Cell, Vol. 98, 859–869, September 17, 1999, Copyright ©1999 by Cell Press

Cdk Phosphorylation Triggers Sequential Intramolecular Interactions that Progressively Block Rb Functions as Cells Move through G1

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Summary

We present evidence that phosphorylation of the C-terminal region of Rb by Cdk4/6 initiates successive intramolecular interactions between the C-terminal region and the central pocket. The initial interaction displaces histone deacetylase from the pocket, blocking active transcriptional repression by Rb. This facilitates a second interaction that leads to phosphorylation of the pocket by Cdk2 and disruption of pocket structure. These intramolecular interactions provide a molecular basis for sequential phosphorylation of Rb by Cdk4/6 and Cdk2. Cdk4/6 is activated early in G1, blocking active repression by Rb. However, it is not until near the end of G1, when cyclin E is expressed and Cdk2 is activated, that Rb is prevented from binding and inactivating E2F.

Introduction

Control of the G1 to S phase transition in the cell cycle is an important checkpoint in regulating cell proliferation. One regulator of this transition is the retinoblastoma protein (Rb) (Ewen, 1994; Weinberg, 1995). Rb can bind to the E2F family of transcription factors (Chellappan et al., 1991; Nevins, 1992; La Thangue, 1994; Lam and La Thangue, 1994; Adams and Kaelin, 1996; Slansky and Farnham, 1996; Dyson, 1998), and the resulting Rb–E2F interaction not only blocks transcriptional activation by E2F but also forms an active transcriptional repressor complex at the promoter of cell cycle genes that can block transcription by recruiting histone deacetylase (HDAC) and remodeling chromatin (Weintraub et al., 1992; Brehm et al., 1998; Luo et al., 1998; Magnaghi et al., 1998). Recent studies suggest that interaction of the Rb–E2F repressor complex with cell cycle control genes is important for growth arrest by Rb (Zhang et al., 1999).

The ability of Rb to interact with E2F and to repress transcription is regulated by phosphorylation catalyzed by cyclin-dependent kinases (Cdks). Rb contains at least 16 consensus sequences for Cdk phosphorylation, but the significance of all of these sites is still unclear. It has been demonstrated that phosphorylation of S-807 or S-811 blocks binding of Rb to c-Abl and that phosphorylation of T-821 or T-826 disrupts interaction with proteins containing the sequence LXCXE (Knudsen and Wang, 1996). Phosphorylation of S-807, S-811, T-821, T-826, and S-780 leads to disruption of binding to E2F (Knudsen and Wang, 1997), which does not contain an LXCXE sequence.

Cdks interact with specific cyclin regulatory subunits, and this interaction is required for kinase activity (Norbury and Nurse, 1992; Reed, 1992; Nasmyth, 1993; Morgan, 1995). Rb can be phosphorylated by several different cyclin–Cdk combinations, including D cyclins (D1, D2, and D3) in combination with Cdk4 or Cdk6, cyclin E associated with Cdk2, and cyclin A associated with Cdk2 or Cdc2 (Hinds et al., 1992; Dowdy et al., 1993; Ewen et al., 1993; Kato et al., 1993; Hatakeyama et al., 1994; Mittnacht et al., 1994; Roberts et al., 1994; Ohtsubo et al., 1995; Weinberg, 1995). Phosphorylation by cyclin D–Cdk4/6 and cyclin E–Cdk2 occurs during G1, whereas cyclin A is not expressed until S phase, and thus cyclin A–Cdk complexes may serve to add to or maintain phosphorylation of Rb during S phase (Sherr, 1996). However, the Cdks that actually phosphorylate Rb in vivo and the phosphoacceptor sites for these kinases are still unclear, as is the precise mechanism by which phosphorylation regulates Rb activity. Recent studies indicate that a "knock in" of the *cyclin E* gene into the *cyclin D1* locus completely reverses the phenotype of the cyclin D1 gene knockout (Geng et al., 1999), suggesting that cyclin E is a major downstream target of cyclin D1 and thus Rb repressor activity. These results then provide a molecular explanation of the sequential expression of cyclin D1 and cyclin E during the cell cycle.

Cdk4/6 becomes active before Cdk2 during G1, and recent evidence suggests that phosphorylation of Rb by Cdk4/6 may be required for its subsequent phosphorylation by Cdk2 (Ezhevsky et al., 1997; Lundberg and Weinberg, 1998). Other studies also support the idea that full hyperphosphorylation of Rb may require multiple Cdks (Hatakeyama et al., 1994; Zarkowska and Mittnacht, 1997). The initial phosphorylation by Cdk4/6 only appears to be responsible for a portion of the phosphorylation of Rb that occurs during G1, and progressive phosphorylation by both Cdk4/6 and Cdk2 seems to be necessary for hyperphosphorylation of Rb and a block of growth arrest (Lundberg and Weinberg, 1998). The targets of Cdk4/6 and Cdk2 phosphorylation in vivo are still unclear, as is the mechanism through which phosphorylation by Cdk4/6 may facilitate subsequent phosphorylation by Cdk2. In addition to the G1/S transition, Rb also seems to regulate progression through S phase, but there are conflicting results as to whether interaction of Rb with E2F is required for S phase regulation (Chew et al., 1998; Knudsen et al., 1998). Phosphorylation by the different Cdks may then regulate Rb functions in different points of the cell cycle.

Here, we present evidence that phosphorylation of Rb by G1 Cdks leads to successive intramolecular interactions that initially block HDAC binding to the pocket and thus active transcriptional repression and then disrupt pocket structure, preventing Rb from binding and inactivating E2F.

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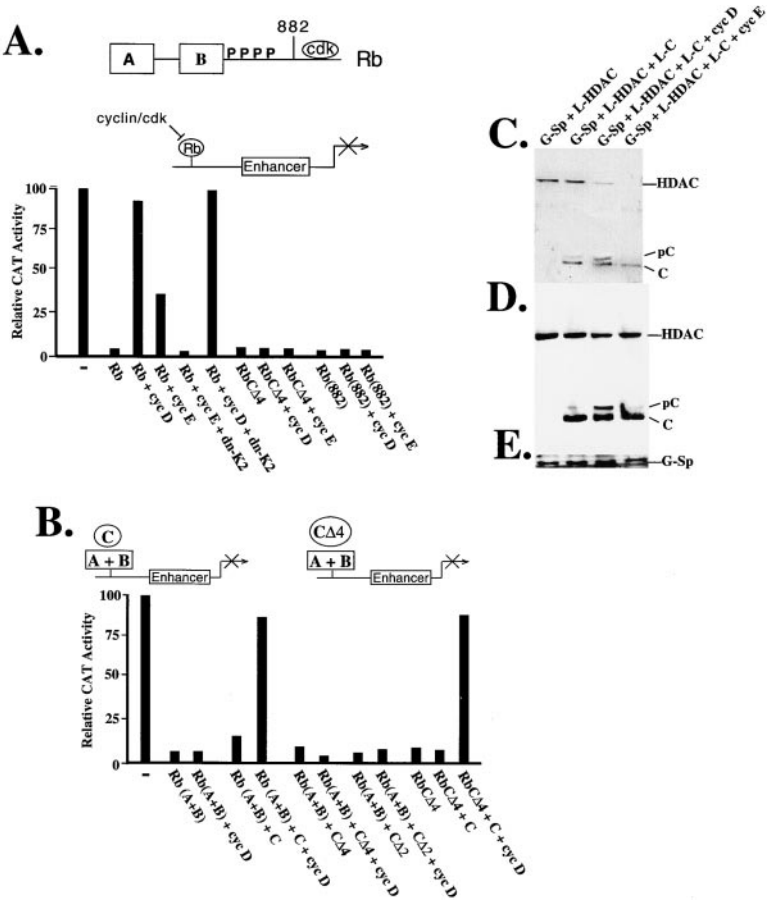

Figure 1. The Phosphorylated C-Terminal Region of Rb Displaces HDAC from the Pocket

(A) The Rb C-terminal region contains docking sites for cyclin–Cdks ("Cdk") (Adams et al., 1999) and Cdk phosphoacceptor sites ("P") (Knudsen and Wang, 1996, 1997; Whitaker et al., 1998; Adams et al., 1999), and both are required for Cdk inhibition of Rb repressor activity. Rb fused to the DNA-binding domain of Gal4 (Weintraub et al., 1995) was coexpressed in Rb(−) C33a cells with the pSVEC-G reporter containing Gal4-binding sites upstream of the SV40 enhancer (Chow and Dean, 1996; Chow et al., 1996) to assay active transcription repression by Rb. CAT activity from the reporter was assayed as described (Weintraub et al., 1995). "cyc" indicates cyclin. Rb(A+B) contains only the A–B pocket (amino acids 379–792). In RbCΔ4, S-807, S-811, T-821, and T-826, Cdk phosphoacceptor sites (P) are mutated to alanine. Rb(882) is truncated at amino acid 882. "dn-Cdk2" indicates an expression vector for dominant-negative Cdk2 (van den Heuvel and Harlow, 1993).

(B) The C-terminal region of Rb in *trans* can block pocket repressor activity. Transfections were done as in (A). "C," "CΔ4," and "CΔ2" indicate that the C-terminal region, the C-terminal region with the CΔ4 mutation, or the C-terminal region with the CΔ2 mutation, respectively, were coexpressed in *trans*.

(C) The phosphorylated C-terminal region of Rb displaces HDAC1 from the pocket. Expression vectors for HDAC1 fused to a LexA tag (L-HDAC), the C-terminal region (amino acids 792–928) fused to LexA (L-C), the Rb small pocket (amino acids 379–792) fused to a Gal4 tag (G-Sp), cyclin D (cyc D), or cyclin E (cyc E) were cotransfected into C33a cells. G-Sp was immunoprecipitated and associated L-HDAC and L-C were detected by Western blot as described (Luo et al., 1998).

(D) Direct Western blot of L-HDAC and L-C. "pC" indicates the more slowly migrating phosphorylated form of the C-terminal region.

(E) Direct Western blot for immunoprecipitated G-Sp.

Results

Mutation of Phosphoacceptor Sites or Deletion of Cdk Docking Sequences in the C-Terminal Region of Rb Prevents a Cdk-Mediated Block in Repressor Activity

The central pocket domain of Rb (amino acids 379–792) is sufficient for active transcriptional repression (Weintraub et al., 1995; Chow and Dean, 1996; Chow et al., 1996). Even though the pocket contains consensus Cdk phosphoacceptor sites, the repressor activity of this region is not inhibited by Cdks; however, when the C-terminal region of Rb (amino acids 792–928) is included with the pocket and G1 Cdks are activated by expression of their cyclin regulator subunits, active repression is inhibited (Figure 1A) (Chow and Dean, 1996). The C-terminal region contains docking sites for both cyclin D and cyclin E within the region C-terminal to amino acid 870 (Adams et al., 1999). Deletion to amino acid 882 (Δ882) removes all of the cyclin D docking sites and all but one potential cyclin E docking site. This Δ882 mutation did not affect repressor activity, but it prevented the inhibition of active repression by Rb when either cyclin D or E was expressed (Figure 1A). Likewise, mutation of four Cdk phosphoacceptor sites in the C-terminal region (S-807, S-811, T-821, and T-826) (CΔ4) had no effect on repressor activity, but this mutation also prevented the block in repression by the G1 Cdks (Figure 1A).

The C-Terminal Region of Rb Can Act in *trans* to Block Repression by the Pocket Domain

How does phosphorylation of the C-terminal region of Rb inhibit active repression by the pocket? One possibility is that phosphorylation of the C-terminal region causes a conformational change that disrupts the adjacent pocket. As another possibility, the C-terminal region, when phosphorylated, may interact with the pocket, blocking its repressor activity. In the first model, the C-terminal region might only function in *cis* (when fused to the pocket); however, in the second model, the C-terminal region may be able to block pocket repressor activity when expressed in *trans* (on a separate protein). To distinguish between these mechanisms, the pocket and the C-terminal region were coexpressed on separate proteins along with cyclin D, and active transcriptional repressor activity was analyzed. This expression of the C-terminal region in *trans* along with cyclin D efficiently blocked repression by the pocket (Figure 1B). Above, we demonstrated that the CΔ4 mutation of phosphoacceptor sites in the C-terminal region of Rb prevented cyclin D–Cdk4/6 from blocking repressor activity

(Figure 1A); however, we found that expression of the wild-type C-terminal region in *trans* restored the ability of cyclin D–Cdk4/6 to block repressor activity of Rb containing the CΔ4 mutation (Figure 1B). It has been demonstrated that mutation of T-821 and T-826 prevents G1 cyclins from blocking binding of Rb to LXCXE proteins (Knudsen and Wang, 1996). We found that introduction of this CΔ2 mutation into the C-terminal region in *trans* (as with the CΔ4 mutation) prevented derepression by cyclin D expression (Figure 1B), providing further evidence that binding to LXCXE proteins (such as HDAC) is important for active repression by Rb.

The above results demonstrate that the phosphorylated C-terminal region of Rb in *trans* can block repressor activity of the pocket, suggesting that phosphorylation of the C-terminal region may cause it to interact with the pocket and disrupt pocket activity.

The Phosphorylated C-Terminal Region Inhibits HDAC Binding to the Pocket

Active transcriptional repression by the Rb pocket is mediated at least in part by recruiting members of the histone deacetylase (HDAC) family (Brehm et al., 1998; Luo et al., 1998; Magnaghi et al., 1998). These enzymes remove inhibitory acetyl groups from the amino-terminal regions of histone octamers, thereby promoting nucleosome assembly that inhibits transcription by limiting accessibility of transcription factors to the promoter (Hassig et al., 1997). HDAC1 and -2 contain an LXCXE-like sequence that interacts with the LXCXE-binding site in domain B of the Rb pocket (Brehm et al., 1998; Magnaghi et al., 1998). E2F binds a separate site on Rb, allowing Rb to be tethered to a promoter through interaction with E2F while simultaneously binding to HDAC (Brehm et al., 1998).

Since the phosphorylated C-terminal region blocks active repression and T-821 and T-826, which regulate binding to LXCXE proteins, are required for this block, we wondered whether the C-terminal region might inhibit HDAC binding to the pocket. Using coimmunoprecipitation assays, we found that coexpression of cyclin D with the C-terminal region in *trans* inhibited binding of HDAC1 to the pocket (Figures 1C–1E). Interestingly, coexpression of cyclin D with the C-terminal region resulted in a more slowly migrating phosphorylated form of the C-terminal region, and preferential binding of this phosphorylated form to the pocket coincided with inhibition of HDAC1 binding (Figure 1C). These results suggest that the phosphorylated C-terminal region can bind to the pocket and inhibit HDAC binding. It is of note, however, that in the absence of cyclin expression the unphosphorylated C-terminal region also bound to the pocket, and expression of cyclin E prevented binding of HDAC to the pocket without binding of the phosphorylated C-terminal region to the pocket (Figure 1C). These latter points will be discussed in detail later in the paper.

A Lysine Patch Encircling the LXCXE-Binding Site in Pocket Domain B Is a Target of the Phosphorylated C-Terminal Region

The crystal structure of the pocket shows that the LXCXE-binding site in domain B is encircled by six lysine residues (Lee et al., 1998). We reasoned that this positively charged "lysine patch" might be a target of phosphoacceptor sites in the C-terminal region, thereby inhibiting HDAC from binding the LXCXE site. A series of lysine patch mutations were created in Rb at K-713, K-720, K-722, and K-729. While none of the mutations affected transcriptional repression, we observed a progressive loss in the ability of Cdk to block Rb repressor activity as more lysine residues were mutated (Figure 2). Thus, we suggest that cyclin D–Cdk4/6 phosphorylates sites in the C-terminal region that then interact with lysine residues encircling the LXCXE-binding site in domain B, preventing HDAC binding and thereby blocking active transcriptional repression.

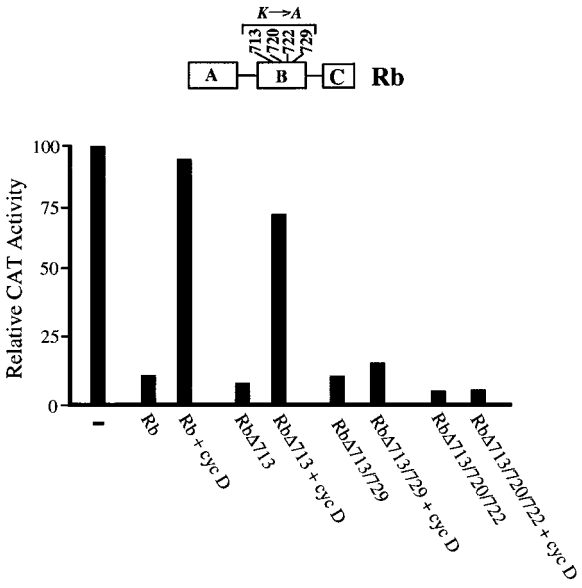

Figure 2. Sequential Mutations in the Lysine Patch surrounding the LXCXE-Binding Site in Pocket Domain B Prevent a Cdk-Mediated Block in Rb Repressor Activity

Transfections were as in Figures 1A and 1B. Lysine to alanine mutations were created sequentially in lysine residues surrounding the LXCXE-binding site (numbers indicate mutated lysine residues). "C" indicates expression of the C-terminal region in *trans*. Similar results were seen with cyclin E expression.

Cyclin D–Cdk4/6 Can Block Active Repression by the Pocket, but Cyclin E–Cdk2 Is Necessary to Prevent Rb from Binding and Inhibiting E2F

Cyclin D–Cdk4/6 becomes active early during G1, while cyclin E–Cdk2 activity peaks near the end of G1 (Sherr, 1996). Phosphorylation of Rb by cyclin D–Cdk4/6 seems to be necessary for its subsequent phosphorylation by cyclin E–Cdk2, and the activity of both kinases is required for complete hyperphosphorylation of Rb (Ezhevsky et al., 1997; Lundberg and Weinberg, 1998). Our above results suggested that expression of cyclin D and thus activation of cyclin D–Cdk4/6 is sufficient to inhibit binding of HDAC to the pocket, leading to a block in active repression. However, expression of cyclin D was inefficient at preventing Rb from inactivating E2F when a reporter containing a minimal promoter with E2F sites was analyzed (Figure 3A). In contrast, expression of

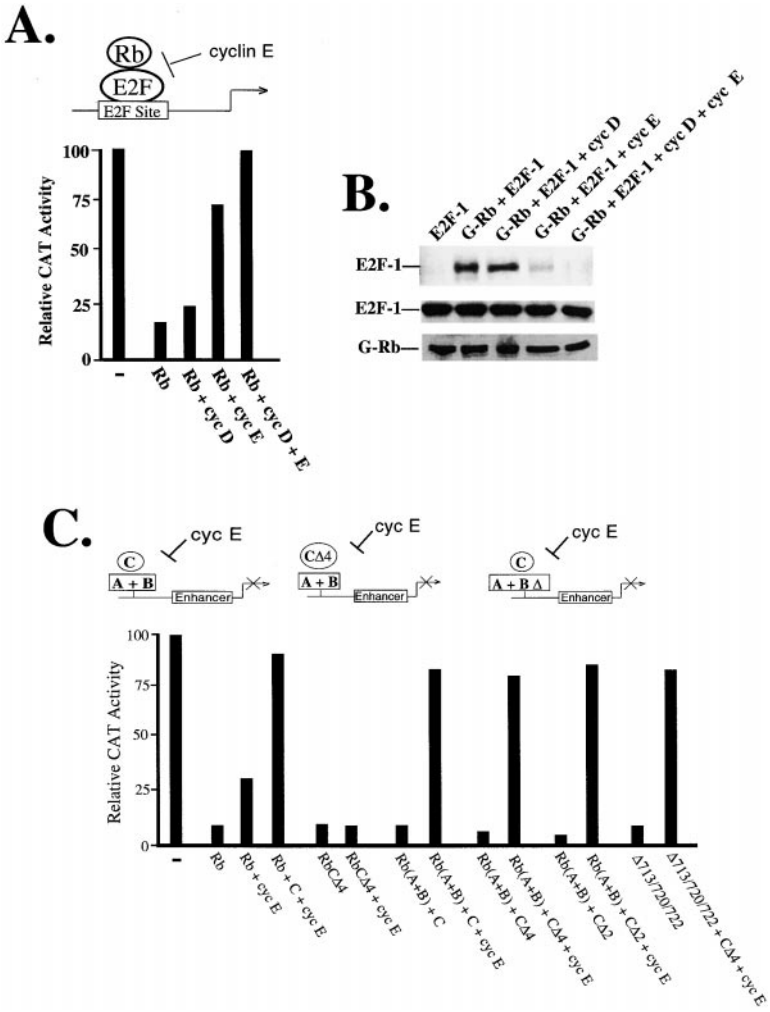

Figure 3. Cyclin E–Cdk4/6 but Not Cyclin D–Cdk4/6 Can Prevent Inactivation of E2F by Rb

(A) Expression of cyclin E but not cyclin D blocks inactivation of E2F by Rb. A minimal promoter containing a TATA box and E2F sites (Weintraub et al., 1992) was cotransfected into Rb(−) C33a cells along with expression vectors for Rb and cyclin D or E.

(B) Expression of cyclin E is required to disrupt the interaction between Rb and E2F-1. Rb (tagged with Gal4) was immunoprecipitated with a Gal4 antibody, and then precipitated proteins were Western blotted for associated E2F-1. "cyc" indicates cyclin. The two blots below show direct Westerns for input E2F-1 and precipitated G-Rb.

(C) Cyclin E–Cdk2 has an additional inhibitory effect on repression by Rb that does not require phosphoacceptor sites in the C-terminal region or the lysine patch in the pocket. The pocket domain of Rb fused to the DNA-binding domain of Gal4 was coexpressed in Rb(−) C33a cells with the pSVEC-G reporter containing Gal4-binding sites upstream of the SV40 enhancer to assess active transcription repression as in Figures 1A and 1B. "cyc" indicates cyclin; A + B Δ is the pocket with lysine patch mutations; and "C" indicates the C-terminal region in *trans*, CΔ4 the C-terminal region in *trans* with four phosphoacceptor sites mutated, and "CΔ2" the C-terminal region with T-821 and T-826 mutated.

cyclin E and thus activation of cyclin E–Cdk2 more efficiently inhibited the inactivation of E2F by Rb. Similarly, in coimmunoprecipitation experiments, expression of cyclin E but not cyclin D was able to inhibit Rb–E2F interaction (Figure 3B). Therefore, we conclude that inhibition of HDAC binding (and thus blocking active repression) as a result of cyclin D–Cdk4 phosphorylation of the C-terminal region is a separate event from inactivation of E2F (which requires cyclin E–Cdk2). Indeed, expression of a dominant-negative form of Cdk2 along with cyclin D had no effect on the block of Rb repressor activity even though it did block the effect of cyclin E (Figure 1A), suggesting that Cdk2 activity is not required for cyclin D–Cdk4/6 to block active repression by Rb.

Even though expression of cyclin D alone had no effect on Rb–E2F interaction, coexpression of cyclin D with cyclin E resulted in further disruption of the Rb–E2F complex and a further inhibition of E2F activity (Figures 3A and 3B). These results suggest that cyclin D–Cdk4/6 may facilitate the inhibitory effect of cyclin E–Cdk2 on Rb–E2F interaction and thus on E2F activity. Our results then reaffirm the previous notion that cyclin E–Cdk2 has an additional effect on Rb, and we initiated studies to examine how cyclin E–Cdk2 affects Rb function.

Cyclin E–Cdk2 Has a Novel Inhibitory Effect on Pocket Activity

Even though expression of cyclin D was more effective than expression of cyclin E in blocking active repression by Rb when the C-terminal region was in *cis*, cyclin E was equally effective as cyclin D when the C-terminal region was expressed in *trans* (Figures 1A and 3C). These results suggest that the activity of cyclin E–Cdk2 is somehow constrained when the C-terminal region is in *cis*, and this constraint is removed when the C-terminal region is expressed in *trans*. Additionally, cyclin E–Cdk2 was not as efficient as cyclin D–Cdk4/6 in phosphorylating the C-terminal region in vivo (Figures 1C and 1D). Together, these results suggested that, when the C-terminal region is expressed in *trans*, cyclin E–Cdk2 may be able to block Rb activity through a mechanism distinct from that of cyclin D–Cdk4/6 and independent of C-terminal phosphorylation.

We reasoned that if inhibition of HDAC binding by the phosphorylated C-terminal region were the only mechanism by which Cdks block Rb repressor activity, then the C-terminal region in *trans* with phosphorylation sites mutated should not block repression. Indeed, this was the case when cyclin D was expressed, but expression of cyclin E still led to derepression with either CΔ4 or

CΔ2 in *trans* (Figure 3C). Likewise, expression of cyclin E also resulted in a block of repressor activity when the C-terminal region in *trans* was coexpressed with the lysine patch mutant. We concluded that cyclin E–Cdk2 has an additional inhibitory effect on Rb that is distinct from inhibition of HDAC binding. This novel activity, in the context of full-length Rb, is dependent upon prior interaction between the lysine patch and the phosphorylated C-terminal region, but this activity becomes constitutive and independent of these sequences when the C-terminal region is expressed in *trans*. Based on these results, we suggest that the initial cyclin D-Cdk4/6-mediated phosphorylation of the C-terminal region and its subsequent interaction with domain B removes a steric constraint imposed on the C-terminal region when it is in *cis*, thereby bringing it into proximity of the pocket. This allows cyclin E–Cdk2, docked to the C-terminal region, to further act on the pocket. Expression of the C-terminal region in *trans* removes the steric constraint and thus eliminates the need for the initial interaction between the phosphorylated C-terminal region and the lysine patch in domain B.

The C-Terminal Region Facilitates Cyclin E–Cdk2 Phosphorylation of S-567 in the Pocket

If relief of steric constraint is all that is required for cyclin E–Cdk2 to block repressor activity by the pocket, it is unclear why the C-terminal region is required, either in *cis* or in *trans*, for cyclin E–Cdk2 to phosphorylate the pocket and block repressor activity. We concluded that in addition to inhibiting HDAC binding, the C-terminal region must have another role in inactivating the pocket through a second cyclin E-Cdk2-dependent mechanism. It seemed likely that this second activity of the C-terminal region might be to facilitate phosphorylation of the pocket by cyclin E–Cdk2. To address this possibility, we performed in vitro phosphorylation assays (Figure 4A). When cyclin E–Cdk2 was incubated with the pocket alone in a 60 min reaction, phosphorylation of the pocket was evident, but inclusion of the C-terminal region in *trans* did not significantly increase the phosphorylation. Mutation of all of the phosphoacceptor sites in the pocket with the exception of S-567 eliminated this phosphorylation of the pocket. When the reaction time was increased to 75 min, we noticed that inclusion of the C-terminal region in *trans* now resulted in increased phosphorylation of the pocket. We found that the pocket containing S-567 as the only Cdk consensus phosphoacceptor site was phosphorylated in a C-terminal-dependent fashion. These results suggest that other phosphoacceptor sites in the pocket are phosphorylated directly by cyclin E–Cdk2, but phosphorylation of S-567 only occurs when the C-terminal region is present. The finding that the other sites are not dependent upon the C-terminal region for phosphorylation suggested that binding of the C-terminal region to the pocket may not simply bring cyclin E–Cdk2 to the pocket; this binding may facilitate access of cyclin E–Cdk2 to S-567, which is normally buried in the A–B interface (Lee et al., 1998). In further support of this possibility, the Δ882 deletion, which removes most of the RXL-like docking sites from the C-terminal region and prevents cyclin D or E from derepressing Rb in transfection assays (Figure 1A), did not prevent this region from augmenting phosphorylation of the pocket by cyclin E–Cdk2 (Figure 4A). We conclude that, in vivo, the C-terminal region facilitates phosphorylation of S-567 both by recruiting cyclin E–Cdk2 to the pocket through the RXL-like docking site and by promoting access to S-567. However, in vitro, the high concentration of cyclin E–Cdk2 may obviate

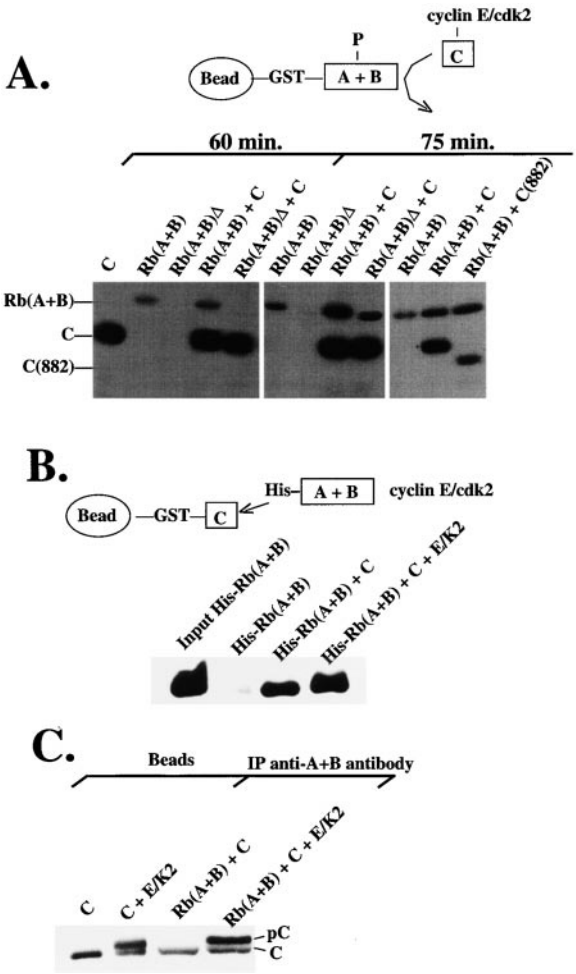

Figure 4. The C-Terminal Region Binds to the Rb Pocket Facilitating Phosphorylation of S-567

(A) Rb(A+B) and the C-terminal region purified from bacteria were incubated as indicated with cyclin E–Cdk2 (expressed in baculovirus) in the presence of [^{32}P]ATP, and proteins were separated by SDS-gel electrophoresis. "60 and 75 min" indicates kinase reaction time. Rb(A+B)Δ is the Rb pocket with phosphoacceptor sites at amino acids 608, 612, 780, and 788 mutated (S-567 is the only remaining consensus site).

(B) Binding of phosphorylated and unphosphorylated C-terminal region to the pocket in vitro appears additive. "E/K2" indicates cyclin E–Cdk2.

(C) Phosphorylated C-terminal region binds to the pocket. Phosphorylation of the C-terminal region in vitro causes the same mobility shift seen in vivo (Figure 1C). "IP" indicates that the complex between the pocket and the C-terminal region was immunoprecipitated with an anti-pocket antibody, and the precipitated proteins were Western blotted with anti-C-terminal region antibody. "Beads" indicates input GST-C, either unphosphorylated or phosphorylated, that was bound to glutathione beads and Western blotted with anti-C-terminal region antibody.

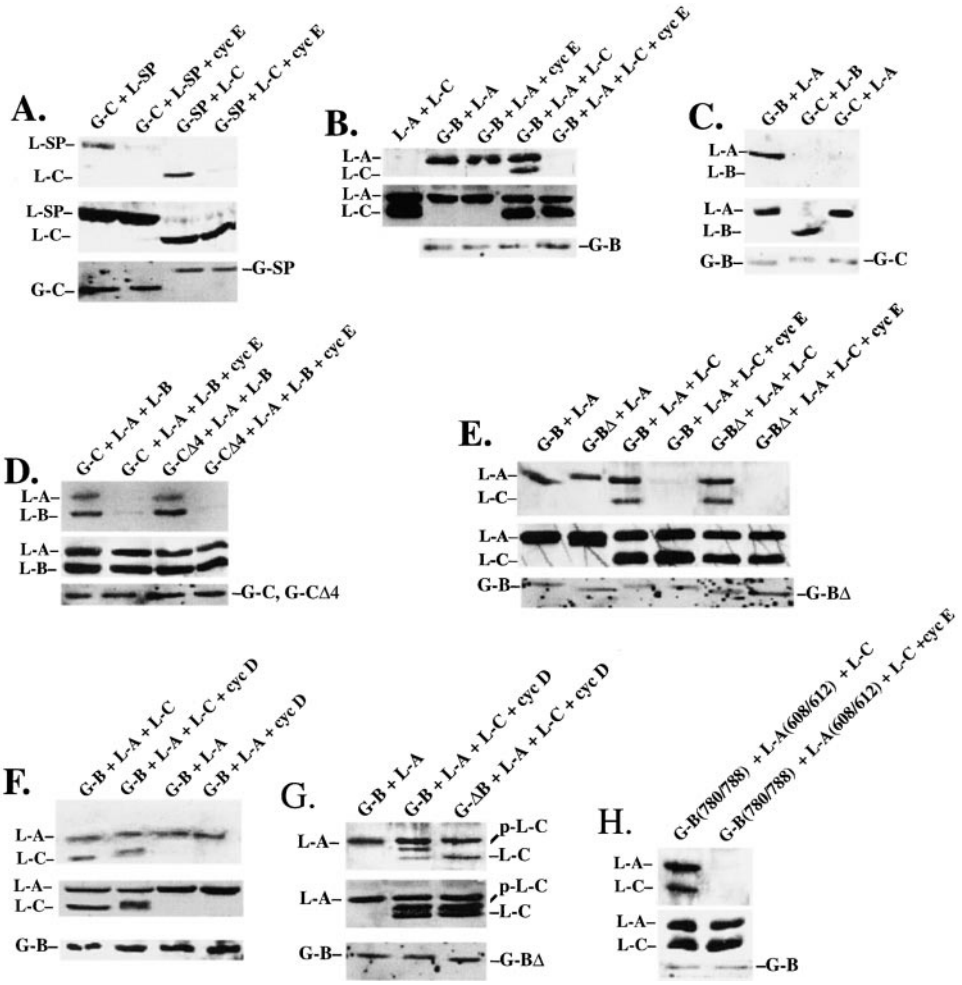

Figure 5. The C-Terminal Region of Rb Cooperates with Cyclin E–Cdk2 to Disrupt the A–B Interaction in the Pocket

"G" and "L" indicate Gal4 and LexA tags used for immunoprecipitation and Western blotting, respectively (Weintraub et al., 1995; Chow and Dean, 1996; Chow et al., 1996; Luo et al., 1998). "A" indicates domain A (amino acids 379–612), "B" indicates domain B (amino acids 620–792), "C" indicates the C-terminal region (amino acids 767–928), and "SP" indicates the small pocket (amino acids 379–792). "ΔB" indicates the lysine patch mutant, and "CΔ4" indicates that phosphoacceptor sites 807, 811, 821, and 826 were mutated to alanine. Numbers in the construct name in (H) indicate mutated phosphoacceptor sites. In (A–H), expression vectors were transfected into Rb(−) C33a cells as indicated and cell extracts were immunoprecipitated with antibody to the Gal4 tag, and associated proteins were detected by Western blot for the LexA tag (Experimental Procedures). In each panel, the top blot is the coimmunoprecipitation, the middle blot is a direct Western showing the level of LexA-binding protein, and the bottom blot is a Western showing the amount of precipitated Gal4 protein.

the need for the RXL-like docking sites. As in vitro, the C-terminal region in *trans* also facilitated phosphorylation of the pocket in vivo (results not shown).

The C-Terminal Region has a Second Phosphorylation-Independent Interaction with the Pocket

In Figures 1C and 1D, we provided evidence that the phosphorylated C-terminal region can bind the pocket and inhibit HDAC binding. However, we also detected binding of the unphosphorylated C-terminal region to the pocket in vivo and in vitro (Figures 1C, 4B, 4C, and 5A). Similarly, when domains A and B were expressed on separate proteins, the unphosphorylated C-terminal region was able to bind the A–B complex that reformed (Figure 5B). However, as with HDAC, no binding was detected to either pocket domain A or B alone (Figure 5C), indicating that binding of unphosphorylated C-terminal region is dependent upon interaction of the two domains. Mutation of the phosphoacceptor sites in the C-terminal region or the lysine patch in pocket domain B did not prevent this binding of unphosphorylated C-terminal region to the pocket (Figures 5D–5G), demonstrating that this binding does not involve the phosphoacceptor sites or the lysine patch. However, when the C-terminal region was phosphorylated, binding to the pocket increased in vitro and in vivo (Figures 4B, 4C, and 5G). Mutation of the lysine patch in the pocket prevented preferential binding to the phosphorylated C-terminal region (Figure 5G). These results provide evidence that the lysine patch mediates interaction of the phosphorylated C-terminal region with the pocket.

Taken together, our results suggest that the C-terminal region makes two contacts with the pocket. Initially,

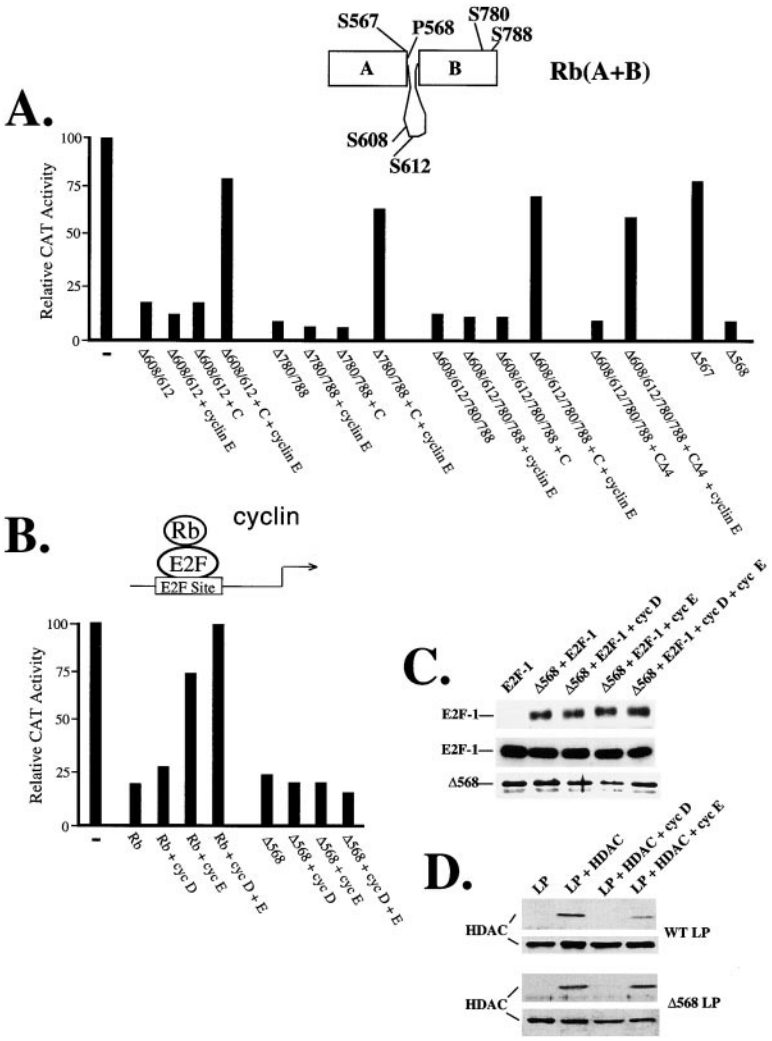

Figure 6. The Cdk Phosphoacceptor Site at S-567 Is Required for Cyclin E–Cdk2 to Prevent Rb from Inactivating E2F

(A) Mutation of phosphoacceptor sites in pocket domain B and in the spacer between A and B does not prevent expression of cyclin D or E from blocking active repression by Rb. The pocket domain of Rb, with the indicated mutations in phosphoacceptor sites, fused to the DNA-binding domain of Gal4 was coexpressed in Rb(−) C33a cells, with the pSVEC-G reporter containing Gal4-binding sites upstream of the SV40 enhancer to assess active transcription repression as in Figures 1A and 1B. "C" and "CΔ4" indicate the C-terminal region and the C-terminal region with four phosphoacceptor sites mutated (in *trans*), respectively. A diagram of the pocket showing the position of mutated residues is shown above.

(B) Elimination of S-567 as a Cdk phosphoacceptor site prevents cyclin E–Cdk2 from blocking the Rb inactivation of E2F. The minimal E2F site reporter (Figure 3A) was cotransfected into Rb(−) C33a cells along with expression vectors for Rb, Δ568, and cyclin D or E, as indicated.

(C) The Δ568 mutation prevents cyclin E–Cdk2 from blocking binding of E2F-1 to Rb. The experiment was similar to that shown in Figure 3B. The second blot is a direct Western showing the level of input E2F-1, and the third blot is a Western showing the level of precipitated Δ568.

(D) The Δ568 mutation prevents cyclin E but not cyclin D expression from inhibiting HDAC binding. "LP" indicates the Rb large pocket (amino acids 379–928); "WT" is wild type. As in Figure 1C, Rb was immunoprecipitated and associated HDAC1 was detected by Western blot. The second blot is a Western showing that the level of input HDAC was similar in each assay.

phosphorylation of the C-terminal region causes it to interact with the lysine patch in pocket domain B and inhibit HDAC binding. This initial interaction facilitates a second phosphorylation-independent interaction with the pocket that is required for cyclin E–Cdk2 phosphorylation of the pocket. These results provide an explanation for how phosphorylation by cyclin D–Cdk4/6 (which triggers the initial interaction) can facilitate phosphorylation by cyclin E–Cdk2 (which is dependent upon the second interaction). It also provides a molecular basis for the observation that both cyclin D–Cdk4/6 and cyclin E–Cdk2 are required for complete hyperphosphorylation of Rb. However, the effect of cyclin E-Cdk2-mediated phosphorylation of the pocket was still unclear.

Cyclin E–Cdk2 Disrupts Interaction of Pocket Domains A and B

In contrast to the effect on active repression by Rb (Figure 1A), expression of cyclin E was more efficient than cyclin D in preventing Rb from inhibiting E2F (Figure 6A). Since it appeared that phosphorylation of the pocket by cyclin E–Cdk2 might mediate this effect, we wondered whether this phosphorylation might prevent Rb from binding and inactivating E2F by disrupting interaction between pocket domains A and B. In coimmunoprecipitation assays, binding of the unphosphorylated C-terminal domain to the pocket did not disrupt the A–B interaction, nor was the A–B interaction significantly affected when cyclin D was coexpressed in these assays (Figures 5B and 5D–5F). However, expression of cyclin E indeed led to disruption of the A–B interaction when the C-terminal region was coexpressed in *trans* (Figures 5B, 5D, and 5E).

The binding site for E2F and HDAC on Rb are distinct, allowing a complex of HDAC-Rb-E2F to be targeted to promoters (Brehm et al., 1998). We suggest that interaction of phosphoacceptor sites on the C-terminal region with the lysine patch blocks binding of Rb to HDAC, thereby preventing active repression. However, the Rb–E2F complex persists, and subsequent phosphorylation of the pocket by cyclin E–Cdk2 is required to disrupt pocket structure and eliminate binding and inactivation of E2F. Therefore, we conclude that the ability of cyclin E–Cdk2 to inhibit HDAC binding to the pocket (Figure 1C) is due to disruption of the A–B interaction, which is required for formation of the LXCXE-binding site in pocket domain B.

Cyclin E–Cdk2 Appears to Disrupt the A–B Interaction by Phosphorylation of S-567 at the A–B Interface

There are five consensus Cdk phosphoacceptor sites within the pocket (S-567 in domain A, S-608 and S-612 in the spacer region between A and B, and S-780 and S-788 in domain B) (Figure 6A). S-608, S-612, S-780, and S-788 could all be mutated without affecting pocket repressor activity (Figure 6A). We then asked whether mutation of these phosphoacceptor sites in the pocket would eliminate the ability of cyclin E–Cdk2 to block repressor activity. For these assays, pocket constructs with the phosphoacceptor sites mutated were coexpressed with cyclin E and the C-terminal region in *trans* (with the four phosphoacceptor sites mutated in order to eliminate the lysine patch interaction and make inhibition of pocket activity totally dependent upon cyclin E–Cdk2). Mutation of these phosphoacceptor sites in the pocket did not prevent cyclin E–Cdk2 from blocking repressor activity (Figure 6A), and furthermore, cyclin E–Cdk2 still disrupted the A–B interaction when these sites were mutated (Figure 5H). These results suggest that phosphorylation of S-608, S-612, S-780, or S-788 by cyclin E–Cdk2 does not affect pocket activity or structure. S-567 is phosphorylated by cyclin E–Cdk2 in vitro, and this is dependent upon the C-terminal region, whereas phosphorylation of the other Cdk phosphoacceptor sites in the pocket is not (Figure 4A).

The phosphoacceptor site at S-567 could not be examined directly because mutation of this residue (Δ567) blocked Rb repressor activity (Figure 6A). In the consensus Cdk phosphorylation sequence, the serine/threonine Cdk phosphoacceptor site is followed by a mandatory proline residue (Songyang et al., 1994; Holmes and Solomon, 1996). Therefore, we mutated the proline at amino acid 568 to an alanine (Δ568), thereby eliminating S-567 as a Cdk phosphoacceptor site. This mutant retained full repressor activity (Figure 6A). To test the importance of S-567 as a potential phosphoacceptor site for cyclin E–Cdk2, we cotransfected an expression vector for Δ568 along with the reporter containing a minimal promoter and E2F sites. Δ568 efficiently blocked E2F transcriptional activation, but in contrast to the wild-type protein, inactivation of E2F by Δ568 was not inhibited by expression of cyclin E (Figure 6B). These results suggest that phosphorylation of S-567 is important for cyclin E–Cdk2 to disrupt pocket structure and block inactivation of E2F.

These results then predict that cyclin E expression should not block binding of Δ568 to E2F. Indeed, we found that neither expression of cyclin E nor the combination of cyclin D and E was able to inhibit binding of E2F-1 to Rb (Figure 6C). However, we reasoned that cyclin D expression should still be able to inhibit HDAC binding, and we found that this was the case (Figure 6D). In Figure 1C, we showed that expression of cyclin E could inhibit HDAC binding to the pocket when the C-terminal region was present in *trans*. In Figure 6D, we show that cyclin E can also disrupt binding of HDAC to Rb (where the C-terminal region is in *cis*), although not as efficiently as cyclin D. Presumably, this effect of cyclin E is to disrupt the A–B interaction in the pocket, resulting in loss of the HDAC-binding site, which is dependent upon the A–B interaction (Luo et al., 1998). In further

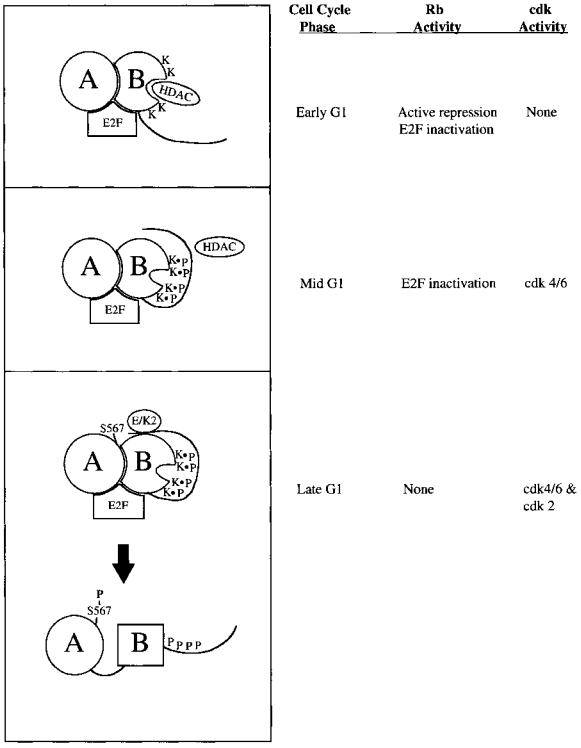

Figure 7. Phosphorylation of Rb Initiates Sequential Intramolecular Interactions between the C-Terminal Region and the Pocket Domain that Result in a Progressive Loss of Rb Functions as Cells Move through G1

See text for additional details.

support of this possibility, we show that the cyclin E effect on HDAC binding is eliminated with the Δ568 mutation (Figure 6D). The finding that cyclin D expression inhibits HDAC binding to Δ568 further emphasized that cyclin D and E are acting through distinct mechanisms and that the Δ568 mutation does not prevent cyclin D–dependent phosphorylation of the C-terminal region and its inhibition of HDAC binding to the pocket.

S-567 is located in domain A at the A–B interface, where it forms contacts with domain B (Lee et al., 1998). Phosphorylation of this site may then destabilize the A–B interface and thus the A–B interaction. The sensitive position of S-567 is further illustrated by the fact that missense mutations occur at this residue in tumors and that these mutations disrupt Rb function (Templeton, et al., 1991).

Discussion

A Model for Inhibition of Rb by G1 Cdks

Cdk4/6 and Cdk2 can both phosphorylate Rb and are activated sequentially as cells progress through G1 (Sherr, 1996). Previous studies have suggested that phosphorylation by Cdk4/6 may facilitate phosphorylation by Cdk2 and that both Cdks may be required for full hyperphosphorylation and inactivation of Rb (Lundberg and Weinberg, 1998). However, little is known of how phosphorylation affects Rb function and why phosphorylation by two different Cdks might be required during

G1. Here, we examined the mechanism of how Cdk phosphorylation regulates Rb activity. Our results suggest that phosphorylation of the C-terminal region of Rb by cyclin D–Cdk4/6 triggers an initial intramolecular interaction with the central pocket domain that inhibits HDAC binding, thereby blocking active transcriptional repression by the pocket (Figure 7). This interaction facilitates a second phosphorylation-independent interaction of the C-terminal region with the pocket. The subsequent interaction is required for cyclin E–Cdk2 to access S-567, which is buried at the A–B interface. Phosphorylation of S-567 disrupts the A–B interface, preventing Rb from binding and inactivating E2F. In this model, the Rb functions of active repression and inactivation of E2F are lost successively through phosphorylation by cyclin D–Cdk4/6 and then cyclin E–Cdk2, respectively. Cyclin D–Cdk4/6 is likely to be the kinase that normally disrupts HDAC binding and blocks active repression in vivo because it is activated in G1 before cyclin E–Cdk2 (Sherr, 1996). If this is the case, then during the interval in G1 between activation of cyclin D–Cdk4/6 and activation of cyclin E–Cdk2, Rb is not able to actively repress transcription, but it can still bind and inhibit E2F. Inactivation of E2F would not be prevented until near the end of G1, when cyclin E–Cdk2 is activated. Such a progressive loss of activities may allow differential regulation of genes involved in cell cycle progression and/or apoptosis.

Potential Rb Target Genes

Recent studies suggest that expression of cyclin E is genetically downstream of cyclin D1 and that cyclin E expression is sufficient to overcome the loss of cyclin D1 (Geng et al., 1996). We have recently used a dominant-negative form of E2F to displace HDAC-Rb-E2F from promoters and provide evidence that this repressor complex has a role in regulation of the G1/S transition (Zhang et al., 1999). We suggest that active repression by HDAC-Rb-E2F may have an important role in repressing cyclin E expression, which is required for assembly of origins of DNA replication and thus for S phase (Hua and Newport, 1998), and this repression is overcome by cyclin D–Cdk4/6. Interestingly, genes such as *cyclin A* also have E2F sites, but their expression is delayed until S phase. This raises the possibility that cyclin A expression may be dependent at least in some cases upon transcriptional activation by E2F, such that its expression is delayed in S phase until cyclin E–Cdk2 disrupts the Rb–E2F complex, thereby releasing free E2F. Indeed, some cell types expressing dominant-negative E2F, which can also displace free E2F from the promoter, are delayed in S phase.

Experimental Procedures

Transfection Assays and Plasmid Constructs

For CAT assays, 0.2 μg of the reporter plasmid pSVEC-G (with Gal4 sites upstream of the SV40 enhancer and E1b TATA box driving the CAT gene) or 2 μg of pE2F-CAT (minimal promoter with E2F sites upstream; Weintraub et al., 1992; Chow and Dean, 1996), along with 0.5 μg of the indicated expression vectors, was transfected into C33A cells in a total of 10 μg of DNA by the calcium phosphate method, as previously described (Weintraub et al., 1995). Empty expression vectors were included in control assays. A phosphoimager was used to quantitate CAT activity. Expression plasmids include the following Gal4-tagged Rb proteins: Rb (amino acids 379–928), Rb(A+B) (amino acids 379–792), RbC (amino acids 767–928), and G-B (amino acids 620–792) (Chow and Dean, 1996). RbCΔ4 was created by subcloning an EcoRI fragment (amino acids 379–928) from pSM.4 (which contains serine-to-alanine substitutions at S-807 and S-811 and threonine-to-alanine substitutions at T-821 and T-826; Knudsen and Wang, 1996) into the Gal4 DNA-binding domain expression vector pM2 (Chow and Dean, 1996). The C-terminal region with the CΔ4 mutation was created by subcloning the SspI/EcoRI (amino acids 767–928) fragment from RbCΔ4 into pM2. RbΔ608/612 was created by deletion of the spacer sequence (amino acids 602–646) between A and B (Chow and Dean, 1996). RbΔ780/788 was created by truncation of Rb(A+B) at amino acid 778 by PCR amplification of the cDNA-encoding amino acids 379–778 and subcloning of the sequence back into pM2. G-BΔ780/788 was created by subcloning amino acids 620–778 from RbΔ780/788 back into pM2. Other Gal4-tagged proteins created by oligonucleotide-directed mutagenesis using the Mutagene in vitro mutagenesis kit (Bio-Rad) include Rb(882) (stop codon introduced at position 882), RbΔ713 (lysine to alanine), RbΔ713/729 (lysine to alanine), RbΔ713/720/722 (lysine to alanine), RbΔ567 (serine to alanine), RbΔ568 (proline to alanine), and RbΔ608/612/780/788, which was created by introducing a stop codon at residue 778 in construct RbΔ608/612. LexA-tagged Rb proteins include L-A (amino acids 379–602) and L-C (amino acids 767–928), described previously (Chow and Dean, 1996). Other vectors include RC.cyclin D2, RC.cyclin E, pCMV-dnCDK2, and pCMV.Cdk4 (van den Heuvel and Harlow, 1993).

In Vitro Phosphorylation

Construction of GST-tagged Rb proteins GST-Rb(A+B) (amino acids 379–792) and GST-C (amino acids 767–928) and their purification from bacteria was described previously (Hinds et al., 1992; Weintraub et al., 1995; Chow and Dean, 1996). Cyclin E–Cdk2 was purified from recombinant baculovirus-infected Sf9 cells (Xu et al., 1994). Kinase reactions were performed in 50 μl of kinase buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 1 mM DTT) with 150 μM ATP, 100 μCi of [γ-^{32}P]ATP, 20 ng of GST-Rb(A+B), and 30 ng of GST-C, as described (Xu et al., 1994). Reactions were incubated at room temperature for 1 hr, resolved by SDS-PAGE, and detected by autoradiography.

In Vivo Phosphorylation

Gal4-tagged constructs (20 μg) were transfected in C33a cells in 150 mm dishes. After 36 hr, media was removed and replaced with 5 ml of phosphate-free MEM containing 5% fetal calf serum dialyzed against HEPES buffer for minimal phosphate content. Five millicuries of [^{32}P]orthophosphate per plate was then added for 6 hr. Cell lysates were then obtained, and Gal4 proteins were collected by immunoprecipitation, resolved by SDS gel, and detected by autoradiography.

Immunoprecipitations

C33A cells were transfected with 9 μg each of the indicated expression vectors, and after 36 hr a protein extract was made, as described previously (Chow et al., 1996; Luo et al., 1998). Ten percent of the extract was used for direct Western blot, and the remainder was immunoprecipitated with a monoclonal anti-Gal4 antibody (Santa Cruz) and Western blotted with a polyclonal anti-LexA antibody (Chow et al., 1996; Luo et al., 1998).

Acknowledgments

We thank H. Piwnica-Worms for baculovirus-expressing cyclin E–Cdk2 and J. Wang for Rb mutant constructs. These studies were supported by grants from the NIH and Research to Prevent Blindness, Inc.

Received April 22, 1999; revised August 11, 1999.

References

Adams, P.D., and Kaelin, W.G., Jr. (1996). The cellular effects of E2F overexpression. Curr. Top. Microbiol. Immunol. *208*, 79–93.

Adams, P.D., Li, X., Sellers, W.R., Baker, K.B., Leng, X., Harper, J.W., Taya, Y., and Kaelin, W.G., Jr. (1999). Retinoblastoma protein contains a C-terminal motif that targets It for phosphorylation by cyclin-cdk complexes. Mol. Cell. Biol. *19*, 1068–1080.

Brehm, A., Miska, E.A., McCance, D.J., Reid, J.L., Bannister, A.J., and Kouzarides, T. (1998). Retinoblastoma protein recruits histone deacetylase to repress transcription. Nature *391*, 597–601.

Chellappan, S.P., Hiebert, S., Mudryj, M., Horowitz, J.M., and Nevins, J.R. (1991). The E2F transcription factor is a cellular target for the RB protein. Cell *65*, 1053–1061.

Chew, Y.P., Ellis, M., Wilkie, S., and Mittnacht, S. (1998). pRB phosphorylation mutants reveal role of pRB in regulating S phase completion by a mechanism independent of E2F. Oncogene *17*, 2177–2186.

Chow, K.N., and Dean, D.C. (1996). Domains A and B in the Rb pocket interact to form a transcriptional repressor motif. Mol. Cell. Biol. *16*, 4862–4868.

Chow, K.N., Starostik, P., and Dean, D.C. (1996). The Rb family contains a conserved cyclin-dependent-kinase-regulated transcriptional repressor motif. Mol. Cell. Biol. *16*, 7173–7181.

Dowdy, S.F., Hinds, P.W., Louie, K., Reed, S.I., Arnold, A., and Weinberg, R.A. (1993). Physical interaction of the retinoblastoma protein with human D cyclins. Cell *73*, 499–511.

Dyson, N. (1998). The regulation of E2F by pRB-family proteins. Genes Dev. *12*, 2245–2262.

Ewen, M.E. (1994). The cell cycle and the retinoblastoma protein family. Cancer Metastasis Rev. *13*, 45–66.

Ewen, M.E., Sluss, H.K., Sherr, C.J., Matsushime, H., Kato, J., and Livingston, D.M. (1993). Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. Cell *73*, 487–497.

Ezhevsky, S.A., Nagahara, H., Vocero-Akbani, A.M., Gius, D.R., Wei, M.C., and Dowdy, S.F. (1997). Hypo-phosphorylation of the retinoblastoma protein (pRb) by cyclin D:Cdk4/6 complexes results in active pRb. Proc. Natl. Acad. Sci. USA *20*, 699–704.

Geng, Y., Eaton, E.N., Picon, M., Roberts, J.M., Lundberg, A.S., Gifford, A., Sardet, C., and Weinberg, R.A. (1996). Regulation of cyclin E transcription by E2Fs and retinoblastoma protein. Oncogene *12*, 1173–1180.

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.

Hassig, C.A., Fleischer, T.C., Billin, A.N., Schreiber, S.L., and Ayer, D.E. (1997). Histone deacetylase activity is required for full transcriptional repression by mSin3A. Cell *89*, 341–347.

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.

Hinds, P.W., Mittnacht, S., Dulic, V., Arnold, A., Reed, S.I., and Weinberg, R.A. (1992). Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. Cell *70*, 993–1006.

Holmes, J.K., and Solomon, M.J. (1996). A predictive scale for evaluating cyclin-dependent kinase substrates. J. Biol. Chem. *41*, 25240–25246.

Hua, X.H., and Newport, J. (1998). Identification of a preinitiation step in DNA replication that is independent of origin recognition complex and cdc6, but dependent on cdk2. J. Cell Biol. *140*, 271–281.

Kato, J., Matsushime, H., Hiebert, S.W., Ewen, M.E., and Sherr, C.J. (1993). Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. Genes Dev. *7*, 331–342.

Knudsen, E.S., and Wang, J.Y. (1996). Differential regulation of retinoblastoma protein function by specific Cdk phosphorylation sites. J. Biol. Chem. *271*, 8313–8320.

Knudsen, E.S., and Wang, J.Y. (1997). Dual mechanisms for the inhibition of E2F binding to RB by cyclin-dependent kinase-mediated RB phosphorylation. Mol. Cell. Biol. *17*, 5771–5783.

Knudsen, E.S., Buckmaster, C., Chen, T.T., Feramisco, J.R., and Wang, J.Y. (1998). Inhibition of DNA synthesis by RB: effects on G1/S transition and S-phase progression. Genes Dev. *12*, 2278–2292.

La Thangue, N.B. (1994). DRTF1/E2F: an expanding family of heterodimeric transcription factors implicated in cell-cycle control. Trends Biochem. Sci. *19*, 108–114.

Lam, E.W., and La Thangue, N.B. (1994). DP and E2F proteins: coordinating transcription with cell cycle progression. Curr. Opin. Cell Biol. *6*, 859–866.

Lee, J.O., Russo, A.A., and Pavletich, N.P. (1998). Structure of the retinoblastoma tumour-suppressor pocket domain bound to a peptide from HPV E7. Nature *391*, 859–865.

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.

Luo, R.X., Postigo, A.A., and Dean, D.C. (1998). Rb interacts with histone deacetylase to repress transcription. Cell *92*, 463–473.

Magnaghi, J.L., Groisman, R., Naguibneva, I., Robin, P., Lorain, S., Le, V.J., Troalen, F., Trouche, D., and Harel, B.A. (1998). Retinoblastoma protein represses transcription by recruiting a histone deacetylase. Nature *391*, 601–605.

Mittnacht, S., Lees, J.A., Desai, D., Harlow, E., Morgan, D.O., and Weinberg, R.A. (1994). Distinct sub-populations of the retinoblastoma protein show a distinct pattern of phosphorylation. EMBO J. *13*, 118–127.

Morgan, D.O. (1995). Principles of CDK regulation. Nature *374*, 131–134.

Nasmyth, K. (1993). Control of the yeast cell cycle by the Cdc28 protein kinase. Curr. Opin. Cell Biol. *5*, 166–179.

Nevins, J.R. (1992). E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. Science *258*, 424–429.

Norbury, C., and Nurse, P. (1992). Animal cell cycles and their control. Annu. Rev. Biochem. *61*, 441–470.

Ohtsubo, M., Theodoras, A.M., Schumacher, J., Roberts, J.M., and Pagano, M. (1995). Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. Mol. Cell. Biol. *15*, 2612–2624.

Reed, S.I. (1992). The role of p34 kinases in the G1 to S-phase transition. Annu. Rev. Cell Biol. *8*, 529–561.

Roberts, J.M., Koff, A., Polyak, K., Firpo, E., Collins, S., Ohtsubo, M., and Massague, J. (1994). Cyclins, Cdks, and cyclin kinase inhibitors. Cold Spring Harbor Symp. Quant. Biol. *59*, 31–38.

Sherr, C.J. (1996). Cancer cell cycles. Science *274*, 1672–1677.

Slansky, J.E., and Farnham, P.J. (1996). Introduction to the E2F family: protein structure and gene regulation. Curr. Top. Microbiol. Immunol. *208*, 1–30.

Songyang, Z., Blechner, S., Hoagland, N., Hoekstra, M.F., Piwnica-Worms, H., and Cantley, L.C. (1994). Use of an orientated peptide library to determine the optimal substrates of protein kinases. Curr. Biol. *4*, 973–982.

Templeton, D.J., Park, S.H., Lanier, L., and Weinberg, R.A. (1991). Nonfunctional mutants of the retinoblastoma protein are characterized by defects in phosphorylation, viral oncoprotein association, and nuclear tethering. Proc. Natl. Acad. Sci. USA *88*, 3033–3037.

van den Heuvel, S., and Harlow, E. (1993). Distinct roles for cyclin-dependent kinases in cell cycle control. Science *262*, 2050–2054.

Weinberg, R.A. (1995). The retinoblastoma protein and cell cycle control. Cell *81*, 323–330.

Weintraub, S.J., Prater, C.A., and Dean, D.C. (1992). Retinoblastoma protein switches the E2F site from positive to negative element. Nature *358*, 259–261.

Weintraub, S.J., Chow, K.N., Luo, R.X., Zhang, S.H., He, S., and Dean, D.C. (1995). Mechanism of active transcriptional repression by the retinoblastoma protein. Nature *375*, 812–815.

Whitaker, L.L., Su, H., Baskaran, R., Knudsen, E.S., and Wang, J.Y.J. (1998). Growth suppression by an E2F-binding-defective retinoblastoma protein (RB): contribution from the RB C pocket. Mol. Cell. Biol. *18*, 4032–4042.

Xu, M., Sheppard, K.A., Peng, C.Y., Yee, A.S., and Piwnica-Worms, H. (1994). Cyclin A/cdk2 binds directly to E2F1 and inhibits the DNA-binding activity of E2F1/DP1 by phosphorylation. Mol. Cell. Biol. *14*, 8420–8431.

Zarkowska, T., and Mittnacht, S. (1997). Differential phosphorylation of the retinoblastoma protein by G1/S cyclin-dependent kinases. J. Biol. Chem. *272*, 12738–12746.

Zhang, H.S., Postigo, A.A., and Dean, D.C. (1999). Active transcriptional repression by the Rb-E2F complex mediates G1 arrest triggered by p16INK4a, TGFβ, and contact inhibition. Cell *97*, 53–61.