Cyclin D1/Cdk4 Regulates Retinoblastoma Protein-mediated Cell Cycle Arrest by Site-specific Phosphorylation

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The retinoblastoma protein (pRb) inhibits progression through the cell cycle. Although pRb is phosphorylated when G₁ cyclin-dependent kinases (Cdks) are active, the mechanisms underlying pRb regulation are unknown. In vitro phosphorylation by cyclin D1/Cdk4 leads to inactivation of pRb in a microinjection-based in vivo cell cycle assay. In contrast, phosphorylation of pRb by Cdk2 or Cdk3 in complexes with A- or E-type cyclins is not sufficient to inactivate pRb function in this assay, despite extensive phosphorylation and conversion to a slowly migrating "hyperphosphorylated form." The differential effects of phosphorylation on pRb function coincide with modification of distinct sets of sites. Serine 795 is phosphorylated efficiently by Cdk4, even in the absence of an intact LXCXE motif in cyclin D, but not by Cdk2 or Cdk3. Mutation of serine 795 to alanine prevents pRb inactivation by Cdk4 phosphorylation in the microinjection assay. This study identifies a residue whose phosphorylation is critical for inactivation of pRb-mediated growth suppression, and it indicates that hyperphosphorylation and inactivation of pRb are not necessarily synonymous.

INTRODUCTION

The retinoblastoma protein (pRb) functions to constrain cell proliferation and exerts its effects during the initial stages of G₁ (Goodrich *et al.*, 1991; Templeton *et al.*, 1991). Although the purpose of the pRb-mediated block to cell proliferation is unknown, it is proposed to be a central component of the restriction point and, therefore, important for normal growth and differentiation (for review, Weinberg, 1995). Consistent with this hypothesis, loss of pRb by mutation causes retinoblastoma, and possibly other neoplasia, as well as defects in terminal differentiation (for review, Chen *et al.*, 1995).

During the latter stages of G₁, pRb is extensively modified by phosphorylation, generating hyperphosphorylated forms that persist until exit from mitosis (Buchkovich *et al.*, 1989; Chen *et al.*, 1989; DeCaprio *et al.*, 1989; Mihara *et al.*, 1989). Hyperphosphorylated

pRb is recognized by its characteristic decrease in electrophoretic mobility, and conditions that favor cell proliferation favor the appearance of these slower migrating forms (Cobrinik *et al.*, 1992; Hinds *et al.*, 1992). The correlation between cell proliferation and pRb phosphorylation suggests that the ability of pRb to constrain cell cycle progression is inhibited by phosphorylation. A model emerges wherein pRb regulates a cell cycle transition late in G₁ that must be traversed to continue with cell division. Appropriate signals lead to activation of regulatory kinases, phosphorylation of pRb, and passage through G₁ (Weinberg, 1995).

Several lines of evidence, albeit indirect, support this model. G₁ cyclin-dependent kinases (Cdks), particularly cyclin D-type/Cdk4 and cyclin E/Cdk2, are maximally active near the time of pRb phosphorylation, and these kinases can phosphorylate pRb in vitro (for review, Sherr, 1994; Weinberg, 1995). Phosphorylation in vitro by cyclin E/Cdk2 affects the ability of pRb to bind and inhibit the transcription factor E2F, a major target of pRb function (Dylnacht *et al.*, 1994).

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The ability of pRb to block transcriptional transactivation can be inhibited by coexpression of cyclin A or E (Bremner *et al.*, 1995). Finally, D-type cyclins, cyclin A, and cyclin E can override pRb-mediated growth arrest upon cotransfection into SAOS-2 cells (Hinds *et al.*, 1992; Dowdy *et al.*, 1993; Ewen *et al.*, 1993; Horton *et al.*, 1995). An additional kinase, Cdk3, is known to be required for S-phase entry, although its cyclin partner and precise function are unknown (van den Heuvel, 1993; Hofmann and Livingston, 1996). Our previous finding that cyclin E/Cdk3 can phosphorylate pRb in vitro (Harper *et al.*, 1995) leaves open the possibility that this kinase participates in pRb regulation.

Attempts have been made to unravel the regulation of pRb function through the use of specific mutations targeted to consensus Cdc2 phosphorylation sites or sites that influence phosphorylation. By using this approach, Hamel *et al.* (1990, 1992) have identified amino acid residues that affect the characteristic change in electrophoretic mobility of pRb seen upon extensive phosphorylation. Mutation of these sites prevents the shift in electrophoretic mobility but does not affect the ability of pRb to bind simian virus 40 tumor antigen or to inhibit transactivation by E2F. Mutation of multiple phosphorylation sites generates *Rb* alleles that, under certain conditions, tend to be more active than the wild type, suggesting that these mutations prevent negative regulation of pRb function. The role of individual phosphorylation sites in the modulation of pRb-induced cell cycle arrest, however, has not been defined. This may be due to inherent limitations in the assays chosen to analyze pRb function or to the possibility that appropriate regulatory kinases have not been identified.

In an attempt to identify relevant regulatory kinases, several reports have characterized phosphorylation of pRb by Cdc2 in vitro (Taya *et al.*, 1989; Lees *et al.*, 1991; Lin *et al.*, 1991) or upon overexpression of cyclins in vivo (Hinds *et al.*, 1992; Dowdy *et al.*, 1993; Ewen *et al.*, 1993; Kato *et al.*, 1993; Horton *et al.*, 1995). Differences in the phosphorylation of pRb by cyclin B/cdc2, cyclin A/Cdk2, cyclin E/Cdk2, and cyclin D-type/Cdk4, however, have not been reported to date; differences might be expected given the distinct characteristics of these kinases. For example, exogenous expression of cyclin D1 and cyclin E has an additive effect in accelerating transit of G₁ in cells containing pRb (Resnitzky and Reed, 1995). Protein inhibitors and antibodies specific for D-type Cdk2s cause G₁ arrest only in cells containing wild-type pRb (Guan *et al.*, 1994; Koh *et al.*, 1995; Lukas *et al.*, 1995; Medema *et al.*, 1995), yet antibodies specific for cyclin E can arrest cells irrespective of the presence of pRb (Ohtsubo *et al.*, 1995). In addition, collaboration between D-type cyclins and cyclin E is required for maximal hyperphosphorylation of pRb expressed in yeast (Hatakeyama *et al.*, 1994). The failure to detect differences in the patterns

of pRb phosphorylation by different Cdk2s may be due to the fact that phosphorylation has been performed in vivo, in the presence of numerous other kinases, or with relatively crude preparations of enzyme.

Our lack of understanding regarding the specific phosphorylation sites important for pRb regulation and the relative contributions of individual Cdk2s to this regulation results primarily from the fact that 12 or more phosphorylation events are observed in pRb isolated from asynchronous cells (Lees *et al.*, 1991). Only a subset of these events, however, may be required for pRb regulation in G₁. Once cells pass the point of pRb inactivation, other kinases, possibly including S-phase and G₂-M Cdk2s, may alter the phosphorylation status of pRb. Since events in the cell cycle are tightly coupled, it is difficult to distinguish the phosphorylation events causing changes in pRb function in G₁ from those that are a consequence of cell cycle progression.

We have exploited the advantages of a microinjection assay using synchronized cells and highly purified protein to examine the phosphorylation of pRb by G₁ Cdk2s in vitro and the functional consequences of this phosphorylation in vivo. The microinjection assay compares the cell cycle arrest activity of pRb preparations that differ only in their state of phosphorylation. A major advantage of this approach is that it directly measures how specific phosphorylation events alter pRb function in the absence of exogenous cyclins and Cdk2s. We have discovered that Cdk4 has kinetically preferred sites of phosphorylation on pRb that are different from those preferred by Cdk2 or Cdk3. These differences in phosphorylation have important functional consequences since in vitro phosphorylation by cyclin D1/Cdk4 inhibits pRb-mediated G₁ arrest upon injection, while phosphorylation by Cdk2 or Cdk3 does not, despite quantitative conversion to a hyperphosphorylated form. Through a biochemical analysis, we have identified a single residue in pRb (S⁷⁹⁵) that is efficiently phosphorylated by Cdk4, but not Cdk2 or Cdk3, in vitro. This residue is found to be phosphorylated in vivo. Mutation of S⁷⁹⁵ to alanine prevents inactivation of pRb by Cdk4. Our results highlight the importance of selective phosphorylation in pRb regulation and are consistent with a model wherein pRb integrates various growth control signals through its different phosphorylation states.

MATERIALS AND METHODS

Cell Culture, Microinjection, and Metabolic Labeling

SAOS-2 osteogenic sarcoma cells were cultured in DMEM containing 10% fetal bovine serum (FBS) at 37°C with 5% CO₂. Cells were synchronized in mitosis by a 12-h treatment with 0.04 µg/ml nocodazole (Sigma, St. Louis, MO). Mitotic cells were collected by shake off, replated, and injected 6–10 h later (Goodrich *et al.*, 1991). This time corresponds to early G₁. Early passage mouse embryonic fibroblasts from *Rb*^{−/−} mice (Lee *et al.*, 1992) were synchronized in

G₀ by incubation in McCoy's 5A medium containing 0.1% FBS for 4 days. Cells were microinjected 1 h prior to refeeding with medium containing 15% FBS. Microinjections were performed on cells growing in 35-mm dishes by using an Eppendorf micromanipulator/injector with femtotip capillary micropipettes as described (Goodrich *et al.*, 1991). Approximately 200,000 molecules of pRb at 0.5–1 mg/ml were injected per cell, on par with endogenous levels in pRB⁺ cells. Bromodeoxyuridine (BrdUrd; 10 μ M, Amersham, Arlington Heights, IL) was added 20–24 h after mitotic release and cells were fixed 6–10 h later. All injected samples contained 1 mg/ml biotinylated rabbit anti-goat antibodies (Vector Laboratories, Burlingame, CA) to identify injected cells. Cells were immunostained with a mouse monoclonal anti-BrdUrd antibody (Amersham) followed by fluorescein-conjugated anti-mouse antibodies (Vector) and Texas red-conjugated avidin (Vector). Specimens were examined by immunofluorescence microscopy using Texas red and fluorescein isothiocyanate filters.

The human T-cell line Molt-4 was grown in RPMI 1640 containing 10% FBS. For labeling, 7×10^7 cells were resuspended in 2 ml of phosphate-free RPMI 1640 containing dialyzed FBS and incubated for 30 min prior to addition of 10 mCi of [³²P]orthophosphate (New England Nuclear, Boston, MA). After 5 h, cells were harvested and RIPA lysates (0.5 ml) were made by using established procedures (Lees *et al.*, 1991). pRb was precipitated with 5 μ g of polyclonal anti-Rb antibodies (Santa Cruz Biotechnology, Santa Cruz, CA; 4°C, 2 h) prior to SDS-PAGE purification using a 12% gel. Rb protein was visualized by autoradiography.

Protein Purification

Rb proteins were expressed in *Escherichia coli*, purified to near homogeneity by established procedures (Hensey *et al.*, 1994), and dialyzed into kinase buffer [150 μ M ATP, 25 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.5, 2% glycerol, 10 mM MgCl₂, 10 mM KCl] prior to phosphorylation and injection. Soluble glutathione S-transferase (GST)-cyclin A/Cdk2, GST-cyclin E/Cdk2, GST-cyclin E/His₆-Cdk3, and cyclin D/GST-Cdk4 complexes were produced in insect cells (High5, Invitrogen, San Diego, CA) and purified on glutathione-Sepharose (GSH-Sepharose, Pharmacia, Uppsala, Sweden) using established procedures (Desai *et al.*, 1992; Matsushima *et al.*, 1992; Harper *et al.*, 1995). The sources of the baculovirus are as described (Harper *et al.*, 1995) except for the E7K cyclin D2 mutant that was kindly provided by C. Sherr (St. Jude Children's Research Hospital). To generate immobilized kinase for phosphorylation, one T-150 flask of infected insect cells was lysed by sonication at 4°C in 6 ml of NETN [20 mM Tris-HCl, pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40 (NP40), 5 mM NaF, 30 mM *p*-nitrophenyl phosphate, 1 μ g/ml leupeptin, 1 μ g/ml antipain, and 1 mM phenylmethylsulfonyl fluoride (PMSF)] for Cdk2 complexes or HBT [50 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid), pH 7.6, 150 mM NaCl, 1 mM EDTA, 2.5 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 0.1% Tween 20, 1 mM dithiothreitol, 5 mM NaF, 30 mM *p*-nitrophenyl phosphate, 25 mM β -glycerol phosphate, 1 μ g/ml leupeptin, 1 μ g/ml antipain, and 1 mM PMSF] for Cdk4 complexes. Lysates were cleared by centrifugation at $14,000 \times g$ for 10 min. Supernatants were rotated with 0.1 ml of GSH-Sepharose for 60 min at 4°C, and the beads were washed three times with 1 ml of the lysis buffer. Prior to use in kinase reactions, beads were washed three times with 0.3 ml of kinase buffer.

Plasmids for expression of GST-E2F-1 and GST-DP-1 were provided by E. Harlow and N. Dyson (Massachusetts General Hospital Cancer Center). E2F gel shift assays were performed essentially as described (Dynlacht *et al.*, 1994). Briefly, 50 ng of p110Rb were treated with 15 ng of the indicated kinase or in the absence of kinase as a control for 20 min at 37°C in the presence of 0.5 mM ATP (volume, 3.5 μ l). Reaction mixtures were then incubated with ~25 ng of GST-E2F and GST-DP-1 in binding buffer containing 100 mM KCl, 0.1% NP-40, and 0.2 mg/ml bovine serum albumin on ice for

15 min prior to addition of ³²P-labeled E2F oligonucleotide. Mixtures were electrophoresed on 4% polyacrylamide gels for 2 h at 4°C at 180 V.

pRb Phosphorylation

To examine the specificity of pRb phosphorylation (Figure 2), purified p110Rb (1.8–9.0 μ M) was phosphorylated with decreasing amounts of each Cdk complex to produce various extents of phosphorylation (low, medium, and high). Reactions were performed in a total volume of 25 μ l and contained 20 mM Tris-HCl (pH 7.5), 30 mM NaCl, 10 mM MgCl₂, 1 μ l of diluted kinase, pRb, and 40 μ M [γ -³²P]ATP for 40 min at 37°C. The relative amount of ³²P incorporated into 300 ng of each pRb sample was quantitated by SDS-PAGE and PhosphorImager analysis.

To generate phosphorylated protein for microinjection, 5 μ l of GSH-Sepharose beads containing the indicated kinase complex were incubated with 10 μ l of p110Rb or p56Rb in a total volume of 15 μ l (37°C for 30 min). The final concentrations of components in these reactions were 150 μ M ATP, 25 mM Tris-HCl (pH 7.5), 2% glycerol, 10 mM MgCl₂, 10 mM KCl, 1 μ M pRb, and 1–2 μ M kinase. To generate ³²P-labeled pRb for peptide mapping, 3 μ l of each kinase reaction were mixed with 1 μ l of [γ -³²P]ATP (3000 Ci/mmol) immediately after addition of pRb. After the reaction period, pRb was recovered from the GSH-Sepharose by using a micropipette and stored at 4°C for injection or boiled in 2 $\times$ SDS-PAGE buffer for mapping. Synthetic pRb-derived peptides (5 μ g) and p110Rb in Figure 7 were phosphorylated by soluble Cdk complexes (typically at 1 μ M) in 20 μ l of 20 mM Tris-HCl (pH 7.5), 15 mM MgCl₂, and 40 μ M ATP containing 1 μ l of [γ -³²P]ATP (3000 Ci/mmol; 45 min at 37°C).

Phosphopeptide Mapping and Phosphoamino Acid Analysis

In vitro-phosphorylated pRb was separated by SDS-PAGE in 12% gels, transferred to nitrocellulose, and trypsinized as described (Luo *et al.*, 1991). In vivo-labeled and immunoprecipitated pRb was trypsinized in the SDS gel slice after washing with 50% methanol, water, and 50 mM ammonium bicarbonate. Trypsinization was accomplished by using 20 μ g of trypsin in 0.4 ml of 50 mM ammonium bicarbonate. The resulting peptides from either gel slices or nitrocellulose filters were oxidized with performic acid and approximately 10,000 cpm were subjected to two-dimensional peptide mapping as described (Boyle *et al.*, 1991) using thin layer cellulose plates 20 $\times$ 20 cm (EM Science, Gibbstown, NJ). Electrophoresis was performed in pH 1.9 buffer [88% formic acid:acetic acid:H₂O, 50:156:1794 (vol:vol)] for 45 min at 1007 V using the Hunter Thin Layer Electrophoresis System (HTLE-7000, CBS Scientific, Del Mar, CA), followed by ascending chromatography in *n*-butanol:pyridine:acetic acid:H₂O, 75:50:15:60 (vol:vol) for 7 to 8 h. The separated phosphopeptides were visualized by autoradiography.

Phosphorylated synthetic peptides were separated by SDS-PAGE on 21% gels. Peptides were excised and processed for mapping using one of two methods which gave equivalent results: 1) Crushed gel slices were boiled for 30 min in 1 ml of 50 mM ammonium bicarbonate (pH 7.5), 0.1% SDS, and 5% 2-mercaptoethanol and agitated for 4 h at 25°C (Lees *et al.*, 1991) prior to reverse-phase high-pressure liquid chromatography on a C₄ column (Vydac). Column fractions were lyophilized, trypsinized, and prepared for mapping as described above. 2) The gel slices were incubated 1 h with 50% methanol, followed by 30 min with water, and two 5-min washes with 50 mM ammonium bicarbonate. The peptides were trypsinized with 20 μ g of trypsin overnight in 0.5 ml of 50 mM ammonium bicarbonate prior to mapping as above. Between 500 and 5000 cpm were used for mapping.

Phosphoamino acid analysis of individual peptides isolated from two-dimensional peptide maps was accomplished using standard procedures (Boyle *et al.*, 1991). Radiolabeled phosphoamino acids

were visualized by autoradiography. Internal standards (phosphoserine, phosphothreonine, and phosphotyrosine) were visualized with ninhydrin.

Partial trypsin cleavage of cyclin D1/GST-Cdk4 phosphorylated p56Rb to generate the 30-kDa and 19-kDa fragments encompassing the A- and B-pockets, respectively, was performed as described (Hensey *et al.*, 1994). Partial digestion products were separated by SDS-PAGE on 12% gels. Autoradiography of these gels revealed the presence of three radiolabeled proteins at 21, 23, and 25 kDa that correspond to intermediate cleavage products containing C-terminal extensions of the B-pocket. Only trace amounts of radioactivity were observed in the A-pocket fragment and labeling of the 19-kDa fragment was not detectable. The fragments were excised from the gel and subjected to peptide mapping, and the maps were compared with maps of the starting p56Rb protein. The map of the 25-kDa fragment was essentially indistinguishable from the map of the p56Rb protein.

Plasmid Constructions and In Vitro Translation

An *EcoRI-HindIII* fragment encoding residues 301–928 of Rb was cloned into M13 to facilitate oligonucleotide-directed mutagenesis by using the Sculptor system (Amersham). Mutant *EcoRI-HindIII* fragments (verified by DNA sequence analysis) were either subcloned into pETRbc (Huang *et al.*, 1991) to regenerate a full-length Rb cDNA for expression in *E. coli* or subcloned into pRSETc (Invitrogen). The pRSET-Rb plasmids produce an 80-kDa His₆-T7 gene 10 Rb fusion protein by in vitro translation (TNT, Promega, Madison, WI). We found that unlabeled in vitro translation products could be used for peptide mapping after immunoprecipitation using anti-gene 10 antibodies (Novagen, Madison, WI). Fifty microliters of Rb translation mixture were added to 200 μ l of 0.5% NP40, 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 5 mM NaF, 30 mM *p*-nitrophenyl phosphate, 1 μ g/ml leupeptin, 1 μ g/ml antipain, and 1 mM PMSF and incubated with 4 μ l of anti-gene 10 antibodies and anti-mouse IgG (Sigma) coupled to 10 μ l of protein A-Sepharose (Pharmacia; 1 h at 4°C). Rb protein contained in washed immunoprecipitates was phosphorylated with cyclin D1/GST-Cdk4 (~500 nM) and subjected to peptide mapping as described above.

Peptide Sequence Analysis

Phosphopeptides were isolated by two-dimensional peptide mapping and eluted from cellulose plates with pH 1.9 buffer. Peptides (2000–20,000 cpm) were subjected to automated Edman degradation, and the radioactivity released in each cycle was determined by liquid scintillation.

RESULTS

Differential Phosphorylation of pRb by Cdks In Vitro

Using purified preparations of full-length wild-type pRb (p110Rb), we examined whether particular sites might be differentially phosphorylated in vitro by purified cyclin/Cdk complexes (Figure 1). The p110Rb was treated with kinase complexes under conditions that yielded low, intermediate, and highly phosphorylated p110Rb, as measured by the amount of ³²P incorporated per unit mass of protein (Figure 2, A and B). Phosphorylated p110Rb preparations were purified by SDS-PAGE, transferred to nitrocellulose, and then subjected to two-dimensional tryptic phosphopeptide mapping (Figure 2C). For each kinase, a 50–100-fold range of p110Rb

Figure 1. Purified proteins used in this study. Recombinant proteins purified as described in MATERIALS AND METHODS were separated by SDS-PAGE on 12% gels and stained with Coomassie blue. Lane 1, cyclin D1/GST-Cdk4; lane 2, GST-cyclin E/Cdk3; lane 3, GST-cyclin E/Cdk2; lane 4, GST-cyclin A/Cdk2; lane 5, pRb; lane 6, p56Rb. The position of insect cell GST is indicated by the asterisk. The molecular masses of the different components are GST-Cdk4 (60 kDa), cyclin D1 (36 kDa), GST-cyclin E (70 kDa), His₆-Cdk3 (40 kDa), Cdk2 (32 kDa), and GST-cyclin A (90 kDa).

phosphorylation was achieved (Figure 2B), but similar amounts of radioactivity were used for mapping to compare the distribution of sites phosphorylated at each level.

At low and intermediate levels of phosphorylation, dramatic differences in kinase specificity were readily evident (Figure 2C). Cdk4 preferentially phosphorylated sites that gave rise to phosphopeptides Y, C, 13, 2, and 21, whereas Cdk2 kinases preferentially gave rise to phosphopeptides F, 22, 9, and 13 (Figure 2C, a, d, and g). At the highest levels of kinase used, differences in specificity were present but less pronounced. For example, phosphopeptide F was prominent in maps of p110Rb phosphorylated by Cdk2 but not by Cdk4. Likewise, phosphopeptides Y and C were weakly phosphorylated by Cdk2 but were phosphorylated efficiently by Cdk4. As expected, sites that were efficiently phosphorylated reached saturating levels of ³²P incorporation at the low or intermediate level reactions, whereas inefficiently phosphorylated sites accumulated significant ³²P in only the most highly phosphorylated p110Rb samples. Even in these

Figure 2. Differential phosphorylation of pRb by Cdk2 and Cdk4 in vitro. (A) Phosphorylation of pRb with low, intermediate, and high amounts of cyclin A/Cdk2, cyclin E/Cdk2, and cyclin D1/Cdk4 as described in MATERIALS AND METHODS. One-third of each reaction was separated by SDS-PAGE on 12% gels and the phosphorylated pRb was visualized by autoradiography. Exposure time was 5 min. (B) The relative cpm incorporated into each Rb sample were quantitated by subjecting the gel in A to PhosphorImager analysis. (C) Cdk2 and Cdk4 preferentially phosphorylate different sites. Equal cpm of each kinase reaction in A were subjected to two-dimensional tryptic phosphopeptide mapping (see MATERIALS AND METHODS). The cyclin/Cdk complex used in each reaction and the amount of ^{32}P incorporation (low, medium, or high) are indicated. The nomenclature used for phosphopeptides is based primarily on previous studies (Lees *et al.*, 1991). Electrophoresis proceeds from left to right and chromatography proceeds from bottom to top.

highly phosphorylated preparations, the level of ^{32}P incorporation at nonpreferred sites was lower than at the preferred sites. These data indicated that Cdk2 and Cdk4 kinases, although capable of phos-

phorylating many of the same sites, had dramatically different preferences for particular sites in p110Rb when examined under conditions of limiting kinase.

Figure 3. In vitro phosphorylation of pRb by cyclin D1/Cdk4 inactivates its growth suppression activity in vivo. (A and B) A representative set of phosphorylated pRb used for microinjection.

Phosphorylation of pRb by Cyclin D1/Cdk4 In Vitro Inhibits Its Growth Suppression Function In Vivo

To examine whether differential pRb phosphorylation is important for its regulation, we have devised an assay that allows the consequences of pRb phosphorylation, performed in vitro, to be assessed in vivo. The assay is based on the previous finding that microinjection of unphosphorylated pRb into SAOS-2 cells in early G₁ blocks S-phase entry (Goodrich *et al.*, 1991). These cells have undetectable levels of D-type cyclins and are highly sensitive to the effects of exogenous pRb. However, appropriate phosphorylation of pRb in vitro before injection may render the protein inactive for G₁ arrest. This assay has several advantages over standard cotransfection approaches. First, pRb with defined changes in phosphorylation status is introduced into cells without exogenous Cdk or cyclins. In principle, the effects of phosphorylation on pRb-mediated growth suppression are assayed without the complication of pRb-independent events caused by ectopic cyclin/Cdk expression. Second, since the phosphorylation status of injected pRb is known, the phosphorylation sites important for pRb regulation are readily defined. Finally, since pRb is injected into synchronized cells in early G₁, the assay monitors the activity of pRb at a time when it is normally undergoing inactivation. Any additional modification of the protein after injection is likely performed by cellular enzymes that are normally relevant to pRb regulation.

Full-length wild-type Rb protein was treated with the various immobilized kinases under conditions that led to similar levels of pRb phosphorylation (Figure 3A). Phosphorylated p110Rb was then recovered from kinase beads prior to microinjection and peptide mapping. In a typical experiment, more than 99% of cells injected with unphosphorylated p110Rb remained in G₁, indicated by lack of BrdUrd incorporation, whereas only 30% of the

Figure 3 (cont). Reactions with the indicated kinases were performed as described in MATERIALS AND METHODS prior to electrophoresis on a 7.5% SDS-polyacrylamide gel. Three different amounts of cyclin D1/Cdk4 used in the kinase reactions are indicated as high (H), medium (M), and low (L). (A) Proteins were visualized by Coomassie blue staining and autoradiography. Extent of phosphorylation was determined with a Betagen Scanner. (B) Growth arrest activity of pRb samples shown in A. SAOS-2 cells were injected with the indicated pRb protein and entry into the S-phase was determined by incorporation of BrdUrd as described in MATERIALS AND METHODS. The percentage of cells in G₁ was calculated using the formula: (percent uninjected in S – percent injected in S) divided by percent uninjected in S. The number of injected cells for each sample ranged from 100 to 150 cells. (C) A compilation of three to seven injection experiments for each different phosphorylated pRb sample is shown, except for p56Rb with cyclin E/Cdk3, which was a single experiment. (D) Phosphorylation of p110Rb by both cyclin D1/Cdk4 and cyclin E/Cdk2 in vitro abolishes association with E2F-1/DP-1 complexes in vitro. pRb phosphorylation and gel shift analysis were performed as described in MATERIALS AND METHODS.

cells injected with inert rabbit IgG were in G₁ 30 h after injection (Figure 3B). However, p110Rb phosphorylated by cyclin D1/Cdk4 was significantly impaired in its ability to block S-phase entry. The percentage of cells that remained in G₁ (29%) was similar to that observed with IgG, and the extent of p110Rb inactivation correlated with the extent of phosphorylation by Cdk4 (Figure 3, A and B). In contrast, an identical concentration of p110Rb phosphorylated by either cyclin A/Cdk2 or cyclin E/Cdk2 was still functional and arrested >98% of the cells, despite the fact that this protein was quantitatively shifted to slower migrating species on SDS-PAGE (Figure 3A).

The results of several experiments using p110Rb and p56Rb (Figure 1), an N-terminal truncated form of pRb capable of inducing cell cycle arrest upon injection (Goodrich *et al.*, 1991), are summarized in Figure 3C. Both p56Rb and p110Rb are inhibited by Cdk4 but not by Cdk2. Cdk3 phosphorylation, like Cdk2 phosphorylation, fails to significantly alter pRb function. Immunofluorescent detection of injected pRb indicates that phosphorylation does not detectably alter its nuclear localization or turnover (our unpublished observation). Although Rb-deficient primary fibroblasts were less susceptible to arrest by pRb injection, qualitatively similar results were obtained when pRb was injected into serum-starved cells and cell cycle entry was monitored upon serum addition. In one experiment using higher concentrations of pRb (~1.5 mg/ml), for example, 25% of the cells injected with cyclin D1/Cdk4-phosphorylated pRb entered the S-phase after serum addition versus 5% for cyclin A/Cdk2-phosphorylated pRb (our unpublished observation). In these experiments, 50% of the cells entered the S-phase with injection of IgG. These results indicated that the consequences of phosphorylation on pRb function were not necessarily cell-type specific.

Since *in vitro* phosphorylation by Cdk2 kinases has little effect on pRb function in our *in vivo* assay, we sought to determine whether this phosphorylation affects the ability of pRb to associate with E2F. Previous studies have shown that phosphorylation of p110Rb *in vitro* by Cdk2 kinases blocks its association with E2F-1/DP-1 complexes, as measured in a gel mobility shift assay (Dynlacht *et al.*, 1994). Consistent with this, we find that phosphorylation of pRb by both cyclin D1/Cdk4 and cyclin E/Cdk2 precludes association with E2F/DP-1 (Figure 3D). Thus, while phosphorylation of pRb by Cdk2 *in vitro* does not inactivate its growth suppression function in our microinjection assay, it is sufficient to block association with E2F.

pRb Inactivation Correlates with Phosphorylation of Particular Sites by Cdk4

Our observation that phosphorylation by Cdk4, but not by Cdk2 or Cdk3, inactivated pRb function might

Figure 4. Inactivation of pRb growth suppression correlates with phosphorylation of cyclin D1/Cdk4 specific sites. (a, b, d, f, and h) Tryptic peptide maps of p56Rb phosphorylated with the indicated Cdk complex. Equal quantities of cpm were used for mapping. (c) Equal quantities of tryptic peptides from p56Rb phosphorylated with cyclin A/Cdk2 and cyclin D1/Cdk4 were mixed prior to mapping. (e and g) Tryptic peptide maps of pRb phosphorylated by cyclin D1/Cdk4 and cyclin A/Cdk2. These samples correspond to those shown in Figure 1C. (h) The cyclin D2 mutant (CyD2 mut) used is E7K. All exposures were 8 h. Electrophoresis proceeds from left to right, and chromatography proceeds from bottom to top.

be explained by the different sites phosphorylated by Cdk4. To confirm that pRb used for injection was also differentially phosphorylated, samples prepared in parallel with those used for injection were analyzed by phosphopeptide mapping. This analysis revealed that the differential phosphorylation detailed in Figure 2C was maintained in p110Rb samples used for injection (Figure 4). Analogous differential phosphorylation was also observed with p56Rb (Figure 4), consistent with the functional data (Figure 3). In particular, phosphopeptides Y and C were prominent in maps of pRb phosphorylated by Cdk4 but were extremely weak in Cdk2/3-

phosphorylated pRb. The Cdk4 preferred sites were likely modified to near saturation since modification of nonpreferred sites was also detected. These data demonstrated that preparations of pRb, differing only in the particular residues phosphorylated, had dramatically different abilities to inhibit S-phase entry. Because of the possibility that phosphorylated pRb underwent additional modification after introduction into the cell, Cdk4 phosphorylation might not be sufficient to inactivate pRb, but modification of Cdk4-specific sites was required to initiate inactivation. Of importance, any modifications that occurred subsequent to injection were unable to convert Cdk2-phosphorylated pRb into an inactive species, despite the overlapping pattern of phosphorylation.

LXCXE Motif Is Not Required for Cyclin D/Cdk4-specific p56Rb Phosphorylation

Previous studies indicated that all three D-type cyclins can physically associate with pRb, but with differing affinities (Dowdy *et al.*, 1993; Ewen *et al.*, 1993; Kato *et al.*, 1993). This association requires an LXCXE motif in the N terminus of D-type cyclins. It has been proposed that these interactions target active cyclin D/Cdk4 complexes to critical phosphorylation sites in pRb (Weinberg, 1995).

In vitro phosphorylation with cyclin D2 or D3 in complex with Cdk4 generated phosphorylation patterns similar to that observed with cyclin D1/Cdk4; the Cdk4-specific phosphopeptides b, C, Y, and W were present irrespective of the particular cyclin D used (our unpublished observation). These data suggested that the identity of the D-type cyclin subunit had little influence on specificity in vitro. To test whether an intact LXCXE motif was required for the observed cyclin D/Cdk4 site-specific phosphorylation, pRb was phosphorylated with cyclin D2 E7K/Cdk4 in vitro. Cyclin D2 E7K (LXCXK), which does not associate efficiently with pRb (Dowdy *et al.*, 1993; Ewen *et al.*, 1993), had no discernible effect on the specific activity of the kinase complex or on the specificity of pRb phosphorylation (Figure 4, a and h). The results indicated that LXCXE-mediated binding of cyclin D to pRb was not required for phosphorylation of sites important for inactivation of pRb function.

Identification of Major Cdk4 Phosphorylation Sites in p56Rb

Although previous studies using pRb-derived peptides have identified several sites that are phosphorylated by cyclin B/Cdk2 in vitro (Lees *et al.*, 1991), the identity of sites phosphorylated by Cdk2, Cdk3, and Cdk4 are unknown. The finding that Cdk4 and Cdk2 demonstrate different specificity in pRb phosphorylation and have different effects on pRb function prompted us to identify the sites of modification by

these kinases. In particular, we sought to identify those residues whose phosphorylation correlates with pRb inactivation by Cdk4. The results of a series of mapping studies, focusing primarily on p56Rb, are summarized in Figure 5A.

Individual point mutations in four candidate phosphorylation sites in pRb (S780A, S788A, S795A, and S807A), as well as a quadruple mutant lacking all four sites, were generated in a form of pRb consisting of residues 301–928 (Figure 5B). These candidate sites were selected for mutagenesis based on the results of 1) partial proteolysis (Hensley *et al.*, 1994) of Cdk4-phosphorylated p56Rb, which revealed that the major sites of phosphorylation were located C-terminal to the B-pocket (see MATERIALS AND METHODS and our unpublished observation); 2) phosphoamino acid analysis, which showed that phosphopeptides W, Y, C, 2, and 21 contained phosphoserine, whereas 13 contained phosphothreonine (see MATERIALS AND METHODS); and 3) the finding that Cdk4 efficiently phosphorylated peptides containing these residues but inefficiently phosphorylated peptides containing S⁵⁶⁷, S⁸⁰⁷, S⁸¹¹, T⁸²¹, and T⁸²⁶ (Figure 6A). Mutations S780A and S807A resulted in the loss of two weakly phosphorylated peptides, b and 1, respectively (Figure 5B, c and f). In contrast, mutation S788A or S795A resulted in the loss of two prominent phosphopeptides: 2 and 21 for S788A and Y and C for S795A (Figure 5B, d and e). In an pRb mutant lacking all four of these sites, phosphopeptide 13 was the only prominent peptide identified upon long exposure of the map (Figure 5B, b).

The loss of multiple phosphopeptides upon mutation of a single amino acid residue could reflect differential proteolysis during the mapping procedure or an ordered reaction pathway in which phosphorylation at a particular site depends on prior phosphorylation at a second site. Ultimately, we were able to demonstrate that the multiple phosphopeptides lost upon mutation of S⁷⁸⁸ or S⁷⁹⁵ represented differential proteolysis. In particular, tryptic mapping of several Cdk4-phosphorylated peptides overlapping S⁷⁸⁸ and S⁷⁹⁵ allowed us to recreate phosphopeptides 2, 21, Y, and C. Three pRb-derived peptides (Rb776–794, Rb766–794, and Rb782–801), all of which contained S⁷⁸⁸, yielded major phosphopeptides that comigrated with spots 2 and 21 when mixed with p56Rb (Figure 6B and our unpublished observation). Similarly, the candidate tryptic peptide containing S⁷⁹⁵ (Rb792–798; FPSSPLR) gave a phosphopeptide that comigrated with spot Y after phosphorylation with cyclin D1/Cdk4, whereas the overlapping peptide derived from a predicted chymotrypsin-like cleavage event (Rb791–798; KFPSSPLR) produced peptide C (Figure 6B). Consistent with these data, Edman degradation of phosphopeptide C showed peak radioactivity at cycle 5 (Figure 5C) whereas phosphopeptide Y released peak

Figure 5. Cyclin D1/Cdk4 preferentially phosphorylates S^{788} and S^{795} in pRb. (A) Identification of major sites of pRb phosphorylation catalyzed by Cdk4 and Cdk2 in vitro. The position of the A- and B-pockets of pRb relative to 16 potential Cdk phosphorylation sites (S or T followed by P) are shown. The C-terminal region of Rb has been enlarged to highlight seven putative Cdk phosphorylation sites. Phosphopeptides produced by phosphorylation at particular sites are indicated. The italic R (Arg and K (Lys) represent predicted trypsin cleavage sites, and the arrowhead located between S^{788} and S^{795} indicates the site of differential cleavage. The size of the asterisks below or above the phosphorylation sites indicates the relative preference of the kinase for that site. Phosphorylation of T^{252}/T^{356} and T^{373} by Cdk4 and Cdk2 have been provisionally assigned based on the characteristic pattern of differential proteolysis products containing each of these residues (Lees *et al.*, 1991). (B) Two-dimensional tryptic phosphopeptide mapping of wild-type and mutant pRb phosphorylated by cyclin D1/Cdk4. Mutant pRbs contained alanine substitutions in candidate phosphorylation sites. In vitro-translated pRb samples were phosphorylated in vitro prior to mapping. a, wild-type pRb; b, quadruple mutant pRb containing S780A:S788A:S795A:S807A; c, S780A pRb; d, S788A pRb; e, S795A pRb; f,

radioactivity at cycle 4 (our unpublished observation). In addition, phosphopeptide Y was absent in the tryptic map of Rb792–810 in which S^{795} was replaced by alanine (Figure 6B). Although S^{807} is weakly phosphorylated in this peptide, the peptide map generated from a larger quantity of phosphorylated Rb792–810 revealed a phosphopeptide that comigrated with spot 1, consistent with the mutagenesis results (Figures 5B and 6B).

These data indicated that S^{788} and S^{795} were the preferred sites of p56Rb modification by cyclin D1/Cdk4 in vitro; S^{795} was inefficiently modified by Cdk2 and Cdk3 (Figure 5). Although we did not analyze N-terminal phosphorylation sites in detail, previous studies have shown that T^{373} and T^{252} potentially gave rise to two sets of characteristic phosphopeptides derived by differential proteolysis (9, for T^{252} or 14 for a peptide also containing phosphoserine S^{249} ; 6 and 12 for T^{373} ; Lees *et al.*, 1991). Phosphorylation of T^{356} also can produce a peptide identical to that produced by phosphorylation of T^{252} (spot 9), making identification of this spot ambiguous. Rb protein phosphorylated extensively by both Cdk4 and Cdk2 contained similar levels of phosphopeptides 6, 9, and 12, consistent with phosphorylation of T^{373} , T^{252}/T^{356} by both kinases (Figure 2C, c, f, and i; Figure 5, e and g). Similarly, p110Rb phosphorylated extensively by Cdk2 contained detectable quantities of phosphopeptides 14 and B, consistent with weak phosphorylation of T^{249} (Figure 4g). In agreement with previous studies (Lees *et al.*, 1991), we found that the prominent phosphopeptide F, generated by phosphorylation with Cdk2, was lost when T^{821} was replaced by alanine (Leng, Harper, and Connell-Crowley, unpublished results). The identity of the phosphorylation site in phosphopeptide W has not been determined because it was not reproducibly lost upon mutation of S^{780} , S^{788} , S^{795} , or S^{807} , and because it was not regenerated with our pRb-derived synthetic peptides.

Phosphorylation of S^{795} Is Required to Inactivate the pRb Cell Cycle Arrest Function

To date, no single phosphorylation event has been shown to be required for inactivation of pRb cell cycle arrest function. Since pRb inactivation correlated with Cdk4 phosphorylation, our results suggested that inactivation required phosphorylation of S^{795} since this

(Figure 5 cont). S807A pRb. Exposure time was 16–24 h. The arrows indicate missing spots. Electrophoresis proceeds from left to right, and chromatography proceeds from bottom to top. (C) Phosphopeptide C is phosphorylated at residue 5. Phosphopeptide C, obtained by phosphorylation of p56Rb with cyclin D1/Cdk4, was purified by two-dimensional tryptic phosphopeptide mapping and sequenced by automated Edman degradation. The cpm released at each cycle are shown.

Figure 6. Selective phosphorylation of synthetic pRb-derived peptides by cyclin D1/Cdk4. (A) Peptides were phosphorylated by either Cdk2 or Cdk4 complexes as described in MATERIALS AND METHODS, resolved by SDS-PAGE on 21% gels, and visualized by autoradiography after a 10-min exposure. (B) The indicated peptides were phosphorylated with cyclin D1/Cdk4 prior to purification and mapping as described in MATERIALS AND METHODS. Peptides in a–d and i were digested with trypsin before mapping. Electrophoresis proceeds from left to right, and chromatography proceeds from bottom to top. Exposure time was 16–48 h. To examine comigration of synthetic peptides with authentic pRb, indicated peptides were mixed with phosphorylated and trypsin digested p56Rb. Rb792–810:S795A is weakly phosphorylated by Cdk4. To visual the products of phosphorylation (i), larger quantities of peptide (50 μ g) were phosphorylated prior to mapping. The arrow indicates the position of phosphopeptide 1, which can be seen on very long exposure of these maps.

was the only residue selectively phosphorylated by Cdk4. We reasoned that if phosphorylation of S⁷⁹⁵ was required for pRb inactivation, then mutation of this residue to alanine would render pRb resistant to inactivation by cyclin D1/Cdk4. The S795A mutant p110Rb was purified to near homogeneity from *E. coli* and was found to block S-phase entry after microinjection in early G₁ (Figure 7, A and B). S795A p110Rb was phosphorylated by cyclin D1/Cdk4 in vitro, although it was a less efficient substrate than wild-type pRb. With higher concentrations of cyclin D1/Cdk4, S795A p110Rb was readily phosphorylated, and it exhibited a greater degree of electrophoretic mobility shift compared with wild-type p110Rb at similar levels of ³²P incorporation (Figure 7A). Tryptic phosphopeptide mapping revealed that phosphopeptides Y and C were absent in the S795A mutant, as expected (Figure 7, C and D). However, other major phosphopeptides including 2, 21, and 13, as well as minor phosphopeptides such as B, b, W, X, 3, 6, 9, 12, and 24, were present at levels comparable to or higher than those found in wild-type pRb phosphorylated under the same conditions. Nevertheless, more than 95% of

the cells injected with Cdk4-phosphorylated S795A p110Rb remained in G₁, indicating that S795A pRb was resistant to inactivation by cyclin D1/Cdk4 phosphorylation (Figure 7B). In contrast, 28% of the cells injected with cyclin D1/Cdk4-phosphorylated wild-type pRb, prepared in parallel, remained in G₁. These results indicated that phosphorylation of S⁷⁹⁵, a major cyclin D1/Cdk4 phosphorylation site, was required for inactivation of pRb cell cycle arrest function.

Phosphorylation of S⁷⁹⁵ In Vivo

The finding that phosphorylation of S⁷⁹⁵ was required for inactivation of pRb by Cdk4 predicts that this site will be phosphorylated in vivo. To examine this question, ³²P-labeled pRb was immunoprecipitated from asynchronous human T-cells, separated by SDS-PAGE, and transferred to nitrocellulose. Since only a small fraction of the total pRb (<10%) was transferred in this experiment, Rb protein remaining in the gel was subjected to in-gel proteolysis (see MATERIALS AND METHODS) and peptide mapped in the presence and absence of an equal number of cpm of pRb

Figure 7. pRb S795A is resistant to inactivation by cyclin D1/Cdk4 phosphorylation in vitro. (A) pRb S795A is phosphorylated by cyclin D1/Cdk4 in vitro. SDS-PAGE analysis of wild-type and S795A pRb before and after treatment with cyclin D1/Cdk4. Phosphorylation reactions were performed using soluble kinase as described in MATERIALS AND METHODS. The extent of phosphorylation was determined by autoradiography. The specific activities of these samples were within a factor of 2 based on quantitation on a Betagen Scanner. (B) Phosphorylation by cyclin D1/Cdk4 does not abrogate S795A pRb-induced cell cycle arrest. pRb and S795A pRb with or without treatment with cyclin D1/Cdk4 were assayed for growth suppression activity in SAOS-2 cells as described in MATERIALS AND METHODS. A compilation of at least three experiments, with samples derived from at least two independent kinase reactions, is shown (see Figure 3B for details). (C and D) S795A pRb extensively phosphorylated by cyclin D1/Cdk4 lacks phosphopeptides Y and C. pRb proteins shown in A were analyzed by two-dimensional peptide mapping. The positions of the absent peptides Y and C are indicated by arrows. Mutation of S⁷⁹⁵ leads to more extensive phosphorylation of peptides 6, 9, and 12, compared with wild-type pRb. Exposure time was 15 h. Electrophoresis proceeds from left to right, and chromatography proceeds from bottom to top.

phosphorylated by Cdk4 in vitro (Figure 8). The pattern of phosphopeptides in the in vivo-labeled pRb sample was indistinguishable from that of its mixture with p110Rb phosphorylated with cyclin D1/Cdk4 in vitro; major phosphopeptides in Cdk4-phosphorylated pRb, including Y plus C and 2 plus 21, were found to comigrate with phosphopeptides derived from in vivo-labeled pRb (Figure 8, a-c). In addition, phosphopeptides corresponding to T³⁷³ and S⁷⁸⁰ (Fig-

Figure 8. Major pRb phosphopeptides phosphorylated by Cdk4 in vitro comigrate with pRb phosphopeptides found in vivo. Molt-4 cells were labeled with [³²P]orthophosphate and pRb was immunoprecipitated prior to separation by SDS-PAGE and transfer to nitrocellulose. SDS gel slices retaining ~90% of the total pRb were subjected to trypsinization, and resultant peptides were mapped alone or in combination with pRb phosphorylated by Cdk4 in vitro and transferred to nitrocellulose (see MATERIALS AND METHODS). Peptides were separated by electrophoresis (left to right) and chromatography (bottom to top). (A) In vivo-labeled pRb (~10,000 cpm), exposure 48 h. (B) In vivo-labeled pRb (~10,000 cpm) mixed with p110Rb (~10,000 cpm) phosphorylated with cyclin D1/Cdk4 in vitro, exposure 20 h. (C) p110Rb (~10,000 cpm) phosphorylated with cyclin D1/Cdk4 in vitro, exposure 8 h. (D) Mixture of p110Rb phosphorylated individually with cyclin E/Cdk2 and cyclin D1/Cdk4 (~10,000 cpm each), exposure 8 h. (E) Small quantities (~2000 cpm) of pRb transferred to nitrocellulose were mixed with an equal quantity of pRb phosphorylated by Cdk4 prior to mapping. The intensely labeled phosphopeptide located between spots 21 and F in the samples derived from in-gel mapping procedures are not present in this map (E).

ure 5A) were also present. However, several phosphopeptides found in vivo-labeled pRb, including F, 22, and 1, were present at low levels in pRb phosphorylated by Cdk4 in vitro. Peptides F and 22 appeared to correspond to sites phosphorylated by Cdk2 in vitro (Figure 8d). Additionally, an intensely labeled peptide, located between peptides 21 and F, was found in the in vivo pRb peptide map but not in the maps of in vitro-phosphorylated pRb. This phosphopeptide was a result of incomplete proteolysis during the in-gel

mapping procedure, since it is absent from maps of in vivo-labeled pRb that was transferred to nitrocellulose prior to mapping (Figure 8e). These data were consistent with the idea that the sites we have identified as being phosphorylated by Cdks in vitro, particularly S⁷⁹⁵, are utilized by these kinases in vivo.

DISCUSSION

pRb Function Is Regulated by Phosphorylation

Using a microinjection-based cell cycle arrest assay, we found that preparations of pRb differing only in their pattern of phosphorylation had dramatically different abilities to constrain cell cycle progression. In vitro, pRb was efficiently phosphorylated by both cyclin E/Cdk2 and cyclin D1/Cdk4 complexes, although their preferred sites of modification differed. The pRb phosphorylated in vitro by Cdk4 was inactive as a growth suppressor upon microinjection, but pRb phosphorylated by Cdk2 or Cdk3 retained cell cycle arrest activity. While these kinases efficiently phosphorylate a common set of residues, including S³⁷³ and S⁷⁸⁸, cyclin D1/Cdk4 was a much more efficient kinase for S⁷⁹⁵ than the other kinases examined. Consistent with a critical role for S⁷⁹⁵ phosphorylation in pRb inactivation, mutation of S⁷⁹⁵ to alanine rendered pRb resistant to inactivation by cyclin D1/Cdk4 phosphorylation in vitro, despite extensive phosphorylation at other sites. The S⁷⁹⁵ residue was also efficiently phosphorylated in vivo.

Our data provide direct confirmation, in living cells, of the hypothesis that the ability of pRb to constrain the cell cycle is inhibited by phosphorylation. Furthermore, our data suggest that phosphorylation of particular sites in pRb by specific Cdks, as opposed to extensive phosphorylation at a large number of sites, is rate limiting for pRb inactivation. These findings provide a framework for beginning to understanding how selective phosphorylation affects interactions of pRb with its cellular targets.

Distinct Roles for G₁ Cdks in pRb Regulation

Progression through G₁ is thought to require the activities of both cyclin D/Cdk4 and cyclin E/Cdk2 complexes. Although overexpression of both D- and E-type cyclins can override Rb-mediated growth arrest in a cotransfection cell cycle arrest assay (Hinds *et al.*, 1992; Dowdy *et al.*, 1993; Ewen *et al.*, 1993), recent data indicate that cyclin E/Cdk2 may catalyze events that are rate limiting for S-phase entry that are independent of pRb phosphorylation. Cyclin E/Cdk2 function is required in both Rb-positive and -negative cells (Ohtsubo *et al.*, 1995). A role for cyclin E in *Drosophila* DNA replication distinct from pRb regulation is also suggested by the inability of ectopic dE2F/dDP expression to initiate the S-phase in the absence

of cyclin E, despite normal induction of dE2F/dDP target genes (Duronio *et al.*, 1996). Moreover, cyclin E overexpression can by-pass a requirement for E2F in S-phase entry (Duronio and O'Farrell, 1995). In contrast, cyclin D/Cdk4 activity is required only in cells containing functional Rb (Guan *et al.*, 1994; Koh *et al.*, 1995; Lukas *et al.*, 1995; Medema *et al.*, 1995), and there is currently little evidence that Cdk4 has essential substrates other than pRb-related proteins.

Our results are consistent with the idea that phosphorylation of pRb by cyclin D/Cdk4 is a rate-limiting event for S-phase entry, but the role of cyclin E/Cdk2 in pRb regulation remains unclear. Although pRb phosphorylated extensively by cyclin E/Cdk2 in vitro can still arrest cells in G₁ upon microinjection, it seems likely that phosphorylation by this kinase plays some role in pRb regulation. First, cyclin E/Cdk2 efficiently phosphorylates sites that are not modified efficiently by cyclin D/Cdk4 (e.g., T⁸²¹). These sites are also efficiently phosphorylated in vivo, intimating that they may contribute to pRb regulation. Second, the observation that *Drosophila* cyclin E genetically interacts with a *Drosophila* Rb homologue and *Drosophila* E2F/DP1 indicates some role for cyclin E in pRb regulation (Duronio and O'Farrell, 1995; Du *et al.*, 1996). Third, phosphorylation of Rb by cyclin E/Cdk2 is sufficient to inhibit in vitro complex formation with E2F1/DP1 (Figure 3D; Dynlacht *et al.*, 1994). Inhibition of complex formation is apparently not sufficient to relieve pRb-mediated cell cycle arrest, but it may be required. Although the lack of correlation between the ability of pRb to bind E2F1/DP1 and to arrest the cell cycle is surprising, it is not unprecedented. Rb transfection into C33A cells is sufficient to inhibit E2F-mediated gene transcription but is not sufficient to arrest cell growth (Zhu *et al.*, 1993).

There are several possible reasons why cyclin E/Cdk2 phosphorylation in vitro has no apparent effect on pRb function, as assayed by microinjection, but can overcome Rb in cotransfection experiments. First, the specificity and/or efficiency of pRb phosphorylation in vitro may not adequately recapitulate pRb phosphorylation upon cyclin E overexpression in vivo. Our experiments utilize purified components and it is conceivable that formation of complexes in the cell alters the site preference of cyclin E/Cdk2 in a manner that allows for pRb inactivation. However, if additional subunits are required in vivo for Cdk2 to inactivate pRb, these factors are not required by cyclin D/Cdk4. Second, as with transfection approaches, the exact fate of pRb introduced into the cell is unknown. Although unlikely, we have not ruled out the possibility that the Cdk2 phosphorylated pRb is subject to dephosphorylation upon injection while Cdk4 phosphorylated pRb is not. Currently, the identities and specificities of Rb phosphatases are not well understood, but it is known that Rb phosphatase activity is

highest during the G2/M transition (Ludlow *et al.*, 1993). Regardless of potential dephosphorylation after microinjection, the finding that S⁷⁹⁵ is not efficiently phosphorylated by Cdk2 in vitro is sufficient to explain the observation that pRb is not inactivated by cyclin E/Cdk2; phosphorylation of this residue is required for pRb inactivation in our assay. Third, ectopic expression of cyclin E may facilitate cell cycle progression through mechanisms that are independent of Rb/E2F, as appears to be the case in *Drosophila* (Duronio and O'Farrell, 1995).

A major question raised by our results concerns the issue of whether phosphorylation of pRb by cyclin D1/Cdk4 is sufficient to inactivate its growth suppression function. It is conceivable that phosphorylation of residues inefficiently modified by Cdk4 are also required for pRb inactivation. Based on phosphopeptide mapping, several residues in pRb that are phosphorylated in vivo are not efficiently phosphorylated by cyclin D1/Cdk4 in vitro. Some of these residues, including T⁸²¹, are phosphorylated efficiently by Cdk2 or Cdk3 in vitro. If true, then phosphorylation by endogenous kinases, possibly Cdk2 or Cdk3, subsequent to microinjection may be required to fully inactivate pRb phosphorylated in vitro by Cdk4. Analyzing the requirement for phosphorylation at Cdk2 preferred sites in pRb inactivation may help to resolve this issue.

A potential advantage of the microinjection-based cell cycle assay over more commonly used cotransfection based assays is that cyclins are not introduced into the cell, thereby avoiding complications that result from pRb independent effects of inappropriate Cdk activation. Furthermore, transfection studies use asynchronously growing cells; therefore, it is not clear whether the pRb phosphorylation observed upon ectopic expression of G₁ cyclins is due to G₁ Cdk activity. Transfection studies with cyclin E have demonstrated an accumulation of S-phase cells in the presence or absence of pRb (Zhu *et al.*, 1993 and our unpublished observation), suggesting that cyclin E expression may advance cells into the S-phase regardless of the phosphorylation status of pRb. Comparative studies using both microinjection and transfection assays in combination with potential dominant pRb mutations may help to resolve the issue of whether pRb phosphorylation by ectopically expressed cyclins is the primary event allowing cell cycle entry or whether pRb-independent events are involved.

Slower-migrating Phosphorylated Forms of pRb Are Not Necessarily Inactive

Highly phosphorylated forms of pRb are typically recognized by their slower mobility on SDS-PAGE. These slower migrating forms of pRb are generally assumed to be inactive based on their appearance in

proliferating cells (Buchkovich *et al.*, 1989; Chen *et al.*, 1989; DeCaprio *et al.*, 1989; Mihara *et al.*, 1989). In addition, pRb-associated proteins bind poorly, if at all, to these slower migrating forms. Our data, however, indicate that the mobility shift in pRb observed by SDS-PAGE does not necessarily correlate with its functional status. In particular, mutation of S⁷⁹⁵ to alanine produces an Rb protein that undergoes a mobility shift upon phosphorylation by Cdk4 in vitro, yet this protein is fully growth inhibitory upon injection. In contrast, wild-type Rb protein phosphorylated to a similar extent by Cdk4 does not appreciably alter its mobility, yet it is essentially inactive as a growth suppressor. Although the appearance of slower migrating forms of pRb do not necessarily correlate with inactivation of protein function, the phosphorylation events critical for initiating pRb inactivation and the events causing electrophoretic mobility shift may be tightly coupled in the cell.

We demonstrate in this study that pRb is phosphorylated differently by two classes of G₁ Cdk, in vitro, with differing functional consequences in vivo. This suggests the possibility that pRb can exist in different functional states defined by the particular subset of sites phosphorylated. Since pRb serves as a substrate for distinct kinases, it could potentially integrate phosphorylation signals derived from different signaling pathways. For example, D-type cyclins are uniquely responsive to the presence of mitogenic signals (Matsushime *et al.*, 1994; Meyerson and Harlow, 1994); addition of growth factors to quiescent cells facilitates assembly of active cyclin D/Cdk complexes (Kato *et al.*, 1994). The ability to integrate various intracellular and extracellular signals is a property required by molecules with a central role in the regulation of the restriction point.

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