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Downregulation of Cyclin D1 Alters cdk 4- and cdk 2-Specific Phosphorylation of Retinoblastoma Protein

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Progression of cells through the G1 phase of the cell cycle requires the assembly and activation of specific cyclin:cyclin-dependent kinase (cdk) complexes in a tightly regulated, sequential fashion. To more clearly define the temporal events leading to the G1/S transition, sequential changes in the expression of cyclin E and cdks 2, 4, and 6, as well as the phosphorylation of the retinoblastoma protein (pRb), were assayed in RA28 cells, a variant of human colon cancer RKO cells which were modified by transfection of an ecdysone-inducible antisense (AS) CD1 expression system. Induction of cyclin D1 antisense mRNA by the ecdysteroid, ponasterone A, resulted in a 55% decrease in cyclin D1 mRNA and a 58% decrease in CD1 protein levels. There was a 2.4-fold decrease in the ratio of hyperphosphorylated pRb (ppRb) to hypophosphorylated pRb, as well as a 60–75% decrease in cdk 2- and cdk 4-specific phosphorylated pRb proteins. Of interest, cyclin E-dependent phosphorylation (cdk2) decreased 2.5-fold at 3 h despite only a 30% decrease in cyclin E protein level. Levels of cdk 2, cdk 4, and cdk 6 decreased 40–70%, while levels of cyclin A and B were unaffected by induction of CD1 antisense. Induction of a CD1 antisense gene in a human colon cancer cell line resulted in rapid, concomitant changes in CD1 mRNA and protein, cyclin E, cdk2, cdk4, and cdk6, as well as the ratio of ppRb to pRb. In this system, growth regulatory events are tightly regulated and the perturbed expression of a single protein, CD1, rapidly alters expression of multiple regulatory proteins involved in the G1/S transition phase of cell cycle progression. **© 2000 Academic Press**

The molecular events controlling the G1 phase of the cell cycle are determined by a series of phosphorylation events that are regulated by the expression of specific cyclins, cyclin-dependent kinases (cdks), and cdk inhibitors. The D-type cyclins, which regulate progression through the early portions of G1, exert their effects by the formation of a holoenzyme complex with either cdk4 or cdk6, which is subsequently activated by cdk activated kinase (CAK) containing cyclin H and cdk7 proteins (1, 2). Entry into the G1/S transition appears to also be controlled by the actions of cyclin E and its catalytic partner cdk2, which are synthesized later than D-type cyclins and peak later in the G1 phase (3, 4).

The expression and activity of cyclin D1 are intimately related to the expression and activity of the retinoblastoma protein (pRb). Typically, the activated cyclin D/cdk 4 or cdk 6 complex phosphorylates pRb, releasing it from its growth-suppressive functions by altering its binding to the E2F family of transcription factors. The activated cyclin E/cdk 2 complex also contributes to the phosphorylation of pRb late in G1 and appears to cooperate with cyclin D1 in this regulatory function (5, 6). Activation of E2F results in increased transcriptional activity and loss of the repressive function of the pRB/E2F complex, with subsequent transcription of the genes necessary for progression into S phase (7–9).

The timely regulation of cyclin D1 is important for normal cell-cycle progression. In normal cells, overexpression of exogenous cyclin D1 results in acceleration of the cell cycle due to a decrease in the length of the G1 phase (10). In contrast, antisense cyclin D1 expression has been shown to be an effective inhibitor of growth in some tumor cell lines, including osteosarcoma (11), esophageal carcinoma (12), colon carcinoma (13), and lung adenocarcinoma (14). We, therefore, postulate that an inducible antisense system would be useful for defining the precise role of cyclin D1 in the cell growth regulatory process. The ecdysteroid hormones are ideal inducers in such an application because they are only active in the presence of the ecdysone receptor, which is not expressed in mammalian cells. In addition, ec-

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dysteroids are neither toxic, teratogenic, nor known to affect mammalian physiology. The short half-life associated with these compounds allows for precise and potent inductions, and favorable pharmacokinetics prevent drug accumulation and expedite clearance (15). The ecdysone-inducible system also has the advantage of providing selective expression in cells in which the ecdysone receptor has been transfected (15–18). We have previously employed this inducible system to overexpress cyclin D1 protein levels in different mammalian cell lines (19, 20).

Our data demonstrate that the ecdysone analog, PA, tightly regulates the stable expression of antisense cyclin D1 in the human colon carcinoma RKO cell line (RA28 cells). This regulation results in decreased levels of endogenous cyclin D1 mRNA and protein at 3 h after PA induction. Changes in cyclin E, cdk 2, cdk 4, cdk 6, as well as in the ratio of ppRb to pRb, suggest that RA28 cell growth is tightly regulated and that the perturbed expression of CD1 rapidly alters expression of other regulatory proteins involved in the G1/S transition phase of cell cycle progression. Furthermore, we have shown that cyclin D1 expression is coordinated with the expression of cyclin E, confirming previous data that the activities of these two growth control proteins are intimately linked.

MATERIALS AND METHODS

Cell lines. The human colon carcinoma cell line RKO1 (ATCC, Rockville, MD) and its derivatives were grown in RPMI 1640 medium supplemented with 1% penicillin/neomycin/streptomycin (PNS) antibiotic mixture (GibcoBRL, Grand Island, NY) and 10% fetal bovine serum (GibcoBRL). RKO cells expressing the antisense cyclin D1 gene (RA28 cells) were transfected with separate plasmids bearing the ecdysone receptor (pVgRXR) and the antisense cyclin D1 gene (pINDCD1AS). Control cells (RV-1) were transfected with pVgRXR and the empty vector pIND. Transfected cells lines were maintained in the above media with the addition of 150 μg/ml zeocin and 360 μg/ml G418 sulfate. All cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Antibodies. Anti-pRb-Ser-780 (169) antibody for the cyclin D1/cdk4-specific phosphorylated (serine 780) form of the pRb protein, and anti-pRb-Thr-356 (172) antibody for the cyclin E/cdk2-specific phosphorylated (threonine 356) form of the pRb protein were kindly supplied by Dr. Yoichi Taya (21). The anti-ecdysone receptor antiserum was generously provided by Dr. Michelle Arbeitman (22). Anti-RXRα (D-20), anti-CD1 (R-124), anti-cyclin E (HE-12), anti-cyclin A (BF-683), anti-cyclin B (GNS1), anti-cdk 2 (D-12), anti-cdk 4 (C-22), anti-cdk 6 (C-21), and anti-pRb (IF8) antibodies were obtained from Santa Cruz Biotechnology Inc., Santa Cruz, CA.

Construction of an inducible antisense cyclin D1 expression system. The entire 1.1 kb human cyclin D1 cDNA coding sequence was subcloned in its antisense (CD1AS) orientation into the EcoRI site of a modified pIND expression vector (Invitrogen Corp., Carlsbad, CA). This vector contains a 5× E/GRE enhancer and the Hsp promoter linked to multiple cloning sites as well as the G418 resistance gene (neo-). The E/GRE enhancer is a hybrid DNA sequence that contains half of the ecdysone response element and half of the glucocorticoid response element (15, 18). It is located immediately upstream of the *Drosophila* minimal heat shock promoter (P-Hsp) on pIND. The vector, pVgRXR (Invitrogen), constituitively expresses the RXRα and the VgEcR receptor subunits which then interact to form a functional ecdysone receptor (VgEcR). The gene for zeocin resistance and the viral transcriptional activator, VP16, are also contained within pVgRXR (16). RXRα and VgEcR both contain DNA-binding domains (DBD) that share in the recognition of a specially constructed *cis*-acting regulatory element, the E/GRE (17). The ecdysone-responsive promoter is located on the second vector, pIND, and ultimately drives expression of antisense cyclin D1 cDNA.

Transfection and selection procedure. The control cells (RV-1) were established by transfecting RKO1 cells first with pVgRXR and then with pIND, using a standard electroporation procedure with the Gene Pulser II Electroporation System (Bio-Rad, Hercules, CA). The plasmids were transfected separately at 350 V and 250 μF capacitance (23). Electroporation efficiency was determined by transfection of a marker plasmid (green fluorescent protein, hGFP). Following electroporation of pVgRXR, transfected RKO cells were selected in complete RPMI medium containing 300 μg/ml of zeocin (Invitrogen) for 2 weeks. Individual drug resistant clones were randomly selected for clonal expansion and then verified with PCR, E/GRE-luciferase assay, and Western blot analysis for EcR construct integration, induction potentiality, expression regulation of EcR, RXRα, and luciferase reporter genes in the presence of ponasterone A. Following EcR clone characterization, pVgRXR positive cells were transfected with either pIND (RV-1 cells) or pIND/ASCD1 (RA28 cells containing the CD1 antisense sequence), and were further selected in complete RPMI medium containing 300 μg/ml zeocin and 500 μg/ml G418 sulfate.

PCR for positive clones. To verify whether pVgRXR and pIND/CD1 antisense plasmids integrated into the genomic DNA of RKO cells, high molecular weight DNA was extracted from positive clone cells with QIAprep Spin Miniprep kit (QIAGEN, Valencia, CA). Two pairs of flanking primers were used to amplify the EcR gene of pVgRXR and the region on pIND that included the Hsp promoter and an additional downstream 900

bp segment encompassing portions of the sense or antisense CD1. The following primers were utilized to identify genomic integration: 5′-AAG GAG AAG GAC AAA ATG AC and 3′-CAC TAC TAC GAC GCA TAC for EcR, 5′-ACC GCC GGA GTA TAA ATA GAG GCG C and 3′-CCA GTA ACG CCG GTC CAA GGT GAA for Hsp promoter/CD1 antisense, and 5′-ACC GCC GGA GTA TAA ATA GAG GCG C and 3′-GGA AGC AAC GGA GAA CAC GGT GT for Hsp promoter/CD1 sense. PCR reactions were carried out in a buffer containing 25 ng of genomic DNA, 20 nM $MgCl_2$, 0.2 mM each of dNTP, 10 mM Tris–HCl (pH 8.6), 50 mM KCl, 5% DMSO, 0.4 M of each primer and 2.5 U of *Taq* polymerase for 30 cycles. A positive control (pIND/ASCD1 antisense plasmid) and negative control (water) were run in parallel for each experiment. The expression of positive clones was further confirmed by Western blots with anti-EcR serum, anti-RXRα, and anti-CD1 antibodies.

Luciferase assay for testing the function of EcR-positive clones. The ecdysone-inducible system requires the nuclear receptor, RXRα, and a mutant ecdysone receptor, VgEcR, which binds to a DNA enhancer sequence (5× E/GRE) with high affinity and specificity in the presence of an ecdysteroid. To assay for inducible EcR expression in pVgRXR positive cells, an E/GRE-luciferase plasmid containing ecdysone response elements linked to a luciferase reporter gene was transfected into EcR positive cells for transient luciferase expression in the presence of 100 nM–10 μM PA (15–18). After 24 h induction with PA, cell samples were processed with the Luciferase Assay System kit (Promega Corp., Madison, WI), and the light intensity of the reaction between the cell extract and assay reagents was measured with a Turner TD-20e luminometer (Promega, Madison, WI).

Protein extraction and Western blot analysis. Subconfluent cells were washed three times in ice-cold PBS, scraped, and lysed in lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 10% (V/V) glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 200 μM Na orthovanadate, 10 mM Na pyrophosphate, 20 mM NaF) on ice (40). For immunoblotting, lysates, 30 μg/lane, were subjected to 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and then electrophoretically transferred to a nitrocellulose. After blocking, nitrocellulose filters were incubated with the anti-target protein murine monoclonal antibody and anti-actin murine monoclonal antibody (Sigma, St. Louis, MO) in 2% nonfat-TBS–Tween buffer for 1 h. HRP-conjugated antimurine secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was added at 1:3000 dilution in TBS-buffer for 50 min. Detection of the immune signal was performed using the Super Signal Western Blotting Detection System (Pierce Chemical Co., Rockford, IL). Intensities of the autoradiographic bands were quantitated by densitometric scanning.

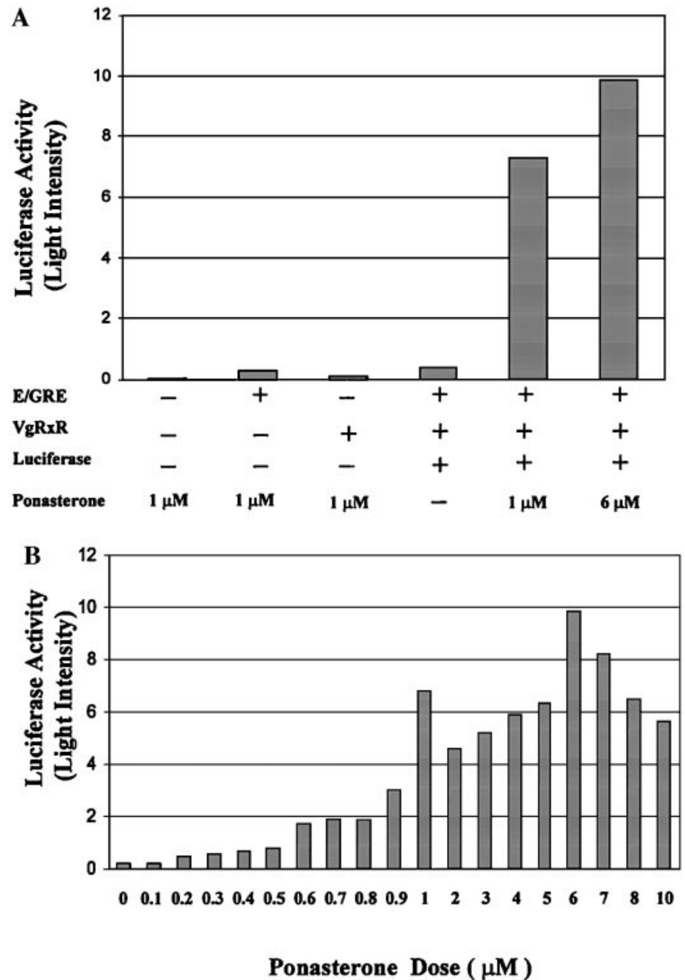

FIG. 1. E/GRE-luciferase activity in ecdysone-inducible cell lines. (A) E/GRE-luciferase assay following treatment with PA. In a transient transfection assay, RKO and RKO-pVgRXR cells were transfected with an equal amount of E/GRE-luciferase plasmid, and treated with 1 or 6 μM PA. After 24 h, cells were lysed and assayed for luciferase activity. Compared with RKO-E/GRE-luciferase cells, luciferase activity increased 42-fold with 1 μM PA and 54-fold with 6 μM PA in RKO-pVgRXR-E/GRE-luciferase cells. (B) PA dose response in RKO-pVgRXR-E/GRE-luciferase cells.

Northern analysis. Total RNA was prepared from exponentially growing cell lines and 15 μg of total RNA was electrophoresed through a 1% denaturing agarose gel containing 6% formaldehyde for Northern analysis. The blots were hybridized with either deoxycytidine 5′-[α-^{32}P]triphosphate labeled cDNA probes for cyclin D1 mRNA (6000 Ci/mmol; Amersham Life Science, Arlington Heights, IL), and then washed at high stringency with the final wash at 65°C in 0.1× SSC, or the blots were concurrently hybridized with five different adenosine 5′-[γ-^{32}P]triphosphate labeled oligonucleotide probes encoding five different regions of the cyclin

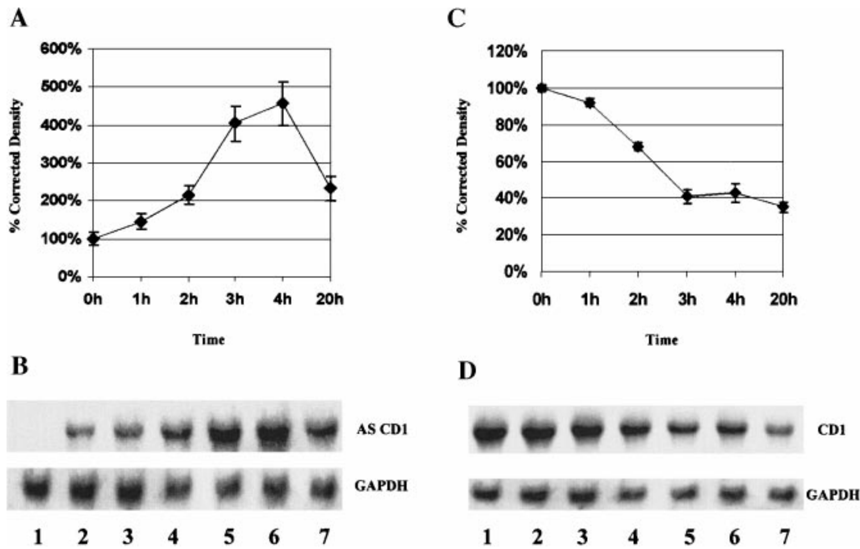

FIG. 2. Northern blot analysis of cyclin D1 mRNA and antisense mRNA in RA28 cells and RV-1 vector cells. (A) Levels of CD1 antisense mRNA following addition of PA, 1 μM, at 0 h. Bars represent mean of 3 experiments ± SEM. The reference level (0 h) represents basal expression of cyclin D1 antisense mRNA in RA28 cells. (B) Representative Northern blot showing changes in levels of 4.8 kb antisense CD1 mRNA following addition of PA. Lane 1, RV-1 cells; lanes 2–7, RA28 cells at indicated time points (0, 1, 2, 3, 4, and 20 h). Blots were reprobed for GAPDH as an internal control. (C) The levels of CD1 mRNA after addition of PA, 1 μM, at 0 h. Bars represent mean of 3 experiments ± SEM. The reference point (0 h) represents basal level of CD1 mRNA expression in RA28 cells. (D) Representative Northern blot of CD1 mRNA after addition of PA. Lane 1, RV-1 control; lanes 2–7, cyclin D1 mRNA levels at 0, 1, 2, 3, 4, and 20 h postinduction with 1 μM PA in RA28 cells.

D1 sense strand (3000 Ci/mmol; Amersham), which is specific for antisense cyclin D1 mRNA (24). Equivalent loading of lanes on Northern blots was confirmed by the hybridization on the same filter with a deoxycytidine 5′-[α-^{32}P]triphosphate labeled GAPDH probe, (6000 Ci/mmol; Amersham) which was synthesized using pTRI-GAPDH-Mouse plasmid template with Strip-EZ DNA probe kit (StripAble DNA Probe Synthesis & Removal Kit, Ambion Inc., Austin, TX).

RESULTS

Integration of the ecdysone receptor and antisense cyclin D1 constructs and functional confirmation of ecdysone-inducible activity after stable cotransfection in RKO cells. After electroporation and zeocin/G418 selection, the genomic DNA extracted from the colonies of RKO transfected cells was screened for the presence of pVgRXR/ecdysone receptor DNA and pIND/CD1AS cDNA by PCR. When amplified with a pair of primers specific for the ecdysone receptor, high levels of the 460 bp exogenous ecdysone receptor DNA bands were observed in 20% of transfected colonies. Positive clones were tested for the expression of the ecdysone and RXRα receptors with Western blot analysis. Although the 120 kDa ecdysone receptor (EcR) and the 46 kDa RXRα were expressed in all positive clones, there were differences in the expression levels of both receptors (data not shown). No amplification signals were observed with either the RKO1 parental or control (RV-1) cell lines. All of the EcR positive colonies were further screened to verify integration of pIND/AS CD1 with another pair of primers specific to the pIND hsp promoter *3′* end 250-bp region and antisense CD1 *5′* end 750-bp region. Strong 1018 bp bands were detected in 40% of positive EcR clones (data not shown), indicating the integration of the antisense cyclin D1 cDNA.

In order to confirm the functionality of the ecdysone inducible system in EcR positive cells, we next determined whether expression of EcR and RXRα could regulate target gene expression by cotransfecting an E/GRE-luciferase plasmid into the same positive clone cells. The luciferase assay was performed to determine the levels of luciferase expression and activities with PA induction, 100 nM to 10 μM (Fig. 1). Luciferase expression and activities increased in a dose-dependent fashion, and maximum expression was 58-fold higher than in uninduced cells.

Inducible expression of antisense cyclin D1 mRNA in transfected positive cells with a mammalian ecdysone inducible system. RKO derived clones, which stably expressed the functional EcR receptor, were transfected with an expression plasmid containing the human antisense cyclin D1 cDNA under the control of a minimal HSP promotor. Colonies were analyzed for inducible expression of antisense cyclin D1. Northern blot analyses of total RNA extracted from the parental RKO cells, control cells transfected with vector only (RV-1 cells), and a RKO antisense cyclin D1 clone

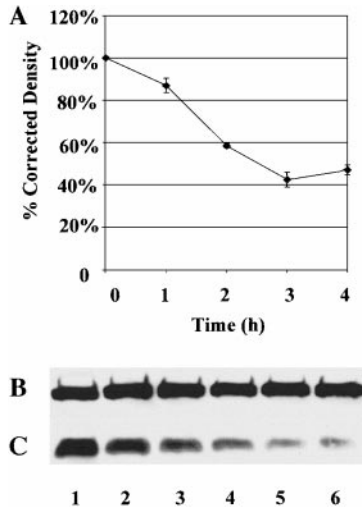

FIG. 3. Cyclin D1 protein level in RA28 cells following treatment with ponasterone A. (A) CD1 protein levels in RA28 cells experiments using basal levels as reference point. (B) Representative Western blot of CD1 protein at specific time points after addition of 1 μM ponasterone. Lane 1, RV-1 cells; lanes 2–6, Cyclin D1 protein level at 0, 1, 2, 3, and 4 h, respectively, after 1 μM treatment in RA28 cells. Equal protein loading per lane (20 μg/lane) was confirmed by probing the 45-kDa region of the blot with an anti-actin antibody as an internal loading control.

(RA28) are shown in Fig. 2. When hybridized concurrently with five specific sense cyclin D1 oligonucleotide probes, a 4.8-kb exogenous antisense cyclin D1 mRNA was detected in the RA28 antisense clone. No hybridization signals were observed in either the RKO parental or RV-1 control cell lines with these probes. Antisense cyclin D1 mRNA expression exhibited a time-dependent response when treated with 1 μM PA. Antisense cyclin D1 mRNA expression peaked at 3–4 h with an increase of 4.5-fold compared with baseline levels after 1 μM PA induction (Figs. 2A and 2B). When the total RNA samples extracted from the same treated RA28 clone were hybridized with a cDNA probe specific for cyclin D1 mRNA, there was a complementary decrease in CD1 sense mRNA. These reductions ranged from 10% at 1 h postinduction to 55% at 4 h postinduction (Figs. 2C and 2D). There was a dose-dependent response in expression levels of sense or antisense cyclin D1 mRNA (data not shown), with maximal induction of antisense cyclin D1 mRNA at 1 μM PA.

Antisense cyclin D1 mRNA reduces the expression level of cyclin D1 protein. Expression of endogenous cyclin D1 protein in response to antisense expression was determined using wild type RKO, RV-1, and RA28 cells treated with PA. Proteins from cell extracts were resolved by SDS-PAGE and were analyzed by Western blot. As shown in Fig. 3, induction with 1 μM PA resulted in a 12% decrease in the expression levels of cyclin D1 protein at 1 h, a 40% decrease at 2 h, and a 59% decrease at 3 h. These data correlated with the temporal changes previously observed in cyclin D1 mRNA sense and antisense levels.

Changes in the levels of expression of pRb, cyclin E:cdk2-, and cyclin D:cdk4-specific phosphorylation of pRb, and the ratio of ppRb to pRb in antisense cyclin D1-transfected cells. Following treatment of RA28 cells for 6 h with 1 μM PA, there was a linear decrease in the expression of pRB protein ranging from 56.16% 1 h to 20.43% at 4 h. In addition, there was also a linear decrease in the ratio of ppRb to pRB ranging from 62% at 1 h to 31% at 4 h (Fig. 4). This decrease was not observed in RKO and RV-1 cells (data not shown). To determine the specific effect of cyclin D1 suppression on pRb independent of cyclin E effects, Western blot analysis was employed to quantitate the levels of the pRb proteins with cdk 2-specific phosphorylation of pRb antibody or with cdk 4-specific phosphorylation of pRb antibody. We employed the anti-pRb-Ser-780 (169) antibody which was generated with the amino acid sequence RPPTLS(p)PIPHIP-COOH, and only detected the cyclin D1/cdk 4-specific phosphorylated (serine 780) form of the ppRb protein. The anti-pRb-Thr-356 (172) antibody was generated with the amino acid sequence FETQRTPRKSNLD-COOH and is specific for the cyclin E/cdk2-specific phosphorylated (threonine 356) form of ppRb protein (21). Both cyclin D1/cdk 4-specific and cyclin E/cdk 2-specific phosphorylated pRb proteins decreased 70 and 60%,

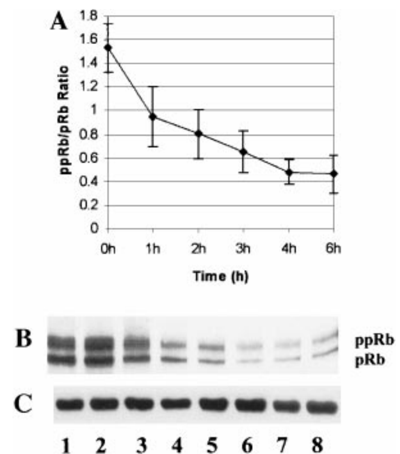

FIG. 4. The effect of antisense cyclin D1 on retinoblastoma protein. (A) Ratios of hyperphosphorylated and hypophosphorylated retinoblastoma protein at 0–6 h. Scanning densitometry was performed on individual bands for pRb and ppRb detected by Western blot. Ratio of ppRb to pRb following exposure to 1 μM decreased by 58% at 3 h when compared to untreated RA28 cells (0 h). Bars represent mean of 4 experiments ± SEM. (B) Representative Western blot of pRb protein at specific time points after addition of 1 μM ponasterone A to RA28 cells. Lane 1, RV-1 cells. Lane 2, RKO cells. Lanes 3–8, retinoblastoma protein level at 0, 1, 2, 3, 4, and 6 h, respectively, after 1 μM ponasterone treatment in RA28 cells. (C) Equal protein loading per lane was confirmed by probing the 45 kDa region of the blot with an anti-actin antibody as an internal loading control.

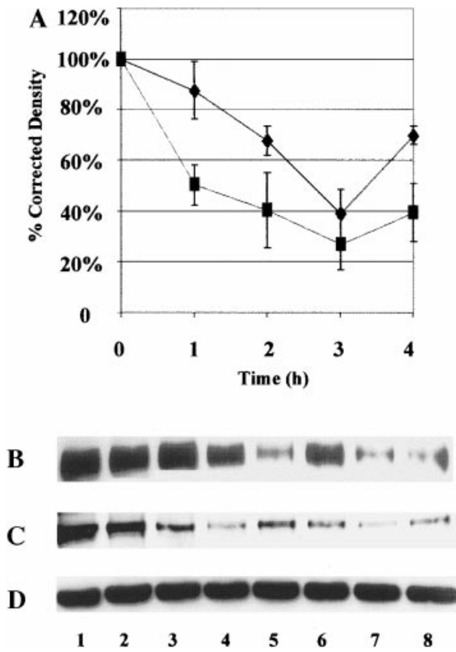

FIG. 5. Effect of antisense cyclin D1 on cdk 2- and cdk 4-specific phosphorylation of retinoblastoma proteins. (A) Scanning densitometry analysis of Western blots. Expression level of the cdk 2/cdk 4-specific phosphorylated pRb decreased following addition of PA at 0 h. Bars represent mean of 3 experiments ± SEM. (B) Representative Western blot of cdk 2-specific phosphorylated pRb and cdk 4-specific phosphorylated 115 kDa pRb proteins. Unlike blots for nonspecific pRb phosphorylation, only a single band is detected with each antibody. Lane 1: RV-1 cells. Lanes 2–8: cdk 2- or cdk 4-specific phosphorylated ppRb proteins at 0 h (lane 2), 1, 2, 3, 4, 6, and 8 h (lanes 3–8) time points after induction with 1 μM PA in RA28 cells. Blots were reprobed for β-actin as an internal control.

respectively, at 3 h (Fig. 5). These changes occurred in a reproducible and sequential fashion. Specifically, levels of cdk 4-specific ppRb decreased rapidly by 50% within the first hour. However, only minimal effects were observed on cdk 2-dependent phosphorylation of ppRb at the same time point. At 2 h, cdk 4-specific ppRb decreased 60% versus a 30% decrease for cdk 2-specific phosphorylation. Maximal suppression for both cdk2 and cdk4 specific phosphorylation occurred at 3 h and partially rebounded at 4 h (Figs. 5A and 5B).

Effects of antisense cyclin D1 on other cell cycle-related proteins. Expression of cyclin E decreased 30% after a 3 h induction with PA (Figs. 6A and 6B). It is interesting that while cyclin E and cyclin D1 decreased over time, the level of suppression for cyclin E was not as great as that of cyclin D1. Conversely, the expression of the S phase-specific cyclin, cyclin A, and the G2/M phase-associated cyclin, cyclin B, were essentially unaffected over time (0–4 h) in both RA28 or RV-1 cells following treatment with PA (data not shown). The expression of the cyclin D1-dependent protein kinases, cdk 4 and cdk 6, decreased 45 and 50%, respectively (Figs. 7A and 7C–7E), while expression of the cyclin E-dependent protein kinase, cdk 2, decreased by 30% after induction with 1 μM PA (Figs. 7A and 7B). Interestingly, the relative change in protein levels over time replicated that of CD1, suggesting that all of these proteins are tightly regulated by changes in CD1 expression.

DISCUSSION

Our studies utilized the ecdysone analogue, Ponasterone A, to induce the expression of antisense cyclin D1 in a unique, stably transfected colon cancer cell line, RA28 cells. This novel, inducible system, markedly reduced CD1 expression and is extremely useful in the elucidation of related temporal events associated with cyclin D1 expression. The goal of our studies was to determine the effect of decreased cyclin D1 levels on cell cycle progression and on the expression of other cell cycle-related proteins in order to more clearly understand the mechanisms associated with cell cycle regulatory events. We were particularly interested in the relationship between cyclin D1 and cyclin E, the predominant cyclins found in G_1. The relationship between these two cell cycle proteins is not clearly understood to date but it is a critical issue in cell cycle regulation. Induction of antisense cyclin D1 expression in our novel RA28 cell line, produced a rapid decrease in cyclin D1 as well as a rapid reduction and almost concomitant suppression of the levels of cyclin E, cdks2, 4 and 6. The reduction of cyclin E levels over time, directly correlated with the induced reduction of

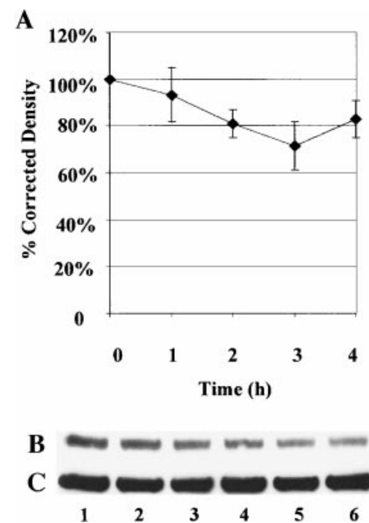

FIG. 6. Western blot analysis for cyclin E protein levels in RV-1 and RA28 cells. (A) Western blots were performed at selected time points after addition of PA and analyzed by scanning densitometry. Bars represent mean of 3 experiments ± SEM. (B) Representative Western blots of cyclin E protein levels following addition of 1 μM ponasterone. Lane 1: RV-1 control. Lanes 2–6: cyclin E levels at 0, 1, 2, 3, and 4 h following PA exposure in RA28 cells.

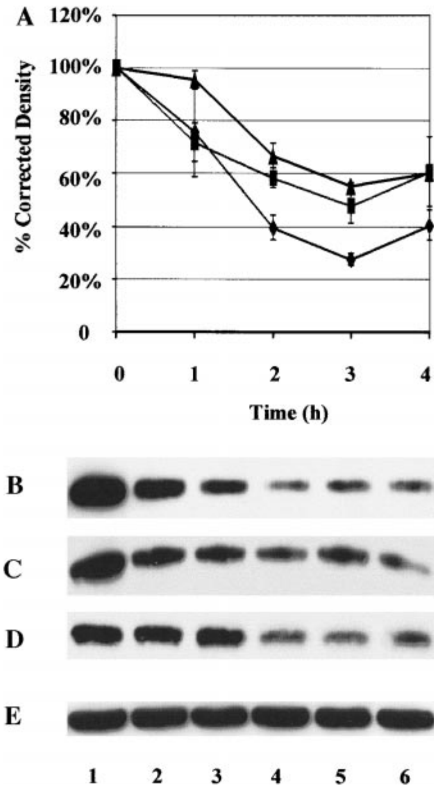

FIG. 7. Cdk 2, cdk 4, and cdk 6 protein levels after ponasterone A induction in RV-1 and RA28 cells. (A) Western blots were performed at selected time points. Results of densitometric analysis is presented relative to basal levels. Bars represent mean of 3 experiments ± SEM. (B–D) Representative Western blots of cdk 2, cdk 4, cdk 6 in RA28 cells. Lane 1: RV-1 cells. Lanes 2–6: cdk 2, cdk 4, or cdk 6 levels at 0 h (lane 2), 1, 2, 3, and 4 h (lanes 3–6) following addition of 1 μM PA in RA28 cells. (E) Blots were reprobed for β-actin levels as the internal control. This is a representative blot demonstrating equal loading.

cyclin D1. This supports the recent theory by Geng and his colleagues (25) that cyclin E is the major downstream target of cyclin D1. The fact that cyclin E does not compensate for the reduction in cyclin D1 protein levels by increasing expression rules out a redundant function between these two G_1 phase proteins.

While it has been reported that some malignant cells may preferentially use only one cyclin to accomplish hyperphosphorylation of pRB, making the other cyclin redundant (26–28), pRB phosphorylation normally requires an active cyclin D/cdk4 complex to perform the initial phosphorylation event followed by additional phosphorylation events initiated by an active cyclin E/cdk2 complex (29, 30). One caveat, however, is that cyclin E is also regulated by E2F-1 in a feedback loop mechanism (31). Therefore, changes in levels of cyclin E in our system may reflect both the effects of cyclin D1 and compensatory effects of E2F-1. Despite the concomitant decrease in CD1 and cyclin E expression, the decrease in cdk2 specific phosphorylation of pRB clearly lagged behind that of cyclin D1/cdk4 specific phosphorylation of pRB. This is most likely due to the fact that there was a greater suppression of cyclin D1 (60%) compared to that of cyclin E (30%). It is also possible that cyclin E/cdk2 complexes are activated by a cyclin D1 independent mechanism. For example, recent studies have shown that two distinct pathways function downstream of E2F to initiate S phase entry (32); one pathway depends on the activity of cdk2 and the other does not.

In summary, we present here new data demonstrating that cyclin D1 and cyclin E share a tightly regulated, temporal relationship in cell cycle events, and provide confirmatory evidence that cyclin E is a major downstream target of cyclin D1. Furthermore, we demonstrate that decreases in cyclin D1 expression (through induction of the antisense cyclin D1 gene) produced not only concomitant decreases in cyclin E, but decreased cyclin D1/cdk4 specific phosphorylation of pRB. Effects on cyclin E/cdk2 specific phosphorylation of pRB were dissociated from effects on cyclin E, suggesting another regulatory pathway. The presence of reduced levels of cyclin D1 and cyclin E may be sufficient to progress the cells through the G_1/S transition. Alternatively, cyclin E/cdk2 complexes may be activated via a cyclin D1 or cdk2 independent mechanism. This mechanism needs to be further elucidated because the cyclin E/cdk2 complex is likely to be an important therapeutic target for the future treatment of cancer. This is especially true since it can be the major complex associated with pRB hyperphosphorylation which results in the activation of the E2F family of transcription factors that are important in the massive task of replicating the genome during S phase.

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REFERENCES

1. Fisher, R. P., and Morgan, D. O. (1994) A novel cyclin associates with MO15/CDK7 to form the CDK-activating kinase. *Cell* **78,** 713–724.
2. Kato, J. Y., Matsuoka, M., Strom, D. K., and Sherr, C. J. (1994) Regulation of cyclin D-dependent kinase 4 (cdk4) by cdk4-activating kinase. *Mol. Cell Biol.* **14,** 2713–2721.
3. Dulic, V., Lees, E., and Reed, S. I. (1992) Association of human cyclin E with a periodic G1–S phase protein kinase. *Science* **257,** 1958–1961.
4. Koff, A., Giordano, A., Desai, D., Yamashita, K., Harper, J. W., Elledge, S., Nishimoto, T., Morgan, D. O., Franza, B. R., and Roberts, J. M. (1992) Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle. *Science* **257,** 1689–1694.
5. Hatakeyama, M., Brill, J. A., Fink, G. R., and Weinberg, R. A.

(1994) Collaboration of G1 cyclins in the functional inactivation of the retinoblastoma protein. *Genes Dev.* **8,** 1759–1771.

6. Hinds, P. W., Dowdy, S. F., Eaton, E. N., Arnold, A., and Weinberg, R. A. (1994) Function of a human cyclin gene as an oncogene. *Proc. Natl. Acad. Sci. USA* **91,** 709–713.
7. Matsushime, H., Ewen, M. E., Strom, D. K., Kato, J. Y., Hanks, S. K., Roussel, M. F., and Sherr, C. J. (1992) Identification and properties of an atypical catalytic subunit (p34PSK-J3/cdk4) for mammalian D type G1 cyclins. *Cell* **71,** 323–334.
8. Nevins, J. R. (1992) E2F: A link between the Rb tumor suppressor protein and viral oncoproteins. *Science* **258,** 424–429.
9. Wu, C. L., Zukerberg, L. R., Ngwu, C., Harlow, E., and Lees, J. A. (1995) In vivo association of E2F and DP family proteins. *Mol. Cell Biol.* **15,** 2536–2546.
10. Quelle, D. E., Ashmun, R. A., Shurtleff, S. A., Kato, J. Y., Bar-Sagi, D., Roussel, M. F., and Sherr, C. J. (1993) Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. *Genes Dev.* **7,** 1559–1571.
11. Sherr, C. J. (1993) Mammalian G1 cyclins. *Cell* **73,** 1059–1065.
12. Zhou, P., Jiang, W., Zhang, Y. J., Kahn, S. M., Schieren, I., Santella, R. M., and Weinstein, I. B. (1995) Antisense to cyclin D1 inhibits growth and reverses the transformed phenotype of human esophageal cancer cells. *Oncogene* **11,** 571–580.
13. Arber, N., Doki, Y., Han, E. K., Sgambato, A., Zhou, P., Kim, N. H., Delohery, T., Klein, M. G., Holt, P. R., and Weinstein, I. B. (1997) Antisense to cyclin D1 inhibits the growth and tumorigenicity of human colon cancer cells. *Cancer Res.* **57,** 1569–1574.
14. Driscoll, B., Wu, L., Buckley, S., Hall, F. L., Anderson, K. D., and Warburton, D. (1997) Cyclin D1 antisense RNA destabilizes pRb and retards lung cancer cell growth. *Am. J. Physiol.* **273,** L941–L949.
15. No, D., Yao, T. P., and Evans, R. M. (1996) Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proc. Natl. Acad. Sci. USA* **93,** 3346–3351.
16. Sadowski, I., Ma, J., Triezenberg, S., and Ptashne, M. (1988) GAL4-VP16 is an unusually potent transcriptional activator. *Nature* **335,** 563–564.
17. Umesono, K., and Evans, R. M. (1989) Determinants of target gene specificity for steroid/thyroid hormone receptors. *Cell* **57,** 1139–1146.
18. Yao, T. P., Forman, B. M., Jiang, Z., Cherbas, L., Chen, J. D., McKeown, M., Cherbas, P., and Evans, R. M. (1993) Functional ecdysone receptor is the product of EcR and Ultraspiracle genes. *Nature* **366,** 476–479.
19. Lane, M. E., Yu, B., Pestell, R., Albanese, C., and Wadler, S. (1999) Characterization of growth and cell cycle properties of RKO cells stably transfected with an inducible cyclin D1 gene. *In* American Association for Cancer Research, Philadelphia, PA.
20. Yu, B., Lane, M., Pestell, R., Albanese, C., and Wadler, C. (1999) Tight regulation of cyclin D1 expression in stable human cell lines with an ecdysone-inducible system. *In* American Association for Cancer Research, p. 26. Philadelphia, PA.
21. Carlson, B., Lahusen, T., Singh, S., Loaiza-Perez, A., Worland, P. J., Pestell, R., Albanese, C., Sausville, E. A., and Senderowicz, A. M. (1999) Down-regulation of cyclin D1 by transcriptional repression in MCF-7 human breast carcinoma cells induced by flavopiridol. *Cancer Res.* **59,** 4634–4641.
22. Talbot, W. S., Swyryd, E. A., and Hogness, D. S. (1993) *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. *Cell* **73,** 1323–1337.
23. Levin, A. A., Sturzenbecker, L. J., Kazmer, S., Bosakowski, T., Huselton, C., Allenby, G., Speck, J., Kratzeisen, C., Rosenberger, M., Lovey, A., *et al.* (1992) 9-*cis*-Retinoic acid stereoisomer binds and activates the nuclear receptor RXR alpha. *Nature* **355,** 359–361.
24. Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K., and Green, M. R. (1984) Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* **12,** 7035–7056.
25. Geng, Y., Whoriskey, W., Park, M. Y., Bronson, R. T., Medema, R. H., Li, T., Weinberg, R. A., and Sicinski, P. (1999) Rescue of cyclin D1 deficiency by knockin cyclin E. *Cell* **97,** 767–777.
26. Courjal, F., Louason, G., Speiser, P., Katsaros, D., Zeillinger, R., and Theillet, C. (1996) Cyclin gene amplification and overexpression in breast and ovarian cancers: Evidence for the selection of cyclin D1 in breast and cyclin E in ovarian tumors. *Int. J. Cancer* **69,** 247–253.
27. Gray-Bablin, J., Zalvide, J., Fox, M. P., Knickerbocker, C. J., DeCaprio, J. A., and Keyomarsi, K. (1996) Cyclin E, a redundant cyclin in breast cancer. *Proc. Natl. Acad. Sci. USA* **93,** 15215–15220.
28. Nielsen, N. H., Emdin, S. O., Cajander, J., and Landberg, G. (1997) Deregulation of cyclin E and D1 in breast cancer is associated with inactivation of the retinoblastoma protein. *Oncogene* **14,** 295–304.
29. Lundberg, A. S., and Weinberg, R. A. (1998) Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin–cdk complexes. *Mol. Cell Biol.* **18,** 753–761.
30. Resnitzky, D., and Reed, S. I. (1995) Different roles for cyclins D1 and E in regulation of the G1-to-S transition. *Mol. Cell Biol.* **15,** 3463–3469.
31. Sherr, C. J. (1996) Cancer cell cycles. *Science* **274,** 1672–1677.
32. Arata, Y., Fujita, M., Ohtani, K., Kijima, S., and Kato, J. (2000) Cdk2-dependent and -independent pathways in E2F-mediated S phase induction. *J. Biol. Chem.* **275,** 6337–6345.
33. Li, Z., Hromchak, R., and Bloch, A. (1997) Differential expression of proteins regulating cell cycle progression in growth vs. differentiation. *Biochim. Biophys. Acta* **1356,** 149–159.