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Direct Interaction of p21 Cyclin-Dependent Kinase Inhibitor with the Retinoblastoma Tumor Suppressor Protein

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The p21CKI forms a physical complex with the retinoblastoma protein (pRb) both *in vitro* and *in vivo*. The A/B pocket region of pRb and the N-terminal region of p21 were indispensable for this interaction. Among p21 family members, p57, but not p27, associated with pRb. Overexpression of cyclin D1, Cdk4, and E2F1 in the cells expressing pRb and p21 did not perturb the interaction between p21 and pRb. Coexpression of p21 in cells expressing pRb, cyclin D1, and Cdk4 prevented pRb hyperphosphorylation by cyclin D1/Cdk4. On the other hand, hyperphosphorylation of pRb by an excess amount of cyclin/Cdk disrupted pRb/p21 complex formation *in vitro*. These findings suggest that pRb may be dynamically regulated by the relative binding and activities of p21 and Cdks. © 1999 Academic Press

Key Words: **Cdk inhibitor; retinoblastoma protein; cell cycle arrest; cyclin D1 kinases; G1 progression.**

The retinoblastoma gene (RB) encodes a nuclear phosphoprotein (pRb) that functions as a critical negative regulator of mammalian cell cycle progression (1). The function of pRb appears to be controlled through cell cycle-dependent phosphorylation and inactivation of pRb by phosphorylation in mid to late G1 is thought to permit progression into the later phases of the cell cycle (2). The cell cycle-dependent phosphorylation of pRb appears to be carried out primarily by D- or E-type cyclin-dependent protein kinases (Cdks) (3–5).

Cdk activities are regulated positively by cyclins and negatively by CKIs (6). Two distinct families of CKIs have been detected in mammalian cells. The Cip/Kip family includes the structurally related proteins p21, p27 and p57, all of which inhibit a variety of Cdk activities *in vitro* (7). The INK4 family includes p15, p16, p18 and p19, is not structurally related to the p21 family (7). The INK4 family has a narrower range of inhibitory activities than the p21 family, specifically inhibiting the cyclin D-dependent kinases *in vitro* and the presence of a functional pRb appears to be necessary for p16-mediated growth arrest (8, 9), suggesting that cyclin D-dependent kinases regulate cell cycle progression through pRb. However, details in the cell cycle arrest caused by p21 remain unclear.

We report here that p21 and p57, but not p27, interact directly with pRb and that the complex formation depends in part upon the integrity of a major protein-binding domain of pRb (the A/B pocket domain). Our results suggest that direct interaction of p21 to pRb may play a role in cell cycle arrest by the Cip/Kip family CKI.

MATERIALS AND METHODS

Plasmids and baculoviruses. Recombinant baculoviruses expressing cyclins A, D1, and E were kindly provided by Dr. D. Morgan, and viruses expressing pRb and E2F1 were kindly provided by Dr. J-Y. Kato. The pGEX-p21-wild type plasmid has been described (10). The pGEX-p21-triple mutant △17-22/W49G/M147A was generated by PCR using common 5′ (pGEX5′) and 3′ (pGEX3′) primers, previously described (11), and the inner primers GGC-AGC-AAG-CCC-CGG-GGC-GGC-CCA-GTG-GAC-AGC-GAG and CAC-TGG-GCC-GCC-CCG-GGG-CTT-GCT-GCC-GCA-TGG-GTT, respectively. pGEX-p27-wild-type was generated by PCR using the 5′ primer GGA-TCC-CCT-CGA-GGG-GAT-CCC-CCC-GCC-ATG-TCA-AAC-GTG-CGA-GTG, the 3′ primer AGG-ACA-GTG-GGA-GTG-GCA-CCT-TCC, and pcDNA3-hp27 (derived from pCS2+-hp27, and kindly provided by Drs. J. Roberts and M. Ohtsubo) (12) as a template. pGEX-p57 was also generated by PCR using the 5′ primer GGA-TCC-CCT-CGA-GGG-GAT-CCC-CCC-GCC-ATG-GAA-CGC-TTG-GCC-TCC, the 3′ primer GGA-TCC-GAA-TTC-CTA-TCA-TCT-CAG-ACG-TTT-GCG, and pcDNA3-mp57 (derived from pBluescript mp57, and kindly provided by Dr. S. Elledge) (13) as a template. pBluescript

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Rb was generated by removing the XhoI/NotI fragment from pSPT18Rb (a gift from Dr. L. Whitaker). pBluescript Rb-377-772, 768-928, and 835-928 were generated by PCR using T7 and T3 primers, and the primers CCT-CTC-GAG-TCA-AGC-ATA-CTG-CAA-AAT-ATT-TGT-TTT-CAG for 377-772, CCT-ACT-AGT-GGC-GCC-ATG-ATT-TTG-CAG-TAT-GCT-TCC-ACC-A and the T3 primer for 768-928, and CCT-ACT-AGT-GGC-GCC-ATG-ATT-GGT-GAA-TCA-TTC-GGG-ACT for 835-928.

Cell culture, nuclear extracts, and immunoprecipitation-Western analyses. Human MJ-90 (normal diploid fibroblasts isolated from neonatal foreskin) (14) cells were grown in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, using standard cell culture conditions, as previously described (14).

Nuclear extracts were prepared from young and senescent MJ-90 cells (70–80% confluence; approximately 5×10^7 cells) by washing the monolayers once with cold phosphate buffered saline (PBS), adding hypotonic lysis buffer (10 mM HEPES [N-2-hydoxyethylpiperazine-N′-2-ethanesulfonic acid], pH 8.0, 1.5 mM $MgCl_2$, 10 mM NaCl, 0.5 mM DTT, 0.3 mM sucrose, 0.1 mM EGTA, 0.1 % NP-40, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM sodium orthovanadate) containing protease inhibitors (20 μg/ml soybean trypsin inhibitor, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride [PMSF]), and scraping the cells from the culture dish. The lysed cells were rotated for 10 min at 4°C and collected by centrifugation at 300*g* for 2 min. The pellet was carefully resuspended in 1 ml high salt nuclear lysis buffer (20 mM Tris, pH 7.4, 0.3 M NaCl, 1 mM EDTA, 0.5% NP-40, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM sodium orthovanadate) containing the protease inhibitors described above and sonicated briefly. The extract was clarified by centrifugation at 27,000*g* for 5 min and the supernatant was incubated with the anti-pRb antibody (C-15, Santa Cruz Biotechnologies) or normal rabbit serum. The mixtures were gently agitated for 1 h at 4°C, 20 μl of Protein A Sepharose beads was added, and incubation was continued for 1 h at 4°C. The beads were washed three times with high salt nuclear lysis buffer, and the proteins bound to them were released and dissolved in Laemmli sample buffer and separated by electrophoresis through a 15% denaturing polyacrylamide gel (SDS-PAGE). The separated proteins were transfered to a nitrocellulose membrane (Amersham Corporation), and p21 was detected using an anti-p21 antibody (H-164, Santa Cruz) (1:1000) and enhanced chemiluminesence (ECL, Amersham).

Production of GST fusion proteins. Stationary cultures of *E. coli* transformed with plasmids encoding glutathione-S-transferase (GST) fusion proteins were diluted 10-fold with fresh medium, cultured for additional 2 h at 37°C, and recombinant proteins were induced by addition of 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After 2 h at 37°C, the induced cells were harvested and lysed by sonication in NETN150 buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40) containing protease inhibitors (20 μg/ml soybean trypsin inhibitor, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 100 μg/ml PMSF). Recombinant proteins were adsorbed to glutathione-Sepharose beads, and washed first with NETN100 buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) and then with 50 mM HEPES, pH 8.0. Proteins were eluted from the beads by 50 mM HEPES (pH 8.0) containing 5 mM reduced glutathione (Sigma Chemical Co.), dialyzed against 50 mM HEPES (pH 8.0), and quantitated by densitometric analysis after separation by SDS-PAGE, using bovine serum albumin (BSA) as a standard.

In vitro binding assays. Truncated pRb proteins were produced by translation using a reticulocyte lysate (Promega) and pBluescript-based plasmids as transcription templates for T7 RNA polymerase. The translation products were labeled using 40 μCi [^{35}S]-methionine per 50 μl reaction. For *in vitro* binding, 20 μl of the translation reaction mix was added to 300 μl of EBC buffer (50 mM Tris, pH 8.0, 120 mM NaCl, 2.5 mM EGTA [ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid], 1 mM EDTA, 1 mM DTT, 0.5% NP-40, 20 μg/ml soybean trypsin inhibitor, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 100 μg/ml PMSF) containing 0.5 μg of GST-CKI proteins. The mixture was gently agitated for 1 h at 4°C, whereupon 20 μl of glutathione-Sepharose beads were added and mixing was continued for 1 h at 4°C. The beads were washed three times with 0.5 ml EBC buffer before the bound proteins were separated by SDS-PAGE and identified by autoradiography.

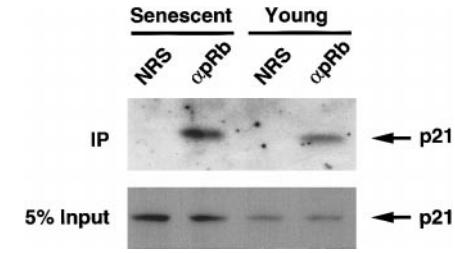

FIG. 1. *In vivo* association between pRb and p21. Nuclear extracts from young (PD = 18) and senescent (PD = 60) MJ-90 cells were immunoprecipitated with a rabbit polyclonal anti-pRb antibody, or normal rabbit serum (NRS) as a control. Immunoprecipitates were collected on protein A-Sepharose beads, and the bound proteins were separated by 15% SDS–PAGE, transferred to a nitrocellulose filter by Western blotting, and probed with the rabbit polyclonal anti-p21 antibody (upper panel). 5% input was immunoblotted with anti–p21 antibody (lower panel). The arrow indicates the position of p21.

Metabolic labeling and immunoprecipitation from insect cells. Spodoptera frugiperda (Sf9) cells (1×10^6 cells) were infected with recombinant baculoviruses encoding pRb, cyclins, E2F1, Cdks or CKIs at multiplicities of infection (moi) of 3 (pRb) or 30. Forty-eight hours after infection, cells were labeled for 4 h with 0.1 mCi/ml of [^{35}S] methionine (1000 Ci/mmol; ICN, Irvine, CA) and lysed in 500 μl of EBC buffer. The lysates were clarified by centrifugation and incubated with the indicated antibody (C-15 anti-pRb, C-19 anti-p21, C-19 anti p27 antibodies from Santa Cruz Biotechnologies) for 1 h at 4°C. Protein A-Sepharose beads were added, and the mixture was further incubated for 1 h at 4°C. The immunocomplexes were collected by centrifugation, washed four times in cold EBC buffer, released in Laemmli sample buffer, separated by SDS-PAGE and detected by autoradiography.

RESULTS AND DISCUSSION

p21 and pRb Interact in Vivo

To detect the association of p21 with pRb *in vivo*, we immunoprecipitated nuclear lysates with an anti-Rb antibody and analyzed the precipitates for the presence of p21 by Western analysis using an anti-p21 antibody. For these experiments, we prepared nuclear lysates from normal human fibroblasts (MJ-90) in order to avoid any possible alterations in pRb regulation or function that might occur in immortal cells, and any nonspecific association of cytosolic proteins with pRb. As shown in Fig. 1, the p21 CKI was detectable at a higher level in immunoprecipitate using lysates derived from senescent cells than that from young cells, consistent with the higher expression of p21 in senescent than in young cells (Fig. 1, lower panel). In contrast, p21 was not detected in immunoprecipitates by normal rabbit serum at all. Thus, we conclude that p21

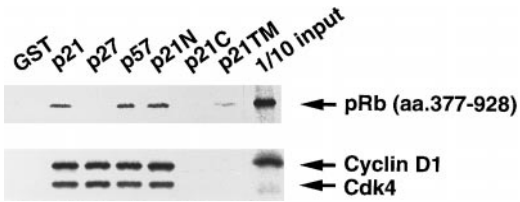

FIG. 2. p21 and p57, but not p27, bind pRb *in vitro*. [^{35}S] labeled pRb (amino acids 377–928), translated in the rabbit reticulocyte lysate system, or [^{35}S] labeled cyclin D1/Cdk4 complex, expressed in insect cells, were incubated with 0.5 μg of GST or GST–fused p21, p27, p57 or p21 mutants (p21N; amino acids 1–71, p21C; amino acids 72–164, p21TM; triple mutant of △17-22/W49G/M147A) at 4°C for 1 h. The mixtures were then incubated with glutathione-Sepharose beads for an additional 1 h at 4°C, and the bound fractions were resolved by 12.5% SDS–PAGE followed by autography.

is physically associated with pRb protein complexes in normal human cells.

p21 and p57, but Not p27, Binds to pRb in Vitro

To study the binding characteristics of p21 and related proteins to pRb, we expressed p21, p27 and p57 as fusion proteins to glutathione-S-transferase (GST) in *E. coli*. The fusion proteins were purified and incubated with [^{35}S]-methionine-labeled human pRb (amino acids 377–928) that had been translated *in vitro* by a reticulocyte lysate. We then assessed the ability of the p21, p27 and p57 CKIs to bind pRb *in vitro* by recovering GST-CKIs on glutathione beads and detecting associated pRb by SDS-PAGE and autoradiography. We performed these experiments using an amino-terminal truncated pRb (amino acids 377–928) because the full-length pRb was not efficiently translated by the rabbit reticulocyte lysate (data not shown). Our analysis showed that p21 and p57 specifically associated with pRb, whereas p27 did not (Fig. 2, upper lane). Control experiments showed that, under the same conditions, all the CKIs expressed in *E. coli* bound cyclin D1/Cdk4 complexes (expressed by baculoviruses). Thus, all the CKIs expressed in *E. coli* were functionally active, although only p21 and p57 physically associated with pRb. These data suggest that the interaction between p21 with pRb may be direct. In addition, among the members of the p21 CKI family, p21 and p57, but not p27, appear capable of this interaction.

The N-Terminus of p21 Is Responsible for Binding pRb

We next determined the region of the p21 molecule that was responsible for binding pRb. GST-fusion proteins of wild-type, truncated or mutant p21 proteins were incubated with radiolabeled, *in vitro* translated pRb (amino acids 377–928), recovered on a glutathione affinity support, and analyzed for bound pRb by SDS-PAGE and autoradiography. As shown in Fig. 2, the N-terminal (1–71), but not the C-terminal (72–164), region of p21 bound pRb. A triple mutant of p21 (△17-22/W49G/M147A), which lacks cyclin, Cdk and PCNA binding activity (data not shown), also bound pRb, although to a lesser extent than wild-type p21 (Fig. 2). Of the three mutations harbored by this mutant, the one that was responsible for decreasing (but not obliterating) the ability to bind pRb was the point mutation of Trp to Gly at amino acid 49 (W49G); neither deletion of amino acids 17–22 (△17–22) nor the point mutation of Met to Ala at amino acid 147 (M147A), altered the ability of p21 to bind pRb (data not shown).

The A/B Pocket of pRb Is Responsible for Binding p21

To map the region of pRb responsible for binding p21, we assayed the ability of various *in vitro* translated pRb fragments to bind the GST-p21 fusion protein (Fig. 3, right panel). Deletion of the N-terminal 376 amino acids of pRb did not affect binding to p21 (data not shown). Thus, the C-terminal two-thirds of pRb (amino acids 377–772), which contains the intact A/B pocket, was sufficient for efficient binding to GST-p21 (Fig. 3, left panel). By contrast, GST alone did not bind this pRb fragment. We noted barely detectable binding between p21 and the C-terminal 161 amino acids of pRb (768–928), which are reported to contain the c-abl (15) binding site. We have not determined whether this weak signal represents non-specific binding to GST-p21 or a bona fide, low affinity interaction. When the C-terminal two-thirds segment of pRb was

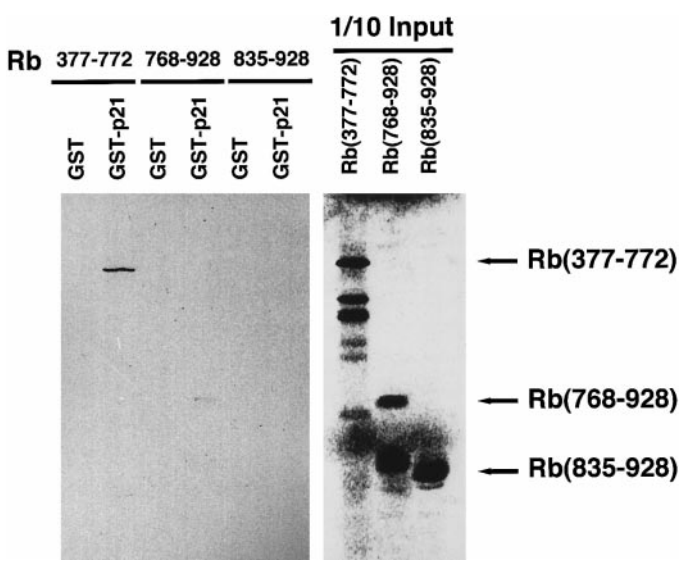

FIG. 3. p21 binds pRb through an A/B pocket region. 0.5 μg of either GST or GST-p21 was incubated for 1 h at 4°C with various truncated pRb fragments which were translated and labeled in the rabbit reticulocyte lysate system (1/10 input; right panel). The mixtures were further incubated with glutathione-Sepharose beads for an additional 1 h at 4°C, and the bound fractions were resolved by 12.5% SDS-PAGE followed by autography (left panel).

cut approximately in half, the p21 binding activity was completely lost (Fig. 3, left panel). Taken together, the above results suggest that the interaction between p21 and pRb is mediated by the N-terminal 70 amino acids of p21 and the A/B pocket region of pRb.

There are at least three ways in which pRb-binding proteins can interact with pRb. First, viral oncoproteins (HPV E7, SV40 large T antigen and adenovirus E1A), as well as the E2F transcription factor, interact with pRb through the A/B pocket domain (16, 17). Second, the mammalian D-type cyclins require both the A/B pocket and C-terminal regions of pRb for binding (3). This is also true for the pRb-E2F-DNA complex in gel mobility shift assays (18–20). Third, the c-abl tyrosine kinase requires the C-pocket of pRb, independently of the A/B pocket (15). Our data indicate strong binding of p21 to the A/B pocket, and weak binding to the C-terminal region, suggesting that p21 interacts with pRb through the second mechanism, similar to cyclin D. However, the exact region(s) in pRb required for p21 binding appears to be different from those required for cyclin D binding.

p21 but Not p27 Forms Stable Complexes with pRb in Insect Cells

To study the p21-pRb interaction in intact cells and analyze the effects of other pRb-binding proteins on this interaction, Sf9 cells were infected with recombinant baculoviruses encoding either full-length pRb, p21 or p27. The p21 and p27 viruses were used at a high multiplicity (moi = 30), whereas the pRb virus was used at a 10-fold lower multiplicity (moi = 3). When [^{35}S] methionine-labeled lysates from cells infected by the pRb virus were immunoprecipitated by an anti-pRb antibody, pRb was readily detected by SDS-PAGE (Fig. 4A, lane 1). When lysates were used from cells coinfected with p21 and pRb viruses, p21 was readily detectable in the anti-pRb immunoprecipitate (Fig. 4A, lane 2). By contrast, lysates from cells coinfected with p27 and pRb viruses showed no p27 in the anti-pRb immunoprecipitates (Fig. 4A, lane 3). Moreover, pRb was readily detected in anti-p21 immunoprecipitates, but not anti-p27 immunoprecipitates (data not shown). The minor mobility shift of pRb protein on SDS-PAGE was detected in the lysates expressing pRb alone (Fig. 4B, lane 1), presumably due to its phosphoyrlation by insect cyclin/Cdk(s) and this shift was inhibited by the co-expression of p21 or p27 (Fig. 4B, lanes 2, 3). When cyclin D1 and Cdk4 were coinfected with p21 or p27, the anti-p21 and anti-p27 immunoprecipitates contained readily detectable cyclin D1 and Cdk4 (data not shown), indicating that the p21 and p27 expressed in these cells were functionally active. These results using cellular lysates support the results of our *in vitro* studies that p21, but not p27, interacts with pRb.

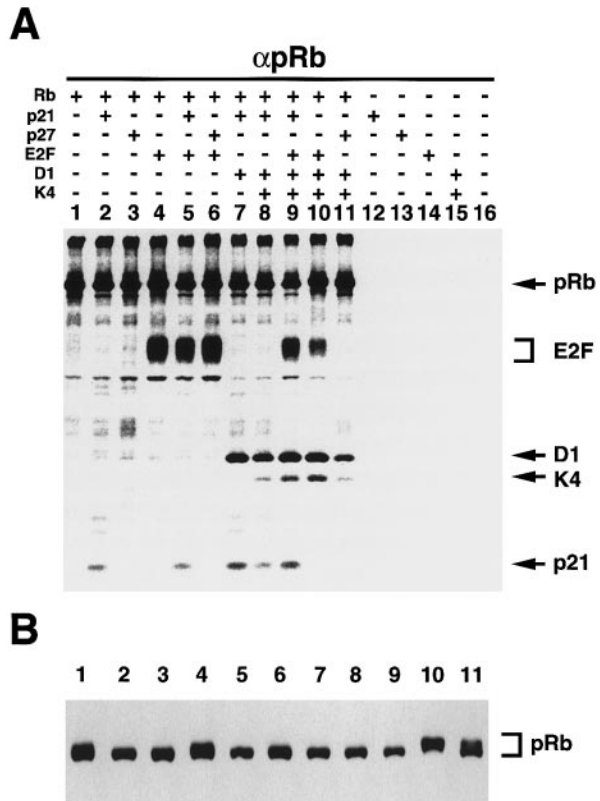

FIG. 4. Effect of pRb–binding proteins on pRb/p21 interaction in intact Sf9 cells. Sf9 insect cells were infected with baculovirus vectors encoding pRb and the indicated combinations of p21, p27, E2F1, cyclin D1, and Cdk4. After 48 h, intact cells were labeled with [^{35}S] methionine for 4 h, and lysates were immunoprecipitated with an anti-pRb polyclonal antibody (A) and (B). Immunoprecipitates using protein A-Sepharose were then separated on SDS-PAGE. (B) was derived from the same gel as (A) with a shorter exposure time.

Because E2F1 and D-type cyclins have also been reported to interact with pRb in Sf9 cells (4), we next asked whether these pRb-binding proteins altered the p21-pRb interaction. Recombinant baculoviruses expressing p21 or p27, cyclin D1, Cdk4, and E2F1 were used at a moi of 30, whereas the pRb virus was used at a moi of 3. As expected, cells coinfected with viruses encoding pRb and E2F1 or cyclin D1 showed readily detectable complexes between pRb and E2F1 and pRb and cyclin D1 (Fig. 4A, lane 4 and data not shown). Cyclin D1 or E2F1 were then expressed in cells that also expressed both p21 and pRb. Neither cyclin D1 nor E2F1 appeared to perturb the interaction between p21 and pRb (Fig. 4A, lanes 5 and 7). Interestingly, coexpression of p21 in cells expressing pRb, cyclin D1 and Cdk4 prevented the hyperphosphorylation of pRb by cyclin D1/Cdk4 (Fig. 4A, lane 8 and Fig 4B, lane 8). By contrast, coexpression of p27 in cells expressing pRb, cyclin D1 and Cdk4 failed to do so (Fig. 4A, lane 11 and Fig. 4B, lane 11). Since equivalent amounts of p21 and p27 were expressed (data not shown), it is likely that the differential effects of p21 and p27 on pRb phosphor-

ylation status reflect their differential abilities to bind pRb. We suggest that because p21 binds directly to pRb, p21 may be a more stringent inhibitor of cyclin D1/Cdk4 phosphorylation than p27; that is, p21 but not p27 can inhibit the kinase activity of cyclin D1/Cdk4 already bound pRb. Since both p21 and p27 forms an active complex with cyclin D/Cdk4 (21), binding of p21 to pRb may enhance its inhibitory activity for pRb phosphorylation by cyclin D/Cdk4. Alternatively, direct interaction of p21 to pRb itself may prevent the phosporylation of pRb by masking its phosphorylation site(s). Recently, Blain *et al.* reported that p27, unlike p21, inhibited the cell cycle progression through inhibiting cyclin A/Cdk2 but not cyclin D/Cdk4 (22). This different mode of inhibition of Cdks by p21 and p27 may be explained by their abilities to bind pRb.

As previously reported, coexpression of pRb, E2F1, cyclin D1 and Cdk4 prevented the interaction of pRb with E2F1 (4), presumably due to phosphorylation of pRb by cyclin D1/Cdk4 (Fig. 4A, lane 10 and Fig. 4B, lane 10). When p21 was expressed in the cells that also expressed pRb, E2F1, cyclin D1 and Cdk4, pRb-E2F1 complexes were readily detectable (Fig. 4A, lane 9).

Effect of pRb Phosphorylation on p21 Binding

Many proteins that bind pRb, such as viral oncoproteins, the E2F transcription factor and cyclin D (2), bind the hypophosphorylated, but not the hyperphosphorylated, form of pRb. Our results described suggest that p21 binding prevents pRb phosphorylation. However, it is also possible that the phosphorylation state of pRb influences its ability to bind p21. The following experiments were designed to test this possibility.

Coinfection of viruses expressing pRb and p21 in Sf9 cells maintained pRb in a predominantly hypophosphorylated state (Fig. 4B, lane 2). The pRb/p21 complex was purified from these cell lysates using an anti-pRb antibody. The immunoprecipitate was then phosphorylated *in vitro* using an excess amount of several cyclin/Cdk complexes expressed in insect cells. The resultant pRb complexes were then analyzed by SDS-PAGE and autoradiography. In control experiments, apyrase was used in place of ATP, in order to degrade any endogenous cellular ATP. Almost complete dissociation of p21 from the pRb immunocomplex was observed when cyclin A or cyclin E/Cdk2 was used; partial dissociation was observed in the case of cyclin D1/Cdk4 or Cdk6 (Fig. 5). Dissociation of p21 from pRb was not observed in control experiments performed in the absence of ATP, suggesting that phosphorylation of pRb —not p21 binding to cyclin/Cdk complexes—was responsible for the dissociation. Thus, pRb/p21 complex may be dynamically regulated by the relative activities of p21 and cyclin A or E/Cdk2.

Finally, the p21 CKIs are involved in a number of diverse cellular processes such as cellular senescence (23), quiescence (14), differentiation (24–27), apoptosis (28) and carcinogenesis (29, 30). Our results suggest that direct interaction of p21 to pRb may play an important role in these processes.

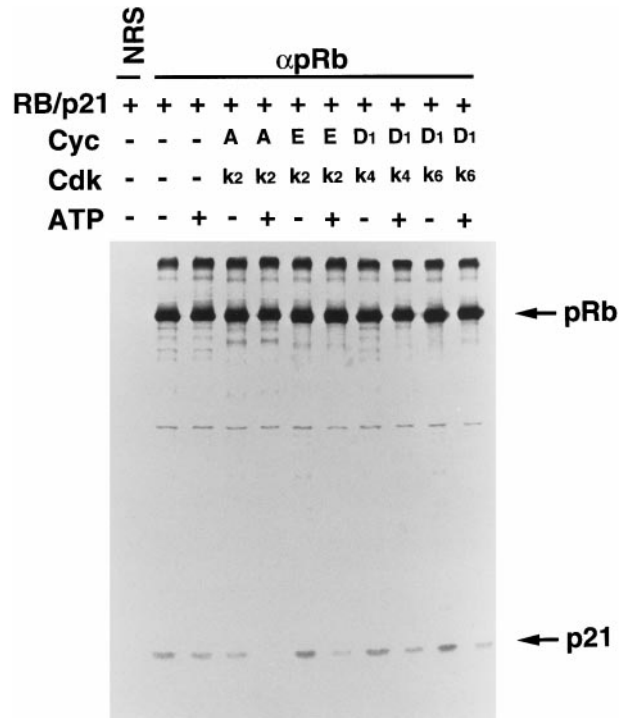

FIG. 5. Phosphorylation of pRb by cyclin/Cdk complexes disrupts the pRb-p21 interaction. Sf9 cells were infected with baculoviruses encoding pRb and p21. [^{35}S] labeled pRb/p21 complexes were immunoprecipitated by an anti-pRb polyclonal antibody, and then incubated with the indicated cyclin/Cdk complexes, expressed in insect cells, in the presence or absence of ATP. To eliminate the effect of endogenous ATP from the cell lysates, apyrase was used for the reactions lacking ATP. Normal rabbit serum (NRS) was used as a negative control. The reaction mixes were then analyzed by SDS-PAGE and autoradiography.

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