Prostate epithelial and adenocarcinoma cells sense androgen through the AR,3 a member of the steroid hormone receptor superfamily of transcription factors (5, 6). The AR contains three functional domains, classified based upon their homology to other known nuclear receptors: a NH2-terminal transactivation domain; a highly conserved DNA binding region; and a COOH-terminal ligand binding pocket (5, 7). The AR differs from other nuclear receptors in that its NH2-terminal domain is the site of its major transactivation function, AF-1 (7). In addition, interaction between the NH2- and COOH-terminal regions of the AR is necessary for complete receptor activity (8). Binding of androgens such as DHT to the AR causes the dissociation of heat shock proteins from the receptor and allows for its dimerization and translocation into the nucleus (9, 10). Within the nucleus, the AR binds to ARES located on target genes such as PSA, which is used clinically to monitor prostate cancer progression (11–13). The gene expression profile initiated by the AR results in a diverse set of biological outcomes, including secretion, differentiation, growth, and survival (11). The specificity of such biological outcomes is hypothesized to hinge upon the cellular environment and availability of AR cofactors. Nevertheless, the precise gene targets involved in these diverse functions remain largely undefined.

Intriguingly, in recurrent androgen independent prostate cancer, the AR is expressed and inappropriately activated (i.e., in the absence of ligand; Ref. 2). This activation event is known to occur through multiple mechanisms, including AR amplification (up to 30% of recurrent tumors) and mutations within the AR itself, which allow alternative steroids (e.g., 17β-estradiol, progesterone) to serve as ligands (2). Also thought to contribute to the androgen-independent phenotype is indirect stimulation of the AR by growth factors and signal transduction pathways (reviewed in Ref. 14). Specifically, EGF, IGF-I, KGF, and IL-6 were previously demonstrated to induce AR activity in the absence of ligand and may synergize with low-level DHT to enhance AR action (15, 16). It has been hypothesized that activation of signal transduction pathways in response to cytokines and growth factors results in phosphorylation of the AR, thus providing a potential mechanism by which receptor activity is modulated (17). It is through these disparate pathways that the AR is thought to be inappropriately activated, facilitating proliferation and tumor progression in the absence of canonical ligand. Thus, inhibition of AR activity is a major goal of therapies used to treat both early and late stage prostate cancers.

We and others have previously shown that cyclin D1 is a potent inhibitor of AR activity (18, 19). Although well characterized for its role in cell cycle transitions, cyclin D1 has been shown to harbor multiple transcriptional functions independent of the cell cycle. Through an LxxLL motif in its COOH terminus and independent of CDK association, cyclin D1 forms a trimeric complex with ERα and the steroid receptor coactivator, SRC-1, to enhance estrogen-responsive transcription (20, 21). Association of cyclin D1 with the AR

Received 12/4/02; revised 6/6/03; accepted 6/9/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by NIH Grant R01CA099996 (to K. E. K.) and the Department of Defense Grant DAMD17-02-1-0037 (to K. E. K.). C. E. P.-D. and C. J. B. are supported by the University Distinguished Graduate Fellowship (University of Cincinnati) and the Albert J. Ryan Foundation.

2 To whom requests for reprints should be addressed, at Phone: (513) 558-7371; Fax: (513) 558-4454; E-mail: Karen.Knudsen@uc.edu.

Prostatic epithelial and adenocarcinoma cells sense androgen through the AR,3 a member of the steroid hormone receptor superfamily of transcription factors (5, 6). The AR contains three functional domains, classified based upon their homology to other known nuclear receptors: a NH2-terminal transactivation domain; a highly conserved DNA binding region; and a COOH-terminal ligand binding pocket (5, 7). The AR differs from other nuclear receptors in that its NH2-terminal domain is the site of its major transactivation function, AF-1 (7). In addition, interaction between the NH2- and COOH-terminal regions of the AR is necessary for complete receptor activity (8). Binding of androgens such as DHT to the AR causes the dissociation of heat shock proteins from the receptor and allows for its dimerization and translocation into the nucleus (9, 10). Within the nucleus, the AR binds to ARES located on target genes such as PSA, which is used clinically to monitor prostate cancer progression (11–13). The gene expression profile initiated by the AR results in a diverse set of biological outcomes, including secretion, differentiation, growth, and survival (11). The specificity of such biological outcomes is hypothesized to hinge upon the cellular environment and availability of AR cofactors. Nevertheless, the precise gene targets involved in these diverse functions remain largely undefined.

Intriguingly, in recurrent androgen independent prostate cancer, the AR is expressed and inappropriately activated (i.e., in the absence of ligand; Ref. 2). This activation event is known to occur through multiple mechanisms, including AR amplification (up to 30% of recurrent tumors) and mutations within the AR itself, which allow alternative steroids (e.g., 17β-estradiol, progesterone) to serve as ligands (2). Also thought to contribute to the androgen-independent phenotype is indirect stimulation of the AR by growth factors and signal transduction pathways (reviewed in Ref. 14). Specifically, EGF, IGF-I, KGF, and IL-6 were previously demonstrated to induce AR activity in the absence of ligand and may synergize with low-level DHT to enhance AR action (15, 16). It has been hypothesized that activation of signal transduction pathways in response to cytokines and growth factors results in phosphorylation of the AR, thus providing a potential mechanism by which receptor activity is modulated (17). It is through these disparate pathways that the AR is thought to be inappropriately activated, facilitating proliferation and tumor progression in the absence of canonical ligand. Thus, inhibition of AR activity is a major goal of therapies used to treat both early and late stage prostate cancers.

We and others have previously shown that cyclin D1 is a potent inhibitor of AR activity (18, 19). Although well characterized for its role in cell cycle transitions, cyclin D1 has been shown to harbor multiple transcriptional functions independent of the cell cycle. Through an LxxLL motif in its COOH terminus and independent of CDK association, cyclin D1 forms a trimeric complex with ERα and the steroid receptor coactivator, SRC-1, to enhance estrogen-responsive transcription (20, 21). Association of cyclin D1 with the AR
coactivator, P/CAF, also has been demonstrated to enhance ER-mediated transactivation and suggests a second, possibly cell type specific, mechanism of cyclin D1 enhancement of ER activity (22). In addition, cyclin D1 has been demonstrated to possess the opposite effect, serving as a corepressor for many transcription factors, including v-Myb, STAT3, DMP1, the thyroid hormone receptor, and the AR (reviewed in Ref. 23). Using the PSA promoter as read-out, we previously established that repression of the AR is CDK and LxxLL independent, dominant to AR coactivators, and is mediated through direct, ligand-independent binding of cyclin D1 to the AR NH2 terminus (18, 19, 24). Because cyclin D1 expression is induced by androgen in androgen-dependent prostatic adenocarcinoma cells (24) and represses receptor activity when overexpressed, the hypothesis was put forth that cyclin D1 serves as a feedback inhibitor of the AR. Indeed, this hypothesis was supported by our observation that androgen-dependent prostatic adenocarcinoma cells (LNCaP) undergo a decrease in cell cycle progression when expressing ectopic cyclin D1 or a mutant form, cyclin D1-KE, which fails to bind CDK4 and cannot regulate cell cycle transitions but is competent to inhibit AR activity (19, 25). In addition to this finding, endogenous AR has been observed in complex with cyclin D1, and AR activity is reduced at the G1-S transition, wherein cyclin D1 levels are highest (26, 27). Taken together, these data demonstrate that cyclin D1 serves as a potent inhibitor of AR activity.

Given that bypass pathways activate the AR in recurrent adenocarcinomas, it is critical to determine whether cyclin D1 corepressor activity can be maintained under these conditions. Here, we determined the specificity of cyclin D1 action, with emphasis upon factors that facilitate the transition of prostastic adenocarcinomas to androgen independence. We now demonstrate that cyclin D1 maintains its ability to repress AR activity in a wide variety of cellular backgrounds including androgen-dependent and -independent prostate cancer cells. We also provide evidence that cyclin D1 regulation of AR activity spans multiple androgen-responsive promoters, inhibiting not only PSA but also MMTV and probasin promoters, indicating that the mechanism of repression is conserved across multiple AR targets. Furthermore, NH2-terminal phospho-mutants of the AR retained cyclin D1 sensitivity. In addition, we show that cyclin D1 corepressor activity regulates AR mutants and polymorphisms associated with prostate cancer susceptibility and with the transition to androgen independence. This function is conserved among tumor-derived AR alleles activated by nonandrogen steroids, indicating that cyclin D1 function is retained with alternate ligands. Lastly, we demonstrate that cyclin D1 is capable of inhibiting wild-type AR induced through cytokine and nonconventional ligand pathways. Taken together, these data represent the first in-depth analysis of an AR corepressor, to date, and demonstrate the potential of cyclin D1 action in the treatment of both androgen-dependent and -independent tumors.

MATERIALS AND METHODS

Cell Culture and Treatment. CV1, MCF-7, LNCaP, and PC-3 cells were obtained from American Type Culture Collection and maintained in 5% CO2 incubators. The 22Rv1 cell line was the gift of Dr. James W. Jacobberger (Case Western Reserve University, Cleveland, OH; Ref. 28). CV1, PC-3, LNCaP, and MCF-7 cells were cultured as described previously (29, 30). 22Rv1 cells were seeded in RPMI supplemented with 5% FBS, 2 mML-glutamine, and 100 μg/ml streptomycin. For steroid-free conditions, cells were seeded in phenol red-free media containing charcoal-dextran-treated FBS (5% for 22Rv1 and LNCaP, 10% for others; HyClone Laboratories). Plasmids. The H2B-GFP, pSG5-AR, PSA61-LUC, CMV-β-galactosidase, pSG5-AR-T877A, RSV-cyclin D1, RSV-cyclin D1-KE, and pGEX-3Xcyclin D1 constructs have been described previously (19). The pSV-AR902, pSV-AR715, pSV-AR721, pSV-AR874, pSV-AR877S, and pSV-AR890 mammalian expression plasmids encoding tumor derived AR alleles were generously provided by Dr. Steven P. Balk (Beth Israel Hospital, Boston, MA; Ref. 31). Plasmid-encoding dominant negative AR (pSG5-ARΔ46-408) was supplied by Jorma Palvimo (University of Helsinki, Helsinki, Finland; Ref. 32). The pEGFP-C1-ARQ48 and pEGFP-C1-ARQ48 plasmids for the expression of polymorphic ARs were gifts of Dr. Michael Mancini (Baylor College of Medicine, Houston, TX; Ref. 33). The pCMVhARSA650, pCMVhARSA81.94, and pGEXAR1-173 expression plasmids were kindly provided by Dr. Elizabeth Wilson (University of North Carolina School of Medicine, Chapel Hill, NC; Ref. 34). pCDNA3 empty vector was obtained from Invitrogen. PBSXERE-LUC and PVM5-HERe were kindly provided by Dr. Sohaib Khan (University of Cincinnati, Cincinnati, OH). Plasmid-encoding myc-tagged, wild-type cyclin E was the gift of Dr. Jim Roberts (Fred Hutchinson Cancer Research Center, Seattle, WA). The MMTV-luciferase reporter construct was obtained from Dr. Richard Pestell (Georgetown University, Washington, DC). ARR2P-LUC was constructed as described previously (35).

Transfection and Transcriptional Reporter Assays. CV1, MCF-7, PC-3, and 22Rv1 cells were seeded for transfection in CDT serum, which lacks steroids but maintains growth factors. The N,N-bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid/calcium phosphate transfection protocol (36) was used for transfection of pSG5-AR (0.5 μg), luciferase reporter (MMTV-LUC, ARR2P-LUC, or PSA61-LUC; 0.75 μg), RSV-cyclin D1 (1.5 μg), CMV-β-galactosidase (0.5 μg), and empty vector (pCDNA3.1; to total of 4 μg/well for a 6-well dish). After transfection, cells were allowed to recover for a period of 5–6 h before stimulation with 0.1% ethanol vehicle, 0.1 μM DHT (Sigma), 0.1 μM testosterone, or 50 ng/ml IL-6 (IL Labs Biotechnology, London, Ontario, Canada) for 24–24 h. For IL-6 assays, low serum (0.1% CDT) conditions were used during the recovery and stimulation periods. After treatment, all cells were harvested and assayed for luciferase and β-galactosidase activity as described previously (19). Reporter data represents at least three independent experiments. Appropriate Ps were obtained using ANOVA and Newman-Keuls Multiple Comparison post tests.

Transfection of LNCaP cells was performed using Lipofectin reagent according to the manufacturers’ protocol (Invitrogen, Carlsbad, CA). For LNCaP transfections in 6-cm dishes, plasmid concentrations of 1.0 μg of pSG5-AR, 1.0 μg of ARR2-LUC, 3.0 μg of RSV-cyclin D1, and 0.5 μg of CMV-β-galactosidase were used and supplemented where necessary with pCDNA3.1 for a total of 5.5 μg. Transfected LNCaP cells were stimulated as indicated for a period of 72 h. β-Galactosidase and luciferase activity were measured as reported previously (19).

Immunoblots. Cells from reporter assays in which H2B-GFP was substituted for CMV-β-galactosidase were pelleted after treatment and lysed in radioimmunoprecipitation assay buffer [150 μM NaCl, 0.5% NP0, 0.5% deoxycholate, 0.1% SDS, and 50 μM Tris (pH 8.0)] solution containing 1 μM phenylmethyl sulfonyl fluoride, 10 μg/ml 1,10-phenanthroline, 10 μg/ml aprotonin, 10 μg/ml leupeptin, 10 μM sodium fluoride, 1 mM sodium vanadate, and 60 μM β-glycerophosphate. After centrifugation, clarified lysates were subjected to SDS-PAGE and transferred to Immobilon (Millipore Corp., Bedford, MA). Immunoblots for AR phosphorylation site mutants were then cut in half and blotted separately using antibodies generated against the AR (N-20) and GFP (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoblots for polymorphic, GFP-tagged ARs were probed with GFP antibody to detect both proteins. Anti-mouse and anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Pierce, Rockford, IL) along with enhanced chemiluminescence enhancer (Perkin-Elmer Life Sciences) were used to visualize proteins.

Endogenous Protein Quantification. GST-AR1-173 and GST-cyclin D1 were purified from Escherichia coli using standard techniques. The concentration of GST fusion proteins was determined by SDS-PAGE electrophoresis using Coomassie staining and BSA (Sigma) as a standard. Known concentrations of GST-AR and GST-cyclin D1 were then used to quantify AR and cyclin D1 molecules in LNCaP cells derived from samples transfected as described for transcriptional reporter assays. Total protein in LNCaP lysate was measured using the Bio-Rad D1 protein assay kit as described by the manufacturer (Bio-Rad Laboratories, Hercules, CA). Purified GST proteins were separated by 12% PAGE at known concentrations indicated, alongside LNCaP lysates, and were subsequently immunoblotted with antibody directed against the NH2 terminus of the AR (N-20; Santa Cruz Biotechnology) and the COOH terminus.

Downloaded from cancerres.aacrjournals.org on April 14, 2017. © 2003 American Association for Cancer Research.
of cyclin D1 (Ab-3; Neomarkers, Fremont, CA). Signal was quantified using Metamorph software (Universal Imaging Corporation, West Chester, PA).

**RESULTS**

Cyclin D1 Inhibits AR Activity in Androgen-dependent Prostatic Adenocarcinoma Cells. We have previously demonstrated that cyclin D1 is a potent AR repressor, inhibiting transactivation of the PSA promoter in both C33A and CV1 cell lines, and leading to cell cycle attenuation in androgen-dependent prostatic adenocarcinoma cells (LNCaP; Refs. 18, 19). In addition, we demonstrated that AR-T877A, a common tumor-derived allele present in LNCaP cells, is effectively repressed by cyclin D1 in the context of CV1 cells (19). To determine whether AR repression by cyclin D1 is effective in the context of an androgen-dependent prostate cancer cell, reporter assays were performed on ectopic and endogenous AR in LNCaP cells. Previous to this study, a titration curve was performed in CV1 cells to assess the ability of cyclin D1 to repress AR activity on the PSA61-LUC reporter (18). The minimal DNA ratio of cyclin D1 to AR was determined to be 3:1. On the basis of these prior data, LNCaP cells were transfected with the ARR2-LUC (probasin) reporter and exogenous AR at a 1:3 ratio. Cotransfection of cyclin D1, however, significantly induced activity of endogenous AR 3.8-fold, whereas addition of ectopic wild-type AR increased this activity to 11.3-fold, as expected (Fig. 1A, top). Cotransfection of cyclin D1, however, reduced activity to near basal levels in both cases (0.67-fold for endogenous and 3.7-fold activation for ectopic). Inhibition of AR transactivation was not caused by general transcriptional inhibition because basal AR activity was not affected by cyclin D1 cotransfection. Thus, cyclin D1 is an effective inhibitor of endogenous and ectopic AR activity as determined by reporter assay.

To determine the level of cyclin D1 required to inhibit AR, molar concentrations of AR and cyclin D1 were examined after transfection. For these experiments, GST-tagged cyclin D1 and AR NH2 terminus (amino acids 1–173) were isolated and quantified using standard techniques. Known concentrations of GST fusion proteins (Fig. 1A, lanes 1–4) were then subjected to SDS-PAGE alongside transfected LNCaP cell lysates (Fig. 1A, lanes 5–8). Using antibodies with defined epitopes, proteins were detected by immunoblot and quantified using densitometry. Calculation of protein content based on GST standards revealed that after transfection, LNCaP cells express ~0.030 nmol cyclin D1/µg total protein and 0.033 nmol AR/µg total protein (Fig. 1A, bottom). This translates to a 0.9:1 molar ratio achieved in vivo and suggests a stoichiometric interaction between corepressor and receptor.

Lastly, we examined the ability of the AR to activate endogenous target gene expression in the presence of cyclin D1. LNCaP cells were transfected with pBABE-Puro and either empty vector (CMV-Neo-Bam), cyclin D1, or dominant negative AR (Δ46–408) in the presence of complete serum. Transfected cells were subjected to rapid selection for 72 h in puromycin, at which time RNA was harvested and converted to cDNA. Radioactive PCR was then performed to detect and accurately quantify PSA levels normalized to GAPDH. As

---

**Figure 1.** Cyclin D1 inhibits endogenous and ectopic AR activity in LNCaP cells. A, top panel, LNCaP cells were transfected in the absence of steroid with the probasin (ARR2) reporter and expression plasmids indicated. After transfection, cells were stimulated as indicated for 72 h. After lysis, luciferase activity was measured and normalized to β-galactosidase. Vehicle-treated AR activity was set to 1. Data shown represent at least three independent experiments. Error bars depict the SD. Lower panel, purified GST-AR1-173 (lanes 1–4, top panel) and GST-cyclin D1 (lanes 1–4, bottom panel) proteins were subjected to SDS-PAGE at the concentrations indicated alongside whole cell lysates (µg as indicated) from LNCaP cells transfected with AR and cyclin D1 at a 1:3 ratio. Using the GST standards, AR and cyclin D1 proteins levels were quantified by densitometry as described in “Materials and Methods.” B, LNCaP cells were transfected in the presence of complete serum as described in “Materials and Methods.” After rapid selection with puromycin, RNA was harvested and converted to cDNA using standard techniques. Amplification of PSA and GAPDH was performed in the presence of [α-32P]dCTP. PCR products were subject to nondenaturing PAGE and quantified using a phosphomager. PSA expression was normalized to GAPDH (internal control) and relative expression is shown (bottom panel).
seen in Fig. 1B, PSA mRNA levels were reduced 43.5% in the presence of dominant negative AR when compared with vector only. Addition of cyclin D1 similarly reduced PSA activity, resulting in a 36.0% decrease in mRNA production compared with control. Taken together, these data conclusively demonstrate that cyclin D1 is an effective inhibitor of AR activity in androgen-dependent prostatic adenocarcinoma cells.

Cyclin D1 Repression of AR Activity Is Independent of Cell Type and Promoter. It was recently reported that another AR corepressor, DAX-1, functions in a cell type-specific manner (37). To further examine the specificity of cyclin D1 corepressor activity in context of cancer, four distinct tumor cell lines were used: PC-3; 22Rv1; MCF-7; and TSUPr1. 22Rv1 cells retain important characteristics of clinical androgen-independent prostate cancer as they maintain both AR expression and activity but have bypassed the requirement for androgen (28). PC-3 cells are derived from prostatic adenocarcinoma but have lost AR and PSA expression (38). TSUPr1 cells were originally believed to be prostatic in origin but were later shown to be identical to T24 bladder carcinoma cells (39). As a result, many early studies characterizing AR comodulators were performed in this cell type. MCF-7 cells were also examined because AR is suspected to play a role in breast cancer, yet their endogenous AR appears nonfunctional (40, 41).

To test the specificity of cyclin D1 in these four cell types, all cells were transfected with plasmids encoding wild-type human AR and either cyclin D1, cyclin D1-KE (a mutant defective in CDK4 binding 25), cyclin E, or vector control (pCDNA3) at a 1:3 ratio. AR activity was measured using the PSA61-LUC reporter construct, which contains 6.1-kb of the PSA promoter fused to luciferase. After transfection, cells were stimulated with either 0.1 nM DHT or 0.1% ethanol vehicle for 22–24 h. Cells were then harvested, lysed, and monitored for both luciferase and β-galactosidase activity. Relative luciferase activity is shown with error bars representing the SD. As expected, addition of 0.1 nM DHT induced AR activity in all cell types examined (Fig. 2, A–D, left bars). Induction of AR in response to DHT was most pronounced in MCF7 cells (~14.7 fold), with TSUPr1, PC3, and 22Rv1 induction remaining slightly lower at ~9.6-, ~6.9-, ~10.0-fold, respectively. However, addition of cyclin D1 or cyclin D1-KE reduced AR transactivation capacity to basal levels in all cell types. Basal activity (in the presence of ethanol vehicle) remained unchanged as reported previously (18, 19). The G1 cyclin, cyclin E, was also examined as a previous report demonstrated that under specified conditions this protein serves as an AR coactivator (42). In our experiments, AR activity was only slightly enhanced in TSUPr1, MCF-7, and 22Rv1 cells. These data together demonstrate that unlike DAX-1, cyclin D1 is a potent AR repressor in multiple cell types, suggesting a highly conserved mechanism of repression.

In addition to cell type specificity of comodulators, promoter-specific effects have been noted for many AR coactivators and repressors. It is hypothesized that the AR binds differentially to AREs within promoters of target genes and that this may contribute to promoter and cell type-specific responses to androgens (43). Specifically, both ARIP3 and PIAS1 are AR coactivators known to enhance transcription from minimal AREs, yet ARIP3 (but not PIAS1) represses the probasin promoter (44). Herein, we determine that cyclin D1 potently represses AR activity in the context of androgen-dependent prostatic adenocarcinomas, monitoring endogenous PSA promoter activity (Fig. 1, A and B). To further examine the promoter specificity of AR inhibition by cyclin D1, we used two well-characterized and widely used androgen responsive promoters, MMTV and probasin. CV1 cells were transfected with expression plasmid encoding wild-type AR, MMTV, or probasin luciferase-based reporters and

![Fig. 2. Cyclin D1 is refractory to cellular context.](https://cancerres.aacrjournals.org)
either cyclin D1, cyclin D1-KE, or vector control (at a 3:1 ratio with the AR). Cells were stimulated, harvested, and monitored for phosphorylation site mutants of the AR [S81,94A (double mutant)] and S650A [34], reporter assays were performed as described in “Materials and Methods.” B. CV1 cells were transfected as in Fig. 2 with ERα, pBS3XERE-LUC, CMV-β-galactosidase, and vector or cyclin D1 at a 3:1 ratio. Cells were washed and stimulated as indicated for 24 h. Lysates were obtained and data collected and reported as in Fig. 2.

Fig. 3. Cyclin D1 demonstrates repressor activity across multiple AR target promoters. A. CV1 cells were transfected with MMTV or ARR2 (probasin) reporter constructs as described in Fig. 2 in the presence or absence of cyclin D1 or cyclin D1-KE at a 3:1 ratio with the AR. After stimulation with ligand as indicated, reporter assays were performed as described in “Materials and Methods.” B. CV1 cells were transfected as in Fig. 2 with ERα, pBS3XERE-LUC, CMV-β-galactosidase, and vector or cyclin D1 at a 3:1 ratio. Cells were washed and stimulated as indicated for 24 h. Lysates were obtained and data collected and reported as in Fig. 2.

Relative luciferase activity is reported with error bars representing SDs. Both the MMTV and probasin promoters were strongly activated by 0.1 nM DHT (~107.5- and ~252.8-fold, respectively) when compared with vehicle control (Fig. 3A). AR activity on each promoter was reduced significantly with the addition of cyclin D1 or cyclin D1-KE. Fold repression by cyclin D1 on the MMTV (14.5 fold) and probasin (8.9 fold) reporters was similar to our previous findings using the PSA promoter (10–12 fold repression; Refs. 18, 19). To further demonstrate the specificity of cyclin D1 corepressor activity for the AR, CV1 cells were transfected as in Fig. 3A with plasmids encoding ERα, pBS3XERE-LUC (an estrogen-responsive reporter), CMV-β-galactosidase, and cyclin D1 or vector. Transfected cells were treated with 10 nM 17β-estradiol, a natural ligand for ERα, or vehicle (Fig. 3B). Consistent with previous studies, at a 3:1 ratio with the receptor, cyclin D1 served as a coactivator for ERα activity enhancing relative luciferase activity from 6- to 15-fold (20–22). Thus, cyclin D1 fails to repress promoters driven by ERα activity but maintains its corepressor function on several androgen-responsive targets. Taken together, these data validate the ability of cyclin D1 corepressor activity to span multiple androgen-responsive promoters and distinguish it from other AR coactivators known to function in a promoter-specific fashion. Moreover, these data demonstrate an equal efficacy with regard to fold repression.

Phosphorylation of Major AR Residues Residing within the NH2 Terminus and DNA Binding Domain Does Not Regulate Cyclin D1 Corepression Activity. We previously demonstrated that cyclin D1 directly binds to the NH2 terminus of the AR to inhibit transactivation (19). In a cell type-independent fashion, it has also been noted that the NH2 terminus and hinge region of the AR have a high frequency of in vivo phosphorylation at specific sites, serines 81, 94 and 650 (34, 45). Because phosphorylation of the AR is hypothesized to play a role in receptor activation and prostate cancer progression, we examined the significance of these sites in cyclin D1-mediated AR repression (34, 45). Using previously described phosphorylation site mutants of the AR [S81,94A (double mutant) and S650A 34], reporter assays were preformed in CV1 cells using the PSA reporter as described for Fig. 2. Relative luciferase activity is reported with error bars representing SDs. In the presence of 0.1 nM DHT, activity of S81,94A and S650A was increased ~7.5- and ~18.0-fold, respectively (Fig. 4, A and B). Addition of cyclin D1 or cyclin D1-KE at a 3:1 ratio with AR phosphorylation site mutants completely abolished transactivation, returning relative luciferase activity to basal levels. Phosphorylation of the AR was originally hypothesized to regulate its turnover in vivo, but recent evidence suggests it may contribute to the modulation of AR stability and nuclear export, thus altering its activity (45). To verify expression of AR phosphorylation site mutants in the presence of ectopic cyclin D1, experiments were conducted in parallel wherein plasmid-encoding H2B-GFP (histone H2B tagged with GFP) was substituted for CMV-β-galactosidase. Cell lysates were prepared and subjected to SDS-PAGE followed by immunoblotting for both the AR and GFP. Expression of AR phosphorylation mutants was not altered by coexpression of cyclin D1 (Fig. 4C), indicating that the repression noted was not because of an increase in protein turnover or decrease in receptor production. These findings are consistent with our previously published data showing that cyclin D1 does not affect stability of the wild-type AR (19). Taken together, these data demonstrate that the phosphorylation state of these critical residues in the NH2 terminus and DNA binding domain of the AR does not affect cyclin D1 corepressor activity.

AR Polymorphisms and Tumor-derived AR Alleles Respond to Cyclin D1 Inhibition. We previously mapped cyclin D1 binding to the NH2 terminus of the AR (19). Within this region of the AR exist two polymorphic repeat domains, the polyglutamine and polyglycine repeats. Although the length of the glycine repeat appears to have little prognostic value, the number of NH2-terminal glutamine repeats has suggested clinical implications (46–48). Expanded repeats (>22) are found in patients with androgen-insensitivity syndrome, whereas individuals with contracted repeats (<22) harbor a putative increased risk for prostate cancer development (46, 48). Current evidence suggests that the contracted repeats result in a more active receptor (49, 50). To examine the ability of cyclin D1 to repress such polymorphic alleles, we used expression constructs encoding GFP-tagged polymorphic ARs with 0 and 48 glutamine repeats (Q0 and Q48, respectively; Ref. 33). Transfections were performed in CV1 cells as described in
Relative luciferase activity is shown (Fig. 5, A and B). As previously reported, ARs with an expanded number of glutamine repeats (Q48) displayed lower activity in the presence of 0.1 nM DHT than the contracted ARQ0 (compare Fig. 5A with Fig. 5B: /H11011 10.6-fold in comparison with /H11011 5.0-fold; Ref. 50). Addition of cyclin D1 at a 3:1 ratio with the AR did not affect basal transactivation in any of the polymorphisms and DHT-induced AR transactivation was completely inhibited. In parallel experiments wherein H2B-GFP expression plasmid was substituted for that encoding CMV-β-galactosidase, immunoblotting revealed that AR expression levels remained unchanged (Fig. 5C). Taken together, these data suggest that cyclin D1 does not require a modal number of NH2-terminal polyglutamine repeats to inhibit AR transactivation (Fig. 5, A and B) and that contracted alleles with enhanced activity are susceptible to cyclin D1 repressor function.

In addition to polymorphisms in the glutamine repeat domain of the AR, mutations within the ligand binding pocket of its COOH terminus have been demonstrated to play a role in prostate cancer development and progression (17). Several mutations within the ligand binding domain are selected for during prostate cancer therapy and result in promiscuous ligand binding and AR activation (17, 31). Given the frequency of AR mutation in late-stage prostate cancer, determining the efficacy of cyclin D1 on these tumor-derived alleles is of obvious importance. To achieve this goal, CV1 cells were transfected as in Fig. 2 with constructs encoding tumor-derived AR alleles (31, 51). Six AR ligand binding pocket mutant alleles were examined, all of which have been reported in recurrent prostatic adenocarcinoma (17, 51). The PSA61-LUC reporter was used as readout for AR activity and normalized as in previous experiments to β-galactosidase. In the presence of ligand (0.1 nM DHT), all mutants demonstrated a significant increase in transactivation of the PSA promoter (Fig. 6, A–F). Addition of cyclin D1 at a 3:1 ratio with the AR-reduced activity to basal levels, consistent with our observations using the wild-type receptor (Figs. 1–3). These data clearly demonstrate that cyclin D1 retains its AR corepressor activity even in the presence of clinically relevant ligand binding mutations known to play a role in prostate cancer progression.

**Cyclin D1 Is Dominant to Alternatively Activated AR.** Conformation of nuclear receptors is known to vary in the presence of differential ligands (52). The ligand molecular structure appears to dictate the position of helix 12 within the ligand binding domain of steroid hormone receptors (52). In this fashion, it is hypothesized that differential AR activity is produced in response to individual ligands. Specifically, it has been demonstrated that the potency and specificity of wild-type AR transactivation differs when activated with testosterone versus DHT (53). We previously showed that cyclin D1 is able to inhibit both methyltrienolone (R1881, a synthetic DHT analogue) and DHT-induced AR activity (18, 19). To test the efficacy of cyclin D1 on testosterone-induced AR transactivation, CV1 cells were transfected as in Fig. 2 and treated with either 0.1 nM testosterone or 0.1%
ethanol vehicle. Stimulation with 0.1 nM testosterone resulted in an 
~5-fold induction of luciferase activity over cells treated with vehicle 
alone (Fig. 7A). CV1 cells lack the enzyme to reduce testosterone to 
DHT and, therefore, PSA61-LUC induction observed was not because 
of metabolic conversion. In addition, decreased AR activity in 
response to testosterone versus DHT was expected because testosterone 
stimulation of the MMTV promoter is reduced in comparison with 
DHT (54). Addition of cyclin D1 or cyclin D1-KE inhibited testos-
terone-induced AR transactivation to basal levels, indicating that 
cyclin D1 corepressor activity is still functional when alternate AR 
natural ligands are used.

The transition of prostate cancer from androgen dependence to 
independence is hypothesized to involve adaptation of the receptor 
such that cytokines, low-level androgens, and growth factors can 
stimulate AR activity (14, 55). This theory is supported by data 
demonstrating that IL-6, EGF, IGF-I, and KGF activate the AR and 
can enhance transactivation driven by low-level androgen (15, 16, 56).
Indeed, high levels of growth factors and cytokines are found proxim-
al to late-stage prostate tumors (57–59). The precise mechanism by 
which the AR is activated through these alternate pathways has yet to 
be uncovered. To determine whether cyclin D1 can inhibit AR activity 
induced by these alternate mechanisms significant to prostate cancer 
progression, reporter assays were performed as described in CV1 cells 
transfected as described in Fig. 2 but after transfection cells were 
placed in 0.1% CDT serum to lower the amount of endogenous 
growth factors and cytokines available. In our system, treatment with 
EGF (50 ng/ml) and IGF (50 ng/ml) produced no significant induction of 
the PSA reporter and failed to synergize with low-level androgen 
(0.1 nM DHT; data not shown). This result is similar to that recently 
published by Ueda et al. (60). In the presence of 50 ng/ml IL-6, a 
slight but not significant increase (~1.5-fold over basal; \( P > 0.05 \)) in 
PSA61-LUC transactivation was observed, which was completely 
inhibited by cotransfection of cyclin D1 (Fig. 7B). IL-6, as previously 
reported, potentiated DHT-mediated AR transactivation, increasing 
PSA61-LUC activity from 10.5- to 16.3-fold (\( P < 0.01 \); Refs. 15, 60).
Cotransfection of cyclin D1 at a 3:1 ratio with the AR, however, 
completely inhibited the combined effect of these two ligands. These 
data imply that cyclin D1 remains functional, even in the presence of 
nonligand activators known to potentiate the transition from androgen 
dependence to independence in late-stage prostate cancers.

Lastly, nonconventional AR ligands are known to play a role in 
prostate cancer progression by acting to stimulate ligand binding 
pocket mutants of the AR in the absence of androgen. As such, 
mutations are found in 5–37% of recurrent prostate cancers, and 
regulation of inappropriate (in the absence of androgen) AR activity 
by cyclin D1 was examined (2). CV1 cells were transfected as in Fig. 
2 with plasmid encoding AR-T887A, a common ligand binding pocket 
mutant of the AR known to be responsive to both progesterone and 
17\( \beta \)-estradiol. As expected, 17\( \beta \)-estradiol- and progesterone-stimu-
lated activity of AR-T887A ~2.6- and 17.4-fold, respectively, in 
comparison to vehicle alone (Fig. 7C). However, addition of ectopic 
cyclin D1 at a 3:1 ratio with AR-T887A resulted in abrogation of AR 
activity in the presence of both steroids. These data additionally 
demonstrate the potency of cyclin D1 corepressor activity and suggest 
that novel therapeutics modeled after such repression would maintain 
their efficacy in androgen-independent prostate cancers.

**DISCUSSION**

Although many coactivators of the AR have been identified, far 
fewer corepressors have been established and well characterized. 
Because the regulation of AR activity is essential to the current 
treatment of prostate cancers as well as the future development of 
novel therapeutics, in-depth characterization of relevant AR corepres-
sors has obvious clinical relevance. In this study, we demonstrate that 
cyclin D1 is a potent AR corepressor, capable of inhibiting receptor
transactivation independent of cell type or AR target promoter analyzed. Cyclin D1 also maintains its corepressor activity on key phosphorylation-site mutants, polymorphisms, and tumor-derived AR alleles. Both ligand (testosterone, DHT, 17β-estriadiol, and progesterone) and nonligand (IL-6) activated AR complexes are repressed by cyclin D1 action. Taken together, our data suggest that cyclin D1 functions not only in androgen-dependent prostate cancers but also in the milieu of AR mutations, polymorphisms, and nontraditional activators predisposing to the development of androgen-independent cancers.

Cyclin D1 Repression Is neither Promoter nor Cell Type Specific. We previously demonstrated that cyclin D1 fully inhibits AR transactivation of the PSA61-LUC reporter in CV1 and C33A cells (18, 19). Recent studies suggest that AR-mediated gene transcription is influenced based upon the cell type and promoter examined (37, 43, 44, 61). Certainly, such differences are biologically essential because the response of AR-containing tissues to androgens is predicted to vary dependent on cellular context. Within each cell type, expression levels of transcription factors and AR accessory molecules are hypothesized to regulate receptor transactivation, leading to a diverse set of biological outcomes. Cell type specificity has also been recognized to modulate PSA promoter activity, wherein PC-3 cells have reduced transactivation in comparison with MCF-7 cells (62). The AR corepressor, DAX-1, also functions in a cell type-dependent manner as its activity diminishes in HeLa cells (50% inhibition) in comparison to that noted in the COS-7 (80% inhibition) cell type (37). It is important to note that specificity of DAX-1 action is observed at even higher repressor to receptor ratios than used in this study (10:1 versus 3:1). These findings lead to the hypothesis that cyclin D1 corepressor activity could also be regulated in a cell type specific fashion. We show that cyclin D1 inhibits ligand-dependent activity of both ectopic and endogenous AR in LNCaP cells (Fig. 1, A and B). Strikingly, this repression event occurs at a 0.91:1.00 molar ratio, indicating that even low levels of cyclin D1 are efficient at tempering AR activity (Fig. 1A). This result is in keeping with the model that androgen-dependent induction of cyclin D1 in LNCaP cells likely serves to regulate the rate of future cell cycle progression.

To assess the cell type specificity of cyclin D1, we examined AR transactivation of the PSA promoter in two additional cell types derived from androgen-independent cancers (PC-3 and 22Rv1) as well as those that were initially thought to be derived from a prostatic adenocarcinoma (TSUPr1) and thus used previously in the characterization of other AR comodulators. In addition, we examined the effect of cyclin D1 on AR activity in breast carcinoma because AR activity in this cell type is thought to contribute to tumor regression upon administration of medroxyprogesterone acetate as therapy (41). In all cell types, cyclin D1 maintained its corepressor activity, reducing AR transactivation to basal levels in 22Rv1, TSUPr1, PC-3, and MCF-7 cells (Fig. 2, A–D). Overall, cyclin D1 inhibition of the AR appears to be conserved in multiple cell types supporting its efficacy as an AR inhibitor.

In addition to cell type specificity, a number of AR comodulators also demonstrate promoter specificity. Both ARIP3 and Pias1 are AR coactivators known to enhance transcription from minimal AREs, yet ARIP3 (but not Pias1) represses the probasin promoter (44). N-C interaction of the AR is essential for both PSA and probasin promoter regulation but is not required for activation of MMTV and sex-limited protein (61). Binding of the AR to AREs on target promoters is sequence specific as the response element sequence dictates receptor binding affinity (43). Thus, examination of cyclin D1 corepressor activity on multiple gene promoters was essential to determine the specificity of its action in vivo. We previously demonstrated cyclin D1 inhibition of AR activity on the PSA promoter in the context of CV1 cells and data shown herein examine its repressor activity on the MMTV and probasin promoters (18, 19). Cyclin D1 effectively inhibited MMTV and probasin transactivation, consistent with our previous result using the PSA promoter (10–12-fold repression, Fig. 3A; Ref. 19). In addition, we demonstrate that cyclin D1 inhibition of androgen-responsive promoters is not because of general transcriptional inhibition as it enhances ERα transactivation of target genes as reported previously (Fig. 3B; Refs. 20–22). Finally, we show that transactivation of endogenous PSA in the presence of steroid is similarly reduced by ectopic cyclin D1 or dominant negative AR expression (Fig. 1B). These data indicate that cyclin D1 corepressor activity targets a wide array of characterized AR promoters.

The Inhibitory Action of Cyclin D1 Is Independent of NH2-Terminal AR Phosphorylation. The AR is a phosphoprotein with modification hypothesized to originate from upstream signal transduction cascades (34, 45, 60). A recent study demonstrated that in vivo phosphorylation of the AR at identified sites (other than at serine 308) has seemingly no effect upon its ability to transactivate target gene promoters (45). Instead, it is hypothesized that the stabilization and/or localization of the AR may be regulated through phosphorylation, yet...
no study, to date, has determined the exact mechanism by which this regulation may occur (45). With the finding that cytokines and growth factors can induce AR phosphorylation and are up-regulated in the vicinity of androgen-independent prostate cancer tumors, elucidation of the role of phosphorylation in AR signaling has become desirable (57–59). In vivo studies have indicated that at least two major phosphorylation sites (serines 81 and 94) exist in the NH2-terminal region of the AR and one in the hinge region (serine 650; Refs. 34, 45). As cyclin D1 binding requires the AR NH2 terminus and phosphorylation of the receptor may play a role in prostate cancer transition toward androgen independence, we examined the ability of cyclin D1 to inhibit PSA transactivation by phosphorylation site mutants of the AR (19). As previously noted, AR activity was not significantly modulated by phosphorylation site receptor mutants (Figs. 4, A and B). Addition of cyclin D1 or cyclin D1-KE, however, diminished transactivation of phosphorylation site AR mutants to basal levels (Fig. 4, A and B). In addition, cyclin D1 corepressor activity was not caused simply by the down-regulation of AR protein levels, as immunoblotting revealed in Fig. 4C. These data demonstrate efficacy for cyclin D1 irrespective of AR phosphorylation at serines 81, 94, and 650.

Cyclin D1 Inhibits Transactivation of AR Polymorphisms and Ligand Binding Pocket Mutants Implicated in Prostate Cancer Development and Androgen Independence. Two polymorphic regions exist within the AR NH2 terminus, the polyglutamine and polyglycine tracts (reviewed in Ref. 7). Polymorphisms within the polyglutamine repeat appear to be clinically insignificant, whereas expansion and contraction of the polyglutamine repeat is reported to result in significant biological outcomes (46–48, 63, 64). Polyglutamine expansion (40–62 repeats) is associated with Kennedy’s disease, dentatorubral and pallidolysiasin atrophy, and spinocerebellar ataxia (63, 64). Reduced androgen sensitivity because of repeat expansion is thought to result in the neurodegeneration in these diseases. Contraction of the polyglutamine repeat (<10 glutamines), however, is associated with increased AR activity, leading to a higher propensity to develop both benign prostatic hyperplasia and prostate cancer (47, 48). It has been reported that men harboring ARs with glutamine repeats fewer than 19 amino acids have a 52-fold greater risk of developing prostate cancer when compared with those with ≥25 glutamine residues (46). Shortened repeats were also shown to correlate with prostate cancer metastasis and higher mortality (48). Two hypotheses have been generated to explain the differential activity of glutamine repeat polymorphisms: (a) expansion of the polyglutamine repeat is known to result in the formation of AR aggregates, thus inhibiting its ability to transactivate target promoters; and (b) ARs with expanded polyglutamine repeats have reduced binding capacity for coactivators and may instead cause corepressor association (33). Evidence exists to support both of these hypotheses that appear to be inclusive, both contributing to the patient phenotypes observed. As we previously mapped the binding of cyclin D1 to the AR NH2 terminus (containing the polyglutamine repeat domain; Ref. 19), examination of its ability to serve as a corepressor on both expanded (Q48) and contracted (Q0) repeats was necessary to assess the efficacy of cyclin D1 as a corepressor. Our data indicate that cyclin D1 maintains its inhibitory activity on ARs of varying glutamine repeat length (Fig. 5, A and B). Using Western blot analysis we also demonstrate that cyclin D1 does not alter AR protein levels to down-regulate AR transactivation (Fig. 5C). These findings classify cyclin D1 as a potent AR corepressor, capable of inhibiting receptor activity irrespective of glutamine repeat polymorphisms.

Androgen ablation therapy, although initially effective, appears to select for a population of tumor cells adept to growing in an androgen-independent fashion. One method by which prostate tumors may subvert androgen dependence is through mutation of the AR itself. In fact, mutation of the AR has been reported to occur in between 5 and 37% of prostate cancer patients, with the higher rates being documented in patients treated with multiple antiandrogen therapies (reviewed in Ref. 65). Mutation of the AR ligand binding domain yields receptors responsive to 17β-estradiol (T877A/S and H874Y), progesterone (T877A/S, H874Y, and V715M), adrenal androgens (V715M, H874Y, and T877S), cortisol (H874Y and T877A) and even antiandrogens such as hydroxyflutamide (T877A/S, V715M, and H874Y), which is used in the treatment of advanced prostate cancers (17, 65). AR mutations at codons 890 (D to N) and 902 (Q to R) have also been identified in flutamide-treated patients (31, 51). Hydroxyflutamide failed to stimulate transactivation of the 890 and 902 mutants, but the efficacy of other alternative ligands has yet to be examined (51). Because ligand binding domain mutations are frequent in androgen-independent prostate cancers, we examined the efficacy of cyclin D1 inhibition of clinically relevant alleles. In all reporter assays wherein AR mutants examined (V715M, A721T, H874Y, T877S, T877A, D890N, and Q902R) cyclin D1 served as a potent inhibitor of AR activity, reducing PSA or probasin transactivation to basal levels (Figs. 1A and 6, A–F). Moreover, activation of AR-T877A by 17β-estradiol or progesterone was ablated by cyclin D1 (Fig. 7C). These results together suggest that cyclin D1 will repress the activity of mutant ARs frequently arising during the transition of prostate cancer tumors to androgen independence.

Cyclin D1 Represses AR Activity Regardless of Ligand Activator. It is known that AR response to individual ligands results in differential gene expression. Previous studies demonstrated that testosterone bound AR exhibits differential regulation of AREs (53, 54). Specifically, DHT was more potent in stimulating MMTV-driven promoters, whereas testosterone bound AR showed increased activity on a multimerized ARE site (53). In addition, binding of individual ligands to the AR is known to trigger specific receptor conformations. For example, DHT binding to the AR causes helix 12 closure over the AR ligand binding pocket, whereas binding of the DHT analogue, R1881, results in a bipartite helix 12 conformation (66). Because the AR is seemingly activated through multiple mechanisms within the prostate cancer patient and the type of activator can modulate AR conformation and activity, we examined the ability of cyclin D1 to inhibit AR activity in the presence of testosterone. Our data indicate that cyclin D1 maintains repression of the AR even in the presence of testosterone (Fig. 7A). In addition, we previously demonstrated that AR activation by R1881 and DHT is inhibited by ectopic cyclin D1 expression (18). Taken together, these data verify the efficacy of cyclin D1-mediated inhibition of the AR in the presence of multiple natural ligand activators.

As discussed above, androgen ablation therapy is thought to select for cancer cells with the ability to grow independently of androgen, yet analysis of these tumors reveals that the AR remains expressed and active (14). Multiple mechanisms have been proposed for overcoming androgen dependence, including mutation of the AR ligand binding domain, amplification of the receptor gene, and activation by nonsteroids (14, 55). Growth factors and cytokines are up-regulated in the milieu of androgen-independent tumors (57, 59). This observation led to the hypothesis that these nonsteroids stimulate AR activity possibly through triggering of AR phosphorylation downstream of specific signal transduction pathways. Previous studies have demonstrated AR activation by EGF, IGFI-I, KGF, and IL-6 (15, 16). As inhibition of androgen-independent AR activity is a major target for the development of novel prostate cancer therapies, we examined the ability of cyclin D1 to inhibit EGF-, IGFI-I, and IL-6-induced AR transactivation. In our hands, EGF and IGFI-I failed to stimulate AR-mediated transactivation of the PSA promoter (data not shown). IL-6, however, slightly but not significantly enhanced AR activity
alone (P > 0.05) and potentiated low-level DHT activity (P < 0.01; Fig. 7B). AR activation by both IL-6 alone and IL-6 plus DHT was completely inhibited by coexpression of cyclin D1. This finding indicates that cyclin D1 inhibition is dominant to nonligand activators and additionally supports its efficacy in inhibiting androgen-independent AR activity.

In summary, cyclin D1 is a uniquely potent co-repressor of the AR with broad specificity for ablation of ligand-dependent transactivation. Cyclin D1 maintains its co-repressor activity independently of cell type, promoter, and agonist examined. Its ability to inhibit clinically relevant polymorphisms and mutations suggests that analogues of cyclin D1 action may be useful in the treatment of initial (androgen dependent) and recurrent (androgen independent) prostate cancers. Taken together, our data provide the impetus to examine the in vivo effects of cyclin D1 on both androgen dependent and independent tumors.

ACKNOWLEDGMENTS

We thank Drs. Steven Balk, Sohaib Khan, Erik Knudsen, and both Knudsen laboratories for their critical reading of the manuscript. We also thank Dr. Randal Morris for his advice concerning statistical analyses.

REFERENCES


