J. Biochem. **137**, 381–386 (2005)
DOI: 10.1093/jb/mvi050

Preferences for Phosphorylation Sites in the Retinoblastoma Protein of D-Type Cyclin–Dependent Kinases, Cdk4 and Cdk6, *In Vitro*

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Received December 9, 2004; accepted January 4, 2005

D-type cyclin–dependent kinases (Cdk4 and Cdk6) regulate the G1 to S phase progression of the mammalian cell cycle. It has been suggested that Cdk4 and Cdk6 may have distinct functions *in vivo*, even though they are indistinguishable biochemically. Here we show that although these Cdks phosphorylate multiple residues in pRB, they do so with different residue selectivities *in vitro*; Thr821 and Thr826 are preferentially phosphorylated by Cdk6 and Cdk4, respectively. This raises the possibility different substrate specificities lead to their different roles in the regulation of cellular events. Furthermore, our results indicate the new concept that Cdk itself contributes to substrate recognition.

Key words: Cdk4, Cdk6, phosphorylation, pRB.

Abbreviations: Cdk, cyclin-dependent kinase; ECL, enhanced chemiluminescence; GST, glutathione S-transferase; pRB, retinoblastoma protein.

Cyclin-dependent kinases (Cdks) are proline-directed serine/threonine kinases and are activated through binding with a regulatory subunit, cyclin. In mammalian cells, at least four types of Cdk, Cdk1, Cdk2, Cdk4, and Cdk6, participate in regulation of the cell cycle. Among them, Cdk4 and Cdk6 form complexes with D-type cyclins (D1, D2, and D3) and regulate G0/G1 to S phase progression (*1*–*3*). Although both Cdk4 and Cdk6 are believed to play redundant roles in cell cycle regulation, these enzymes are expressed in different tissues or cell lines (*4*, *5*), and some information indicates they have distinct physiological roles *in vivo*. Indeed, exogenous expression of Cdk4 or Cdk6 causes distinct phenotypic changes in some cell lines (*6*–*8*). In addition, Cdk4 and Cdk6 null mice showed almost redundant, but some distinct phenotypes (*9*, *10*). These observations suggest Cdk4 and Cdk6 play different roles *in vivo*, but their biochemical differences *in vitro* as well as *in vivo* have not been clarified yet.

Retinoblastoma protein (pRB) is one of the important Cdk substrates for the transition of the restriction point of the mammalian cell cycle (*2*, *4*, *11*–*15*). In the G0 or early G1 phase, pRB exists as a hypophosphorylated form and forms a complex with E2F to repress its transcriptional activity. In the late G1 phase, pRB becomes hyperphosphorylated, and this phosphorylation causes the release and activation of E2F, allowing transcription of genes necessary for progression to the S phase (*16*, *17*).

Cyclin/Cdk complexes in cell cycle regulation exhibit different substrate specificities *in vitro*. pRB contains 16 potential phosphoacceptor sites (Ser/Thr-Pro motif) (*14*). Among them, Cdk2 and Cdk4 phosphorylate pRB at different sites *in vitro* (*18*, *19*). Furthermore, although Cdk2 and Cdc2 phosphorylate both pRB and histone H1 *in vitro*, Cdk4 and Cdk6 phosphorylate pRB but not histone H1 (*20*).

During biochemical analysis of pRB phosphorylated with either Cdk4 or Cdk6, we found it migrated differently on SDS–polyacrylamide gel electrophoresis (SDS-PAGE). In this manuscript, we report Cdk4 and Cdk6 phosphorylate pRB with different residue selectivities *in vitro*.

MATERIALS AND METHODS

Plasmids—Plasmids for the production of pRB (amino acid residues 379 to 928) fused with glutathione S-transferase (GST-pRB) were generated as described previously (*21*). Deletion mutants of pRB that had amino acid residues 768–928 and 379–835 were generated by utilizing the *Ssp*I and *Mfe*I sites in the *RB* gene, respectively. Single amino acid mutations in the phosphorylation sites in pRB were introduced by PCR-based mutagenesis.

Antibodies—The polyclonal antibodies against phospho-Ser608, Ser811, Thr821, and Thr826 will be described elsewhere (Taya *et al.*, unpublished). The specificity of each antibody was determined using alanine-substituted GST-pRB mutant proteins.

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Expression and Purification of Proteins—GST-pRB and GST-p19^{INK4d} expressed in *Escherichia coli* (BL21) were prepared as described previously (*21*, *22*).

Cyclin D2/Cdk4, cyclin D2/Cdk6 and cyclin A/Cdk2 were purified from Sf9 cells co-infected with recombinant baculoviruses as described previously (*18*). The purity of each cyclin/Cdk exceeded 90%, as judged on SDS-PAGE.

In Vitro Phosphorylation of GST-pRB—The phosphorylation of GST-pRB was performed as described previously (*21*). The reaction was terminated by the addition of 10 μl of SDS-containing sample buffer (*23*). Samples were resolved by SDS-PAGE and visualized with a BAS2000 (Fuji).

Western Blot Analysis—The phosphorylation of GST-pRB was performed as described above without [γ-^{33}P]ATP. The phosphorylated products were subjected to 12.5% SDS-PAGE, transferred to polyvinylidene fluoride microporous membranes, and then probed with phospho-specific antibodies, which were detected with anti–rabbit IgG-HRP (Amersham Biosciences) and visualized with an ECL system (Amersham Biosciences).

Two-Dimensional Tryptic Phosphopeptide Mapping of Phosphorylated GST-pRB—Two-dimensional tryptic phosphopeptide mapping was performed as described previously (*24*). GST-pRB was phosphorylated with Cdk4 or Cdk6 as described above, but using [γ-^{32}P]ATP instead of [γ-^{33}P]ATP. Following SDS-PAGE, radiolabeled GST-pRB was cut out from the gel, and extracted and resolved for each map. Radiolabeled spots were analyzed with a BAS2000.

Phosphoamino Acid Analysis—Each radiolabeled spot was scraped from the thin layer chromatography plate after two-dimensional tryptic phosphopeptide mapping. Phosphoamino acid analysis was performed as described previously (*24*). ^{32}P labeled spots were analyzed with a BAS2000 and then a phosphoamino acid markers were stained with ninhydrin.

RESULTS

Different Migration of pRB Phosphorylated by Cdk4 and Cdk6 on SDS-PAGE—To determine the phosphorylation site-specificities of Cdk4 and Cdk6 for pRB *in vitro*, we purified Cdk4 and Cdk6 in complexes with cyclin D2. The dose-response experiments revealed both Cdk4 and Cdk6 phosphorylated GST-pRB (Fig. 1A). The specific activities were 2.1×10^4 units/mg Cdk4 protein and 1.4×10^4 units/mg Cdk6 protein (1 unit was defined as 100 pmol of ^{33}P incorporated into GST-pRB at 30°C for 20 min). Interestingly, when GST-pRB phosphorylated with either Cdk4 or Cdk6 was subjected to SDS-PAGE, the migration patterns were different (Fig. 1B). pRB phosphorylated with Cdk4 comprised mainly two species, represented by the faster and slower migrating bands after 10 min or later incubation (Fig. 1B, lanes 4–7). In contrast, pRB phosphorylated with Cdk6 comprised mainly the slower one (lanes 12–14).

To rule out the possibility that this difference was due to inactivation of the enzyme during incubation, two step reaction experiments were performed (Fig. 1C). In the first step, pRB was phosphorylated with Cdk4 or Cdk6 for 20 min, and in the second step the same amount of Cdk4 or Cdk6 was added to the reaction mixture, followed by incubation for a further 20 min. Most of the faster migrating band for pRB phosphorylated with Cdk4 changed to the slower one on the second incubation with Cdk6, but not on that with Cdk4. Moreover, the migration pattern of pRB phosphorylated with Cdk6 was not affected by additional incubation with either Cdk4 or Cdk6.

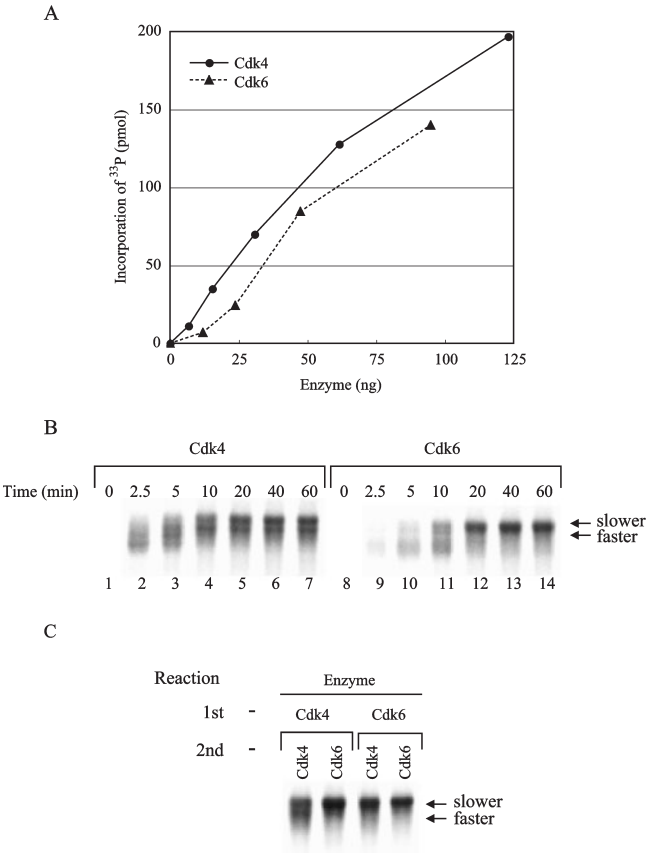

Fig. 1. **pRB phosphorylated by Cdk4 or Cdk6 shows different migration on SDS-PAGE.** (A) The phosphorylation activity of recombinant Cdk4 or Cdk6 was investigated *in vitro* using GST-pRB as a substrate. GST-pRB was incubated with various amounts of either Cdk4 (circles) or Cdk6 (triangles) at 30°C for 20 min. Samples were subjected to 12.5% SDS-PAGE and then the incorporation of ^{33}P into pRB was determined with a BAS2000. (B) GST-pRB was phosphorylated with either Cdk4 (60 ng) or Cdk6 (100 ng) for the indicated times with [γ-^{33}P]ATP at 30°C *in vitro*, and then subjected to 6% SDS-PAGE. The phosphorylated bands were analyzed with a BAS2000. (C) GST-pRB was first phosphorylated with Cdk4 or Cdk6 and [γ-^{33}P]ATP at 30°C for 20 min, and then as the second step, further phosphorylated with Cdk4 or Cdk6 for another 20 min at 30°C. The phosphorylated pRB was analyzed by 6% SDS-PAGE.

We also found that pRB phosphorylated with either Cdk6 associated with cyclin D3 or Cdk4 associated cyclin D1 showed a similar migration pattern on SDS-PAGE to that of Cdk4 or Cdk6 associated with cyclin D2 (data not shown). These results indicated that the differences in the migration pattern of pRB phosphorylated with either Cdk4 or Cdk6 were independent of the associated cyclin D.

Determination of Cdk4 and Cdk6 Phosphorylation Sites in pRB by Phosphopeptide Mapping Analysis—Our results suggested the differences in migration pattern between Cdk4 and Cdk6 were not due to inactivation of the enzymes, and the phosphorylation status of pRB dif-

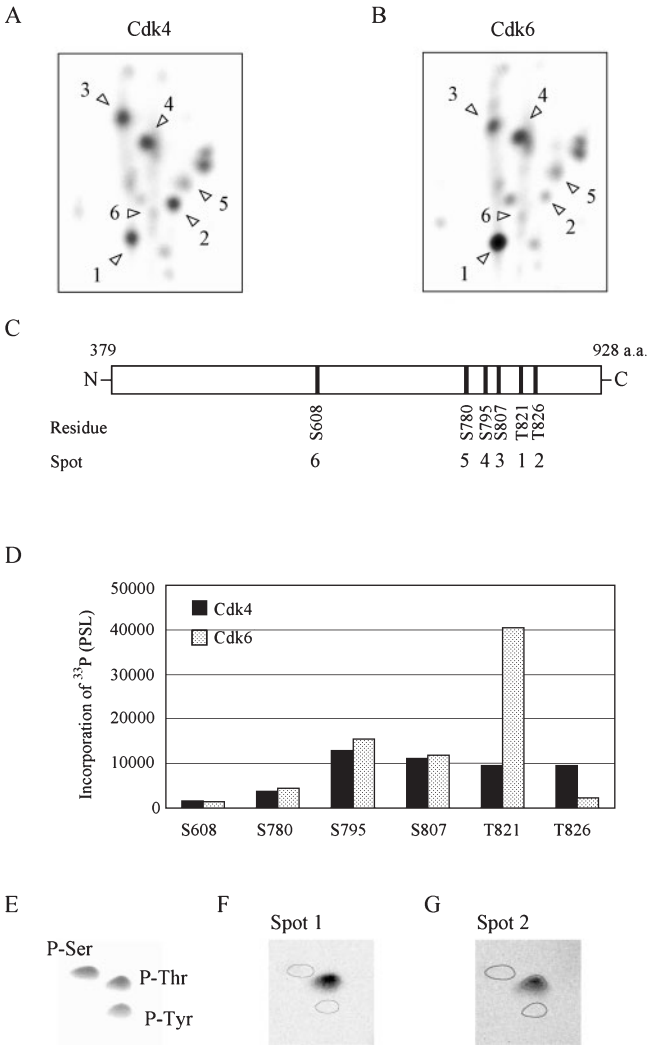

Fig. 2. **Determination of phosphorylation sites by phosphopeptide mapping analysis.** (A and B) Two-dimensional typtic phosphopeptide mapping analysis of GST-pRB phosphorylated with Cdk4 (A) or Cdk6 (B) and [γ-^{32}P]ATP. (C) The phosphorylated residues in GST-pRB corresponding to spots 1 to 6 in A and B were identified using alanine-substituted mutants of GST-pRB and are illustrated. (D) The radioactivity in spots 1 to 6 in A (Cdk4, black bars) and B (Cdk6, gray bars) was quantified with a BAS2000. PSL (photo-stimulated luminescence) indicates arbitrary units of radioactivity. (E–G) Phosphoamino acid analysis of spots 1 (F) and 2 (G). (E) The migration pattern of phospho-serine, threonine, and tyrosine visualized with ninhydrin. Circles in (F) and (G) indicate the overlaying of phosphoamino acids stained with ninhydrin.

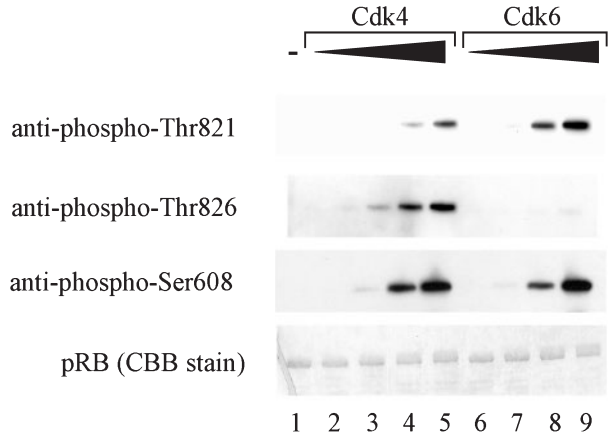

Fig. 3. **Determination of phosphorylation site by Western blot analysis with phospho-specific antibodies.** GST-pRB phosphorylated by Cdk4 or Cdk6 *in vitro* was subjected to 12.5% SDS-PAGE and then immuno-stained using the indicated anti–phospho-specific antibodies. Lanes 1, 2, 3, 4, and 5 contained GST-pRB phosphorylated with 0, 7.5, 15, 30, and 60 ng of Cdk4. Lanes 6, 7, 8, and 9 contained GST-pRB phosphorylated with 15, 25, 50, and 100 ng of Cdk6. The lower panel shows the GST-pRB used in this assay stained with CBB R-250.

fered between them. To determine the residues in pRB phosphorylated by Cdk4 or Cdk6, two-dimensional tryptic phosphopeptide mapping was performed. GST-pRB phosphorylated with either Cdk4 or Cdk6 *in vitro* gave multiple radiolabeled phosphopeptide spots (Fig. 2, A and B). These spots should be derived from the phosphorylated pRB because GST phosphorylated by both Cdks did not give any spot (data not shown). The patterns of spots for pRB phosphorylated by Cdk4 and Cdk6 were nearly identical. Among them, spot 1 was phosphorylated more potently by Cdk6 rather than Cdk4, while spot 2 was more remarkably phosphorylated by Cdk4 (Fig. 2, A and B). To determine which residue in pRB gave each spot, we constructed six site-directed mutant pRB proteins in which each consensus serine or threonine residue was changed to alanine (Fig. 2C). On phosphopeptide map analysis of these mutant pRB, we found spots 1 and 2 corresponded to Thr821 and Thr826, respectively (data not shown). Further phosphoamino acid analysis revealed the phosphorylated amino acids in both spots 1 and 2 were threonine (Fig. 2, E, F, and G). This supports our conclusion became Thr821 and Thr826 are the only threonine residues in pRB(379–928) to be phosphorylated by Cdks. Quantification of the radioactivity in spot 2 showed Cdk4 phosphorylated the Thr826 residue 4.1 times more efficiently than Cdk6, and Cdk6 phosphorylated the Thr821 residue (spot 1) 4.3 times more than Cdk4 (Fig. 2D).

On further analyses of alanine-substituted mutant pRB, spots 3, 4, 5, and 6 were identified as Ser807, Ser795, Ser780 and Ser608, respectively (Fig. 2C). These residues were phosphorylated comparably by both Cdks (Fig. 2D). There were several other minor spots exhibiting no significant differences between Cdk4 and Cdk6.

Confirmation of Selective Cdk4 and Cdk6 Phosphorylation of Thr826 and Thr821 in pRB—Western blot analysis with phospho-specific antibodies was performed to confirm the site-specific phosphorylation of pRB by Cdk6 and Cdk4. Consistent with the results of tryptic phosphopeptide mapping, Thr826 was phosphorylated by Cdk4 extensively, while Thr821 was phosphorylated efficiently by Cdk6 (Fig. 3). Besides, Ser608 was phosphorylated comparably by both Cdks (Fig. 3).

We carried out the same experiment using full length, non-tagged pRB instead of GST-pRB as a substrate. Principally, site-specific phosphorylation by Cdk4 and Cdk6 was also observed with full length, non-tagged pRB, indicating the above results are not due to tagging of GST or deletion of residues 1–378 of pRB (data not shown).

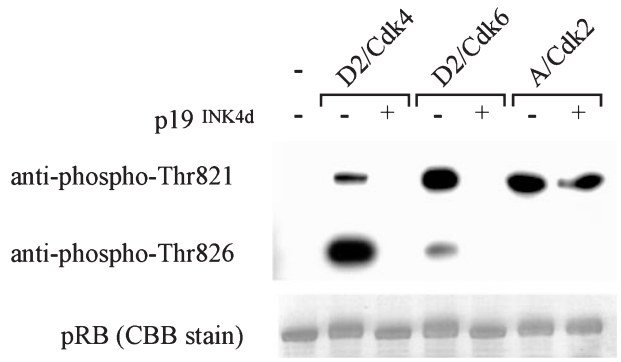

Fig. 4. **Phosphorylation of the Thr821 and Thr826 sites is inhibited by GST-p19^{INK4d}.** GST-pRB was phosphorylated with cyclin D2/Cdk4, Cdk6 or cyclin A/Cdk2, with or without GST-p19^{INK4d} (3 μg), at 30°C for 20 min. The phosphorylation of Thr821 and Thr826 in pRB was detected by Western blot analysis using phospho-specific antibodies. The lower panel shows GST-pRB stained with CBB R-250 to confirm equal loading of pRB.

Thr821 in pRB has been reported to be phosphorylated by Cdk2 *in vitro* (*19*). Thus, we examined whether or not the phosphorylation of Thr821 in pRB was due to minor contaminating, if any, Cdk2 activity in our Cdk4 and Cdk6 preparations. p19^{INK4d} is a specific Cdk4 and Cdk6 inhibitor protein, and does not inhibit Cdk2 activity *in vitro* (*22*). We investigated the effect of GST-p19^{INK4d} on the phosphorylation activity of Cdk4 and Cdk6. As shown in Fig. 4, p19^{INK4d} completely inhibited the pRB phosphorylation by Cdk4 or Cdk6, whereas Cdk2 was only slightly inhibited by p19^{INK4d}. These results confirmed that Cdk4 and Cdk6 themselves phosphorylated Thr821 and Thr826. Notably, Cdk2 did not phosphorylate Thr826 at all, indicating that, at least *in vitro*, the phosphorylation of this site is preferred by Cdk4.

Motifs in pRB for Preferential Phosphorylation—Some reports have stated pRB has two important motifs for interaction with cyclins/Cdks (*1*). One is the RXL motif (X is typically basic) found in various cyclin-Cdk interacting proteins such as E2F and Cdk inhibitor family proteins, and pRB has an RXL-related sequence in the C-terminal region (*25*). This motif is believed to be recognized by cyclins, and determines the substrate specificity of Cdks. Indeed, mutations in the RXL motif decrease the substrate activity toward pRB of Cdks (*25*). The second motif is a pocket region recognized by proteins containing the LXCXE motif such as cyclin Ds or SV40 large T antigen (*26*, *27*). To determine whether or not these two domains affect the site-specific phosphorylation of pRB, we obtained two deletion mutants; pRB(379–835) and pRB(768–928), which lacked the RXL motif and pocket region, respectively. Both Cdk4 and Cdk6 could not phosphorylate GST-pRB when pRB(379–835) was used as the substrate (Fig. 5). This result is consistent with the previous report that the RXL motif is necessary for Cdk2 to recognize pRB, and suggests the RXL motif is also necessary for Cdk4 and Cdk6. Meanwhile, the Thr821 residue in pRB(768–928), which does not have a pocket region, was phosphorylated by Cdk4 as well as Cdk6 (Fig. 5). In contrast, the preferential phosphorylation of Thr826 by Cdk4 was preserved even in pRB(768–928).

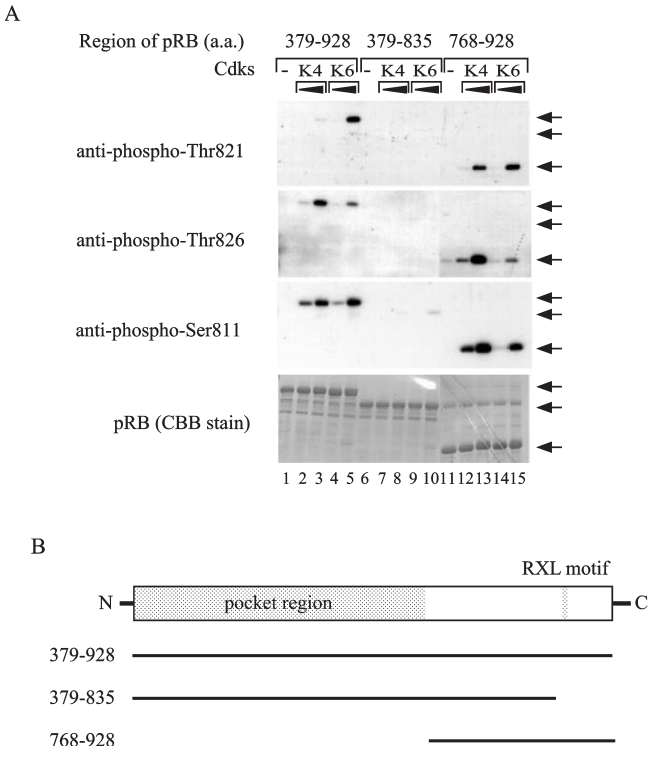

Fig. 5. **The pocket region in pRB is necessary for preferential phosphorylation by Cdk6.** (A) Deletion mutant forms of GST-pRB containing 379–928 (lanes 1–5), 379–835 (lanes 6–10), and 768–928 (lanes 11–15) were phosphorylated with Cdk4 or Cdk6 *in vitro*. Samples were analyzed by Western blot analysis as described in the legend to Fig. 3. Lanes 1, 6, and 11 were without a Cdk. Lanes 2, 7, and 12, and 3, 8, and 13, incubation with 30 and 60 ng of Cdk4, respectively. Lanes 4, 9, and 14, and 5, 10, and 15, incubation with 50 and 100 ng of Cdk6, respectively. The lower panel shows the pRB used in this assay stained with CBB R-250. Arrows indicate the GST-pRB mutants. (B) Construction map of deletion mutant pRB used in A.

DISCUSSION

We have shown that Cdk4 and Cdk6 phosphorylate different residues in pRB *in vitro*; Thr821 and Thr826 were preferentially phosphorylated by Cdk6 and Cdk4, respectively, and both Cdks needed a distinct region of pRB for the site-specific phosphorylation.

The site-specific pRB phosphorylation by Cdk2 and Cdk4 has previously been reported (*18*, *19*). Our results indicate that, as expected, Cdk6 exhibits residue selectivity like Cdk4. But Cdk6 acts like Cdk2 in the selective phosphorylation of Thr821 and Thr826 in pRB. It remains unclear whether or not this preferential phosphorylation also occurs *in vivo*. Recently, Kozar *et al.* showed that phosphorylation of Thr826 in pRB decreased in cyclin D (D1, D2, and D3)–deficient cells (*10*). This suggests a D-type cyclin-dependent kinase, Cdk4 or Cdk6, is at least in part, involved in phosphorylation at this residue. Moreover, phosphorylation of pRB at residue Thr821 is often used as an indicator of Cdk2 activity in cells, while Thr826 phosphorylation is linked to Cdk4 activity. Our data suggest one should be cautious as to the involvement of Cdk6 in the phosphorylation of Thr821 *in vivo*. Today, associated cyclins, but not Cdk itself, are

believed to determine the substrate specificity (*28*). Our results indicate the new concept that Cdk itself may contribute to substrate recognition.

We also found the selective phosphorylation of Thr821 by Cdk6 disappeared when the pocket region was deleted in pRB. In contrast, the selective phosphorylation of Thr826 by Cdk4 was preserved even with this mutant pRB. Although the mechanism is unknown, our results imply that site-specific pRB phosphorylation by Cdk4 and Cdk6 is not simply determined by the surrounding amino acid residues, but that the overall structure of the substrate is required. In addition, a mutant pRB lacking the pocket region was equally phosphorylated by Cdk4 and Cdk6 as in the case of pRB(379–928). This indicates the binding activity of cyclin/Cdk as to pRB *via* the LXCXE motif is not essential for recognizing pRB.

Cdk2 and Cdk4 phosphorylate pRB with different preferences and have distinct effects on pRB (*19*, *21*, *29–31*). Indeed, Knudsen *et al.* indicated phosphorylation of Thr821 and Thr826 in pRB was required to inhibit the binding to LXCXE-containing proteins (*32*). We have shown Cdk4/cyclin D2 inhibited the binding activity of pRB to HDAC protein, which contains the LXCXE motif, *in vitro* (*21*). But for the mutant pRB which had an alanine substitution at Thr826, the effect of Cdk4/cyclin D2 was decreased, but not that of Cdk2/cyclin A or cyclin E (*21*). Thus phosphorylation of Thr821 and Thr826 appeared to be involved in regulation of the binding activity toward LXCXE-containing proteins *in vitro*. On the other hand, we did not see a marked difference in E2F-binding activity for pRB phosphorylated by either Cdk4 or Cdk6 (data not shown). This means that the limited differences in the phosphorylation site of Thr821 or Thr826 do not affect the E2F-binding activity. The *in vivo* biological significance of preferential phosphorylation of Thr821 and Thr826 has not been clarified yet. Further investigation of these differences *in vivo* as well as *in vitro* is important, especially as to control LXCXE protein–binding, as Knudsen mentioned (*32*).

Recently, some reports have indicated that Cdk4 and Cdk6 may have different functions in cells (*5*, *6*, *8*). Taken together, our results suggest that Cdk4 and Cdk6 exhibit biological differences in phosphorylation substrate selectivity and cellular functions.

We wish to thank Drs. K. Kurokawa, T. Miki, and K. Sekimizu for the helpful discussions.

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