Signal transduction pathways in androgen-dependent and -independent prostate cancer cell proliferation

Paramita M Ghosh1,4, Shazli N Malik1, Roble G Bedolla1, Yu Wang3, Margarita Mikhailova1, Thomas J Prihoda2, Dean A Troyer2 and Jeffrey I Kreisberg1

1Department of Surgery, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900, USA
2Department of Pathology, University of Texas Health Science Center at San Antonio, TX, USA
3Department of Molecular Medicine, University of Texas Health Science Center at San Antonio, TX, USA
4South Texas Veterans Health Care, San Antonio, TX 78229, USA

(Restrictions for offprints should be addressed to P M Ghosh at the Department of Surgery, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900, USA; Email: ghosh@uthscsa.edu)

Abstract

In a previous report, we showed that increased activation of Akt, a downstream effector of phosphoinositide 3-kinase (PI3K) together with decreased activation of extracellular-signal-regulated kinase (ERK), a member of the mitogen-activated protein kinase (MAPK) family, predicted poor clinical outcome in prostate cancer (Kreisberg et al. 2004 Cancer Research 64 5232–5236). We now show that Akt activation, but not ERK activation, is correlated with proliferation in human prostate tumors as estimated by the expression of the cell proliferation antigen Ki67. We verified these results in vitro, using the androgen-dependent prostate cancer cell line LNCaP and its androgen-independent clone C4-2 as models of prostate cancer of good and poor clinical outcome, respectively. C4-2 cells expressed higher Akt activation, lower ERK activation and increased proliferation compared with LNCaP cells, similar to cases of poor clinical outcome. The PI3K inhibitor LY294002, but not the MAPK/ERK kinase inhibitor PD98059, induced growth arrest in both cell lines. Transient transfection with constitutively active Akt increased proliferation while dominant negative Akt decreased it, thus showing that Akt plays an important role in prostate cancer proliferation. Akt regulates the expression and activation of the androgen receptor. Androgen receptor inhibition with Casodex induced growth arrest in LNCaP cells, but not in C4-2 cells. Another PI3K downstream effector, p70 S6 kinase, requires prior phosphorylation by mammalian target of rapamycin (mTOR) for complete activation. Activation of p70 S6 kinase was higher in C4-2 compared with LNCaP cells. Rapamycin, an mTOR inhibitor, had a growth-inhibitory effect in C4-2 cells, but not in LNCaP cells. Our data suggest a shift from a Casodex-sensitive proliferation pathway in LNCaP cells to a rapamycin-sensitive pathway in C4-2 cells.

Introduction

Prostate cancer is the most commonly diagnosed cancer among men in the Western hemisphere, and is second only to lung cancer as a leading cause of male cancer deaths (American Cancer Society, 2004). Prostate cancer is currently diagnosed by elevated serum levels of prostate-specific antigen (PSA) and histological grading. By the Gleason grading scheme, tumors are classified as well-differentiated (Gleason score 2–4), moderately differentiated (Gleason score 5–7) or poorly differentiated (Gleason score 8–10; Gleason & Mellinger 1974). In general, tumors with a Gleason score of ≥7 show a poor prognosis (Roach et al. 1994, Rees et al. 1997). The majority of prostate tumors are dependent on androgens for growth in the initial stages, and are effectively treated by androgen-ablation therapy; however, in most cases the tumor
eventually progresses to an androgen-independent phenotype (Feldman & Feldman 2001). In spite of being insensitive to hormone-withdrawal therapy, the majority of these tumors continue to express the androgen receptor (AR), and androgen-regulated genes such as PSA, indicating that the AR pathway is active (Denmeade et al. 2003). Androgen-independent prostate cancer (AIPC) tends to progress and metastasize, and has a low survival rate (Feldman & Feldman 2001). There is currently no consensus on therapy for such tumors.

In the normal prostate there is a balance between the rate of proliferation and the rate of apoptosis; however, in prostate cancer this balance is lost, leading to tumor growth (Denmeade et al. 1996). Growth factor stimulation of receptor tyrosine kinases activates different cell-signaling pathways ultimately resulting in proliferation and survival. Of the known pathways, the two most significant and well-investigated pathways are those downstream of the small GTP-binding protein p21Ras and phosphoinositide 3-kinase (PI3K). Receptor tyrosine kinase autophosphorylation results in the phosphorylation of non-receptor tyrosine kinases such as Shc and Grb2, which activate Ras. Activation of Ras triggers a multitude of signaling cascades ultimately resulting in the activation of mitogen-activated protein kinases (MAPK) including extracellular-signal-regulated kinase (ERK)1/2 (p42/44MAPK; Bonni et al. 1999). Receptor tyrosine kinases also have binding sites for p85PI3K, the regulatory domain of PI3K. p85PI3K phosphorylation activates the p110PI3K catalytic domain, which catalyses the conversion of phosphatidylinositol bisphosphate to phosphatidylinositol trisphosphate. Phosphatidylinositol trisphosphate phosphorylates PI3K-dependent kinases 1 and 2, which has multiple substrates, including Akt. For complete activation, Akt requires phosphorylation at two sites, Ser-473 and Thr-308 (Burgering & Coffer 1995).

Akt has often been implicated in the stimulation of both cell proliferation and survival. In the phosphorylated state, Akt promotes cell survival by phosphorylating and inactivating the pro-apoptotic proteins BAD and caspase-9 (Kandel & Hay 1999). In addition, Akt can stimulate cell-cycle progression by phosphorylating and inactivating the AFX/Forkhead family of transcription factors (Kops et al. 1999, Tang et al. 1999) that in turn suppresses AFX-mediated transcription of target genes such as p27Kip1 (Medema et al. 2000). Another substrate of Akt, glycogen synthase kinase 3β (GSK3β) is phosphorylated and inactivated by Akt, thereby promoting the downregulation of p27Kip1 (Appleman et al. 2002). ERK accumulation in the nucleus regulates transcription factors leading to DNA synthesis (Pouyssegur et al. 2002). ERK is not only known to be a stimulator of cell proliferation, but also of cellular differentiation (Ochi et al. 2003, Bai et al. 2004). The role of Akt and ERK in the progression of prostate cancer to an androgen-independent state was discussed in a recent review (Ghosh et al. 2003).

Another downstream target of PI3K, p70 S6 kinase, is an important regulator of cell growth (Grewe et al. 1999). p70 S6 kinase phosphorylates the ribosomal protein S6 and is involved in translational control of 5′-oligopyrimidine tract mRNAs (Pullen & Thomas 1997). The regulation of p70 S6 kinase includes phosphorylation at multiple sites (Grewe et al. 1999). p70 S6 kinase is phosphorylated by a phosphoinositide kinase-related kinase, mammalian target of rapamycin (mTOR) at Thr-389. This causes re-folding of the protein resulting in exposure of the Thr-229 site, which is then phosphorylated by PI3K-dependent kinase 1, resulting p70 S6 kinase activation. Earlier studies suggested that Akt mediated p70 S6 kinase activation, since p70 S6 kinase was stimulated by active mutants of Akt in co-transfection assays (Kohn et al. 1996, Reif et al. 1997). However, it now appears that Akt mediates p70 S6 kinase activation only as a function of constitutive membrane localization (Dufner & Thomas 1999).

In the prostate, the AR is also regulated by Akt both with respect to expression (Manin et al. 2002) and transactivation (Wen et al. 2000, Lin et al. 2001, Sharma et al. 2002). Androgens are required for the normal growth and functional activities of the prostate. In men the primary androgen is testosterone. In the prostate, testosterone is converted to a more-potent form, 5α-dihydrotestosterone, which has a 10-fold higher affinity for the AR (Debes & Tindall 2002). In the normal prostate, the AR is activated by androgen binding (Ridley 2001). The AR is a transcription factor and regulates the transcription of many known genes including PSA. The AR is present in the majority of prostate cancers at both the primary and metastatic sites regardless of androgen dependence, stage or grade (Marcelli & Cunningham 1999, Culig et al. 2000). The AR gene was amplified in about 30% of androgen-independent tumors (Visakorpi et al. 1995, Koivisto et al. 1997). Concurrent overexpression of the AR was associated with a higher clinical stage, higher PSA levels and earlier relapse after radical prostatectomy (Henshall et al. 2001). While mutations in the ligand-binding domain of the AR have been identified in many cases of prostate cancer (Shi et al. 2002), the frequency of AR mutations in prostate cancers is low (Newmark et al. 1992). The commonly used androgen-dependent cell line LNCaP harbors a T877A mutation.
in the AR, which is also seen in all its androgen-independent mutants, including C4-2. The C4-2 cell line is not known to have any further mutations.

We previously showed activation of Akt and inactivation of ERK in high-Gleason-grade prostate cancer (Malik et al. 2002). Akt activation was found to be an excellent predictor of poor clinical outcome in prostate cancer (Kreisberg et al. 2004). In this study we show that the PI3K/Akt cell-signaling pathway mediates proliferation in androgen-dependent and -independent prostate tumor cell lines, as well as in human prostate tumors in situ. In addition, we show that Akt-induced proliferation is mediated by AR transcriptional activity in androgen-dependent cells, whereas proliferation is mediated by p70 S6 kinase in androgen-independent cells. Our studies not only emphasize the importance of Akt in prostate cancer proliferation, but also indicate that development of the androgen-independent phenotype arises from a change in the cell-signaling pathways leading to proliferation.

Materials and methods
Prostate cancer tissues
A total of 74 formalin-fixed, paraffin-embedded human primary prostate cancer specimens were studied from the archival files of South Texas Veterans Health Care System, Audie L. Murphy Division, San Antonio, TX, USA. 53 samples were obtained from radical prostatectomies, and 21 samples were obtained from transurethral resections. In a majority of the cases, adjacent areas of normal prostatic epithelium, benign prostatic hyperplasia and prostatic intraepithelial neoplasia (PIN) were also available for review along with infiltrating carcinoma. The proportion of carcinoma and PIN staining, and the intensity of staining seen in different areas of the same slide were analyzed according to criteria described previously in the literature (Allred et al. 1993).

Immunohistochemistry
Immunohistochemical studies were conducted as described earlier (Malik et al. 2002). Briefly, sections were heated to 60°C, and rehydrated in xylene and graded alcohols. Antigen retrieval was performed with 0.01 M citrate buffer at pH 6.0 for 10 min in a pressure chamber at 121°C. Slides were allowed to cool for another 30 min, followed by sequential rinsing in PBS and 50 mM Tris/HCl, pH 7.6, 150 mM NaCl and 0.1% Tween-20 (TBS-T). Endogenous peroxidase activity was quenched by incubation in TBS-T containing 3% hydrogen peroxide. Each incubation step was carried out at room temperature and was followed by three sequential washes (5 min each) in TBS-T. Sections were incubated in primary antibody diluted in TBS-T containing 1% BSA and 0.1% sodium azide (overnight), followed by incubations with biotinylated secondary antibody for 15 min, peroxidase-labeled streptavidin for 15 min (LSAB-2 System; Dako Corp, Carpenteria, CA, USA) and diaminobenzidine substrate for peroxidase-based immunohistochemistry (Dako Corp) along with diaminobenzidine enhancer (Signet Laboratories Inc., Dedham, MA, USA) for 10 min. Slides were counter-stained with hematoxylin and mounted. The negative control was rabbit Ig fraction or mouse IgG.

Data interpretation and analysis
For phospho-ERK (pERK) and phospho-Akt (pAkt), total staining was scored as previously described (Malik et al. 2002). Ki67 labeling index (Ki67-LI) was determined by counting 500 cells and determining the percentage of cells staining positively for Ki67. To compare the expression levels of pAkt, pERK and Ki67-LI, the r-test and the non-parametric Wilcoxon test were considered. Pearson’s and Spearman’s correlation coefficients were used to determine if individual expression levels were related to each other. The analyses were performed using a statistical analysis system on a PC-compatible computer with SAS 6.12 software (SAS Institute, Cary, NC, USA).

Cell culture and transfections
LNCaP (ATCC, Manassas, VA, USA) and C4-2 (Urocor, Oklahoma City, OK, USA) cell lines were cultured in RPMI 1640 medium with 10% fetal bovine serum and 1% antibiotic/antimycotic solutions. LNCaP cells used were early passage only as they are known to develop spontaneous androgen independence in the late passages (Denmeade et al. 2003). The androgen dependence of these cells was tested periodically by Casodex treatment and the cells were discarded when they became resistant to this drug. Cells were transiently transfected using Lipofectamine PLUS reagent (Invitrogen, Grand Island, NY, USA) according to manufacturer’s specifications based on established protocols (Zhang et al. 2002) using 1 μg plasmid DNA plus 100 ng pEGFP-C1 (a plasmid coding for green fluorescent protein (GFP)); BD Biosciences Clontech Palo Alto, CA, USA); controls received empty vector (pCMV-HA, also from Clontech) plus pEGFP-C1. GFP-expressing cells were sorted by flow cytometry 48 h post-transfection. The following plasmids were used in the transfections: constitutively active Akt (pCMV-6-yr-Akt-HA) and dominant-negative Akt (pCMV-6-Akt-K179M) were kindly provided by
Dr Thomas F. Franke, Columbia University, New York, NY, USA, while a human PSA-luciferase construct (hPSA-luc) was kindly provided by Dr Bandana Chatterjee, University of Texas Health Science Center at San Antonio.

Pharmacological agents and antibodies

PD98059, LY294002, AG1478 and rapamycin were obtained from Calbiochem, San Diego, CA, USA. All these reagents were dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich) and stored at –20°C. Epidermal growth factor (EGF) was obtained from Invitrogen. Casodex (bicalutamide) was kindly provided by AstraZeneca, Cheshire, UK. The following antibodies were used: rabbit polyclonal pAkt (Ser-473), pERK (Thr-202/Tyr-204), phospho-p70 S6 kinase (Thr-389), phospho-S6 ribosomal protein (Ser-235/-236), ribosomal protein S6 (Cell Signaling Technology, Beverly, MA, USA), Akt1/2 (H-136), ERK1 (K-23), p70 S6 kinase (C-18; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a mouse monoclonal antibody to the cell-proliferation antigen, Ki67 (clone MIB-1; Dako, Carpenteria, CA, USA).

Western blotting

Cells were grown on 100 mm dishes at 2 × 10^6 cells/dish and serum starved for 48 h before the experiments. Whole-cell extracts were prepared by washing the cells twice in PBS and lysing cells in 250 μl cell-lysis buffer (50 mM Tris/Cl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40 and the protease inhibitors 0.1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride and 10 mg/ml P-40 and the protease inhibitors 0.1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride and 10 mg/ml each of phenanthroline, leupeptin, aprotinin and pepstatin A) and phosphatase inhibitors: 20 mM β-glycerol phosphate, 1 mM sodium orthovanadate and 10 mM NaF. Proteins were quantitated using a BCA assay (Pierce, Rockford, IL, USA) and fractionated on 29:1 acrylamide/bisacrylamide SDS/PAGE. Electrophoresis was performed at 45 mA for 45 min using minivertical electrophoresis cells (Mini-PROTEAN II Electrophoresis Cell; BioRad Laboratories, Hercules, CA, USA). The gels were electroblotted for 1.5 h at 200 mA using a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad) on to 0.2 μm poly(vinylidene difluoride) membrane (Osmonics, Westborough, MA, USA). The blots were stained with primary antibodies at a dilution of 1:500. The staining was detected by enhanced chemiluminescence (Pierce) after incubation with a peroxidase-labeled secondary antibody (donkey anti-mouse IgG from Chemicon, Temecula, CA, USA; goat anti-rabbit IgG, Fc-specific, from Jackson Immunoresearch Laboratories, West Grove, PA, USA).

Analysis of cell proliferation using flow cytometry

Cells were grown under desired conditions in 100 mm dishes at 0.5 × 10^6 cells/dish as described previously (Ghosh et al. 2002). Flow cytometry was conducted on FACStar Plus (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Cells were illuminated with 200 mW of 488 nm light produced by an argon-ion laser. Fluorescence was read through a 630/22 nm band-pass filter (for propidium iodide) or a 530/30 nm band-pass filter (for annexin V–FITC). Data was collected on 20000 cells as determined by forward and right-angle light scatter and stored as frequency histograms; data used for cell-cycle analysis were analyzed further using MODFIT (Verity Software, Topsham, ME, USA).

MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) cell proliferation were performed as shown previously (Ghosh et al. 1999). Cells were plated in 24-well plates and treated as necessary. Following treatment, each well was incubated with 30 μl 5 mg/ml MTT (Sigma-Aldrich) for 1 h in a CO₂ incubator at 37°C. MTT permeates the plasma membrane and is reduced by a mitochondrial dehydrogenase enzyme in proliferative cells to yield a purple formazan product which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. The medium was aspirated and 1 ml DMSO added per well. The DMSO dissolves the plasma membrane, and releases the formazan, which then is dissolved in DMSO to yield a purple liquid. Proliferation rates were estimated by colorimetric assay reading formazan intensity in a plate reader at 570 nm.

AR transcriptional activity

Reporter-gene activity was determined by luciferase assay (Hartig et al. 2002). LNCaP and/or C4-2 cells were transfected with 20 μg pGL3-MMTV-luc with or without co-transfection of 2 μg mutant Akt using Lipofectamine PLUS according to the manufacturer’s recommendations. After 24 h, cells were trypsinized and 100 000 cells plated in a six-well tissue-culture plate. Cells grown in phenol-free medium containing charcoal-stripped serum for 24 h were treated as required for an additional 24 h. Cells were harvested, and cell lysates prepared to perform luciferase assays using a luciferase enzyme assay system (Promega). Each transfection experiment was performed in duplicate or triplicate on at least three separate occasions. Results represent an average of independent experiments with
Results

Proliferation in human prostate cancer correlates with Akt activation

We showed recently that prostate tumors from patients with poor clinical outcome are characterized by increased expression of pAkt (Ser-473) and decreased expression of pERK (Tyr-202/Thr-204) compared with those of good clinical outcome (Kreisberg et al. 2004). Earlier we showed that high-Gleason-grade prostate cancer (≥7), which is considered to have a poor clinical outcome, expressed higher pAkt and lower pERK compared with low-Gleason-grade prostate cancer (Malik et al. 2002). We have now investigated whether Akt or ERK phosphorylation regulated proliferation in prostate tumors. To determine proliferation, the same prostate cancer specimens were stained with a mouse monoclonal antibody to the cell-proliferation antigen, Ki67 (n = 74). The rate of proliferation was expressed as the percentage of Ki67-expressing cells out of 500 cells counted (Ki67-LI). Normal and hyperplastic tissues did not express Ki67, well and moderately differentiated tumors had low Ki67-LI (≤10%) while poorly differentiated tumors had high Ki67-LI (≥20%; Fig. 1A). There was a highly
A significant progressive increase in Ki67-LI with increasing Gleason scores (Pearson coefficient, 0.47 for Gleason scores of 2–10; \( P < 0.0001 \)). Figure 1B demonstrates a typical case of high Ki67-LI. The staining for pAkt and pERK in the same tissues has been described previously (Malik et al. 2002). Comparison of Ki67-LI with pAkt expression showed a significant positive correlation (Pearson coefficient, 0.36; \( P \leq 0.003 \); Fig. 1C). There was good correlation between Ki67-LI and pAkt in both high (\( \geq 7 \)) and low Gleason scores. In contrast, a comparison of Ki67-LI with staining for pERK showed poor correlation (Pearson coefficient, –0.2; \( P > 0.1 \); Fig. 1D). The above results clearly demonstrate that proliferation in prostate cancer correlates with Akt phosphorylation.

**LNCaP and C4-2 cells as models for good and poor clinical outcome in prostate cancer**

In order to study the signaling pathways characterizing tumors of good and poor clinical outcome in prostate cancer, we turned to the androgen-dependent LNCaP prostate cancer cell line and its androgen-independent clone, C4-2. Expression and activation of Akt and ERK were compared in LNCaP and C4-2 cells to determine whether they reflected the characteristics of human prostate cancer of good and poor clinical outcome, respectively. Both cell lines expressed active Akt (pAkt) under baseline conditions; with C4-2 displaying higher levels of pAkt (Fig. 2). Growth factor stimulation of both cell lines increased pAkt levels further (Fig. 2A). In contrast to Akt, ERK was not activated in the basal (unstimulated) state; however, growth factor stimulation transiently activated ERK with peak activation at 5 mins post-stimulation (Fig. 2A). The level of ERK activation in C4-2 cells was reduced compared with LNCaP. Growth factor stimulated ERK phosphorylation, but not Akt phosphorylation, was inhibited by the tyrosine kinase inhibitor AG1478, which selectively inhibits EGF receptor kinase activity (Fig. 2B), further demonstrating constitutive Akt activation in these cells. pAkt and pERK levels in these cells mimicked that observed in human prostate cancer; therefore, these cells are appropriate for studying the mechanisms of prostate tumor progression.

**Proliferation in LNCaP and C4-2 cells is mediated by the PI3K/Akt pathway**

Next we compared cell signaling pathways leading to proliferation in LNCaP and C4-2 cells (Fig. 3). Cells were plated in the presence of control (DMSO), 25 \( \mu \)M LY294002, an inhibitor of PI3K activity or 20 \( \mu \)M PD98059, an inhibitor of MAPK/ERK kinase 1 (MEK1) activity, which is directly upstream of ERK1/2. Figure 3 shows that 20 \( \mu \)M PD 98059 did not significantly affect cell growth in either LNCaP or C4-2 cells over a period of 3 days (Fig. 3A and B). In contrast, LY294002 significantly reduced cell growth in both LNCaP and C4-2 cells over a period of 3 days (Fig. 3A and B). Our previous observations indicated that LY294002 caused significant apoptosis in prostate cancer cells (Ghosh et al. 2002); hence, to ensure that the reduction in cell numbers reflected growth arrest, and not simply increased apoptosis, we further analyzed proliferation by flow cytometry following propidium iodide staining of DNA to measure the fraction of cells in the S-phase (Fig. 3C). Flow-cytometric analysis confirmed that proliferation was mediated by the PI3K pathway and...
not the MAPK pathway. Figure 3D demonstrates the specificity of the inhibitors used in this study. In these experiments rapamycin was used as a control and shows no effect on the phosphorylation of either Akt or ERK.

Akt regulates proliferation in both LNCaP and C4-2 cells

Next, we determined the downstream effectors of PI3K involved in regulation of proliferation in LNCaP and C4-2 cells. LNCaP and C4-2 cells were transiently transfected with dominant-negative Akt (dn-Akt; Songyang et al. 1997), constitutively activated Akt (myr-Akt; Stoica et al. 2003) or an empty vector, together with pEGFP. Transfected cells were sorted by flow cytometry using GFP-expressing cells as a marker. dn-Akt-transfected cells had a reduced proliferation rate compared with controls, while myr-Akt-transfected cells had an increased proliferation rate in both LNCaP and C4-2 cells (Fig. 4A and B). Expression of the mutant plasmids was confirmed by immunoblotting using a pAkt antibody (Fig. 4C). These data indicate that Akt plays an important role in the proliferation of both LNCaP and C4-2 cells, thus confirming our in vivo results.

Akt-induced proliferation is mediated by AR in LNCaP cells but not in C4-2 cells

Next, we investigated the molecular mechanisms by which Akt regulates proliferation in LNCaP and C4-2 cells. It was shown previously that transcriptional activity (Wen et al. 2000, Lin et al. 2001, Sharma et al. 2002) and expression (Manin et al. 2002) of AR is regulated by Akt. To determine whether Akt-induced proliferation is mediated by AR transactivation, LNCaP and C4-2 cells were transiently transfected

Figure 3 LNCaP and C4-2 cells were plated in the presence of one of the following: DMSO (vehicle control), 25 μM LY294002 (LY), an inhibitor of PI3K activation, or 20 μM PD98059 (PD), an inhibitor of MAPK/ERK kinase 1 (MEK1) activation, which is directly upstream of ERK1/2. (A) LNCaP and (B) C4-2 cells were plated in 24-well plates and cultured for 1–3 days in the presence of the inhibitors listed above. At the end of 1, 2 or 3 days, MTT was added to the cells for 1 h and proliferation rates estimated by colorimetric assay. (C) Cells were plated in 60 mm dishes and cultured in the presence of the inhibitors for 48 h. At the end of that period, cells were trypsinized, fixed in 70% ethanol, stained with propidium iodide and DNA content analyzed by flow cytometry. For each experiment, the percentage of cells in the S-phase was normalized to the percentage of cells in the S-phase in DMSO-treated LNCaP cells. Bars represent means ± S.E.M. from three individual experiments. * Represents changes from control that yield a statistical significance (P < 0.001) by Student's t-test. (D) C4-2 cells were serum starved for 48 h prior to treatment with inhibitors for 2 h, followed by EGF stimulation (10 ng/ml) for 5 min. In this series of experiments, cells were also treated with the mTOR inhibitor rapamycin (100 nM) as a control. Similar treatment in LNCaP cells produced identical results (not shown).
with GFP-empty vector or GFP+myr-Akt, and GFP-expressing cells sorted by flow cytometry. Control and myr-Akt-transfected cells were treated for 24 h with DMSO or 10 μM Casodex (bicalutamide), which inhibits AR transactivation by preventing interaction between the AR N- and C-terminal domains (Masiello et al. 2002). Figure 5A shows that treatment with 10 μM Casodex prevented basal and Akt-induced proliferation in LNCaP, but not in C4-2 cells, indicating that Akt-induced proliferation is mediated by AR transactivation in LNCaP cells, but not in C4-2 cells. Next, we investigated whether this lack of response in C4-2 cells was due to an inability of Casodex to inhibit AR transcriptional activity, as has been reported in certain androgen-independent clones of LNCaP cells (i.e. LNCaP-abl; Culig et al. 1999). We transfected LNCaP and C4-2 cells with plasmids coding for GFP, and a human PSA-tagged luciferase reporter plasmid (hPSA-luc), together with control or myr-Akt. Transfected, GFP-expressing cells were sorted by flow cytometry and collected for luciferase assays. Figure 5B shows that myr-Akt potently induced AR transcriptional activity on hPSA-luc. AR transcriptional activity was inhibited by 10 μM Casodex in both LNCaP and C4-2 cells. These results indicate that the AR is functional and sensitive to Casodex in both cell lines; however, in the LNCaP cells, proliferation is mediated by an AR-dependent pathway, whereas in C4-2 cells they are not.

**Figure 4** LNCaP and C4-2 cells were transfected with plasmids expressing GFP together with pCMV6 (empty vector) or vectors expressing myr-Akt or dn-Akt. (A, B) Collected cells were fixed, stained with propidium iodide and analyzed for phases of the cell cycle. (A) Results from a typical experiment are shown. (B) Mean results from three individual experiments. Proliferation index indicates the percentage of cells in S-phase normalized to controls for each cell type to indicate relative effect of Akt on the two cell lines. Bars represent means ± S.E.M. from three individual experiments. (C) Expression of the mutant Akt plasmids was confirmed by immunoblotting using a pAkt antibody. Note that total Akt (tAkt) levels were increased in cells overexpressing mutant Akt cDNAs. Hence, expression of β-tubulin was used as a loading control.

**Differential effect of rapamycin on LNCaP and C4-2 cell proliferation**

Next, we investigated whether other downstream effectors of PI3K played a role in the regulation of proliferation in prostate cancer. p70 S6 kinase, a downstream target of PI3K, is an important regulator of cell growth (Grewe et al. 1999). Similar to Akt and ERK, p70 S6 kinase is activated by phosphorylation in response to growth factor stimulation (Fig. 6A). C4-2 cells expressed higher levels of p70 S6 kinase activation compared with LNCaP, as determined by increased phosphorylation of p70 S6 kinase at Thr-389 (Fig. 6A). For complete activity, p70 S6 kinase is phosphorylated by PI3K as well as by mTOR, a PI3K-related kinase (Dufner & Thomas 1999, Grewe et al. 1999). To determine the role of p70 S6 kinase in proliferation, LNCaP and C4-2 cells were treated with the mTOR inhibitor rapamycin. 100 nM rapamycin did not significantly affect LNCaP cell-proliferation rates, as demonstrated by flow-cytometric estimations of the fraction of cells in the S-phase; however, rapamycin caused a 50% reduction in C4-2 cell proliferation (Fig. 6B). Phosphorylation of p70 S6 kinase at Thr-389 was not affected by PD98059 treatment, but was completely inhibited by LY294002 or rapamycin (Fig. 6C), confirming that p70 S6 kinase is downstream of both mTOR and PI3K, but independent of ERK activation. Since phosphorylation at multiple sites on p70 S6...
kinase is necessary for its complete activation, we investigated whether the ribosomal protein S6, which is a substrate for p70 S6 kinase, was phosphorylated in the presence of these inhibitors. As expected, both rapamycin and LY294002 inhibited S6 phosphorylation at Ser-235/236 (Fig. 6D). PD98059 did not affect S6 phosphorylation (Fig. 6D), thus confirming that activation of both mTOR and PI3K are required for p70 S6 kinase activity. Our results indicate that whereas PI3K mediates proliferation of both LNCaP and C4-2 cells (Fig. 3), mTOR mediates the proliferation of C4-2 cells but not LNCaP cells (Fig. 6). Additionally, we show that AR mediates the proliferation of LNCaP cells but not C4-2 cells (Fig. 5). A comparison of the growth characteristics of LNCaP and C4-2 cells in the presence of rapamycin and Casodex over a period of 3 days confirmed these observations (Fig. 7). Our results suggest that androgen independence arises from an alteration in the cell signaling pathways mediating proliferation, from proliferation that is Casodex sensitive in LNCaP cells to proliferation that is rapamycin sensitive in C4-2 cells. This is summarized in the scheme shown in Fig. 8.

Discussion

Recently we showed that increased activation of Akt and decreased activation of ERK predict poor clinical outcome in prostate cancer (Kreisberg et al. 2004). In addition, in 74 human prostate cancer specimens, we showed that high-Gleason-grade prostate cancer is characterized by increased activation of Akt and decreased activation of ERK compared with low-Gleason-grade cancer (Malik et al. 2002). In this paper, we immunostained the same 74 tumor samples with an antibody to the cell-proliferation antigen Ki67, and showed that Akt activation, but not ERK activation, correlated with proliferation in human prostate tumors. Based on these results, we hypothesized that cell proliferation in prostate cancer is mediated by an Akt-dependent, but not an ERK-dependent, pathway. To prove this hypothesis, we used the androgen-dependent prostate cancer cell line LNCaP and its androgen-independent clone C4-2 as models of good and poor clinical outcome, respectively. C4-2 cells expressed higher Akt activation, lower ERK activation and increased proliferation compared with LNCaP cells. We showed that proliferation in both cell lines was
mediated by the PI3K/Akt pathway. Next, we investigated the cell-signaling pathways leading to proliferation downstream of Akt. We show that the anti-androgen Casodex arrested LNCaP cell growth, whereas the mTOR/p70 S6 kinase inhibitor rapamycin caused growth arrest in C4-2 cells. Thus, our data suggest a shift from a Casodex-sensitive proliferation pathway in LNCaP cells to a rapamycin-sensitive pathway in C4-2 cells.

Our observation that Akt phosphorylation increases with prostate cancer progression is supported by several reports in the literature. Androgen ablation in LNCaP cells stimulated the activation of Akt, which eventually resulted in androgen-independence of the cell line in culture (Murillo et al. 2001). In addition, expression of constitutively active Akt in LNCaP cells resulted in a 6-fold increase in xenograft tumor growth (Graff et al. 2000). Our observation that Akt activity increases with tumor progression has been confirmed by others (Paweletz et al. 2001). In contrast to the activated form of Akt, total Akt expression does not change with tumor development and progression. Immunohistochemical examination revealed that all three isoforms of Akt (Akt-1, -2, -3) were expressed in both normal and malignant prostate tissues (Zinda et al. 2001).

In contrast to Akt, ERK inactivation with increasing Gleason grades was not widely established. Reports from other laboratories showed an increase in ERK activation in primary prostate cancer compared with normal prostate and benign prostatic hyperplasia (Royuela et al. 2002), and in PIN versus normal prostate (Price et al. 1999). These reports suggested that ERK activation was necessary for tumor initiation.

Figure 6 (A) LNCaP and C4-2 cells were serum starved for 48 h and then re-stimulated with 10 ng/ml EGF for 5, 15 and 30 min. Cell lysates were run on SDS/PAGE (10% gels). Immunoblot was stained with phospho-p70 S6 kinase (Thr-389; pp70S6K) antibody (upper panel). The blot was stripped and restained with an antibody to total p70 S6 kinase (tp70S6K; lower panel). (B) LNCaP and C4-2 cells were cultured in the presence of DMSO (vehicle control) or 100 nM rapamycin for 48 h. For each experiment, the percentage of cells in the S-phase was estimated by flow cytometry, and normalized to the percentage of cells in the S-phase in DMSO-treated LNCaP cells. Bars represent means ± S.E.M. from three individual experiments. (C, D) Cells were serum starved for 48 h prior to treatment with 20 μM PD98059 (PD), 25 μM LY294002 (LY) or 100 nM rapamycin for 2 h, followed by EGF stimulation (10 ng/ml) for 5 min. (C) Lysates were run on SDS/PAGE (10% gels) and immunoblotted with pp70 S6K (upper panel). Note that the antibody cross-reacts with the related phospho-p85 S6 kinase (pp85S6K), which also phosphorylates S6 and is an isoform of p70 S6 kinase, arising from the same gene but with 23 extra amino acids in its N-terminal domain, which encodes a nuclear-localization signal. The blot was stripped and restained with an antibody to total p70 S6K (tp70S6K; lower panel). (D) Lysates were run on SDS/PAGE (10% gels) and immunoblotted with a rabbit polyclonal antibody to phospho-ribosomal protein S6 (Ser-235/236; prpS6; upper panel). The blot was stripped and restained with an antibody to total S6 (trpS6; lower panel).
Among primary prostate tumors, we and others observed a steady decline in ERK phosphorylation from PIN to poorly differentiated prostate cancer (Paweletz et al. 2001, Malik et al. 2002, Uzgare et al. 2003). The decline in the activation levels of ERK with tumor grades may indicate that ERK in these cells was involved in the state of cell differentiation, as suggested in other tissues (Ochi et al. 2003, Bai et al. 2004). In support of this argument, activation of ERK stimulated neuroendocrine differentiation in LNCaP cells (Kim et al. 2002). Further progression leading to metastasis may require ERK activation in the tumor tissues, as

Figure 7 LNCaP and C4-2 cells were plated in the presence of one of the following: DMSO (vehicle control), 10 μM Casodex, an anti-androgen, or 100 nM rapamycin, an inhibitor of mTOR activation, which is directly upstream of p70 S6 kinase. LNCaP (left-hand panels) and C4-2 (right-hand panels) cells were plated in 24-well plates and cultured for 1–3 days in the presence of the inhibitors listed above. At the end of 1, 2, or 3 days, MTT was added to the cells for 1 h and proliferation rates estimated by colorimetric assay.

Figure 8 Scheme showing possible signaling mechanisms leading to cell-cycle progression in LNCaP and C4-2 cells.
ERK was also required for cell motility and metastasis in prostate tumors (Suthiphongchai et al. 2003, Zayzafoon et al. 2004). A previous report showed a significant increase in ERK activation in metastatic tumor obtained from prostate cancer patients (stage IV) compared with localized tumors (Gioeli et al. 1999). In this paper, we use the androgen-dependent cell line LNCaP and its androgen-independent clone C4-2 as models of non-aggressive and aggressive prostate cancer, respectively. LNCaP cells were isolated from a lymph-node metastasis in a 63-year-old Caucasian male (Horoszewicz et al. 1980). These cells are not aggressively tumorigenic. The C4-2 cells were isolated from a second-generation tumor developed in castrated nude mice injected with LNCaP cells together with a bone stromal (also non-tumorigenic) cell line (Wu et al. 1994). C4-2 cells are tumorigenic and metastasize to bone and lymph nodes (Thalmann et al. 1994). Unlike other commonly used AIPC cell lines, such as PC-3 and DU-145, the C4-2 cells express a functional AR, similar to the majority of human AIPC, and hence may be a better model of prostate cancer progression.

One of the significant findings of this paper is the observation that proliferation in both androgen-dependent and -independent prostate cancer cells is mediated by Akt activation and not ERK activation. We previously showed in a cell line derived from a tumor in a transgenic mouse model for prostate cancer (TRAMP) that PI3K mediated both proliferation and survival (Ghosh et al. 2002). The TRAMP-C2 cell line used was highly dependent on androgens for growth, and hence gave no clues regarding the cell-proliferation pathways in AIPC. In this paper, we extend our previous observations and compare cell-signaling pathways leading to proliferation in both androgen-dependent and -independent cell lines. Previous studies have indicated the importance of PI3K and Akt in LNCaP cell survival relative to other signaling pathways. The PI3K inhibitor LY294002, but not the ERK inhibitor PD98059, inhibited LNCaP cell survival (Lin et al. 1999). Another recent paper showed that Akt was required for cell growth in both normal and malignant prostate cancer cell lines (Uzgare & Isaacs 2004). The current model used may not reflect the events in other prostate cancer cell lines, as shown by a previous report demonstrating that EGF-stimulated proliferation in PC-3 cells is mediated by ERK (Guo et al. 2000). However, strong similarities between our results in LNCaP and C4-2 cells and those in human prostate tumors demonstrate that our model may be representative of human prostate tumors that progress from an androgen-dependent to an androgen-independent phenotype.

In this paper we also show that constitutively active Akt increases proliferation while a dominant-negative Akt decreases it. Unlike PI3K inhibition by LY294002, expression of dominant-negative Akt in these cell lines did not cause complete growth arrest. This may reflect problems with the transient transfection methodology, and currently we are in the process of developing a stable cell line expressing a tetracycline-repressible dominant-negative Akt to address this problem. Alternatively, PI3K-dependent proliferation in these cells may be mediated partially by Akt and also by another PI3K downstream effector. Hence in this study we also examined other downstream effectors of PI3K, such as p70 S6 kinase.

Another significant finding of this paper is the observation that although Akt mediates proliferation in both LNCaP and C4-2 cells, downstream of Akt, cell-signaling pathways leading to proliferation in the two cell lines are different. Our data demonstrate that in both LNCaP and C4-2 cells activation of Akt stimulates AR transcriptional activity. Akt-induction of AR transcriptional activity has been shown previously and may be mediated by direct phosphorylation of the AR by Akt (Wen et al. 2000, Lin et al. 2001) or by β-catenin nuclear localization (Sharma et al. 2002). We show that Akt-induced proliferation is mediated by AR transactivation in LNCaP cells but not in C4-2 cells. These results reflect the phenotype (androgen-dependent versus -independent) of these two cell lines. Previous reports suggested that increasing Akt activation in LNCaP cells may confer androgen independence to these cells (Graff et al. 2000, Murillo et al. 2001). However, we show that transient transfection of constitutively active Akt does not result in androgen-independence of LNCaP cells. This may be because the degree of Akt activation was not high enough or because long-term increase in Akt activation, such as that achieved with stable transfection, as shown in (Graff et al. 2000), results in further changes that result in androgen-independence.

We showed previously that p70 S6 kinase and Akt are two independent downstream effectors of PI3K in prostate cancer cells (Ghosh et al. 2002). Like Akt, p70 S6 kinase activity was also increased in C4-2 cells compared with LNCaP cells. The mTOR inhibitor, rapamycin, which inhibits p70 S6 kinase phosphorylation, inhibited proliferation of the androgen-independent C4-2 cells, but not of the parental androgen-dependent line, suggesting that proliferation in C4-2 cells is mediated by p70 S6 kinase as shown by others (Mousses et al. 2001). We hypothesize that AR regulates the cell cycle in LNCaP cells while in C4-2 cells androgen independence arises due to cell-cycle
regulation by p70 S6 kinase rather than AR. AR is a transcription factor that transcriptionally regulates the expression of certain components of the cell cycle. Importantly, AR binds to a T-cell factor 4 (TCF-4)-responsive element in the c-Myc promoter in LNCaP cells (Amir et al. 2003), showing that AR regulates c-Myc expression in androgen-dependent cells. It was recently shown that in the presence of high levels of active Akt, the mTOR inhibitor rapamycin was able to inhibit c-Myc expression in androgen-dependent LAPC-4 prostate cancer cells, while at low Akt phosphorylation levels, rapamycin was unable to inhibit c-Myc expression (Gera et al. 2004). These results suggest that in androgen-dependent cells, where Akt activation is not high, c-Myc expression is regulated by androgens and is independent of p70 S6 kinase. In contrast, in androgen-independent cells, where Akt activation is high, c-Myc expression is regulated by a pathway involving p70 S6 kinase. In support of this hypothesis, it was previously shown that the androgen-independent human prostate cancer cell line PC-3, which lacks a functional AR, depends on p70 S6 kinase for cell proliferation (Kiefer & Farach-Carson 2001).

In Fig. 8, we show the major signaling pathways involved in proliferation in LNCaP and C4-2 cells. In both cell lines, proliferation is mediated by PI3K activation. PI3K activates Akt and, consequently, AR. AR transactivation by Akt results in transcription of non-cell-cycle-related AR target genes such as PSA. However, in LNCaP cells, AR transactivation also triggers transcriptional regulation of the cell cycle, whereas in the C4-2 cells, the cell cycle is regulated by p70 S6 kinase. A possible explanation for this phenomenon is that perhaps in both cell lines, AR transcriptional activity is intact, but the downstream targets of p70 S6 kinase, which are translation regulators, prove to be stronger regulators of the cell cycle than AR, hence masking the effects of AR on the cell cycle.

In conclusion, in this paper we provide important clues regarding the nature of changes in the molecular mechanisms resulting in the progression of prostate cancer to an androgen-independent state. We show conclusively that the PI3K/Akt pathways mediate proliferation in both androgen-dependent LNCaP and androgen-independent C4-2 cell lines. Downstream of Akt, proliferation is mediated by AR in LNCaP cells whereas in C4-2 cells, it is mediated by p70 S6 kinase. Our results suggest that androgen-independence in the C4-2 cells arises from an alteration in the cell-signaling pathways mediating proliferation. Further studies are needed to elucidate the molecular mechanisms resulting in this alteration.

Acknowledgements

We thank Dr Barry Furr, Chief Scientist and Head of Project Evaluation Group, AstraZeneca, UK, for the gift of the bicalutamide and for critical reading of the manuscript. Dr Thomas Franke, Columbia University, New York, NY, USA, for the mutant Akt plasmids and Dr Bandana Chatterjee, University of Texas Health Science Center in San Antonio, San Antonio, TX, USA, for the hPSA-luc expression vector. This work was supported by a grant from Veterans Administration, and the San Antonio Area Foundation.

References


Denmeade SR, Lin XS & Isaacs JT 1996 Role of programmed (apoptotic) cell death during the
Ghosh et al.: Akt mediates proliferation in prostate cancer

progression and therapy for prostate cancer. Prostate 28 251–265.
Kohn AD, Takeuchi F & Roth RA 1996 Akt, a pleckstrin homology domain containing kinase, is activated primarily by phosphorylation. Journal of Biological Chemistry 271 21920–21926.
Kreisberg JI, Malik SN, Prihoda TJ, Bedolla RG, Troyer DA, Kreisberg S & Ghosh PM 2004 Phosphorylation of Akt (Ser473) is an excellent predictor of poor clinical outcome in prostate cancer. Cancer Research 64 5232–5236.
Malik SN, Brattain M, Ghosh PM, Troyer DA, Prihoda T, Bedolla R & Kreisberg JI 2002 Immunohistochemical demonstration of phosho-Akt in high Gleason


Pullen N & Thomas G 1997 The modular phosphorylation and activation of p70s6k. FEBS Letters 410 78–82.


Uzgare AR, Kaplan PJ & Greenberg NM 2003 Differential expression and/or activation of P38MAPK, erk1/2, and jak during the initiation and progression of prostate cancer. *Prostate* 55 128–139.


Zayzafoon M, Abdulkadir SA & McDonald JM 2004 Notch signaling and ERK activation are important for the osteomimetic properties of prostate cancer bone metastatic cell lines. *Journal of Biological Chemistry* 279 3662–3670.
