Cyclin A Is a Functional Target of Retinoblastoma Tumor Suppressor Protein-mediated Cell Cycle Arrest*

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Although RB inhibits the G1-S transition, the mechanism through which RB prevents cell cycle advancement remains unidentified. To delineate the mechanism(s) utilized by RB to exert its anti-proliferative activity, constitutively active RB proteins (which cannot be inactivated by phosphorylation) or p16ink4a (which prevents RB inactivation) were utilized. Both proteins inhibited the G1-S transition, whereas wild-type RB did not. We show that active RB acts to attenuate cyclin A promoter activity, and that overexpression of cyclin E reverses RB-mediated repression of the cyclin A promoter. Although cyclin A is an E2F-regulated gene, and it has been long hypothesized that RB mediates cell cycle advancement through binding to E2F and attenuating its transactivation potential, cyclin E does not reverse dominant negative E2F-mediated repression of the cyclin A promoter. Although active RB repressed both cyclin A and two other paradigm E2F-regulated promoters, only cyclin A transcription was restored upon co-expression of cyclin E. Additionally, we show that RB but not dominant negative E2F regulates the cyclin A promoter through the CCRE element. These data identify cyclin A as a downstream target of RB-mediated arrest. Consistent with this idea, ectopic expression of cyclin A reversed RB-mediated G1 arrest. The findings presented suggest a pathway wherein cyclin A is a downstream target of RB, and cyclin E functions to antagonize this aspect of RB-mediated G1-S inhibition.

The retinoblastoma tumor suppressor protein (RB),1 is functionally inactivated in over 60% of human tumors (1–3). The role of RB as a tumor suppressor has been well established, and it is known that RB can inhibit cellular proliferation by halting cell cycle progression (4–6). RB carries out this growth inhibition through its ability to assemble and modulate a host of multiprotein complexes (5, 7, 8). At least four distinct protein-binding domains of RB have been identified and extensively characterized, including: the A/B pocket, the large A/B pocket, the C-pocket, and the N-terminal domain (5, 7, 9). The large A/B pocket is the minimal growth suppressing region of RB and is required to bind the E2F family of transcription factors (10, 11).

Binding of RB to proteins such as E2F is regulated by cyclin-dependent kinase (Cdk)-mediated phosphorylation (4, 5, 12). The full-length RB protein contains 16 consensus Cdk-phosphorylation sites, and phosphorylation at specific sites inhibits the binding of RB to cellular proteins, thereby disrupting the anti-proliferative activity of RB (13–19). Not surprisingly, therefore, overexpression of proteins which cause excessive or deregulated phosphorylation of RB is a common event in human tumors (2, 3, 20). For example, amplification of Cdk4 and/or its regulatory partner, cyclin D1, are frequently observed in human tumors. In either case, excessive Cdk4/cyclin D kinase activity results in deregulated phosphorylation and inactivation of RB. Similarly, loss of the tumor suppressor p16ink4a, which acts to attenuate Cdk4/cyclin D activity, is a common event in human tumors (2, 3, 20). Loss of p16ink4a also results in excessive phosphorylation and inactivation of RB. In normal cells, Cdk4/cyclin D complexes promote early G1 cell cycle progression upon growth factor stimulation, and it has been shown that the principle cell cycle role for Cdk4/cyclin D complexes is to phosphorylate RB (21–24). For example, Cdk4/cyclin D is dispensable for cell cycle progression in RB-deficient cells (21). In mid to late G1, Cdk2/cyclin E and Cdk2/cyclin A complexes are sequentially activated, and also phosphorylate RB (3, 25). Cdk2 complexes clearly have other roles in addition to phosphorylating RB, since Cdk2 activity is required irrespective of RB status. Activation of all three complexes, Cdk4/cyclin D, Cdk2/cyclin E, and Cdk2/cyclin A is required for entry into S-phase (26, 27).

Although it is clear that Cdk/cyclin complexes act upstream to modulate the anti-proliferative activity of RB through phosphorylation (12), the downstream effectors of RB-mediated arrest have yet to be unequivocally identified. Studies aimed directly at identifying the downstream effectors of RB have been hindered by the fact that ectopically expressed RB is rapidly phosphorylated and inactivated by endogenous Cdk-cyclin kinase activity (14–16). As a result, RB acts as a poor growth inhibitor in most cell types. Most studies aimed at understanding the role of RB in growth inhibition have been carried out in the osteosarcoma cell line SAOS-2; wild-type RB is not phosphorylated in this cell line and therefore these cells are growth arrested by introduction of RB (10, 28). To circumvent the problem of RB phosphorylation and extend RB studies to non-tumorigenic cell lines, we previously designed phosphorylation site-mutated RB proteins, or PSM-RB (13–15). One such protein, PSM.7LP, acts as a constitutively active RB pro-
tein, and binds cognate cellular proteins such as E2F regardless of phosphorylation status. As such, this protein mimics unphosphorylated RB. We have previously shown that this protein acts as a potent growth inhibitor in a large number of tumorigenic (29) and non-tumorigenic cell lines, and can be used as a powerful tool to study RB function and identify the downstream effectors of RB (14, 15).

It has been previously hypothesized that RB inhibits cell cycle progression by binding the E2F family of transcription factors (4, 30). E2F is known to activate transcription of a number of genes required for S-phase, including cyclin A, cyclin E, dihydrofolate reductase (DHFR), and thymidylate synthase (6, 30, 31). RB represses E2F transcriptional activity by directly binding to E2F and recruiting histone deacetylases to E2F-specific promoters (8, 32). The importance of this interaction was demonstrated by Sellers et al. (33), who created a chimeric protein wherein the transrepression function of RB was provided in cis to the DNA-binding and dimerization domains of E2F. Introduction of such chimeric molecules into cells caused cell cycle arrest (33). Due to the import of the RB/E2F interaction, a model for how RB inhibits cell cycle progression has been proposed. It has been postulated that prior to Cdk/cyclin activation, RB inhibits the expression of cyclin E through the E2F-binding site in the cyclin E promoter (34, 35). The role of RB as a regulator of cyclin E is well established, in that in Rb−/− murine embryo fibroblasts, cyclin E expression is deregulated (expressed in quiescence) (36, 37). Furthermore, it has been shown that ectopic expression of cyclin E overrides the G1 arrest induced by unphosphorylated RB (15, 16, 38). These findings suggest that cyclin E is a downstream effector of RB activity.

However, recent studies suggest that E2F binding is dispensable for RB-mediated growth inhibition. For example, mutants RB proteins incapable of binding to E2F still inhibit cell cycle progression in SAOS-2 cells (39, 40). Furthermore, it has been shown that in cells arrested in G1 by unphosphorylated RB (through introduction of PSM.7LP or p16ink4a), the expression and activity of cyclin E is not down-regulated (15, 19, 38). These findings suggest that cyclin E acts to antagonize RB function, and that downstream effectors other than cyclin E must implement the RB-mediated G1 arrest. However, these downstream effectors have yet to be identified.

In this report, we sought to delineate the mechanisms through which RB inhibits cell cycle progression and determine how this function is antagonized by cyclin E. We identify cyclin A as a downstream target of RB function, as active RB inhibited cyclin A promoter activity and resulted in reduced cyclin A protein levels. As would be expected for a functional target of RB, ectopic expression of cyclin E lifted RB-mediated cyclin A promoter repression, concomitant with a restoration of cell cycle progression. This action of cyclin E occurred without RB phosphorylation. Although the cyclin A promoter is regulated by E2F, RB and dominant negative E2F repressed cyclin A promoter activity through distinct motifs, and cyclin E did not lift dominant negative E2F-mediated repression of cyclin A. Importantly, the expression of cyclin A was sufficient to overcome RB-mediated arrest. These data suggest a model wherein RB exerts its anti-proliferative activity by repressing cyclin A expression, and cyclin E acts to antagonize this function of RB.

MATERIALS AND METHODS

Cell Culture—CV1 cells were obtained from American Type Culture Collection and passages 33–40 were utilized for the experiments described. For regular passage, cells were grown in Dulbecco’s modified Eagle’s medium (Mediatech) supplemented with 10% heat inactivated fetal bovine serum (Hyclone), 100 units/ml penicillin-streptomycin, and 2 mM l-glutamine at 37 °C in a humidified atmosphere of 5% CO2.

Plasmids—The pH2B-GFP plasmid, encoding histone H2B fused to the green fluorescent protein, was obtained from Dr. Geoff Wahl (Salk Institute). The pPSM.7LP and pWT-LP plasmids have been previously described (14). The cyclin E expression construct was obtained from Dr. James Roberts (Fred Hutchinson Cancer Research Center). The p16ink4a, OX2EFLUC, and DHFR-LUC expression plasmids were obtained from Dr. Jean Wang (University of California at San Diego). The 608Cya reporter was obtained from Kinichiro Oda (Science University of Tokyo) (41). The pCycALUC and MCCCRe reporters have been previously described (42). The E2F-A/B plasmid was kindly provided by Dr. William Kaelin (Dana-Farber Cancer Institute) (33). The E1A expression plasmid was supplied by Dr. Gilbert Morris (Tulane University).

Plasmid—Incorporation—CV1 cells were seeded on coverslips at a density of 6 × 104 cells per well of a six-well dish. Twenty-four hours later the cells were transfected with 4 μg of total plasmid DNA (as indicated) by the BES-buffered saline/calcium phosphate method as described previously (43). Forty-eight hours post-transfection, Cell Proliferation Labeling Reagent (Amersham Pharmacia Biotech) was added according to the manufacturer’s protocol. Sixteen hours later, cells were fixed with formaldehyde and processed for indirect immunofluorescence to detect BrdUrd incorporation, as described previously (44). For each experiment at least 150 transfected (GFP positive) and untransfected (GFP negative) cells were counted. Data shown reflects the average of at least two to three independent experiments. Images were captured using a Nikon Axioptih at ×20 magnification and a SpotCam digital camera.

Reporter Assays—CV1 cells were seeded at a density of 1.2 × 105 cells/6-cm dish. Cells were transfected 24 h later with 8 μg of total plasmid DNA (as indicated) by the BES-buffered saline/calcium phosphate method as described previously (43). Thirty-six to forty-eight hours post-transfection the cells were harvested and processed for luciferase activity using the Luciferase Assay System (Promega) according to the manufacturer’s protocol. β-Galactosidase activity was also quantitated as an internal control for transfection efficiency. Reported relative luciferase activity reflects luciferase activity normalized to β-galactosidase activity. Data shown reflects the average of at least three independent experiments.

Quiescence Studies—To monitor reporter activity during quiescence, cells were transfected as stated above during a state of active growth. After transfection, cells were washed and placed in media containing only 0.1% for 48 h to achieve quiescence, as described previously (45). At this time one-half of the transfected dishes were then stimulated with media containing 10% fetal bovine serum, and the other half remained in 0.1% fetal bovine serum. Twenty hours after stimulation (or not) the cells were harvested and processed as above to monitor luciferase and β-galactosidase activities.

Rapid Selection and Immunoblots—CV1 cells were seeded at a density of 7.5 × 104 cells per 10-cm dish. Twenty-four hours later the cells were transfected with 16 μg of total plasmid DNA (as indicated) by the BES-buffered saline/calcium phosphate method (43). After removal of transfection precipitate, cells were recovered for 8 h in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum. At this time puromycin was added at a final concentration of 8 μg/ml. Forty-eight hours later, cells that were mock-transfected were dead, and cells from the transfected dishes were harvested by trypsinization and processed for immunoblots. For immunoblotting, cell pellets were resuspended in RIPA buffer containing protease inhibitors (10 μg/ml 1,10-phenanthroline, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (10 μM sodium fluoride, 0.1 mM sodium vanadate, and 60 μg/ml β-glycerophosphate) for 15 min on ice. Following overnight sonication, equal protein was resolved by SDS-polyacrylamide gel electrophoresis. Following electrophoresis, protein was transferred to Immobilon-P (Millipore) by standard methods. Blots were probed for cyclin A protein with the sc-751 antibody (Santa Cruz Biotechnology), and for cyclin E using the sc-198 antibody (Santa Cruz Biotechnology). Goat anti-rabbit horseradish peroxidase (Bio-Rad) was used for antibody visualization via enhanced chemiluminescence (Amersham Pharmacia Biotech).

RESULTS

Cyclin E Antagonizes the Anti-proliferative Activity of RB—We and others have shown that RB-mediated inhibition of the G1-S transition can be alleviated by overexpression of cyclin E (15, 16, 38). To verify that this function of cyclin E is conserved in non-tumorigenic cells, CV1 cells were transfected with H2B-GFP and constitutively active RB, PSM.7LP, in the presence or absence of cyclin E (Fig. 1). As we and others had
previously observed in rodent and osteosarcoma cells (15, 16, 38), CV1 cells transfected with PSM.7LP were inhibited for the G1-S transition, as scored by a marked decrease in BrdUrd incorporation (Fig. 1), and identify cyclin A as a downstream target of RB action.

**Active RB Inhibits Cyclin A Transactivation**

To test this hypothesis, CV1 cells were transfected with one of two cyclin A reporter constructs (Fig. 2). The human p-608CycA construct contains nucleotides -608 to +177 fused to a luciferase reporter (41). Alternatively, cells were transfected with a similar cyclin A promoter of mouse origin, pCycA-LUC, which contains nucleotides -177 to +100 fused to a luciferase reporter (42). Both promoters act as expected in CV1 cells, in that they are both repressed during quiescence (Fig. 2A). In addition, p-608CycA and pCycA-LUC promoters were both repressed upon co-transfection with either PSM.7LP or p16ink4a (Fig. 2, B and C). However, this transcriptional inhibition was effectively reversed by co-transfection of cyclin E with PSM.7LP or p16ink4a expression plasmids at an approximate 1:1 ratio (Fig. 2, B and C). Co-transfection of cyclin E had no effect on PSM.7LP or p16ink4a protein expression (data not shown). These data are consistent with the biological response of CV1 cells to active RB and cyclin E (Fig. 1), and identify cyclin A as a downstream target of RB action.

**E2F-dependent Transcription Is Not Reversed by Cyclin E**—It has long been hypothesized that the growth inhibitory activity of RB is manifested through its ability to cause transcriptional repression of E2F-dependent promoters (4). Since the cyclin A promoter is known to be regulated by E2F (46), we questioned whether co-expression of cyclin E with PSM.7LP would restore expression of other E2F-dependent promoters. To test this hypothesis, reporter assays were carried out using the well characterized 3XE2F promoter fused to a luciferase reporter gene (39, 40). The 3XE2FLUC promoter contains three consensus binding sites for the E2F family of transcription factors, and has been utilized previously to monitor E2F activity (40). As expected, transfection of PSM.7LP (constitutively active RB) or p16ink4a (prevents inactivation of endogenous RB) expression plasmids reduced transcriptional activity from the 3XE2FLUC promoter by as much as 90% (Fig. 3A). However, co-transfection of cyclin E expression plasmids at 1:1 ratios with those encoding either PSM.7LP or p16ink4a did not result in restoration of 3XE2FLUC transcriptional activity (Fig. 3A). Ectopic cyclin E expression was verified by immunoblot (data not shown). These data suggest that although cyclin E abrogates the ability of RB to inhibit cyclin A promoter activity and the G1-S transition, cyclin E does not abrogate the ability of RB to cause transcriptional repression of an E2F-dependent promoter. To verify these findings, a physiological E2F-dependent promoter, the DHFR promoter, was utilized. Like the cyclin A promoter, the DHFR promoter is cell cycle regulated and is known to be transcriptionally repressed during quiescence (47). These observations were verified in CV1 cells using the DHFR-LUC reporter plasmid (Fig. 3B). Moreover, transcriptional activity from the DHFR promoter is E2F-dependent (47). As we observed using the 3XE2FLUC promoter, both PSM.7LP and p16ink4a inhibited transactivation from the DHFR-LUC reporter (Fig. 3C). Again, co-expression of cyclin E in these cells did not restore transcriptional activity of the DHFR promoter. These data suggest that although cyclin E abrogates the ability of RB to inhibit both cyclin A promoter activity and the G1-S transition, cyclin E does not abrogate the ability of RB to cause transcriptional repression of other E2F-dependent promoters.

**Active RB inhibits Cyclin A Expression in a Manner That Is Distinct from Dominant Negative E2F**—To test the mechanism by which RB represses the cyclin A promoter, the chimeric molecule E2F-A/B was utilized (33). E2F-A/B consists of the E2F DNA-binding and dimerization domains fused to the A/B pocket of RB, and acts as a dominant negative protein. As has been previously described, the A/B pocket provides transcriptional repression activity *in cis* to E2F (33). As expected, E2F-
FIG. 2. Cyclin A is a target of RB, and cyclin E reverses RB-mediated inhibition of the cyclin A promoter. A, CV1 cells were transfected with 1 μg of CMV-β-gal, 0.5 μg of cyclin A promoter (either human −608CycA or mouse CycA-LUC) and 6.5 μg of blank vector. Transfected cells were rendered quiescent and one-half of the transfected dishes were subsequently stimulated with serum for 24 h. Cells were then harvested and analyzed for luciferase and β-galactosidase activities. For each reporter, relative luciferase activity for stimulated (cycling) cells was set to 100%. Data shown are the results of at least three independent experiments.

B, CV1 cells were transfected with 1 μg of CMV-β-gal, 0.5 μg of 2608LUC, and designated combinations of the indicated effector plasmid(s) PSM.7LP (3 μg) or p16ink4a (3 μg) and cyclin E (3.5 μg). All transfections were brought to 8 μg of total plasmid DNA using parental vector (CMVNeoBam). Cells were harvested 48 h post-transfection and analyzed for luciferase and β-galactosidase activities. Relative luciferase activity for cells transfected with CMV-β-gal, −608LUC, and vector alone was set to 100%. Data shown are the results of three to five independent experiments.

C, CV1 cells were transfected with 1 μg of CMV-β-gal, 0.5 μg of CycA-LUC, and designated combinations of the indicated effector plasmid(s) PSM.7LP (3 μg) or p16ink4a (3 μg) and cyclin E (3.5 μg). All transfections were brought to 8 μg of total plasmid DNA using parental vector (CMVNeoBam). Cells were harvested 48 h post-transfection and analyzed for luciferase and β-galactosidase activities. Relative luciferase activity for cells transfected with CMV-β-gal, CycA-LUC, and vector alone was set to 100%. Data shown are the results of at least three independent experiments.
FIG. 3. Cyclin E does not reverse RB-mediated inhibition of other E2F-dependent promoters. A, CV1 cells were transfected with 1 μg of CMV-β-gal, 1 μg of 3XE2FLUC, and designated combinations of the indicated effector plasmid(s) PSM.7LP (3 μg) or p16ink4a (3 μg) and cyclin E (3 μg). All transfections were brought to 8 μg of total plasmid DNA using parental vector (CMVNeoBam). Cells were harvested 48 h post-transfection and analyzed for luciferase and β-galactosidase activities. Relative luciferase activity for cells transfected with CMV-β-gal, 3XE2FLUC, and vector alone was set to 100%. Data shown are the results of at least three independent experiments. B, CV1 cells were transfected with 2 μg of DHFR reporter, 1 μg of CMV-β-gal and 5 μg of blank vector (CMVNeoBam). Transfected cells were rendered quiescent and one-half of the transfected dishes were subsequently stimulated with serum for 24 h. Cells were then harvested and analyzed for luciferase and β-galactosidase activities. Relative luciferase activity for stimulated (cycling) cells was set to 100%. Data shown are the results of at least three independent experiments. C, CV1 cells were transfected with 1 μg of CMV-β-gal, 1 μg of DHFR, and designated combinations of the indicated effector plasmid(s) PSM.7LP (3 μg) or p16ink4a (3 μg) and cyclin E (3 μg). All transfections were brought to 8 μg of total plasmid DNA using parental vector (CMVNeoBam). Cells were harvested 48 h post-transfection and analyzed for luciferase and β-galactosidase activities. Relative
A/B inhibits transcription from the 3XE2F-LUC (E2F-dependent) promoter (Fig. 4A). Like RB-mediated repression of 3XE2F-LUC (Fig. 3A), this inhibition was not reversed by co-expression of cyclin E (Fig. 4A). E2F-A/B also inhibited transcription from the DHFR (E2F-dependent) promoter in a manner that could not be reversed by cyclin E (Fig. 4B). Co-transfection of adenovirus E1A expression constructs at an approximate 1:1 ratio with E2F-A/B did restore transcriptional activity at the DHFR promoter (Fig. 4B), demonstrating that transcriptional repression by E2F-A/B can be reversed.

Since E2F is thought to modulate the cyclin A promoter (46), it was not surprising to find that E2F-A/B inhibits transcription from both the p-608CycA and pCycALUC promoters (Figs. 4, C and D). Unlike PSM.7LP and p16ink4a mediated repression of these promoters, however, repression mediated by E2F-A/B could not be reversed by cyclin E (Fig. 4, B and C). Transcriptional repression of cyclin A by E2F-A/B was reversed by co-expression of adenovirus E1A (Fig. 4, C and D). These data suggest that the manner in which active RB inhibits cyclin A expression is distinct from dominant negative E2F. We reasoned that RB likely inhibits cyclin A promoter activity through cell cycle regulatory elements. To test this hypothesis, a mutant cyclin A promoter was utilized which is defective for cell cycle regulation, pMCCRE. This promoter was derived from the wild-type pCycA-LUC promoter utilized in Figs. 2C and 4D. In the MCCRE promoter, the cell cycle regulatory element (CCRE), also known as the cell-cycle dependent element (CDE), was mutated (42). As a result, transcriptional repression in quiescence was eliminated (Fig. 5A) (42). As shown in Fig. 5B, both PSM.7LP and p16ink4a failed to inhibit transcription of the pMCCRE promoter. By contrast, E2F-A/B retained the ability to repress transcription of the MCCRE promoter. These data suggest that RB but not dominant negative E2F acts through the CCRE to modulate cyclin A transcription.

**Cyclin A Reverses RB-mediated Growth Inhibition**—Since the data presented suggest that cyclin A represents an important functional target for RB-mediated growth inhibition, we hypothesized that active RB would result in decreased cyclin A protein. To test this hypothesis, CV1 cells were transfected with PSM.7LP or p16ink4a expression plasmids and a puromycin resistance plasmid. After rapid selection with puromycin (PSM.7LP or p16ink4a expression plasmids and a puromycin resistance plasmid), these data suggest that cyclin A represents an important E2F acts through the CCRE to modulate cyclin A promoter activity. These data indicate that RB but not dominant negative E2F acts through the CCRE to repress cyclin A transcription.

**DISCUSSION**

In this report, we investigated the mechanism through which RB inhibits the G1/S transition and how this function is antagonized by cyclin E (Fig. 1). We show that introduction of constitutively active RB or p16ink4a into a non-tumorigenic cell line resulted in repression of the cyclin A promoter (Fig. 2). As would be expected for a functional target of RB, ectopic expression of cyclin E alleviated RB-mediated repression of the cyclin A promoter (Fig. 2). Although cyclin A is known to be an E2F-regulated gene, we show that RB-mediated inhibition of other two E2F-dependent promoters cannot be alleviated by cyclin E (Fig. 3), consistent with previous reports (15, 16, 38). Therefore, we hypothesized that RB-mediated repression of cyclin A may occur through an E2F-independent manner. To test this hypothesis, a chimeric E2F molecule was utilized wherein the transcriptional repression function of RB was provided in *cis* to E2F, E2F-A/B. Although E2F-A/B did inhibit cyclin A promoter activity, transcriptional repression could not be lifted by co-expression of cyclin E (Fig. 4). In addition, we demonstrated that RB-mediated repression of the cyclin A promoter requires the CCRE element, whereas dominant negative E2F-mediated repression of this promoter acts independently of the CCRE (Fig. 5). Consistent with the idea that cyclin A is a critical target of RB growth inhibitory activity, we show that active RB causes a dramatic reduction in cyclin A but not cyclin E protein levels (Fig. 6). The functional significance of cyclin A as an effector of RB-mediated cell cycle arrest was demonstrated by the fact that ectopic expression of cyclin A enabled entry into S-phase (Fig. 6). These data identify cyclin A as a downstream target of RB action, and provide a rationale for how cyclin E reverses RB-mediated cell cycle arrest.

Cyclin E is known to antagonize the function of RB as a cell cycle inhibitor (15, 16, 38, 48, 49). Initially, it was shown by Hinds *et al.* (48) that cyclin E promotes the phosphorylation of RB in SAOS-2 cells, leading to the reversal of RB-mediated cell cycle arrest. This finding is consistent with the observation that cyclin E forms active kinase complexes with Cdk2 and phosphorylates/inactivates RB. Based on the data presented here and the work of others, cyclin E must also antagonize RB function independently of its ability to phosphorylate and inactivate RB. First, the constitutively active form of RB utilized in our and other studies cannot be inactivated by phosphorylation (14, 49). Second, in p16ink4a-arrested cells RB is not phosphorylated, even in the presence of ectopically expressed cyclin E (19, 38). Third, if cyclin E were antagonizing RB function by phosphorylation, then cyclin E would have also disrupted E2F binding/repression, as has been previously observed for wild-type RB (14, 50). As such, cyclin E must employ a mechanism besides RB phosphorylation to disrupt RB function.

Although RB is known to arrest cell cycle progression and this arrest is alleviated by cyclin E, the functional targets of RB have not been defined. We and others have observed that cyclin E expression and associated kinase activity is not attenuated in RB-arrested cells (15, 16, 19, 38). By contrast, reduced cyclin A expression correlates with RB-mediated cell cycle arrest (15, 38, 42). In this report we identify cyclin A as a functionally luciferase activity for cells transfected with CMV-ß-gal, DHFR, and vector alone was set to 100%. Data shown are the results of at least three independent experiments.
Fig. 4. Inhibitory effects of dominant negative E2F can be rescued by E1A but not by cyclin E. A, CV1 cells were transfected with 1 μg of CMV-β-gal, 1 μg of 3XE2FLUC, and designated combinations of the indicated effector plasmid(s) E2F-A/B (3 μg) and cyclin E (3 μg). All transfections were brought to 8 μg of total plasmid DNA using parental vector (CMVNeoBam). Cells were harvested 48 h post-transfection and analyzed for luciferase and β-galactosidase activities. Relative luciferase activity for cells transfected with CMV-β-gal, 3XE2FLUC, and vector alone was set to 100%. Data shown are the results of at least three independent experiments. B, CV1 cells were transfected with 1 μg of CMV-β-gal, 1 μg of DHFR reporter, and designated combinations of the indicated effector plasmid(s) E2F-A/B (3 μg), cyclin E (3 μg), and E1A (3.5 μg). All transfections were brought to 8 μg of total plasmid DNA using parental vector (CMVNeoBam). Cells were harvested 48 h post-transfection and analyzed for luciferase and β-galactosidase activities. Relative luciferase activity for cells transfected with CMV-β-gal, DHFR, and vector alone was set to 100%. Data shown are the results of at least three independent experiments.
RB inhibits Cyclin A Transactivation

Fig. 5. RB but not dominant negative E2F requires the CCRE for repression of the cyclin A promoter. A, CV1 cells were transfected with 0.5 μg of mCCRE reporter, 1 μg of CMV-β-gal, and 6.5 μg of blank vector. Transfected cells were rendered quiescent and one-half of the transfected dishes were subsequently stimulated with serum for 24 h. Cells were then harvested and analyzed for luciferase and β-galactosidase activities. Data shown are results of at least three independent experiments. B, CV1 cells were transfected with 1 μg of CMV-β-gal, 0.5 μg of mCCRE reporter, and designated combinations of the indicated effector plasmid(s) PSM.7LP (3 μg) or p16ink4a (3 μg) and E2F A/B (3.5 μg). All transfections were brought to 8 μg of total plasmid DNA using parental vector (CMVNeoBam). Cells were harvested 48 h post-transfection and analyzed for luciferase and β-galactosidase activities. Relative luciferase activity for cells transfected with CMV-β-gal, mCCRE, and vector alone was set to 100%. Data shown are results of at least three independent experiments.

significant target in RB-mediated arrest. This conclusion is based on several critical observations. First, RB inhibits cyclin A promoter activity, leading to a down-regulation of cyclin A protein. Although it could be suggested that the reduction of cyclin A expression is not causative but is a consequence of RB-mediated arrest, this supposition is unlikely based on the additional observations that: (i) cyclin E reverses RB-mediated growth arrest and specifically restores cyclin A promoter activity without influencing other E2F-dependent promoters; and (ii) ectopic expression of cyclin A alleviates RB-mediated G1/S inhibition. Together, these data demonstrate that cyclin A is a functionally significant target of RB. These data also agree well with the known biological role of cyclin A. For example, it has been well documented that cyclin A and cyclin A-associated kinase activity is required for entry into S-phase, and the observations presented herein explain why RB-arrested cells cannot traverse the G1-S transition (26, 51). Moreover, cyclin A is known to be deregulated in Rb(−/−) MEFs (36, 37, 42).

Since cyclin A is proposed to be an E2F-regulated gene, it has been hypothesized that deregulation of cyclin A in Rb(−/−) MEFs is a result of E2F dysregulation (36, 37). We show that RB-mediated but not E2F-A/B-mediated inhibition of the cyclin A promoter requires an intact CCRE element. The CCRE does resemble a variant E2F site, but whether E2F actually binds this site is controversial (42, 46, 52–54). In fact, recombinant E2F capable of binding in vitro to a genuine E2F probe was shown to be incapable of binding the cyclin A CCRE under identical conditions (52). Instead, it has been proposed that non-E2F transcription factors which have yet to be defined bind this site and regulate the expression of cyclin A (53, 55). While it has been shown that a RB family member, p107, can repress cyclin A transcription (56), no evidence of direct RB binding to the cyclin A promoter has been reported. As such, whether RB acts directly or indirectly to inhibit cyclin A promoter activity has yet to be established; however, this function of RB has a significant impact on cell cycle progression. Several scenarios can be envisioned to explain how cyclin E can restore cyclin A expression. For example, it has been shown that cyclin E can directly bind the cyclin A promoter and activate transcription (57). It is possible that through direct binding, cyclin E reversed the effect of RB on cyclin A transcription. Alternatively, cyclin E-Cdk2 complexes could phosphorylate and mod-

set to 100%. Data shown are results of at least three independent experiments. C, CV1 cells were transfected with 1 μg of CMV-β-gal, 0.5 μg of −608CycA reporter, and designated combinations of the indicated effector plasmid(s) E2F-A/B (3 μg), cyclin E (3 μg), and E1A (3 μg). All transfections were brought to 8 μg of total plasmid DNA using parental vector (CMVNeoBam). Cells were harvested 48 h post-transfection and analyzed for luciferase and β-galactosidase activities. Relative luciferase activity for cells transfected with CMV-β-gal, −608Luc, and vector alone was set to 100%. Data shown are results of the at least three independent experiments. D, CV1 cells were transfected with 1 μg of CMV-β-gal, 0.5 μg of CycALUC reporter, and designated combinations of the indicated effector plasmid(s) E2F-A/B (3 μg), cyclin E (3 μg), and E1A (3 μg). All transfections were brought to 8 μg of total plasmid DNA using parental vector (CMVNeoBam). Cells were harvested 48 h post-transfection and analyzed for luciferase and β-galactosidase activities. Relative luciferase activity for cells transfected with CMV-β-gal, CycALUC, and vector alone was set to 100%. Data shown are results of at least three independent experiments.
ulate substrates other than RB through which RB repression is mediated. Importantly, this function of cyclin E is specific to the cyclin A promoter and is not employed against E2F. Future experiments will be directed at defining the effect of RB and cyclin E on the transcriptional machinery which regulates the cyclin A promoter.

In summary, this report puts forth a model (Fig. 6B) wherein active RB inhibits the G1-S transition by repressing cyclin A promoter activity and reducing cyclin A protein levels. This cell cycle inhibition can be abrogated directly through ectopic expression of cyclin A, or indirectly through ectopic expression of cyclin E, which acts upstream to abrogate RB function and de-repress cyclin A expression. Intriguingly, this function of cyclin E occurs independently of RB phosphorylation, and provides the basis for future investigation into the pleiotropic effects of cyclin E on RB. Moreover, the identification of the cyclin A promoter represents a significant advance toward understanding RB function, and provides the impetus for determining how RB influences the CCRE element and cyclin A promoter activity.

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