

Differential Phosphorylation of the Retinoblastoma Protein by G₁/S Cyclin-dependent Kinases*

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The retinoblastoma tumor suppressor protein, pRB, is inactivated by phosphorylation. While existing evidence is strong that such phosphorylation is mediated by one or more cyclin-dependent kinases (CDKs) active during G₁/S, it remains unclear which of the various CDKs is responsible. We show here that three candidate pRB-inactivating kinases, CDK4-cyclin D1, CDK2-cyclin E, and CDK2-cyclin A, phosphorylate pRB differentially, each on a subset of authentic pRB phosphorylation sites. Notably, two neighboring pRB phosphate acceptors, threonine 821 and threonine 826, which have previously been implicated in the regulation of LXCXE protein binding, are phosphorylated by different CDKs. We demonstrate that phosphorylation by either CDK2-cyclin A, which phosphorylates T821, or CDK4-cyclin D1, which phosphorylates threonine 826, can disable pRB for subsequent binding of an LXCXE protein. However, only one of these two kinases, CDK2-cyclin A, can dissociate a pre-existing LXCXE protein-pRB complex. We provide evidence that prior binding of an LXCXE protein blocks access to certain residues specifically targeted by CDK4-cyclin D1, explaining the inability of this kinase to resolve such complexes. While these results are not direct proof of the relevance of differential pRB phosphorylation in cells, our findings support a model whereby full phosphorylation of pRB may require the action of more than one kinase and explains how such differential phosphorylation by different CDKs might translate into a differential regulation of downstream effector pathways.

The retinoblastoma tumor suppressor gene encodes a nuclear phosphoprotein, pRB, which plays a central role in control of the cell cycle. pRB seems to act as a check during the G₁ phase of the cell cycle, ensuring that cells do not enter S phase until such a time as is appropriate. In some differentiating cells this function is extended, with pRB mediating their permanent exit from the cell cycle (see review in Ref. 1). These functions of pRB seem to rely on its ability to bind to, and alter the activity of, transcription factors including E2F-1, PU.1, ATF-2, UBF, Elf-1, MyoD, and BRG-1 (reviewed in Ref. 2). pRB appears to inhibit transcription from genes necessary for cell cycle progression by binding to certain of these transcription factors (the best studied of which is E2F-1) while promoting transcription from differentiation-specific genes through binding others (*e.g.* MyoD).

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Hyperphosphorylation of pRB is thought to inactivate its functioning by disabling its transcription factor-binding abilities, thus allowing progression of cells through the division cycle (3). The likely mediators of this phosphorylation are members of the family of proline-directed, serine/threonine, cyclin-dependent kinases (CDKs)¹; they phosphorylate authentic sites on pRB and are themselves thought to play a key role in cell cycle control (4, 5).

Hyperphosphorylation of pRB is accompanied by release of pRB from nuclear tethering and a change in pRB's mobility in SDS-containing gels (6–10, 29). These changes are first seen during the late G₁ phase of the cell cycle, implicating cyclin-dependent kinases active at around this time to be responsible. These include CDK2-cyclin E, CDK2-cyclin A, and the CDK4/CDK6-cyclin D subfamily of CDKs (reviewed in Ref. 11). However, the involvement of any single one of these CDKs in pRB hyperphosphorylation remains controversial. Expression of either D type cyclins, cyclin A, or cyclin E can induce retarded migration in SDS gels, released from nuclear tethering and inactivation of pRB's growth suppressing function in SAOS2 osteosarcoma cells (12, 13). However, while ectopic expression of either cyclin D1 or cyclin E shortens the G₁ phase in NIH 3T3 cells, only cyclin D1 expression concomitantly leads to premature pRB hyperphosphorylation (14). Other evidence suggests that successive phosphorylation by cyclin D1- and cyclin E-directed kinases may be necessary to achieve hyperphosphorylation of pRB (15).

Regulation of pRB by phosphorylation might indeed be quite complex. There are 16 consensus CDK phosphorylation sites (Ser/Thr-Pro motifs) on pRB and phosphopeptide analysis suggests that many, if not all, of them serve as bona fide phosphate acceptors in cells (4). Moreover, a number of differentially phosphorylated forms of pRB appear to exist in cells (as judged by differential mobility in SDS gels and phosphopeptide patterns) and the state of phosphorylation appears to alter even after pRB's initial hyperphosphorylation in late G₁ (16).

Evidence is emerging that phosphorylation at particular residues of pRB may affect the binding of pRB to only particular subsets of its interacting partners (17). This suggests that different arrays of pRB phosphorylation may result in differential regulation of downstream effector pathways.

We have recently shown evidence that one of pRB's phosphorylated residues, serine 608, is a target for some, but not all, CDKs (18). This raises the possibility that different CDKs may phosphorylate pRB differentially. Here, we have extended this observation and provide evidence that the same CDKs also target other pRB residues differentially. We also demonstrate how such differential phosphorylation may affect the ability of these CDKs to dissociate pre-existing pRB-protein complexes.

¹ The abbreviations used are: CDK, cyclin-dependent kinase; GST, glutathione S-transferase.

MATERIALS AND METHODS

Plasmids and Antibodies—Vectors for the production of glutathione S-transferase (GST)-pRB fusion proteins were constructed by insertion of the entire pRB coding sequence (or fragments thereof) into the *Bam*HI site of pGEX 2T (Pharmacia). The threonine at position 826 was converted into alanine using polymerase chain reaction-based mutagenesis.

Baculovirus encoding human CDK4 was a gift of C. Sherr (HHMI, St Jude's Hospital, Memphis, TE). Cloned versions of human CDK2 and cyclin A (obtained from E. Harlow, MGH Cancer Center, Charlestown, MA), human cyclin D1 (obtained from A. Arnold, Harvard Medical School, Boston, MA), and human cyclin E (obtained from S. Reed, University of California San Francisco, San Francisco, CA) were inserted into the baculovirus shuttle vectors pVL1392/3 to produce recombinant baculovirus. Baculoviruses were generated using the baculovirus Gold™ kit (PharMingen) according to the manufacturer's instruction. A vector allowing expression of 9E10 (myc)-tagged cyclin E in COS cells was constructed by engineering (by polymerase chain reaction-directed mutagenesis) a *Not*I site in front of the natural cyclin E stop codon into which a cloned version of the 9E10 epitope (coding for amino acids EQLISEEDL-STOP) was subsequently inserted. Cyclin E modified in this way was cloned into the COS cell expression vector RccMV (Invitrogen).

Anti-pRB antisera were raised in rabbits using human GST-pRB fusion proteins as immunogens. Monoclonal antibodies specific for underphosphorylated pRB (14441A) and SV40 large T antigen (14111A) were purchased from PharMingen. Monoclonal α -human cyclin A antibody (BF683) was a gift of E. Harlow. The tag-specific 9E10 antibody was purified from tissue culture supernatant of 9E10 Hybridoma cells (a gift of G. Evan, ICRF, London) using DEAE-Sepharose chromatography.

Cell Culture and Labeling—Human HaCat cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were labeled with orthophosphate and pRB immunoprecipitated as described previously (10). COS cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and transfected with DNA using DEAE-dextran as described by others (27).

Kinase Preparation from Baculovirus-infected Insect Cells and Mammalian Tissue Culture Cells—Insect Sf9 cells and baculoviruses were cultured and used based on the detailed, published procedures of others (25). Enzyme preparation was essentially as described by others (26). Briefly, Sf9 cells were infected at a multiplicity of infection of >10 plaque forming units per cell with baculoviruses encoding either cyclin or CDK subunits for control lysates, or both viruses concomitantly to generate active enzymes. Cells were harvested 48 h post-infection and lysed at 4 °C in 250 μ l of kinase buffer (50 mM HEPES, pH 7.0, 10 mM MgCl₂, 10 mM MnCl₂, 1 mM dithiothreitol, containing protease inhibitors leupeptin, phenylmethylsulfonyl fluoride, and aprotinin, the phosphatase inhibitor β -glycerophosphate, and 0.1 mM protein kinase A inhibitor). Cell lysates were clarified by centrifugation and stored in aliquots at -70 °C. Kinases from mammalian tissue culture cells were purified by immunoprecipitation as described in Ref. 20.

Expression and Purification of Recombinant Proteins from Bacteria—Glutathione S-transferase fusion proteins were expressed and purified from cultures of *Escherichia coli* BL21 (DE3) P-Lys transformed with plasmids as described recently (18). Purified proteins were either stored in kinase buffer (see above) and bound to glutathione-Sepharose beads (for phosphorylation by insect cell-produced kinases) or eluted (for binding assays and phosphorylation by CDKs immunopurified from mammalian cells). Elution was performed with elution buffer (100 mM Tris-Cl, pH 8.0, 250 mM NaCl, 0.1% Triton X-100, 25 mM reduced glutathione (Sigma), and protease inhibitors). Eluted proteins were stored in 50% glycerol at -20 °C.

CDK Phosphorylation of GST-pRB Proteins for Tryptic Phosphopeptide Mapping—GST fusion proteins (300 ng), bound to glutathione-Sepharose, were incubated with 0.5–2 μ l of insect cell expressed enzymes (or equivalent amounts of control lysate) in kinase buffer (see above) containing 25 μ M ATP and 3 μ Ci of [γ -³²P]ATP for 30 min at 25 °C. Following phosphorylation, the pRB-containing beads were washed once with TNET (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.1% Triton X-100) and resuspended in SDS-containing sample buffer. Phosphorylated proteins were resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. The level of phosphorylation achieved by individual kinase preparation was characteristically 20–50-fold above the levels achieved using control lysates. Phosphorylation by immunopurified cyclin-associated kinases was essentially performed identically, except that eluted GST fusion proteins

were used as substrates to aid access to protein G-Sepharose bound kinase.

For phosphorylation of pRB in the presence of E1A, full-length GST-pRB (GST-pRB(1–928)) was mixed with a molar excess of purified E1A and, following incubation on ice for 10 min, phosphorylated using CDK4-cyclin D1 as described above. E1A was expressed as a full-length product in bacteria and purified as described by others (28).

Two-dimensional Tryptic Phosphopeptide Mapping—Two-dimensional tryptic phosphopeptide maps were performed essentially as described recently (10). Typically, an amount equivalent to 300–1000 cpm of the digested proteins was resolved for each map. Numbers were assigned to individual peptides by comparison with standard maps run in parallel and/or further maps where material from two sources was comigrated. pRB labeled *in vivo* was immunoprecipitated from HaCat cells as described previously (10).

Phosphoamino Acid Analysis—Individual tryptic phosphopeptides were scratched from thin layer chromatography plates using the autoradiograph as a template. Peptides were eluted from the cellulose matrix by resuspension in 0.5 ml of pH 1.9 buffer (88% formic acid/ acetic acid/water, 25:78:897 (by volume) containing, for a carrier, 0.1 μ g/ml unlabeled phosphoserine and phosphothreonine). Elutions were performed at room temperature for 30 min after which time the cellulose was pelleted by centrifugation (15,000 \times g, 5 min). The supernatant, containing the eluted peptide, was removed and the cellulose pellet subjected to one further round of elution. The eluates were combined, lyophilized, hydrolyzed in 6 N HCl at 110 °C for 1 h under nitrogen, lyophilized again, resuspended in pH 1.9 buffer (as above), loaded onto cellulose thin layer chromatography plates (Kodak), and subjected to electrophoresis. Electrophoretic separation was performed toward the anode at 800 V for a duration of 40 min after which time the plates were dried and stained with ninhydrin to visualize the position of unlabeled carrier phosphoserine and phosphothreonine. Radiolabeled amino acids were visualized by exposing the plates to autoradiographic film.

SV40 Large T Antigen Binding Assays—COS cell lysate, equivalent to 2 \times 10⁶ cells, was mixed with 50 ng of full-length GST-pRB (GST-pRB(1–928)) and pRB-containing complexes were collected on glutathione-Sepharose beads. COS cell lysates were prepared in ELB (50 mM Hepes-KOH, pH 7.0, 5 mM EDTA, 250 mM NaCl, 0.2% Triton X-100, 0.5 mM dithiothreitol, containing the protease inhibitors leupeptin, aprotinin, and phenylmethylsulfonyl fluoride and the phosphatase inhibitors sodium fluoride, β -glycerophosphate, and sodium vanadate) and stored at -70 °C. Beads containing the pRB complexes were washed twice in 0.5 \times ELB and analyzed for the presence of pRB and T(Ag) by Western blotting or, alternatively, used as a substrate for subsequent CDK phosphorylation. In the latter case the Sepharose beads were washed a further two times in kinase buffer and treated with insect cell-expressed CDKs (for phosphorylation) or buffer alone (for a control). The conditions for phosphorylation were as described for protein labeling except that radioactive ATP was omitted and the amount of unlabeled ATP was increased to 50 μ M. To ensure complete phosphorylation, the incubation period was increased to 45 min and fresh kinase and ATP were added every 15 min. Prior to the reaction, the different kinase-containing extracts were diluted with kinase buffer to contain equal concentrations of kinase activity. To remove any proteins that may have been released as a consequence of the kinase treatment the Sepharose beads were washed twice in 0.5 \times ELB and subsequently analyzed by Western blotting using antibodies specific for pRB or T(Ag).

To test for the impact of pRB phosphorylation on complex assembly, pRB was phosphorylated prior to addition of COS cell extract. The conditions for phosphorylation were identical to those for phosphorylation of pRB complexes.

RESULTS

Different CDKs Phosphorylate Distinct Subsets of pRB Residues

We have previously demonstrated that one of pRB's consensus CDK phosphorylation sites, serine 608, is part of the epitope recognized by antibodies that specifically detect the underphosphorylated, G₁ form of pRB and that this site is phosphorylated by CDK4-cyclin D1 and CDK2-cyclin A but not by CDK2-cyclin E. To establish whether differences in phosphorylation by these three CDKs occur on other Ser/Thr-Pro motifs, we performed two-dimensional tryptic phosphopeptide maps of pRB following its phosphorylation by these kinases *in*

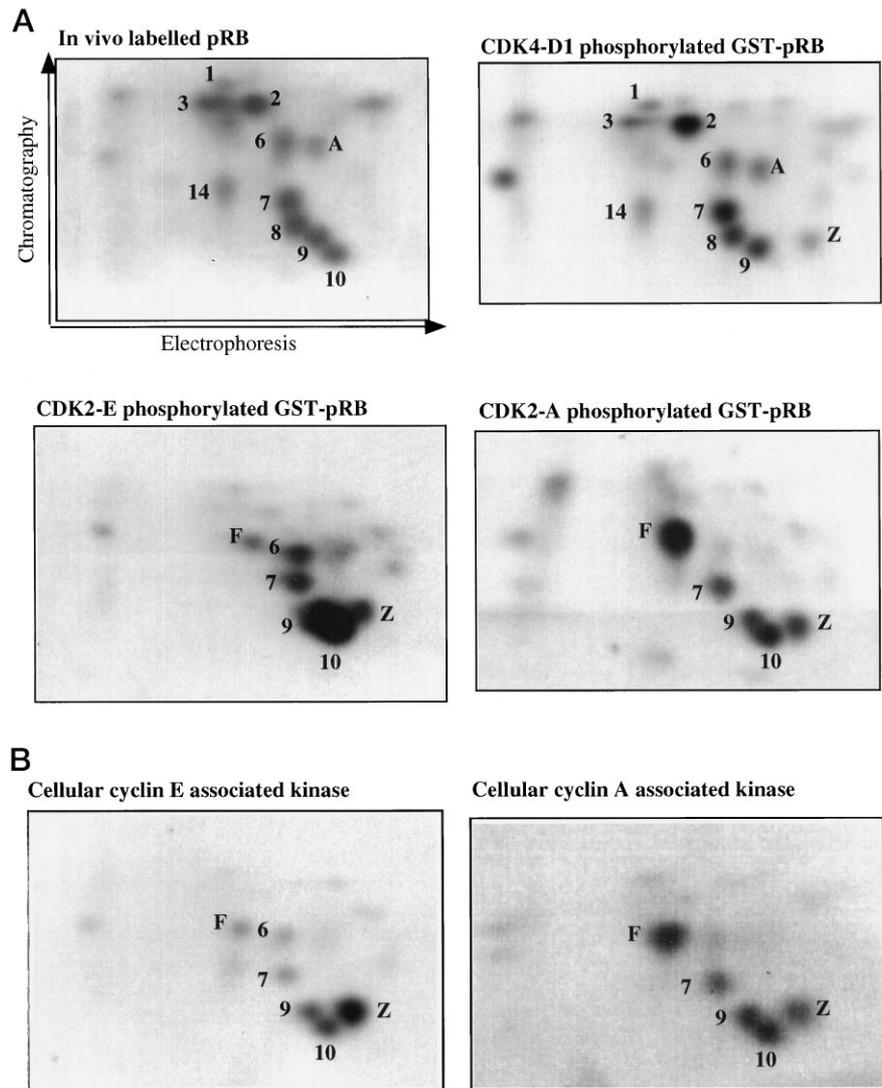


FIG. 1. Two-dimensional tryptic phosphopeptide analysis of pRB phosphorylated by different CDKs. *A*, tryptic phosphopeptide maps of cellular pRB labeled *in vivo* or bacterially produced full-length pRB (GST-pRB) phosphorylated by various CDKs *in vitro*. CDKs were produced in insect Sf9 cells coinfecting with individual baculoviruses encoding either CDK4 and cyclin D1 (CDK4-D1), CDK2 and cyclin A (CDK2-A), or CDK2 and cyclin E (CDK2-E). *B*, GST-pRB phosphorylated *in vitro* by cyclin A-associated kinase immunopurified from human HaCat cells or cyclin E-associated kinase immunopurified from COS cells transfected with an expression plasmid encoding 9E10(myc)-tagged human cyclin E. Maps were resolved by electrophoresis toward the cathode and ascending chromatography as indicated. The identity of individual phosphopeptides was confirmed through comigration with maps from cellular pRB. Phosphopeptides are numbered according to Ref. 4.

in vitro. As a substrate, we used purified full-length pRB produced as a glutathione *S*-transferase fusion protein (GST-pRB(1-928)) in *E. coli*. The kinases were derived from insect cells infected with recombinant baculoviruses encoding the various cyclin and CDK subunits. The resulting phosphopeptide maps demonstrate that serine 608 is not the only phosphorylation acceptor differentially targeted by these different CDKs (Fig. 1A). Phosphorylation by any one of the three CDKs generates authentic phosphopeptides, demonstrated by comigration with peptides derived from cellular pRB labeled *in vivo* with radioactive phosphate (data not shown). Yet the array of peptides phosphorylated by these three kinases is remarkably different.

Cellular pRB (labeled by exposing growing HaCat cell to [³²P]orthophosphate) gives a characteristic pattern of phosphopeptide spots, each of which has been previously named to aid comparison between maps (4). In general, the most hydrophobic phosphopeptides migrate furthest in the chromatography dimension, such as the upper diamond of spots seen in *in vivo* maps (embracing spots 1, 2, and 3) (see Fig. 1A, *top left panel*). Smaller phosphopeptides generally migrate furthest in the electrophoresis direction, such as those making up an “ear” shaped configuration on the right of the map (spots A and 6–10). To the left of the ear migrates spot 14. Although the maps generated by phosphorylation with each CDK contain

many of these same phosphopeptides, none of the CDKs alone can reproduce the full pattern of spots seen for maps of cellular pRB.

Most of the authentic pRB phosphopeptide spots are generated when pRB is phosphorylated by CDK4-cyclin D1 (Fig. 1A, *top right panel*), including spot 14, most of the upper diamond (spots 1, 2, and 3) and nearly all the ear (spots A, 6, 7, 8, and 9). Only spot 10 (the lowest of the ear phosphopeptides) is absent from this map. In addition, one aberrant phosphopeptide is generated, spot Z (see below). Interestingly, of the three kinases tested, CDK4-cyclin D1 is the only one that is able to generate all three of the hyperphosphorylation-specific spots (3, 9, and 14) which are under-represented in maps of active, nuclear tethered pRB (10).

Phosphorylation of pRB by CDK2-cyclin A or CDK2-cyclin E results in phosphopeptide maps very different from the one generated by CDK4-cyclin D1 (Fig. 1A, *lower panels*). Both of these maps consist of far fewer phosphopeptide spots (immediately apparent is that neither map contains spot 14 nor spots 1, 2, and 3 from the upper diamond). Phosphorylation of pRB by both of these kinases gives rise to three phosphopeptides also seen to be produced upon CDK4-cyclin D1 phosphorylation (spots 7, 9, and Z). Additionally, both give rise to spot 10 (the one major phosphopeptide of cellular pRB that is absent when recombinant pRB is phosphorylated by CDK4-cyclin D1) and

spot F (an authentic, *in vivo* tryptic phosphopeptide of pRB, migrating below spot 2 and to the left of spot 6, which is represented only at low levels in maps of cellular pRB labeled in growing cells (4)). While the maps of pRB phosphorylated by CDK2-cyclin A and CDK2-cyclin E are very similar they are not, however, identical. One phosphopeptide, spot 6, is produced upon phosphorylation by CDK2-cyclin E kinase but not upon phosphorylation by CDK2-cyclin A.

It is perhaps surprising that CDK2-cyclin A and CDK2-cyclin E phosphorylate so few residues on pRB, since both cyclin A and cyclin E trigger kinase activity that results in full hyperphosphorylation and inactivation of pRB upon transfection into SAOS2 cells (12). However, both these cyclins have been reported to associate with, and activate, CDK subunits other than CDK2 (19, 20). We therefore reasoned that, by combining with additional CDK subunits in cells, these cyclins might direct modification of additional residues of pRB, resulting in a more complete phosphorylation. We therefore sought to assess whether the kinase activity associated with these cyclins isolated from cells would result in a tryptic phosphopeptide pattern equivalent or different to that generated by our recombinant enzymes.

We immunopurified cyclin A, for which a highly specific monoclonal antibody is available, from growing HaCat keratinocytes. For a cyclin E source, we transfected COS cells with a vector encoding 9E10 epitope-tagged cyclin E and purified kinase activity with tag-specific antibody. The map of recombinant pRB phosphorylated by α -cyclin A associated kinases is identical to that using insect cell-expressed CDK2-cyclin A (Fig. 1B, right panel). Similarly, maps using either 9E10 (anti-tag) associated kinases or CDK2-cyclin E expressed in insect cells are also identical (Fig. 1B, left panel). Thus the distinct patterns of phosphates installed on pRB by recombinant CDK2-cyclin A and CDK2-cyclin E genuinely reflect the kinase activities associated with the respective cyclins in cells.

Mapping of Residues Phosphorylated by Individual CDKs

CDK2-Cyclin A-phosphorylated Residues—The phosphopeptide maps of GST-pRB phosphorylated by CDK4-cyclin D1, CDK2-cyclin A, and CDK2-cyclin E kinases clearly imply that each of these CDKs phosphorylates a distinct subset of residues on pRB. We therefore set out to unravel which residues of pRB are phosphorylated by these different kinases. This task was greatly aided by the results of previous work from Lees and co-workers (4). They provided evidence for the identity of many of the tryptic phosphopeptide spots generated from cellular pRB by demonstrating their co-migration with synthetic pRB peptides phosphorylated by mitotic CDK (4). We have used two-dimensional tryptic phosphopeptide mapping of phosphorylated GST-pRB fragments in combination with phosphoamino acid analysis to confirm many of their predictions and to map previously unassigned spots.

Using these two, complementary methods, we first established the identities of pRB residues phosphorylated by CDK2-cyclin A. We subjected each tryptic phosphopeptide of full-length pRB phosphorylated by CDK2-cyclin A to phosphoamino acid analysis to identify the nature of the phosphorylated residue(s) (Fig. 2A). We also used two-dimensional maps of pRB fragments to determine the region of pRB from which each phosphopeptide arises (Fig. 2B).

The phosphopeptide giving rise to spot 10 has previously been shown to co-migrate with phospho-SPK, suggesting that it arises from phosphorylation of serine 612, the only serine within pRB's primary amino acid sequence positioned to give such a peptide upon tryptic cleavage (4). We have confirmed the identity of spot 10 by demonstrating that it contains only

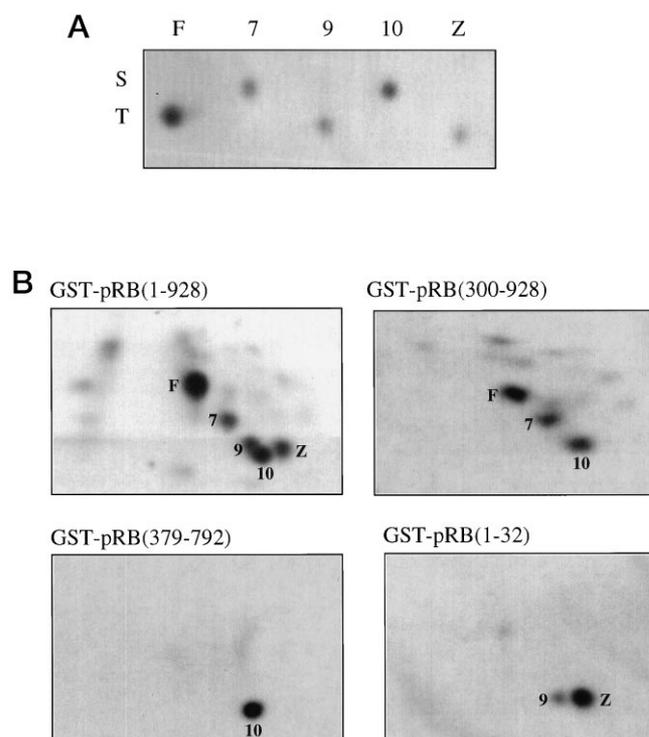


FIG. 2. Localization and assignment of CDK2-cyclin A phosphorylated residues using phosphoamino acid analysis and phosphorylation of pRB subfragments. A, phosphoamino acid analysis of individual tryptic pRB peptides. Individual tryptic phosphopeptides were recovered after two-dimensional separation of trypsin-digested full-length GST-pRB phosphorylated by CDK2-cyclin A. The peptides were subjected to acid hydrolysis and resolved by electrophoresis on cellulose. S indicates the position for phosphoserine and T indicates that of phosphothreonine. Numbers denote the identity of the phosphopeptide spots. B, two-dimensional tryptic phosphopeptide maps of GST-fused pRB subfragments phosphorylated by CDK2-cyclin A. The numbers in parentheses denote the pRB amino acids encompassed.

phosphoserine (Fig. 2A) and that it arises from CDK2-cyclin A phosphorylation of a pRB fragment spanning amino acids 379–792 (the pocket region) (Fig. 2B, bottom left panel). Similarly, we have confirmed that spot F represents phosphorylation at threonine 821 of pRB, since it contains only phosphothreonine (Fig. 2A) and, while not arising from the pocket region alone, is generated upon phosphorylation of a fragment that also contains the COOH terminus of pRB (amino acids 300–928; top right panel). Spot 7, which has not been previously mapped, is also generated only from this larger region (see below).

The origins of certain pRB tryptic phosphopeptide spots are ambiguous since identical tryptic peptides can be derived from different regions of pRB (4). For example, phosphopeptides generated from three distinct regions of pRB can give rise to spot 9. This spot has previously been shown to co-migrate with phospho-TPR, the phosphopeptide generated upon tryptic cleavage around Thr⁵, Thr²⁵², or Thr³⁵⁶ of pRB. Thus, although spot 9 is seen in maps of pRB phosphorylated by each of the three CDKs investigated here, we cannot automatically infer that they phosphorylate the same residue. We have confirmed that spot 9, generated upon phosphorylation of full-length pRB by each of these CDKs, contains only phosphothreonine (Figs. 2A and 3A, and data not shown) and have used maps of smaller regions of pRB to determine, in some instances, which of the three sites are phosphorylated.

CDK2-cyclin A does not appear to phosphorylate Thr³⁵⁶ of pRB, since spot 9 is not represented in maps of GST-pRB(300–928) phosphorylated by this kinase (Fig. 2B, top right panel). Spot 9 is, however, present in maps of GST-pRB(1–32) phos-

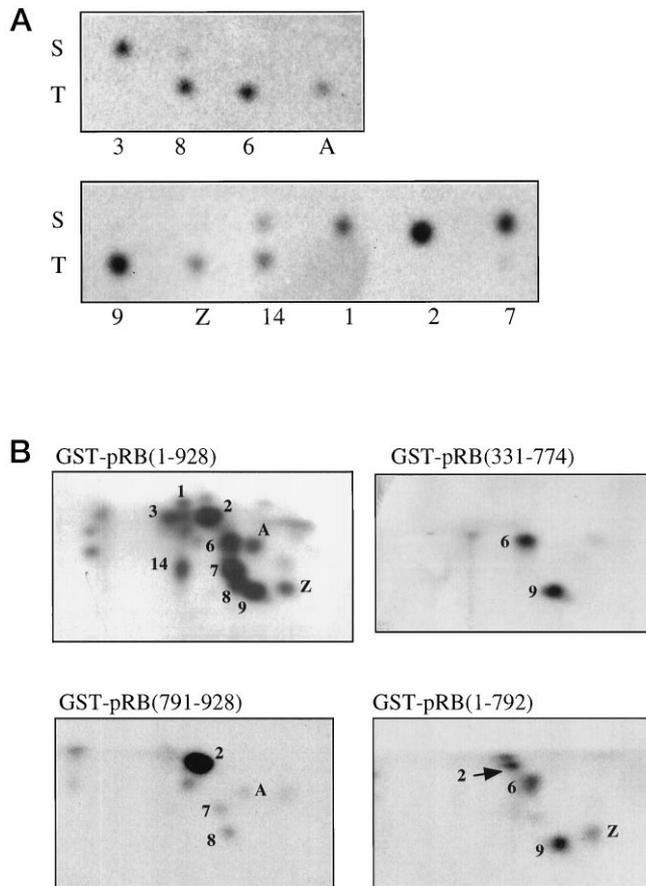


FIG. 3. Localization and assignment of CDK4-cyclin D1 phosphorylated residues. A, phosphoamino acid analysis of individual tryptic pRB peptides. Individual tryptic phosphopeptides were recovered after two-dimensional separation of trypsin-digested full-length GST-pRB phosphorylated by CDK4-cyclin D1. All procedures and labeling as described for Fig. 2. B, two-dimensional tryptic phosphopeptide maps of GST-fused pRB subfragments phosphorylated by CDK4-cyclin D1. The numbers in parentheses denote the pRB amino acids encompassed.

phosphorylated by CDK2-cyclin A (*bottom right panel*). On this short NH_2 -terminal fragment of pRB, the presence of spot 9 can only indicate phosphorylation at Thr⁵.

The aberrant spot Z also arises from this short, NH_2 -terminal fragment (GST-pRB(1–32)). Phosphoamino acid analysis of spot Z reveals that, like spot 9, it too contains only phosphothreonine and thus presumably also results from phosphorylation at Thr⁵. We infer that spot Z does not arise from the GST portion of this fusion protein since: 1) the only proline-directed site on GST is a Ser/Pro motif; 2) GST alone is not a phosphate acceptor for CDK phosphorylation; and 3) spot Z is not generated by phosphorylation of GST fusions of other regions of pRB. Phosphorylation at Thr⁵ may give rise to two tryptic phosphopeptide spots if trypsin is not efficiently cutting at one of the surrounding target residues. Spot Z may therefore represent a partial tryptic phosphopeptide spanning the region where pRB is fused to GST, providing an explanation for its non-authenticity. Nevertheless, the presence of spot Z seems to be indicative of phosphorylation at Thr⁵ on GST-pRB.

Besides the sites described above, we have previously shown evidence that serine 608 of pRB is targeted by CDK2-cyclin A (18). However, the corresponding Ser⁶⁰⁸-containing tryptic phosphopeptide cannot be seen on two-dimensional maps, presumably since its large size (predicted to be 35 amino acids in length) does not facilitate solubilization under the conditions

used for two-dimensional separation.² Such insolubility of tryptic phosphopeptides is not uncommon (21) and serves as a demonstration that other potential CDK phosphorylation sites of pRB that cannot be described in terms of phosphopeptide spots should not be ruled out as authentic phosphate acceptors. In summary, we have presented evidence that CDK2-cyclin A can phosphorylate residues Ser⁶¹², Thr⁸²¹, and Thr⁵ of pRB, as well as the residue that gives rise to spot 7 (see below).

CDK4-Cyclin D1 Phosphorylated Residues—Phosphorylation of pRB fragments by CDK4-cyclin D1 combined with phosphoamino acid analysis have, similarly, enabled us to describe the identity of residues phosphorylated by this kinase (Figs. 3, A and B, respectively). The phosphopeptide giving rise to spot 6 has previously been shown to co-migrate with the phosphopeptide containing Thr³⁷³ of pRB (4). We have confirmed this by demonstrating that spot 6 contains only phosphothreonine (Fig. 3A) and is generated when an NH_2 -terminally extended pocket region of pRB is phosphorylated by CDK4-cyclin D1 (amino acids 331–774; Fig. 3B, *top right panel*).

Spot 9, the phosphopeptide generated from tryptic cleavage around three distinct threonines, is also generated from this NH_2 -terminally extended pocket region. Thus CDK4-cyclin D1 kinase, unlike CDK2-cyclin A, can presumably phosphorylate Thr³⁵⁶, the sole residue on this fragment whose phosphorylation can give rise to spot 9. CDK4-cyclin D1 phosphorylation of a larger pRB fragment, containing the complete NH_2 -terminal region, additionally gives rise to spot Z (amino acids 1–792; *lower right panel*). As discussed, this is indicative of phosphorylation at another residue from which spot 9 can originate, Thr⁵ of pRB.

A second phosphopeptide with more than one possible origin gives rise to spot 2. This spot has previously been shown to co-migrate with phospho-SFYK, and could therefore arise from tryptic cleavage around either Ser⁷⁸⁸ or Ser⁸¹¹ of pRB. Phosphoamino acid analysis of spot 2 from maps of full-length pRB phosphorylated by CDK4-cyclin D1 confirms that spot 2 contains only phosphoserine (Fig. 3A), but clearly does not reveal which of the two residues is phosphorylated. Maps of GST-pRB(1–792) and GST-pRB(791–928) phosphorylated by CDK4-cyclin D1 are more informative; since both contain spot 2 we can predict that both Ser⁷⁸⁸ and Ser⁸¹¹ are phosphorylated by this enzyme (Fig. 3B, *lower two panels*).

The above analysis leaves various CDK4-cyclin D1 tryptic phosphopeptides unassigned. These include spots A and 8, both of which are specifically produced upon CDK4-cyclin D1 phosphorylation, and spot 7 which is also produced upon phosphorylation by CDK2-cyclin A and CDK2-cyclin E. All three of these phosphopeptide spots are generated from the COOH-terminal region of pRB when phosphorylated by CDK4-cyclin D1 (amino acids 791–928; Fig. 3, *lower left panel*). There are two consensus CDK phosphorylation sites on this fragment, Ser⁷⁹⁵ and Thr⁸²⁶, for which no tryptic phosphopeptides have yet been ascribed (4). However, based on our phosphoamino acid analysis, we can predict which phosphorylated residue each of these phosphopeptide spots contains (Fig. 3A). We find that spots 8 and A contain phosphothreonine, while spot 7, in agreement with our previous result using CDK2-cyclin A (Fig. 2A), contains phosphoserine. We therefore predict that spots 8 and A are generated by phosphorylation at Thr⁸²⁶, and spot 7 from that at Ser⁷⁹⁵.

Spots 8 and A were of particular interest to us since they represent two of the CDK4-cyclin D1-specific tryptic phosphopeptides. To confirm their identity, we made and analyzed full-length GST-pRB with a threonine to alanine substitution

² T. Zarkowska, unpublished data.

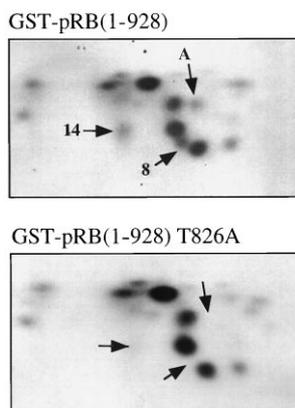


FIG. 4. Identification of a CDK4-cyclin D1 specific phosphorylation site by mutagenesis. Two-dimensional tryptic phosphopeptide maps of full-length GST-pRB (GST-pRB(1-928)) and a mutated version in which the threonine at position 826 was converted into an alanine (GST-pRB(1-928)/T826A). Analysis of wild type and mutant proteins were performed in parallel using CDK4-cyclin D1 for phosphorylation.

at residue 826. GST-pRB(1-928)/T826A can be efficiently phosphorylated by CDK4-cyclin D1 and tryptic phosphopeptide analysis reveals that spots 8 and A are indeed absent from its map (Fig. 4), confirming that both spots arise from phosphorylation at Thr⁸²⁶.

We also note that spot 14 is consistently absent when GST-pRB(1-928)/T826A is phosphorylated by CDK4-cyclin D1. Spot 14 has previously been shown to derive from a phosphopeptide spanning Ser²⁴⁹ and Thr²⁵² on which both of these residues are phosphorylated (4). Phosphoamino acid analysis of CDK4-cyclin D1-phosphorylated material confirms that spot 14 contains both serine and threonine (Fig. 3A). However, this peptide is not generated by CDK4-cyclin D1 phosphorylation of GST-pRB(1-792), as one might expect, or indeed from a fusion encompassing the remainder of pRB, GST-pRB(791-928) (Fig. 3B). We therefore suspect that spot 14 does arise from phosphorylation at Ser²⁴⁹-Thr²⁵², but that this phosphorylation may be dependent on the prior phosphorylation of Thr⁸²⁶.

Spots 1 and 3, two other phosphopeptides specifically produced upon CDK4-cyclin D1 phosphorylation, do not arise from maps of the NH₂-terminal fragment of pRB (amino acids 1-792) or the COOH-terminal fragment (amino acids 791-928) either. This may similarly indicate that prior phosphorylation at distal residues is required for modification at the associated sites. Spot 1 has previously been predicted to arise from phosphorylation at Ser⁸⁰⁷ (4). Since we find that it contains only phosphoserine we tentatively agree with this prediction (Fig. 3A). Spot 3, on the other hand, has not been previously assigned. Since it contains only phosphoserine, we would hazard that it probably arises from phosphorylation at one of the unassigned Ser/Pro motifs of pRB; Ser²³⁰, Ser⁵⁶⁷, or Ser⁷⁸⁰ (Figs. 3A and 5). In summary, we have shown evidence that CDK4-cyclin D1 phosphorylates Thr⁵, Ser²⁴⁹, Thr²⁵², Thr³⁵⁶, Thr³⁷³, Ser⁷⁸⁸, Ser⁷⁹⁵, Ser⁸⁰⁷, Ser⁸¹¹, and Thr⁸²⁶ of pRB.

Summary of Phosphorylation Analysis

If we collate all the evidence described above with our previous indications of the kinases that phosphorylate Ser⁶⁰⁸ of pRB (18), a fairly complete description emerges of the pRB residues targeted by CDK4-cyclin D1 and CDK2-cyclin A (Fig. 5). In addition, CDK2-cyclin E sites can also be predicted on the basis of this work. CDK2-cyclin E phosphorylation of GST-pRB gives rise to phosphopeptide spots F, 6, 7, 10, and Z which, from the analysis above, enables us to predict that it phosphorylates Thr⁸²¹, Thr³⁷³, Ser⁷⁹⁵, Ser⁶¹², and Thr⁵, respectively. Spot 9 is also generated, which may be purely due to the phosphoryla-

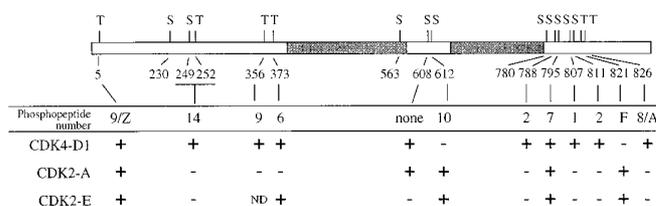


FIG. 5. Summary of pRB residues phosphorylated by individual CDKs. A schematic of human pRB is shown, with the shaded regions denoting the pRB pocket. Bars indicate consensus sites for CDKs (Ser/Thr-Pro motifs). Indicated above is the nature of the potential phosphate-accepting residue and below, their amino acid number. Our analysis is summarized by depicting tryptic phosphopeptide numbers and indicating their status with regards to phosphorylation by CDK4-cyclin D1, CDK2-cyclin A, and CDK2-cyclin E.

tion of Thr⁵, but may also be indicative of phosphorylation at Thr²⁵² and/or Thr³⁵⁶.

As predicted by the pattern of phosphopeptides generated by each of the CDKs, only a few residues are phosphorylated by all three kinases (*e.g.* Ser⁷⁹⁵ and Thr⁵), while others are differentially targeted by the CDKs described. We were particularly intrigued by the pattern of CDK phosphorylation of two clustered pairs of CDK target sites, Ser⁶⁰⁸-Ser⁶¹² and Thr⁸²¹-Thr⁸²⁶. In each case, CDK4-cyclin D1 phosphorylates only one of the two adjacent phosphorylation sites, while the other one is phosphorylated by a CDK2-containing kinase.

The Role of Differential CDK Phosphorylation in Regulating the Interaction with LXCXE Proteins

One clustered pair of pRB phosphorylation sites, Thr⁸²¹-Thr⁸²⁶, has previously been implicated in regulating the association of pRB with proteins containing an LXCXE motif (17). Thus, pRB-LXCXE protein interactions seemed ideal as a model system to test the impact of differential CDK phosphorylation. According to our analysis, Thr⁸²¹ is phosphorylated by CDK2-cyclin A and CDK2-cyclin E, but not by CDK4-cyclin D1, while for its neighbor, Thr⁸²⁶, the converse is true. One attractive possibility is that phosphorylation at both Thr⁸²¹ and Thr⁸²⁶ is required to disable pRB for LXCXE protein binding. Hence, phosphorylation by either CDK4-cyclin D1 or a CDK2-containing kinase would be insufficient to inactivate this particular function of pRB, rather, their co-operation would be required.

To test this possibility we investigated whether phosphorylation by either CDK2-cyclin A or CDK4-cyclin D1 would suffice to disable pRB for binding to SV40 large T antigen (T(Ag)), which binds to pRB via an LXCXE motif (22). When unphosphorylated, full-length GST-pRB is mixed with excess T(Ag)-containing COS cell lysate, T(Ag) co-purifies with GST-pRB on glutathione-Sepharose (Fig. 6A, lane 1). However, prior phosphorylation of GST-pRB by either CDK4-cyclin D1 or CDK2-cyclin A abolishes T(Ag) recovery (Fig. 6A, lanes 2 and 3, respectively). This suggests that co-operation of different CDKs is not required to render pRB incapable of binding to this LXCXE protein and, presumably, that phosphorylation at either Thr⁸²¹ or Thr⁸²⁶ is equivalent in inactivating this particular function of pRB.

Another, perhaps physiologically more relevant, assay would be to test release of an LXCXE protein once complexed to pRB. We therefore performed a parallel experiment in which we used a preformed pRB-T(Ag) complex as a substrate for the same two kinases. Following phosphorylation, we washed the pRB-T(Ag)-containing beads to remove any T(Ag) released during the procedure. We find that treatment with CDK2-cyclin A results in loss of the majority of T(Ag) attached to pRB, while treatment with CDK4-cyclin D1 is without effect (Fig. 6B). Together, these experiments suggest that both CDK4-cyclin D1 and

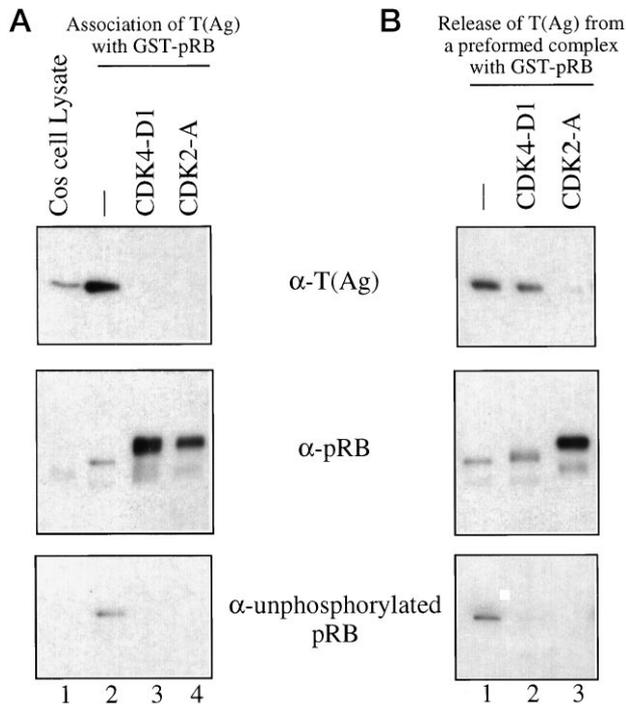


FIG. 6. Affect of CDK phosphorylation on pRB/SV40 large T antigen complexes. *A*, assembly of pRB-T(Ag) complex *in vitro*. Full-length GST-pRB was phosphorylated either by CDK4-cyclin D1 (CDK4-D1) or CDK2-cyclin A (CDK2-A) or treated with buffer only for a control (-). Following incubation with T(Ag)-containing COS cell lysate the different forms of pRB were recovered on glutathione-Sepharose beads. Material bound to the beads was separated on SDS-containing gels and subjected to Western blot analysis using either an anti-T(Ag) monoclonal antibody (α -T(Ag)), rabbit sera directed against human pRB (α -pRB), or a monoclonal antibody specific for underphosphorylated pRB (α -underphosphorylated pRB). *B*, complexes between GST-pRB and T(Ag) were pre-formed on glutathione-Sepharose beads and subsequently treated with CDK4-cyclin D1, CDK2-cyclin A, or buffer alone. Beads were then washed and bound material analyzed by Western blotting as described in *A*.

CDK2-cyclin A are capable of disabling free pRB for binding T(Ag), but that only CDK2-cyclin A is capable of resolving a pre-existing complex with this LXCXE protein.

Significantly, the inability of CDK4-cyclin D1 to resolve pRB-T(Ag) complexes does not coincide with a complete inability of this kinase to phosphorylate pRB in the presence of T(Ag). We find that such pRB is no longer recognized by underphosphorylated pRB-specific antibodies (Fig. 6*B*, lower panel), which measure phosphorylation of serine 608 (18). Thus CDK4-cyclin D1 appears to retain its ability to phosphorylate at least one residue of pRB, Ser⁶⁰⁸, to completion. Furthermore, such pRB displays a slightly retarded migration on SDS-polyacrylamide electrophoresis gels when compared with unphosphorylated sample (indicative of some degree of phosphorylation). However, this change in migration is not equivalent to the more pronounced change seen when free pRB is phosphorylated by CDK4-cyclin D1 or when pRB, free or complexed, is phosphorylated by CDK2-cyclin A (Fig. 6, *A* and *B*, middle panels). The migration of pRB phosphorylated in the presence of T(Ag) suggests that phosphorylation of certain CDK4-cyclin D1 targeted residues may still occur, while phosphorylation of others may be inhibited by prior T(Ag) binding.

We therefore reasoned that CDK4-cyclin D1 may be unable to disrupt pRB-T(Ag) complexes because access to certain CDK4-cyclin D1 specific pRB phosphorylation sites is blocked in the presence of T(Ag). In contrast, CDK2-cyclin A, which phosphorylates a different subset of pRB residues, may be

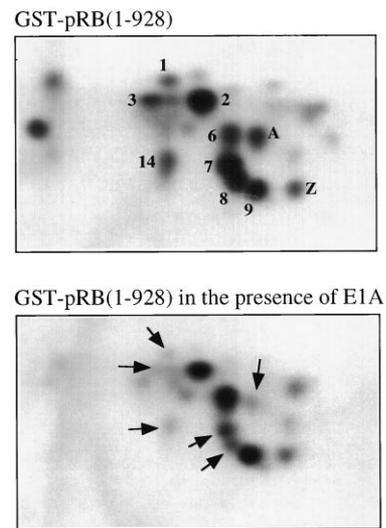


FIG. 7. Prebound E1A prevents phosphorylation of Thr⁸²⁶ and other pRB residues by CDK4-cyclin D1. Two-dimensional tryptic maps showing full-length pRB (GST-pRB(1-928)) phosphorylated by CDK4-cyclin D1 in the absence (upper panel) or presence (lower panel) of recombinant E1A.

competent to dissolve such complexes if these sites remained accessible in the presence of T(Ag).

LXCXE Protein Binding Prevents Phosphorylation of T826

To directly test whether the inability of CDK4-cyclin D1 to disrupt an LXCXE protein-pRB complex is due to a specific blocking of CDK4-cyclin D1 phosphorylation sites, we investigated whether phosphorylation of a subset of pRB sites by CDK4-cyclin D1 might be inhibited by the binding of another LXCXE protein, E1A. This protein can be purified in large amounts from a recombinant source, enabling us to phosphorylate sufficient GST-pRB(1-928) in its presence to perform two-dimensional tryptic phosphopeptide mapping and visualize phosphorylations at specific residues. Additionally, purified recombinant E1A has the advantage (over COS cell lysate) of being free of other pRB-binding proteins.

Two-dimensional tryptic phosphopeptide maps of pRB phosphorylated by CDK4-cyclin D1 in the absence or presence of E1A reveal that phosphorylation of a subset of pRB sites is specifically prevented by E1A (Fig. 7). While the level of phosphorylation at Thr³⁷³, for example (spot 6), remains high, phosphorylation of other sites is inhibited. Among these, spots 7, 1, 8/A, and 14 (indicated by arrows) are under-represented suggesting that access to Ser⁷⁹⁵, Ser⁸⁰⁷, Thr⁸²⁶, and Ser²⁴⁹-Thr²⁵², respectively, is blocked by the binding of E1A. This analysis provides direct evidence that LXCXE protein binding to pRB can block access to certain CDK4-cyclin D1-targeted phosphorylation sites on pRB, including Thr⁸²⁶, while allowing others to be modified. These data support a model whereby the phosphorylation of a distinct set of pRB residues by CDK4-cyclin D1, while allowing this kinase to disable free pRB for subsequent binding to LXCXE proteins, does not allow such proteins to be released from pre-formed complexes.

DISCUSSION

Cyclin-dependent kinases have been implicated in the inactivation of pRB. The cell cycle regulatory CDKs activated in late G₁ and early S phase are suspected to catalyze the initial conversion of active pRB into a form that is inactive, since their activation is concurrent with the first appearance of hyperphosphorylated, inactive pRB. Furthermore, known regulatory events that interfere with activation of these kinases result in

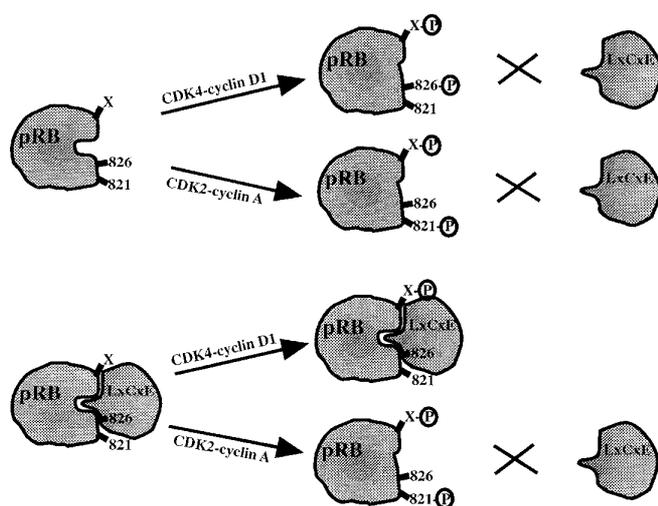


FIG. 8. Model of CDK regulation of pRB-LXCXE complexes. Activation of either CDK4-cyclin D1 or CDK2-cyclin A disables free, uncomplexed, pRB for subsequent binding of LXCXE proteins. The same conformational change is achieved by both CDKs although they phosphorylate different residues on pRB. When pRB is complexed to an LXCXE protein, certain residues (such as Thr⁸²⁶) are inaccessible, while others (such as Thr⁸²¹) remain accessible. This results in a differential ability of CDK4-cyclin D1 and CDK2-cyclin A to dissociate pRB-LXCXE complexes.

an inability of cells to execute pRB hyperphosphorylation (reviewed in Ref. 3).

Our results demonstrate, however, that *in vitro* the CDKs suspected of causing pRB inactivation do not phosphorylate pRB identically. The array of sites phosphorylated by CDK4-cyclin D1, for example, is very distinct from that phosphorylated by CDK2-cyclin A or CDK2-cyclin E. Of the 13 bona fide phosphate accepting residues for which we have demonstrated CDK phosphorylation, only two (threonine 5 and serine 795) appear to be phosphorylated by all three of the CDKs. More importantly, no single one of these CDKs can phosphorylate all the pRB residues seen phosphorylated in cells. Since almost all authentic pRB residues are phosphorylated at some point during late G₁/early S phase (8), it can be inferred that none of the kinases investigated here may singly be responsible for pRB phosphorylation at this stage of the cell cycle. Thus, either two or more kinases operate together to fully phosphorylate pRB or, alternatively, a very different kinase (capable of phosphorylating all the sites) is responsible.

Recent evidence from Hatakeyama and co-workers (15) would support a model for kinase co-operation. They describe that human pRB, expressed in yeast cells lacking G₁ cyclins, cannot be fully phosphorylated by coexpression of either cyclin D1 or cyclin E, but requires the concomitant expression of both (15). Our demonstration that different sites are phosphorylated by different CDK-cyclin complexes may provide the molecular explanation for their observation.

The biological significance of differential CDK phosphorylation depends on the contribution to pRB inactivation that phosphorylation by each CDK has. One theoretical possibility is that pRB is rendered inactive only when fully phosphorylated and that partial phosphorylation by any single CDK is without biochemical impact. Alternatively, phosphorylation by each CDK may have a different impact on pRB's biochemical functioning. The data presented in this paper support the latter alternative.

Knudsen and Wang (17) have recently implicated two neighboring pRB threonines, Thr⁸²¹ and Thr⁸²⁶, in regulating complex formation of pRB with SV40 T(Ag) and other, cellular LXCXE proteins. We have demonstrated here that phosphoryl-

ation at each of these two residues is directed by different CDKs and that prior phosphorylation by either CDK2-cyclin A, which phosphorylates Thr⁸²¹, or CDK4-cyclin D1, which phosphorylates Thr⁸²⁶, can disable pRB for binding T(Ag). This result suggests that phosphorylation by either CDK4-cyclin D1 or CDK2-cyclin A, although occurring on a different subset of pRB's residues, has the same effect on pRB's ability to bind LXCXE proteins. More specifically, the redundancy of the two CDKs hints that phosphorylation at either Thr⁸²¹ or Thr⁸²⁶ may be equivalent in terms of inhibiting LXCXE protein interactions.

While prior phosphorylation by either CDK4-cyclin D1 or CDK2-cyclin A may be equivalent in disabling subsequent T(Ag) binding, only CDK2-cyclin A can dissociate a pre-formed complex between these two proteins. We show two pieces of evidence that suggest that the inability of CDK4-cyclin D1 to resolve a pRB-T(Ag) complex is due to blocking of access to the necessary CDK4-cyclin D1 phosphorylation sites by LXCXE protein binding. First, CDK4-cyclin D1 phosphorylation of pRB-T(Ag) results in an altered phosphorylation of the pRB that is best explained by an inhibition of phosphorylation at only a subset of target sites. Second, the presence of another LXCXE protein, E1A, specifically inhibits the CDK4-cyclin D1 phosphorylation of certain pRB residues, including Thr⁸²⁶.

We therefore propose a model whereby CDKs, whether they phosphorylate Thr⁸²¹ or Thr⁸²⁶ of pRB, can inhibit free pRB from forming complexes with LXCXE motif proteins, while only those that phosphorylate Thr⁸²¹ can release pRB from a pre-formed LXCXE protein-containing complex (Fig. 8). Since the key to pRB inactivation is assumed to be the release of pRB bound proteins at the end of G₁ phase, CDK4-cyclin D1 may be unable to fulfill this function, at least with regards to LXCXE proteins. Interestingly cyclin D1 itself contains an LXCXE motif that can mediate binding to pRB under certain conditions (23). The physiological significance of this motif is, however, unclear; it does not appear to be necessary for pRB phosphorylation since cyclin D1 mutated at this motif is not compromised in its ability to direct CDK4 phosphorylation of pRB (24).

pRB forms complexes with various other cellular proteins, many of which do not possess an LXCXE motif but instead interact with pRB via other sequence modules. Recent evidence suggests that modification of different subsets of phosphorylation sites disables pRB for interaction with different cellular factors (17). It is therefore conceivable that different CDKs may also affect other pRB-containing complexes differentially.

The wider implications of such a model are profound. Differential phosphorylation may be a mechanism by which pRB can integrate signaling transmitted through various CDKs. Progression into S phase might thus depend on the prior activation of both CDK4-cyclin D1 and CDK2-cyclin E, each one regulating complementary sets of pRB-containing complexes. Future work, aimed at assessing the significance of differential pRB phosphorylation *in vivo* and its effects on different pRB-containing complexes *in vitro* will serve to test this model.

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