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Androgen Stimulated Cellular Proliferation in the Human Prostate Cancer Cell Line LNCaP is Associated With Reduced Retinoblastoma Protein Expression

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Abstract To elucidate the mechanism of androgen-dependent cellular proliferation in prostate cancer, androgen-dependent alterations of individual cell cycle regulatory proteins in the androgen-sensitive prostate cancer cell line LNCaP were evaluated. LNCaP cells were deprived of androgens by culture in steroid-depleted media for 5 days, which resulted in the maximal accumulation of cells in G_0/G_1 phase of the cell cycle. The mitogenic concentration of the synthetic androgen R1881 was established as 0.1 nM using cell proliferation assay. Protein and mRNA levels of particular cyclins, cyclin-dependent kinases (Cdks), cyclin-dependent kinase inhibitors (Ckis), and the retinoblastoma proteins (Rb) were assessed. Androgen stimulation resulted in a post-transcriptional reduction in Rb protein levels, an increase in Rb phosphorylation at serine 780 and an accumulation of high molecular weight Rb protein species. Androgen stimulation also induced the expression of the Cdk2 and Cdk1 as well as their regulatory partners, cyclin A and cyclin B, resulting in a corresponding increase in cyclin A/Cdk2 activity in vitro. Pulse-chase showed decreased Rb protein stability in androgen-treated LNCaP cells. Collectively, our findings suggest a novel mechanism of androgen-dependent prostate cancer growth in which androgen stimulation results in decreased Rb protein expression in LNCaP cells. The observation of decreased Rb protein stability in the setting of increased phosphorylation supports the concept of phosphorylation mediated protein degradation. We propose that the observed reduction in Rb protein level occurs through Rb degradation via the ubiquitin/proteasome pathway, and is preceded by selective Rb phosphorylation by cyclin A/Cdk2 and cyclin B/Cdk1. J. Cell. Biochem. 84: 188–200, 2002.

Key words: retinoblastoma protein; androgen; prostate cancer; cell cycle; ubiquitination; 26S proteasome

Androgens transduce their cellular signals through the androgen receptor (AR), a ligand-regulated transcription factor. Upon ligand binding, AR translocates to the cell nucleus, and activates or represses target gene expression [Jenster et al., 1991, 1993; Kemppainen et al., 1992; Chang et al., 1995]. This gene activation or repression likely results in the cell-specific effects of androgens such as proliferation in prostate cancer [Gordon et al., 1995]. In the case of prostate cancer, the AR target genes ultimately transducing the androgen mitogenic signal have not been identified.

Regulation of prostate development and normal epithelial cell proliferation occurs via a paracrine pathway initiated by androgen signaling in the AR-expressing stromal cells [Cunha and Donjacour, 1987, 1989; Hayward et al., 1997, 1998]. In contrast, androgen-stimulated proliferation of prostate cancer appears to be initiated by AR directly, within the epithelial cells themselves [Gao and Isaacs, 1998].

Clinical and epidemiological evidence implicates androgens in the development and

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progression of prostate cancer [Franksson, 1966; Huggins, 1967; Huggins and Hodges, 1972; Noble, 1977a,b; Bosland et al., 1995; Bosland, 1996; McCormick et al., 1998]. The mechanisms by which androgens exert this mitogenic effect on prostate cancer are indeed poorly understood. At the time of diagnosis, the majority of prostate cancers are androgen dependent for growth, and upon removal of androgens by surgical or pharmacologic means, up to 85% of tumors demonstrate complete regression [Crawford, 1990; Daneshgari and Crawford, 1993]. Inevitably, the regression is of limited durability, and patients progress to a hormone-refractory stage.

Mitogenic signaling by extracellular messengers is generally mediated through effects on cell cycle regulators of the G_1 to S transition. Cell cycle progression is regulated through the expression, modification, and degradation of cyclins and their catalytic partners the cyclin-dependent kinases (Cdks) [Nasmyth, 1996]. A family of Cdk inhibitory proteins (Ckis) binds to cyclin-Cdk complexes and inhibits their catalytic activity, ultimately impeding cell cycle progression [Hengst and Reed, 1996; Hengst et al., 1998; Niculescu et al., 1998]. Targets of cyclin–Cdk action include a family of 'pocket proteins', p107, p130, and the retinoblastoma gene product (Rb), all of which are inactivated by Cdk phosphorylation during the G_1 phase of the cell cycle [Hatakeyama and Weinberg, 1995; Mulligan and Jacks, 1998]. Down-regulation of specific G_1 kinases or inhibition of their activity by Ckis results in Rb hypophosphorylation, assembly of a repressor complex involving Rb and a histone deacetylase, which, is in turn, tethered to the promoters of cellular genes required for DNA replication and cell division through a heterodimeric transcriptional activator comprised of E2F and DP1 [Brehm et al., 1999]. Conversely, phosphorylation of Rb by the Cdks dissociates the histone deacetylase repressor complex, resulting in an open chromatin structure and activation of genes involved in the G_1 to S-phase transition. Various members of the cyclin, Cdk families are active at specific points within the cell cycle. The collective activity of such members is to maintain the phosphorylation of Rb protein, thereby driving the cell cycle through individual restriction points.

To delineate the growth signaling pathway of androgens in prostate cancer, we studied the effect of mitogenic concentrations of androgen on the expression of individual cell cycle regulatory proteins in the androgen-responsive prostate cancer cell line, LNCaP. Our results suggest a novel mechanism of growth regulation in which specific Rb phosphorylation events result in increased Rb protein degradation, thereby facilitating cellular proliferation.

MATERIALS AND METHODS

Cell Lines and Treatments

Human prostate cancer cell lines LNCaP were obtained from the ATCC and maintained in RPMI-1640 (Life Technologies) supplemented with 10% fetal bovine serum (FBS; HyClone), 50 U/ml of each Penicillin and Streptomycin, and 2 mM L-Glutamine (Life Technologies). To examine the effects of AR activation on cellular proliferation, protein expression, and steady state mRNA levels, cells were cultured in RPMI (phenol-red free) and 10% charcoal-treated FBS(cFBS) in the absence or presence of the synthetic androgen, R1881 (New England Nuclear) dissolved in 100% ethanol, or an equal volume of 100% ethanol for the indicated times.

Cell Proliferation Assays and Flow Cytometry

LNCaP cells were seeded into 6-well plates (15,000 cells/well) on Day 0 and cultured in RPMI (phenol red free) supplemented with 10% FBS charcoal-treated FBS containing either R1881 or the ethanol vehicle. On indicated days, cells were trypsinized, resuspended in DMEM, stained by the trypan blue exclusion method, and counted using a hemocytometer.

Cell cycle distribution was assessed using flow cytometry analysis. On indicated days, 1.0×10^6 of cells were trypsinized, pelleted, resuspended in 0.5 ml of 1% FBS in phosphate-buffered saline (PBS) and fixed by adding 5 ml of ethanol (-20°C; 80%) dropwise with continuous vortexing. After overnight incubation at 4°C, cells were pelleted and resuspended in 0.75 ml of 1% FBS–PBS. The DNA was stained by adding 0.25 ml of 4× propidium iodide solution (200 μg/ml propidium iodide in 38 mM sodium citrate) followed by a 2 h incubation at 37°C in the presence of 100 μg/ml RNAse A. Nuclear emitted fluorescence was measured with a FACScan flow cytometer and the percentage of cells in each phase of the cell

cycle was determined using the "Modfit" computer program.

Western Blotting

To analyze changes in protein expression, LNCaP cells were cultured in steroid-depleted media for 3–5 days and then treated with R1881 for 72 h, harvested in PBS and lysed in 25–100 μl of the lysis buffer (150 mM NaCl, 50 mM HEPES pH 7.5, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM NaF, 25 μM $ZnCl_2$) supplemented with protease inhibitors 1 mM phenylmethylsulfonylfluoride (PMSF; Sigma), and 1 μg/ml each of aprotinin, pepstatin A and leupeptin (Boehringer Mannheim) for 15 min on ice. Cell lysates were clarified by centrifugation (10,000*g* for 15 min at 4°C), total protein concentration adjusted with the lysis buffer and samples were boiled in an equal volume of 2 × SDS–sample buffer. For Western blotting, protein extracts were separated on 10% SDS–polyacrylamide gels, transferred to Immobilon paper (Millipore Corp.) and probed with rabbit polyclonal antibody against Rb (sc-50), a mouse monoclonal against Cyclin E (sc-247, Santa Cruz Biotechnology, Inc.) and Cyclin A (h-432, Santa Cruz Biotechnology), Cyclin B1 (C23420), Cyclin D1, Cdk1(C12720), Cdk2 (C18520), Cdk4 (C18720), $p27^{Kip1}$ (K25020), $p21^{Cip1}$ (C24420), p16 (Transduction Laboratories), or rabbit polyclonal antiserum against ERK (sc-94, Santa Cruz Biotechnology, Inc.). Rb phosphorylation site specific antibodies were purchased from New England Biolabs. The blots were developed using horseradish peroxidase-coupled goat anti-mouse or donkey anti-rabbit antibodies (Kierkegard-Perry Labs) and the enhanced chemoluminescence (ECL) substrate (Amersham Pharmacia Biotech) as per the manufacturer's instructions.

Northern Blotting

Cells were cultured in 100 mm dishes for indicated periods of time with appropriate treatments (see "Figure legends"), the media aspirated and cells lysed directly on the dishes by adding 3 ml/dish of Ultraspec RNA reagent (BIOTEXC Laboratories). Total RNA was isolated from cell homogenates as per the manufacturer's instructions, denatured at 65°C for 15 min, chilled on ice, and separated on a 1.2% agarose–6% formaldehyde denaturing gel (5–8 μg RNA/lane). Equivalent loading was verified by ethidium bromide staining of ribosomal RNA. RNA was transferred to "Duralon" (Stratagene), UV-crosslinked to the membrane and hybridized to a cDNA probe using QuikHyb hybridization mix (Stratagene) as described by the manufacturer. cDNA fragments coding for human Rb, cyclin A, cyclin B1, Ckd1 and Cdk2 were labeled with (γ-^{32}P) dCTP using RediPrime random priming labeling kit (Amersham Pharmacia Biotech) using the manufacturer's instructions. Blots were washed and exposed to Kodak BioMax film at −80°C for autoradiography.

Immune Complex Kinase Assay

The cyclin A/Cdk2 complex was immunoprecipitated from LNCaP cells (1×10^7 cells) for 1 h on ice with 5 μg of a monoclonal antibody against cyclin A (h-432, Santa Cruz Biotechnology). Immune complexes were immobilized on Protein A/G agarose beads (Santa Cruz Biotechnology) for 1.5 h at 4°C, washed three times in 1 ml of lysis buffer (described above), once with 1 ml of lysis buffer without Triton X-100 and once with DK buffer (50 mM potassium phosphate, pH 7.15/10 mM $MgCl_2$/5 mM NaF/4.5 mM DTT). The histone H1 substrate (approximately 10 μg in 100 μl) was added to the immobilized kinase complex, the kinase reaction was initiated by adding 25 μM ATP, 10 mM $MgCl_2$, 1 mM DTT and (γ-^{32}P) ATP (100 μCi) in a total volume of 300 μl and allowed to proceed for 30 min at RT with continuous shaking. The reaction was terminated by the addition of equal volume of 2 × SDS–sample buffer, placed at 100°C for 3 min and fractionated on 10% SDS–PAGE. The gels were stained with Coomassie Blue, dried, and the phosphorylation of substrate was examined by autoradiography.

Pulse-chase. LNCaP (passage 23; 1×10^6) cells were plated into 60 mm^2 plates. After 24 h, the media was replaced with phenol red free RPMI 1640 media (Life Technologies, Inc.) supplemented with 10% cFBS (charcoal stripped serum). After 72 h of androgen deprivation, cells were treated with 0 nM or 0.1 nM R1881 (NEN Life Science Products, Inc.) for an additional 72 h. To deplete cells of endogenous methionine, cells were washed two times with methionine free RPMI 1640 media supplemented with 5% dialyzed FBS (Life Technologies, Inc.) and 1% penicillin-streptomycin and then incubated for 15 min (37°C; 5% CO_2) in methionine free media. Cells were incubated for 2 h at 37°C in short term labeling media,

consisting of methionine free RPMI 1640, 0 nM or 0.1 nM R1881, and S^{35} methionine (0.1 mCi/ml). Unincorporated S^{35} methionine was washed away with phenol red free RPMI 1640 with 10% cFBS. Cells were then cultured in the presence or absence of androgens for an additional 6, 12, 24, and 36 h. Protein extraction was carried out as above. Lysates were incubated in 3 μg of a polyclonal antibody against Rb (c-15; Santa Cruz Biotechnology) for 1 h at 4°C.

Immunoprecipitation was carried out as above, and an equivalent volume of 2 × SDS–sample buffer was added to each lysate. Bound proteins were eluted from the Protein A/G beads and separated on a 7.5% SDS–polyacrylamide gel. The gel was then dried and exposed to a Phosphoimaging cassette (Molecular Dynamics) and Rb protein levels were quantitated using ImageQuant software. Statistical significance between the conditions was determined by one-way ANOVA *t*-test using JMP software (SAS, Cary, NC). Standard error of the mean was calculated for each point, generated as a mean of repetitive experiments.

RESULTS

Effect of Androgen Stimulation and Depletion on LNCaP Proliferation

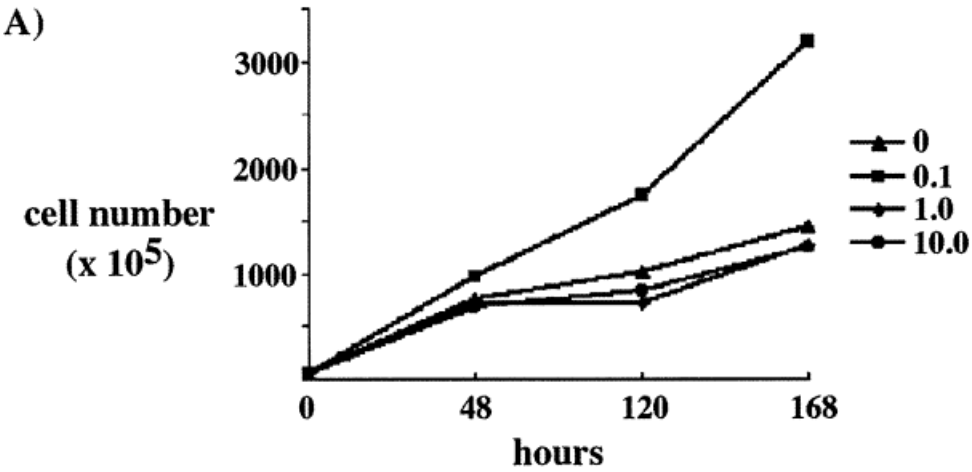
To identify the concentration of androgen optimal for growth stimulation, LNCaP cells were treated with increasing concentrations of the synthetic androgen R1881 following androgen depletion. Treatment of steroid-depleted LNCaP cells with 0.1 nM R1881 resulted in an approximately 2-fold increase in cell number relative to untreated cells (Fig. 1A). Higher doses of R1881 did not induce a mitogenic response, with the proliferation profile resembling those of non-stimulated cells. Our results are consistent with previous findings demonstrating that androgen stimulation of LNCaP cells is biphasic with respect to the dose of androgen used [Lee et al., 1995]. While the mechanism of this dose-dependent response to androgen is unclear, our findings indicate that stimulation of LNCaP cells with 0.1 nM R1881 results in a proliferative response. This suggests that stimulation of LNCaP cells with this concentration of androgen will result in selective expression of those AR target genes involved in androgen mitogenic signaling.

Prior to examining the effects of androgens on individual cell cycle regulators, we determined the cell cycle distribution of LNCaP cells cultured in media depleted of steroids by flow cytometry. In doing so, we were able to demonstrate that androgen stimulation not only results in increased LNCaP proliferation, but also that androgen withdrawal results in decreased proliferation and an accumulation of cells in G_0/G_1 phase. Table I demonstrates that androgen depletion of LNCaP cells results in the accumulation of cells in the G_1 phase of the cell cycle with a simultaneous reduction in the fraction of cells in the S-phase. LNCaP cells start to accumulate in G_1 within 24 h of androgen deprivation. The percentage of cells in G_1 continues to increase over the next 5 days (compare 70% of cells in G_1 in non-depleted media to 81, 85, and 90% in steroid-depleted media at days 1, 3, and 5, respectively). The proportion of cells in S-phase progressively decreases from 17% in non-depleted media to 3% by Day 5 of androgen depletion, whereas the percentage of cells undergoing mitosis declines from 13 to 7%. These results indicate that androgen deprivation of LNCaP cells results in an accumulation of cells in the G_0/G_1 phase of the cell cycle, and further demonstrates that androgen depletion can serve as a means of G_1 cell cycle synchronization in this cell line.

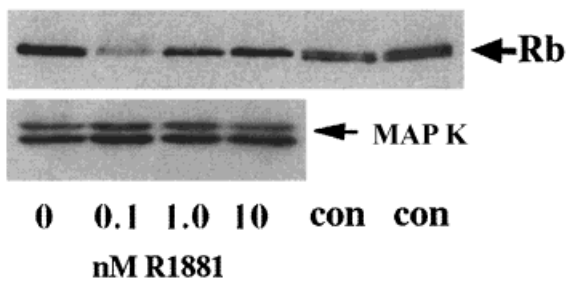
Effect of Androgen Stimulation on the Retinoblastoma Gene Product in LNCaP Cells

LNCaP cells were deprived of androgen for 5 days (described above) and were then cultured for 3 days in the absence or presence of 0.1, 1, and 10 nM R1881. Whole cell extracts were prepared and subjected to immunoblotting with a polyclonal antibody against Rb. Treatment of LNCaP cells with a mitogenic dose of androgen of 0.1 nM R1881 markedly reduced the steady-state Rb protein levels (Fig. 1B; Upper Panel). In contrast, treatment with non-mitogenic concentrations of R1881 (1 and 10 nM) resulted in Rb expression comparable to non-treated cells, consistent with the concentration-dependent effect on cellular proliferation. The polyclonal Rb-specific antibody used in these experiments recognizes both the hypophosphorylated and hyperphosphorylated forms of Rb, as shown by the doublet seen in the control lane (U2OS cells), suggesting that the loss of Rb immunoreactivity observed upon treatment of LNCaP cells with 0.1 nM R1881 reflects the loss of Rb protein rather than reduced affinity of the antibody to the hyperphosphorylated Rb

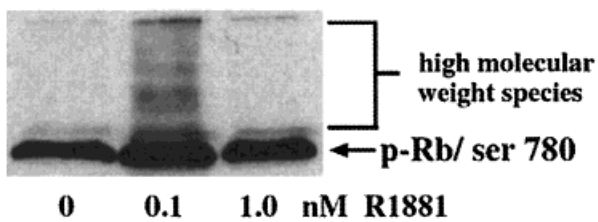
A)


3000
2000
1000
cell number
(x 10^5)
0
48
120
168
hours
0
0.1
1.0
10.0

B)


Rb
MAP K
0 0.1 1.0 10 con con
nM R1881

C)


high molecular
weight species
p-Rb/ ser 780
0 0.1 1.0 nM R1881

D)

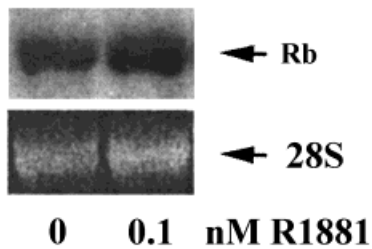

Rb
28S
0 0.1 nM R1881

TABLE I. Changes in Cell Cycle Distribution of LNCaP Cells Undergoing Androgen Withdrawal

Days following androgen withdrawal	Percent fluorescence at cell cycle phase		
	G_0/G_1	S	G_2/M
0	70	17	13
1	81	10	9
3	85	7	8
5	90	3	7

species. No change in MAPK protein expression at any of androgen concentrations utilized is observed (Fig. 1B; Bottom Panel). Likewise, the level of the Rb family member Rb2 (p130) was unaffected by androgen treatment (Fig. 2).

To determine if alterations in Rb phosphorylation accompany the reduction in Rb protein expression observed with androgen-dependent cellular proliferation, we examined Rb phosphorylation in LNCaP cells in the presence and absence of growth stimulatory concentrations of androgen using Rb phosphorylation site-specific antisera. As shown in Figure 1C, an increase in Rb-immunoreactivity is noted in LNCaP cells treated with 0.1 nM R1881 when antiserum recognizing the serine 780 phosphorylated form of Rb is used. A group of proteins with decreased electrophoretic mobility also reacts with the phospho-Rb-serine-780 antibody, suggesting the presence of cross-reacting high molecular weight Rb species within the 0.1 nM treated cells. This result is specific for the serine 780 phosphorylated form of Rb, since other phospho-Rb site-specific antisera that distinguish Rb phosphorylation at residues serine 249/252, threonine 373, serine 795, and serine 807/811, show no increased Rb-immunoreactivity in response to androgen (not shown). Therefore, while the total level of Rb protein, as recognized by a polyclonal Rb antibody, is significantly reduced, specific Rb phosphorylation events are ongoing, resulting in an increase in hyperphosphorylated forms of the protein.

To determine whether the effect of androgen on Rb protein level is a result of a corresponding decrease in the steady-state Rb mRNA levels, total RNA was extracted from LNCaP cells cultured in the presence or absence of 0.1 nM R1881 for 3 days and Northern hybridization was performed. Interestingly, the expression of Rb mRNA was unchanged by androgen treatment, suggesting that the observed androgen-dependent decrease in Rb protein levels is not a function of a reduction in the steady-state mRNA level, but rather reflects post-transcriptional changes in Rb expression (Fig. 1D). These findings also suggest that androgen's mitogenic effect in LNCaP cells may, in part, be mediated through alterations in Rb protein synthesis or degradation.

Androgen Treatment Induces the Expression of Specific Cyclins and Cdks in LNCaP Cells

Since androgen stimulation of LNCaP cells results in alterations in the level of Rb, perhaps as a result of changes in Rb phosphorylation, we next examined the protein levels of the Rb kinases, including the Cdk catalytic subunits

Fig. 1. Cell growth kinetics and expression of Rb androgen-treated LNCaP cells. **A**: Cell growth kinetics of androgen-stimulated LNCaP cells. LNCaP cells were seeded at hour 0 and cultured in steroid-depleted media in the absence or presence of the synthetic androgen R1881 at the indicated concentrations. Total numbers of viable cells were determined by the trypan blue exclusion method at 0, 48, 120, and 168 h. The graph represents one of three independent experiments. Note that androgen-dependent cell proliferation is observed at 0.1 nM R1881. **B**: Decreased Rb expression is associated with androgen-dependent growth stimulation. LNCaP cells were cultured in steroid-depleted media (phenol red-free RPMI-1640 supplemented with 10% charcoal-treated fetal bovine serum) for 72 h and refed with media containing the indicated concentrations of synthetic androgen R1881. After 72 h whole cell extracts were prepared and Rb expression was evaluated. Upon adjustment of the total protein concentration in each sample, the proteins were resolved on 7.5% SDS–PAGE and transferred to Immobilon paper (see Materials and Methods). The blot was probed with a polyclonal antibody against Rb (top panel). The membrane was stripped and probed with anti-MAPK antiserum to control for equal loading (Bottom Panel). A reduction of Rb protein level is observed upon stimulation of LNCaP with 0.1 nM R1881, the growth-stimulatory concentration. The control lanes, representing U2OS cells demonstrate the antibodies' affinity for both hyper- and hypo-phosphorylated forms of Rb protein. **C**: Prepared extracts were then probed with an antibody specific for the serine 780-phosphorylated form of Rb. Note the accumulation of immunoreactive bands of decreased electrophoretic mobility in cells treated with 0.1 nM R1881. **D**: Rb steady state mRNA levels as a function of androgen stimulation. Total RNA was extracted from LNCaP cells in the absence or presence of 0.1 nM R1881. Equal amounts of RNA were separated on denaturing formaldehyde-agarose gels (see Materials and Methods), transferred to Duralon and hybridized to a ^{32}P-labeled-cDNA probe corresponding to Rb (Top Panel). Equal loading for each lane is demonstrated by ethidium bromide staining of 28S rRNA (Bottom Panel). Each panel is representative of at least two independent experiments.

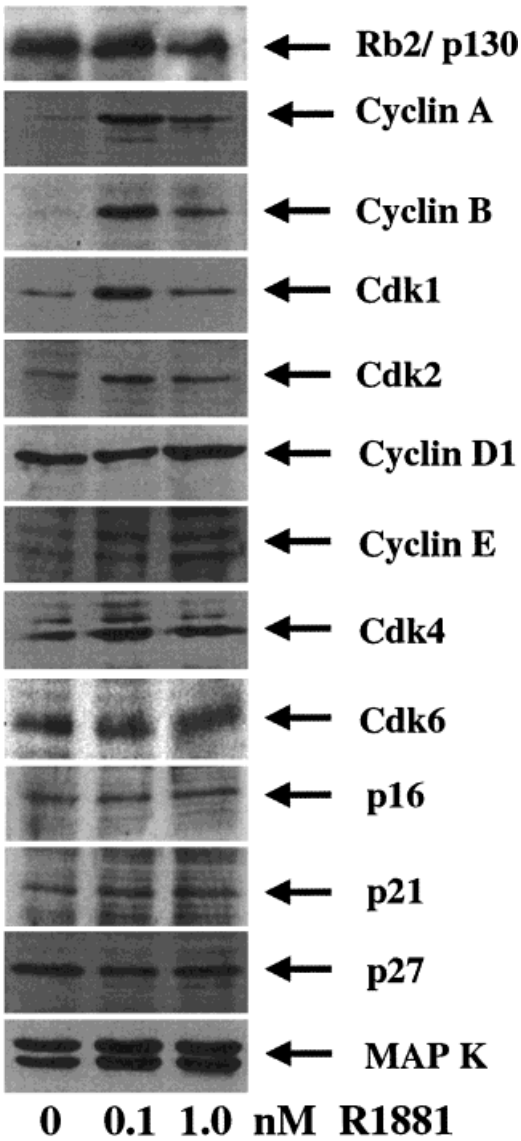


Fig. 2. Expression of cell-cycle regulatory proteins in LNCaP cells. LNCaP cells were cultured in steroid-depleted media, as described in Figure 1, for 5 days to allow maximal androgen depletion, and refed with media containing increasing concentrations of R1881 for 72 h. Whole cell lysates were prepared, total protein concentration adjusted among samples and proteins resolved on 10 or 12.5% SDS–PAGE, transferred to Immobilon and probed with antibodies against Rb2 (p130), cyclin A; cyclin B1; Cdk1; Cdk2; cyclin D1; cyclin E; Cdk4; Cdk6; p16; p21; p27; and MAPK. The antibodies used in each experiment are described in Materials and Methods. Each blot is representative of at least two independent experiments.

Cdk4, Cdk6, Cdk2, their regulatory subunits, cyclin D1, cyclin A, cyclin E, and cyclin B1 as well as the cyclin-dependent kinase inhibitors (Cki's), $p21^{CIP}$, $p27^{KIP}$, and $p16^{INK}$. LNCaP cells were cultured for 3 days in the absence or presence of androgen, then whole cell extracts were prepared and subjected to immunoblot analysis with antibodies against the above mentioned cell cycle regulatory proteins. Figure 2 demonstrates an induction of cyclin A and cyclin B1 as well as an increase in Cdk1 and Cdk2 protein levels upon treatment with 0.1 nM R1881, a mitogenic dose of androgen. Cells treated with 1 nM R1881, which displayed no mitogenic response exhibit a lower level of cyclin A and cyclin B1 expression relative to the growth stimulatory dose of androgen but higher than that of non-treated cells. These findings are particularly provocative in view of the fact that Rb is a potential substrate for the cyclin A/Cdk2 complex in vivo.

Immunoblot analysis of cyclin D1, cyclin E, Cdk4 and Cdk6, as well as p27, p21, and p16 revealed no change in the level of these proteins upon stimulation of LNCaP cells with either 0.1 or 1 nM R1881 (Fig. 2). This is contrary to previous observations of others that the effects of extracellular mitogens are generally mediated through alterations in G_1/S cell cycle regulatory proteins. Thus, our results indicate that cyclin A, cyclin B1, Cdk1 and Cdk2 are induced maximally at an androgen concentration that elicits a proliferative response in LNCaP cells and that this effect appears specific for these cell cycle regulatory proteins.

To determine whether androgen-dependent induction of cyclin A, cyclin B1, Cdk1 and Cdk2 is a result of an increase in their steady-state mRNA levels, Northern hybridization of total RNA extracted from LNCaP cells cultured in the presence or absence of 0.1 nM R1881 was performed. Figure 3 demonstrates that the expression of cyclin A, cyclin B1, and Cdk1 mRNAs was induced by 0.1 nM R1881 treatment, suggesting that the observed androgen-dependent changes in cyclin A, cyclin B, and Cdk1 protein levels likely occur at the level of transcription initiation or mRNA stability. In contrast, no androgen-dependent change in Cdk2 mRNA levels was observed, suggesting that upon androgen stimulation Cdk2 expression is regulated post-transcriptionally, perhaps through a mechanism of increased protein stability.

Catalytic Activity of the Cyclin A/Cdk2 Complex Increases in Response to Androgen Treatment in LNCaP Cells

Cyclin A associates with Cdk2 to form an active kinase in late G_1 and S-phase. Because

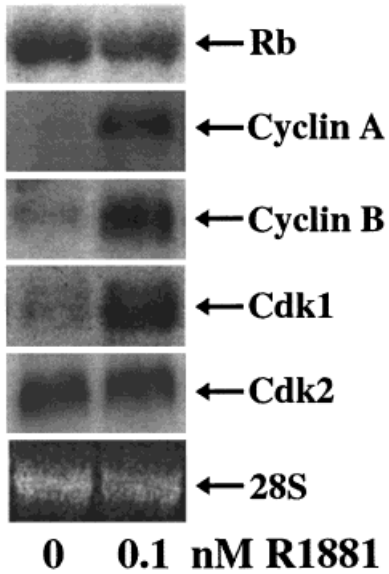


Fig. 3. Changes in steady-state mRNA levels of cell cycle regulators in LNCaP cells. Total RNA was extracted from LNCaP cells treated as in Figure 3. Equal amounts of RNA was separated as described in Figure 2 probed with ^{32}P-labeled cDNAs for Rb; cyclin A; cyclin B; Cdk1; and Cdk2. Equal loading for each lane is demonstrated by ethidium bromide staining of 28S rRNA.

this kinase is known to phosphorylate Rb at unique sites maintaining it in an inactive hyperphosphorylated state [Zarkowska and Mittnacht, 1997], we performed an immune complex kinase assay with cyclin A/Cdk2 complex purified from androgen-treated LNCaP cells to determine if the observed increase in cyclin A and Cdk2 protein resulted in increased kinase activity. Cyclin A/Cdk2 complexes were immunoprecipitated with a cyclin A-specific monoclonal antibody from whole cell extracts of LNCaP cells treated with 0.1 nM R1881 for 3 days or from control androgen-depleted cells and the kinase activity was analyzed in vitro using histone H1 as the substrate. An increase in histone H1 phosphorylation is observed when the cyclin A/Cdk2 complex purified from LNCaP cells is treated with 0.1 nM R1881 compared to non-treated cells (not shown). This increase in cyclin A/Cdk2 activity parallels the increase in protein expression for each component of this kinase complex. These findings indicate that growth stimulatory concentrations of androgen result in greater cyclin A/Cdk2 activity, likely resulting in increased Rb phosphorylation.

Androgens Modulate Rb Protein Half-Life in LNCaP Cells

To determine if the stability of Rb protein was affected by androgen stimulation in LNCaP cells, a pulse-chase experiment was performed. Androgen-depleted LNCaP cells were pulse-labeled with 35S-methionine for 2 h, washed and chased by culturing in media with and without R1881 for 12, 24, 36 h. Rb was immunoprecipitated from cell lysates with anti-Rb antibody, resolved on SDS–PAGE and quantitated. As seen in Figure 4, the pulse-chase experiment shows that the decay of Rb was accelerated in androgen-stimulated cells. Androgen treatment resulted in a 39 and 50% reduction in Rb protein at 24 and 36 h, respectively, as compared to non-stimulated cells. The reduction in Rb protein at 36 h was statistically significant by one-way ANOVA *t*-test ($P < 0.05$). This finding suggests that

A.

Hours	0	6	12	24	36
0 nM R1881	100%	71%	56%	34%	24%
0.1 nM R1881	100%	56%	39%	21%	12%*

* $p < .05$

B.

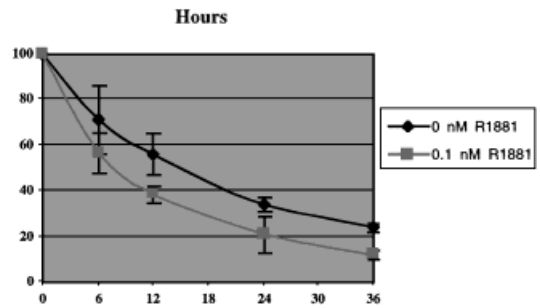


Fig. 4. In vivo Rb protein stability in LNCaP cells. LNCaP cells either treated with 0.1 nM R1881 or androgen depleted for 72 h, were labeled with S^{35} methionine, and washed free of unincorporated S^{35} methionine. Cells were then cultured in the presence or absence of androgens for an additional 6, 12, 24, and 36 h. Protein extraction was carried out, and Rb protein immmunoprecipitated from each extract was separated by SDS–PAGE and exposed to a phosphoimaging cassette (Molecular Dynamics). Rb protein levels were quantitated using ImageQuant software. Statistical significance was determined by ANOVA one way *t*-test using JMP software (SAA, Cary, NC). Error bars are representative of standard error of the mean. Data presented in tabular form (**A**) and in graph form (**B**) as % Rb remaining. The difference in remaining Rb protein in androgen-depleted and androgen-treated cells was determined to be statistically significant ($P < 0.05$).

androgen treatment affects Rb protein stability resulting in a reduction in Rb protein in androgen-treated LNCaP cells.

DISCUSSION

Androgens exert their cellular effects through the AR, which upon ligand binding modulates the activity of selected promoters through transcriptional activation or repression. [Jenster et al., 1991, 1993; Kemppainen et al., 1992; Chang et al., 1995]. In the case of prostate cancer, the cumulative effect of AR activation is cellular proliferation. [Lee et al., 1995]. Unlike the pattern of stromal-epithelial interactive growth noted in BPH, [Cunha and Donjacour, 1987, 1989; Hayward et al., 1997, 1998] uncontrolled proliferation seen in prostate cancer may be morphologically limited to the epithelial compartment. For example, the growth of an androgen-dependent prostate cancer xenograft, PC-82, is supported by exogenous androgens administered to an AR-null mouse carrying AR-null stroma [Gao and Isaacs, 1998]. Such observations suggest that malignant transformation of prostate epithelium leads to an alteration in cellular milieu allowing AR to signal growth in the absence of paracrine stromal factors.

The relevance of delineating the mechanism of AR mediated growth in prostate cancer has tremendous potential impact. A number of studies have demonstrated that AR expression persists or is even increased in a number of hormone-refractory prostate cancers [van der Kwast et al., 1991; Ruizeveld de Winter et al., 1994; Koivisto et al., 1996; Gregory et al., 1998; Sweat et al., 1999]. Evidence exists to support the idea that AR may serve as a mediator of androgen-independent prostate cancer growth by one of several ligand-independent mechanisms [Veldscholte et al., 1990a,b, 1992; Brinkmann and Trapman, 1992; Taplin et al., 1995; Koivisto et al., 1996, 1997; Culig et al., 1994; Nazareth and Weigel, 1996; Tilley et al., 1996; Yeh et al., 1999]. Collectively, these studies support the idea that identification of the targets of AR growth signaling in prostate cancer will reveal novel points of intervention to be exploited in the development of new therapies for controlling not only androgen-dependent prostate cancer, but a large subset of androgen-independent tumors as well.

In this study we compared the expression of a variety of cell cycle regulatory proteins from LNCaP cells depleted of androgen with those treated with a mitogenic dose of androgen. In doing so, we hoped to identify the endpoints of cellular growth signaling responsible for exerting androgen's mitogenic effects. A significant post-transcriptional reduction in Rb protein levels was observed upon treatment of cells with a growth stimulatory dose of androgen. We also observed additional Rb-immunoreactive species with decreased electrophoretic mobility when using a Rb phosphoserine-780 site-specific antibody [Mittnacht, 1998] in cells treated with a growth-stimulatory dose of androgen. The presence of an increase in Rb phosphorylation in the setting of a decrease in total protein levels is quite suggestive of Rb degradation in vivo, as in many cases, protein degradation is preceded by a phosphorylation event. Upon metabolically labeling cells with S^{35} methionine, a more rapid degradation of Rb protein was indeed noted in androgen treated cells, confirming a reduction in protein stability as the likely basis of the observed decrease in Rb protein levels. These results suggest that the mitogenic response of LNCaP cells to androgens is uniquely mediated through the degradation of Rb protein resulting in unregulated cell cycle progression.

Post-transcriptional reduction of Rb protein levels resulting in increased cell growth, represents a potentially novel mechanism of mitogen-induced cellular proliferation. Scant evidence exits for translational control of Rb protein expression [Pedley et al., 1996]. There is, however, precedent for ubiquitin-mediated proteolysis of Rb family members in transformed mammalian cells. Several studies indicate that the HPV E7 gene product is associated with a decrease in Rb protein levels through ubiquitin-mediated proteolysis [Boyer et al., 1996; Jones et al., 1997]. Higashitsuji et al. [2000] recently demonstrated a reduction in Rb protein stability in hepatocellular carcinomas due to overexpression of an oncolytic gene, gankyrin, isolated by subtractive hybridization. The authors postulated, based upon the ubiquitin binding properties of gankyrin, that the observed degradation was mediated by the 26S proteasome [Higashitsuji et al., 2000]. Jang and Choi [1999] demonstrated that p107 protein, a member of the retinoblastoma family, is degraded by the ubiquitin-proteasome

pathway, and that the addition of lactacystin, a specific inhibitor of the 26S proteasome, reverses the observed reduction in p107 levels.

Clinical correlates substantiate a variability in Rb expression in human prostate cancer samples. A number of authors have noted mutations in up to 35% of primary prostate cancers, corresponding with loss of Rb protein upon immunohistochemistry [Bookstein et al., 1990; Ittmann and Wieczorek, 1996; Melamed et al., 1997; Latil et al., 1999]. In those specimen not demonstrating genetic mutation or loss of heterozygosity, variability in Rb protein expression is noted on immunohistochemistry, and this variability correlates strongly with prognosis [Theodorescu et al., 1997]. Those tumors displaying decreased Rb immunostaining display a worse clinical prognosis.

Since ubiquitination of cellular proteins is generally preceded by a phosphorylation event [Willems et al., 1996, 1999], we examined the effects of androgen stimulation on cell cycle regulatory proteins known to influence the phosphorylation state of Rb. In addition to loss of Rb protein, we observed induction of cyclins A and B as well as Cdks 1 and 2 at the growth stimulatory concentration of androgen. Importantly, induction of these cell cycle regulatory molecules appears selective, since no androgen-dependent changes in the expression of cyclin D1, cyclin E, Cdk 4, 6 or p27, p21, and 16 were observed at the timepoints studied. This selective induction of those cyclin/Cdk complexes predominantly active late in the cell cycle differs from the more commonly observed mitogen induction of G_1/S cyclins.

Interestingly, androgen depletion of the LNCaP cells resulted in G_1 arrest suggesting that perhaps charcoal depletion of the serum removes other critical growth factors involved in G_1/S transition. Others have demonstrated an induction of D type cyclins in LNCaP cell upon androgen stimulation [Lu et al., 1997; Knudsen et al., 1998] suggesting perhaps that our experiments utilized too late a timepoint for assay of G_1/S cyclins, or that we did not study those cyclins critical for G_1/S transition in our cell line. Additionally, qualitative changes such as release of cell cycle inhibitors from cyclin/Cdk complexes cannot be assessed by our current methodology. The downstream effects of androgen stimulation are likely to be multiple, and the expression of local autocrine factors involved in G_1/S transition may be under androgen regulation as well.

Lu et al. [1997] demonstrated an induction of both Cdk2 and Cdk4 as well as a downregulation of the Cki p16 as a function of androgen treatment. Under conditions of our experiments, we do not observe increased Cdk4 expression or decreased p16 levels upon androgen stimulation, but differences between this study and ours may be due, in part, to the dose of androgen utilized by Lu et al. [1997] (1.0 nM dihydrotestosterone), which we and others [Lee et al., 1995] have not found to be mitogenic for LNCaP cells. Knudsen et al. [1998] observed that androgen depletion of LNCaP cells reduces cyclin D3/Cdk4 and cyclin A/Cdk2 expression and activities. Upon repletion with 0.1 nM DHT, conditions comparable to ours, the authors observed a marked induction of cyclin A/Cdk2 and what appears to be a lesser effect on cyclin D3/Cdk4. Ultimately, differences between these studies and ours likely reflect variation among LNCaP cell lines, culture conditions, or alternatively, the steroid depletion and repletion regimens used.

It is clear from our experiments, and those of others, that cyclin A and Cdk2 are strongly induced by androgen stimulation of LNCaP cells by a yet unknown mechanism. Since cyclin A, cyclin B and Cdk1 genes are E2F-responsive, their induction may be a consequence of reduced Rb protein level, which in turn allows free E2F to activate these target genes. Recently, it was shown that constitutively active Rb mutants, which are incapable of being inactivated by phosphorylation, attenuate the cyclin A promoter [Knudsen et al., 1999]. Alternatively, cyclins and Cdks may represent direct AR target genes, a hypothesis we are currently testing. Many proteins undergo a specific phosphorylation event prior to ubiquitin mediated degradation [Willems et al., 1996; Sheaff et al., 1997], and the Rb protein is thought to contain at least 16 serine/threonine phosphorylation sites which are known to be differentially phosphorylated by individual cyclin/Cdk complexes [Lees et al., 1991; Zarkowska and Mittnacht, 1997]. Selective induction of cyclin A/Cdk 1 or 2 and cyclin B/Cdk 1 in LNCaP cells may result in a unique phosphorylation pattern for Rb, thereby making the protein vulnerable to degradation by cellular mechanisms such as ubiquitination (Fig. 5). Finally, while a phosphorylation

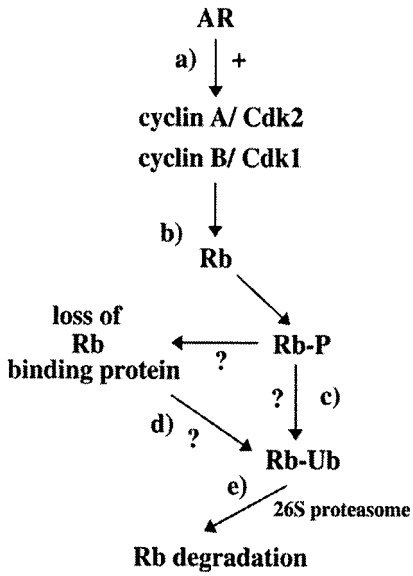


Fig. 5. A possible mechanism of retinoblastoma (Rb) protein degradation in androgen stimulated LNCaP cells is shown. (**a**) Androgens stimulate the activity of cyclin A/Cdk 2 and Cyclin B/ Cdk 1, which in turn (**b**) phosphorylate Rb. Selective phosphorylation of Rb by cyclin A/Cdk2 or Cyclin B/Cdk1 may potentially: (**c**) result in targeting of Rb for ubiquitination, or (**d**) cause dissociation of a bound, protective protein. Either of these events may, in turn, result in Rb ubiquitination. Ubiquitinated Rb is then degraded by the 26S proteasome.

mediated mechanism of Rb protein degradation is a possibility, alternate mechanisms exist including androgen-stimulated alterations in the proteins which mediate the ubiquitin-proteasome pathway. Specific substrates such as Rb protein could be ubiquitinated [Bartel et al., 1990] or deubiquitinated by enzymes known to be regulated by growth suppressive signals or steroids [Zhu et al., 1996, 1997],

Our results suggest a novel mechanism of cellular proliferation in prostate cancer cells reflecting an androgen-dependent post-transcriptional reduction in Rb protein stability. While inactivation of Rb protein binding ability through hyperphosphorylation is the usual mechanism of cell cycle progression, the degradation of Rb protein represents a novel mechanism of mitogen induced cell cycle progression. The reduction in Rb protein stability may occur through unique Rb phosphorylation events or alterations in the expression of proteins in the ubiquitin-proteasome pathway. Therapeutic strategies that stabilize Rb, such as those utilizing inhibitors of the proteasome pathway, might reverse the effect of androgen-dependent cellular proliferation and inhibit the growth of both androgen-dependent and androgen-independent prostate cancer.

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