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Cdk-mediated phosphorylation of pRB regulates HDAC binding in vitro

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Abstract

Retinoblastoma protein (pRB) controls the G1/S transition in the cell cycle by binding and inactivating E2F transcription factor. pRB changes the chromatin structure at the E2F-responsive promoter by recruiting histone deacetylase (HDAC) to the pRB–E2F complex, thus controlling the transcriptional activity of E2F. Cyclin-dependent kinases (Cdks) phosphorylate pRB and disrupt association between pRB and E2F. We investigated the effects of pRB phosphorylation on HDAC-1 binding in vitro. Phosphorylation of pRB by Cdk4-cyclin D2, Cdk2-cyclin E, and Cdk2-cyclin A inhibited association of pRB with HDAC. Among these Cdks, Cdk4-cyclin D2 showed particularly effective inhibition of pRB–HDAC complex formation. Using pRB mutants with various deletions in the N- and C-terminal domains, we found that both the pocket and C-terminal domains are important for regulating association between pRB and HDAC.

Keywords: pRB; Cdk4; Cdk2; HDAC; E2F; LXCXE motif

Retinoblastoma protein (pRB) regulates cell cycle progression at G1/S phase [1,2]. pRB is known to interact with members of the E2F family, which are transcription factors, and suppress their transcriptional activity [3–5]. Recently, it has been shown that pRB regulates E2F transcriptional activity via association with histone deacetylase protein (HDAC) [6–11].

The interaction of pRB and E2F is regulated by cell-cycle-dependent phosphorylation of pRB [3–5,12,13]. In the G0 and G1 phases, pRB is hypophosphorylated and forms stable complexes with E2F to repress its transcriptional activity. In late G1, pRB becomes hyperphosphorylated and E2F then dissociates from pRB. This phosphorylation of pRB is mediated by cyclin-dependent kinases (Cdks) [14–18]. At the G0/G1 to S transition, Cdk4/Cdk6-cyclin D, Cdk2-cyclin E, and Cdk2-cyclin A are activated, and thus it is thought that these Cdks are involved in the regulation of the pRB–E2F pathway.

Numerous pRB-binding proteins, such as SV40 large T antigen, possess a conserved LXCXE motif, which is thought to be important for binding to pRB [13,19]. HDAC-1 also possesses the LXCXE motif and interacts with pRB though this motif [6,20]. However, it is not well understood how association between pRB and HDAC-1 is regulated. Some studies have reported that the binding of pRB with HDAC-1 is disrupted by SV40 large T antigen binding with pRB in vitro and that E2F transcriptional activity previously suppressed via pRB–HDAC is recovered by expression of cyclin D1 in vivo [7,21]. However, a detailed mechanism has not yet been documented for the regulation of pRB–HDAC binding. The pRB–E2F complex is disrupted by Cdk-mediated phosphorylation of pRB. We therefore investigated whether phosphorylation of pRB by Cdks had any effect on pRB–HDAC-1 complex formation.

Materials and methods

Plasmids. Plasmids containing HDAC (pTA-HDAC-1) were constructed for this study. HDAC-1 was amplified by PCR using HL60 cDNA (Clontech) as a template. An expression plasmid containing a fusion protein of pRB (amino acids: 379–928) with glutathione-*S*-transferase (GST–pRB) and deletion mutants of pRB (379–835, 612–928, and 768–928) were constructed previously [22]. Other deletion mutants of pRB (379–612, 379–657, 379–767, 379–784, and 379–802) were constructed by insertion of the respective fragments amplified by PCR.

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Purification of proteins. Recombinant Cdk4-cyclin D2, Cdk6-cyclin D2, Cdk2-cyclin E, and Cdk2-cyclin A were purified from Sf9 cells co-infected with baculoviruses. Purification of these proteins was performed as described elsewhere [16].

Expression and purification of GST–pRB. Wild-type and mutant GST–pRB were expressed in *Escherichia coli* BL21 (DE3) at 30 °C for 1 h with 1 mM IPTG. The cell pellet was collected by centrifugation, suspended in cold PBS containing 1% (v/v) Triton X-100 (PBS-T), and lysed by sonication. Cell lysate was centrifuged (3000 rpm for 10 min at 4 °C) and the supernatant was recovered (GST–pRB sol.). Purification of GST–pRB was performed by pull-down with glutathione–Sepharose 4B beads (Amersham–Pharmacia Biotech). GST–pRB sol. was suspended in cold PBS-T and incubated with glutathione–Sepharose 4B beads at 4 °C for 1 h with rotation. The incubated mixture was centrifuged and the pellet was washed twice with PBS-T.

In vitro phosphorylation of GST–pRB and association with HDAC-1 in vitro translated. Purified GST–pRB was incubated with each Cdk-cyclin at 30 °C for 20 min in 40 μl reaction buffer [16] containing 200 μM ATP and 2 μCi [γ-^{33}P]ATP.

After phosphorylation, pRB conjugated with glutathione–Sepharose 4B beads was recovered by centrifugation and washed twice with PBS-T. pRB and HDAC-1, which were translated in vitro with [^{35}S]methionine using a Promega TNT system, were incubated at 4 °C for 1 h in 200 μl B buffer (150 mM NaCl, 1 mM EDTA, pH 8.0, 0.5% (v/v) NP-40, and 20 mM Tris–HCl, pH 8.0). The suspension was centrifuged and the glutathione–Sepharose 4B beads were collected. Beads were washed three times in PBS-T, pelleted, and boiled in 15 μl SDS-sample buffer. Samples were resolved by SDS–polyacrylamide gel electrophoresis and visualized using a BAS2000 image analyzer.

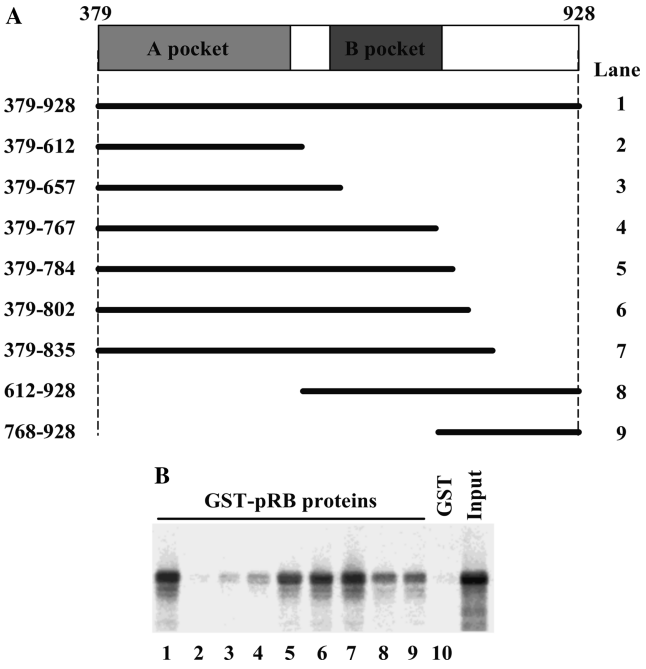

Fig. 1. Association between pRB and HDAC-1 required the entire pocket region and the C-terminal region in vitro. (A) Construction map of GST–pRB proteins. (B) Wild-type and deletion mutant GST–pRB proteins were incubated with ^{35}S-labeled HDAC-1 translated in vitro and analyzed by SDS–PAGE. Binding of pRB to HDAC-1 was detected using a BAS2000 image analyzer. (B) Lane numbers correspond to the numbers indicated on the right of (A).

Results and discussion

Association of pRB with HDAC-1 in vitro

We investigated whether Cdk-mediated phosphorylation of pRB affected its binding activity with HDAC-1. Fig. 1 shows that a fusion protein of pRB (amino acids 379–928) with glutathione-*S*-transferase (GST–pRB) is able to associate with HDAC-1 translated in vitro, but not with GST (Fig. 1A; lanes 1, 10). This shows that GST–pRB is able to associate with HDAC-1 in vitro.

Importance of pocket and C terminal domains for association of pRB with HDAC-1

It is reported that the pocket domains, the A pocket and B pocket, are necessary for pRB to bind with LXCXE proteins [13]. We therefore investigated which regions of pRB are important for binding with HDAC-1 in vitro. We prepared various pRB proteins containing deletions in the N- and C-terminal domains (Fig. 1A). In accordance with other reports, the entire pocket region was necessary for binding with HDAC-1 (Fig. 1B; lanes 1–7). Interestingly, mutant pRB proteins which lacked pocket region were able to bind (weakly) with HDAC-1 (lanes 8, 9). This suggests that the pocket region of pRB is important for association with HDAC-1 and the C-terminal region also plays some role in this activity.

Inhibition of pRB-binding activity to HDAC-1 by Cdk-dependent phosphorylation

In Fig. 2, we observed that GST–pRB phosphorylated by recombinant Cdk4-cyclin D2 (lanes 4–7), Cdk2-cyclin E (lanes 8–11), or Cdk2-cyclin A (lanes 12–15) exhibited weaker binding activity toward HDAC-1 when compared with non-phosphorylated GST–pRB (lane 3). In addition, their inhibition activities were proportional to the phosphorylation level. All Cdks were able to inhibit the association of pRB with HDAC-1, but Cdk4-cyclin D2 inhibited pRB-HDAC complex formation by about 80%, while Cdk2-cyclin E and Cdk2-cyclin A gave about 40% inhibition at a similar phosphorylation status of pRB (Fig. 2B). Cdk6-cyclin D2 and Cdk4-cyclin D2 gave equal inhibition activity (data not shown). It was recently reported that inhibition of HDAC activity resulted in expression of cyclin E [6,10,11]. Taken together with these observations, our results suggest that phosphorylation of pRB by Cdk4/6-cyclin D2 releases HDAC from pRB–E2F complexes, and promotes the expression of cyclin E.

Phosphorylation of Thr826 in pRB is important for regulating HDAC binding

We then investigated which phosphorylation sites in pRB are important for disruption of pRB and HDAC-1

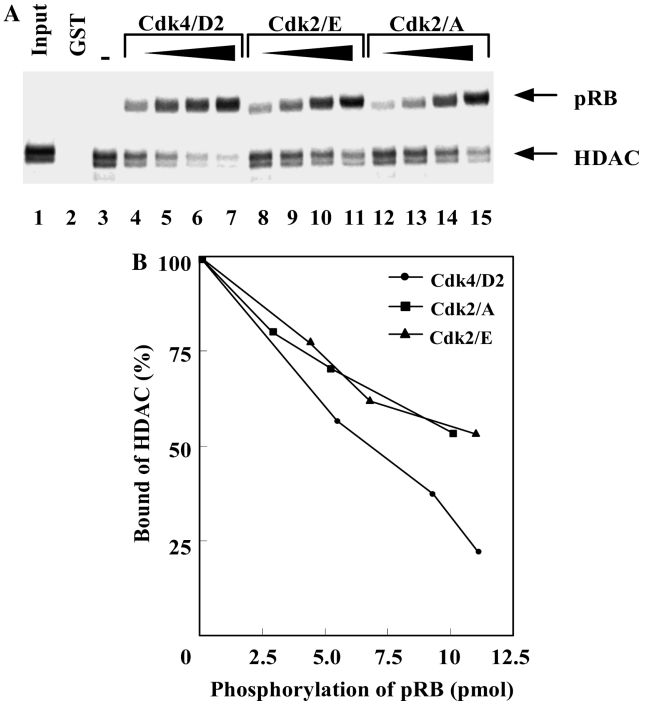

Fig. 2. Phosphorylation by Cdk-cyclin inhibited the binding of pRB to HDAC-1 in vitro. (A) GST–pRB was phosphorylated by Cdk4-cyclin D2, Cdk2-cyclin E, or Cdk2-cyclin A under conditions including [γ-^{33}P]ATP, and incubated with ^{35}S-labeled HDAC-1 translated in vitro. Phosphorylation of pRB and binding of pRB with HDAC-1 were visualized and quantified using a BAS2000 image analyzer. Input HDAC-1 (lane 1), GST (lane 2), GST–pRB phosphorylated by different amounts of Cdk4-cyclin D2 (lanes 3–7), Cdk2-cyclin E (lanes 8–11), and Cdk2-cyclin A (lanes 12–15). (B) Phosphorylation level and amount of bound HDAC-1 were quantified using a BAS2000 image analyzer and plotted. Circles, triangles, and squares indicate phosphorylation by Cdk4-cyclin D2, Cdk2-cyclin E, and Cdk2-cyclin A, respectively.

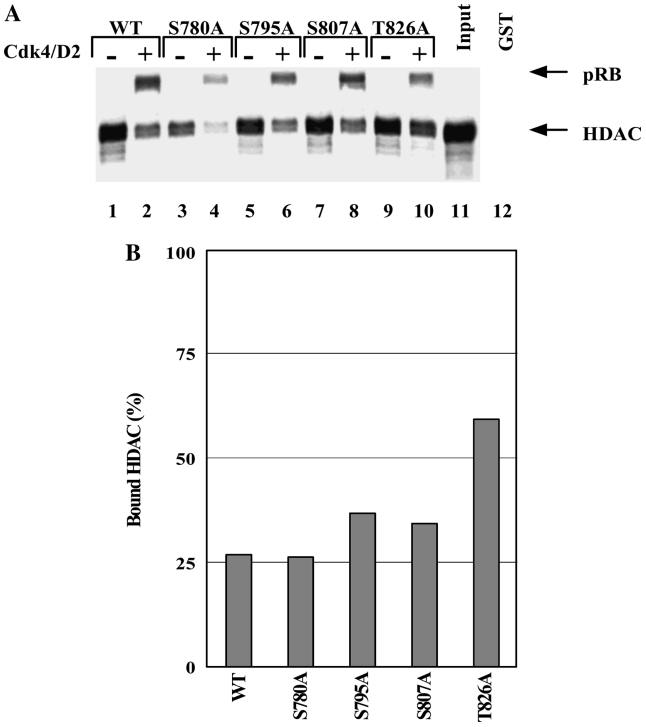

Fig. 3. Identification of phosphorylation sites important for regulating binding of pRB with HDAC-1. (A) Wild-type and mutant pRB proteins were phosphorylated by Cdk4-cyclin D2 under conditions including [γ-^{33}P]ATP and incubated with ^{35}S-labeled HDAC-1 translated in vitro. Phosphorylation of pRB and association of pRB with HDAC-1 were visualized and quantified using a BAS2000 image analyzer. (B) Quantification of bound HDAC-1 to each GST–pRB.

complex formation. We prepared several mutant GST–pRB proteins containing substitutions of serine (S) or threonine (T) with alanine (A) residues at each Cdk phosphorylation site (S780A, Ser795A, S807A, and T826A). Fig. 4 shows that mutant GST–pRBs, except for GST–pRB T826A, exhibited reduced binding activity for HDAC-1 that was similar to that of wild-type GST–pRB (Figs. 3A; lanes 1–8, B). The effects of phosphorylation by Cdk4-cyclin D2 on association between pRB T826A and HDAC-1 was about 2-fold weaker than that of the other GST–pRB. Cdk4-cyclin D2 may have preferential phosphorylation sites in pRB that are different from those of Cdk2-cyclin E and Cdk2-cyclin A. Ser780 and Thr826 are specifically phosphorylated by Cdk4-cyclin D2 but not by Cdk2-cyclin E and Cdk2-cyclin A [15–17,22]. Our results indicate that phosphorylation at Thr826 is involved in regulating pRB and HDAC-1 binding. Zarkowska et al. [15] reported that phosphorylation at Thr821 and Thr826 is important for the binding of SV40 large T antigen to pRB. Both SV40 large T antigen and HDAC-1 are LXCXE motif-containing proteins, so phosphorylation of Thr826 may be important for regulating the binding of such proteins with pRB. Furthermore, these results indicate sequential regulation of the pRB–E2F pathway at the G1–S transition. First, Cdk4-cyclin D partially phosphorylates pRB, which inhibits pRB–HDAC complex formation. This leads to expression of cyclin E and then Cdk2-cyclin E completely phosphorylates pRB. Subsequently, E2F induces transcription of its target genes and the cell cycle progress from G1 to S phase.

Combination of pRB pocket and C-terminal domains in regulation of binding with HDAC-1

Next, we investigated the relationship between pRB domains and regulation of association with HDAC-1 via Cdk-mediated phosphorylation. Mutant pRB proteins that possessed the entire pocket domain but not the C-terminal region were not inhibited in binding with HDAC-1 after phosphorylation by Cdk4-cyclin D2 (Fig. 4; lanes 3–8). In contrast, a mutant pRB that possessed only the C-terminal domain was inhibited (lanes 11, 12). Interestingly, one mutant pRB that contained only the B pocket (incomplete pocket) and the C-terminal region was not inhibited, which was similar to

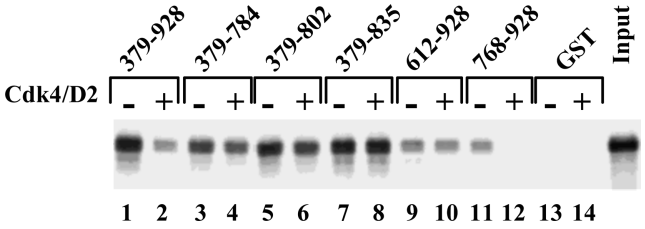

Fig. 4. Regulation of binding activity of pRB to HDAC-1 by Cdks requires the C-terminal region. Wild-type and deletion mutant GST–pRB proteins treated with or without Cdk4-cyclin D2 were incubated with ^{35}S-labeled HDAC-1 translated in vitro and analyzed by SDS–PAGE. Binding of pRB to HDAC-1 was detected using a BAS2000 image analyzer.

the behavior of the C-terminal deletion mutants (lanes 9, 10). These results suggest that the C-terminal region is important for regulating the association of pRB with HDAC-1 via Cdk-mediated phosphorylation, and that the pocket region functions as a repressive domain.

Implication of Cdks in epigenetic control of E2F

There have been many recent reports about the epigenetic control of transcription via DNA methylation, histone acetylation, and histone methylation [23–25]. In addition to HDAC, pRB is able to associate with SUV39H1, which is a histone methyltransferase [23,26–28], and p300/CBP, which the E2F transcriptional activity through histone acetylation [29]. The present study indicates that Cdks may also play a role in epigenetic control via phosphorylation of pRB.

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