein under the control of the CMV promoter in the context of a first generation replication-defective adenovirus. This construct was a gift from M Ittmann (Baylor College of Medicine), and was used to identify the ideal multiplicity of infections (MOI) for the various cell lines and as a negative control when required.

Cell lines
Prostate cancer-derived LNCaP (Horoszewicz et al. 1980) (maintained in RPMI-1640, 10% FBS and 1% penicillin and streptomycin (P&S)), LNCaP LP (maintained in RPMI-1649, 10% FBS and 1% P&S), LAPC-4 (Klein et al. 1997) (provided by Dr C Sawyers, UCLA; maintained in Iscove’s modified Dulbecco’s medium (Invitrogen Corporation; 15% FBS and 1% P&S) and PC-3 (Kaighn et al. 1979) (maintained in F12+10% FBS+1% P&S) were used for the experiments reported in this paper. LNCaP, LNCaP LP and LAPC-4 were chosen because they contain the AR, which is wt in LAPC-4 (Klein et al. 1997), and contains a well-characterized (T877A) mutation in LNCaP and LNCaP LP cells (Veldscholdte et al. 1990). The difference between LNCaP and LNCaP LP cells is that while the former have been continuously passaged in our laboratory for the last 8 years, LNCaP LP (low passage) were recently purchased from the ATCC (Manassas, VA, USA), and used immediately after thawing in the experiments described below. PC-3 was chosen because this cell line is an example of an AR (−) (Tilley et al. 1990) PTEN (−) (Li et al. 1997) cell line of prostatic derivation.

Cell proliferation assay
LNCaP cells (5 × 10^5) were seeded per well in a 24-well plate and then infected with adenovirus Av-CMV or Av-CMV-PTEN (MOI 100:1) in media with charcoal-stripped serum. Twenty-four hours after infection, R1881 (0.05 nM) or vehicle was added to the media. This dose of ligand was used because AR agonists are known to have a biphasic effect on LNCaP cell proliferation, consisting of a stimulatory activity at subsaturating doses, and an inhibitory activity at saturating doses (Sonnenschein et al. 1989, Lee et al. 1995, Zhao et al. 1997). Cell proliferation was determined using the colorimetric [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide] (MTT) assay at 24-h interval. Results shown are the MTT optical density (OD) readings of triplicate wells expressed as the means ± s.d. and are representative of at least three independent experiments.

Measurement of PSA production by LNCaP cells
LNCaP cells (10^6 per well) were seeded in a six-well plate. After 24 h, cells were washed with phosphate-buffered saline and then incubated with 2 ml serum-free medium. Then DHT (2 nM) or LY294002 (10 or 20 µM) or vehicle as indicated was added to the medium. After 24 h, the supernatant was collected and analyzed for PSA by a commercially available enzyme-linked immunosorbent assay (ELISA) (ICN Pharmaceutical, Costa Mesa, CA, USA). PSA levels in the collected supernatant are expressed as ng/ml and represent the means ± s.d. of two independent experiments.

Experimental protocols
Two days before adenoviral infection, 1 × 10^5 cells were seeded in each well of a six-well plate. On the day of infection, cells from one well were detached with trypsin and counted. This information was used to infect each cell line at the desired MOI. Infections were carried out with 500 µl infection medium (the same medium used for each cell line with 2% FBS and 1% P&S) in a 5% CO₂ incubator at 37 °C for 1 h on a rocker. Pilot experiments with an adenovirus containing the green fluorescent protein (GFP) cDNA (Av-CMV-GFP) determined the optimal MOI for the cell lines used in this investigation. Based on this, LAPC-4 and LNCaP were infected with an MOI of 100:1 with every adenovirus used, except Av-CMV-PTEN which was used at an MOI of 1000:1 to achieve complete dephosphorylation of Akt in LNCaP cells.

These experiments were performed in regular FBS. The use of regular FBS did not have any consequences on AR transcriptional activation, as we have found that the concentrations of
testosterone or DHT determined by radioimmunoassay in the FBS from Invitrogen are extremely low (17 ng/dl (59 pM) and 3 pg/ml (0.01 pM) for testosterone and DHT respectively) and unable to induce ARR2PB activity under the experimental conditions used throughout these studies (data not shown; Y Zhang & M Marcelli, unpublished observations).

LNCaP, LNCaP LP or LAPC-4 cells were infected with Av-CMV-PTEN, Av-CMV-PTEN(mut) or Av-CMV-LacZ on day 0. In some experiments, Adeno(dn)Akt or Adeno(myr)Akt were infected simultaneously with the PTEN construct. After 48 h, cells were infected with Av-CMV-PTEN, Av-CMV-PTEN(mut), or Av-CMV-LacZ. The infection was performed for 24 h of hormonal stimulation, cells were harvested and immunoblot analysis was performed for: PTEN (to control for successful infection with Av-PTEN or Av-PTEN(mut)), total and 473Ser-phospho-Akt (to control for wt PTEN activity, but not total Akt levels), β-actin (to control for equal loading in each lane), Bax or HA to control for hormonal induction of Av-ARR2PB-HA-Bax (the AR-inducible construct in which HA-Bax is controlled by the exogenous ARR2PB promoter). In some experiments, immunoblot analysis was performed for PSA (to control for DHT induction of an endogenous AR-responsive gene). In each experiment the same number of micrograms of cell lysate was loaded. When precise quantitation was required, densitometric analysis was performed to correct expression of the protein of interest with that of β-actin, which was immunodetected in the same sample (Li et al. 2001b). Densitometry was done by importing images to a Macintosh G4 personal computer using the Chemi Doc Documentation System, and the Quantity One quantitation software (both from BioRad, Hercules, CA, USA). Arbitrary densitometric units of the protein of interest were then corrected for the densitometric units of β-actin.

Site-directed mutagenesis

S213 and S791 are two putative Akt phosphorylation sites identified in the sequence of AR (Wen et al. 2000). One possible mechanism used by PTEN to control AR transcriptional activation may be by modulating phosphorylation of these sites by preventing Akt activation. To test this hypothesis, we performed site-directed mutagenesis to obtain an AR cDNA in which S213 and S791 were changed into alanine residues, using the QuickChange XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). The procedure was performed according to the specifications of the manufacturer, using as a template the wt pCMV-AR expression plasmid, and primers 5'-CGA GGG AGC GCG CGG GGG CTC CCA C-3' and 5'-GTG GGA GCC CCC GCG CGC TCC CTC G-3' to obtain S213A-AR, and 5'-TGA GCC ACC TCG CTC AAG AGT TTG G-3' and 5'-CCA AAC TCT TGA GCG AGG TGC CTC A-3' to obtain S791A. The presence of the desired nucleotide substitutions and absence of unwanted PCR-related mutations in the resulting plasmids was confirmed by sequence analysis with a published sequence of wt AR (Tilley et al. 1989). This analysis confirmed that S213 (TCTG) and S791 (TCT) were mutagenized into alanine residues (GGG and GCT respectively). The two resulting plasmids CMV-AR S213A and CMV-AR S791A were subsequently digested, and the two mutated fragments ligated to obtain plasmid CMV-AR S213A&S791A in which both mutations were correctly inserted within the same AR cDNA.

Transient transfections to evaluate the transcriptional activity of CMV-AR S213A&S791A

Non-recombinant adenoviral-mediated DNA transfer technique

To study whether PTEN affects transcription of an AR cDNA mutated in its two putative Akt-phosphorylation sites, AR (−) and PTEN (−) PC-3 cells were infected with a virus encoding wt PTEN (or a control virus encoding LacZ) at MOIs of 5000:1. After 48 h, cells were transiently transfected with the non-recombinant adenoviral-mediated DNA transfer technique (Allgood et al. 1997) using 10 ng CMV-AR or CMV-AR S213A&S791A in association with 0.5 µg of the androgen-inducible GREpE1b-Luc or PSA P/E-luc reporters (expressing firefly luciferase activity upon induction), and 10 ng of the constitutively active pRL-CMV-TK plasmid (expressing renilla luciferase activity). Plasmids were incubated with the coupled virus (at an MOI of 500:1) for 30 min. Subsequently, additional poly-L-lysine (1:3 µg/µg DNA) was added to shrink the DNA onto the viral
surface. The virus–DNA complex was added to the cells and allowed to infect them for 2 h in serum-free medium after which time the medium was supplemented with charcoal-stripped serum to a final concentration of 5%. Each experiment was performed a minimum of three times.

**Cell treatment**

Twenty-four hours after transfection, transfected PC-3 cells were treated with 2 nM DHT or 0.2% ethanol vehicle for 24 h.

**Western analysis for AR**

Cell lysates from each well were divided into two aliquots. The first was used for the detection of luciferase activity (described below). Cell lysate volumes from the second aliquot equal to 10 µg proteins were utilized for the immunodetection of AR and β-actin by Western analysis. Arbitrary densitometric units of the AR band of each well were corrected for the densitometric units of the corresponding β-actin band (AR/β-actin DU ratio).

**Luciferase activity**

Luciferase activity was measured using the Dual-Luciferase Reporter (DLRRTM) Assay System (Promega). Results are expressed as luciferase units/s (LU/s), and represent the ratio of the firefly (representing DHT-inducible luciferase activity from plasmids GRE2Elb-Luc or PSA P/E-luc) and renilla (representing the constitutive luciferase activity from plasmid pRL-CMV-TK) luciferases activities detected in the cell lysate. Renilla luciferase activity was generated by a constitutively active plasmid and it was measured to correct firefly luciferase activity for differences of transfection efficiency among the various plates. The LU/s units obtained after this initial correction were further corrected for the AR/β-actin DU ratio derived from the cell lysates of the same well, to normalize for differences in AR expression detected by the Western analysis step described above.

**Results**

**PI3K signaling inhibition prevents known effects of AR in LNCaP cells**

PTEN (−) LNCaP cells were infected with adenoviral constructs Av-CMV-PTEN or Av-CMV and stimulated with R1881 (0.05 nM) or vehicle. Proliferation was significantly enhanced in Av-CMV-infected cells after stimulation with R1881 in comparison with Av-CMV-infected cells treated with vehicle alone. In contrast, R1881-stimulated proliferation was significantly inhibited in Av-CMV-PTEN-infected cells (Fig. 1A). In additional experiments, we determined DHT-stimulated PSA concentration in the supernatant of LNCaP cells treated with 0, 10 or 20 µM of the PI3K inhibitor LY294002. Under these experimental conditions, DHT induced a significant increase of PSA only in control cells, while increasing concentrations of LY294002 inhibited production of this surrogate marker of endogenous AR activation (Fig. 1B). These experiments suggested the possibility that inhibition of PI3K signaling may reduce AR activity. To rule out that reduced AR activity was due to decreased AR expression, quantitation of immunoreactive AR was performed during inhibition of PI3K signaling through LY294002 treatment or adenoviral-mediated PTEN re-expression, and no changes were detected compared with vehicle-treated cells (Fig. 1C).

**PTEN reduces AR transcriptional activity in a cell line- and promoter-independent fashion**

Further experiments were performed to determine how PTEN interferes with AR function. LNCaP cells were infected with Av-CMV-PTEN or Av-CMV-LacZ as a control, followed after 48 h by infection with Av-ARR2PB-HA-Bax and treatment for 24 h with 2 nM DHT. Western analysis of the resulting cell lysates are shown in Fig. 2. Adenoviral-mediated expression of wt PTEN in high and low passage LNCaP cells inhibited Akt phosphorylation, while no effect on total Akt expression was detected (Fig. 2A: compare lanes 1 and 2 (Av-CMV-LacZ infected) with 3 and 4 (Av-CMV-PTEN infected), and lanes 5 and 6 (Av-CMV-LacZ infected) with 7 and 8 (Av-CMV-PTEN infected) in LNCaP low and high passage respectively). Following infection with Av-ARR2PB-HA-Bax, treatment with DHT induced significant expression of HA-Bax in the absence of PTEN (recognizable by the appearance of the larger HA-Bax band in lanes 2 and 6 which were infected with the control virus Av-CMV-LacZ). However, when DHT was administered to LNCaP cells previously infected with Av-CMV-PTEN,
induction of HA-Bax expression was significantly lower than in control cells infected with Av-CMV-LacZ (compare lanes 4 and 8 (infected with Av-CMV-PTEN (wt)) with lanes 2 and 6 (infected with Av-CMV-LacZ)). At least eight experiments were carried out looking at PTEN-induced inhibition of HA-Bax expression under these experimental conditions, and an average of 65% inhibition was seen. For instance, the experiment in Fig. 2A (lanes 4 and 8 compared with 2 and 6 respectively) shows 100% inhibition, while the experiment in Fig. 2C (lane 2 compared with 4) shows 55% inhibition. Essentially similar results (i.e. PTEN-mediated decreased expression of HA-Bax in both LNCaP and LNCaP LP cells by 80 and 70% respectively) were obtained in experiments where Western analysis was done using an anti-HA antibody to trace the amount of HA-Bax (and not of endogenous Bax) expressed under the same experimental conditions (Fig. 3).

In addition to Av-CMV-LacZ, the inactive lipid phosphatase-deficient form of PTEN was also used to control these experiments (Yuan & Whang 2002). As shown in Fig. 2C, despite its dramatic overexpression, this form of PTEN was functionally inactive (shown by its inability to prevent Akt phosphorylation (compare lanes 1 and 2 which were infected with Av-CMV-PTEN with lanes 3...
and 4 which were infected with AvCMV-PTEN(mut)). HA-Bax expression from the androgen-responsive virus Av-ARR2PB-HA-Bax was inhibited by the wt (Fig. 2C, lanes 1 and 2) but not the mutant form of PTEN (Fig. 2C, lanes 3 and 4), or the control virus Av-CMV-LacZ (Fig. 2C, lanes 5 and 6) following treatment with DHT.

PTEN-mediated inhibition of AR activity was also observed with PSA, an endogenous AR-regulated gene. The experiment in Fig. 2B shows that PSA expression was dramatically stimulated by DHT in LNCaP cells, and that this effect was prevented by adenoviral-mediated expression of wt PTEN. This experiment was further controlled

Figure 2. PTEN inhibits expression of exogenous and endogenous AR-regulated genes. (A) LNCaP cells (high and low passage clones) were infected with MOI 1000:1 of adenovirus Av-CMV-PTEN (wt) or Av-CMV-LacZ on day 0. After 48 h, cells were infected with Av-ARR2PB-HA-Bax (MOI 100:1) and incubated with DHT or vehicle for an additional 24 h. Immunoblot analysis was performed for β-actin (to control for equal loading), phospho-Akt (p-Akt), total Akt, PTEN and Bax (the gene placed under the control of the AR-responsive promoter ARR2PB). Note that exogenous Bax contains an HA tag, and runs slightly higher than endogenous Bax. HA-Bax is indicated by an asterisk. (B) LNCaP cells were infected with Av-CMV-PTEN (lanes 1 and 2) or a control adenovirus Av-CMV-LacZ (lanes 3 and 4) and treated with 2 nM DHT or vehicle for 24 h. Immunoblot analysis was done for PTEN, β-actin (to control for equal loading), and PSA. The experiment in (B) was performed with the same cell lysates as (A). (C) LNCaP (high passage) were infected with Av-CMV-PTEN, Av-CMV-PTEN(mut) or Av-CMV-LacZ (MOI 1000:1) at time-point 0. After 48 h, cells were infected with Av-ARR2PB-HA-Bax and incubated with 2 nM DHT or vehicle for the following 24 h. Cell lysates were utilized to perform Western analysis of β-actin, total Akt, p-Akt, PTEN, Bax and PSA. The data suggest that wt (lanes 1 and 2) but not mutant PTEN (lanes 3 and 4) or the control adenovirus Av-CMV-LacZ (lanes 5 and 6) inhibits DHT-mediated expression of the exogenous (HA-Bax) and endogenous (PSA) AR-dependent genes. HA-Bax is indicated by an asterisk.
using the mutated PTEN adenovirus. Figure 2C shows that PSA expression is inhibited by the wt PTEN, but not by the mutated PTEN or the control adenovirus Av-CMV-LacZ.

These experiments suggested, therefore, that in LNCaP cells wt PTEN antagonizes the ability of AR to induce expression of a reporter gene (HA-Bax) driven by an exogenous androgen-responsive promoter, and of a native gene (PSA) driven by an endogenous AR-responsive promoter. These experiments also demonstrated that the lipid phosphatase function of PTEN was required to achieve inhibition of DHT-induced expression of HA-Bax and PSA.

PTEN-mediated inhibition of AR activity is not cell line dependent

We utilized AR (+) and PTEN (+) LAPC-4 cells to determine if the inhibitory effect of PTEN on AR activity is present in CaP cell lines other than LNCaP cells. The experiments in Fig. 4 show that adenoviral-mediated PTEN overexpression is associated in LAPC-4 cells with decreased DHT-dependent induction of HA-Bax and PSA. Thus PTEN-induced antagonism of AR transcription is not cell line dependent. Note that LAPC-4 cells express minimal amounts of endogenous Bax, and the Bax band present in Fig. 4 is uniquely HA-Bax with the exception of lane 2, which contains a smaller (and fainter) band representing endogenous Bax.

PTEN-mediated inhibition of AR activity is Akt dependent

The fact that the lipid phosphatase activity of PTEN is necessary to antagonize Akt activation and AR transcription led to the hypothesis that Akt is the mediator of the observed inhibitory effect of PTEN on AR transcription. Additional experiments were performed to demonstrate this point. LNCaP cells were again infected with Av-CMV-LacZ (MOI 100:1) or Av-CMV-PTEN (1000:1) for 48 h, followed by Av-ARR2PB-HA-Bax (100:1) for 24 h and treatment with 2 nM DHT for 24 h. As shown in Fig. 5, under control conditions (lanes 3 and 4) DHT induced significant amount of HA-Bax expression, while the presence of wt PTEN inhibited HA-Bax induction by 85% (lanes 5 and 6). In additional experiments (shown in Fig. 5), LNCaP cells were infected with Av-CMV-PTEN (MOI 1000:1) and Adeno-dnAkt (MOI 100:1) or Adeno-myrAkt (MOI 100:1) for 48 h, followed by treatment for 24 h with 2 nM DHT. Association of PTEN with the dominant negative Akt construct completely prevented HA-Bax expression (lanes 1 and 2), while association of wt
PTEN with the constitutively active Akt construct (lanes 7 and 8) was able to revert (at least partially) the inhibitory effect of PTEN on HA-Bax expression observed in lanes 5 and 6. HA-Bax expression was rescued by 62% when LNCaP cells were infected with PTEN+myrAkt compared with PTEN alone. These experiments showed that a dominant negative form of Akt contributed with PTEN to inhibit AR transcriptional activity. In contrast, the constitutively active form of Akt antagonized this effect of PTEN. Together with the observation that the phosphatase-deficient form of PTEN did not have an effect on Akt activation and AR transcription (Fig. 2C), these experiments supported the hypothesis that PTEN inhibits AR activity in an Akt-dependent way.

Figure 4 PTEN inhibits the AR-induced expression of endogenous (PSA) and exogenous (HA-Bax) AR-dependent genes in the cell line LAPC-4. Cells were seeded on day 0. After 24 h, cells were infected with adenovirus Av-CMV-PTEN or Av-CMV-LacZ (MOI 100:1). After 48 h, cells were infected with adenovirus Av-ARR2PB-HA-Bax (MOI 100:1), followed by incubation in the presence of DHT or vehicle for an additional 24 h. Cell lysates were subjected to Western analysis for β-actin (to control for equal loading), Bax (the gene placed under the control of the AR-responsive promoter ARR2PB), PTEN, total Akt, p-Akt, and PSA (the endogenous AR-responsive gene). Note that the endogenous level of Bax in this cell line is almost undetectable, and that a band migrating below HA-Bax is visible only in lane 2. The data suggest that DHT-mediated induction of HA-Bax and PSA is inhibited in the cells infected with the PTEN virus (compare lane 2 (addition of DHT and absence of PTEN) with lane 4 (addition of DHT and PTEN)).
Does Akt modulate AR activity through its direct phosphorylation?

Investigators have reported that two putative Akt phosphorylation sites within the sequence of AR undergo Akt-mediated phosphorylation (Wen et al. 2000, Lin et al. 2001). We reasoned that if PTEN modulates AR transcription by inhibiting Akt activation, absence of these putative Akt phosphorylation sites should prevent inhibition of AR by PTEN. We performed site-directed mutagenesis of these two putative phosphorylation sites to produce plasmid CMV-AR S213A&S791A, in which serine residues 213 and 791 are replaced by alanines. PC-3 cells were initially infected with Av-CMV-PTEN (or Av-CMV-LacZ as a control) for 48 h, and subsequently transfected with pCMV-AR or pCMV-AR S213A&S791A and with reporter plasmids GRE_E1b-Luc or PSA P/E-luc and pRL-CMV-TK. Vehicle or vehicle plus DHT were then added to the culture plates for 24 h. These experiments (Fig. 6A and B) show that, regardless of the AR-inducible promoter, transcriptional activity of both AR plasmids was similarly inhibited by PTEN, therefore inhibition of AR transcriptional activity does not depend on prevention by PTEN of Akt-mediated phosphorylation, at least in the two putative sites mutated in this experiment and in the cell line PC-3.

Discussion

This paper provides evidence in support of the hypothesis that in CaP cell lines PTEN antagonizes AR transcriptional activity through inhibition of Akt activation, and that this effect is not cell line or promoter dependent. Recent papers have suggested that two serines (S213 and S791) located in the midst of two Akt-consensus sites in the coding sequence of AR are phosphorylated by Akt, and that AR activity is affected by Akt-mediated phosphorylation. Based on this, we devised an experiment to test the hypothesis that the observed
ability of PTEN to antagonize AR transcription is due to direct Akt-mediated AR phosphorylation. An AR construct with alanine residues inserted in place of S213 and S791 was prepared and transfected in AR (+) and PTEN (+) PC-3 cells. PTEN exerted a similar inhibitory effect on transcriptional activity of both wt AR or AR S213A&S791A, suggesting that lack of the two putative Akt phosphorylation sites does not affect PTEN-mediated inhibition of AR transcription.

Figure 6 PTEN similarly inhibits DHT-induced luciferase activity from PC-3 cells transfected with a wt AR construct or an AR construct with mutagenized Ser213 and 791. PC-3 cells were infected with a control virus (Av-CMV-LacZ), or with Av-CMV-PTEN on day 0. After 48 h, cells were transfected with pCMV-AR (wt AR) or pCMV-AR S213A&S791A (mut AR), the reporter plasmid GRE2E1b-Luc (A) or PSA P/E-luc (B), and the constitutively active plasmid pRL-CMV-TK expressing Renilla luciferase. Cells were then incubated with 2 nM DHT or vehicle for additional 24 h. Luciferase activity was determined and corrected for transfection efficiency of AR expression as described in Materials and Methods. Data are compared with cells infected with Av-CMV-LacZ+wt AR+DHT set at 100% and represent means±S.D. of six wells. One of three experiments is shown. With both reporter plasmids and with both wt AR or mut AR, there was a statistically significant difference between the luciferase activity detected in the absence vs the presence of PTEN (two tailed paired t-test).
Akt regulates its target molecules by phosphorylation, and its activity results in survival, proliferation and cellular growth (Vivanco & Sawyers 2002). Some of the activities resulting in survival consist of direct inactivation (by phosphorylation) of factors mediating cell death such as the apoptotic proteins Bad (Zha et al. 1996) and caspase-9 (Cardone et al. 1998). Alternatively, Akt-mediated phosphorylation stimulates survival by activating other factors such as Mdm2, a molecule whose ability to function as a survival factor depends on facilitating degradation of the pro-apoptotic tumor suppressor gene p53 (Mayo & Donner 2001, Zhou et al. 2001). A third mechanism through which Akt affects survival is by activating or inhibiting transcription factors responsible for the synthesis of anti-apoptotic or pro-apoptotic genes respectively. For instance, Akt indirectly (through phosphorylation of IkB) activates the transcription factor nuclear factor-κB (NF κB) (Romashkova & Makarov 1999), which affects survival by transcribing the antiapoptotic genes TRAF1, TRAF2, c-IAP1, cIAP2 and c-FLIP (Wang et al. 1998, Micheau et al. 2001). An example of a transcription factor responsible for the transcription of proapoptotic molecules such as FAS ligand (Brunet et al. 1999) and Bcl-2 interacting mediator of apoptosis (BIM) (Dijkers et al. 2000) is the forkhead transcription factor FHKR. Akt inhibits FHKR by anchoring it to the cytosol through phosphorylation (Brunet et al. 1999). In addition to regulating cell survival, Akt also induces cellular proliferation and growth by phosphorylating a variety of substrates using the same general mechanisms (Vivanco & Sawyers 2002). Based on this, one can conclude that PTEN-mediated inhibition of Akt activation has several potential ways to affect AR transcription. Akt could affect AR transcription by post-translationally modifying substrates required for AR activation or repression, or alternatively could modulate in a positive or negative way the transcription of such factors. Identification of these Akt-regulated regulators of AR transcriptional activity is one of the projects currently going on in our laboratories.

A number of studies have recently examined the interaction existing between AR and PTEN/Akt signaling, and the conclusions are controversial. Wen et al. (2000) were the first to identify the presence of two Akt consensus sites in AR in Ser213 and 791, and to show that Akt can directly bind to and phosphorylate AR. Lin et al. (2001) demonstrated Akt-mediated AR phosphorylation in Ser213 and 791. These authors also described that active Akt inhibits AR transcriotional activity, and that this effect is mimicked by the constitutively active form of Akt, and inhibited by the dominant negative Akt construct. According to these authors, inhibition of AR activity goes through two steps, a first step of Akt-mediated phosphorylation and a second step of Mdm2-mediated ubiquitination (Lin et al. 2002). The reasons for the discrepancy between our data and that of Lin et al. (2002) are not clear, but have probably to do with the fact that we used assays measuring the mitogenic and anti-apoptotic effects of AR, while Lin et al. (2002) used a model of AR-induced apoptosis.

Post-translational modifications of AR such as phosphorylation have been suggested to be an important mechanism modulating AR activity for a number of years (Kemppainen et al. 1992, Blok et al. 1996). Using a combination of peptide mapping, Edman degradation and mass spectrometry (Gioeli et al. 2002), Gioeli et al. (2002) have mapped the phosphorylation sites of AR, which do not include S213 and S791, possibly due to the non-selectivity of the in vitro kinase reactions which were utilized to identify these two sites. In agreement with the data of Gioeli et al. (2002), we did not find differences in the transcriptional activity of CMV-AR and CMV-AR S213A&S791A and detected similar degrees of suppression when the experiments were done in the presence of PTEN, suggesting that these two phosphorylation sites are not used by Akt to modulate AR activity, at least in PC-3 cells.

The negative interaction between inhibition of the PI3K pathway and AR transcriptional activity described in this paper is supported in the literature by the papers of Li et al. (2001a) and Sharma et al. (2002). Similarly to us, Li et al. (2001a) found that PTEN antagonizes AR signaling, and that this occurs in an Akt-dependent way. Sharma et al. (2002) not only described a negative interaction between PI3K inhibition and AR signaling, but also that this is mediated by down-regulation of β-catenin, an AR co-activator (Truica et al. 2000).

In conclusion, our studies support the theory that PTEN functions as a transcriptional inhibitor of AR by preventing Akt activation, and that a downstream effect of the protein kinase Akt mediates this interaction. Unchecked Akt activation, which is frequently observed in advanced CaP, may be associated with uncontrolled AR
signaling, which may explain why androgen-independent CaP cells are insensitive to hormonal manipulation, but still require AR for their survival/proliferation. Furthering our knowledge on the Pten (Akt)–AR axis will most likely create new therapeutic targets for androgen-independent CaP.

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