Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4

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The product (pRb) of the retinoblastoma gene (*RB-1*) prevents S-phase entry during the cell cycle, and inactivation of this growth-suppressive function is presumed to result from pRb hyperphosphorylation during late G_1 phase. Complexes of the cyclin-dependent kinase, cdk4, and each of three different D-type cyclins, assembled in insect Sf9 cells, phosphorylated a pRb fusion protein in vitro at sites identical to those phosphorylated in human T cells. Only D-type cyclins activated cdk4 enzyme activity, whereas cyclins A, B1, and E did not. When Sf9 cells were coinfected with baculovirus vectors encoding human pRb and murine D-type cyclins, cyclins D2 and D3, but not D1, bound pRb with high stoichiometry in intact cells. Introduction of a vector encoding cdk4, together with those expressing pRb and D-type cyclins, induced pRb hyperphosphorylation and dissociation of cyclins D2 and D3, whereas expression of a kinase-defective cdk4 mutant in lieu of the wild-type catalytic subunit yielded ternary complexes. The transcription factor E2F-1 also bound to pRb in insect cells, and coexpression of cyclin D–cdk4 complexes, but neither subunit alone, triggered pRb phosphorylation and prevented its interaction with E2F-1. The D-type cyclins may play dual roles as cdk4 regulatory subunits and as adaptor proteins that physically target active enzyme complexes to particular substrates.

[*Key Words*: G_1 cyclins; D-type cyclins; cyclin-dependent kinases (cdks); retinoblastoma gene product (pRb)]

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The functional inactivation of the retinoblastoma gene (*RB-1*) in many human cancers suggests that it operates ubiquitously as a tumor suppressor (Weinberg 1991). DNA tumor virus oncoproteins, including SV40 T antigen (T), adenovirus E1A, and human papillomavirus E7, abrogate retinoblastoma gene product (pRb)-induced growth suppression by binding selectively to its unphosphorylated and underphosphorylated forms, which are manifested during the G_1 interval of the cell cycle (DeCaprio et al. 1988, 1989; Whyte et al. 1988, 1989; Buchkovich et al. 1989; Chen et al. 1989; Dyson et al. 1989; Mihara et al. 1989; Ludlow et al. 1990, 1992). Hyperphosphorylation of pRb in late G_1 is presumed to be required for cells to enter S-phase, and such forms accumulate and persist until the cells exit mitosis. Although pRb localizes to the nucleus throughout interphase, hyperphosphorylated forms of the protein can be extracted more readily, suggesting that they are less tightly tethered to other nuclear proteins (Mittnacht and Weinberg 1991).

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Some of the pRb protein expressed during G_1 is found in association with a known transcription factor, E2F (Bagchi et al. 1991; Bandara and La Thangue 1991; Chellappan et al. 1991; Chittenden et al. 1991). The complex can bind to DNA and appears to actively repress transcription of certain genes containing E2F-binding sites (Hamel et al. 1992b; Hiebert et al. 1992; Weintraub et al. 1992). Like T antigen and E1A, E2F selectively associates with hypophosphorylated forms of pRb (Chellappan et al. 1991; Helin et al. 1992; Kaelin et al. 1992), implying that pRb hyperphosphorylation can prevent E2F–pRb interactions, thereby releasing E2F from an inhibitory constraint and enabling it to promote transcription from a subset of cellular genes, at least some of which are expressed during S-phase (Blake and Azizkhan 1989; Hiebert et al. 1989, 1991; Mudryj et al. 1990).

Mapped sites of pRb phosphorylation in vivo correspond to those recognized by cyclin-dependent kinases (cdks) (Lees et al. 1991; Lin et al. 1991), so that cdks associated with one or more of the mammalian G_1 cyclins, including cyclins C, D1, D2, D3, and E (Koff et al. 1991; Lew et al. 1991; Matsushime et al. 1991b; Motokura et al. 1991; Xiong et al. 1991), are the most likely

to represent the physiologic pRb kinase(s). In *RB*-negative human Saos-2 osteosarcoma cells, cotransfection of *RB-1* expression plasmids, together with genes encoding cyclins A, E, D2, or D3 (but not B, D1, or C), induces pRb hyperphosphorylation, prevents its interaction with E2F, and allows the cells to enter S-phase (Hinds et al. 1992; M.E. Ewen, H.K. Sluss, C.J. Sherr, D.M. Livingston, and H. Matsushime, in prep.). Because cyclin A is degraded near the G_2/M transition and is not re-expressed until cells enter S-phase (Minshull et al. 1990; Pines and Hunter 1990, 1991), it is unlikely that cyclin A-associated kinase(s) would normally function to hyperphosphorylate pRb during the G_1 interval. Cyclin A-containing immunoprecipitates contain a pRb kinase (Hu et al. 1992), but cyclin A does not bind to pRb (Ewen et al. 1992; Faha et al. 1992). The D- and E-type cyclins are more reasonable candidates for regulating this function, being expressed during the interval when pRb phosphorylation first occurs (Koff et al. 1991, 1992; Lew et al. 1991; Matsushime et al. 1991a,b; Dulic et al. 1992).

Unlike cyclins A and E, an unusual feature of cyclins D2 and D3 is their ability to bind directly to pRb in vitro (Matsushime et al. 1992; M.E. Ewen, H.K. Sluss, C.J. Sherr, D.M. Livingston, and H. Matsushime, in prep.). Cyclin D1, which binds less well than cyclins D2 or D3 to pRb in vitro (M.E. Ewen, H.K. Sluss, C.J. Sherr, D.M. Livingston, and H. Matsushime, in prep.), does not trigger pRb hyperphosphorylation in Saos-2 cells but paradoxically induces some S-phase entry (Hinds et al. 1992). The associations between cyclins D2 or D3 and pRb require pRb subdomains that include both the functional T/E1A/E7-binding pocket and additional carboxy-terminal sequences, as well as an amino-terminal Leu-*X*-Cys-*X*-Glu motif found both in the D type cyclins and pRb-binding oncoproteins. Importantly, the same regions of pRb are required for its interaction with E2F (Hiebert et al. 1992; Qian et al. 1992) and for G_1-specific growth-suppressive activity in vivo (Goodrich et al. 1991; Qin et al. 1992; M.E. Ewen, H.K. Sluss, C.J. Sherr, D.M. Livingston, and H. Matsushime, in prep.). However, attempts to identify complexes between pRb and the D-type cyclins in intact cells have been unsuccessful so far. Here, we show that stable complexes between pRb and cyclin D2 or D3, but not D1, formed in insect *Spodoptera frugiperda* (Sf9) cells, are disrupted after pRb hyperphosphorylation by cdk4, a recently identified cyclin D-dependent kinase (Matsushime et al. 1992). The physical interaction of D-type cyclins with pRb may therefore help to direct cdks to this substrate, resulting in pRb phosphorylation at multiple sites and the subsequent destabilization of the multimeric complexes.

Results

Cyclins D1, D2, and D3 activate cdk4 protein kinase activity in vitro

Lysates of insect Sf9 cells coinfected with baculovirus vectors encoding D-type cyclins and cdk4, but not those containing the regulatory or catalytic subunits alone, exhibited a protein kinase activity that phosphorylated a glutathione *S*-transferase (GST)–pRb fusion protein in vitro (Fig. 1A). Complexes of cdk4 with each of the three D-type cyclins were equally active (lanes 4,6,8), and proteolytic removal of the GST fragment confirmed that only the pRb moiety underwent phosphorylation (85% P-Ser, 15% P-Thr) (Matsushime et al. 1992). Removal of D-type cyclins, together with bound cdk4 catalytic subunits from the insect cell lysates by immunoprecipitation with specific antisera to cyclin D1 or D3, reduced GST–pRb kinase activity to background levels (Fig. 1B). An antiserum directed to cdk4 itself was equally effective. Therefore, cdk4/cyclin D complexes did not activate an endogenous pRb kinase in Sf9 cells but phosphorylated pRb directly.

More cyclins than cdk4 were produced in Sf9 cells coinfected with both classes of vectors (see Fig. 4 below). When such cells were metabolically labeled and the lysates were precipitated with antiserum to cdk4, equivalent quantities of D-type cyclins and cdk4 were recovered, implying that their molar ratio in the complexes was 1 : 1. In the reactions shown in Figure 1A, the ratio of the active enzyme complex to pRb substrate was 1 : 5 to 1 : 10, so that net pRb phosphorylation occurred within the first 5 min after the addition of ATP. When enzyme concentrations were lowered 200- to 600-fold, measurement of the relative kinetics of pRb phosphorylation indicated that complexes containing cdk4 and either of the D-type cyclins phosphorylated GST–pRb in vitro at indistinguishable rates. The cyclins themselves were not phosphorylated by cdk4 in these reactions.

Cyclin D/cdk4 complexes phosphorylate pRb at physiologically relevant sites

The GST–pRb substrate used in the above experiments contained pRb residues 379–928, which are sufficient for its growth-suppressive activity in vivo (Goodrich et al. 1991; Qin et al. 1992). Carboxy-terminal deletion of residues 793–928 removes sites of potential cdk phosphorylation encoded by *RB-1* exon 23 and results in a protein that is refractory to hyperphosphorylation (Hamel et al. 1992a,b), unable to bind E2F (Hiebert et al. 1992; Qian et al. 1992) or D-type cyclins (Matsushime et al. 1992; M.E. Ewen, H.K. Sluss, C.J. Sherr, D.M. Livingston, and H. Matsushime, in prep.), and nonfunctional in vivo (Goodrich et al. 1991; Qin et al. 1992), but that retains the ability to bind T antigen and E1A (Hu et al. 1990; Huang et al. 1990; Kaelin et al. 1990). As shown in Figure 1C (lanes 2–4), a GST–pRb fusion protein lacking these carboxy-terminal residues was also very poorly phosphorylated by cdk4/cyclin D complexes in vitro. Serines 807 and 811 within the carboxy-terminal domain are known sites of pRb phosphorylation in human cells (Lees et al. 1991). Point mutations of homologous sites in murine pRb do not prevent its hyperphosphorylation in vivo but inhibit the attendant shift in pRb electrophoretic mobility that is otherwise observed (Hamel et al. 1992a). In agreement, phosphorylation by cdk4/cyclin D complexes led to retarded migration of a significant fraction

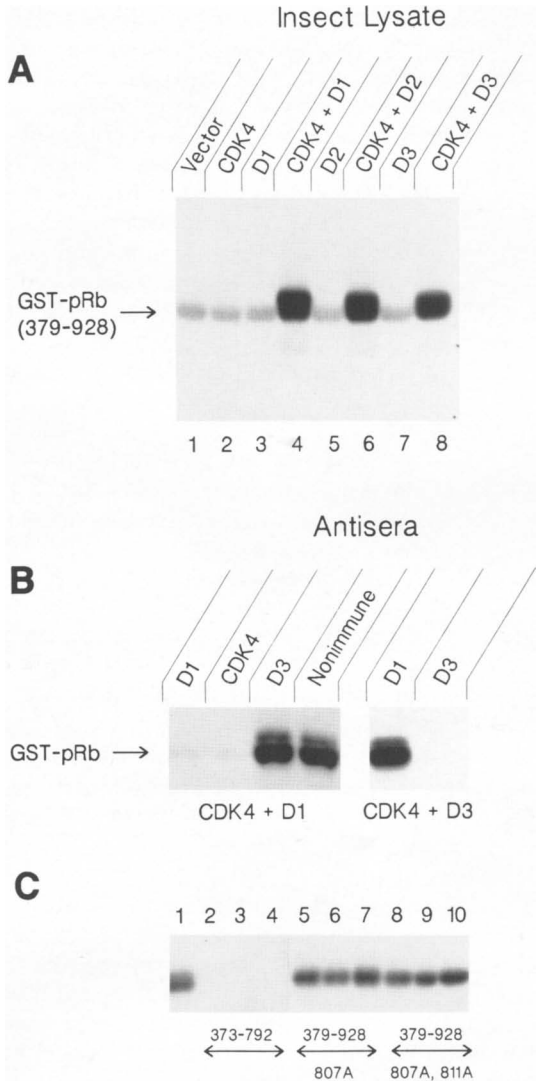

Figure 1. pRb kinase activity of cdk4–cyclin D complexes. (*A*) Lysates of insect Sf9 cells coinfected with wild-type baculovirus (control) or vectors encoding the indicated D-type cyclins and/or cdk4 were incubated with a bacterial GST–pRb fusion protein for 30 min at 30°C in kinase buffer containing [γ-^{32}P]ATP. The GST–pRb protein, recovered on glutathione–Sepharose beads, was eluted and separated on denaturing polyacrylamide gels (autoradiographic exposure time, 45 min). (*B*) Lysates of Sf9 cells coinfected with baculoviruses encoding cdk4 and D-type cyclins (indicated below) were precleared using antisera to the indicated proteins (*top*) before performing kinase assays as in *A* (autoradiographic exposure time, 1 hr). Antisera to cyclins D1 and D3 do not cross-react. (*C*) Kinase reactions were performed as above, using the same GST–pRb fusion protein (residues 379–928) as in *A* (lane *1*), a GST–pRb carboxy-terminal truncation mutant (residues 373–792) (lanes *2–4*), or the indicated point mutants containing alanine instead of Ser-807 (lanes *5–7*) or both Ser-807 and Ser-811 (lanes *8–10*). The sources of enzyme were cdk4 plus cyclin D1 (lanes *2,5,8*), cdk4 plus cyclin D2 (lanes *3,6,9*), and cdk4 plus cyclin D3 (lanes *1,4,7,10*). The exposure time was 15 min.

of the longer GST–pRb (379–928) fusion protein on denaturing gels (Fig. 1C, lane 1; also see Fig. 5A below). However, pRb mutants containing alanine substitutions at Ser-807 alone, or at Ser-807 plus Ser-811, did not manifest characteristic mobility shifts in spite of being hyperphosphorylated (lanes 5–10). Thus, cdk4/cyclin D complexes appeared to phosphorylate pRb at a known cdk consensus site (Ser-807) and presumably at others (Fig. 1C, lanes 5–10).

Identical tryptic phosphopeptide maps were obtained from GST–pRb proteins phosphorylated in vitro by complexes of cdk4 with cyclin D1, D2, or D3 (Fig. 2A shows a representative map). Mixing of pRb tryptic digests from each enzyme reaction shown in Figure 1A reinforced this conclusion (data not shown). Four major phosphopeptides (Fig. 2A, spots A–D) and many minor ones (spots E, X, Y, and Z and other undesignated peptides) were common to each map. To determine whether the multiple phosphopeptides were reminiscent of those phosphorylated in mammalian cells, pRb was immunoprecipitated from a [^{32}P]orthophosphate-labeled human T-cell leukemia line, Molt-4, and the pRb tryptic phosphopeptides were compared with those from GST–pRb products phosphorylated by cyclin D/cdk4 complexes in vitro. The pRb peptides phosphorylated in vivo (Fig. 2B) revealed each spot (including undesignated ones) detected in the in vitro product (Fig. 2A), and mixing experiments again confirmed their identity (data not shown). The only differences concerned the relative intensity of the spots. Spot E was more prominent in the map of the in vivo product, whereas spots B and C were less so (Fig. 2A,B). No additional phosphopeptides were detected in the map of intact pRb versus that of the GST–pRb fusion protein, suggesting that major phosphorylation sites were confined to pRb residues 379–928.

When GST–pRb proteins lacking Ser-807 were phosphorylated by cyclin D/cdk4 complexes in vitro and subjected to similar analyses, spots designated A and Y were no longer visualized (Fig. 2C). Additionally, spot X was lost from the map of the double mutant (data not shown). Because poorer peptide separation was obtained in the experiment shown in Figure 2C, the spots are more closely clustered, and spots D and E overlap. Mixing experiments confirmed the identity of the designated spots with those shown in Figure 2A. It is not surprising that Ser-807 was contained within two peptides, because the flanking basic residues are not equally susceptible to trypsin cleavage (Lees et al. 1991). Therefore, (1) cyclin D/cdk4 complexes phosphorylate pRb at multiple sites, identical to those detected in vivo; (2) based on mutational analysis, one of these sites appears to be Ser-807, whereas less phosphorylation was detected at Ser-811; and (3) the major phosphorylation sites were contained within the GST–pRb fusion protein lacking residues 1–378.

Specificities of cyclin interactions with p34^{cdk4} and p34^{cdc2}

Infection of Sf9 cells with viruses encoding D-type cyclins alone did not yield pRb kinase activity (Fig. 1A), suggesting that they could not activate endogenous in-

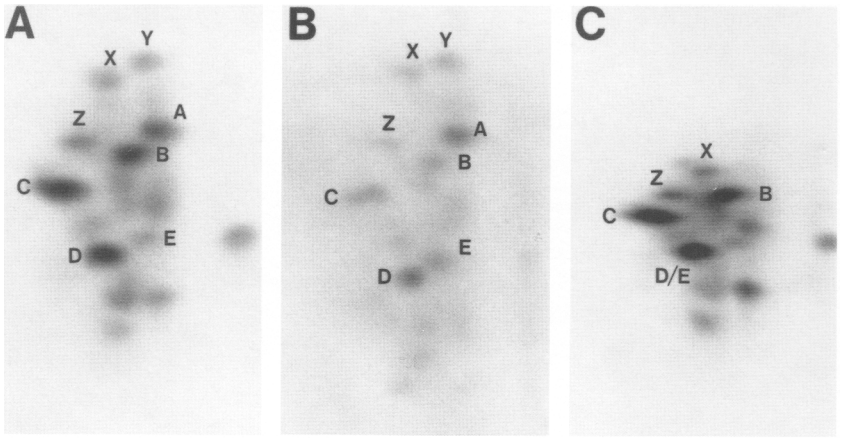

Figure 2. Tryptic phosphopeptide maps of pRb phosphorylated in vitro and in vivo. (*A*) Purified GST–pRb, phosphorylated in vitro by cyclin D2/cdk4 from Sf9 cell lysates (Fig. 1A), was digested with trypsin, and separated into two dimensions by electrophoresis (right to left) and ascending chromatography (bottom to top). Only the pRb moiety (residues 379–928) was phosphorylated (see text). The autoradiographic exposure time was 4 days. (*B*) pRb immunoprecipitated from the human Molt-4 T cell line metabolically labeled with [^{32}P]orthophosphate was purified, digested, and analyzed as in *A*. The autoradiographic exposure time was 8 days. (*C*) A GST–pRb mutant containing an alanine for Ser-807 substitution was phosphorylated in vitro and analyzed as in *A*. The autoradiographic exposure time was 4 days. In each panel, four major phosphorylation sites are designated *A–D*, and four minor spots are designated *E*, *X*, *Y*, and *Z*. Other undesignated spots in *A* and *B* are also concordant in their map positions.

sect cyclin-dependent kinases. Low levels of pRb kinase activity were recovered from cells producing exogenous cyclin A, B1, or E, but D-type cyclins again yielded background levels (Fig. 3, top). Cyclin A was more effective than cyclins E or B1 in activating endogenous insect cdk activity. Staining of the gels with Coomassie blue confirmed that equivalent amounts of the different cyclins were synthesized, so the observed differences were likely to be significant.

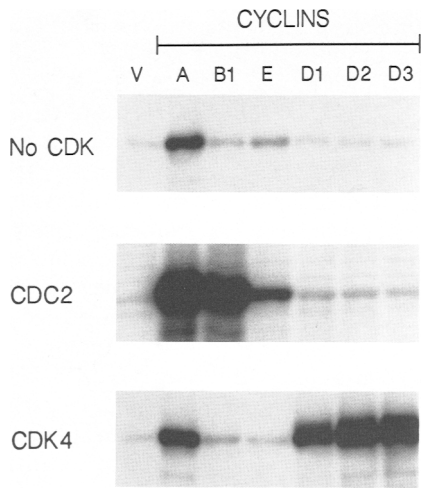

Figure 3. Specific activation of cdk4 by D-type cyclins. Insect cells were infected with baculoviruses encoding cyclins A, B1, D1, D2, D3, or E alone (*top*) or with combinations of vectors encoding the indicated cyclins and either cdc2 (*middle*) or cdk4 (*bottom*). The lanes labeled V (for vector) show results with extracts lacking exogenous cyclins. Lysates containing equal quantities of the engineered cyclins were incubated in a kinase assay with a GST–pRb, as in Fig. 1. The autoradiographic exposure time for the *top* and *bottom* panels was 2 hr. The *middle* panel was overexposed (6 hr) to illustrate both the ability of cyclin E to inefficiently trigger the activity of p34^{cdc2} in this assay and the corresponding failure of D-type cyclins to functionally interact with this catalytic subunit under the same conditions.

When Sf9 cells were coinfected with baculovirus vectors encoding cyclin A, B1, or E together with vectors encoding either p34^{cdc2} or p34^{cdk4}, kinase reactions performed with the GST–pRb substrate demonstrated that cyclins A, B1, and E could activate p34^{cdc2}, whereas cyclins D1, D2, and D3 could not (Fig. 3, middle). Cyclin E was less effective than cyclins A and B1 in activating p34^{cdc2}, consistent with the data of others (Koff et al. 1992). In control experiments, we confirmed that cyclin E was a much more potent activator of the p33^{cdk2} kinase (Dulic et al. 1992; Koff et al. 1992), which was also stimulated by cyclins A, B1, D2, and D3. In contrast, neither cyclin B1 nor E activated p34^{cdk4}, under conditions where each of the D-type cyclins was active (Fig. 3, bottom). The level of cdk4 kinase activity observed using cyclin A (Fig. 3, bottom) was similar to that recovered from cells infected with the cyclin A vector alone (Fig. 3, top), suggesting that like cyclins B1 and E, cyclin A and cdk4 do not functionally interact. Lysates of insect cells expressing either cyclin A or D-type cyclins were therefore precleared of endogenous cdk activity with p13^{suc1} beads, and the supernatants were then mixed with 10-fold-diluted lysates containing cdk4 to regenerate enzyme activity. Under these conditions, background cdk activity was minimal and assays of the recombined extracts demonstrated that cyclin A could not activate cdk4, whereas the three D-type cyclins remained highly effective (data not shown).

Phosphorylation of pRb in intact Sf9 cells by cdk4–cyclin D complexes

To determine whether pRb might undergo phosphorylation by cyclin D/cdk4 in intact cells, Sf9 cells were coinfected with combinations of baculovirus vectors encoding pRb, cdk4, and different D-type cyclins. When

[^{35}S]methionine-labeled lysates from infected cells were separated on gels, pRb was readily detected in whole-cell lysates (Fig. 4A) or after specific precipitation with a monoclonal antibody (Fig. 4B). Equivalent levels of cyclins D1, D2, and D3 were expressed in greater quantity than pRb in corresponding infected cultures (Fig. 4A, lanes 3–8) and were distinguished by differences in their electrophoretic mobilities, as predicted from their molecular masses (Matsushime et al. 1991a). Comparable amounts of the cdk4 catalytic subunit were also synthesized in each of the appropriately infected cultures (Fig. 4A, lanes 2,4,6,8). Given its molecular mass and methionine content, the amount of cdk4 produced approximated that of pRb. We observed two forms of cdk4 of 34 and 35.5 kD in Sf9 cells (arrowheads, lanes 2), of which only the faster migrating species corresponded to that seen in mammalian cells (see legend to Fig. 4A). In cells expressing pRb alone (Fig. 4A,B, lanes 1), pRb plus cdk4 (lanes 2), or pRb and different D-type cyclins (lanes 3,5,7), several electrophoretically distinguishable forms of pRb were observed, consistent with previous reports that Sf9 cells have endogenous kinases that induce pRb hypophosphorylation (Lin et al. 1991). However, coexpression of pRb, D-type cyclins, and cdk4 generated more slowly migrating pRb species (lanes 4,6,8), suggesting that pRb had undergone hyperphosphorylation.

Metabolic labeling with [^{32}P]orthophosphate showed that pRb was hyperphosphorylated when coexpressed with D-type cyclins and cdk4 (Fig. 5A, bottom). Again, no phosphorylation of cyclin D was detected under these conditions. When parallel cultures were labeled with [^{35}S]methionine, the hyperphosphorylated forms of pRb migrated more slowly than their hypophosphorylated counterparts (Fig. 5A, top). The only difference between these data and those shown in Figure 4B is that the pro-

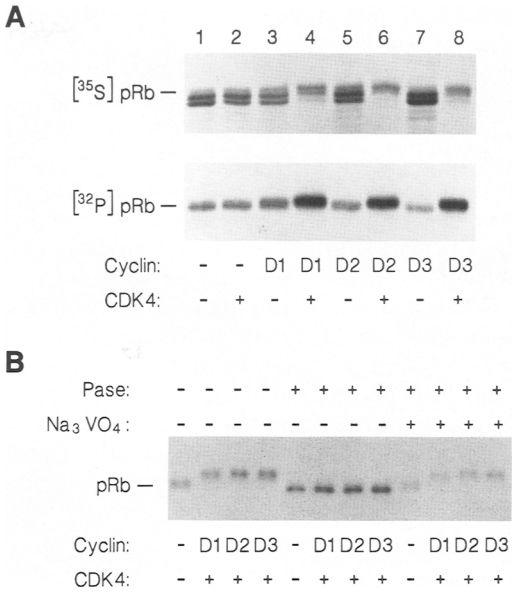

Figure 5. Phosphorylation of pRb by cdk4–cyclin D complexes in insect cells. Insect cells were infected with different baculovirus vectors as in Fig. 4 (indicated *below*), labeled with either [^{35}S]methionine or [^{32}P]orthophosphate (as shown at *left*), and immunoprecipitated with antibody to pRb. In *A*, the order of lanes is identical to that shown in Fig. 4. The [^{35}S]methionine-labeled pRb proteins (*top*) were separated on a 6% polyacrylamide slab gel to better resolve the different phosphorylated forms of pRb. [^{32}P]Orthophosphate-labeled proteins (*bottom*) were separated on 10% polyacrylamide gels. In *B*, cells infected with vectors encoding pRb and those indicated below were precipitated with antibody to pRb, and the immune complexes were then incubated with CIP with or without sodium orthovanadate (as indicated at the *top*). Denatured proteins were separated on gels and visualized by immunoblotting with anti-pRb. Exposure times were 2 hr (*A*, *top*), 1 hr (*A*, *bottom*), or 18 hr (*B*).

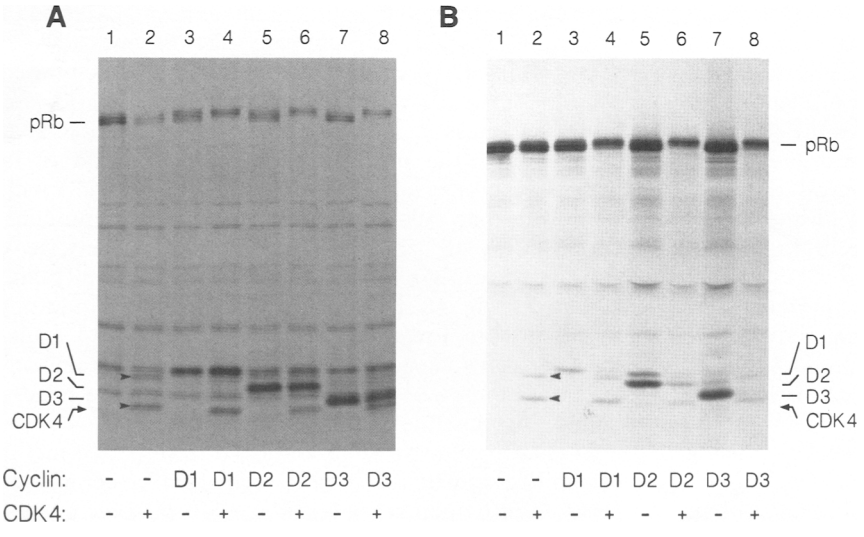

Figure 4. Cyclin D–pRb associations and cdk4-mediated pRb phosphorylation in intact Sf9 cells. Insect cells were infected with baculovirus vectors encoding pRb and the combinations of cdk4 and D-type cyclins indicated below. After 40 hr, intact cells were labeled with [^{35}S]methionine for 4 hr, and lysates were prepared and separated on gels. (*A*) The total metabolically labeled protein in unfractionated cell lysates. The positions of pRb, the three D-type cyclins, and the major cdk4 species (34 kD) are indicated at *left*. The positions of 35.5- and 34-kD forms of cdk4 are designated by arrowheads in lane 2. Both were detected by immunoblotting with antiserum to cdk4 (data not shown) and were observed after transcription and translation of cdk4 plasmids in a cell-free system (Matsushime et al. 1992). In contrast, we do not detect the slower migrating form in mammalian cells. Because the plasmid or baculovirus vectors lack initiation codons 5′ of the one predicted from the cdk4 cDNA sequence, the 35.5-kD isoform probably results from inefficient translational termination. (*B*) An autoradiograph of the proteins recovered in pRb immunoprecipitates. The positions of the various proteins are noted at *right*, and the order of lanes is identical to that in *A*. The autoradiographic exposure time was 2 hr at room temperature.

teins were separated on gels of lower porosity, enabling the different forms of pRb to be better resolved. Hyperphosphorylation of pRb was also observed when total cellular proteins were first immunoprecipitated and then immunoblotted with antibodies to pRb (Fig. 5B). Treatment of pRb immunoprecipitates with calf intestinal phosphatase collapsed the pRb bands into a single faster migrating species (Fig. 5B, lanes 5–8), but the addition of sodium vanadate inhibited dephosphorylation. Under the conditions used, this enzyme dephosphorylates P-Ser, P-Thr, and P-Tyr, and high concentrations of sodium vanadate completely inhibit these activities. These results also confirmed that the antibody to pRb can bind to its different phosphorylated forms. In principle, the high levels of enzyme expression achieved and the high ratio of enzyme to pRb concentration might obscure potential differences in rates of pRb phosphorylation.

Cyclins D2 and D3 form stable complexes with pRb in the absence of cdk4

Cyclins D2 and D3, transcribed and translated in vitro, form stable complexes with GST–pRb, but cyclin D1 binds poorly, and cyclin A not at all (M.E. Ewen, H.K. Sluss, C.J. Sherr, D.M. Livingston, and H. Matsushime, in prep.). Similarly, cyclins D2 and D3 produced in Sf9 cells also bound to GST–pRb in vitro, but stoichiometric concentrations of cyclin D1 bound weakly and cyclin A, B1, or E at concentrations as high as 30 μg/ml did not bind (data not shown). In intact Sf9 cells coexpressing D-type cyclins and full-length pRb (but not cdk4), cyclins D2 and D3 were preferentially recovered in anti-pRb immunoprecipitates (Fig. 4B, lanes 5,7). Much lower quantities of cyclin D1 were coprecipitated with pRb (Fig. 4B, lane 3), in spite of the fact that equally high levels of cyclin D1 were produced in the cells (Fig. 4A, lane 3). Considerably less pRb than D-type cyclins was detected in unfractionated cell lysates (Fig. 4A, lanes 3–8); but in anti-pRb immune complexes in which quantitative pRb precipitation could be obtained with a monoclonal antibody, the molar ratios of pRb to cyclin D2 or D3 were considerably higher (Fig. 4B, lanes 5,7). Densitometric measurements revealed that the signal ratio of pRb to cyclin D2 or D3 in these immunoprecipitates was 3–4 to 1. Given the approximately threefold greater mass and methionine content of pRb (105 kD) versus that of the cyclins (34–36 kD), the binding appeared to be nearly stoichiometric. Similar data were obtained by immunoblotting the precipitated proteins with antibodies to pRb and the cyclins (data not shown). We estimate that as much as 50% of the pRb produced in Sf9 cells under these conditions was complexed to cyclin D2 or D3, whereas <5% formed complexes with cyclin D1. Significant quantities of pRb were also recovered using antisera to cyclins D2 and D3, but not D1 (Fig. 6, lanes 2,5,8). As expected, the ratio of labeled cyclins to pRb in the latter immune complexes (Fig. 6) was higher than that seen in whole-cell lysates (Fig. 4A), confirming that unbound cyclins were synthesized in substantial molar excess.

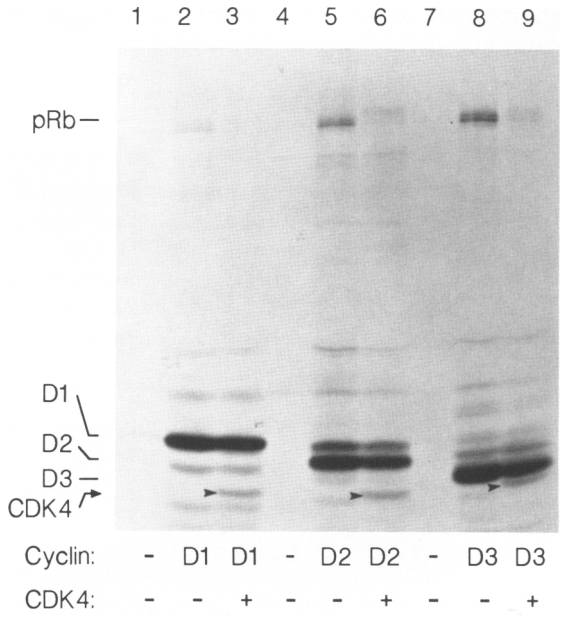

Figure 6. Cyclin D–pRb complexes and cdk4-mediated pRb phosphorylation in insect cells. Insect cells were infected with baculovirus vectors encoding pRb and different combinations of cdk4 and D-type cyclins (as indicated below). The conditions of the experiment were identical to those described in Fig. 4, except that insect lysates were precipitated with rabbit antisera to cyclin D1 (lanes *1–3*), D2 (lanes *4–6*), or D3 (lanes *7–9*). The autoradiographic exposure time was 5 hr.

pRb–cyclin D complex formation is inhibited by cdk4-mediated phosphorylation

In the absence of cdk4, stable complexes were formed between pRb and cyclins D2 and D3; pRb remained underphosphorylated, whereas coexpression of cdk4 and D-type cyclins not only induced pRb phosphorylation but resulted in the virtually complete destabilization of the complexes (Fig. 4B, lanes 6,8; Fig. 6, lanes 6,9). In cells lacking D-type cyclins, some cdk4 coprecipitated with pRb (Fig. 4B, lane 2). Although normalization for molecular mass and methionine content revealed that similar quantities of cdk4 and pRb were expressed in whole-cell lysates (e.g., Fig. 4A, lane 2; see above), much more pRb than cdk4 was detected in pRb immunoprecipitates (Fig. 4B, lane 2), indicative of a relatively weak interaction similar to that observed between pRb and cyclin D1 (Fig. 4B, lane 3). In agreement, we could not assemble complexes between radiolabeled cdk4 translated in vitro and bacterial GST–pRb fusion proteins under conditions where cyclins D2 and D3 were readily bound (Matsushime et al. 1992).

Reasoning that phosphorylation of pRb per se might destabilize and/or inhibit the formation of complexes between pRb and cyclin D2 or D3, Sf9 cells were coinfected with an enzymatically inactive cdk4 mutant in which the ATP-binding site (Lys-35) in the catalytic subunit was mutated to a methionine residue (Matsushime et al. 1992). Coexpression of cdk4 (K35M) with pRb and

either cyclin D2 (Fig. 7A) or D3 (Fig. 7B) in insect cells did not lead to pRb phosphorylation but, instead, facilitated the formation of stable ternary complexes (lanes 3). As expected, fewer complexes were detected when an equivalent level of the wild-type catalytic subunit was coexpressed with the D-type cyclins, and only under these conditions was pRb hyperphosphorylated (lanes 2). Like the wild-type catalytic subunit, mutant cdk4 did not bind to pRb with high affinity (data not shown). Thus, the failure of an inactivated cdk4 subunit to catalyze pRb phosphorylation enabled it to be trapped in ternary complexes from which cyclin D2 or D3 was not dissociated. Phosphorylation must therefore interfere with the ability of pRb to bind cyclins D2 and D3.

Cyclin D/cdk4-mediated phosphorylation of pRb prevents its association with E2F-1

A recently cloned E2F-1 cDNA (termed RBP3 or RBAP1) encodes a protein that binds to pRb (Helin et al. 1992; Kaelin et al. 1992), but this interaction should be inhibited if pRb were phosphorylated by a cyclin D/cdk4 complex. When insect cells were infected with baculovirus vectors encoding pRb and E2F-1, complexes between the two proteins could be observed (Fig. 8A). Unlike the interactions between pRb and cyclins D2 and D3, however, only a small percentage of expressed E2F-1 (~1–5%) formed complexes with immunoprecipitated pRb (data not shown), and these were best detected by the immunoblotting of proteins in the pRb immunoprecipitates with an antiserum to E2F-1 (Fig. 8A, lane 3). As expected,

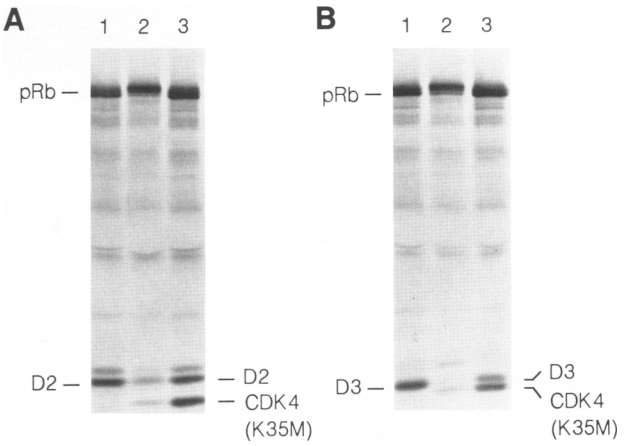

Figure 7. Stabilization of ternary complexes among pRb, mutant cdk4, and cyclin D2 or D3. Insect cells were coinfected with vectors encoding pRb and D-type cyclin (lanes *1*), pRb, D-type cyclin, and wild-type cdk4 (lanes *2*), or pRb, D-type cyclin, and mutant cdk4 (K35M) (lanes *3*). Lysates were precipitated with antibody to pRb, and the denatured complexes were separated on a gel. Results with cyclins D2 (*A*) and D3 (*B*) are shown, and the positions of the relevant proteins are labeled. pRb exhibits a slower mobility in lanes *2* compared with lanes *3*, consistent with its hyperphosphorylation by wild-type, but not mutant, cdk4. The autoradiographic exposure time was 2 hr.

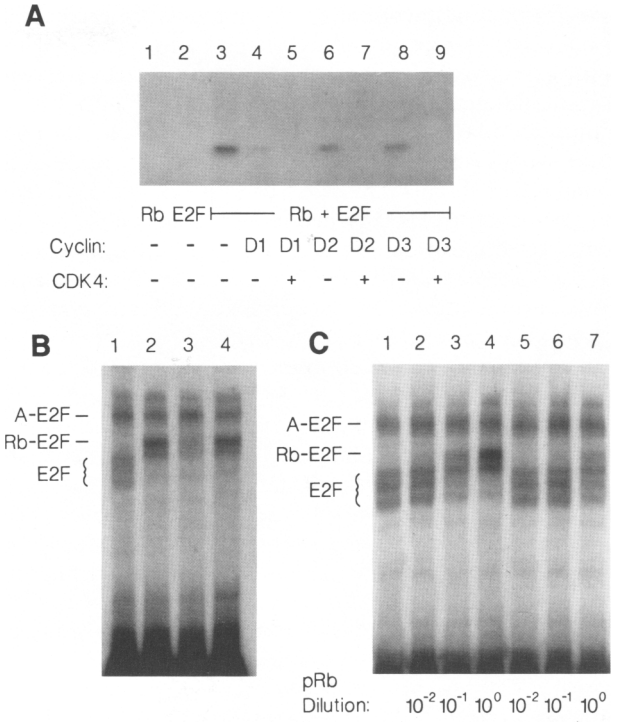

Figure 8. Reconstitution of the E2F–Rb complex. (*A*) Sf9 cells were coinfected with vectors encoding pRb, E2F-1, and combinations of D-type cyclins and cdk4, as indicated below. Lysates precipitated with a monoclonal antibody to pRb were separated on denaturing gels and immunoblotted with an antiserum to an E2F-1 peptide (exposure time, 18 hr). (*B*) Extracts of Rb-negative Saos-2 cells were incubated with extracts from Sf9 cells infected with a wild-type baculovirus (lane *1*) or with a vector encoding pRb (lanes *2–4*), and the pRb–E2F complex was detected by gel mobility shift, using a radiolabeled DNA fragment from the adenovirus E2 promoter. The positions of the pRb–E2F complex, free E2F, and a complex containing E2F and cyclin A are noted at *left*. The pRb–E2F complex was dissociated with an antibody to pRb (lane *3*) but not with an antiserum to an E2F peptide that includes sequences from the pRb-binding site (lane *4*). (*C*) Extracts from Saos-2 cells (lane *1*) were incubated with the indicated dilutions of Sf9 cell lysates containing pRb alone (lanes *2–4*) or with extracts containing pRb, cyclin D3, and cdk4 (lanes *5–7*). E2F complexes were detected by gel mobility shift as in *B*. Immunoblotting analysis using a pRb-specific rabbit antiserum confirmed that each undiluted extract (lanes *4,7*) contained equal quantities of pRb.

no E2F-1 was detected in pRb immunoprecipitates obtained from Sf9 cells expressing pRb (lane 1) or E2F-1 (lane 2) alone. Possible explanations for the weak pRb–E2F-1 interactions in coinfected cells are that the formation of these complexes normally depends on accessory factors (Hiebert et al. 1992; Ray et al. 1992) or requires E2F-1 heterodimerization with other E2F isoforms (Helin et al. 1992; Kaelin et al. 1992) that are not expressed in insect cells.

In cells coexpressing E2F-1, pRb, and various D-type cyclins, pRb–E2F-1 complexes were still detected (Fig. 8A, lanes 4,6,8), but the addition of cdk4 prevented these interactions (lanes 5,7,9). Immunoblotting confirmed

that similar amounts of pRb were expressed in each case. In contrast, coinfection of these cells with vectors containing D-type cyclins and the inactivated cdk4 (K35M) mutant did not abrogate the formation of pRb–E2F-1 complexes (data not shown). Coexpression of pRb and E2F-1 with cyclin D1 appeared to reduce the fraction of bound E2F-1 (lane 4), whereas cyclins D2 and D3 were without effect in the absence of cdk4 (lanes 6,8). We do not as yet understand the basis for these differences nor have we attempted to establish whether quaternary complexes among pRb, E2F-1, cyclin D2 or D3, and inactive cdk4 (K35M) can be formed.

Incubation of insect cell extracts containing pRb with lysates of human Rb-negative Saos-2 osteosarcoma cells allowed reconstitution of pRb–E2F complexes capable of interacting with a radiolabeled DNA fragment containing E2F-binding sites (Fig. 8B, lane 2). No such complex was observed using control lysates (lane 1). However, two other complexes were seen under both conditions, corresponding to free E2F and an E2F–cyclin A complex (Mudryj et al. 1991). The pRb–E2F complex was disrupted by antibodies to the recombinant pRb protein (lane 3) but not to the pRb-binding domain of E2F-1 itself (lane 4). Although it is possible that pRb might interact with products of other E2F genes present in the Saos-2 cell lysates, the epitope in E2F-1 detected by the latter antibody is masked in the pRb–E2F-1 complex (Helin et al. 1992; Kaelin et al. 1992). Complexes between E2F and the radiolabeled E2 DNA fragment could be competed with unlabeled oligonucleotide, whereas a fragment containing mutated E2-binding sites neither yielded detectable complexes itself, nor competed with the wild-type oligonucleotide (data not shown).

When the E2F-binding DNA fragment was mixed with a constant amount of Saos-2 lysate and different dilutions of baculovirus-encoded pRb, formation of pRb–E2F complexes was detected at 1 : 10 dilution (Fig. 8C, lane 3) but not at a 1 : 100 dilution (lane 2). In contrast, incubation of insect cell lysates containing hyperphosphorylated pRb (i.e., those coinfected with pRb, cyclin D3, and cdk4), together with Saos-2 extracts and the labeled E2 oligonucleotide, yielded significantly lower amounts of the pRb–E2F complex (Fig. 8C, lanes 5–7). Because most, but not all, pRb undergoes hyperphosphorylation in Sf9 cells (e.g., see Fig. 5A), it is likely that the small amount of underphosphorylated pRb remaining in the insect cell extracts contributed to the residual complexes detected in the undiluted sample (lane 7). Together, these results indicate that hyperphosphorylation of pRb at physiologically relevant sites by cyclin D–cdk4 can change the conformation of pRb in such a manner as to abrogate its association with a pRb-binding protein normally expressed in mammalian cells.

Discussion

We provide evidence, albeit indirect, implicating D-type cyclin-dependent kinases in the control of pRb function. Unlike cyclins A and E, D-type cyclins can bind directly to GST–pRb fusion proteins in vitro (Matsushime et al. 1992; M.E. Ewen, H.K. Sluss, C.J. Sherr, D.M. Livingston, and H. Matsushime, in prep.), and these interactions, which preferentially involve cyclins D2 and D3, depend on the integrity of pRb subdomains required both for E2F-1 binding (Hiebert et al. 1992; Qian et al. 1992) and suppression of G_1 exit in vivo (Goodrich et al. 1991; Qin et al. 1992). As shown here, complexes between D-type cyclins and pRb were also formed in intact insect cells, again with the preferential incorporation of cyclins D2 and D3 versus D1. These associations occurred with high stoichiometry, approaching a 1 : 1 molar ratio between cyclin D2 or D3 and pRb and involving a significant percentage of the total pRb pool. Although complexes between pRb and D-type cyclins have not been detected in mammalian Saos-2 cells where a functional interaction between them has been documented (M.E. Ewen, H.K. Sluss, C.J. Sherr, D.M. Livingston, and H. Matsushime, in prep.), a potential explanation may be that the D-type cyclins can target cdks to pRb, thereby inducing pRb hyperphosphorylation and destabilization of the complexes. Cdk4-mediated phosphorylation, and not simply cdk4 binding, was required for disruption of these complexes, because an inactive mutated kinase was recruited into stable ternary complexes. Thus, D-type cyclins may be multifunctional regulators, which not only govern the enzymatic activity of certain cdks but also direct the kinase to pRb and possibly to other pRb-like proteins. Similarly, B-type cyclins interact with both $p34^{cdc2}$ (cdk1) and a class of tyrosine phosphatases whose action is required for triggering $p34^{cdc2}$ kinase activity (Galaktionov and Beach 1991).

Because very high levels of vector-encoded proteins were produced in insect cells, these might obscure potential differences in the rates of pRb phosphorylation catalyzed by cdk4 complexes containing different D-type regulatory subunits. However, kinetic analyses in vitro did not reveal such differences, suggesting that only under conditions in which these components are limiting is the putative targeting function of the regulatory cyclin D subunits likely to be critical. The D-type cyclins did not functionally interact with $p34^{cdc2}$, and only they (but not cyclin A, B1, or E) were able to activate cdk4 in vitro, arguing for strong type specificity in the activation of this catalytic partner. However, these results do not preclude interactions between D-type cyclins and other catalytic subunits, and they can form complexes with both cdk2 and cdk5 in human fibroblasts (Xiong et al. 1992). On the basis of the data now available, cdk2 appears to be relatively promiscuous in its ability to form active complexes with cyclins A, B1, E, and at least some D-types, whereas cdc2 and cdk4 are more restricted in their functional interactions.

Given that cyclins D2 and D3 bind preferentially to pRb, they might be more effective than cyclin D1 as regulators of pRb phosphorylation in mammalian cells. When these cyclins were cotransfected with pRb into Rb-negative, human Saos-2 cells, cyclin D2, but not D1, induced significant pRb phosphorylation and preferentially overrode its ability to suppress G_1 exit (M.E. Ewen,

H.K. Sluss, C.J. Sherr, D.M. Livingston, and H. Matsushime, in prep.). Hinds et al. (1992) reported that enforced expression of cyclins A and E, but not B or C, also induced pRb phosphorylation and abrogated its growth-suppressive effects in Saos-2 cells. Although cyclin D1 neither induced pRb phosphorylation nor its release from its nuclear tether, it facilitated S-phase entry in a lower percentage of cotransfected cells, possibly by targeting the underphosphorylated forms for degradation. Whatever the mechanistic interpretation, cyclin D1 appears to exhibit different properties from cyclins D2 and D3 in its interaction with pRb, both in vitro and in intact insect or mammalian cells. Because cyclin E (Dulic et al. 1992; Koff et al. 1992), cyclin A (Giordano et al. 1989; Pines and Hunter 1990; Tsai et al. 1991; Cao et al. 1992; Desai et al. 1992; Elledge et al. 1992; Pagano et al. 1992a), and D1 or D3 (Xiong et al. 1992) associate with cdk2 in separate complexes during late G_1- and S-phase, reconstitution experiments of this type are unlikely to be sufficiently stringent to define the cdk(s) that normally phosphorylate pRb. Moreover, more than one of the cyclins might regulate pRb function(s), possibly triggering its phosphorylation at different sites or times or affecting its activity in a more subtle manner.

Complexes between pRb and E2F-1 could also be generated in insect cells, but unlike the interactions of pRb with cyclins D2 and D3, E2F-1 was bound at low stoichiometry, possibly because the cells lack, or express limiting quantities of, accessory factor(s) (Hiebert et al. 1992; Ray et al. 1992) or other E2F isoforms that are normally required for high-affinity E2F binding. In mammalian cells, only hypophosphorylated forms of pRb bind E2F (Chellappan et al. 1991; Helin et al. 1992; Kaelin et al. 1992; Shirodkar et al. 1992). Coexpression of cyclin D2 or D3 with pRb in insect cells did not significantly disrupt pRb–E2F-1 complexes, but the addition of cdk4 again led to pRb phosphorylation and completely abrogated E2F-1 binding. Therefore, not only can cyclin D/cdk complexes associate with and phosphorylate pRb, but they can affect its interactions with a cellular transcriptional regulator.

Complexes of cdk4 and D-type cyclins phosphorylate pRb in vitro at many sites, which correspond to those normally observed in proliferating human T cells. It remains unclear how phosphorylation might lead to the changes in pRb conformation that alter its biologic functions. pRb can repress transcription from several different promoters containing E2F-binding sites (Weintraub et al. 1992; Hamel et al. 1992b; Hiebert et al. 1992), suggesting that pRb–E2F complexes negatively regulate certain genes whose transcription might be stimulated by free E2F (Blake and Azizkhan 1989; Mudryj et al. 1990; Hiebert et al. 1991). A mutant form of pRb altered at eight potential phosphorylation sites acts as a much stronger repressor, thereby implicating pRb phosphorylation in governing this switch (Hamel et al. 1992b). The shift in pRb mobility on denaturing gels can be eliminated by mutations at Ser-800 and Ser-804 in murine pRb (Hamel et al. 1992a), which correspond to Ser-807 and Ser-811 in its human counterpart. However, phosphorylation at these sites affects neither T-antigen binding nor pRb repression of the E2F-responsive adenovirus early promoter, EIIaE, indicating that more complex modifications are required to disrupt E2F–pRb interactions (Hamel et al. 1992b). In agreement, we found that human pRb mutants lacking Ser-807, or Ser-807 and Ser-811, did not undergo characteristic mobility shifts on gels but were still hyperphosphorylated. Carboxy-terminally truncated pRb lacking residues 793–928 was not phosphorylated at all, suggesting that other carboxy-terminal cdk sites in addition to Ser-807 and Ser-811 might be important or that the deletion compromises pRb conformation so as to prevent its phosphorylation.

In mammalian cells, E2F also associates independently with an Rb-like protein, p107, and such complexes can include either cyclin A (Cao et al. 1992; Devoto et al. 1992; Ewen et al. 1992; Faha et al. 1992; Pagano et al. 1992b) or cyclin E (Lees et al. 1992) and their common catalytic partner cdk2 (see above). If, by analogy to results given here, phosphorylation of p107 interferes with its ability to form complexes with E2F and cyclins, we might infer that those cdk2/cyclin complexes that are stably associated with p107 are either catalytically inactive or do not phosphorylate p107 itself. p107 can also bind in vitro to cyclins D2 and D3 (M.E. Ewen, H.K. Sluss, C.J. Sherr, D.M. Livingston, and H. Matsushime, in prep.) and can serve as a cdk4/cyclin D substrate (Matsushime et al. 1992), but its interactions with the D-type cyclins depend on p107 subdomains other than those necessary for its binding to cyclin A (Ewen et al. 1992). Complexes containing p107 and E2F first appear during G_1 (Lees et al. 1992; for review, see Nevins 1992) and might potentially involve different combinations of E2F family members, pointing to further complexity in understanding how such proteins regulate transcription.

Several substrates for $p34^{cdc2}$ have been well characterized (for review, see Moreno and Nurse 1990; Nigg 1991), and independent complexes between different D-type cyclins and their catalytic partners should similarly phosphorylate many target molecules. The replication factor, proliferating cell nuclear antigen (PCNA), a subunit of the δ DNA polymerase, as well as an as yet uncharacterized 21-kD protein, coprecipitate with cyclin D1 from lysates of human fibroblasts (Xiong et al. 1992) and mouse macrophages (our confirmatory observations). PCNA does not appear to be a phosphoprotein, but D-type cyclins might indirectly modulate its function, possibly by assembling into multiprotein complexes that include additional cdk substrates. Given the potential targeting function of D-type cyclins, it would be of interest to determine whether PCNA, "p21," and pRb share structural motifs that enable them to bind to these regulators.

Materials and methods

Insect cell culture and baculovirus infection

Sf9 cells were maintained at 27°C in Grace's medium containing 10% fetal bovine serum (FBS), yeastolate, lactalbumin hy-

drolysate, and gentamicin in 100-ml spinner bottles. Virus infections and plaque assays were performed in 60-mm-diam. dishes (Summers and Smith 1987). Vectors encoding mouse D-type cyclins and cdk4 were described previously (Matsushime et al. 1992), and those encoding human cyclins A, B1, and E were provided by Dr. David Morgan (Univerity of California, San Francisco). cDNA fragments containing the entire coding regions of human pRb and human E2F-1 (supplied by Dr. William G. Kaelin, Dana Farber Cancer Institute) were inserted into the transfer vector pAcYM1 (Matsuura et al. 1987) and cotransfected into Sf9 cells with linearized baculovirus DNA (Pharmingen, San Diego, CA) using a liposome-mediated transfection kit (Invitrogen, San Diego, CA). Recombinant viruses purified from plaques were assayed for expression of their encoded proteins by metabolic labeling and immunoprecipitation or by immunoblotting (Matsushime et al. 1992).

Antibodies

Antisera to mouse cyclins D1, D2, D3, and cdk4 were prepared by immunizing rabbits with full-length recombinant proteins produced in bacteria (Matsushime et al. 1991b, 1992). The antiserum to cdk4 was raised against the complete protein fused to GST at its amino terminus and is able to coprecipitate D-type cyclins in complexes with $p34^{cdk4}$ from mammalian cells. Mouse monoclonal antibody (C36) and rabbit antisera against human pRb were from Oncogene Sciences (Manhasset, NY). A rabbit antiserum to E2F-1 was raised against a carboxy-terminal peptide (NH_2-LDYHFGLEEGEGIRDLFD) corresponding to codons 409–426 (Helin et al. 1992; Kaelin et al. 1992).

Metabolic labeling, immunoprecipitation, and protein detection

Sf9 cells (1×10^6) were infected with combinations of recombinant viruses, each at a m.o.i. of 30. Cells were labeled for 4 hr 40 hr post-infection with 200 μCi/ml of [^{35}S]methionine (1000 Ci/mmole) in 1 ml of methionine-free medium (supplemented with 5% dialyzed FBS) or with 1 mCi of carrier-free [^{32}P]orthophosphate (9000 Ci/mmole) in 1 ml of supplemented phosphate-free medium. Labeled cells were lysed for 1 hr at 4°C in 1 ml of EBC buffer [50 mM Tris-HCl at pH 8.0, 120 mM NaCl, 0.5% NP-40, 1 mM EDTA, and 1 mM dithiothreitol (DTT)] containing 5 μg/ml of aprotinin, 5 μg/ml of leupeptin (both Sigma Biochemicals), 0.1 mM NaF, 10 mM β-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 mM sodium orthovanadate. Centrifuged lysates were incubated with the designated monoclonal antibody or antisera for 3 hr at 4°C, and protein A–Sepharose beads (precoated with anti-mouse IgG for precipitation with monoclonal antibody) were added and incubated for an additional hour. Immunoprecipitates were collected by centrifugation, washed five times in EBC buffer at 4°C, and separated electrophoretically on polyacrylamide gels containing SDS (Anderson et al. 1984). To isolate phosphorylated pRb from mammalian cells, 5×10^8 Molt-4 human T lymphocytes were metabolically labeled in phosphate-free medium with 2.5 mCi/ml [^{32}P]orthophosphate (Matsushime et al. 1991b); pRb was then immunoprecipitated from detergent cell lysates and separated on denaturing gels using identical methods. Where indicated, phosphorylated pRb was eluted from gels and digested with trypsin, and phosphopeptides were separated into two dimensions by electrophoresis and ascending chromatography (Rettenmier et al. 1985). Immunoblotting was performed with ^{125}I-labeled protein A for detection (Downing et al. 1988).

In vitro kinase assay

Forty-eight hours postinfection, 1×10^7 infected Sf9 cells were lysed at 4°C in 250 μl of kinase buffer [50 mM HEPES at pH 7.5, 10 mM $MgCl_2$, 1 mM DTT containing protease and phosphatase inhibitors (see above)] and then cleared by centrifugation. Aliquots (25 μl) were mixed with bacterially expressed GST–pRb fusion proteins, prepared, and immobilized on glutathione–Sepharose beads (Matsushime et al. 1992). Where indicated, lysates were precleared of cyclins or cdk4 by use of appropriate antisera or $p13^{suc1}$ beads (Oncogene Science, Manhasset, NY). Kinase reactions were initiated at 30°C by adding 10 μCi of [γ-^{32}P]ATP (6000 Ci/mmole) adjusted with unlabeled ATP to a final concentration of 25 μM. After incubation for the indicated times, the beads were washed three times with ice-cold TNEN buffer (20 mM Tris HCl at pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40), and the phosphorylated GST–pRb fusion proteins were resolved on gels.

Phosphatase assay

Immunoprecipitates bound to protein A–Sepharose beads were washed and equilibrated in 50 μl of calf intestinal phosphatase (CIP) buffer (10 mM Tris HCl at pH 8.0, 1 mM $MgCl_2$, 0.14 U/ml of aprotinin, and 1 mM PMSF). CIP (40 units, Sigma Chemicals, St. Louis, MO), with or without sodium orthovanadate at a fully inhibitory concentration (1 mM), was added for 3 hr at 37°C where indicated. Beads were washed three times with ice-cold CIP buffer supplemented with sodium orthovanadate and then processed for gel electrophoresis.

Gel retardation assay for E2F

High salt (450 mM NaCl) extracts of Saos-2 cells and virus-infected Sf9 cells were prepared by microextraction (Schöler et al. 1989). For reconstitution of the E2F–pRb complex, 10-fold serial dilutions of Sf9 extracts were added to 5 μg of Saos-2 extract and diluted 1 : 2.5 in 1× gel-shift buffer (20 mM HEPES at pH 7.85, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, and 40 mM KCl) containing 1 mg/ml of bovine serum albumin. After 10 min of incubation on ice, E2F DNA-binding activity was measured by gel retardation of a ^{32}P-end-labeled probe from the adenovirus 5 E2 promoter, which lacks an ATF-binding site (Hiebert et al. 1991). For antibody disruption, 0.2 μg of an anti-pRb monoclonal antibody, or 1 μl of antiserum directed against a peptide representing the pRb-binding domain of E2F-1, was added just after the addition of radiolabeled DNA. DNA–protein complexes were separated on a 4% polyacrylamide gel buffered with Tris-borate/EDTA (Hiebert et al. 1991).

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