Androgen receptor expression is regulated by the phosphoinositide 3-kinase/Akt pathway in normal and tumoral epithelial cells

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The androgen receptor (AR) is a ligand-responsive transcription factor known to play a central role in the pathogenesis of prostate cancer. However, the regulation of AR gene expression in the normal and pathological prostate remains poorly understood. This study focuses on the effect of the phosphoinositide 3-kinase (PI 3-kinase)/Akt axis on AR expression in vas deferens epithelial cells (VDEC), a suitable model to study androgen regulation of gene expression, and LNCaP cells (derived from a metastasis at the left supraclavicular lymph node from a 50-year-old patient with a confirmed diagnosis of metastatic prostate carcinoma). Taken together, our data show for the first time that the PI 3-kinase/Akt pathway is required for basal and dihydrotestosterone-induced AR protein expression in both VDEC and LNCaP. Inhibition of the PI 3-kinase/Akt pathway reduced AR expression and the decline in AR protein level correlated with a decrease in AR mRNA in VDEC but not in LNCaP. Since PI 3-kinase/Akt axis is active in prostate cancer, cross-talk between PI 3-kinase/Akt and AR signalling pathways may have implications for endocrine therapy.

Key words: androgens and growth factor signalling, cell differentiation, epithelial cells.

INTRODUCTION

Androgens are required for the development, growth and function of reproductive organs such as the prostate. Androgen action is mediated through the androgen receptor (AR), which regulates target gene transcription. During the initial stages of morphogenesis, prostatic stromal cells are AR-positive, whereas epithelial cells remain negative until shortly after birth, suggesting that the effects of androgens on prostatic epithelial development are mediated by paracrine activities of the associated mesenchyme [1]. In the adult prostate, the luminal epithelial cells express high levels of the AR, while the basal cells, considered to be luminal-cell precursors, lack AR [2]. Tumoral development results from a multistep process that leads successively to the formation of low- and high-grade prostatic intraepithelial neoplasia, which are mainly under androgenic control [3]. As a result, androgen ablation induces tumour regression, but, after a limited period of relapse, cancer recurs as an androgen-independent disease [4]. Most androgen-independent prostate cancer cells still express AR as well as the androgen-inducible prostate-specific antigen (PSA), suggesting that these cells maintain an active AR signalling pathway [5]. The mechanisms involved in the escape of prostate cancer from androgen control include mutation and overexpression of the AR gene or ligand-independent activation by other signalling pathways [6,7]. Epigenetic changes leading to the overexpression of the erbB1 and erbB2/neu receptors are also involved in the tumoral progression (for reviews see [8–10]). To better understand the molecular mechanisms involved in this deregulation of tumoral cells, it is important to previously determine how AR expression and activity are regulated in non- tumoral differentiated epithelial cells.

We have previously shown that epidermal growth factor (EGF) and insulin have a permissive effect on the androgenic response of the vas deferens epithelial cells (VDEC) [11]. The binding of these polypeptide growth factors or polypeptide hormones such as insulin to their cell-surface receptors triggers the recruitment of numerous molecules to form a localized signalling complex at the plasma membrane. Multiple transduction pathways can in turn be activated, amongst which the mitogen-activated protein kinase (MAPK)/extracellular-signal-related kinase (ERK) pathway, the protein kinase C (PKC) pathway and the phosphoinositide 3-kinase (PI 3-kinase)/Akt pathway. The aim of the present work was to investigate the possible role of tyrosine kinase receptor signalling on AR expression and activity in VDEC, a model of proliferation and differentiation of androgen-responsive cells, and in LNCaP prostatic cancer cells.

EXPERIMENTAL

Life Technologies (Cergy Pontoise, France) supplied the Dulbecco's modified Eagle (DMEM)/Ham's F12 medium, OPTI-MEM medium, glutamine, Hepes, transferrin, 10-fold concentrated PBS and gentamicin. Bovine insulin and EGF were from Roche (Le Pont de Claix, France). Cholera toxin, mitomycin C, dibutyryl cAMP, selenium, cortisol ('hydrocortisone'), dihydrotestosterone (DHT), LY294002 and Matrigel were from Sigma Chemical Co. Microporous PET membranes were from Falcon (Becton Dickinson Labware) and PD 098059 was from New England Biolabs.

Cell-culture conditions

Epithelial cells derived from primary culture of mouse vas deferens explants were propagated as previously described [12]

Abbreviations used: DHT, dihydrotestosterone; EGF, epidermal growth factor; ERK, extracellular-signal-related kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, haemagglutinin; (h)AR, (human) androgen receptor; IGF-I, insulin-like growth factor I; luc, luciferase; MAPK, mitogen-activated protein kinase; MVDP, mouse vas deferens protein; PARP, poly(ADP-ribose) polymerase; PI 3-kinase, phosphoinositide 3-kinase ('phosphatidylinositol 3-kinase'); PKC, protein kinase C; PSA, prostate-specific antigen; VDEC, vas deferens epithelial cells.

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in a basal mixture of DMEM/Ham F12 (1:1, v/v) containing transferrin (10 μg/ml), cholera toxin (10 ng/ml), selenium (17.3 ng/ml), cAMP (1.5 μg/ml), glutamine (2 mM), ethanolamine (0.6 μg/ml), Hepes (20 mM), gentamicin (50 μg/ml) supplemented or not with EGF (10 ng/ml), insulin (5 μg/ml). For proliferation, cortisol (1 μM) was added in the medium. After trypsinization, epithelial cells were seeded in the same medium at confluent density on to matrigel-coated microporous membranes, which allow cell polarization. After 24 h, the medium was replaced by the basal medium containing only cortisol at 10 nM to prevent further cell proliferation. After 3 days the whole cell population was homogeneously polarized. Then polarized cells were cultured in the basal medium containing either EGF (1 ng/ml) or insulin (5 μg/ml) or insulin-like growth factor I (IGF-1; 1 or 50 ng/ml). To study gene expression during cell proliferation, cells were seeded without 3T3 feeder layer on serum fibronectin-coated plastic dishes. Inhibitors of the PI 3-kinase (LY 294002) and MAPK (PD 098059) pathways were always added 1 h before DHT induction at the respective concentrations of 50 μM and 20 μM. Mouse vas deferens protein (MVDP) expression increases as a function of DHT concentration, giving optimal expression at 1 μM DHT [12]. This DHT concentration was used for short-term induction experiments.

LNCaP cells were cultured in RPMI medium, containing 10 % (v/v) fetal-calf serum. Cells were seeded at 80 % confluent density in the same medium to allow cell attachment. The next day, the medium was replaced by DMEM/Ham F12 (1:1) complemented as indicated for epithelial cells without EGF and insulin. The day after the cells were incubated in the same medium supplemented with either EGF or insulin at various concentrations in the absence or in the presence of LY294002 and PD098059.

Transfections

HEK-293 cells were plated the day before transfection at a density of 4×10^5 cells/well in six-well plates. The cells were co-transfected in OPTI-MEM medium with a luciferase (luc) reporter plasmid (0.1 μg of 0.5mvdp-luc) [13], a human androgen receptor expression vector (0.3 μg of pSG5-hAR) and a dominant negative Akt–haemagglutinin (HA)-tagged expression vector [14] or empty vector (0.4 μg each) using polyethylenimine (Exgen 500; Euromedex, Mundolsheim, France). After transfection, cells were starved for 8 h in a basal medium without growth factors and then they were cultured in DMEM/Ham F12 medium with 1 % charcoal-treated fetal-calf serum and 5 μg/ml of insulin in the absence or the presence of R1881 (10 nM) for 24 h. The cells were harvested and the luc activity of each sample was measured with the luc assay kit (Promega).

RNA extraction and Northern-blot analysis

Total RNA was extracted using RNAzol solution (Quantum, Montreuil-sous-bois, France) and subjected to electrophoresis in denaturing formaldehyde/formamide agarose gels then transferred to Hybond N filters (Amersham Pharmacia Biotech). The Northern blots were prehybridized overnight at 42 °C in a solution containing 50 % formamide, 5 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), 5 × Denhardt's solution, 0.5 % SDS and 200 mg/ml salmon sperm DNA carrier. Hybridization was performed in an identical solution containing 10^6 c.p.m./ml of [α-^{32}P]dCTP-labelled MVDP, AR, PSA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or 18 S rRNA cDNA probes. Membranes were washed twice in 1 × SSC, then in 1 × SSC/0.1 % SDS and finally in 0.1 × SSC/0.1 % SDS for 10 min at 65 °C. Northern blots were subjected to autoradiography, and signals were measured by densitometric analysis using Molecular Analyst software (Bio-Rad).

Protein analysis

Cells were rinsed once with PBS and then scraped into 0.75 ml of PBS containing 3.5 mM β-mercaptoethanol. After centrifugation at 200 ***g***, the pellets were resuspended in 1 vol. of buffer C [20 mM Hepes (pH 7.6)/0.42 M NaCl/1.2 mM $MgCl_2$/0.2 M EDTA/25 % (v/v) glycerol plus a cocktail of protease inhibitors (Complete™ protease inhibitor cocktail tablets; Roche) and 2 mM β-mercaptoethanol]. After mixing the suspension, the NaCl concentration was adjusted to 0.42 M. Cell lysis was achieved by thawing on ice; then the extracts were sonicated for 10 s, centrifuged at 20000 ***g*** for 20 min at 4 °C and the supernatants were saved. Proteins were separated by one-dimensional electrophoresis and transferred to nitrocellulose. MVDP was detected using the polyclonal immune serum (1:1500), whereas AR was detected using a rabbit anti-AR antibody diluted to 1:4000 [15]. Anti-Akt, Anti-phospho-Ser^{473}-Akt (New England Biolabs), anti-HA (Babco; Eurogentec, Belgium), anti-β-actin and anti-MAPK (Sigma) antibodies were respectively used at dilution 1:1000, 1:500, 1:2500, 1:2000 and 1:20000. Anti-phospho-MAPK (ERK1/2) (Upstate Biotechnology, Euromedex) and anti-poly(ADP-ribose) polymerase (PARP) (Trevigen; Interchim, Montluçon, France) antibodies were used at dilution 1:500. The specific complexes were detected using the enhanced chemiluminescence (ECL®) system from Amersham Pharmacia Biotech.

RESULTS

The AR signalling pathway in VDEC is mainly regulated by epithelial-cell polarization and insulin action

Characterization of molecular events mediating androgen-responsive gene expression has been hampered by the lack of appropriate cell-culture systems. Transformed or tumour cells have been used extensively in these studies, but can be considered somewhat artificial, since they do not necessarily express endogenous AR, and since they may be deficient for certain transcription factors involved in hormone-specific gene activation. In the present study we have used a culture system (Figure 1) in which VDEC, cultured in a serum-free defined medium containing EGF and insulin, are able to differentiate and to express MVDP in response to androgen and in a dose-dependent manner. Thus MVDP is used as a marker of VDEC differentiation [12].

Cell polarization and insulin influence androgen-regulated gene expression

MVDP expression is detectable only in polarized cells and is markedly induced by DHT. It is correlated with a stimulation of the AR protein accumulation. As shown in Figure 2(A), the specific band corresponding to AR was clearly visible only when cells were polarized and was greatly enhanced after addition of DHT. Northern-blot analysis (Figure 2B) also revealed that AR mRNA was almost undetectable in proliferative cells, but accumulated markedly after cell polarization in the absence of hormone. DHT addition did not change AR mRNA levels after 18 h induction, suggesting that DHT regulates AR expression mainly at the post-transcriptional level, as already described [16].

EGF and insulin are the major components of the culture medium. These polypeptides are both necessary for cell

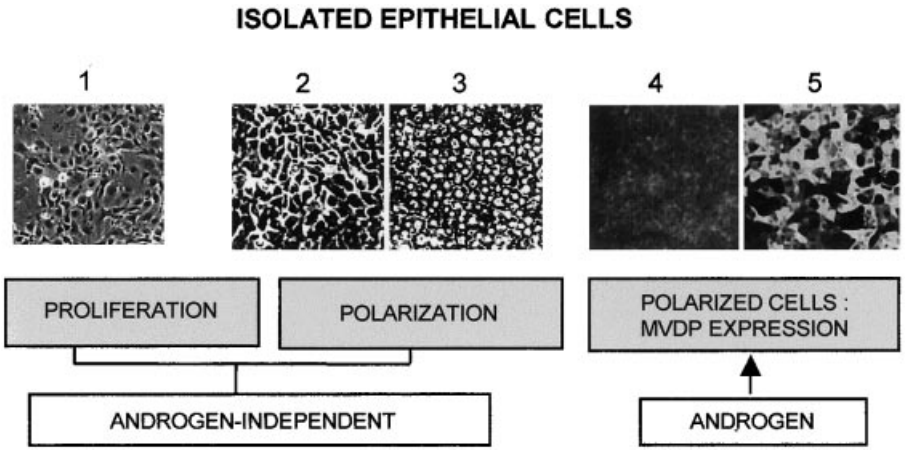

Figure 1 Mouse VDEC culture

Isolated epithelial cells are propagated on a 3T3 fibroblastic feeder layer (1) in a serum-free medium containing EGF and insulin. Cell differentiation is achieved by seeding cells at confluent density on matrix-coated microporous membranes that allow cells to polarize. Homogeneous polarization of the cell population occurs within 3 days and is associated with a typical morphological change observable under inverse microscope (2 and 3). It is only when the cells are polarized that they are able to express MVDP in response to DHT (5). In the absence of DHT, MVDP expression is almost undetectable using immunocytochemistry (4).

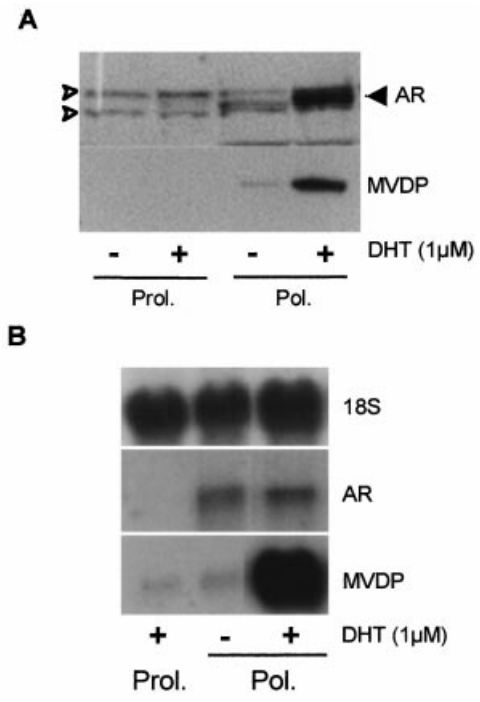

Figure 2 Regulation of AR and MVDP expression during epithelial cell differentiation and DHT induction

Proliferative and polarized cells were cultured in a medium containing EGF and insulin in the absence or in the presence of 1 μM DHT. (**A**) Western-blot analysis of AR and MVDP proteins using 30 μg of the protein extracts. Immunodetection was performed using a polyclonal immune serum against AR (1:4000) and MVDP (1:1500). The specific complexes were detected using an ECL® system. (**B**) Northern-blot analysis from 30 μg of total RNA extracts. Hybridization was performed using labelled 18 S rRNA, MVDP, AR cDNA probes. The Figures are representative of at least two independent experiments. The black arrowhead indicates non-specific staining.

proliferation and for expression of differentiated functions, and especially for androgen-regulated functions. Treatment of VDEC with increasing concentrations of insulin, but not EGF, enhanced DHT-induced levels of MVDP in a dose-dependent manner (results not shown). As shown in Figure 3(A), insulin stimulated androgen-induced AR protein expression in a dose-dependent manner. It is known that insulin at high concentration can act via insulin binding to IGF-I receptor [17]. Therefore, the effect of insulin and its related factor IGF-I on AR expression was analysed in differentiated VDEC. As shown in Figure 3(B), insulin (5 μg/ml) increased AR as well as did IGF-I at a lower concentration (1 ng/ml), indicating that both insulin and IGF-I receptors could be involved in this effect.

AR expression is dependent on the PI 3-kinase signalling pathway

To gain insights into the signalling pathway involved in the control of AR expression following cell polarization and DHT induction, specific inhibitors of the PI 3-kinase (LY294002) and MAPK (PD098059) pathways were tested. The cells were seeded on matrix-coated microporous membranes and then cultured with insulin for 3 days to reach homogeneous polarization of the cell population. Then they were incubated in the same medium with the inhibitors in the absence or in the presence of DHT. As indicated in Figure 4(A), treatment with LY294002 inhibited androgen-induced AR protein accumulation as a function of dose, the complete blockade being obtained with the highest concentration (50 μM). The latest is known to completely abolish PI 3-kinase activity without cell toxicity [18,19] and was used in kinetic studies. AR protein levels were increased significantly as a function of time in the presence of DHT (Figure 4B), and this effect was inhibited by a pretreatment with LY294002. To better characterize the differential expression of AR, polarized VDEC were incubated for 18 h with or without inhibitors in the absence or in the presence of DHT.

To demonstrate that Akt, the main target of PI 3-kinase, mediates the induced expression of AR, we checked the levels of

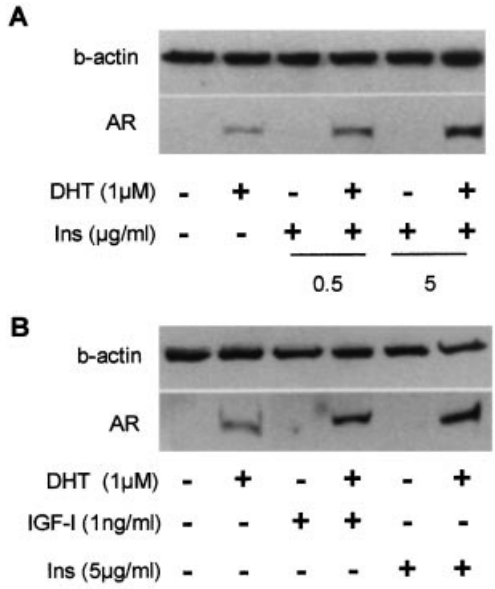

Figure 3 Effect of insulin and IGF-I on AR expression in differentiated VDEC

(**A**) Polarized cells were cultured with increasing concentrations of insulin. (**B**) Polarized cells were cultured without or with insulin (5 μg/ml) and IGF-I (1 ng/m)l and then for 2 additional days in the presence of 1 μM DHT. Western-blot analysis was performed as described previously. Data are representive of two individual experiments.

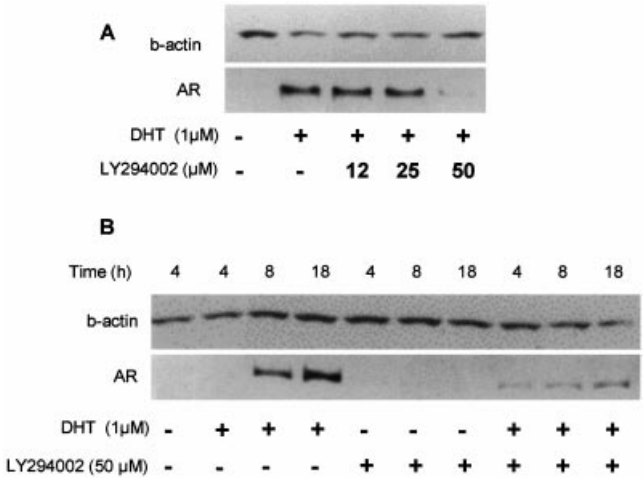

Figure 4 Dose- and time-dependent effects of the PI 3-kinase pathway inhibitor (LY 294002) on AR expression in differentiated VDEC

(**A**) Polarized cells cultured in the presence of insulin (5 μg/ml) were treated or not with increasing concentrations of LY294002 for 1 h and then for additionnal 17 h in the presence of 1 μM DHT. (**B**) Polarized cells were treated for 1 h with 50 μM LY294002, and then for various additional times in the presence or in the absence of 1 μM DHT. Western-blot analysis was performed as described previously.

phosphorylated Akt in the presence or in the absence of insulin. As shown in Figure 5(A), Akt phosphorylation was readily detectable in the presence of insulin. The addition of LY294002 completely eliminated phospho-Akt. Only minimal effects were observed after addition of PD098059. As expected, IGF-I also

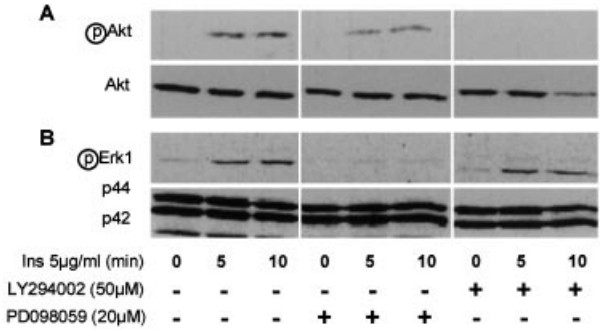

Figure 5 Inhibition of insulin-induced phosphorylation of Akt and ERK by the specific inhibitors of the PI 3-kinase (LY294002) and MAP kinase (PD098059) pathways respectively in differentiated VDEC

Cells were cultured for 2 h in basal medium in the absence or in the presence of LY294002 (50 μM) or PD098059 (20 μM). Then they were cultured in the same medium with insulin (5 μg/ml) for 5 and 10 min. Phosphorylated proteins were detected on Western blots using a rabbit polyclonal anti-phospho-Ser^{473}-Akt (1:500) or a mouse monoclonal anti-phospho-ERK1 (1:500) antibody. Non-phosphorylated forms were detected using a rabbit polyclonal anti-Akt (1:1000) and a rabbit polyclonal anti-ERK1/2 (p42/p44) (1:20000) antibodies. Data are representative of two individual experiments.

induced Akt phosphorylation (results not shown). The phospho-ERK blot shown in Figure 5(B) established that this pathway was indeed becoming activated by insulin. ERK phosphorylation was completely abolished by PD098059, but unaffected by LY294002 treatment. Inhibition of the PI 3-kinase pathway strongly decreased both basal and DHT-induced levels of AR (Figure 6A). Consequently, when expressed as fold increase over basal values, the ability of DHT to induce AR expression remained unchanged (Figure 6B). In contrast, AR levels were not affected by inhibition of the MAPK pathway in these cells. Taken together, our data show that regulation of AR protein levels by insulin is specifically mediated by the PI 3-kinase/Akt pathway. To determine whether the decline in AR protein is the result of a decrease in AR mRNA, Northern-blot analyses were performed. Inhibition of the PI 3-kinase axis resulted in a 60% decrease in steady-state levels of AR mRNA, whereas down regulation of the MAPK had no effect (Figure 6A). This effect was specific, since hybridization of the blots with the GAPDH probe showed no significant change. These data indicate a possible regulation of AR gene expression by the PI 3-kinase pathway in VDEC. Addition of DHT reduced AR mRNA levels, except in the presence of LY294002. As a consequence of decreased expression of AR, DHT-induced MVDP mRNA levels were greatly reduced in the presence of LY294002 (Figure 6A).

Involvement of the PI 3-kinase pathway in the regulation of AR expression in LNCaP prostatic carcinoma cells

Growth factors such IGF-I, keratinocyte growth factor and EGF are known to activate androgen-dependent expression and secretion of PSA in LNCaP cells [20]. We checked whether or not AR expression in tumoral LNCaP cells could be sensitive to the inhibition of the PI 3-kinase pathway.

To induce depletion in AR proteins, LNCaP cells were found to be more resistant to LY294002 treatment than VDEC. Thus treatments with PI 3-kinase and MAPK inhibitors were applied 3 h prior to DHT induction (16 h). Inhibition of PI 3-kinase signalling pathway with LY294002 dramatically decreased both basal and DHT-induced AR protein levels, whereas inhibition of the MAP kinase pathway had no significant effect. The effect

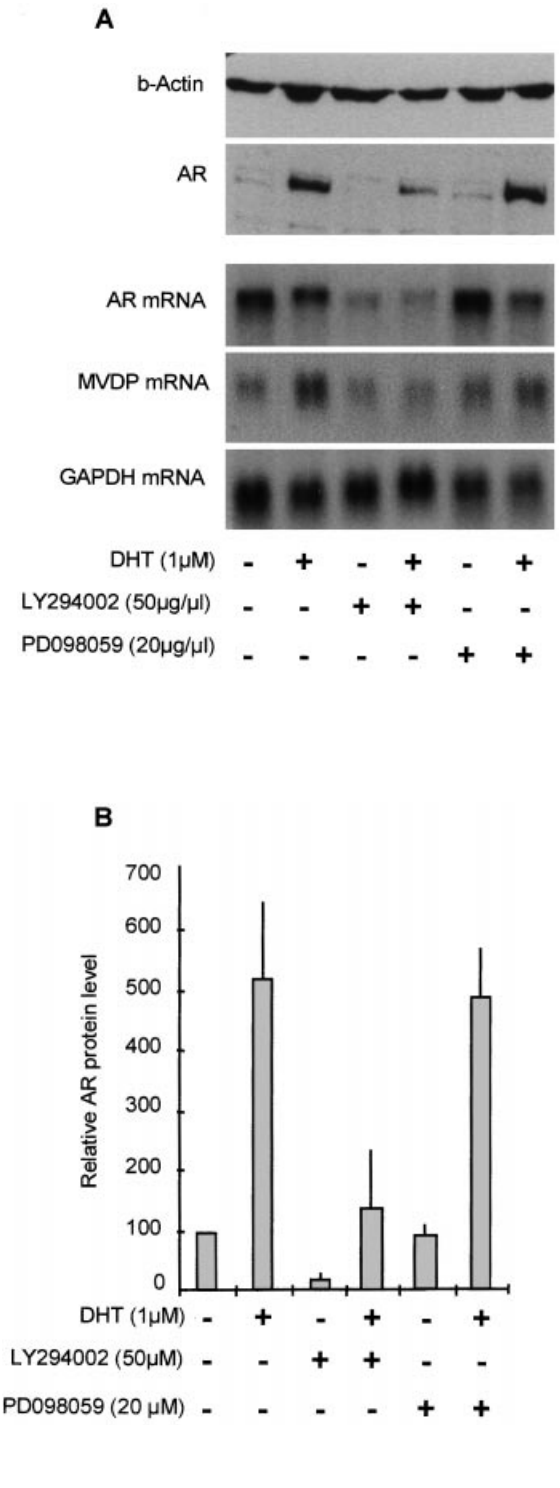

Figure 6 Effect of inhibition of the PI 3-kinase and of the MAPK signalling pathways on basal and DHT-induced AR protein and AR mRNA expression in differentiated VDEC

(**A**) Cells cultured with insulin (5 μg/ml) were treated with the vehicle or 50 μM LY294002 or with 20 μM PD098059 for 18 h in the presence or in the absence of 1 μM DHT. AR and β-actin expression was analysed as described in Figure 3. Specific mRNA accumulation was studied in Northern blotting using AR-, MVDP- and GAPDH-labelled cDNA probes. Autoradiography and histograms are representative data from three individual experiments. (**B**) Histograms show cumulative data as means $\pm$ S.E.M. from at least three independent experiments for each culture condition with insulin. AR protein levels were expressed relative to the value obtained with vehicle alone used (= 100%).

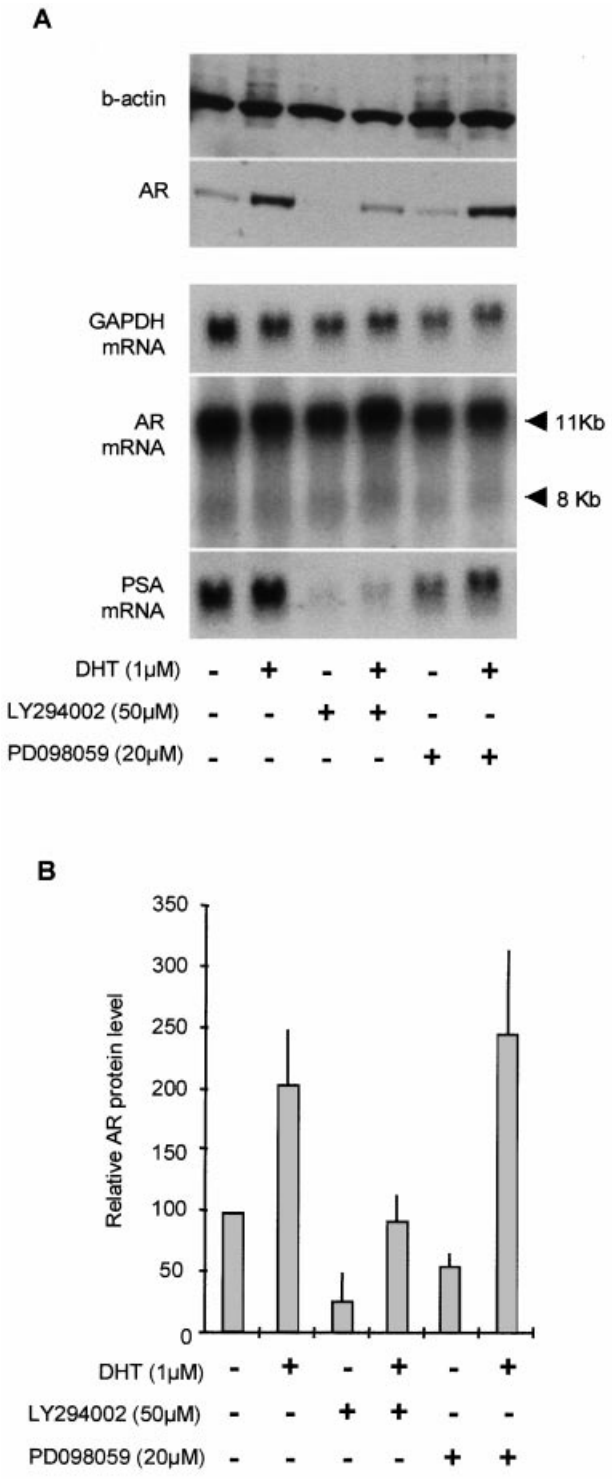

Figure 7 Effect of the inhibition of the PI 3-kinase and the MAPK signalling pathways on the expression of AR and PSA genes in LNCaP tumoral cells

Cells cultured in the same serum-free medium containing insulin as for VDEC were incubated for 18 h without or with DHT (1 μM) in the absence or in the presence of either the vehicle or LY294002 or PD98059. (**A**) AR and β-actin protein levels from 30 μg of protein extracts were analysed on a Western blot as described in Figure 3. PSA, GAPDH and the AR mRNA expression was detected on Northern blots from 30 μg of total RNAs. The data regarding protein analysis are representative of three independent experiments. The data regarding mRNA analysis are representative of three independent experiments. (**B**) The histograms show cumulative data obtained from Western-blot analysis. AR protein levels were expressed relative to the value obtained with vehicle alone (= 100%).

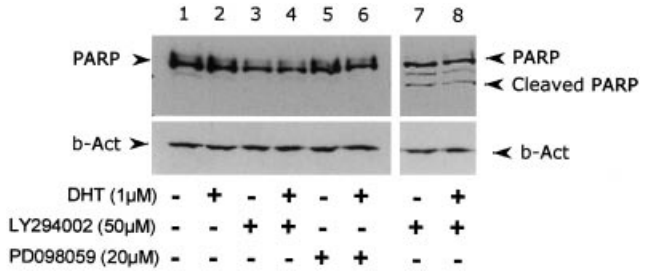

Figure 8 Effect of inhibition of the PI 3-kinase and MAPK pathways on PARP cleavage as a marker of apoptosis in LNCaP cells

Cells were cultured in a defined medium supplemented as previously described in the Experimental section (lanes 1–6) or in a DMEM/F12 medium only supplemented with glutamine and antibiotics (lanes 7 and 8) and treated with LY294002 or PD098059 for 18 h. PARP cleavage was detected using a mouse monoclonal anti-PARP antibody (clone C2-10, 1:500) recognizing the 116 kDa full-length protein and the 85 kDa cleavage fragment. Data are representative of two independent experiments. b-Act, β-actin.

induced by LY294002 on AR is specific, as the actin expression was not modified by the treatment (Figure 7A). To determine whether or not down-regulation or AR induced by LY294002 influences the expression of androgen-regulated genes, PSA mRNA levels were measured. Exposure of LNCaP to LY294002 strongly inhibited the DHT-induced overexpression of PSA mRNA levels (Figure 7A). In contrast, inhibition of the MAPK pathway had no effect. Taken together the results demonstrate that in LNCaP, as in VDEC, activation of the PI 3-kinase signalling pathway is required for constitutive and DHT-induced AR expression. The effect of the PI 3-kinase inhibitor on AR and GAPDH mRNA levels was analysed using Northern blotting. In contrast with VDEC, we found that the steady-state levels of AR mRNA in LNCaP cells were not significantly affected following treatment with LY294002 or with an inhibitor of the MAPK pathway (Figure 7A), suggesting that AR expression is regulated at a post-transcriptional level by the PI 3-kinase signalling pathway.

LY294002 is known to induce cell apoptosis by preventing the anti-apoptotic protection provided by the PI 3-kinase axis. To test whether the changes in AR expression in response to LY294002 are a result of cell apoptosis, we analysed PARP cleavage using the anti-PARP antibody. PARP expression was higher in LNCaP cells than in VDEC (results not shown), but there was no change in PARP expression and no PARP cleavage in both cell lines after a 18 h treatment either with LY294002 or PD098059. Both cell types were cultured in a defined medium (see the Experimental section) containing cAMP and cholera toxin which are necessary for cell polarization. PARP cleavage was observed when LNCaP cells were treated with LY294002 in the basal DMEM/Ham F12 mixture containing only glutamine and antibiotics (Figure 8, lanes 7 and 8). Thus, under our culture conditions, LY294002 did not induce apoptosis and the change observed in AR protein levels is a direct consequence of the PI 3-kinase axis inhibition.

AR expression is dependent upon Akt activity

Our results show that PI 3-kinase inhibition reduced AR expression and activity, as measured by decreased AR protein levels and expression of AR-regulated genes. To determine the role of Akt, one of the downstream targets of PI 3-kinase, HEK-293

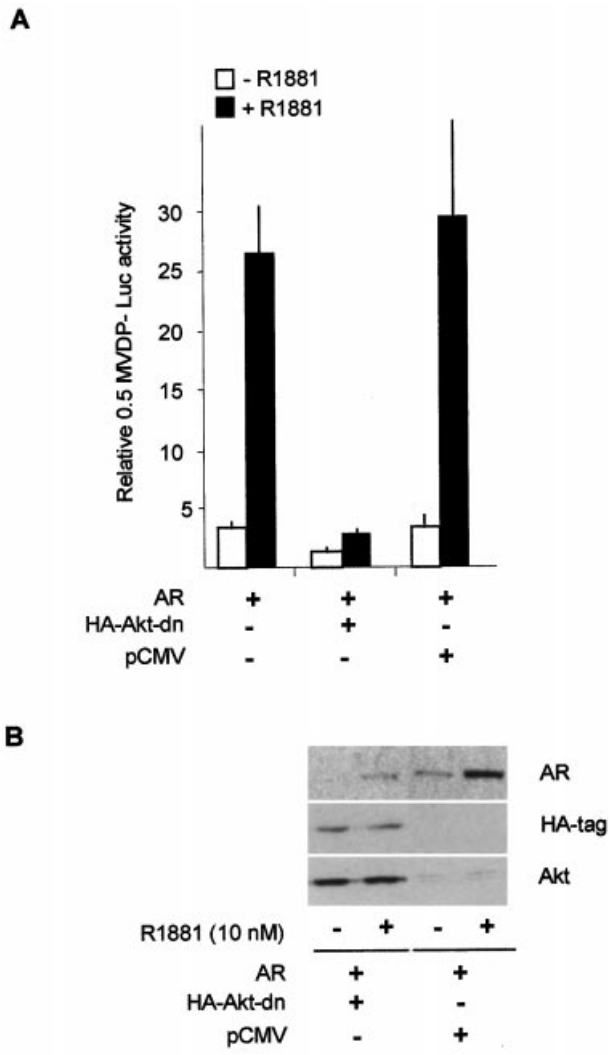

Figure 9 Regulation of AR expression is dependent upon Akt

HEK-293 cells were co-transfected with 0.1 μg of the androgen-inducible 0.5mvdp-luc reporter construct, 0.3 μg of hAR expression vector (pSG5-hAR) and 0.4 μg of dominant negative Akt-HA tagged expression vector (Akt d/n) or empty vector (pCMV) without or with R1881 at 10 nM. (**A**) luciferase activity; (**B**) Western-blot analysis of AR and dominant-negative Akt protein. Data are representative of eight individual experiments.

cells were co-transfected with an hAR expression vector, the AR-inducible MVDP-luc construct and an HA-tagged dominant negative (dn) form of Akt (HA-Akt-dn). As shown in Figure 9(A), the transcriptional responsiveness of the androgen-inducible reporter to AR is abolished in the presence of HA-Akt-dn, whereas transfection with the control vector had no effect. The presence of HA-tagged dominant-negative Akt was confirmed by Western blotting (Figure 9B). AR protein expression is strongly enhanced by R1881 (Figure 9B). This could result from an increase in AR stabilization by R1881, but is also in agreement with previous data showing that androgenic up-regulation of AR mRNA is reproduced in cells expressing a human AR (hAR) cDNA and that this response is due to androgen response elements within the cDNA [21]. The androgen-induced AR expression is significantly blocked in cells expressing the HA-Akt-dn, but not in cells harbouring the empty vector. Thus AR protein down-regulation induced by the dominant-negative form of Akt

would explain the decrease observed in transcriptional responsiveness of the androgen-inducible reporter construct.

DISCUSSION

Although several factors are known to control AR gene expression, the specific factors that induce overexpression of AR in both normal and malignant reproductive tissues remain unknown. The results reported here, using VDEC and LNCaP cells as an experimental paradigm, establish a critical role for the PI 3-kinase/Akt pathway in regulating AR expression. We have shown that basal and DHT-induced AR protein levels strongly decreased following treatment of VDEC and LNCaP with the PI 3-kinase pathway inhibitor LY294002. Decrease of AR expression was not the result of non-specific sensitivity of cells to treatment with kinase inhibitors, since treatment with a specific inhibitor of MAP kinase pathway did not affect AR expression. Moreover changes in AR expression are not due to cell apoptosis, as no PARP cleavage was observed under our cell-culture conditions. The data suggest that EGF and/or insulin are the factors normally acting on VDEC and LNCaP, because the medium is devoid of other factors acting via the PI 3-kinase pathway. Effects of extracellular signals from growth factors are mediated principally by two signalling pathways: the MAPK pathway and the PI 3-kinase pathway. In the present paper we demonstrate that regulation of AR protein contents by insulin and IGF-I is specifically mediated by PI 3-kinase pathway. It has been suggested that up-regulation of growth-factor production, and, in particular, the appearance of several autocrine pathways in epithelial cells, is an adaptative response to androgen ablation in the growth regulation of androgen-independent tumours [22]. Therefore the PI 3-kinase pathway may represent a link between exogenous growth factors, acting via cell-surface tyrosine kinase receptors and AR protein expression.

Our studies, using a dominant-negative Akt mutant, corroborate the results obtained with LY294002, implicating the PI 3-kinase/Akt axis as a key regulator of AR expression. Regulation of the AR gene expression by PI 3-kinase/Akt in VDEC can be related both to transcriptional and post-transcriptional events, as inhibition of the PI 3-kinase pathway strongly reduces AR mRNA steady-state levels and AR protein. In tumoral LNCaP cells, LY294002 did not alter AR mRNA accumulation, suggesting that translational or post-translational mechanisms might be the main factors responsible for increased AR expression. Consistent with this possibility, it has been reported that PI 3-kinase and its downstream effector Akt activate mRNA translation by phosphorylation and inactivation of the translational repressors eIF4E binding proteins [23]. The mammalian target of rapamycin ('mTOR'), which is important in the regulation of protein translation, is a direct target for Akt [24], and might be the mediator of the growth factors effects on AR protein expression.

Collectively the data clearly demonstrate in physiological and pathological cell lines that disruption of the PI 3-kinase signalling pathway reduces the levels of AR protein under basal and DHT-induced conditions. The increased expression of androgen-regulated genes such as MVDP in the VDEC and PSA in LNCaP cells indicate that overexpression of AR induced by the PI 3-kinase/Akt pathway is physiologically relevant. However, whether the overexpression of MVDP and PSA genes is attributable to increased AR protein contents and/or to enhanced AR transactivation remains to be determined.

To search for the biological consequences of the interaction between PI 3-kinase/Akt and AR signalling pathways, we tested whether Akt was able to directly alter AR-mediated transcription. In transient transfection experiments we showed that a dominant-negative form of Akt inhibits ligand-dependent AR-mediated transactivation. However, interpretation of the data was hampered by the AR protein down-regulation observed in cells transfected with kinase-defective Akt. These results emphasize the importance of analysing AR protein levels and AR transactivation simultaneously, because variations observed in AR-transactivating capacities may be due in part to changes in AR protein contents in this type of experiment. The activity of transcription factors is frequently regulated by multiple phosphorylation and dephosphorylation events. Furthermore, steroid receptors are phosphoproteins, and many of them become hyperphosphorylated upon hormone binding, suggesting a link between the phosphorylation status and the activation of these receptors [25]. It has been recently reported that HER2/Neu, a member of the EGF family of receptor tyrosine kinases, could induce AR transactivation and PSA expression through the MAP kinase pathway [26]. It is possible that the effects of Akt on AR are mediated by one of its downstream targets, but it is also possible that Akt directly phosphorylates AR and, thereby, alters transcriptional activity. Akt has been shown to phosphorylate several transcription factors and transcriptional co-regulators such as CREB [27], nuclear factor κB [28], Forkhead [29], Nur 77 [30] and BRCA1 [31]. It was demonstrated that AR is also a direct Akt target. Akt phosphorylates AR at Ser^{210}, leading to inhibition of AR target genes such as p21 and to a decrease of androgen/AR-mediated apoptosis [32,33].

AR expression also appears largely influenced by the state of the culture and therefore of cell differentiation, which, in turn, is probably associated with changes in the PI 3-kinase-signalling-pathway activity. It has been shown that (1) cell-growth arrest by contact inhibition can be associated with a rise in the level of PI 3-kinase [34]; (2) epithelial cell attachment to laminin-rich matrix enhances the PI 3-kinase activity and cell survival [35,36]; (3) the PI 3-kinase signalling pathway is also involved in insulin-induced glucose transport, glycogen synthesis and protein synthesis [37]. VDEC have been shown to produce their own laminin matrix when seeded on microporous membranes [11]. In non-tumoral epithelial cells AR expression is regulated during cell differentiation and polarization, leading to an increased androgen responsiveness and to expression of androgen-regulated markers such as MVDP. The difference in AR expression between proliferative and polarized cells could be explained by major changes in the PI 3-kinase pathway activity induced by attachment and growth arrest of the confluent cell monolayer on the matrix-coated microporous membrane that allows cell differentiation and polarization.

Of interest is the fact that, in both cell lines, the PI 3-kinase signalling pathway plays a critical role in the regulation of AR expression. In LNCaP prostate-cancer cells the PTEN phosphoinositide phosphatase is inactivated, leading to constitutive activation of Akt and resistance to apoptosis [38]. Constitutive activity of the PI 3-kinase pathway could induce increasing AR protein expression independently of the presence of growth factors and explain the high AR levels in the LNCaP cells. Nevertheless, as in VDEC, it remains possible to inhibit this signalling pathway by PI 3-kinase inhibitor.

To our knowledge this is the first report showing that the PI 3-kinase pathway is required for AR protein expression in non-tumoral (VDEC) and in tumoral cells (LNCaP), an experimental paradigm for human prostate cancer. Identification of cross-talk between the PI 3-kinase signalling and the AR pathway may have clinical implications. Studies are currently being developed to understand the molecular mechanisms involved in this interaction, and the therapeutic potential of inhibiting the PI 3-kinase pathway and AR expression will be considered.

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