Androgens Induce Prostate Cancer Cell Proliferation through Mammalian Target of Rapamycin Activation and Post-transcriptional Increases in Cyclin D Proteins

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Abstract

Androgen receptor (AR) plays a central role in prostate cancer, with most tumors responding to androgen deprivation therapies, but the molecular basis for this androgen dependence has not been determined. Androgen [5α-dihydrotestosterone (DHT)] stimulation of LNCaP prostate cancer cells, which have constitutive phosphatidylinositol 3-kinase (PI3K)/Akt pathway activation due to PTEN loss, caused increased expression of cyclin D1, D2, and D3 proteins, retinoblastoma protein hyperphosphorylation, and cell cycle progression. However, cyclin D1 and D2 message levels were unchanged, indicating that the increases in cyclin D proteins were mediated by a post-transcriptional mechanism. This mechanism was identified as mammalian target of rapamycin (mTOR) activation. DHT treatment increased mTOR activity as assessed by phosphorylation of the downstream targets p70 S6 kinase and 4E-BP1, and mTOR inhibition with rapamycin blocked the DHT-stimulated increase in cyclin D proteins. Significantly, DHT stimulation of mTOR was not mediated through activation of the PI3K/Akt or mitogen-activated protein kinase/p90 ribosomal S6 kinase pathways and subsequent tuberous sclerosis complex 2/tuberin inactivation or by suppression of AMP-activated protein kinase. In contrast, mTOR activation by DHT was dependent on AR-stimulated mRNA synthesis. Oligonucleotide microarrays showed that DHT-stimulated rapid increases in multiple genes that regulate nutrient availability, including transporters for amino acids and other organic ions. These results indicate that a critical function of AR in PTEN-deficient prostate cancer cells is to support the pathologic activation of mTOR, possibly by increasing the expression of proteins that enhance nutrient availability and thereby prevent feedback inhibition of mTOR. (Cancer Res 2006; 66(15): 7783-92)

Introduction

Androgen receptor (AR) regulates the transcription of multiple genes in prostate and plays a central role in prostate cancer development, with most prostate cancers expressing high levels of AR and responding to androgen deprivation therapies (1). Androgen-stimulated prostate cancer cell growth has been studied most extensively in the LNCaP prostate cancer cell line, one of the few available AR-expressing and androgen-responsive prostate cancer lines (2). The AR in LNCaP cells has a point mutation in the ligand-binding domain (T877A) but responds normally to physiologic ligands, such as testosterone and 5α-dihydrotestosterone (DHT; ref. 3). Similarly to many advanced prostate cancer, LNCaP cells are also PTEN deficient and thus have constitutive activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (4). LNCaP growth is repressed by androgen deprivation, which causes a retinoblastoma protein (pRb)–dependent G_0-G_1 cell cycle arrest (5–7). Conversely, androgen stimulates cell cycle progression with increased activity of cyclin-dependent kinases (CDK) and pRb hyperphosphorylation (8–10).

Studies in other prostate cancer cell lines and in mouse prostate have similarly shown that androgens can stimulate the increased expression of G_1 cyclins and CDKs and decrease the expression of CDK inhibitors (11–15). However, the mechanisms by which AR stimulates cell cycle progression in prostate cancer cells remain unclear. The p21 CDK inhibitor has been identified as a direct AR transcriptional target, but genes directly regulated by AR that stimulate growth of prostate cancer cells remain to be identified (16). Moreover, studies in other cell types expressing low levels of AR indicate that androgens can stimulate proliferation by non-transcriptional mechanisms through activation of the PI3K/Akt pathway (17–19). In this study, we show that AR stimulates LNCaP proliferation through PI3K/Akt-independent activation of mammalian target of rapamycin (mTOR) and subsequent post-transcriptional increases in cyclin D protein expression.

Materials and Methods

Cell culture and reagents. LNCaP cells obtained from the American Type Culture Collection (Manassas, VA) were cultured in a 5% CO_2 incubator in RPMI 1640 with 10% fetal bovine serum (FBS) or 10% steroid hormone-depleted charcoal dextran-stripped FBS (CDS-FBS; Hyclone, Logan, UT). RPMI 1640 without L-glutamine and leucine was from MP Biomedicals (Aurora, OH). Amino acid solutions (50×) without glutamine were from Cellgro (Herndon, VA). L-Glutamine (200 mmol/L, 100×) was from Invitrogen (Carlsbad, CA). Hormones, actinomycin D, α-amanitin, and compound C were from Sigma (St. Louis, MO). LY294002 and rapamycin were from Calbiochem (La Jolla, CA).

Cell cycle analysis. LNCaP cells were stained using 10 μg/mL propidium iodide (PI; Sigma) in the presence of 250 μg/mL RNase A (Sigma) in PBS after fixation in ethanol. PI-stained samples were run on FACScan (BD Bioscience, Rockville, MD) and the cell cycle data were analyzed using ModFit 3.0 (Verity, Topsham, ME).

Microarray analysis. Total RNA was extracted from duplicate DHT (1 nmol/L)–stimulated or control LNCaP cells at times 0, 2, 4, 8, and 24 hours using Trizol (Invitrogen). Biotin-labeled cRNA was generated and hybridized to Affymetrix U133A oligonucleotide microarrays as described (20). The expression files were normalized across the arrays, and genes expressed below 10 were filtered out. For each gene, the average expression level in the control-treated samples (no DHT) was set as 1.0, and expression

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doi:10.1158/0008-5472.CAN-05-4472

at each time point in the treated samples was expressed as fold change relative to this value.

Real-time reverse transcription-PCR. Real-time reverse transcription-PCR (RT-PCR) was done using an ABI-PRISM 7700 Sequence Detector and the One-Step RT-PCR Master Mix Reagent kit (PE Applied Biosystems, Foster City, CA), with 18S rRNA as an internal control. The 18S rRNA primers and VIC-labeled probe were from PE Applied Biosystems. The primers and probes of specific genes were synthesized by Biosources International (Camarillo, CA). The cyclin D1 primers were forward primer 5′-CCAAATCTCAATGAAGCCAGC-3′, reverse primer 5′-AATTCTTTTGGTTCGGCAGCT-3′, and probe FAM-TGCTGTGTGCCCCGGTCATCTAGC-TAMRA. Cyclin D2 primers were forward primer 5′-CCGACAACTCCATCAAGCCT-3′, reverse primer 5′-CCACTTCAACTTCCCCAGCA-3′, and probe FAM-AGGAGCTGCTGGAGTGGGAACTGGT-TAMRA. Cyclin D3 primers were forward primer 5′-CAGGGATCACTGGCACTGAA-3′, reverse primer 5′-TGAGTGCAGCTTCGATCTGC-3′, and probe FAM-ACTGCCTGCGGGCCTGTCAGG-TAMRA. Cyclin E1 primers were forward primer 5′-TGAAGAAATGGCCAAAATCGA-3′, reverse primer 5′-ATTGTCCCAAGGCTGGCTC-3′, and probe FAM-ATTGTCCCAAGGCTGGCTC-TAMRA. Cyclin E2 primers were forward primer 5′-CCATTTTACCTCCATTGAAGTGGT-3′, reverse primer 5′-ACCATCCAATCTACACATTCTGAAATA-3′, and probe FAM-AGAAAGCCTCAGGTTTGGAGTGGGACA-TAMRA. All the treatments at different time points were in duplicate and experiments were done at least twice with comparable results.

Immunoblotting. Cellular proteins were collected in 1% SDS, standardized for total protein content, and separated on NuPAGE Novex high-performance precast gels (Invitrogen). After transfer to nitrocellulose membranes, blots were blocked with 5% nonfat milk in TBST (0.05% Tween 20 in TBS) for 1 hour and then incubated with primary antibody at 4°C overnight. Detection was carried out with either anti-mouse or anti-rabbit horseradish peroxidase (Promega, Madison, WI) and enhanced chemiluminescence detection reagents (Perkin-Elmer Life Sciences, Boston, MA). Antibodies against cyclin D1 (A-12), cyclin D2 (M-20), cyclin D3 (C-16), and β-tubulin (D-10) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-pRb (Ser^{780}), phospho–AMP-activated protein kinase (AMPK; Thr^{172}), S6, phospho-S6 (Ser^{235}/Ser^{236}), phospho–p70 S6 kinase (p70S6K; Thr^{421}/Ser^{424}), phospho-p70S6K (Thr^{389}), phospho-4E-BP1 (Thr^{37}/Thr^{46}), p44/42 mitogen-activated protein kinase (MAPK; Erk1 and Erk2), phospho-p44/p42 MAPK (Thr^{202}/Tyr^{204}), Akt, phospho-Akt (Ser^{473}), phospho-tuberin (Ser^{939}), phospho-tuberin (Thr^{1462}), and phospho-p90 ribosomal S6 kinase (Rsk; Ser^{380}) were purchased from Cell Signaling Technology (Beverly, MA). In some cases, developed blots were stripped in 62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, and 0.85% β-mercaptoethanol at 55°C for 30 minutes and reused for immunoblotting to confirm equal protein loading.

Transfections and cell sorting. Plasmids encoding mutant 4E-BP1 (pACTAG2/3HA-F114A-4E-BP1) and p70S6K short hairpin RNA (shRNA; pBS/U6-S6K1) were kindly provided by Dr. John Blenis (Harvard Medical School, Boston, MA; ref. 21), and the enhanced green fluorescent protein (EGFP) vector (pIRES2-EGFP) was from Clontech (Mountain View, CA). LNCaP cells in 10-cm plates and cultured for 2 days in medium with 10% CDS-FBS were transfected with 4 μg pIRES2-EGFP and 20 μg 4E-BP1 or p70S6K shRNA (or 10 μg of both) using LipofectAMINE for 24 hours. The cells were then hormone treated for 24 hours, trypsinized, and MoFlow sorted (EGFP-positive and EGFP-negative populations) directly into cell lysis buffer.

Results

Androgen stimulates post-transcriptional increases in cyclin D proteins. LNCaP cell proliferation is suppressed in CDS-FBS medium, and addition of DHT (1 nmol/L) can rapidly (within 24 hours) increase the S-phase fraction (Fig. 1*A*). As shown previously, the proliferative response is biphasic, with higher DHT concentrations being less effective (Fig. 1*A*). To determine whether there were genes directly regulated by AR that may mediate this proliferative response, we used Affymetrix oligonucleotide microarrays to identify genes that were rapidly induced by androgen. Among the G_1 regulatory cyclins and CDKs, the earliest change was a small increase in cyclin D3 (20% increase at 8 hours), with a further increase at 24 hours (40%; Table 1). At 24 hours, there were also increases in CDK2 (60%), CDK4 (40%), cyclin E1 (50%), cyclin E2 (60%), and cyclin A2 (80%), but there were no increases in cyclin D1 or D2.

Real-time RT-PCR was next used to confirm the Affymetrix results. This similarly showed a small increase in cyclin D3 message and more substantial increases in cyclin E1 and E2 at 24 hours (2.5- to 4-fold) and confirmed that there were no changes in cyclin D1 or D2 messages (Fig. 1*B-F*). In contrast, immunoblotting showed substantial increases in cyclin D1 and D2 proteins (as well as in cyclin D3) at 16 to 24 hours (Fig. 1*G*). The phosphorylation of pRb on Ser^{780}, which is mediated by a cyclin D-CDK4/CDK6 complex, was also increased concurrently (Fig. 1*G*). These results, based on both Affymetrix microarrays and independent real-time RT-PCR analyses, showed that androgen stimulation was increasing cyclin D protein levels primarily by a post-transcriptional (translational or post-translational) mechanism.

We next carried out DHT dose-response experiments to determine whether the biphasic proliferative response to androgens was due to biphasic increases in cyclin D proteins. Consistent with the cell cycle data, pRb hyperphosphorylation peaked at 1 nmol/L DHT and declined at higher concentrations (Fig. 1*H*). Cyclin D protein expression also reached maximal levels at 1 nmol/L DHT, but there was no decline at higher DHT concentrations. These results indicate that increased cyclin D protein expression mediates the proliferative response that peaks at ~1 nmol/L DHT and that the subsequent decrease in proliferation is mediated by one or more distinct mechanisms.

AR signaling increases mTOR activation. One potential mechanism for increasing cyclin D expression post-transcriptionally is through mTOR activation. mTOR stimulates protein synthesis by phosphorylating ribosomal p70S6K and 4E-BP1 and has been shown to enhance the translation of cyclin D mRNA (22–25). mTOR activity is increased by Akt through phosphorylation and inactivation of tuberin (tuberous sclerosis complex 2), which functions as a GTPase-activating protein to negatively regulate Rheb, the small GTPase that activates mTOR (26–29). Significantly, previous studies have indicated that AR can stimulate PI3K and subsequently Akt through direct binding of the PI3K regulatory subunit and c-Src (17–19). LNCaP cells have high constitutive Akt activity due to PTEN deficiency and subsequent enhanced PI3K signaling, but it was possible that this could be further enhanced by androgen stimulation. Therefore, we next determined whether mTOR activation may mediate the DHT-stimulated increase in cyclin D proteins and proliferation.

mTOR activity was assessed by activation of p70S6K using phosphospecific antibodies recognizing $pThr^{421}/pSer^{424}$ in the activated protein and phosphorylation of S6, the major substrate for p70S6K. Phosphorylation of p70S6K and S6 was increased after 8 to 16 hours of DHT treatment (Fig. 2*A*). Comparable results were obtained using another phosphospecific p70S6K antibody recognizing $pThr^{389}$, which seems to be the direct mTOR target site (Fig. 2*A*; ref. 30). Phosphorylation of 4E-BP1 was also increased, further indicating that DHT was stimulating mTOR activation (Fig. 2*A*). This mTOR activation correlated with the increase in cyclin D proteins observed at ~16 hours. An AR antagonist (bicalutamide) failed to stimulate mTOR activity and in most experiments suppressed basal mTOR activity, with a corresponding

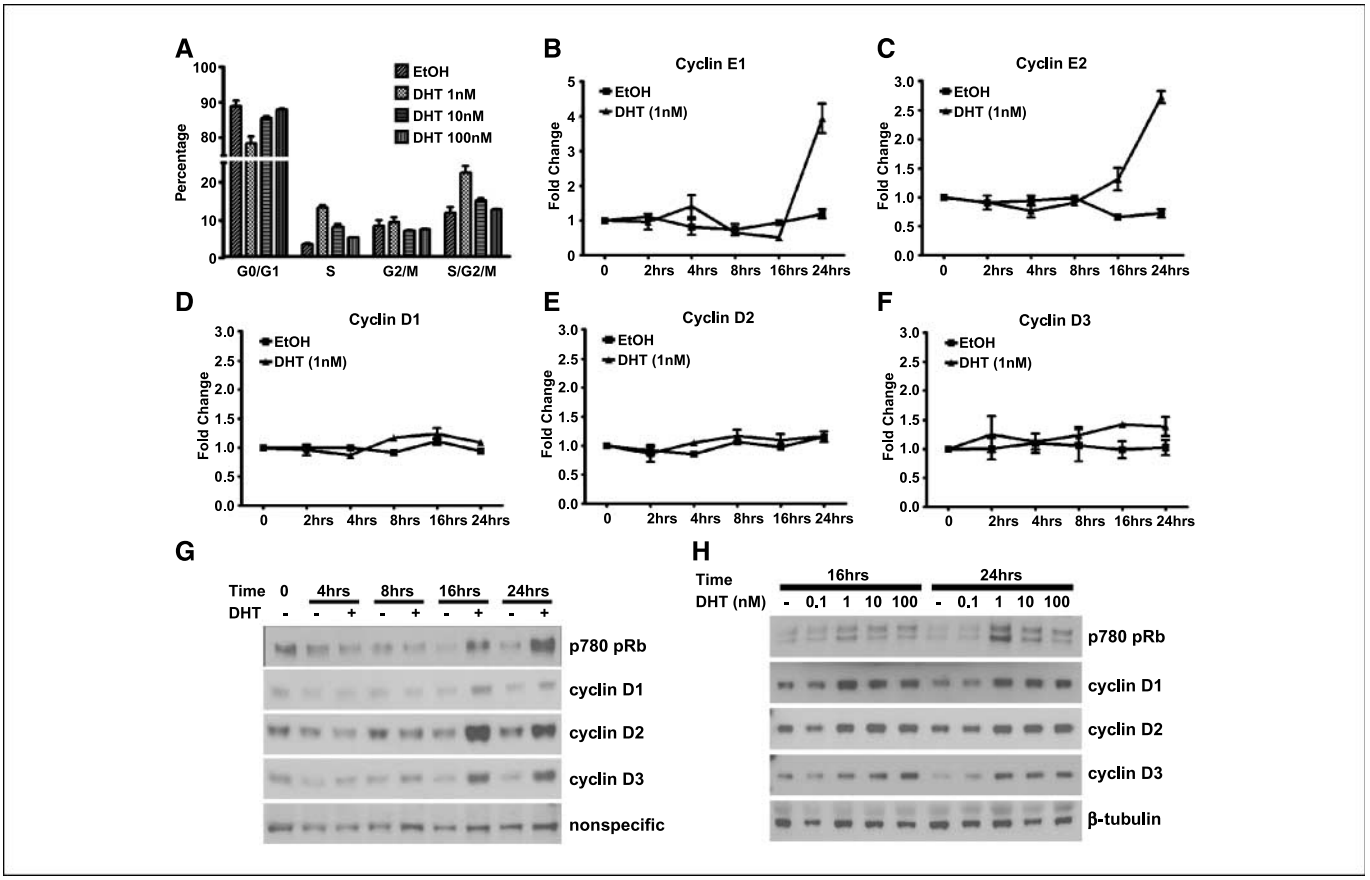

Figure 1. DHT increases cyclin D proteins without increases in cyclin D1 or D2 messages. *A,* LNCaP cells were cultured in medium with 10% CDS-FBS for 3 days and then stimulated with vehicle or different concentration of DHT for 24 hours (final ethanol vehicle concentrations were identical at 0.01%). The fraction of cells in G_0-G_1, S, and G_2-M phases was determined by PI staining. *B* to *F,* LNCaP cells were pretreated as in (*A*) and then stimulated with 1 nmol/L DHT or vehicle in duplicate for various times. RNA was then extracted from treated cells and used for real-time RT-PCR analysis of cyclin E1, E2, D1, D2, and D3. *G,* LNCaP cells in CDS-FBS were treated with 1 nmol/L DHT or vehicle and equal amounts of total protein were immunoblotted for phospho-pRb (Ser^{780}) and cyclin D1, D2, and D3. *H,* LNCaP cells in CDS-FBS were treated with DHT and immunoblotted as above at 16 or 24 hours.

decline in basal cyclin D protein levels (Fig. 2*B*). This mTOR suppression presumably reflects inhibition of AR activated by residual steroid hormones in the medium and/or bicalutamide-mediated corepressor recruitment. Significantly, bicalutamide effectively blocked the DHT stimulation of mTOR, further indicating that mTOR activation by DHT was AR mediated (Fig. 2*B*).

mTOR increases cyclin D protein expression in LNCaP cells. The mTOR antagonist, rapamycin, was next used to determine whether mTOR-regulated cyclin D protein expression in LNCaP cells. Consistent with the high basal PI3K/Akt activity and subsequent mTOR activation in LNCaP cells, rapamycin strongly repressed phosphorylation of p70S6K and S6 (Fig. 3*A*). Cyclin D proteins similarly declined within 4 hours, with a further marked decrease in cyclin D3 levels after 16 and 24 hours of rapamycin treatment (Fig. 3*A*).

To determine whether the DHT-stimulated increase in cyclin D proteins was dependent on mTOR activation, we next tested whether mTOR blockade by rapamycin prevented the DHT-stimulated increase in cyclin D proteins. As expected, rapamycin prevented the DHT-stimulated increase in p70S6K and S6 phosphorylation (Fig. 3*B*). Significantly, rapamycin also blocked the increase in cyclin D proteins in response to DHT stimulation, indicating that mTOR activity was required for the DHT-stimulated increase in cyclin D protein expression.

As an alternative approach to block mTOR, we used transfection with plasmids encoding p70S6K shRNA or dominant-negative 4E-BP1 (F114A mutant that does not associate with mTOR; ref. 21). LNCaP cells were transiently transfected with either or both of these plasmids in conjunction with an EGFP encoding plasmid (pIRES2-EGFP) to allow for selection by fluorescence-activated cell sorting (FACS). The transfections were carried out for 24 hours in steroid hormone-depleted medium, and the cells were then treated for another 24 hours with DHT or vehicle. EGFP-positive and EGFP-negative cells were then sorted by FACS into cell lysis buffer and immunoblotted for cyclin D2. LNCaP cells transfected with the EGFP vector alone showed the expected DHT-stimulated increase in cyclin D2 (Fig. 3*C, lanes 1-4*). In contrast, cyclin D2 expression was not increased in cells transfected with the dominant-negative F114A-4E-BP1 encoding plasmid or in cells expressing the p70S6K shRNA. Similarly to rapamycin treatment, cotransfection with both plasmids further decreased cyclin D2 expression below the basal levels observed in the absence of DHT.

Taken together, these results showed that mTOR was regulating cyclin D protein expression in LNCaP cells and that the DHT-stimulated increase in cyclin D proteins was dependent on mTOR signaling. In conjunction with the DHT-stimulated increase in mTOR activity, these results indicated that the increased cyclin D protein expression in response to DHT was mediated through

Table 1. Expression of cell cycle genes after DHT (1 nmol/L) stimulation based on Affymetrix oligonucleotide microarrays

Probe	Gene	2 h	4 h	8 h	24 h
208712	*Cyclin D1*	1.1	0.8	1.0	0.9
208711	*Cyclin D1*	0.8	1.1	1.0	0.9
200953	*Cyclin D2*	0.7	1.0	1.1	1.0
201700	*Cyclin D3*	0.9	1.0	1.2	1.4
213523	*Cyclin E1*	—	1.1	1.1	1.5
211814	*Cyclin E2v1*	0.6	1.0	1.1	1.6
205034	*Cyclin E2*	0.7	0.9	0.7	1.9
205899	*Cyclin A1*	1.1	1.0	1.2	0.9
203418	*Cyclin A2*	1.1	1.3	1.2	1.8
211804	*CDK2*	0.6	1.2	1.1	1.6
202246	*CDK4*	0.9	1.0	1.0	1.4
207143	*CDK6*	0.7	1.2	0.6	1.0
202284	*p21, Cip1*	1.1	1.0	0.8	0.7
209112	*p27, Kip1*	0.8	0.8	0.9	0.9

NOTE: All values are average of duplicate samples. Results for cyclin E1 at 2 hours were uncertain due to large differences in the replicates.

mTOR. However, we cannot yet rule out the possibility that there are additional mechanisms contributing to the DHT-mediated increase in cyclin D proteins, with these mechanisms being dependent on a basal level of mTOR activity. In any case, our subsequent studies focused on the mechanism of androgen-stimulated mTOR activation.

mTOR activation by DHT is not mediated through activation of the PI3K/Akt pathways. We next determined whether mTOR activation by DHT was mediated through activation of Akt. Consistent with PTEN loss and activation of PI3K signaling, LNCaP cells cultured in medium with 10% CDS-FBS had high levels of phospho-Akt that could be markedly decreased by treatment with a PI3K antagonist (LY294002; Fig. 4*A*). However, there was no clear increase in Akt phosphorylation after DHT stimulation. To further address whether DHT may be stimulating a subtle increase in Akt, we also examined tuberin phosphorylation at Akt target sites (Ser^{939} and Thr^{1462}; refs. 26, 27, 29). DHT weakly increased tuberin phosphorylation at Ser^{939} (with no clear effect on Thr^{1462}), but this occurred at early time points (1-4 hours) and was not sustained at later times when mTOR activation was observed (Fig. 4*A*; data not shown). As expected, LY294002 treatment caused a marked decline in tuberin phosphorylation.

Finally, we carried out DHT dose-response experiments to determine whether the weak early DHT-stimulated increase in tuberin phosphorylation correlated with the later mTOR activation. Significantly, although the proliferative response to DHT is biphasic, DHT-stimulated mTOR activation is not biphasic, with DHT at 10 and 100 nmol/L stimulating equal or greater phosphorylation of p70S6K and S6 than 1 nmol/L DHT (Fig. 4*B*). In contrast, tuberin phosphorylation was not enhanced by the increased concentration of DHT at early or later time points (Fig. 4*B*). These results indicate that the modest early increase in tuberin phosphorylation may reflect the previously reported rapid stimulation of PI3K/Akt activity by AR but that an alternative Akt-independent mechanism mediates the robust mTOR activation observed 16 to 24 hours after DHT stimulation.

mTOR activation by DHT is not mediated through Rsk or AMPK. The AR and other steroid receptors may also mediate rapid nongenomic activation of the Ras/Raf/Erk1 and Erk2 MAPK pathway. Recent studies have shown that this pathway can inactivate tuberin via activation of p90 Rsk, which phosphorylates tuberin on a site distinct from the Akt target sites (31). To determine whether DHT stimulation of mTOR may be mediated by this pathway, we first examined Erk1 and Erk2 activation. Whereas DHT treatment of LNCaP cells cultured in serum-free medium may rapidly stimulate Erk1 and Erk2 (data not shown), this was not observed in medium containing 10% serum (CDS-FBS medium; Fig. 5). Moreover, immunoblotting with phosphospecific antibodies recognizing activated Rsk ($pSer^{380}$) similarly indicated that Rsk was not activated by DHT (32). As a positive control, serum stimulation of serum-starved LNCaP cells could stimulate both Erk1/Erk2 and Rsk.

Finally, we determined whether DHT had an effect on AMPK. AMPK phosphorylates tuberin on a distinct site that enhances its ability to suppress mTOR, so a decrease in AMPK activity can enhance mTOR (33). However, DHT treatment did not cause a decrease in AMPK activity as assessed using a phosphospecific antibody against the activated protein (Fig. 5). As a positive control, AMPK could be inhibited by treatment with compound C. Taken together, these data and the results above indicated that AR was stimulating mTOR by a mechanism that was independent of Akt, Rsk, and AMPK.

DHT stimulation of mTOR is dependent on new mRNA synthesis. The delayed activation of mTOR (~ 8 hours after DHT)

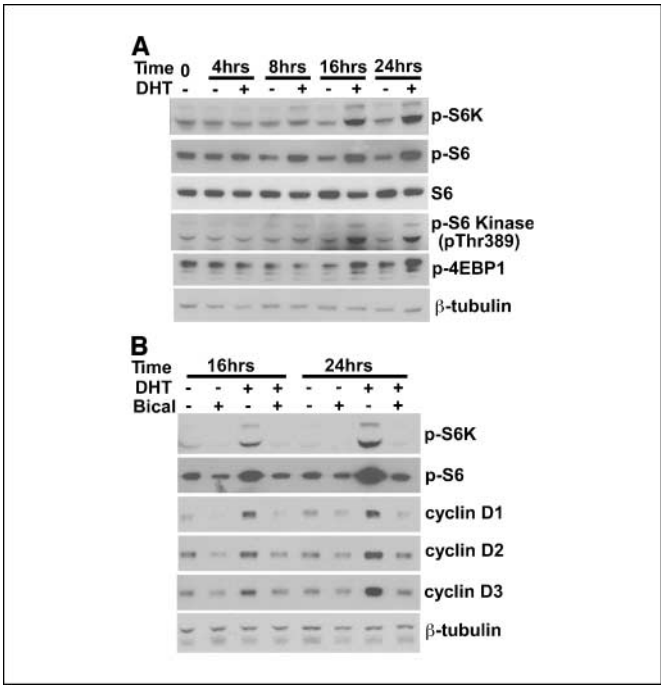

Figure 2. AR signaling increases mTOR activation. *A,* LNCaP cells were cultured in 10% CDS-FBS medium for 3 days and then stimulated with 1 nmol/L DHT. Lysates containing equal amounts of protein were immunoblotted for phospho-p70S6K (Thr^{421}/Ser^{424}; *p-S6K*), phospho-S6 (Ser^{235}/Ser^{236}; *p-S6*), total S6 (*S6*), phospho-p70S6K [Thr^{389}; *p-S6 Kinase (pThr^{389})*], and phospho-4E-BP1 (*p-4E-BP1*). The top band in the phospho-p70S6K blot is consistent with an isoform (p85 S6 kinase) that is identical to p70S6K except for 23 extra residues at the NH_2 terminal that encode a nuclear localization signal. β-Tubulin was used to confirm equal protein loading. *B,* LNCaP cells cultured in medium with 10% CDS-FBS for 3 days were stimulated with vehicle, DHT (1 nmol/L), and/or bicalutamide (*Bical*; 10 μmol/L) for various times, and lysates were immunoblotted for phospho-p70S6K (Thr^{421}/Ser^{424}), phospho-S6 (Ser^{235}/Ser^{236}), and β-tubulin.

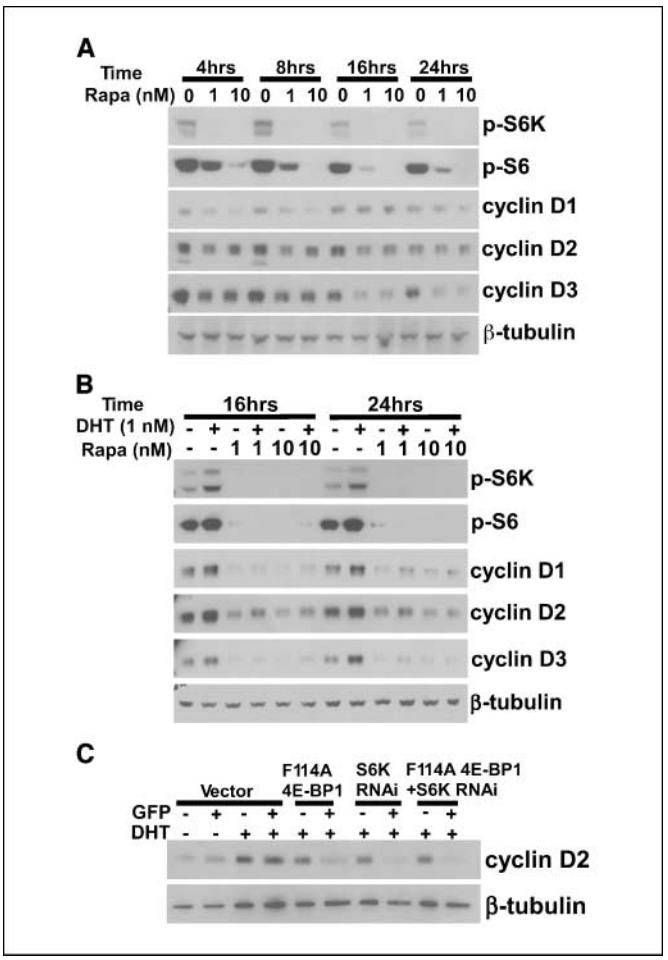

Figure 3. mTOR activation stimulates the expression of cyclin D proteins in LNCaP cells. *A,* LNCaP cells were cultured in medium with 10% FBS for 2 days and then treated with vehicle or 1 or 10 nmol/L rapamycin (*Rapa*) for various times. Lysates were immunoblotted for phospho-p70S6K (Thr^{421}/Ser^{424}), phospho-S6 (Ser^{235}/Ser^{236}), cyclin D1, D2, and D3, and β-tubulin. *B,* LNCaP cells cultured in medium with 10% CDS-FBS for 3 days were stimulated with vehicle, DHT (1 nmol/L), and/or rapamycin (1 or 10 nmol/L) for various times and cell lysates were then immunoblotted for phospho-p70S6K (Thr^{421}/Ser^{424}), phospho-S6 (Ser^{235}/Ser^{236}), cyclin D1, D2, and D3, and β-tubulin. *C,* LNCaP cells were plated in 10-cm plates in medium with 10% CDS-FBS for 2 days and transiently cotransfected with pIRES2-EGFP vector (4 μg) and the pACTAG2/3HA-F114A-4E-BP1 and/or pBS/U6-S6K1 construct (24 μg total DNA per plate). After 24 hours, the cells were treated for 24 hours, trypsinized, and sorted by FACS into GFP-positive and GFP-negative populations. Transfection efficiencies based on GFP expression were ~3%, and ~15,000 GFP-positive cells were collected under each condition. Equal amounts of protein from the GFP-positive and GFP-negative cells were used for immunoblotting of cyclin D2 and β-tubulin on the same blot.

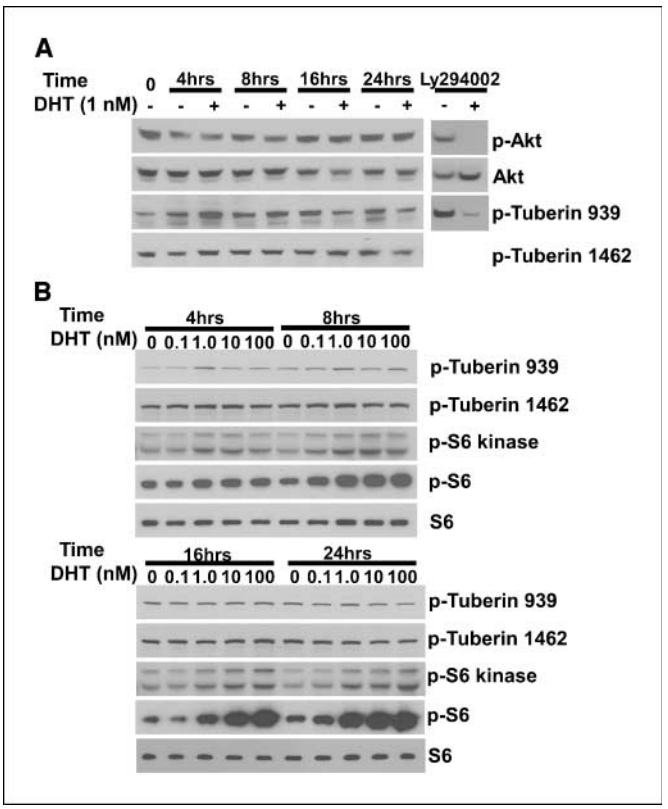

Figure 4. mTOR activation by DHT is not mediated through activation of PI3K/Akt. *A,* LNCaP cells cultured for 3 days in CDS-FBS medium were stimulated with 1 nmol/L DHT and lysates were immunoblotted for phospho-Akt (Ser^{473}), total Akt, phospho-tuberin (Ser^{939}), and phospho-tuberin (Thr^{1462}). A control was LNCaP cells in 10% FBS treated with 10 μmol/L LY294002 for 2 hours. *B,* LNCaP cells cultured in medium with 10% CDS-FBS for 3 days were stimulated with vehicle and various concentration of DHT for different times, and cell lysates were collected and quantified by immunoblotting for phospho-tuberin (Ser^{939}), phospho-tuberin (Thr^{1462}), phospho-p70S6K (Thr^{421}/Ser^{424}), phospho-S6 (Ser^{235}/Ser^{236}), and S6.

was consistent with a requirement for AR stimulation of new mRNA synthesis. Therefore, we determined whether blocking mRNA synthesis with actinomycin D would prevent DHT stimulation of mTOR. Actinomycin D treatment alone caused an increase in mTOR activity, but direct comparisons of cells treated with actinomycin D with or without DHT showed that actinomycin D effectively blocked the DHT-stimulated activation of mTOR (Fig. 6*A*).

As a further control for nonspecific effects of actinomycin D treatment, we determined whether the drug blocked mTOR stimulation in response to amino acids. Previous studies have shown that mTOR activity is strongly regulated by amino acid levels. Although the precise mechanisms by which amino acids regulate mTOR remain to be established, they are nontranscriptional and Akt independent (25). To assess responses to amino acids, LNCaP cells grown in RPMI 1640 with 10% CDS-FBS were supplemented with a concentrated mixture of essential and nonessential amino acids to raise the final concentration by 2- or 3-fold. This caused an increase in mTOR activity within 2 hours (Fig. 6*B*). However, in contrast to mTOR stimulation by DHT,

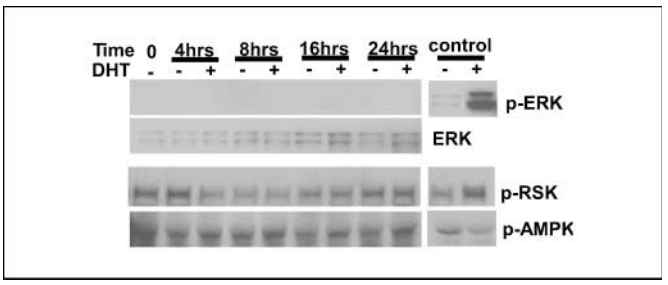

Figure 5. mTOR activation by DHT is not mediated through Rsk or AMPK. LNCaP cells cultured for 3 days in CDS-FBS were stimulated with 1 nmol/L DHT and lysates were immunoblotted for phospho-p44/p42 Erk (Thr^{202}/Tyr^{204}; *p-ERK*), total p44/42 Erk (Erk1 and Erk2; *ERK*), phospho-p90 Rsk (Ser^{380}; *p-RSK*), and phospho-AMPK (Thr^{172}; *p-AMPK*). The control for p-Erk and p-Rsk was lysate from LNCaP cells that were serum stimulated after serum starvation. The control for AMPK was treatment with an inhibitor (compound C, 20 μmol/L) for 2 hours.

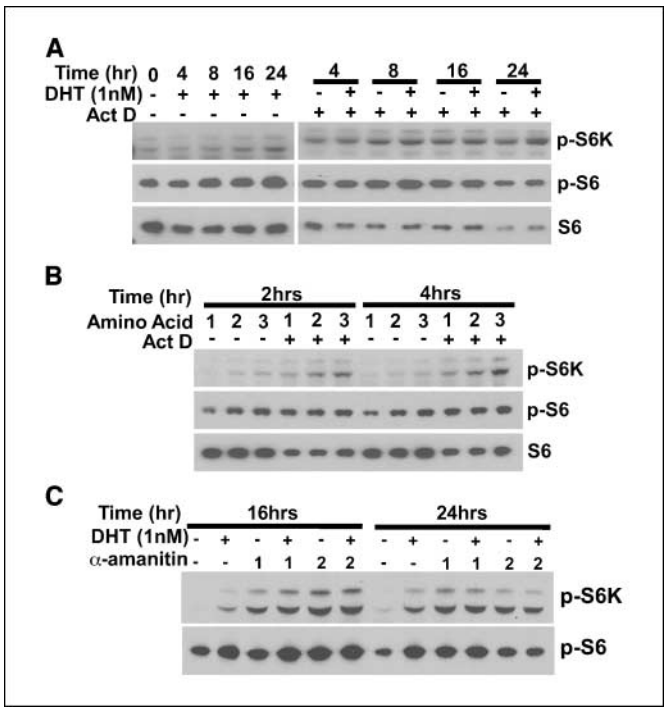

Figure 6. DHT stimulation of mTOR is prevented by actinomycin D. *A,* LNCaP cells cultured in medium with 10% CDS-FBS for 3 days were stimulated with vehicle, 1 nmol/L DHT, and/or 10 μg/mL actinomycin D (*Act D*) for various times, and equal amounts of protein from cell lysates were immunoblotted for phospho-p70S6K (Thr^{421}/Ser^{424}), phospho-S6 (Ser^{235}/Ser^{236}), and total S6. *B,* LNCaP cells cultured in medium with 10% CDS-FBS for 3 days were pretreated with 10 μg/mL actinomycin D for 4 hours and then supplemented to 2× or 3× amino acid levels for various times. Cell lysates were then immunoblotted as above. *C,* LNCaP cells in medium with 10% CDS-FBS were stimulated with vehicle, 1 nmol/L DHT, and/or α-amanitin at 1 or 2 μg/mL for 16 or 24 hours. Equal amounts of protein from cell lysates were then immunoblotted for phospho-p70S6K (Thr^{421}/Ser^{424}) and phospho-S6 (Ser^{235}/Ser^{236}).

mTOR phosphorylation of p70S6K in response to amino acid supplementation was not blocked by actinomycin D, showing that this treatment did not nonspecifically block mTOR activation mediated by a nontranscriptional mechanism (Fig. 6*B*).

Interestingly, although actinomycin D did not block p70S6K phosphorylation in response to amino acids, we did not observe a further increase in the phosphorylation of S6. This may reflect an alternative p70S6K-independent mechanism for increasing S6 phosphorylation by actinomycin D. In any case, the actinomycin D–mediated loss of p70S6K phosphorylation in response to DHT, but not amino acids, indicated that AR transcriptional function was required for the DHT-mediated activation of mTOR.

As an alternative approach to block mRNA synthesis, we also used α-amanitin. Similarly to actinomycin D, treatment with α-amanitin stimulated an increase in p70S6K activity (Fig. 6*C*). However, DHT did not further increase p70S6K activation in the presence of α-amanitin, supporting the conclusion that new mRNA synthesis was required for DHT stimulation of mTOR.

DHT rapidly stimulates expression of multiple genes regulating cellular metabolism. To determine whether there were direct AR transcriptional targets that may mediate mTOR activation, we further examined Affymetrix oligonucleotide microarrays to identify genes whose expression was rapidly stimulated by 1 nmol/L DHT. Consistent with previous studies, androgen treatment stimulated the expression of multiple genes. Table 2 lists those genes whose expression was increased by at least 2-fold within 2 hours of DHT stimulation and remained elevated at 4 to 8 hours. Some of these have been identified previously as androgen-regulated genes, including *C1orf116, SARG, SGK, TMPRSS2, TMEPAI*, and *NKX3.1*, but none have been directly linked to mTOR activation (34–39). However, some of the DHT-induced genes could possibly regulate mTOR through effects on cellular metabolism and nutrient levels. In particular, two solute carrier proteins (*SLC1A3*, a high-affinity aspartate/glutamate transporter, and *SLC01B1*) were strongly induced (Table 2), and multiple additional members of the solute carrier family that transport glucose, amino acids, and other nutrients were also increased to varying extents in response to DHT (Table 3). A previous study also showed that testosterone could increase expression of a high-affinity aspartate transporter in prostate epithelial cells (40).

Further genes rapidly induced by DHT that could possibly contribute to mTOR activation included *SGK, GRWD1*, and *DIO2*. Among other functions, *SGK* can regulate the expression and activities of multiple transport proteins (41). *GRWD1*, a homologue of a yeast protein that mediates ribosome biogenesis, stimulates growth and global protein synthesis in mammalian cells (42). The *type 2 iodothyronine deiodinase* (*DIO2*) enhances *thyroid hormone receptor* (*TR*) activity through conversion of T4 to the more potent T3 and is one of several TR-related genes increased by DHT (Table 3). Significantly, previous studies have shown that thyroid hormone can stimulate proliferation of prostate cancer cells, and recent data support the hypothesis that the regulated expression of deiodinase modulates thyroid hormone action in specific tissues (43–46). Finally, DHT-stimulated increases in multiple insulin-insulin-like growth factor (IGF)-I signaling proteins, which may regulate mTOR activity through the PI3K/Akt pathway in cells with intact PTEN (Table 3).

Role of amino acids in DHT stimulation of mTOR. As mTOR is strongly regulated by intracellular amino acid levels, we next considered whether DHT may be stimulating mTOR by increasing amino acid production or transport (25, 47). Consistent with the hypothesis that DHT stimulates mTOR by increasing amino acid transport, depletion of leucine and glutamine reduced basal mTOR activity and abrogated DHT stimulation of mTOR (Fig. 7*A*). Therefore, we next determined whether DHT treatment would further stimulate mTOR activity when cells were cultured in amino acid–supplemented medium.

LNCaP cells in 10% CDS-FBS medium were supplemented with essential and nonessential amino acids with or without DHT, and mTOR activation was assessed after 2 to 16 hours. Significantly, increasing amino acids from 2- to 3-fold further enhanced mTOR activity at 2 hours but not at later times, indicating that the amino acid supplementation had saturated the intracellular amino acid pools by 4 hours (Fig. 7*B*). Nonetheless, DHT treatment further enhanced mTOR activation in the amino acid–supplemented cells. These results indicated that although amino acid depletion could strongly suppress mTOR activation, mechanisms other than (or in addition to) increasing amino acid levels were mediating DHT stimulation of mTOR.

Finally, we compared the effects of DHT stimulation and amino acid supplementation on cyclin D protein expression. LNCaP cells grown in RPMI 1640 with 10% CDS-FBS were supplemented with a concentrated mixture of essential and nonessential amino acids to raise the final concentration by 2- or 3-fold. As shown above, this caused a rapid increase in mTOR activity (Fig. 7*C*). Significantly, there were concurrent increases in cyclin D1 and increases in cyclin D2 and D3 at 4 to 8 hours. However, these were distinct from the

pattern of increased cyclin D expression induced by DHT (compare with Fig. 1*C*). Taken together, these results indicate that although enhancing amino acid availability may be one mechanism by which androgen stimulates mTOR, other mechanisms also contribute to this response.

Discussion

Previous studies have shown that androgen deprivation causes a G_0-G_1 cell cycle arrest, whereas androgen stimulates proliferation and enhances the expression of multiple G_1-S regulatory proteins (5–15). We similarly found that DHT-stimulated pRb phosphorylation and cell cycle progression within 24 hours and showed a corresponding late increase in cyclin E and A transcripts. DHT also stimulated a small early increase in cyclin D3 message, but there were no increases in cyclin D1 or D2 transcripts. However, there was a marked increase in the expression of all cyclin D proteins at 8 to 16 hours, implicating a post-transcriptional (translational or post-translational) mechanism in DHT-stimulated proliferation.

This post-transcriptional mechanism for increasing cyclin D proteins was found to be mTOR activation. The DHT-stimulated increase in mTOR activity was not mediated by increased PI3K/Akt activation but was dependent on DHT-stimulated gene expression. Significantly, the major function of AR in normal prostate epithelium is to stimulate expression of proteins needed for seminal fluid production. Consistent with the metabolic challenge this imposes, previous expression studies and our Affymetrix microarray results show that androgens also enhance the expression of proteins that increase cellular metabolism (34–39). These observations indicate that a physiologic and critical function of AR is to enhance nutrient availability by the coordinated regulation of multiple metabolic genes. Although these nutrients do not stimulate proliferation in normal prostate epithelium, we propose that they are essential in prostate cancer to support dysregulated cellular proliferation, so that androgen deprivation causes cell cycle arrest essentially as a result of cellular starvation. As decreased nutrient levels cause feedback inhibition of mTOR, the apparent DHT-mediated stimulation of mTOR activity would then reflect loss of feedback inhibition due to repletion of nutrients.

An important feature of the androgen-stimulated proliferative response is that it is biphasic, with higher DHT levels (>1-10 nmol/L) being less effective and strongly suppressing cell growth in some cases (2, 48, 49). However, although DHT-stimulated LNCaP proliferation declined at concentrations above 1 nmol/L, there were no decreases in the activation of mTOR or in cyclin D proteins at higher DHT levels. Significantly, many of the androgen-stimulated

Table 2. Genes stimulated by at least 2-fold after 2, 4, and 8 hours of DHT treatment

Symbol	2 h	4 h	8 h	24 h	Gene description
C1orf116	7.3	8.4	10.1	5.9	*Chromosome 1 open reading frame 116 (SARG)*
SGK	4.9	7	8.1	0.8	*Serum/glucocorticoid-regulated kinase*
TMPRSS2	2.1	6.1	7.7	5.5	*Transmembrane protease, serine 2*
TMEPAI	3.9	4.5	6	3.4	*Transmembrane, prostate androgen induced (STAG1)*
NKX3.1	2.8	4.3	4.4	3.6	*NK3 transcription factor related, locus 1*
C1orf21	2.0	4.3	2.8	0.9	*Proliferation-inducing protein 13*
C16orf3	3.9	4.1	3.8	4.4	*Chromosome 16 open reading frame 3*
SNAI2	2.6	4	5.7	1.4	*Snail homologue 2 (SLUG)*
CHI3L1	2.1	3.9	3.1	1.3	*Chitinase 3-like 1 (cartilage glycoprotein-39)*
SLCO1B1	2.0	3.7	2.3	0.2	*Solute carrier organic anion transporter family, member 1B1*
HIST1H3J	2.8	3.6	4.7	2.8	*Histone 1, H3j*
GPR20	3.3	3.5	2.9	1.3	*G protein-coupled receptor 20*
SULT2A1	2.2	3.2	2.4	1.2	*Sulfotransferase, cytosolic, 2A, DHEA-preferring, member 1*
CASP8	2.1	3.1	2.3	2.8	*Caspase-8, apoptosis-related cysteine protease (FLICE)*
ADRB3	2.4	3	2.8	1.2	*Adrenergic, β3, receptor*
STAT5A	2.5	2.9	2.9	2	*Signal transducer and activator of transcription 5A*
DIO2	2	2.9	2.6	1.1	*Deiodinase, iodothyronine, type II, transcript variant 1*
ASCL1	2.1	2.8	2.2	1.2	*Achaete-scute complex-like 1 (Drosophila)*
PTGER4	2.1	2.7	2.7	1.8	*Prostaglandin E receptor 4 (subtype EP4)*
DPP4	2.3	2.6	2.4	1.6	*Dipeptidylpeptidase 4 (CD26)*
PMAIP1	2.3	2.5	2	1.5	*PMA-induced protein 1 (APR, NOXA)*
SLC1A3	2.9	2.4	2.1	3	*Glial high-affinity glutamate transporter (EEAT1)*
PGDS	2.3	2.2	2.5	1.7	*Prostaglandin D2 synthase, hematopoietic*
CCL16	2.3	2.1	2	0.9	*Chemokine (C-C motif) ligand 16*
ROM1	2	2.1	2.1	1.8	*Retinal outer segment membrane protein 1*
KPNA1	3.4	2	3.4	2.7	*Karyopherin α1 (importin α5)*
DNCLI2	2.7	2	2.5	0.6	*Dynein, cytoplasmic, light intermediate polypeptide 2*
NFKBIA	2.6	2	2.2	1	*Nuclear factor-κB inhibitor, α*
GRWD1	2.3	2	2.5	3.7	*Glutamate-rich WD repeat containing 1*
RAB3B	2.2	2	2	2.9	*RAB3B, member RAS oncogene family*
STARD8	2.1	2	2	1.7	*START domain containing 8*

NOTE: Some highly DHT-stimulated genes, such as PSA, are not included because their induction was <2-fold at 2 hours (PSA was induced 1.9-fold at 2 hours but strongly induced at later times).

Table 3. Groups of related genes stimulated by DHT

Symbol	2 h	4 h	8 h	24 h	Gene description
Solute carrier family-facilitated glucose transport					
SLC2A4	1.6	4.2	2.4	1.7	*Family 2 (facilitated glucose transporter), member 4*
SLC2A5	1.3	1.6	1.3	1.1	*Family 2 (facilitated glucose/fructose transporter), member 5*
SLC5A4	1.4	1.2	2.4	1.9	*Family 5 (low-affinity glucose cotransporter), member 4*
SLC2A1	1.1	1.4	1.2	1.1	*Family 2 (facilitated glucose transporter), member 1*
SLC2A9	1.1	1.3	1.2	1.0	*Family 2 (facilitated glucose transporter), member 9*
Solute carrier family-amino acids and other ions					
SLC1A3	2.9	2.4	2.1	3.0	*Glial high-affinity glutamate transporter (EEAT1)*
SLCO1B1	2.0	3.7	2.3	0.2	*Organic anion transporter family, member 1B1*
SLC22A16	1.9	4.0	2.0	1.3	*Organic cation transporter*
SLC3A2	1.7	1.4	1.3	1.0	*Dibasic and neutral amino acid transporter*
SLC16A6	1.5	3.2	3.7	2.9	*Monocarboxylic acid transporter*
SLC19A2	1.5	2.0	2.1	1.5	*Thiamine transporter*
SLC11A2	1.5	1.4	2.0	1.9	*Proton-coupled divalent metal ion transporter*
SLC25A16	1.5	1.8	1.5	1.5	*Mitochondrial solute carrier protein*
SLC16A7	1.2	1.4	1.8	2.2	*Monocarboxylic acid transporter*
SLC25A37	1.1	1.5	1.8	1.9	*Mitochondrial solute carrier protein*
SLC7A7	1.1	1.4	1.2	1.0	*Cationic amino acid transporter*
SLC32A1	0.9	1.3	1.2	1.2	*γ-Aminobutyric acid vesicular transporter*
SLC7A9	0.8	1.3	1.0	0.8	*Cationic amino acid transporter*
SLC7A6	0.9	1.3	1.5	1.2	*Cationic amino acid transporter*
SLC1A4	0.9	1.3	1.2	1.0	*Glutamate/neutral amino acid transporter*
SLC1A4	0.8	1.3	1.1	1.0	*Glutamate/neutral amino acid transporter*
MCFP	0.7	1.4	1.3	1.3	*Mitochondrial carrier family protein*
SLC7A8	0.6	1.5	1.3	1.5	*Cationic amino acid transporter*
SLC4A7	0.4	1.4	1.3	1.6	*Sodium bicarbonate cotransporter*
Insulin-IGF signaling					
IGFBP7	2.5	1.8	0.5	1.6	*Insulin-like growth factor–binding protein 7*
IRS2	1.8	2.2	2.3	1.1	*Insulin receptor substrate 2*
IRS4	1.9	1.6	1.7	1.4	*Insulin receptor substrate 4*
IGF1	1.2	2.0	1.8	1.2	*Insulin-like growth factor-I*
IGF1R	1.1	1.8	2.4	1.6	*Insulin-like growth factor-I receptor*
INSR	1.0	1.4	0.9	0.9	*Insulin receptor*
IGFBP5	0.9	1.9	1.7	1.0	*Insulin-like growth factor–binding protein 5*
IGFBP2	0.8	1.3	1.3	1.8	*Insulin-like growth factor–binding protein 2*
Thyroid hormone signaling					
DIO2	2.0	2.9	2.6	1.1	*Deiodinase, iodothyronine, type II, transcript variant 1*
FOXE1	1.6	2.0	2.7	1.8	*Forkhead box E1 (thyroid transcription factor 2)*
THRAP5	1.4	2.0	1.8	1.3	*Thyroid hormone receptor–associated protein 5*
TRIP13	1.0	1.0	1.2	2.0	*Thyroid hormone receptor interactor 13*

genes regulate cellular differentiation and some, such as *NKX3.1*, can clearly function to suppress cell growth. Similarly to mTOR activation, higher levels of DHT do not suppress these genes and can further enhance their expression (data not shown). These observations indicate that the biphasic proliferative response may be due to a dominant effect of mTOR activation and cyclin D protein expression at lower DHT concentrations ($\sim$1 nmol/L), with higher DHT concentrations failing to further increase cyclin D proteins and instead repressing proliferation by further inducing the expression of genes driving differentiation. Previous studies have similarly suggested that the biological function of increased DHT generated by 5α-reductase in prostate is to drive terminal differentiation of the epithelium (48, 49).

In summary, these data in conjunction with previous studies indicate that AR can function by several mechanisms to stimulate or support prostate cancer proliferation. The rapid nontranscriptional activation of PI3K and/or other kinases seems to mediate androgen stimulation of oocyte maturation and proliferation of fibroblasts expressing low levels of AR, but the physiologic importance of this mechanism in prostate cancer (particularly in PTEN-deficient tumors) is uncertain (17, 50). Androgen-stimulated expression of genes mediating insulin-IGF signaling may enhance responses to these hormones in prostate cancer cells with intact PTEN, but the extent to which responses to these hormones can be further increased in PTEN-deficient cells is not clear. AR may also directly contribute to the transcription of some cell cycle regulatory genes, including cyclin D3. However, the major mechanism by which androgen stimulates proliferation in PTEN-deficient LNCaP cells is through mTOR activation, which may reflect a coordinated increase in the expression of multiple genes that enhance nutrient availability. Importantly, this coordinated gene expression orchestrated by the AR may be very difficult for cells to achieve by AR-independent mechanisms, which may explain why AR transcriptional activity seems to remain essential in tumors that

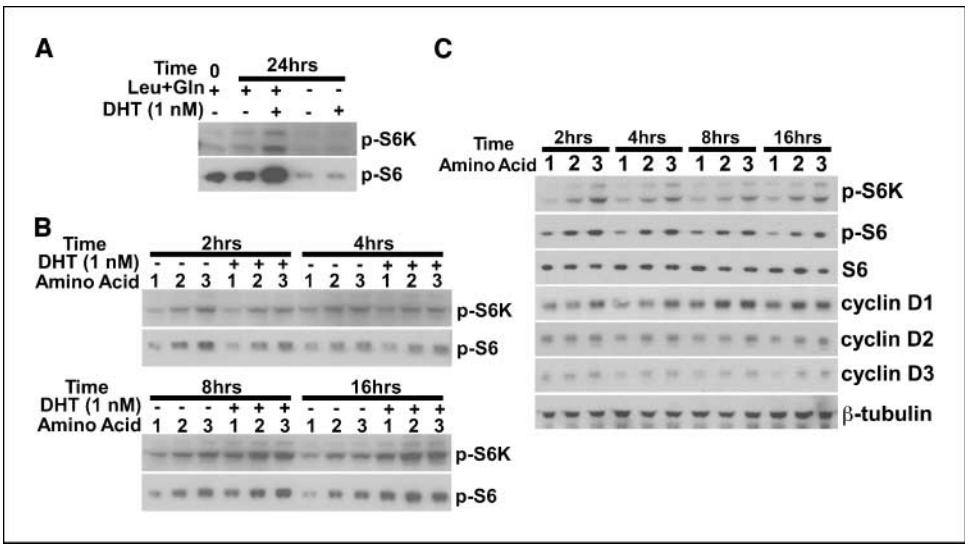

Figure 7. Role of amino acids in mTOR stimulation by DHT. *A,* LNCaP cells were cultured in medium with 10% CDS-FBS for 3 days and then switched to RPMI 1640 ± leucine and glutamine (*Leu+Gln*) with 10% CDS-FBS ± DHT (1 nmol/L) for 24 hours. Equal amounts of protein from cell lysates were immunoblotted for phospho-p70S6K (Thr^{421}/Ser^{424}) and phospho-S6 (Ser^{235}/Ser^{236}). *B,* LNCaP cells cultured in RPMI 1640 with 10% CDS-FBS for 3 days were supplemented with amino acids to yield double (*2*) or triple (*3*) the basal level (*1*) ± DHT for 2 to 16 hours. Cell lysates were immunoblotted as in (*A*). *C,* LNCaP cells cultured in RPMI 1640 with 10% CDS-FBS for 3 days were supplemented with amino acids to double (*2*) or triple (*3*) the basal levels (*1*). Cell lysates collected at the indicated times were then immunoblotted for phospho-p70S6K (Thr^{421}/Ser^{424}), phospho-S6 (Ser^{235}/Ser^{236}), total S6, cyclin D1, D2, and D3, and tubulin.

relapse after androgen deprivation therapy (51, 52). Nonetheless, progression to androgen independence may still in part reflect AR-independent activation of one or more metabolic genes, resulting in a decreased requirement for AR-stimulated nutritional support. In either case, identification of the AR-regulated genes that are most critical for nutritional support remains as an important objective, as they would represent potential new targets for prostate cancer therapy.

Acknowledgments

Received 12/14/2005; revised 5/5/2006; accepted 6/2/2006.

Grant support: NIH, Department of Defense, Dana-Farber/Harvard Cancer Center Specialized Programs of Research Excellence in Prostate Cancer, and Hershey Family Prostate Cancer Research Fund.

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We thank Drs. Phil Febbo for assistance with microarrays, John Blenis for plasmids, and Glenn Bubley for helpful suggestions and review of the article.

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Cancer Res 2006;66:7783-7792.