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Identification of a substrate-targeting domain in cyclin E necessary for phosphorylation of the retinoblastoma protein

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ABSTRACT **Considerable advances have been made in characterizing the cyclins and cyclin-dependent kinases (CDKs) that are necessary for progression through the cell cycle, but there has been relatively lesser success in identifying the specific biochemical pathways and cell cycle events that are directly under CDK control. To identify physiologically significant CDK substrates we generated mutations in cyclin E that altered the ability of the cyclin to direct the cyclin–CDK holoenzyme to specific *in vivo* substrates. We show that one of these mutations defines a domain in cyclin E necessary for phosphorylation of the retinoblastoma protein (Rb). These observations confirm the idea that cyclins contribute to substrate recognition by cyclin–CDK complexes, demonstrate the utility of targeting mutants in the identification of essential cyclin–CDK substrates, and put cyclin E squarely into the family of proteins designed to regulate Rb.**

The identification of G_1 cyclin–cyclin-dependent kinase (CDK) substrates is beginning to provide a clearer picture of how CDKs promote progression through G_1 and into S phase. G_1 cyclin–CDK complexes appear to activate S-phase entry through two consecutive pathways, the first of which requires cyclin D–CDK4/6 and the second, cyclin E–CDK2. The basic idea is that D-type cyclin–CDK complexes phosphorylate the retinoblastoma protein (Rb), which releases Rb-sequestered transcription factor E2F and derepresses the E2F-dependent transcriptional program (1). A major outcome of this sequence of events is activation of cyclin E gene expression by E2F (2–4). Cyclin E–CDK2 then catalyzes S phase entry through a poorly characterized pathway that is, at least in part, independent of Rb (5, 6). Thus, the cyclin D- and cyclin E-dependent pathways are linked to each other through their relationships to Rb. The data in support of this model are briefly summarized below.

Genetic experiments suggest that Rb is one and perhaps the only essential cell cycle target for D-type cyclin–CDK4/6 enzyme (at least in cell culture). Thus, cyclin D-associated kinase activity is required for transit through G_1 in Rb-positive cells, but not in Rb-negative cells (7–13). Cyclin D–CDK4/6 phosphorylates Rb both in cells (14, 15) and *in vitro* (16), and this is mediated by direct binding of the D-type cyclins to Rb (16). Phosphorylation of Rb decreases its affinity for E2F, and this presumably then causes the accumulation of free E2F and the start of the E2F-dependent transcriptional program (1).

The cyclin E gene is positively regulated by E2F (2–4). During a normal mitotic cell cycle a major function of Rb phosphorylation may be to promote expression of cyclin E itself. Indeed, when expression of many E2F-responsive genes is measured in Rb-negative mouse embryo fibroblasts, only expression of cyclin E is significantly elevated (17). Moreover, neither Rb phosphorylation nor the E2F transcriptional program is necessary for S phase entry in cells that constitutively express cyclin E (18, 19). This observation implies that under certain conditions cyclin E can be the limiting target of E2F-activated gene transcription.

It is not understood how cyclin E–CDK2 catalyzes the G_1 to S phase transition. At present, all that is known is that the S phase promoting function of cyclin E is at least in part independent of Rb. Microinjection of antibodies directed against cyclin E arrest both Rb-positive and Rb-negative cell lines in G_1 (5). One non-Rb substrate of cyclin E–CDK2 is $p27^{Kip1}$, an inhibitor of CDKs. Phosphorylation of $p27^{Kip1}$ on T187 by cyclin E–CDK2 initiates $p27^{Kip1}$ turnover and its elimination from the cell (20). Elimination of $p27^{Kip1}$ will presumably enhance the activation of cyclin E–CDK2 itself, as well as downstream CDK2 enzymes. Nevertheless, it is unlikely that phosphorylation of $p27^{Kip1}$ is the only function of cyclin E in initiating chromosome replication. Therefore, we initiated a screen to identify additional *in vivo* substrates of the cyclin E–CDK2 complex.

A means to identify CDK substrates was suggested by previous work showing that a single CDK can recognize different substrates when associated with different cyclins (21, 22). For instance, the transcription factor DP-1 can be phosphorylated by cyclin A–CDK2 but not by cyclin E–CDK2 (22). In the case of cyclin D2 it was shown that a specific domain, the so-called LxCxE motif, promoted its binding to and efficient phosphorylation of the Rb protein (14). These observations suggested that it might be possible to construct mutations in cyclin E that impair substrate recognition, and that these would aid in the identification of cyclin E–CDK2 substrates. These are designated substrate-targeting mutations, and are operationally identified by the following two criteria: a substrate-targeting mutant should (*i*) be able to assemble into a catalytically active complex with CDK2 when assayed against a nonspecific substrate such as histone H1, and (*ii*) will nevertheless be unable to promote S phase entry when assayed for biological activity *in vivo*. We describe the isolation and characterization of a cyclin E targeting mutant and show that this mutant is defective for an interaction with Rb. This mutant sheds light on the role of cyclin E–CDK2 in Rb phosphorylation *in vivo* and also demonstrates the utility of using cyclin mutants to identify substrates of cyclin–CDK complexes.

MATERIALS AND METHODS

Cloning. Site-directed mutagenesis of the human cyclin E gene (23) was done by using the Sculptor *in vitro* mutagenesis kit (Amersham). The most severe of the alanine scanning mutants had three closely spaced charged amino acids, starting at amino acid 273, changed to alanines, and is called E273 throughout the text (Table 1). The 5′ ends of the cyclin E genes were joined in-frame to 6 copies of the myc9E10 epitope in the

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Abbreviations: CDK, cyclin-dependent kinase; Rb, retinoblastoma protein.

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Table 1. Overexpression phenotypes of cyclin E alanine scanning mutants in NIH 3T3 cells

Amino acids	Histone H1 kinase	G_1 acceleration
Wild type	+++	+++
12–14 (RSR)	+++	++
15–17 (KRK)	+++	+++
26–30 (DPDEE)	+++	+++
33–36 (KIDR)	+++	+++
39–40 (RD)	++	++
64–67 (DKED)	−	−
68–70 (DDR)	+++	+++
78–80 (KPR)	+++	+++
98–100 (REE)	+++	+/−
108–110 (KEK)	−	−
114–117 (RDQH)	+++	+++
246–249 (DLHE)	+++	+/−
273–278 (DVDCLE)	++	−
324–325 (RE)	++	++
330–335 (KLKHFR)	+++	++
339–341 (DED)	+++	++
348–350 (HRD)	+++	++
353–357 (DLLDK)	+++	+/−
359–362 (RAKK)	+++	++

plasmid CS2+MT (24). The cyclin E C276G and ΔVDCLE mutants were generated as described (25). Both strands of all cyclin E mutants were sequenced in their entirety.

***In Vivo* Assays.** NIH 3T3 cells, SAOS-2 cells, and 293 cells were grown on Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. Transient transfections were done by using calcium phosphate precipitation. In NIH 3T3 cells, 2.5 μg of CMVCD20 plasmid (26) was transfected with 20 μg of a control vector or 5–20 μg of CS2+MT cyclin E vector. Control vector was added to keep the total amount of transfected DNA constant. Cells were harvested 40 hr after transfection by treatment with EDTA (26, 27). A minimum of 5,000 transfected cells were scored by flow cytometry, and the data were analyzed by using MultiCycle AV (Phoenix Flow Systems, San Diego) to determine the percentage of cells in G_1. Myc-tagged cyclin E expression levels were determined by Western blot analysis using the 9E10 monoclonal antibody. For histone H1 kinase assays, 250 μg of cell lysate was immunoprecipitated with the 9E10 antibody. Immune complexes were collected on protein G beads as described previously (23).

For analysis of Rb phosphorylation SAOS-2 cells were transfected with 5 μg of CMVpRb (28), 2.5 μg of pCMVCD20, and various amounts of cyclin plasmid or a control plasmid. A 350-μg sample of cell lysate was immunoprecipitated by using a polyclonal Rb antibody (C15; Santa Cruz Biotechnology). Immunopreciptated proteins were separated by electrophoresis in a denaturing 6% polyacrylamide gel and were subjected to Western blot analysis using the C15 antibody.

NIH 3T3 cells were transfected with 2.5 μg of pCMVCD20, 2 μg of pCMVp16 (11), and 20 μg of cyclin E. For *in vivo* phosphorylation of large pocket Rb, 5 μg of pCMV large pocket Rb (28), 2 μg of CMVp16, and 5 or 10 μg of cyclin E were transfected into NIH 3T3 cells. An 80-μg sample of cell lysate was electrophoresed on a 7.5% denaturing polyacrylamide gel with SDS and subjected to Western blot analysis using the C15 antibody.

For *in vivo* binding studies, 293 cells or NIH 3T3 cells were transfected with 5 μg of CS2+myc-tagged cyclin, 10 μg of CMVpRb, and 2 μg of CMVwtCDK2, CMVdnCDK2 (26), or empty vector. Cells were harvested and lysed by sonication in PBS-Nonidet P-40 (NP40) buffer (PBS/0.1% NP40/25 mM NaF/10 μg/ml leupeptin/10 μg/ml aprotinin/2 mM Na_3VO_4), immunoprecipitated by using the C15 antibody, and analyzed by denaturing polyacrylamide gel electrophoresis followed by Western blotting using the 9E10 antibody.

***In Vitro* Assays.** Cyclin E and CDK2 baculovirus were coinfected in Sf-9 insect cells. Lysates were prepared as described (29). Lysates containing equivalent amounts of cyclin E–CDK2 complexes, as determined by immunoprecipitation and Western blotting, were mixed with 3 μM histone H1 (Boehringer Mannheim) or 5 μM His-tagged Rb and incubated at 37°C in a total volume of 10 μl containing 50 mM Tris·HCl at pH 8.0, 10 mM MgCl2, and 1 mM DTT (assay buffer) with 50 μM ATP and 1.5 μCi (1 μCi = 37 kBq) of [γ-^{32}P]ATP (3,000 Ci·mmol^{-1}, NEN). After separation by denaturing polyacrylamide gel electrophoresis, incorporation of ^{32}P into protein was quantified by a PhosphorImager and IMAGEQUANT software (Molecular Dynamics). His-tagged full-length Rb was made in insect cells and purified on Ni-nitrilotriacetate (NTA) resin (Qiagen). Control experiments using lysates from Sf-9 cells expressing only CDK2 or only cyclin E showed that both the histone H1 and Rb kinase activities in the crude Sf-9 cell lysates required both cyclin E and CDK2.

Cyclin E–CDK2 complexes generated in Sf-9 insect cells were incubated with purified His-tagged Rb and assay buffer with or without 5 mM ATP for 20 min at 30°C. Immunoprecipitations were done by using protein A beads and polyclonal antibodies directed against cyclin E. Samples were subjected to denaturing polyacrylamide gel electrophoresis followed by Western blotting using a monoclonal antibody directed against Rb (IF8; Santa Cruz Biotechnology). To analyze binding of the deletion mutant to Rb, immunoprecipitations were done by using the C15 polyclonal anti-Rb antibody and a cyclin E monoclonal antibody (HE12; Santa Cruz Biotechnology) was used for Western blot analysis.

RESULTS

VxCxE: A Putative Rb Interaction Motif in Cyclin E. To identify regions of the cyclin E protein involved in targeting substrate molecules, clusters of charged amino acids were changed to alanine, because charged clusters are most likely to reside on the exposed surface of the protein and therefore to be sites of interaction with other proteins (30). The middle third of the cyclin E protein containing the cyclin box, a protein domain required for binding CDK2, was not mutagenized.

Wild-type and mutant cyclins were tagged with the myc9E10 epitope (31) and transiently overexpressed in Rb-positive NIH 3T3 cells. In all the experiments described below the biochemical activities of the mutant cyclins were distinguished from endogenous, wild-type cyclin E by using antibodies that specifically recognize the myc9E10 epitope tag. The effect of cyclin overexpression on the distribution of cells within the cell cycle was determined by flow cytometry, and the ability of the mutant cyclin to bind to and activate CDK2 was determined by immunoprecipitation of epitope-tagged cyclin–CDK complexes from cell extracts (Table 1). By immunofluorescence, wild-type cyclin E and all of the mutants were primarily located in the nucleus (not shown). Transient overexpression of wild-type cyclin E resulted in a decreased proportion of cells in G_1 in comparison with either control transfected cells or untransfected cells (not shown; see Fig. 3*A*). As a control, it was shown that a cyclin E mutated in the CDK2 interaction domain did not activate CDK2 after transfection into NIH 3T3 cells, nor was it able to alter cell cycle progression (not shown).

Six of the 19 cyclin E mutants were markedly impaired in their ability to promote cell cycle progression, four of which retained the ability to activate CDK2 when assayed using histone H1 as a substrate (Table 1). We report here the characterization of the most severely affected of the cyclin E alanine scanning mutants, cyclin E273 (273 DVDCLE → 273 AVACLA). Although this mutation essentially eliminated the ability of cyclin E to promote cell cycle progression in NIH 3T3 cells (Fig. 1*A*), expression of cyclin E273 protein and its

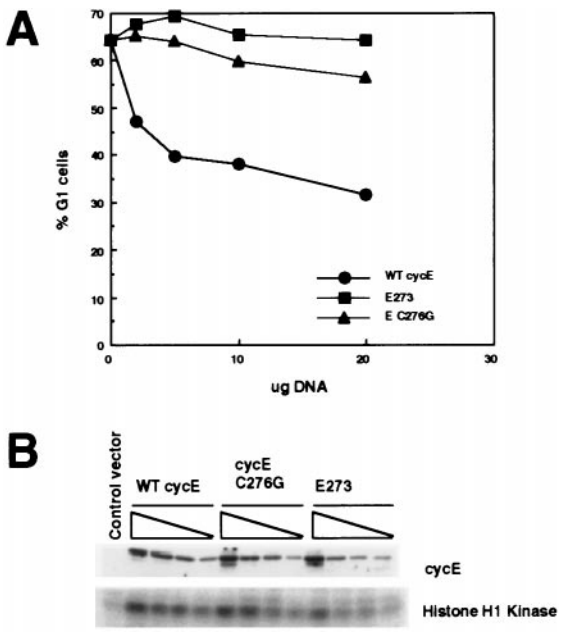

FIG. 1. Transfection of wild-type and mutant cyclin E proteins into NIH 3T3 cells. NIH 3T3 cells were transiently transfected with increasing amounts of myc-tagged wild-type or mutant cyclin E. (*A*) Percentage of cells in G_1 phase of the cell cycle versus amount of transfected plasmid. (*B*) Cyclin E protein expression and associated histone H1 kinase activity from cells depicted in *A*.

associated histone H1 kinase activity were close to those of wild-type cyclin E (Fig. 1*B*). The amino acids altered in cyclin E273 (VxCxE) are phylogenetically conserved and the sequence is similar to a Rb-binding domain found in viral oncoproteins and the D-type cyclins (LxCxE) (14, 32, 33). Single amino acid substitutions in the Rb-binding domain functionally inactivate viral oncoproteins (32, 34–36) and D-type cyclins (14, 37, 38). Similar mutations in cyclin E also abrogated G_1 acceleration (Fig. 1*A*.) Thus cyclin E C276G (Fig. 1) was unable to accelerate cell cycle progression, although it was expressed at levels equivalent to wild-type cyclin E and had wild-type levels of associated histone H1 kinase activity. We emphasize that the decreased biological activity of cyclin E C276G cannot be explained by cyclin E C276G activating CDK2 less well than does wild-type cyclin E. Note in particular that high levels of cyclin E C276G have almost no effect on the cell cycle, even though the amount of histone H1 kinase activated by this mutant cyclin exceeds that of biologically active doses of wild-type cyclin E.

Testing the Role of the VxCxE Motif in the Genetic Interaction Between Cyclin E and Rb. We performed genetic, biochemical, and molecular studies to determine whether the VxCxE motif in cyclin E defined a Rb interaction domain. First, we compared the effects of mutant and wild-type cyclin E protein on Rb-positive NIH 3T3 cells to their effects on SAOS-2 cells, which do not express functional Rb (39). Mutant and wild-type cyclin E proteins were equally effective at promoting G_1 acceleration in SAOS-2 cells (Fig. 2*A*), suggesting that the VxCxE mutants fail to promote cell cycle progression in NIH 3T3 cells because of an impaired interaction with Rb. However, Rb cannot be the only cyclin E substrate, because cyclin E overexpression shortens G_1 in Rb-negative SAOS-2 cells. Moreover, because the VxCxE mutants are active in SAOS-2 cells, the mutations must affect only a subset of cyclin E–CDK2 functions.

We further compared the abilities of mutant and wild-type cyclin E proteins to overcome a G_1 block imposed directly by overexpression of Rb itself. SAOS-2 cells were blocked in G_1 after transient transfection with a plasmid expressing full length Rb (40) (Fig. 2*B*). Co-transfection of Rb with wild-type cyclin E resulted in hyperphosphorylation of the transfected Rb (Fig. 2*D*) and reversal of the G_1 block (40) (Fig. 2*B*). Co-transfection with mutant cyclin E did not result in Rb hyperphosphorylation (Fig. 2*D*) and did not overcome the G_1

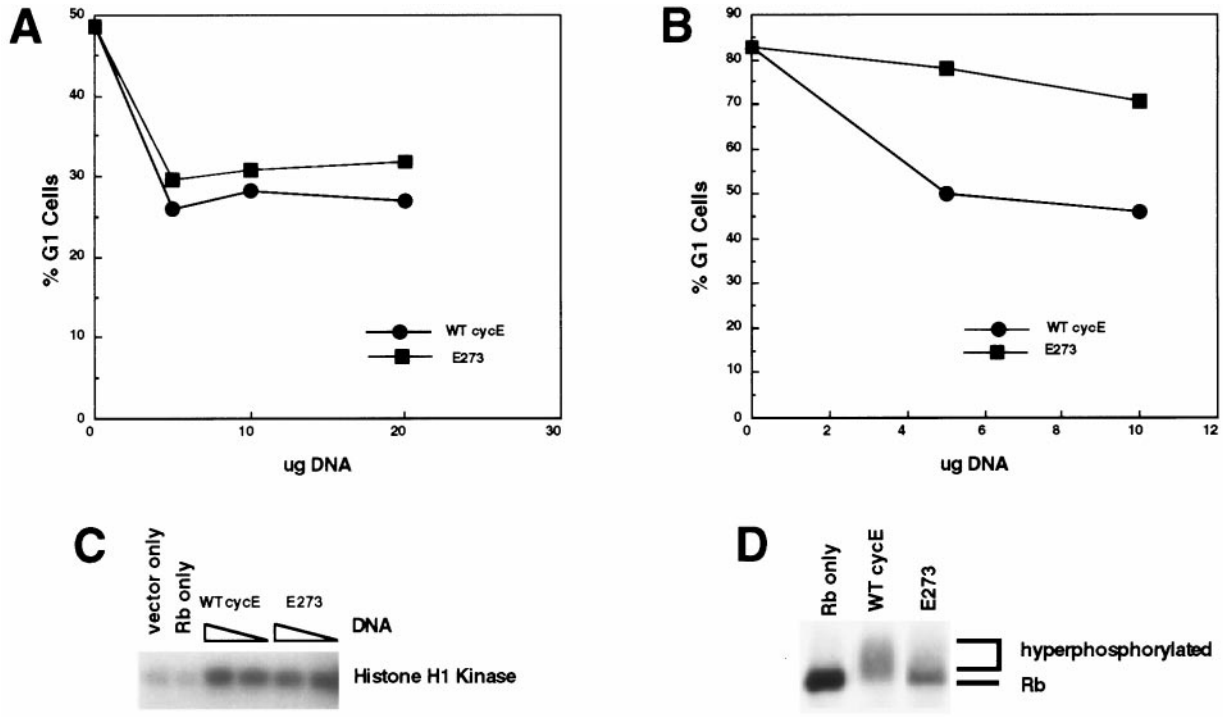

FIG. 2. Transfection of wild-type and mutant cyclin E proteins into SAOS-2 cells. (*A*) Percentage of cells in G_1 phase of the cell cycle versus amount of transfected plasmid. (*B*) SAOS-2 cells transfected with pCMVpRb plasmid and increasing amounts of cyclin E: percentage of cells in G_1 phase of the cell cycle versus amount of transfected plasmid. (*C*) Cyclin E-associated histone H1 kinase activity from cells transfected with pCMVpRb plus cyclin E (*D*) Phosphorylation of transfected full-length Rb by cyclin E (5 μg of transfected DNA) in SAOS-2 cells.

block (Fig. 2*B*), even though mutant and wild-type cyclin E proteins had equivalent levels of histone H1 kinase activity (Fig. 2*C*).

As an alternative approach we used the INK4 protein, p16, to create a specific lesion in the normal pathway of Rb phosphorylation in NIH 3T3 cells. p16 is a specific inhibitor of cyclin D–CDK4/6 complexes (41, 42) and it arrests Rb-positive cells in G_1 when overexpressed *in vivo* (11, 13, 43). Enforced expression of wild-type cyclin E overcame the p16-induced G_1 arrest in NIH 3T3 cells (Fig. 3*A*); cyclin E273 was unable to overcome this G_1 block (Fig. 3*A*), thereby suggesting a specific impairment in the inactivation of Rb. When plasmids encoding p16 and Rb were cotransfected into NIH 3T3 cells, the exogenously expressed Rb was hypophosphorylated (Fig. 3*B*). The ability of wild-type cyclin E to hyperphosphorylate Rb paralleled its ability to overcome the G_1 arrest imposed by p16 expression in NIH 3T3 cells (Fig. 3 *A* and *B*). In contrast, both cyclin E273 and cyclin E C276G were not able to catalyze the complete hyperphosphorylation of Rb in cells overexpressing p16 (Fig. 3*B*) and were not able to overcome the p16-imposed G_1 block (Fig. 3*A* and not shown).

Testing the Role of the VxCxE Motif in Rb Phosphorylation by Cyclin E. The abilities of wild-type and mutant cyclin E proteins to phosphorylate Rb were quantitated *in vitro*. Mutant and wild-type cyclin E genes were cloned into baculoviral expression vectors and coexpressed with CDK2 in Sf-9 insect cells. In four separate experiments K_m values for histone H1 for wild type, cyclin E273, and cyclin E C276G were found to be identical (5 mM histone H1, not shown), and the rates of histone H1 phosphorylation were the same for the wild-type and mutant cyclin E–CDK2 complexes (Fig. 4*A*). This result confirmed that the mutant cyclins were not deficient in binding to or activating CDK2. However, in side-by-side experiments both of the mutant cyclin E proteins reproducibly showed a reduced rate of Rb phosphorylation *in vitro* compared with wild-type cyclin E (Fig. 4*B*), directly demonstrating an impaired interaction between Rb and these mutant enzymes.

Testing the Role of the VxCxE Motif in Binding of Cyclin E to Rb. We found that cyclin E bound stably to Rb, both *in vivo* and *in vitro*, and that this interaction was disrupted by mutation of the VxCxE motif in cyclin E. Cyclin E–CDK2 complexes were generated by coinfection of Sf-9 cells with baculoviral expression vectors and then were mixed with purified recombinant Rb. The assembly of a complex containing cyclin E and Rb was demonstrated by immunoprecipitation with antibodies to cyclin E (Fig. 5*A*) or Rb (Fig. 5*B*). Addition of ATP abolished the binding of cyclin E–CDK2 to Rb, suggesting that phosphorylation of the Rb protein destabilized the cyclin E–CDK2-Rb complex. This idea was confirmed by experiments showing that a complex composed of cyclin E and a catalytically inactive form of CDK2 bound Rb regardless of the presence or absence of ATP (Fig. 5*A*). Similar results were obtained *in vivo* after transfection of both NIH 3T3 cells and

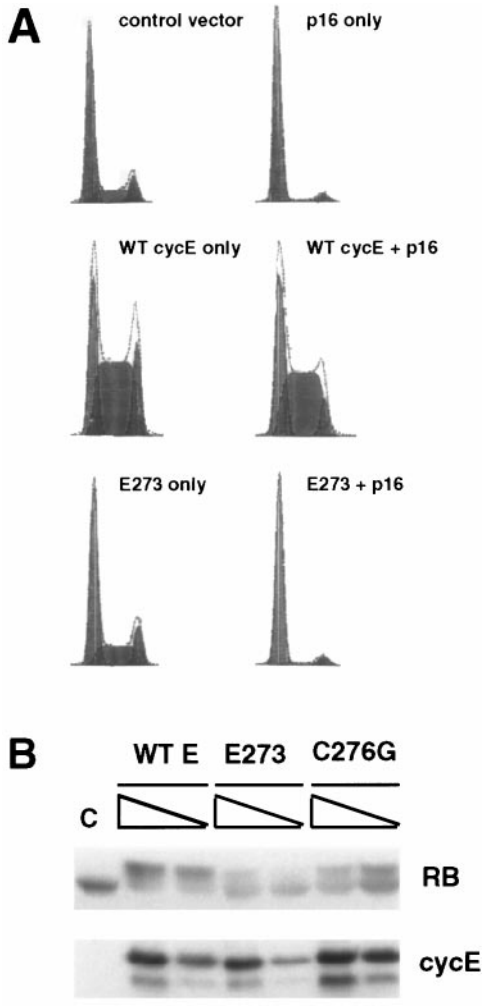

FIG. 3. Mutant cyclin E proteins cannot overcome a p16 cell cycle block in NIH 3T3 cells. (*A*) NIH 3T3 cells were transfected with cyclin E in the presence or absence of p16. For each transfected population, DNA content is shown on the *x* axis and cell number on the *y* axis. (*B*) Transient transfection of NIH 3T3 cells comparing *in vivo* phosphorylation of exogenous large pocket Rb by cyclin E in the presence of exogenous p16.

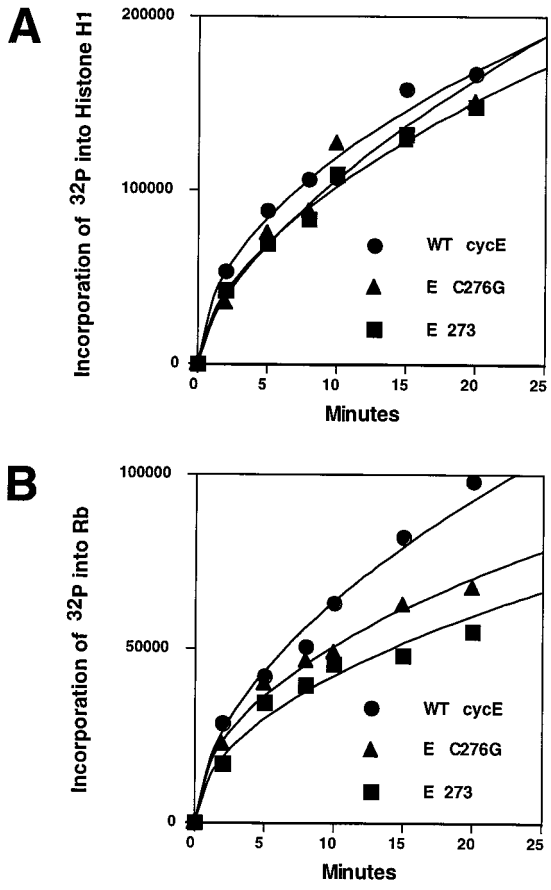

FIG. 4. VxCxE mutants are defective for Rb but not histone H1 phosphorylation *in vitro*. (*A*) Recombinant cyclin E–CDK2 complexes made in baculovirus-infected Sf-9 insect cells were incubated with [γ-^{32}P]ATP and either histone H1 (*A*) or Rb (*B*) for increasing amounts of time. Results shown are representative of three different experiments.

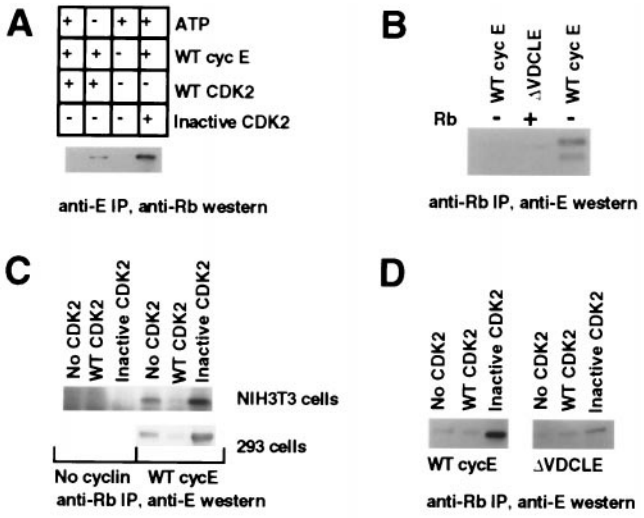

FIG. 5. Binding of cyclin E to Rb *in vivo* and *in vitro*. (*A*) Cyclin E associated with either wild-type or catalytically inactive CDK2 was mixed with recombinant Rb in the presence and absence of ATP, as indicated. Stable binding of Rb to cyclin E was determined by coimmunoprecipitation using anti-cyclin E antibodies. (*B*) Lysates of Sf-9 cells infected with inactive CDK2 and either wild-type or ΔVDCLE cyclin E were mixed with recombinant Rb. Binding of Rb to cyclin E was determined by coimmunoprecipitation using anti-cyclin Rb antibodies. (*C*) Wild-type cyclin E, Rb, and the indicated CDK2 proteins were expressed in NIH 3T3 cells and 293 cells. Binding of Rb to cyclin E was determined as in *B*. (*D*) Wild-type or mutant cyclin E, Rb, and the indicated CDK2 protein were expressed in 293 cells. Binding of Rb to cyclin E was determined as in *B*. In this experiment binding of Rb to cyclin E in the absence of CDK2 is not as evident as in *C*.

293 cells with vectors that express cyclin E, CDK2 (or inactive CDK2), and Rb (Fig. 5*C*). In the absence of exogenous CDK2 or in the presence of inactive CDK2, Rb could be coimmunoprecipitated with cyclin E. However, in the presence of exogenous wild-type CDK2 no Rb was detected in complex with cyclin E. Thus we have demonstrated a specific binding interaction *in vivo* between cyclin E and the Rb protein.

We extended our analysis to cyclin E proteins mutated in the VxCxE motif. We constructed a cyclin E deletion mutant, ΔVDCLE, that removed the putative Rb interaction domain. We were not able to detect binding of the ΔVDCLE mutant to Rb *in vitro*, even in the presence of catalytically inactive CDK2 (Fig. 5*B*) and we obtained similar results after transfection of the appropriate vectors into NIH 3T3 (data not shown) and 293 cells (Fig. 5*D*). Interestingly, deletion of the VxCxE motif also prevented binding of cyclin E to CDK2 (data not shown), demonstrating an unexpected relationship between the binding of cyclin E to a substrate and its ability to stably assemble into a holoenzyme with its catalytic subunit.

DISCUSSION

We have assembled genetic, biochemical, and molecular evidence that the VxCxE motif in cyclin E is a phylogenetically conserved Rb-binding domain. In the course of these studies we have also uncovered evidence that cyclin E can bind directly to Rb in cells. The discovery of an Rb-binding domain in cyclin E that is conserved both among cyclin E proteins from different species and among different proteins that all associate with Rb leaves little doubt that cyclin E is a member of the family of proteins designed to regulate Rb. Although our experiments show that the VxCxE motif is important for the interaction of cyclin E with Rb, they do not address whether this motif may also be important for its interaction with other substrates.

D cyclin–CDK4/6 complexes are the prototypical Rb kinases, and the interaction between cyclin D and Rb is very similar to the one we now describe between cyclin E and Rb. Mutations in the LxCxE motif in cyclin D (14), and the VxCxE motif in cyclin E, decreased their ability to bind to and phosphorylate Rb both in cells and *in vitro*. However, unlike the D-type cyclins, complete deletion of the cyclin E VxCxE motif abrogates binding to both Rb and its CDK catalytic partner. It is possible that substrate recognition and CDK binding by cyclin E might be coordinated with each other through this domain of the cyclin E protein.

The idea that Rb is a cyclin E–CDK2 substrate offers further insights into regulation of G_1 progression by mitogenic signals. Growth factors are required through the D-type cyclins to start the program of Rb phosphorylation and E2F-dependent gene expression (44). Once initiated, however, Rb phosphorylation might be maintained independently of cyclin D (and hence independently of mitogenic growth factors) by means of an autonomous loop linking Rb to cyclin E. In this loop cyclin E promotes its own expression by phosphorylating Rb, which activates E2F-dependent cyclin E gene expression (2–4, 44). Inherent in this scheme is a transition from a mitogen-dependent (cyclin D) to a mitogen-independent (cyclin E) route for maintaining Rb phosphorylation and cyclin E gene expression, and this may therefore represent one pathway underlying commitment to cell cycle progression at the restriction point. Of course, this does not imply that the sole function of cyclin E–CDK2 is to phosphorylate Rb. On the contrary, our results and those of others (5, 6, 18, 19, 45–47) show that cyclin E catalyzes additional essential and rate-limiting steps in the initiation of chromosome replication.

The roles of different cyclin–CDK complexes in phosphorylating Rb have remained an important, unresolved, question. When cyclin D-, cyclin E-, and cyclin A-directed kinases are overexpressed they are all capable of hyperphosphorylating Rb and promoting entry into S phase (6, 48, 49). However, examination of *in vitro* Rb phosphorylation by cyclin D–CDK4/6, cyclin E–CDK2, or cyclin A–CDK2 suggested that no single G_1 cyclin–CDK complex could recapitulate all the *in vivo* phosphorylation sites on Rb (50–52). Interaction of Rb with other proteins, including E2F (51–54), cAbl (53), E1A (52), and simian virus 40 large T-antigen (52, 53), appears to be regulated by phosphorylation of specific amino acids, which in turn may be phosphorylated by specific G_1 cyclin CDK complexes. Therefore, complete phosphorylation and inactivation of Rb *in vivo* may require the combined action of different G_1 cyclin–CDK complexes, each of which may affect a subset of Rb interactions and biological functions.

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