D-Type Cyclins Complex with the Androgen Receptor and Inhibit Its Transcriptional Transactivation Ability

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Abstract

type cyclins regulate distinct cellular processes, such as mitotic cell cycle control, differentiation, and transcription. We have previously shown that the t-type cyclins are critical for the androgen-dependent proliferation of prostate cells. Here, we sought to determine whether cyclin D1 directly influences the transactivation potential of the androgen receptor, a transcription factor that strongly influences androgen-dependent proliferation. We found that ligand-mediated transcriptional activation of a physiological target, prostate-specific antigen, by the androgen receptor was inhibited by cyclins D1 and D3. The ability of t-type cyclins to inhibit androgen receptor transactivation was not shared with other cyclins, and cyclin D1 was as effective as dominant negative mutants of the androgen receptor in inhibiting transactivation. This function of cyclin D1 was independent of its role in cell cycle progression and is likely elicited through its ability to form a specific complex with the androgen receptor. These data underscore the various mechanisms through which the androgen receptor is regulated and also point to a negative feedback role for cyclin D1 in controlling androgen-dependent growth.

Introduction

Multiple cellular functions have been proposed for the t-type cyclins, including control of cell cycle regulation, differentiation, and transcriptional regulation (1–4). Of these, the role of t-type cyclins in cell cycle control is the best characterized. The t-type cyclins (cyclins D1, D2, and D3) bind and activate the CDKs (CDK4 and CDK6 (1). In turn, active cyclin D/CDK4(6) complexes phosphorylate the retinoblastoma tumor suppressor protein (RB). Overt activation of cyclin D1 and/or CDK4 is observed in many tumor cells (1, 5) and contributes to their uncontrolled proliferation. Similarly, overexpression of cyclin D1 in normal cells accelerates the G 1 phase of the cell cycle (6).

Recent evidence also points to a direct role for t-type cyclins in transcriptional activation and repression. For example, it has been shown that cyclin D1 inhibits transcriptional activation by the DMP1 transcription factor (4). Conversely, a multitude of evidence now shows that cyclin D1 binds the estrogen receptor and activates its transactivation potential in a ligand-independent manner (2, 3). In both cases, the influence of cyclin D1 on transcription has been shown to occur independently of its role in cell cycle progression, thus defining a novel function for t-type cyclins as transcriptional regulators.

Prostatic epithelial cells are dependent on specific steroidal hormones for proliferation, and this dependency is commonly lost in prostatic adenocarcinoma cells (7, 8). Growth of normal prostate cells is dependent on the presence of androgen, which elicits its biological activity via the androgen receptor, a member of the nuclear steroid hormone receptor family (9). DHT is a high-affinity ligand for the androgen receptor, and upon DHT binding, the transcriptional transactivation potential of the androgen receptor is activated (9). Upon activation, the androgen receptor stimulates the transcription of a number of target genes. Although many of these target genes have yet to be identified, the best characterized to date is that encoding PSA (9). PSA expression correlates well with active androgen receptors and the growth of prostate cells and, as such, is used clinically as a measure of prostate growth (10). We have previously shown that t-type cyclins play a key regulatory role in the androgen-dependent proliferation of prostate cells (11). To test whether t-type cyclins influence the transactivation potential of the androgen receptor, we investigated the effects of t-type cyclins on the androgen receptor using the PSA promoter as a reporter of androgen receptor function. Surprisingly, we found that t-type cyclins repress activation of androgen receptor-mediated transcription and do so in a direct, cell cycle-independent manner. These findings may explain the low frequency of cyclin D1 amplification in prostatic adenocarcinomas.

Materials and Methods

Cell Culture and Conditions. Twenty-four to 48 h prior to transfection, CV1 and SAOS-2 cells were cultured in a 5% CO 2 incubator in phenol red-free DMEM supplemented with 10% charcoal dextran-treated fetal bovine serum (CDT; HyClone Laboratories, Logan, UT), 2 mM t-glutamine, and 100 units/ml penicillin/streptomycin (Mediatech, Herndon, VA).

Transcriptional Activation Assays. CV1 and SAOS-2 cells were transfected with the indicated plasmids using the X, N-bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid/calcium phosphate method (12) in 60-mm dishes using 8 μg of total DNA per transfection. Approximately 16 h posttransfection, cells were washed with PBS and replaced in phenol red-free DMEM-10% charcoal dextran-treated fetal bovine serum with or without 0.1 nM R1881 (DuPont/NEN, Boston, MA), a DHT analogue. Approximately 30 h later, cells were harvested and luciferase activity was monitored using commercially available reagents (Promega, Madison, WI). β-Galactosidase activity was also monitored as an internal control for transfection efficiency.

Plasmids. The CMV-β-galactosidase, CMV-CDK4, and CMV-cyclin A constructs were gifts of Dr. Jean Wang (University of California at San Diego, La Jolla, CA). The pAR0 human androgen receptor construct was a gift of Dr. A. O. Brinkmann (Erasmus Universiteit, Rotterdam, the Netherlands), and the pSG5-AR androgen receptor expression construct was a gift of Dr. Chawns Chang (University of Rochester, Rochester, NY). The PSA-LUC reporter construct, which contains 6.1 kb of the human PSA promoter, was a gift of Dr. Kitty Cleutjens (Erasmus Universiteit; Ref. 13). The dominant negative androgen receptor constructs pSG5-rAR.D46–408 and pSG5-rAR.A38–296 were provided by J. Palvimo and O. Janne (University of Helsinki, Helsinki, Finland; Ref. 14). The RSV-cyclin D1 and RSV-cyclin D3 constructs were kindly provided by Dr. C. Sherr (St. Jude Children’s Research Hospital, Memphis, TN). The pRcCyclin D1-GH and pRcCyclin D1-KE constructs were gifts of Dr. Robert Weinberg (Whitehead Institute for Biomedical Research, Cambridge, MA). The cyclin E expression construct (expressed from a long
terminal repeat) was obtained from Dr. J. Roberts (Fred Hutchinson Cancer Research Center, Seattle, WA).

**Immunoprecipitation and Immunoblots.** CV1 cells were cultured in 10-cm dishes and cotransfected with 8 μg of pAR0 and 8 μg of RSV-cyclin D1. Approximately 48 h posttransfection, cells were harvested and lysed in NETN [20 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA (pH 8.0), and 0.5% NP40] supplemented with 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml 1,10-phenanthroline, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 mM sodium fluoride, 1 mM sodium vanadate, and 60 mM β-glycerophosphate. Following brief sonication and clarification, lysates were subjected to immunoprecipitation with antibodies against the androgen receptor and cyclin D1, following transfer to Immobilon (Millipore), the membrane was split at the M, 50,000 marker, the top half was probed using the anti-androgen receptor antibody, and the lower half was probed using the antisera against cyclin D1. For immunoprecipitates from cells transfected with cyclin D1 and the androgen receptor, following transfer to Immobilon (Millipore), the membrane was split at the Mr 50,000 marker, the top half was probed using the anti-cyclin D1 antibody. From immunoprecipitates from cells transfected with cyclin A and the androgen receptor, following transfer to Immobilon (Millipore), samples were run in duplicate to probe for cyclin A and the androgen receptor. Proteins were visualized using horseradish peroxidase-conjugated protein A (Bio-Rad) and enhanced chemiluminescence (Amersham).

**Results**

**Cyclin D1 Represses Androgen Receptor Activity.** The 12-type cyclins have been shown to play a key regulatory role in the androgen-dependent proliferation of prostate cells (11). To determine whether cyclin D1 affects the transactivation potential of the androgen receptor, initial reporter assays were performed using CV1 cells. These cells were chosen as they are nontumorigenic and express no measurable androgen receptor or androgen receptor activity (data not shown). The androgen receptor was introduced by transfection into CV1 cells using one of two wild-type androgen receptor expression constructs, pAR0 or pSG5-AR. To activate ectopically expressed androgen receptors when appropriate, we treated transfected cells with R1881, a potent ligand of the androgen receptor. To monitor androgen receptor activation, the PSA61LUC reporter construct was used. PSA61LUC was constructed using 6.1 kb of the PSA promoter cloned upstream of luciferase (13). Transcription from the PSA promoter is activated by ligand-bound androgen receptors in vivo, and the promoter contains at least three consensus androgen-responsive elements. Activation of PSA61LUC in the presence of R1881 was measured by normalizing luciferase activity to β-galactosidase activity as an internal control for transfection efficiency.

In CV1 cells, introduction of PSA61LUC resulted in only low-level background luciferase activity in the presence or absence of R1881 (Fig. 1A), as was expected, based on our observation that no endogenous androgen receptor can be detected in these cells (data not shown). These results also confirm that activation of the PSA promoter is specific to introduction of an activated androgen receptor. Likewise, CV1 cells transfected with the androgen receptor (pAR0 or pSG5-AR) but maintained in a steroid-free environment also demonstrated only low-level PSA61LUC activity (Fig. 1A). By contrast, cells transfected with the androgen receptor and empty vector RcRsv, and treated posttransfection with R1881 demonstrated a significant (5-10-fold) increase in PSA61LUC activity. Strikingly, we observed that cotransfection of RSV-cyclin D1 at a 3:1 ratio with the androgen receptor resulted in a dramatic inhibition of PSA61LUC activity (Fig. 1A). This reduced androgen receptor transactivation potential was observed with both the pAR0 and pSG5-AR constructs. Interestingly, cyclin D1 specifically inhibited the ligand dependent activity of PSA61LUC and not the basal activity.

To verify these observations, titration experiments were performed, wherein cells were transfected with constant amounts of total plasmid at 1:0, 1:1, 1:3, or 1:5 ratios of androgen receptor to cyclin D1. As observed in Fig. 1B, cyclin D-mediated inhibition of androgen receptor activation could be observed at a 1:1 ratio (2-fold decrease), was more pronounced at a 1:3 ratio (3-fold decrease), and forced measurable PSA61LUC activity to below background at a 1:5 ratio.

To determine whether this transcriptional modulation was specific to cyclin D1 or whether it was a function of cyclins in general, CV1 cells were cotransfected with cyclin D1, E, or A and the lower half was probed using the anti-cyclin D1 antibody. As shown in Fig. 1C, PSA61LUC activation was not significantly affected by cotransfection with cyclin E or A but was inhibited by cyclin D1. All cyclin constructs have been previously shown to encode functional cyclin gene products (11). Therefore, inhibition of androgen receptor transactivation potential was not mediated by cyclin E or cyclin A but was mediated by cyclin D1. To determine whether this function of cyclin D1 was common to the 12-type cyclin family, similar experiments were performed using cyclin D3. As shown in Fig. 1D, cyclin D3 was also capable of inhibiting PSA61LUC activation, albeit to a lesser extent than cyclin D1. These results indicate that the ability to inhibit androgen-mediated receptor transactivation potential was shared by specific members of the 12-type cyclin family.

Several classes of proteins have been reported to repress the transactivation function of the androgen receptor. Among these are dominant negative androgen receptor proteins, which lack transactivation potential and prevent transactivation from wild-type receptors by heterodimerization (14). We compared the transcriptional antagonistic activities of two such mutant proteins, ARΔ38—296 and ARΔ46—408, with that exhibited by cyclin D1. At equivalent ratios of androgen receptor:effector, all three inhibitory proteins reduced PSA61LUC activation to just above background levels (Fig. 2). Therefore, transcriptional inhibition associated with cyclin D1 was similar to that observed using known dominant negative androgen receptor proteins.

**Transcriptional Repression Is Independent of the Cell Cycle Function of Cyclin D.** 12-type cyclins play a significant role in cell cycle progression, and activation of CDK4 complexes is imperative in normal cells for progression into S phase. To address whether the transcriptional inhibition function of cyclin D1 is related to its role in cell cycle progression, several approaches were used. First, simple reporter assays using the androgen receptor and cyclin D1 were performed in SAOS-2 cells. It has been shown that the only cell cycle function of CDK4/cyclin D complexes is to phosphorylate and inactivate RB (15). Because SAOS-2 cells do not express functional RB (16), they have lost the requirement for CDK4/cyclin D, and unlike normal cells, overexpression of CDK4 and/or cyclin D in this cell line has no effect on cell cycle progression. Although these cells are refractory to the cell cycle effects of cyclin D1, androgen receptor transactivation potential was still repressed in these cells by a 3:1 ratio of cyclin D1 to androgen receptor (Fig. 3A). These data indicate that this attribute of cyclin D1 was independent of its role in cell cycle progression.

To test this hypothesis, mutants of cyclin D1 were used that either lost the ability to bind to CDK4 (mutant CycD-KE) or to RB (mutant CycD-GH; Ref. 17). As can be seen in Fig. 3B, these mutants of cyclin D1 were able to suppress PSA61LUC activation as effectively as wild-type cyclin D1, again suggesting that this function of cyclin D1 was independent of its cell cycle role. As such, it would be predicted that cyclin D1 may bind to proteins other than CDK4 to carry out this function, and so CDK4 could potentially then compete for this function of cyclin D1. To test this, we cotransfected CDK4 along with cyclin D1 and the androgen receptor (3:3:1 ratio, respectively) and monitored reporter activity. Strikingly, cotransfection of CDK4 was able to partially restore the PSA61LUC activation that was inhibited in the presence of cyclin D1 alone (Fig. 3C). In addition, cotransfection of dominant-negative, kinase-defective CDK4 also partially restored PSA61LUC activation. Taken together, the data in Fig. 3 suggest that...
cyclin D1 inhibited androgen-mediated receptor transactivation potential in a cell cycle-independent manner. Moreover, the data in Fig. 3C suggest that the cyclin D1 complexes required for transcriptional squelching may be distinct from CDK4-containing complexes.

**Cyclin D1 Interacts Directly with the Androgen Receptor.** Because of these observations and because of the functional relationship we observed between cyclin D1 and the androgen receptor, we questioned whether these two proteins may physically interact. To test this, we cotransfected CV1 cells at equal ratios with androgen receptor and cyclin D1 expression plasmids. Transfected cells were lysed, and protein complexes were recovered using antisera generated against either cyclin D1, the androgen receptor, or MDM2 (negative control). Immunoprecipitated complexes were subjected to SDS-PAGE and immunoblotted for either the androgen receptor or cyclin D1. As can be seen in Fig. 4A, complexes immunoprecipitated using anti-cyclin D1 antisera contained both cyclin D1 and the androgen receptor. Conversely, complexes immunoprecipitated using anti-androgen receptor antisera contained both the androgen receptor and cyclin D1 proteins. Lysates immunoprecipitated using anti-MDM2 antisera failed to recover either cyclin D1 or the androgen receptor. To further verify the specificity of this interaction, CV1 cells were cotransfected with expression constructs for cyclin A and the androgen receptor, and similar immunoprecipitation experiments were performed. As can be seen in Fig. 4B, immunoprecipitates recovered using anti-androgen receptor antisera contained only the androgen receptor protein and not cyclin A. Conversely, immunoprecipitates recovered using anti-androgen receptor antisera contained only the cyclin A protein and not the androgen receptor. Lysates immunoprecipitated using anti-cyclin E antisera also failed to recover either cyclin A or the androgen receptor (Fig. 4B). Thus, cyclin D1 forms a specific complex with the androgen receptor.

**Discussion**

The data presented here demonstrate a new function for cyclin D1 in attenuating the transactivation potential of the androgen receptor. Cyclin D1 inhibited transactivation from an androgen receptor-
specific reporter, PSA61LUC, in a dose-dependent manner (Fig. 1). The intensity of androgen receptor transcriptional inhibition was comparable to that observed with known dominant negative androgen receptors (Fig. 2). The ability to inhibit PSA61LUC transactivation was shared with cyclin D3, but not with cyclin E or cyclin A (Fig. 1). Interestingly, the ability of cyclin D1 to limit androgen receptor transactivation potential was shown to be independent of the cell cycle role of cyclin D because transcriptional repression was observed in cells that are refractory to the growth-promoting effects of cyclin D1 and with mutants of cyclin D1 that are defective for binding to CDK4 or RB (Fig. 3). Also, the effect of cyclin D1 on PSA61LUC transactivation could be partially competed by overexpression of CDK4 or kinase-defective CDK4 (Fig. 3). Finally, we observed a specific, direct interaction between cyclin D1 and the androgen receptor, suggesting that the effect of cyclin D1 on androgen-dependent transactivation is direct (Fig. 4).

Our finding that cyclin D1 inhibits the transactivation potential of the androgen receptor directly contrasts with its ability to activate another steroid hormone receptor, the estrogen receptor, in a ligand-independent manner (2, 3). The finding that factors/proteins that act as a coactivators for one nuclear receptor act as corepressors for another receptor is not unprecedented. For example, the coactivator SRC-1 enhances transcriptional activity of many steroid hormone receptors (e.g., estrogen, progesterone, and glucocortocoid receptors) but represses transcriptional activity of the androgen receptor (18). Breast epithelial cells are dependent on estrogen for growth and, consequently, antiestrogens (e.g., 4-hydroxytamoxifen) are relied upon to treat breast carcinoma (19). Because amplification or overexpression of cyclin D1 is observed in >50% of breast carcinomas, this may provide the means for breast cancer cells to bypass the estrogen requirement (5, 20). Consistent with this and unlike estrogen-mediated activation, cyclin D1-induced activation of the estrogen receptor is only slightly perturbed by 4-hydroxytamoxifen (2, 3). These studies underscore the importance of understanding how hormone-dependent cells regulate the requirement for steroids and how cancer cells evade this requirement.

The studies presented here point to an important role for cyclin D1 in regulating the transactivation potential of the androgen receptor. We have previously shown that, in LNCaP cells, which are dependent on androgen for growth, a decrease in α-type cyclin expression is observed upon androgen withdrawal (11). Conversely, such cells cultured in the presence of androgen demonstrate high levels of α-type cyclin expression and associated kinase activity (11). On the basis of the studies presented here, we propose that a negative feedback loop may exist in androgen-dependent prostate cells, wherein ligand-dependent activation of the androgen receptor results in stimulation of cyclin D expression. This net increase in cyclin D would promote cell cycle progression but may also act to attenuate the transcriptional activity of the androgen receptor, thus modulating the rate of future cell cycle...

Fig. 2. Cyclin D-dependent transcriptional inhibition is similar to that observed with dominant negative androgen receptors. CV1 cells were transfected as described in the legend to Fig. 1 with the plasmids/ratios indicated. Constructs pSG5-rARΔ46–408 and pSG5-rARΔ38–296 encode known dominant negative AR proteins.

Fig. 3. Transcriptional inhibition is independent of the cell cycle function of cyclin D. A, inhibition of androgen receptor transactivation potential is observed in SAOS-2 cells, which are resistant to the growth-promoting activity of cyclin D. SAOS-2 cells were transfected as in Fig. 1. B, mutants of cyclin D1 that have lost specific cell cycle functions of cyclin D1 retain the ability to repress androgen receptor transactivation potential. CV1 cells were transfected as in Fig. 1 using wild-type cyclin D1, cyclin D1-GH (defective in RB binding), or cyclin D1-KE (defective in binding or activation of CDK4) at the ratios shown. C, androgen receptor transactivation potential can be partially restored by cotransfection with CDK4. CV1 cells were cotransfected as in Fig. 1 with constructs expressing the androgen receptor, cyclin D1, and either wild-type CDK4 or a kinase-defective CDK4, CDK4-K35M, at the ratios given.
progression. This hypothesis may explain the biphasic response of prostate cells to androgen (21), wherein androgen-dependent prostate cells undergo growth arrest either in the absence of androgen (i.e., in the absence of cyclin D) or in the presence of excess androgen (i.e., in the presence of excess cyclin D).

Deregulation of cell cycle control is a common component of cancer. Specifically, the RB/cyclin D1/p16ink4a pathway is targeted for disruption in >60% of studied tumors (1, 22). Typically, the mutations that target this pathway are tumor type specific (1, 5, 23). For example, in small cell lung carcinoma, RB is commonly inactivated, whereas in non-small cell lung carcinoma, p16ink4a is lost. The basis behind this specificity is largely not understood. The finding that cyclin D1 synergizes with the estrogen receptor indicates that the frequent amplifications of cyclin D1 in breast cancer confer a specific growth advantage both through ubiquitious activation of CDK4 and through activation of the estrogen receptor. Our finding that cyclin D1 subverts androgen receptor signaling provides an explanation for why cyclin D1 amplifications are not commonly observed in prostate carcinomas, whether they are androgen-dependent or androgen independent (24). In contrast, inactivating mutations of RB are frequently observed in human prostate carcinomas.

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References

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