Biochemical and Biophysical Research Communications **283,** 97–101 (2001)
doi:10.1006/bbrc.2001.4738, available online at http://www.idealibrary.com on IDEAL®

E2F Activity Is Biphasically Regulated by Androgens in LNCaP Cells

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Received March 12, 2001

Androgens exert a peculiar biphasic dose-dependent influence on the proliferation of LNCaP cells, a widely used model to study androgen effects on prostate cancer cells. Low concentrations of androgen stimulate proliferation, but high concentrations inhibit proliferation and induce strong expression of differentiation markers. In order to gain more insight into the molecular mechanisms that underlie these changes we studied the influence of a wide concentration range of the synthetic androgen R1881 on several cell cycle- and differentiation-related parameters. Low concentrations (0.1 nM), known to promote LNCaP cell proliferation, induce an increase of Retinoblastoma protein phosphorylation, accompanied by an increase of E2F-1 protein levels and E2F activity and by increased expression of the E2F-target gene products E2F-1 and cyclin A. High concentrations of R1881 (10 nM) induce strong expression of the differentiation marker prostate-specific antigen. Retinoblastoma protein is largely hypophosphorylated, resulting in low E2F activity and low concentrations of E2F-1 and cyclin A mRNA. Finally, there is a strong increase of $p27^{KIP1}$ protein, but not of $p27^{KIP1}$ mRNA. These results indicate that the biphasic dose response of LNCaP proliferation to androgen is closely reflected in Rb phosphorylation, E2F activity and $p27^{KIP1}$ protein expression.

Key Words: **E2F; LNCaP; androgens; prostate cancer; proliferation; differentiation; retinoblastoma; $p27^{KIP1}$.**

In Western males prostate cancer is a major cause of cancer death. The human adenocarcinoma cell line LNCaP, an androgen sensitive cell line derived from a lymph node metastasis of prostate cancer, is widely used to study the response of prostate cancer cells to androgens. LNCaP cells display a striking biphasic growth response to androgens. At low concentrations (0.1 nM) of the synthetic androgen R1881 proliferation is stimulated, as shown by [3H]-thymidine incorporation and cell number measurements, whereas high concentrations (10 nM) inhibit proliferation and further promote differentiation as shown by strong expression of differentiation markers, such as Prostate Specific Androgen (PSA) (1, 2). Only fragmentary information is available on the molecular mechanisms that underlie these changes, since previous reports have focused on the effects of either very low or very high androgen concentrations. Knudsen *et al.* (3) described an increase in the phosphorylation state of Retinoblastoma (Rb), a key control step in the regulation of cell proliferation, when they treated LNCaP cells with 0.1 nM of 5α-dihydrotestosterone (DHT), a concentration that promotes LNCaP cell proliferation. Tsihlias *et al.*, on the other hand, using a 1000-fold higher concentration of DHT (100 nM) observed a marked decrease of Rb phosphorylation and inhibition of LNCaP cell proliferation (4).

In the present study we treated LNCaP cells with a wide concentration range of androgen and studied their effect on Rb phosphorylation. In view of recent reports that hypophosphorylated Rb binds the E2F-1 transcription factor, a major gatekeeper of the transition from the G1- to S-phase of the cell cycle, and thereby inhibits its transcriptional activity (5–10) we also studied E2F activity and E2F1 levels. We show that the typical bell-shaped dose response curve of LNCaP cell proliferation is clearly reflected both in the degree of phosphorylation of Rb and in the activity of E2F. Moreover, the similarity of the dose response curves for the induction of $p27^{KIP1}$ and for the disappearance of hyperphosphorylated Rb strongly suggest that the low degree of Rb phosphorylation observed at high androgen concentrations is due, at least in part, to increased expression of this kinase inhibitor.

MATERIALS AND METHODS

Plasmids. Dr. Kouzarides (Cambridge University, UK) kindly provided pcDNA3-Rb and pcDNA3-E2F-1. The pcDNA3 expression

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0006-291X/01 $35.00

constructs for cyclin A and cyclin D1 were a gift from Dr. Nakanishi (Nagoya City University Medical School, Japan).

DNA manipulations. The E2F reporter construct was generated by cloning a double stranded oligo containing two E2F binding sites in the NheI site of a minimal TK-TATA luciferase reporter vector. The following oligonucleotides were used: 5′-CTAGCA-**TTTCGCGC**AAACTTGACAA **TTTCGCGC**CAAAGGG-3′ and 5′-CTAGCCCTTTGGCGCGAAATTGTCAAGTTTGG CGCGAAATTG-3′. The bold sequences are the E2F consensus sites. The E2F-TK-TATA luciferase reporter used in the transfection experiments contained a triple repeat of the double stranded oligonucleotide.

Transfection of LNCaP cells. LNCaP cells were maintained in culture as described by Swinnen *et al.* (11). Transfection experiments were performed using the Transfast transfection reagent (Promega) according to the manufacturer's protocol. After 16 h, the cells were washed with RPMI medium supplemented with 5% fetal calf serum (treated with dextran-coated charcoal (5% CT-FCS)) and incubated with 5 ml of RPMI medium containing 5% CT-FCS and the indicated concentrations of R1881 (methyltrienolone, a synthetic androgen). Ethanol (EtOH) was used as vehicle at a constant final concentration of 0.1%. Forty-eight hours (or the time indicated) after addition of the androgen, cells were collected in 500 μl of passive lysis buffer (Promega) and assayed for luciferase activity as described by Hofman *et al.* (12). Protein concentrations of the lysates were determined as follows: 40 μl of the lysate was incubated on ice for 10 min with 0.5 ml 0.03% sodium deoxycholate. Thereafter, 0.75 ml 15% trichloroacetic acid was added and the samples were incubated on ice for another 10 min. Proteins were pelleted by 20 min of centrifugation at 13,000*g* and at 4°C. The protein pellets were resuspended in 0.1 N NaOH and protein concentrations were determined using the BCA method (Pierce) according to the manufacturer's protocol. Luciferase activities were corrected accordingly.

Western blotting. For each condition, an equal amount of cell extract was separated by means of SDS–PAGE on a 3% stacking and a 7% resolving gel. Proteins were electroblotted onto a nitrocellulose membrane. The membrane was subsequently blocked and probed with a commercially obtained monoclonal antibody raised against Rb, E2F-1, cyclin D1, or $p27^{KIP1}$ (Becton-Dickinson Pharmingen). Immunoreactive proteins were visualized using a chemiluminescence detection system (NEN).

RNA-isolation and Northern analysis. LNCaP cells (3×10^6 in a 15 cm dish) were treated for 48 h with different concentrations of R1881. Northern blot analysis was performed as described by Swinnen *et al.* (11) on 15 μg of total RNA isolated from these cells. To detect E2F-1, cyclin A, and cyclin D1 mRNA, ^{32}P-radiolabeled PCR fragments were generated using the following forward and reverse primers: 5′-TTTCCAGAGTAGCTCACCTTG-3′ and 5′-TGTATGTT-CACCTTCATTCCCC-3′ for E2F-1; 5′-GAAAGCAAACAGT-AAACAGCC-3′ and 5′-CCTATCAATGTAGTTCACAGCC-3′ for cyclin A; 5′-TAAGATGAAGGAGACCATCCC-3′ and 5′-AAATGAACTTCACATCTGTGGC-3′ for cyclin D1. The detection of the Prostate Specific Antigen (PSA) and Fatty Acid Synthase (FAS) transcripts was performed as described in Swinnen *et al.* (1, 13). For the detection of $p27^{KIP1}$ transcripts, the complete ORF of $p27^{KIP1}$ was amplified by means of RT-PCR on normal human prostate RNA, using respectively 5′-ATGTCAAACGTGCGAGTGTCTAACGG-3′ and 5′-TTACGTTTGACGTCTTCTGAGGCCAGG-3′ as forward and reverse primer. The amplified product was cloned in pGEM-T (Promega) and sequenced. An 18 S probe, used to verify RNA integrity and equal sample loading was labeled using the oligolabeling kit from Amersham-Pharmacia.

RESULTS

Androgen-induced changes in Retinoblastoma phosphorylation and $p27^{KIP1}$ protein levels. To study the effects of androgens on Rb phosphorylation, LNCaP cells were treated with a wide concentration range of R1881. This synthetic androgen was chosen because of its slower metabolisation and to allow a direct comparison with our previous data on LNCaP cell growth and differentiation. As shown in Fig. 1, up to 0.1 nM of R1881 there is an increase of both hyper- and hypophosphorylated Rb. At 1 nM of R1881, however, the Rb signal is markedly weaker and almost completely in the hypophosphorylated form. Since the phosphorylation of Rb is partially controlled by the cyclin D1 dependent kinase CDK4 we also looked at the effect of androgens on cyclin D1. As shown in Fig. 1, cyclin D1 protein levels were not affected, although a slight upregulation of cyclin D1 messenger mRNA was observed at 0.01 and 0.1 nM R1881 (Fig. 2). Another way of controlling Rb phosphorylation is via the regulation of the cyclinE/CDK2 activity by the kinase inhibitor $p27^{KIP1}$. As shown in Fig. 1, $p27^{KIP1}$ protein levels were hardly influenced by low concentrations of R1881. At higher androgen concentrations, however, $p27^{KIP1}$ protein levels were markedly stimulated. These changes are most probably due to a posttranscriptional mechanism, such as an increased stability of the protein. Indeed, in striking contrast to the strong induction of the mRNAs encoding PSA and FAS at high androgen concentrations, $p27^{KIP1}$ mRNA levels were not influenced by androgen treatment (Fig. 2).

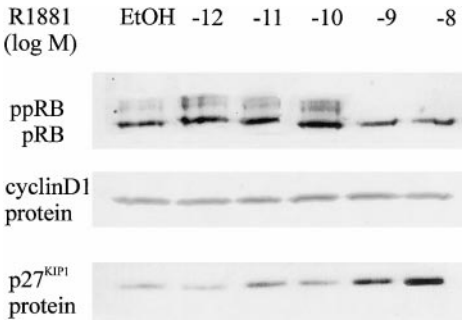

FIG. 1. Influence of androgen treatment on the phosphorylation of Retinoblastoma and on cyclin D1 and $p27^{KIP1}$ protein levels. Cell lysates were prepared from LNCaP cells (5×10^5 cells per 6-cm dish) treated for 48 h with the indicated concentrations of R1881. Equal amounts of these lysates were separated by means of SDS–PAGE and electroblotted onto a nitrocellulose membrane. The membranes were probed with commercial antibodies for the corresponding proteins and immunoreactive proteins were visualized using a chemiluminescence detection system. The monoclonal antibody used for the detection of Rb recognizes both the hypo- (pRb) and hyperphosphorylated (ppRb) forms of Rb.

Androgen effects on E2F activity. To investigate whether these changes of Rb phosphorylation are accompanied by changes of E2F activity, LNCaP cells were transiently transfected with an E2F reporter construct containing 6 E2F responsive sites. Sixteen hours later, the transfected cells were treated for 48 h with different doses of R1881 and assayed for luciferase

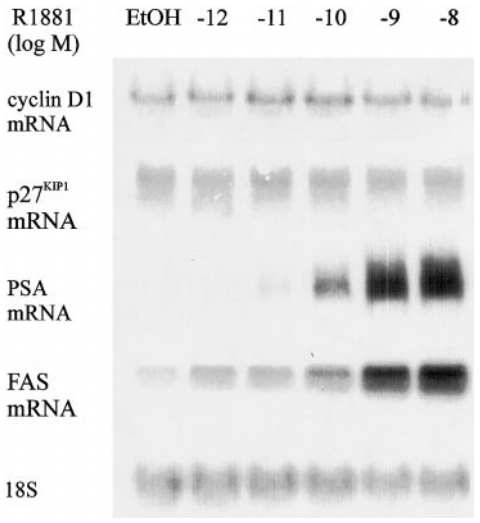

FIG. 2. Influence of androgen treatment on specific mRNAs in LNCaP cells. LNCaP cells (3×10^6 cells per 15-cm dish) were treated for 48 h with the indicated concentrations of R1881. Northern blot analysis of total RNA (15 μg) from these cells was performed with radiolabeled probes for cyclin D1, $p27^{KIP1}$, PSA, and FAS. RNA integrity and equal sample loading were verified using a 18S probe.

activity. As shown in Fig. 3A, marked changes in E2F-controlled luciferase activity are observed as a function of androgen concentration. LNCaP cells treated with low concentrations of R1881 show an increase in E2F activity as compared to the basal activity. The highest E2F activity is observed at 0.1 nM R1881, the concentration that also induces maximal Rb phosphorylation and optimal growth. At higher concentrations of R1881 (1 nM or more) there is a drastic decrease in E2F activity. This decrease in E2F activity parallels the growth arrest and the strong expression of differentiation markers previously demonstrated at these concentrations. As shown in Fig. 3B, the influence of androgen on E2F activity is already observed after 12 h, is maximal between 24 and 36 h and decreases somewhat after 48 h.

Androgen induced changes of E2F activity are reflected in the expression of E2F-1 target genes. E2F-1, a key controller for the G1 to S-phase transition, is responsible for the transcriptional initiation of several S-phase target genes such as E2F-1 itself (by autoregulation), cyclin A, cyclin E, cdc2, dihydrofolate reductase, or thymidine kinase. We looked at the expression of two of these target genes, E2F-1 and cyclin A, in LNCaP cells treated with different doses of R1881 (Fig. 4). Both E2F-1 and cyclin A mRNA are markedly stimulated by R1881 at low concentrations of R1881 with maximal stimulation at 0.1 nM R1881, but are strongly suppressed at higher concentrations. Moreover, a similar regulation could be demonstrated for E2F-1 protein expression by means of Western blotting (Fig. 4).

DISCUSSION

The human prostatic cancer cell line LNCaP shows a remarkable biphasic response to androgen. In fact, low concentrations of androgen stimulate cell proliferation, when compared to androgen depleted medium, whereas high concentrations of the hormone result in strong inhibition of proliferation, and at the same time induce cell differentiation as demonstrated by the increased expression of PSA and other differentiation markers (1, 2, 11, 13). Several reports have already studied in some detail how either very low (3) or very high (4) concentrations of androgen affect a number of regulatory proteins, involved in the control of the cell cycle. In the present study we performed a more com-

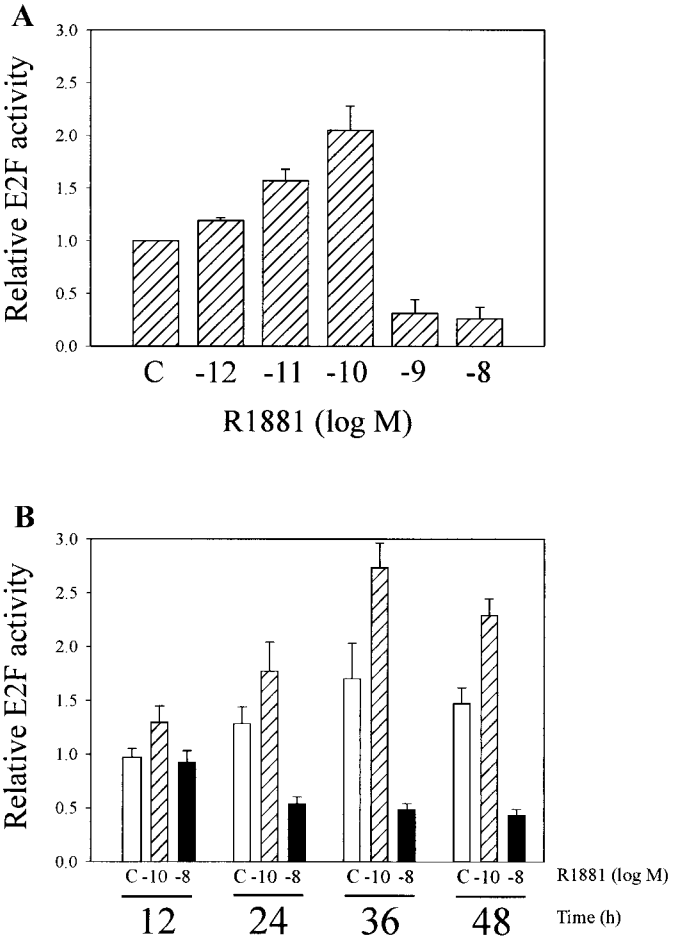

FIG. 3. Influence of R1881 on E2F activity. LNCaP cells (5×10^5 cells per 6-cm dish) were transiently transfected with 3 μg of the E2F reporter construct. (A) Dose response curve. Cells were treated for 48 h with the indicated concentration of R1881. (B) Time course. LNCaP cells were treated for the indicated time with EtOH (C), 10^{-10} M R1881, or 10^{-8} M R1881. Thereafter, luciferase activities were measured and corrected for protein concentration. Relative luciferase values were calculated with respect to the luciferase activity in the presence of EtOH (A) or in untreated cells (B), arbitrarily set to 1. The results shown are the mean + SEM of four experiments.

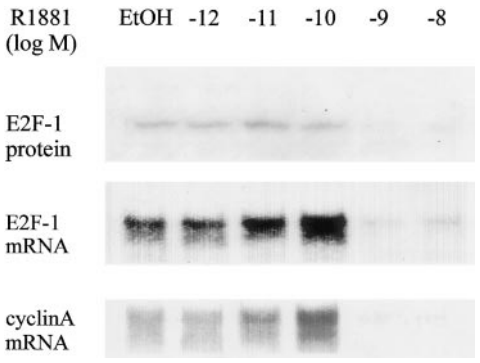

FIG. 4. Influence of androgen on E2F-1 and cyclin A mRNAs and on E2F-1 protein levels. E2F-1 and cyclin A mRNAs and E2F-1 protein levels were measured as described respectively in the legends to Fig. 2 and Fig. 1.

plete dose response curve, covering both the low and high concentration range. Moreover, we used the synthetic androgen R1881 as in our previous studies on LNCaP cell proliferation and differentiation allowing a direct comparison with these results.

Our data show that the effect of different concentrations of R1881 on LNCaP cell proliferation is closely reflected in the degree of phosphorylation of Rb and in E2F activity. Indeed, at low concentrations of R1881 (up to 0.1 nM) we observed a moderate increase of Rb phosphorylation confirming results obtained by Knudsen *et al.* (3) using 0.1 nM DHT. At 1 nM of R1881, however, we already observed a strong and abrupt decrease of the phosphorylation state of Rb, in line with the results of Tsihlias *et al.* using 100 nM of DHT (4). Major targets of Rb are the E2F-transcription factors, which are rendered inactive by binding to Rb. This interaction of E2F with Rb is lifted by phosphorylation of the latter resulting in activation of E2F pathways. Our data on androgen induced changes of E2F activity, as measured by a luciferase-based reporter assay are in agreement with this hypothesis. Up to a concentration of 0.1 nM R 1881 the E2F activity increases in parallel with Rb phosphorylation, whereas E2F1 protein levels only show a moderate increase. A similar stimulation is observed for the E2F-1 regulated gene products cyclin A mRNA and E2F-1 mRNA, which is controlled by an autoregulatory pathway. On the other hand, at higher concentrations of R1881, the marked dephosphorylation of Rb is accompanied by lower E2F-activity, lower E2F-1 protein levels and lower expression of both E2F-regulated gene products. Our data therefore support a key role of the Rb-E2F pathway in the androgen regulation of prostate cancer cell proliferation. The observations of Agus *et al.* (14) on the CWR22 human prostate xenograft in nude mice point in the same direction. These authors demonstrated that androgen-withdrawal led to a gradual increase of E2F-1 expression, as shown by immunohistological staining in parallel with the appearance of hyperphosphorylated RB products on Western blots.

Most probably the regulation of Rb phosphorylation by androgen is mediated by changes in the activity of cyclin-dependent kinases, such as CDK2 and CDK4. Upregulation of the activity of these kinases at low concentration of androgens has been described previously (15–17). On the other hand, we observed a marked increase of the kinase inhibitor $p27^{KIP1}$ at all androgen concentrations that resulted in low Rb phosphorylation. Inhibition of the activity of CDK2 by $p27^{KIP1}$ may thus explain, at least in part, the lack of phosphorylation of Rb under these conditions (18, 19). Interestingly, our data show that androgens do not influence $p27^{KIP1}$ mRNA, in striking contrast to the transcriptional regulation of differentiation markers such as PSA, which follow a similar dose response curve as $p27^{KIP1}$ at the protein level. For that reason the regulation of $p27^{KIP1}$ most probably occurs at a posttranscriptional level, a common finding for the regulation of this protein (20, 21), although there is also evidence for transcriptional regulation (22). A substantial role for $p27^{KIP1}$ in the regulation of prostate cancer cell proliferation is evoked also by other observations. Indeed a strongly diminished $p27^{KIP1}$ expression was observed in LNAI cells, which are originally derived from LNCaP cells but became androgen-independent (23). Moreover, distinct patterns of $p27^{KIP1}$ expression involving both differences in mRNA and protein levels have been described between normal prostatic cells, benign prostatic hyperplasia and prostatic cancer. In the latter group, a low $p27^{KIP1}$ expression was found to be associated with more aggressive prostate carcinoma (24).

Multiple elements probably play a role in the regulation of LNCaP cell proliferation by androgens and their importance may be different in the stimulatory and inhibitory phase of the dose response curve. Although the androgen effects may be based primarily on transcriptional regulation by the androgen receptor, they most probably involve considerable cross talk with other pathways (25, 26) such as Ras/ERK, which mediate the regulation of Rb phosphorylation by mitogenic factors. In the opposite direction, such cross talk has been well documented for the activation of the androgen receptor and other steroid receptors by nonsteroidal stimuli (27). A number of observations show that androgens may influence these ERK-pathways by rapid nongenomic effects, possibly mediated by the androgen receptor (28). Very recently, it has been proposed that the simultaneous interaction of androgen and estradiol receptor with Src triggers LNCaP cell proliferation (26).

In conclusion our results indicate that the biphasic dose response of LNCaP cells proliferation to androgen is closely reflected in a number of cell cycle parameters, such as Rb phosphorylation, E2F activity and $p27^{KIP1}$

protein levels. The mechanisms by which the androgen signal affects these parameters are still poorly understood and may be different for the response to low and high androgen concentrations.

ACKNOWLEDGMENTS

The excellent technical assistance of Hilde Geeraerts is kindly acknowledged. We also thank Dr. Kouzarides and Dr. Nakanishi for their generous gifts of plasmids. This work was supported by a grant from "Geconcerteerde Onderzoeksactie van de Vlaamse Gemeenschap," by a research grant from the Fund for Scientific Research, Flanders, Belgium (F.W.O.), and by a cancer research grant from VIVA (FB Insurances).

REFERENCES

1. Esquenet, M., Swinnen, J. V., Heyns, W., and Verhoeven, G. (1996) Control of LNCaP proliferation and differentiation: Actions and interactions of androgens, 1alpha,25-dihydroxycholecalciferol, all-trans retinoic acid, 9-cis retinoic acid, and phenylacetate. *Prostate* **28,** 182–194.
2. Henttu, P., Liao, S. S., and Vihko, P. (1992) Androgens upregulate the human prostate-specific antigen messenger ribonucleic acid (mRNA), but down-regulate the prostatic acid phosphatase mRNA in the LNCaP cell line. *Endocrinology* **130,** 766–772.
3. Knudsen, K. E., Arden, K. C., and Cavenee, W. K. (1998) Multiple G1 regulatory elements control the androgen-dependent proliferation of prostatic carcinoma cells. *J. Biol. Chem.* **273,** 20213–20222.
4. Tsihlias, J., Zhang, W., Bhattacharya, N., Flanagan, M., Klotz, L., and Slingerland, J. (2000) Involvement of p27Kip1 in G1 arrest by high dose 5 alpha-dihydrotestosterone in LNCaP human prostate cancer cells. *Oncogene* **19,** 670–679.
5. La Thangue, N. B. (1996) E2F and the molecular mechanisms of early cell-cycle control. *Biochem. Soc. Trans.* **24,** 54–59.
6. Flemington, E. K., Speck, S. H., and Kaelin, W. G., Jr. (1993) E2F-1-mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product. *Proc. Natl. Acad. Sci. USA* **90,** 6914–9618.
7. Weintraub, S. J., Chow, K. N., Luo, R. X., Zhang, S. H., He, S., and Dean, D. C. (1995) Mechanism of active transcriptional repression by the retinoblastoma protein. *Nature* **375,** 812–815.
8. Dyson, N. (1998) The regulation of E2F by pRB-family proteins. *Genes Dev.* **12,** 2245–2262
9. Helin, K., Harlow, E., and Fattaey, A. (1993) Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein. *Mol. Cell. Biol.* **13,** 6501–6508.
10. Harbour, J. W., and Dean, D. C. (2000) Rb function in cell-cycle regulation and apoptosis. *Nat. Cell Biol.* **2,** E65–E67.
11. Swinnen, J. V., Esquenet, M., Heyns, W., Rombauts, W., and Verhoeven, G. (1994) Androgen regulation of the messenger RNA encoding diazepam-binding inhibitor/acyl-CoA-binding protein in the human prostatic adenocarcinoma cell line LNCaP. *Mol. Cell. Endocrinol.* **104,** 153–162.
12. Hofman, K., Swinnen, J. V., Claessens, F., Verhoeven, G., and Heyns, W. (2000) Apparent coactivation due to interference of expression constructs with nuclear receptor expression. *Mol. Cell. Endocrinol.* **168,** 21–29.
13. Swinnen, J. V., Esquenet, M., Goossens, K., Heyns, W., and Verhoeven, G. (1997) Androgens stimulate fatty acid synthase in the human prostate cancer cell line LNCaP. *Cancer Res.* **57,** 1086–1090.
14. Agus, D. B., Cordon-Cardo, C., Fox, W., Drobnjak, M., Koff, A., Golde, D. W., and Scher, H. I. (1999) Prostate cancer cell cycle regulators: Response to androgen withdrawal and development of androgen independence. *J. Natl. Cancer. Inst.* **91,** 1869–1876.
15. Lu, S., Tsai, S. Y., and Tsai, M. J. (1997) Regulation of androgen-dependent prostatic cancer cell growth: Androgen regulation of CDK2, CDK4, and CKI p16 genes. *Cancer Res.* **57,** 4511–4516.
16. Fribourg, A. F., Knudsen, K. E., Strobeck, M. W., Lindhorst, C. M., and Knudsen, E. S. (2000) Differential requirements for ras and the retinoblastoma tumor suppressor protein in the androgen dependence of prostatic adenocarcinoma cells. *Cell Growth Differ.* **11,** 361–372.
17. Chen, Y., Robles, A. I., Martinez, L. A., Liu, F., Gimenez-Conti, I. B., and Conti, C. J. (1996) Expression of G1 cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors in androgen-induced prostate proliferation in castrated rats. *Cell Growth Differ.* **7,** 1571–1578.
18. Kokontis, J. M., Hay, N., and Liao, S. (1998) Progression of LNCaP prostate tumor cells during androgen deprivation: Hormone-independent growth, repression of proliferation by androgen, and role for p27Kip1 in androgen-induced cell cycle arrest. *Mol. Endocrinol.* **12,** 941–953.
19. Graff, J. R., Konicek, B. W., McNulty, A. M., Wang, Z., Houck, K., Allen, S., Paul, J. D., Hbaiu, A., Goode, R. G., Sandusky, G. E., Vessella, R. L., and Neubauer, B. L. (2000) Increased AKT activity contributes to prostate cancer progression by dramatically accelerating prostate tumor growth and diminishing p27Kip1 expression. *J. Biol. Chem.* **275,** 24500–24505.
20. Pagano, M., Tam, S. W., Theodoras, A. M., Beer-Romero, P., Del Sal, G., Chau, V., Yew, P. R., Draetta, G. F., and Rolfe, M. (1995) Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. *Science* **269,** 682–685.
21. Macri, E., and Loda, M. (1999) Role of p27 in prostate carcinogenesis. *Cancer Metastasis Rev.* **17,** 337–344.
22. Medema, R. H., Kops, G. J. P. L., Bos, J. L., and Burgering, B. M. T. (2000) AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27Kip1. *Nature* **404,** 782–787.
23. Cordon-Cardo, C., Koff, A., Drobnjak, M., Capodieci, P., Osman, I., Millard, S. S., Gaudin, P. B., Fazzari, M., Zhang, Z. F., Massague, J., and Scher, H. I. (1998) Distinct altered patterns of p27Kip1 gene expression in benign prostatic hyperplasia and prostatic carcinoma. *J. Natl. Cancer Inst.* **90,** 1284–1291.
24. Gnanapragasam, V. J., Robson, C. N., Leung, H. Y., and Neal, D. E. (2000) Androgen receptor signalling in the prostate. *BJU International* **86,** 1001–1013.
25. Culig, Z., Hobisch, A., Bartsch, G., and Klocker, H. (2000) Androgen receptor—an update of mechanisms of action in prostate cancer. *Urol. Res.* **28,** 211–219.
26. Migliaccio, A., Castoria, G., Di Domenico, M., de Falco, A., Bilancio, A., Lombardi, M., Barone, M. V., Ametrano, D., Zannini, M. S., Abbondanza, C., and Auricchio, F. (2000) Steroid-induced androgen receptor-oestradiol receptor beta-Src complex triggers prostate cancer cell proliferation. *EMBO J.* **19,** 5406–5417.
27. Culig, Z., Hobisch, A., Bartsch, G., and Klocker, H. (2000) Androgen receptor—An update of mechanisms in prostate cancer. *Urol. Res.* **28,** 211–219.
28. Peterziel, H., Mink, S., Schonert, A., Becker, M., Klocker, H., and Cato, A. C. (1999) Rapid signalling by androgen receptor in prostate cancer cells. *Oncogene* **18,** 6322–6329.