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Androgens target prohibitin to regulate proliferation of prostate cancer cells

Simon C Gamble[1], Michael Odontiadis[1], Jonathan Waxman[1], Jules A Westbrook[2], Michael J Dunn[3], Robin Wait[4], Eric W-F Lam[1] and Charlotte L Bevan*[,1]

[1]*Department of Cancer Medicine, Imperial College London, Hammersmith Hospital, London W12 0NN, UK;* [2]*Heart Science Centre, Harefield Hospital (Imperial College School of Medicine), Harefield, Middlesex, UK;* [3]*Department of Neuroscience, Institute of Psychiatry, Box P045, De Crespigny Park, London SE5 8AF, UK;* [4]*Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College London, Aspenlea Road, Hammersmith, London W6 8LH, UK*

Proteins involved in the growth response of prostate cancer cells to androgen were investigated by comparing the proteomes of LNCaP cells treated with vehicle or androgen. Whole-cell lysates were separated by two-dimensional PAGE, and HPLC-MS/MS was used to identify androgen-regulated proteins. Prohibitin, a protein with cell-cycle regulatory activity, was shown to be downregulated by 50% following androgen stimulation. Western blot and reverse transcription–PCR experiments confirmed the result and showed that regulation occurs at the level of transcription. To determine the importance of prohibitin in androgen-stimulated growth, we used transient transfection to overexpress the protein and RNA interference to knock down the protein. Subsequent FACS analysis showed that cells with reduced levels of prohibitin showed a slight but reproducible increase in the percentage of population in cell cycle, while cells with increased prohibitin levels showed a clear reduction in the percentage entering cell cycle, following dihydrotestosterone stimulation, when compared to untransfected controls. Confocal microscopy showed localization of prohibitin in the nucleus as well as the mitochondria of LNCaP cells. It therefore seems that the regulation of prohibitin is a vital part of the cellular growth response to androgen stimulation in LNCaPs and prohibitin may have a nuclear regulatory role in cell-cycle progression.
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Introduction

Prostate cancer is now the most commonly diagnosed cancer in Western males (Greenlee *et al*., 2001). Prostate tumours are initially dependent on growth stimulation by circulating androgens (Koivisto *et al*., 1998). The major circulating androgen is testosterone, which is converted to dihydrotestosterone (DHT) in prostate cells by the enzyme 5α-reductase. DHT is the major androgen in prostate growth and development. Androgen response is mediated by binding of DHT or other androgens to the androgen receptor (AR), a ligand-activated transcription factor. This then translocates to the nucleus, binds response elements in the promoters of target genes and activates transcription from these promoters, resulting in the production of proteins involved in mitosis and differentiation of the prostate (Koivisto *et al*., 1998). Treatment for advanced prostate cancer primarily involves compounds that interfere with this signalling process, by blocking the production of circulating androgen and/or by inhibiting the AR itself (Grayhack *et al*., 1987). It is now well documented that these treatments fail after prolonged use, and growth of the cancer then recurs (Koivisto *et al*., 1998). Evidence suggests that this recurrence of growth is often facilitated by modification of the original androgen-responsive pathway in the cancer cells, either by AR amplification (Visakorpi *et al*., 1995) or mutations of the receptor that allow it to be activated by noncanonical ligands (Gottlieb *et al*., 1997) and frequently by the very anti-androgens used as treatment. When this adaptation occurs, the patient relapses and no effective further therapies are currently available.

New treatments that regulate the function of an androgen-regulated, growth-related protein would be an ideal second line of therapy. This would allow treatment with current therapies to be followed, after relapse, by therapy designed to uncouple the growth signal downstream from the AR. However, few *bona fide* targets of the AR have yet been identified. We used two-dimensional polyacrylamide gel electrophoresis (2-DE) to determine which proteins are regulated following androgen stimulation in the androgen-responsive prostate cancer cell line LNCaP. This provides a nonbiased approach allowing visualization of a large number of cellular proteins. Regulation of the protein may be detected not only at the level of total amounts of protein but also that of protein modification, such as acetylation and phosphorylation. The 30 kDa protein prohibitin was identified as being downregulated over 50% by androgen treatment.

*Correspondence: CL Bevan; E-mail: charlotte.bevan@imperial.ac.uk
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Prohibitin appears to have dual roles in the cell, being ubiquitously expressed in the mitochondria and also located in the nucleus of certain cell types (Thompson *et al*., 2001; Wang *et al*., 2002a). Prohibitin was initially suspected to be a tumour suppressor gene, due to the ability of the mRNA to inhibit DNA synthesis and cell cycle (McClung *et al*., 1989). Current data show two likely roles for the protein, firstly as a mitochondrial chaperone protein in a complex with BaP37/REA (Repressor of oEstrogen receptor Activity) (Coates *et al*., 1997), and secondly as a regulator of cell cycle (Nuell *et al*., 1991). The mitochondrial role of prohibitin appears to be as part of a 'holdase', tightly bound in a complex with REA, with 12–14 of these subunits forming a barrel-like structure (Steglich *et al*., 1999). This structure protects mitochondrial proteins required for protein complexes from degradation by proteases until import of further cytoplasmic components has occurred (Coates *et al*., 1997). It is possible that this mitochondrial function is regulated in response to cell growth, as prohibitin protein levels appear to increase in differentiated cells (Thompson *et al*., 2001). It has also been suggested that prohibitin may protect against apoptosis at times of metabolic stress in both yeast and mammalian cells, a function possibly related to its localization in the mitochondria (Vander Heiden *et al*., 2002). The cell-cycle function of prohibitin is less well understood and there is some dispute over the precise role of the protein (Coates *et al*., 2001). Injection of the mRNA results in cell-cycle arrest in some cell types (McClung *et al*., 1989; Nuell *et al*., 1991), and this may in part be due to the 3′ untranslated region (3′UTR) of the mRNA (Jupe *et al*., 1996). Even the localization of the protein is not clear, with some groups maintaining that the protein is purely mitochondrial (Coates *et al*., 2001), and others demonstrating a secondary localization in the nucleus (Thompson *et al*., 2001; Wang *et al*., 2002b). However, recent observations that prohibitin can interact with members of the E2F transcription factor family (Wang *et al*., 1999a) support the concept of a nuclear role in cell-cycle regulation. It has been demonstrated, using transfection assays, that prohibitin can reduce the amount of transcription mediated by E2F1 by interacting directly with E2F1 and also recruiting Rb, histone deacetylases and the corepressor NCoR (Wang *et al*., 2002b). This complex may form an alternative to the Rb-mediated repression of E2F, as Rb and prohibitin appear to bind E2F at different sites on the E2F protein and recruit different corepressors, prohibitin apparently linking E2F to HDACs via NCoR (Wang *et al*., 2002a). As the current literature suggests a cell-cycle role in other hormone-responsive cells, possibly via the regulation of transcription and post-translational modification (Thompson *et al*., 2001), and we saw changes in prohibitin expression in prostate cancer cells simulated to grow by androgen, we have investigated its function further, with an aim of understanding its role in the cell cycle and growth responses to androgen stimulation in prostate cells.

Results

Prohibitin is downregulated by androgen treatment

LNCaP total cell extract was separated by isoelectric focusing (IEF) and 12% SDS–PAGE. A protein feature of 30 kDa and pI 5.7 showed a decrease of 50% detectable protein in the cells exposed to 100 nM DHT for 16 h (Figure 1a and b). This protein was identified as prohibitin using HPLC-MS/MS (Figure 1c), and confirmation of this decrease was obtained by standard SDS–PAGE Western blotting using the commercially available antibody to prohibitin, again showing a significant decrease in protein levels of around 50% following 16 h DHT exposure (Figure 2a). At 4 h exposure to the hormone, no significant change in the prohibitin protein level was detected. A 48 h exposure resulted in a decrease of prohibitin by around 30%. To confirm that this effect was indeed downstream of the AR signalling pathway, the cell line PC3 (an AR-negative

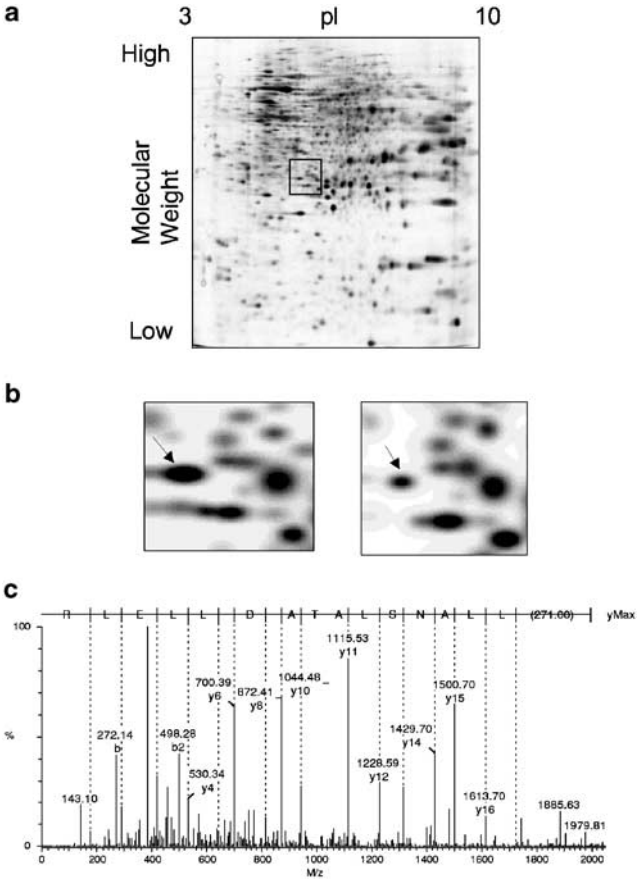


Figure 1 2-DE image of LNCaP total protein. (**a**) 2-DE silver-stained image of LNCaP protein extract separated over pI range 3–10 and 12% PAGE. Boxed area contains prohibitin, expanded in panel b. (**b**) Prohibitin protein regulation following hormone treatment: left-hand panel, ethanol-treated control; right-hand panel, treated with 100 nm DHT for 16 h. (**c**) Spot 3315 was identified as prohibitin (PHB human) by tandem mass spectrometry. In all, 12 tryptic peptides were sequenced, comprising 104-amino-acid residues (39% coverage). A typical spectrum, from which the sequence AAELLANSLATAGDGLLELR was deduced, is shown

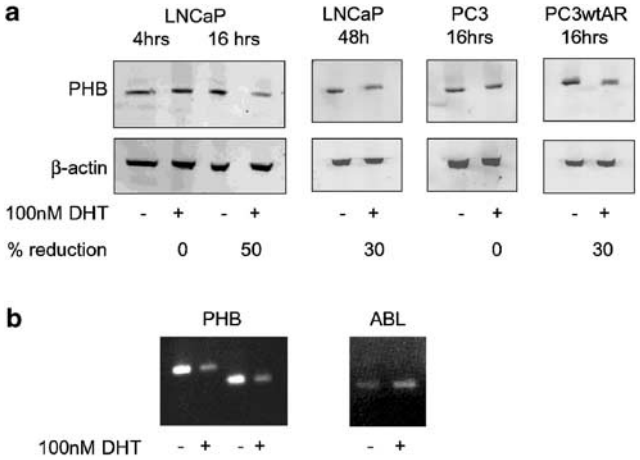


Figure 2 Confirmation of regulation of prohibitin by DHT in prostate cancer cells. (**a**) Western blot of prohibitin in LNCaPs treated with 100 nm DHT for 4, 16 and 48 h and PC3 cells transfected with AR (wtAR) or empty vector, exposed to 100 nM DHT for 16 h. Upper panel, prohibitin protein; lower panel, β-actin. The figure shows a representative blot from two separate experiments. (**b**) Semiquantitative RT–PCR of prohibitin mRNA (left-hand panel) from LNCaP cells exposed to DHT for 16 h and ABL (right-hand panel) as loading control

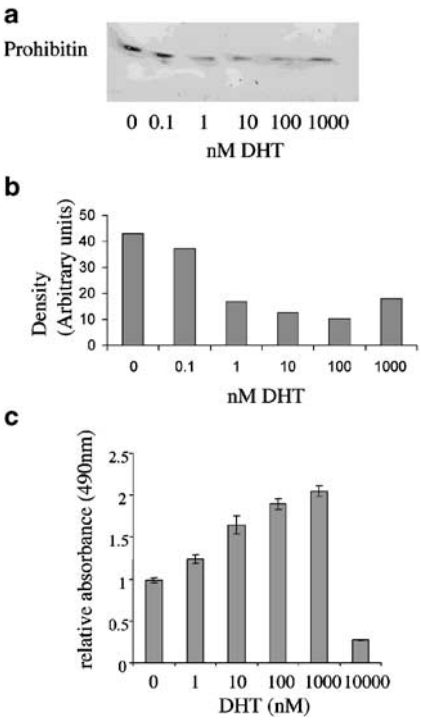


Figure 3 Prohibitin dose–response curve and DHT growth-response curve. (**a**) Western blot of prohibitin expression in LNCaP cells exposed to DHT for 16 h. Results are representative of two independent experiments. (**b**) Signal density of prohibitin Western results; values are means of two independent experiments. (**c**) Sulphorhodamine B assay results of cells exposed to varying concentrations of DHT for 48 h. Values are mean $\pm$ standard error of three independent experiments, $n = 8$ per experiment. Absorbance at 490 nm is expressed relative to the unexposed control

prostate cancer cell line), stably transfected with either empty vector or vector containing wild-type AR, was exposed to 100 nM DHT for 16 h. This exposure resulted in a decrease of prohibitin of around 30% in AR-positive cells, whereas exposure of the untransfected cell line did not cause any decrease in prohibitin. Using primers designed to the first exon of the prohibitin RNA, reverse transcription–PCR (RT–PCR) also showed a decrease of mRNA in the exposed LNCaP cells of 50% following 16 h exposure (Figure 2b), while the control RT–PCR showed no reduction in the levels of ABL message. This demonstrates that regulation of prohibitin by DHT exposure is probably at the level of transcriptional control, although we cannot rule out a specific effect on prohibitin mRNA stability.

Prohibitin regulation occurs at concentrations of DHT that promote growth of LNCaP cells

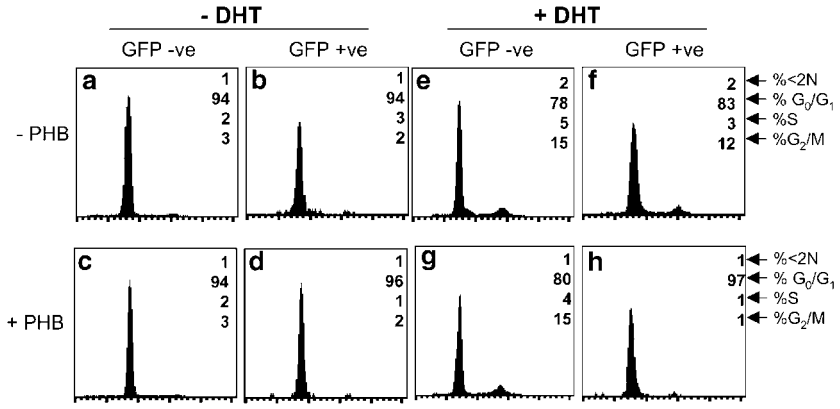
The Western blot of prohibitin demonstrated a clear dose–response relationship between 16 h DHT exposure and prohibitin levels (Figure 3a and b), with maximum repression of prohibitin expression occurring at 10 and 100 nM DHT. At concentrations above 100 nM DHT, repression of prohibitin was not as pronounced, an effect mirrored at concentrations of less than 10 nM DHT. Exposure of LNCaP cells to 1, 10, 100 and 1000 nM DHT (Figure 3c) demonstrated increased growth of LNCaP cells after 48 h compared to control, as measured by sulphorhodamine B assay, with repression at 10 μM.

Prohibitin inhibits stimulation of growth by androgens in LNCaPs

LNCaP cells were cotransfected with spectrin-GFP and a prohibitin expression vector or empty vector at a molar ratio of 1 : 10. Stimulation of LNCaP cells not overexpressing prohibitin with 100 nM DHT (Figure 4, compare a–c with e–g) resulted in a shift of cells from G_0/G_1 to S and G_2/M, with approximately 15% increase in the cell population actively in cell cycle upon stimulation. The overexpression of prohibitin (Figure 4d) did not appear to alter dramatically the number of cells entering into cell cycle without stimulation (compare Figure 4d with a–c), probably due to the low percentage of unstimulated cells in cycle, although there was a small drop in the number of cells in S and G_2/M. In comparison, upon stimulation with DHT, the GFP-negative population of cells entered into the cell cycle as described above (Figure 4e and g), whereas the GFP-positive prohibitin-overexpressing population failed to enter the cell cycle (Figure 4h), with 97% of the transfected population remaining in G_1 compared to approximately 80% of untransfected cells (Figure 4, compare e–g with h).

Silencing of prohibitin using RNAi causes a small increase in cell-cycle entry in LNCaPs

Given the observed regulation of prohibitin in LNCaP cells in response to concentrations of DHT that cause cell growth, we next investigated the importance of



		GFP status	<2N	G_0/G_1	S	G_2/M
-DHT	-PHB	*-ve (a)*	0.4±0.5	96.1±1.3	1.0±0.6	2.4±0.6
		+ve (b)	0.5±0.5	95.1±1.4	2.0±0.7	2.3±0.9
	+PHB	*-ve (c)*	0.6±0.6	96.5±2.2	0.9±0.6	1.9±1.4
		+ve (d)	0.5±0.4	94.5±1.6	2.2±0.9	2.8±1.6
+DHT	-PHB	*-ve (e)*	0.7±1.2	80.0±1.8	6.0±1.1	12.7±2.2
		+ve (f)	0.7±1.2	83.7±1.2	4.9±1.7	10.7±1.5
	+PHB	*-ve (g)*	0.3±0.6	79.8±3.7	5.6±1.5	14.6±2.6
		+ve (h)	0.3±0.6	95.0±2.1	1.8±0.7	2.6±1.4

Figure 4 FACS analysis of LNCaP cells transfected with prohibitin and GFP. Panels e–h represent cells treated with 100 nM DHT for 48 h, and panels a–d represent cells exposed to ethanol only. Left-hand panels are GFP-negative controls, right-hand figures are gated for GFP. Panels c, d, g and h are transfected with pSG5-PHB, panels a, b, e and f with an empty vector. Transfection of GFP to PSG5-PHB or empty vector was in the ratio 1 : 10. The table shows mean ± standard deviation of three independent experiments

prohibitin downregulation to the cell cycle. Two sets of small inhibitory RNA (siRNA) oligos were designed complementary to the mRNA for prohibitin, one to the main body of the mRNA in exon 1 (siPHB1) and a second to the 3′UTR of the mRNA (siPHB2). As a control, a commercially available random oligo of same length with no homology to known mammalian genes was used. Transfection for 48 h, followed by 72 h incubation in RPMI medium supplemented with charcoal-stripped serum, resulted in a knockdown of the prohibitin protein by siPHB2 of 80% as detected by Western blot compared to β-actin loading (Figure 5a). No reduction in prohibitin protein was observed after transfection with control oligo (not shown) or siPHB1, so only siPHB2 was used in all further experiments.

FACS analysis of transfected cells reproducibly showed a small but consistent increase in cells entering cell cycle upon silencing of prohibitin (Table 1), with a small but significant decrease in the G_0/G_1 population being mirrored by an increase in the $S/G_2/M$ population. In all, 2% more cells above control values were detected in the $S/G_2/M$ phase following RNA interference (RNAi), compared with a 10% increase following DHT treatment. This proportional increase in cell cycling was verified using the more sensitive technique of BrdU incorporation (Figure 5b and c), with the increase in DNA-replicating, BrdU-positive cells after siRNA treatment (average 1.2%) being roughly 20% of the increase seen in DHT-treated cells (average 6.2%).

Prohibitin is present in the mitochondria and the nucleus of LNCaP cells

One way in which prohibitin may be affecting cell cycle is by direct interaction with transcription factors in the nucleus. To establish if this was possible in LNCaPs, we used confocal microscopy to determine whether prohibitin was present in the nucleus. Dual staining of LNCaP cells with antibodies to prohibitin and cytochrome *c*, a mitochondrial marker (Figure 6), demonstrated colocalization of endogenous prohibitin and cytochrome *c* in the mitochondria of the cells, and the presence of prohibitin staining in the nucleus was also noted. Z-stacking analysis confirmed that this staining represented discrete localization of the prohibitin protein within the nucleus.

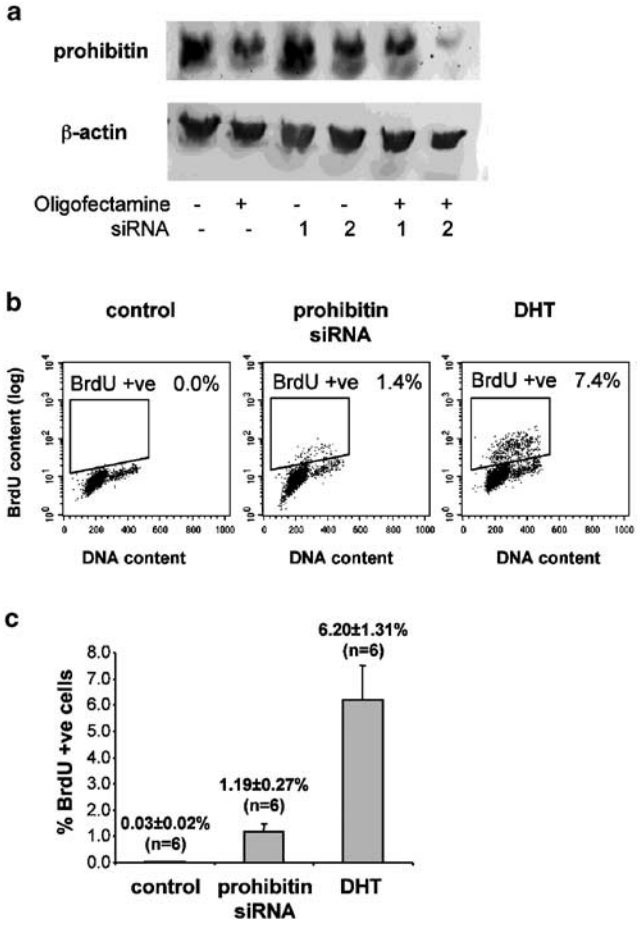


Figure 5 Effect of silencing prohibitin by RNAi on LNCaP growth. (**a**) Western blot showing knockdown of prohibitin protein. Cells were transfected with one of two siRNA oligos or treated with oligo or transfection reagent alone. Upper panel, PHB protein; lower panel, the same blot probed for β-actin. Images are representative of two independent experiments. (**b**, **c**) Cell-cycle analysis of LNCaP cells transfected with siRNA for prohibitin. Cells were incubated in stripped serum medium for 48 h, concomitant with siRNA transfection. Subsequent incubation with 100 nM DHT or ethanol for 48 h was followed by 30 min incubation with BrdU. The harvested cells were fixed and stained with FITC-conjugated anti-BrdU antibody and subjected to FACS analysis to measure DNA content and cells in DNA synthesis. (**b**) A representative cell-cycle analysis; data are represented as BrdU content (log scale) against DNA content (linear scale). (**c**) Percentage of cells synthesizing DNA; values are combined means $\pm$ standard deviation of triplicate experiments performed on two separate occasions

Table 1 Cell-cycle effects of prohibitin silencing on LNCaP cells

	$<2N$	G_0/G_1	$S/G_2/M$
Control	1.9 $\pm$ 1.1	93.7 $\pm$ 1.2	4.4 $\pm$ 0.06
siRNA	2.9 $\pm$ 0.7	91.0 $\pm$ 0.3	6.2 $\pm$ 0.87
DHT	1.6 $\pm$ 0.5	83.5 $\pm$ 1.3	14.6 $\pm$ 1.7

FACS analysis of prohibitin siRNA-treated LNCaPs at 48 h. Values are mean $\pm$ standard deviation of triplicate samples, stained with propidium iodide. DHT concentration was 100 nM

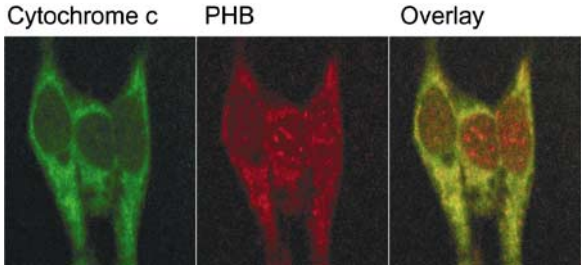


Figure 6 Fluorescent confocal microscopy images demonstrating localization of endogenous prohibitin in LNCaP cells. (Left) Mitochondrial marker cytochrome *c* detected with FITC (green)-labelled secondary antibody. (Middle) Prohibitin detected with TRITC (red)-labelled secondary antibody. (Right) Overlay of images to demonstrate areas of colocalized staining (yellow) and nuclear staining of prohibitin alone (red)

Discussion

The AR signalling pathway is fundamental to the hormonal therapy of prostate cancer and is also implicated in the ultimate failure of that treatment. Greater knowledge of the pathway linking stimulation of the AR to cell growth is therefore vital in our drive to find new treatments for prostate cancer. In a study to find proteins regulated by exposure to androgens, we identified prohibitin, a protein with a possible role in cell-cycle control (McClung *et al*., 1989) that also shows sequence similarity to a known coregulator of the oestrogen receptor, REA (Nijtmans *et al*., 2002).

The downregulation of prohibitin at the protein level after 16 h exposure to DHT was detected by 2-DE, and confirmed by immunoblotting. This regulation was absent at 4 h exposure and still present at 48 h, although not as strongly as at 16 h, suggesting an effect of desynchronization of the cell cycle or possibly degradation of the DHT in culture. The regulation of prohibitin levels in response to hormone stimulation has been reported in other cell types (Thompson *et al*., 1999, 2001; Dixit *et al*., 2003), and regulation of prohibitin in response to growth stimuli and cellular differentiation is well documented (Liu *et al*., 1994; Coates *et al*., 1997; Woodlock *et al*., 2001; Fellenberg *et al*., 2003). In addition, another prostate cancer cell line (PC3) stably transfected with wild-type AR also demonstrated an androgen-dependent decrease in prohibitin of around 30%, at 16 h exposure to 100 nM DHT, and this effect was not seen in the parental AR-negative cells. This confirms that the effects of prohibitin are directly related to AR action following stimulation by DHT, corroborated by the dose-dependent regulation of prohibitin caused by varying the concentration of DHT added to LNCaP cells. Although PC3 cells are AR-negative, androgen-insensitive cells, we were not surprised to see prohibitin expression in these cells, since the mitochondrial role of prohibitin has been demonstrated in cells with no hormone sensitivity and appears to be a fairly ubiquitous role, and further our confocal microscopy demonstrated that the majority of prohibitin even in LNCaP cells is mitochondrial. LNCaPs exposed to 100 nM DHT for 16 h also showed a reduction of

prohibitin mRNA, indicating that the control of prohibitin protein levels in the cell is at least partially at the level of transcription. Although no canonical direct repeat androgen response elements (AREs) have been identified in the 5′ regulatory region or first exon of the prohibitin gene, it has recently been shown that a wide variation of sequences may act as AREs (Verrijdt *et al*., 2003), so direct regulation of the prohibitin promoter is a possibility.

Maximum repression of prohibitin occurred between 10 and 1000 nM DHT exposure, corresponding to the maximum growth increase observed in LNCaP cells exposed to DHT. This indicates that prohibitin protein has a negative effect on cell cycle in prostate epithelial cells. To determine the effect of increasing prohibitin levels, we transfected LNCaP cells with a prohibitin expression vector. FACS analysis showed that while increased expression of prohibitin did not have a significant effect on cell cycle in the absence of androgen, the cells were not capable of entering cell cycle upon stimulation with DHT. Prohibitin downregulation therefore appears to be a vital part of the sequence of events necessary for androgen stimulation of growth. As cell cycle *per se* was not affected by prohibitin overexpression, it appears that the cell-cycle effects of prohibitin may be solely related to the androgen-stimulated growth pathway. This suggests that prohibitin is part of a regulatory system that is present only in some cell types and requires hormone stimulation for its action. Hormone regulation of prohibitin has been documented in other cell types, and other laboratories have shown that prohibitin is capable of interacting with and inhibiting transcription by E2F in a breast cancer cell line, in a manner independent of the Rb protein (Wang *et al*., 1999a, b). It is therefore possible that the role of prohibitin in LNCaP cells is to repress E2F activity and thus the expression of genes required for hormone-stimulated growth. Upon stimulation with hormone, downregulation of prohibitin protein and subsequent de-repression of E2F activity would allow transcriptional activation of those genes required for growth that are not directly under the control of AREs. The results of the RNAi experiments indicate that removal of prohibitin results in a small but reproducible increase in the cell population entering the cell-cycle phase. Although this increase is small, it is significant, especially in the light of the observation that only 15% of the cells enter the cell cycle following optimal DHT stimulation and prohibitin is unlikely to be the only cell-cycle regulator targeted by androgen to mediate cell-cycle entry. The fact that silencing of prohibitin partially mimics the effects of androgen stimulation, together with the observation that overexpression of prohibitin inhibits DHT stimulation of growth, supports our hypothesis that prohibitin is a major effector of androgen-regulated growth.

Prohibitin has long been known to localize to the mitochondria (Ikonen *et al*., 1995; Coates *et al*., 2001), but nuclear localization has also somewhat controversially been reported for various cell types (Wang *et al*., 1999a; Thompson *et al*., 2001). Nuclear prohibitin is reported chiefly in cells that are responsive to steroid hormones (Thompson *et al*., 1999, 2001; Wang *et al*., 1999a; Dixit *et al*., 2003), in agreement with an additional nuclear, cell-cycle regulatory role in these cells as well as that of mitochondrial chaperone.

In conclusion, it appears that prohibitin is an important target of androgens in prostate epithelial cells, regulated at the level of transcription and mediating androgen signalling through its effects on cell cycle. The nuclear localization and inhibitory effect of prohibitin on DHT-stimulated growth indicate that prohibitin has a major role to play in the repression of growth in unstimulated cells, possibly via E2F-mediated gene activation. This role appears to be specifically hormone related and may be distinct from the mitochondrial functions of prohibitin.

Materials and methods

Cell culture and treatments

The LNCaP prostate cancer cell line was obtained from the American Type Culture Collection and cultured at 37°C, 5% CO_2 RPMI medium (Gibco) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 10% foetal bovine serum. PC3 cells transfected with an empty vector or wild-type AR (Peterziel *et al*., 1999), were cultured as above with the addition of 4 μg/ml geneticin (Life Technologies). DHT (Sigma) was resuspended in ethanol, diluted to 1000 × working strength (100 mM) in ethanol and stored at −20°C until use. Flasks (25 cm^2) of cells were transferred to phenol red-free RPMI supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 10% charcoal-stripped foetal bovine serum for 24 h (unless otherwise stated) before exposure to androgen or ethanol. DHT or ethanol was added to spent medium from concurrently seeded flasks, mixed by inversion and added to the flasks. Cells were incubated as above for 4 or 16 h, then placed on ice and washed twice in 330 mM sucrose before lysis in IEF buffer.

Two-dimensional gel electrophoresis

2-DE was performed using immobilized pH gradient (IPG) strips (Amersham Biosciences) of pH range 3–10 (linear). The solubilized protein sample was applied to the strips during gel rehydration, according to the manufacturer's instructions. The samples were diluted with rehydration solution containing 8 M urea, 0.5% CHAPS, 0.2% DTT and 0.2% pharmalyte (pH 3–10) prior to loading, and for analytical gels total protein loaded was 250 μg in 450 μl, and for preparative gels 3 mg in 450 μl. The strips were focused at 0.05 mA/IPG strip for 60 kVh at 20°C. After IEF, the strips were equilibrated in 1.5 M Tris pH 8.8 buffer containing 6 M urea, 30% glycerol, 2% SDS and 0.01% bromophenol blue, with the addition of 1% DTT for 15 min, followed by the same buffer with the addition of 4.8% iodoacetamide for 15 min. SDS–PAGE was performed using 12% T, 2.6% C separating polyacrylamide gels without a stacking gel, using the Iso-Dalt system (Amersham Biosciences). The second-dimension separation was carried out overnight at 20 mA/gel, 15°C. Five samples were prepared as per experimental condition.

Protein visualization

Gels were fixed in a methanol, acetic acid and water solution (4 : 5 : 1 v/v/v) overnight prior to silver staining. Analytical gels

were stained using the Owl silver stain kit (OWL Separation Systems Inc.), while preparative gels were stained using a modified mass spectrometry-compatible kit (Plus-One, Amersham Biosciences), modified according to Yan *et al*. (2000).

Densitometry and gel image analysis

Silver-stained gels were scanned using a Biorad GS-710 calibrated imaging densitometer and analytical images were analysed using PDQuest v6.2.1 (Biorad) (Krauss *et al*., 1990; Corbett *et al*., 1994). After detection of spots, the gels were aligned, landmarked and matched. Gels were then placed into the appropriate experimental class and differential analysis was performed. The Student's *t*-test was used to detect all spots that differed significantly between the control and exposed groups ($P<0.05$); all significantly different spots were then checked manually to eliminate any artefactual differences due to gel pattern distortions and inappropriately matched or badly detected spots.

Tandem mass spectrometry

In-gel digestion with trypsin was performed according to published methods (Jeno *et al*., 1995; Shevchenko *et al*., 1996; Wilm *et al*., 1996) modified for use with a robotic digestion system (Investigator ProGest, Genomic Solutions) (Wait *et al*., 2001). Silver-stained gel pieces were first washed with 30 μl of 15 mM potassium ferricyanide/50 mM sodium thiosulphate (Gharahdaghi *et al*., 1999), followed by successive rinses with deionized water, 50 mM ammonium hydrogen carbonate buffer and acetonitrile. Cysteine residues were reduced with DTT and derivatized by treatment with iodoacetamide. After further washing with ammonium hydrogen carbonate buffer, the gel pieces were again dehydrated with acetonitrile and dried at 60°C, prior to addition of modified trypsin (Promega; 10 μl at 6.5 ng/μl in 25 mM ammonium hydrogen carbonate). Digestion proceeded for 8 h at 37°C and products were recovered by sequential extractions with 25 mM ammonium hydrogen carbonate, 5% formic acid and acetonitrile. The pooled extracts were lyophilized and then redissolved in 0.1% formic acid for mass spectrometry. Tandem electrospray mass spectra were recorded using a Q-Tof hybrid quadrupole/orthogonal acceleration time-of-flight spectrometer (Micromass) interfaced to a Micromass CapLC capillary chromatograph. Samples were dissolved in 0.1% aqueous formic acid, and 6 μl injected onto a Pepmap C18 column (300 μm $\times$ 0.5 cm; LC Packings), and washed for 3 min with 0.1% aqueous formic acid (with the stream select valve diverting the column effluent to waste). The flow rate was then reduced to 1 μl/min, the stream select valve was switched to the data acquisition position and the peptides were eluted into the mass spectrometer with an acetonitrile/0.1% formic acid gradient (5–70% acetonitrile over 20 min). The capillary voltage was set to 3500 V, and data-dependent MS/MS acquisitions were performed on precursors with charge states of 2, 3 or 4 over a survey mass range 540–1000. Known trypsin autolysis products and keratin-derived precursor ions were automatically excluded. The collision voltage was varied between 18 and 45 V depending on the charge and mass of the precursor. Product ion spectra were charge-state de-encrypted and de-isotoped with a maximum entropy algorithm (MaxEnt 3, Micromass). Proteins were identified by correlation of uninterpreted tandem mass spectra to entries in SwissProt/TREMBL, using ProteinLynx Global Server (Version 1, Micromass). One missed cleavage per peptide was allowed, and an initial mass tolerance of 50 ppm was used in all searches. Cysteines were assumed to be carbamidomethylated, but other potential modifications were not considered in the first pass search. If this approach failed, amino-acid sequences were deduced manually from the charge-state de-encrypted spectra (Wait *et al*., 2002), and were used as queries for searches using BLAST (Altschul *et al*., 1997) and FASTS (Mackey *et al*., 2002).

Western blot analysis

Cells were lysed in 2-DE lysis buffer and the protein concentration was determined by modified Bradford assay (Weekes *et al*., 1999). In all, 50 μg of protein was loaded onto a 12% SDS polyacrylamide gel and electrophoretically transferred onto a nitrocellulose membrane (Transblot, Biorad). Blocking was carried out overnight in PBS with 5% nonfat milk and incubated with mouse anti-human prohibitin primary antibody (AbCam Ltd) at 1 : 5000 and mouse anti-human β-actin antibody at 1 : 3000 for 1 h. After washing, detection was carried out using the alkaline phosphatase-conjugated anti-mouse IgG diluted to 1 : 5000, incubating for 1 h followed by further washes and incubation with the substrate for ECF™ (Amersham Biosciences) as per the manufacturer's instructions. Visualization was carried out using the fluorescence detection mode of the Typhoon phosphorimager (Amersham Biosciences).

Reverse transcription–PCR

Total RNA samples were prepared using trizol reagent (Gibco BRL) and quantified by measuring the absorbance at 260 nm. For RT–PCR, mRNA was isolated from the total RNA obtained, using the oligotex mRNA kit (Qiagen) according to the manufacturer's instructions. In all, 0.5 μg of sample was used for the RT reaction (Invitrogen) and 2 μl of this reaction was used in a PCR mix using Taq Gold (Applied Biosystems) and the primer pairs 5′-ctg cct tat ata atg tgg atg ctg-3′ and 5′-gct ctc tct ggg tga tta gtt ctc-3′; 5′-cgt ggg tac aga aac caa tta tct-3′ and 5′-ctc tct ctg gct gat tag ttc tcc-3′, giving product sizes of 360 and 257 bp, respectively. Loading control PCR reactions were carried out using primers to the ABL cDNA as described previously (Slade *et al*., 1999).

RNA interference

siRNA oligonucleotide duplexes were designed to exon 1 (5′-aac ucu gcc uua uau aau gug-3′) and the 3′UTR (5′-aac aca gcc uuc cuu cug cuc-3′) of the mRNA for prohibitin, and manufactured by MWG-Biotech AG. As a control, a random oligo sequence of 21 nucleotides was used (Qiagen) that has no homology to known mammalian genes. Transfection was carried out using oligofectamine (Invitrogen) according to the manufacturer's instructions, for 48 h in phenol red-free RPMI supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 10% charcoal-stripped foetal bovine serum. Following transfection as previously described, the medium was replaced and hormone treatment was carried out.

Sulphorhodomine assay of cell culture growth

To determine cell number following DHT treatment, the sulphorhodamine B assay was used (Skehan *et al*., 1990). Cells were plated into 96-well plates at a density of 10^4 cells/well, medium was changed to phenol red-free, stripped serum medium for 48 h and subsequently hormone or carrier molecule added for 48 h. Eight samples were measured per concentration and the experiment was repeated three times.

Transfection of LNCaP cells with prohibitin

The following primers were used to amplify the prohibitin cDNA fragment from IMAGE clone 3010198, obtained from the HGMP-RC: Fwd 5′-taggaattccccgggatggctgccaaagtg-3′ and Rev 5′-taattggatcctcactggggcagctggag-3′. The product obtained was then cloned into pSG5 expression vector using *EcoR1*/*Bam*H1 restriction site. LNCaP cells were transfected with 2 μg spectrin-GFP and 18 μg of empty PSG5 (control) or pSG5-prohibitin using Fugene 6 transfection reagent (Roche), as per the manufacturer's instructions. Cells were incubated for 48 h in transfection reagent in stripped serum medium, before being dosed with hormone for 48 h.

Cell-cycle analysis

Cell-cycle analysis was performed by propidium iodide staining with or without bromodeoxyuridine (BrdU) staining. At 30 min before harvesting cells, 10 μM BrdU (Sigma) was added to the medium. Cells were then trypsinized, washed twice in PBS and fixed overnight at 4°C in 70% ethanol. The cells were then incubated with 2 M HCl, followed by 0.5% Triton X-100 for 30 min at room temperature, and then with a 1 : 3 dilution of fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody (BD Biosciences) for 30 min. Between each treatment, cells were washed with PBS. Cells were then incubated with 5 μg/ml propidium iodide and 50 μg/ml RNase A in PBS for 1 h at room temperature. FACS analysis was carried out using a Beckton-Dickinson FACS Calibur machine using linear scale representation of forward and side scatter during flow analysis. Samples were prepared in triplicate after 48 h and analysed using CellQuest-Pro software. A total of 10 000 events were measured per sample.

Immunofluorescence and confocal microscopy

LNCaP cells were grown on sterile glass coverslips in 24-well plates to 30% confluence in RPMI media before being washed three times in PBS. Cells were fixed in methanol at −20°C for 10 min. Coverslips were washed a further three times in PBS and treated with 10% whole goat serum and 10% whole rabbit serum (Dako Cytomation) for 30 min. Antibodies to prohibitin and sheep anti-human cytochrome *c* (1 : 100 dilution) were applied in 10% whole goat serum, 10% whole rabbit serum for 1 h and coverslips were washed three times in PBS. In all, 10% whole goat and rabbit serum was applied to the coverslips for a further 15 min prior to incubating with either an FITC isomer I or tetramethylrhodamine isothyocyanate (TRITC)-conjugated secondary antibody (Sigma) in the dark for 1 h. Cells were washed five times in PBS, mounted on glass slides with Vectorshield containing dapi (Vector Laboratories Inc.) and visualized on a Zeiss Meta 512 confocal microscope.

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References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W and Lipman DJ. (1997). *Nucleic Acids Res.*, **25,** 3389–3402.

Coates PJ, Jamieson DJ, Smart K, Prescott AR and Hall PA. (1997). *Curr. Biol.*, **7,** 607–610.

Coates PJ, Nenutil R, McGregor A, Picksley SM, Crouch DH, Hall PA and Wright EG. (2001). *Exp. Cell. Res.*, **265,** 262–273.

Corbett JM, Wheeler CH, Baker CS, Yacoub MH and Dunn MJ. (1994). *Electrophoresis*, **15,** 1459–1465.

Dixit VD, Sridaran R, Edmonsond MA, Taub D and Thompson WE. (2003). *Endocrinology*, **144,** 1496–1505.

Fellenberg J, Dechant MJ, Ewerbeck V and Mau H. (2003). *Int. J. Cancer*, **105,** 636–643.

Gharahdaghi F, Weinberg CR, Meagher DA, Imai BS and Mische SM. (1999). *Electrophoresis*, **20,** 601–605.

Gottlieb B, Trifiro M, Lumbroso R and Pinsky L. (1997). *Nucleic Acids Res.*, **25,** 158–162.

Grayhack JT, Keeler TC and Kozlowski JM. (1987). *Cancer*, **60,** 589–601.

Greenlee RT, Hill-Harmon MB, Murray T and Thun M. (2001). *CA Cancer J. Clin.*, **51,** 15–36.

Ikonen E, Fiedler K, Parton RG and Simons K. (1995). *FEBS Lett.*, **358,** 273–277.

Jeno P, Mini T, Moes S, Hintermann E and Horst M. (1995). *Anal. Biochem.*, **224,** 75–82.

Jupe ER, Liu XT, Kiehlbauch JL, McClung JK and Dell'Orco RT. (1996). *Exp. Cell. Res.*, **224,** 128–135.

Koivisto P, Kolmer M, Visakorpi T and Kallioniemi OP. (1998). *Am. J. Pathol.*, **152,** 1–9.

Krauss MR, Collins PJ and Blose SH. (1990). *Biotechniques*, **8,** 218–223.

Liu XT, Stewart CA, King RL, Danner DA, Dell'Orco RT and McClung JK. (1994). *Biochem. Biophys. Res. Commun.*, **201,** 409–414.

Mackey AJ, Haystead TA and Pearson WR. (2002). *Mol. Cell Proteomics*, **1,** 139–147.

McClung JK, Danner DB, Stewart DA, Smith JR, Schneider EL, Lumpkin CK, Dell'Orco RT and Nuell MJ. (1989). *Biochem. Biophys. Res. Commun.*, **164,** 1316–1322.

Nijtmans LG, Artal SM, Grivell LA and Coates PJ. (2002). *Cell. Mol. Life Sci.*, **59,** 143–155.

Nuell MJ, Stewart DA, Walker L, Friedman V, Wood CM, Owens GA, Smith JR, Schneider EL, Dell' Orco R, Lumpkin CK, Danner DB and McClung JK. (1991). *Mol. Cell. Biol.*, **11,** 1372–1381.

Peterziel H, Mink S, Schonert A, Becker M, Klocker H and Cato AC. (1999). *Oncogene*, **18,** 6322–6329.

Shevchenko A, Wilm M, Vorm O and Mann M. (1996). *Anal. Chem.*, **68,** 850–858.

Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S and Boyd MR. (1990). *J. Natl. Cancer Inst.*, **82,** 1107–1112.

Slade MJ, Smith BM, Sinnett HD, Cross NC and Coombes RC. (1999). *J. Clin. Oncol.*, **17,** 870–879.

Steglich G, Neupert W and Langer T. (1999). *Mol. Cell. Biol.*, **19,** 3435–3442.

Thompson WE, Branch A, Whittaker JA, Lyn D, Zilberstein M, Mayo KE and Thomas K. (2001). *Endocrinology*, **142,** 4076–4085.

Thompson WE, Powell JM, Whittaker JA, Sridaran R and Thomas KH. (1999). *Anat. Rec.*, **256,** 40–48.

Vander Heiden MG, Choy JS, VanderWeele DJ, Brace JL, Harris MH, Bauer DE, Prange B, Kron SJ, Thompson CB and Rudin CM. (2002). *J. Biol. Chem.*, **277,** 44870–44876.

Verrijdt G, Haelens A and Claessens F. (2003). *Mol. Genet. Metab.*, **78,** 175–185.

Visakorpi T, Hyytinen E, Koivisto P, Tanner M, Keinanen R, Palmberg C, Palotie A, Tammela T, Isola J and Kallioniemi OP. (1995). *Nat. Genet.*, **9,** 401–406.

Wait R, Gianazza E, Eberini I, Sironi L, Dunn MJ, Gemeiner M and Miller I. (2001). *Electrophoresis*, **22,** 3043–3052.

Wait R, Miller I, Eberini I, Cairoli F, Veronesi C, Battocchio M, Gemeiner M and Gianazza E. (2002). *Electrophoresis*, **23,** 3418–3427.

Wang S, Fusaro G, Padmanabhan J and Chellappan SP. (2002a). *Oncogene*, **21,** 8388–8396.

Wang S, Nath N, Adlam M and Chellappan S. (1999a). *Oncogene*, **18,** 3501–3510.

Wang S, Nath N, Fusaro G and Chellappan S. (1999b). *Mol. Cell. Biol.*, **19,** 7447–7460.

Wang S, Zhang B and Faller DV. (2002b). *EMBO J.*, **21,** 3019–3028.

Weekes J, Wheeler CH, Yan JX, Weil J, Eschenhagen T, Scholtysik G and Dunn MJ. (1999). *Electrophoresis*, **20,** 898–906.

Wilm M, Shevchenko A, Houthaeve T, Breit S, Schweigerer L, Fotsis T and Mann M. (1996). *Nature*, **379,** 466–469.

Woodlock TJ, Bethlendy G and Segel GB. (2001). *Blood Cells Mol. Dis.*, **27,** 27–34.

Yan JX, Wait R, Berkelman T, Harry RA, Westbrook JA, Wheeler CH and Dunn MJ. (2000). *Electrophoresis*, **21,** 3666–3672.