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The Retinoblastoma Protein Physically Associates with the Human cdc2 Kinase

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The protein product (pRB) of the retinoblastoma susceptibility gene functions as a negative regulator of cell proliferation, and its activity appears to be modulated by phosphorylation. Using a new panel of anti-human pRB monoclonal antibodies, we have investigated the biochemical properties of this protein. These antibodies have allowed us to detect a pRB-associated kinase that has been identified as the cell cycle-regulating kinase $p34^{cdc2}$ or a closely related enzyme. Since this associated kinase phosphorylates pRB at most of the sites used in vivo, these results suggest that this kinase is one of the major regulators of pRB. The associated kinase activity follows the pattern of phosphorylation seen for pRB in vivo. The associated kinase activity is not seen in the G_1 phase but appears in the S phase, and the levels continue to increase throughout the remainder of the cell cycle.

The retinoblastoma susceptibility gene (*RB-1*) is the best studied and characterized of the tumor suppressor genes. The retinoblastoma protein (pRB) is thought to function as a negative regulator of cellular proliferation (reviewed in reference 77). Support for this view comes from a number of different experimental approaches, but perhaps the strongest evidence is the observation that both alleles of the *RB-1* gene are mutated in all retinoblastomas examined to date (10, 20, 25–27, 49, 82). It is believed that the loss of a functional pRB cripples one key element in growth control in these cells. Moreover, analogous *RB-1* mutations have also been identified in a number of other tumors, including osteosarcomas, small-cell lung carcinomas, breast cancers, bladder carcinomas, and prostate carcinomas (6, 25–27, 32, 36, 38, 39, 48, 65, 71, 73, 75, 76, 83).

Further interest in the function of the retinoblastoma gene product has come from the observation that some of the DNA tumor virus transforming proteins, such as the adenovirus E1A proteins, simian virus 40 (SV40) large T antigen, and human papillomavirus E7 protein, form specific complexes with pRB (15, 22, 78). Genetic studies have shown that the transforming activity of these oncoproteins is dependent on regions that are essential for binding to pRB (reviewed in reference 30). Taken together, these observations suggest that the transforming abilities of these oncoproteins may depend, at least in part, on their ability to bind to and sequester pRB. Therefore, it is believed that the binding of these viral oncoproteins to pRB may mimic the loss of the *RB-1* gene seen in naturally occurring tumors.

Mapping studies have shown that two discrete regions of pRB are required for interaction with E1A or large T antigen (41, 42, 44). Since these regions correspond to those that are most frequently mutated in naturally occurring tumors, this finding suggests that E1A, large T antigen, and E7 may bind to and inactivate an important functional domain within pRB. This hypothesis is supported by the observation that in vivo expression of the SV40 large T antigen in the retinas of transgenic mice produces heritable tumors that resemble retinoblastomas (79).

To date, very little is known about the mechanism by which pRB controls cellular proliferation. It is known that pRB is found in most mammalian cells, even those that are rapidly dividing (8, 12, 16, 57). These same studies have shown that the levels of pRB do not vary substantially during the cell cycle, suggesting that whatever the mechanism by which pRB exerts its negative control, it must be regulated by some type of posttranslational modification. This concept is supported by the observation that there is a correlation between the phosphorylation state of pRB and cell cycle progression (8, 12, 16, 57). Unphosphorylated pRB is found in the G_0 and G_1 phases of the cell cycle, while phosphorylated pRB is found in the S, G_2, and M phases. Moreover, SV40 large T antigen binds exclusively to the un- or underphosphorylated form of pRB found at G_0 and G_1 (54, 55). This finding has led to the proposal that it is the unphosphorylated form of pRB that is the active tumor suppressor and that phosphorylation of pRB may act as a key switch in allowing cells to progress through the cell cycle.

To understand the role of pRB in the control of cellular proliferation, we have used anti-pRB monoclonal antibodies to study the biochemical properties of pRB. Here we report that pRB can form a specific complex with $p34^{cdc2}$, a serine/threonine kinase which is required for cell cycle progression in all eukaryotic cells (1, 5, 7, 18, 19, 21, 28, 46, 64, 66, 69). Furthermore, tryptic mapping shows that this associated kinase phosphorylates pRB at sites that are utilized in vivo, suggesting that $p34^{cdc2}$ or a related kinase acts to regulate pRB in vivo.

MATERIALS AND METHODS

Cell culture and labeling. All cells were cultured at 37°C on 100-mm tissue culture plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For

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labeling proteins, cells were incubated at 37°C in serum-free labeling medium for 20 min prior to addition of any labeling materials. Then the cells were labeled for 4 h at 37°C with 500 μCi of Trans[^{35}S]-label (ICN) per plate in 2 ml of methionine-free medium or with 2 mCi of $^{32}P_i$ (carrier free; ICN) per plate in 2 ml of phosphate-free medium.

Immunoprecipitation. Cells were collected by centrifugation and lysed on ice for 30 min in 1 ml of lysis buffer (250 mM NaCl, 0.1% Nonidet P-40, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.0], 5 mM EDTA, 50 mM NaF, 0.1 mM sodium orthovanadate, 50 μg of phenylmethylsulfonyl fluoride per ml, 1 μg of leupeptin per ml, 1 μg of aprotinin per ml, and 1 mM dithiothreitol). The lysate was precleared with 40 μl of normal rabbit serum and 100 μl of fixed and killed *Staphylococcus aureus* (Zymed), and the supernatant was used in immunoprecipitation. Fifty microliters of tissue culture supernatant from monoclonal antibody hybridoma cells was added in each reaction mixture as described by Harlow et al. (34). The proteins were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and the gel was processed by fluorography for ^{35}S-labeled proteins and visualized by autoradiography (4, 35, 47).

For preclearing experiments, lysates were treated by using standard immunoprecipitation conditions except that the levels of antibody and immunoadsorbent were increased 10-fold. The supernatants from the first preclearing immunoprecipitation step were then precleared a second time under identical conditions. After the second preclearing, a standard immunoprecipitation was carried out as described above.

In vitro kinase reaction. Unlabeled cell lysate was immunoprecipitated as described above. The protein concentration of the lysate was adjusted to about 300 μg/ml. One hundred microliters of tissue culture supernatant of monoclonal antibody hybridoma cells (XZ77, XZ91, and PAb419) or 4 μl of a polyclonal antiserum (G6) was added to each reaction mixture. The immunoprecipitation beads were washed twice with the lysis buffer and once with kinase buffer (50 mM HEPES [pH 7.0], 10 mM $MgCl_2$ 5 mM $MnCl_2$, 1 mM dithiothreitol). These conditions were optimized for the kinase activity found associated with adenovirus E1A immunoprecipitations (37), but identical results were found by using the buffers described by Simanis and Nurse (69). Kinase reactions were carried out at 25°C for 20 min in 50 μl of the kinase buffer and 5 μCi of [γ-^{32}P]ATP with 2.5 μg of histone H1 per reaction. The reaction was stopped by adding 50 μl of 2× Laemmli sample buffer (35, 47). The proteins were resolved on SDS-polyacrylamide gels and visualized by autoradiography. For quantitation, the autoradiograms were used as templates to excise the gel slices that contained the phosphorylated proteins. The amount of incorporated ^{32}P in each gel slice was then determined by Cerenkov counting.

For cell cycle experiments, cells were separated and analyzed by centrifugal elutriation as described by Buchkovich et al. (8). Briefly, after an asynchronous population of cells was separated into fractions based on cell size, the number of cells in each fraction was determined, and equal number of cells from each fraction were lysed and immunoprecipitated for kinase assays or immunoblotting. Samples from each sample were processed for propidium iodide staining to determine the relative DNA content. DNA content was measured with an EPICS C flow cytometer as described by Buchkovich et al. (8).

Two-dimensional tryptic mapping of the phosphorylated RB protein. The samples were excised from unfixed SDS-polyacrylamide gels, using the autoradiogram as a template. The proteins were then eluted, digested with trypsin, and resolved by two-dimensional electrophoresis as described by Lees et al. (50).

RESULTS

pRB immunoprecipitations contain an associated protein kinase activity. We have recently described a new set of anti-human pRB monoclonal antibodies (termed the XZ antibodies) that were raised against the portion of the pRB polypeptide that contains the E1A/large-T-antigen binding sites (40). All of these antibodies can immunoprecipitate native pRB from lysates of [^{35}S]methionine-labeled human cells. Several antibodies, such as XZ77 and XZ91, appear to recognize all forms of the protein, while others, such as XZ78 and XZ121, are specific for the un- or underphosphorylated forms of pRB (40) (Fig. 1A).

Since a number of experiments have suggested that phosphorylation is important for the regulation of pRB (8, 12, 16, 57), we investigated whether these monoclonal antibodies would allow us to detect pRB-associated kinases. Unlabeled myeloid leukemia cells (ML-1) were lysed, and proteins were immunoprecipitated with a panel of the XZ monoclonal antibodies, the anti-human cyclin A antibody C160 (29, 63, 78), the anti-human p34^{cdc2} antibody G6 (18), or the anti-SV40 large-T-antigen antibody PAb419 (33). The immunoprecipitated proteins were then tested for their ability to phosphorylate any of the polypeptides in the immune complex or exogenously added histone H1 (Fig. 1B). As expected, immunoprecipitations prepared with the G6 antiserum, which directly recognizes the human p34^{cdc2} kinase, showed high levels of histone H1 kinase activity. Similarly, the anti-cyclin A monoclonal antibody C160 also immunoprecipitated an active histone H1 kinase, as previously reported (29). The negative control antibody, PAb419, had little or no associated kinase activity. Somewhat surprisingly, immunoprecipitations with several of the XZ antibodies, such as XZ77, XZ91, XZ104, and XZ133, contained a strong kinase activity that phosphorylated both histone H1 and another prominent protein of approximately 105 kDa. To determine whether this 105-kDa substrate was pRB, we compared this phosphoprotein with authentic pRB that had been immunoprecipitated from ^{32}P-labeled ML-1 cells (Fig. 2). The 105-kDa protein phosphorylated in vitro had the same mobility on one-dimensional gels as did the in vivo-labeled pRB (Fig. 2A), and partial proteolytic mapping using *Staphylococcus aureus* V8 protease (13) showed that the major substrate in the in vitro kinase assay was pRB. Taken together, these experiments show that a subset of the XZ anti-pRB monoclonal antibodies immunoprecipitated a kinase that was able to phosphorylate both histone H1 and pRB itself.

Although the V8 maps shown in Fig. 2 indicated that the 105-kDa protein phosphorylated in the immunoprecipitation kinase assay was pRB, they also revealed one curious difference between the patterns of the in vivo- and in vitro-phosphorylated pRB. All of the fragments of pRB that were phosphorylated in vitro were also detected in the in vivo-phosphorylated pRB; however, the converse was not true. At least one of the partial proteolytic fragments that was phosphorylated in vivo was not obtained from the in vitro kinase reaction. This was the smallest fragment from the in vivo preparation of pRB (Fig. 2B). Therefore, at least at this level of resolution, the in vitro phosphorylations appear to represent a subset of the authentic in vivo-

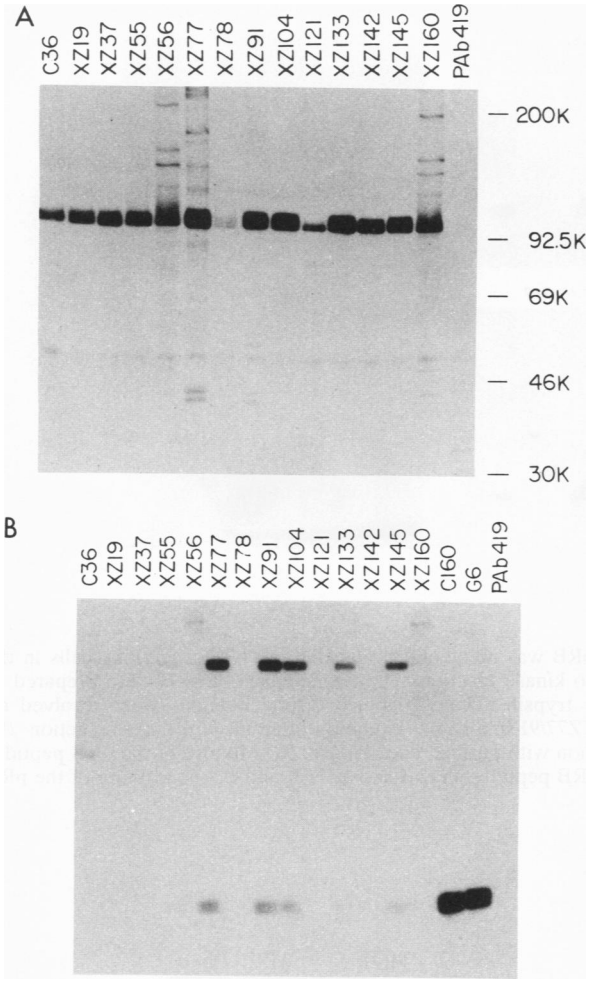

FIG. 1. Protein kinase in immunoprecipitations of pRB from ML-1 cells. (A) Lysates of ^{35}S-labeled ML-1 cells were immunoprecipitated with a panel anti-pRB monoclonal antibodies (C36 and XZ series) or with the anti-SV40 large-T-antigen monoclonal antibody PAb419 as a negative control. The proteins were resolved on a 6% polyacrylamide gel and visualized by fluorography and autoradiography. (B) Lysates of unlabeled ML-1 cells were immunoprecipitated with a panel of anti-pRB monoclonal antibodies (C36 and XZ series), the anti-cyclin A monoclonal antibody C160 and anti-p34^{cdc2} peptide antiserum G6 as positive controls, or the anti-SV40 large-T-antigen antibody PAb419 as a negative control. The immune complexes were used for in vitro kinase reactions that were performed in the presence of [γ-^{32}P]ATP and histone H1. The phosphorylated proteins were resolved on a 10% polyacrylamide gel and visualized by autoradiography.

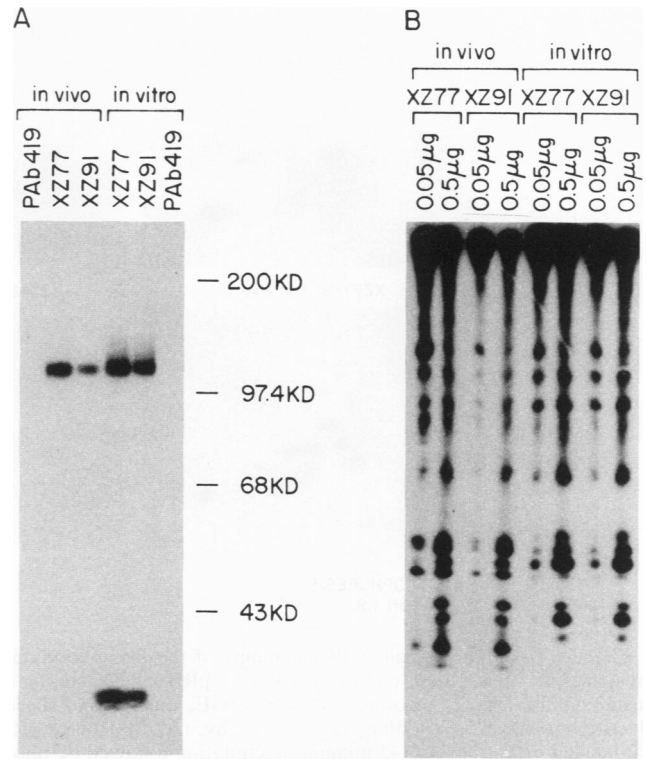

FIG. 2. Evidence that the 105-kDa kinase substrate is pRB. (A) Lysates of ^{32}P-labeled ML-1 cells were immunoprecipitated with the anti-pRB monoclonal antibody XZ77 or XZ91 or the anti-SV40 large-T-antigen monoclonal antibody PAb419 (labeled "in vivo"). The antibodies were used to immunoprecipitate proteins from lysates of unlabeled ML-1 cells and used for in vitro kinase reactions (labeled "in vitro"). Proteins were resolved on a 6% polyacrylamide gel and visualized by autoradiography. (B) The approximately 105-kDa ^{32}P-labeled protein bands were excised and compared by V8 partial proteolytic mapping. Each sample was digested with either 0.05 or 0.5 μg of V8 per lane, as noted. The digested proteins were resolved on a 15% polyacrylamide gel and visualized by autoradiography.

phosphorylated sites. To analyze these differences at a higher resolution, we compared these two sources of pRB by tryptic two-dimensional phosphopeptide mapping. As can be seen in Fig. 3A, D, and F, all of the phosphopeptides from the in vitro kinase reaction were found in vivo, but as expected, these were only a subset of the total phosphopeptides detected in vivo. Thus, the in vitro kinase assays allow phosphorylation of a subset of the authentic in vivo sites. These results suggest that either there is more than one pRB kinase in vivo or the conditions of the in vitro kinase reaction allow the modification of only certain of the pRB residues. Several of the phosphorylated sites have now been identified (50), and we know that serine residues at 249 and 811 as well as the threonine residues at 252 and 373 are phosphorylated both in vivo and in vitro. As described by Lees et al. (50), these sites appear to be consensus sites for p34^{cdc2} or p34^{cdc2}-related kinase phosphorylation.

Since this pRB kinase was detected by immunoprecipitation with anti-pRB antibodies, there were three possible ways to account for pRB phosphorylation in these experiments. First, pRB may be a kinase itself, and the phosphorylation that we have detected is due to an autocatalytic activity. Second, the XZ antibodies may bind to an unknown kinase by an immunological cross-reaction, and this unknown kinase is able to phosphorylate the pRB molecules in the immune complex. Third, the kinase may be coprecipitated indirectly by the XZ antibodies through an association with pRB. Since pRB shares no homology with either the catalytic domains or ATP-binding sites of any known kinase, we considered the first possibility to be unlikely. To distinguish the cross-reaction model from the association model, we chose to analyze a cell line with a homozygous deletion of the *RB-1* gene. If the kinase was precipitated by cross-reaction, then the XZ77 and XZ91 antibodies should be able to bind to the kinase in the absence of pRB. This kinase could then be detected by screening for phosphorylation of

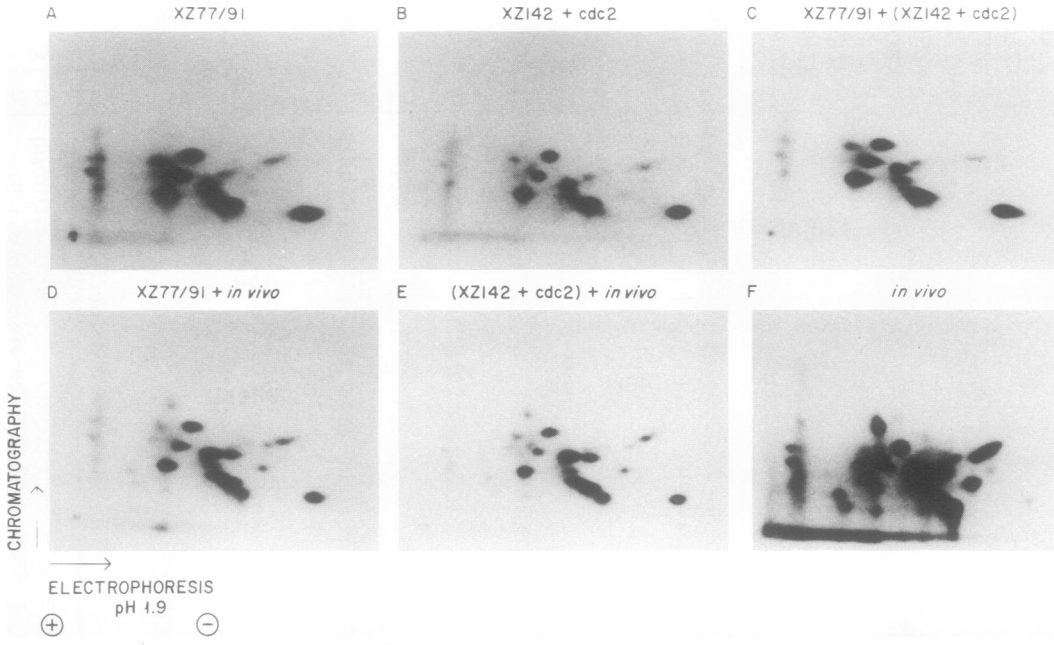

FIG. 3. Two-dimensional tryptic mapping of the phosphorylated pRB. pRB was metabolically labeled in vivo by growing cells in the presence of $^{32}P_i$ and then compared with the pRB labeled during an in vitro kinase reaction. The two samples of pRB were prepared as described for Fig. 2, resolved by SDS-PAGE, eluted, and digested with trypsin. The ^{32}P-labeled tryptic peptides were resolved by electrophoresis and ascending chromatography. (A) ^{32}P-labeled pRB from XZ77/91 immunoprecipitation in an in vitro kinase reaction. (B) ^{32}P-labeled pRB from XZ142 immunoprecipitation followed by phosphorylation with purified cdc2 kinase. (C) Mixture of the pRB peptides shown in panels A and B. (F) In vivo ^{32}P-labeled pRB. (D) Mixture of the pRB peptides shown in panels A and F. (E) Mixture of the pRB peptides shown in panels B and F.

the exogenously added substrate, histone H1. In WERI-1 cells, which have a homozygous deletion of the *RB-1* gene, both C160 and G6 were able to precipitate a potent histone H1 kinase, while immunoprecipitations with either XZ77 or XZ91 had no detectable kinase activity (Fig. 4). We have tested for the XZ77- and XZ91-associated kinase activity in a number of other cell lines; although the level of histone H1 phosphorylation varied from one line to another, in each case the detection of a kinase activity with XZ77 or XZ91 was dependent on the presence of functional pRB protein (data not shown). The suggestion that cross-reaction might account for the kinase activity is further discredited by the observation that multiple monoclonal antibodies recognizing independent epitopes on pRB (40) can precipitate this kinase activity.

To test the association model, we determined whether the kinase activity would copurify with the pRB protein (Fig. 5A). Lysates from the ML-1 myeloid leukemia cell line were layered on a 15 to 35% glycerol gradient, and after centrifugation, half of each fraction was immunoprecipitated with XZ91. Histone H1 kinase activity was precipitated with the anti-pRB monoclonal antibody only in those fractions that showed phosphorylation of pRB (fraction 15 to 17). This finding strongly supports the suggestion that the pRB kinase and the histone H1 kinase were coprecipitated through association with pRB.

The pRB-associated kinase is $p34^{cdc2}$ or a member of the $p34^{cdc2}$ family. The $p34^{cdc2}$ kinase is known to phosphorylate histone H1 in vitro, and recently it has been demonstrated that purified $p34^{cdc2}$ will also phosphorylate pRB in vitro (50, 53). Therefore, $p34^{cdc2}$ was a good candidate for the pRB-associated kinase. To test for associated $p34^{cdc2}$ kinase activity, the other half of the glycerol gradient fractions

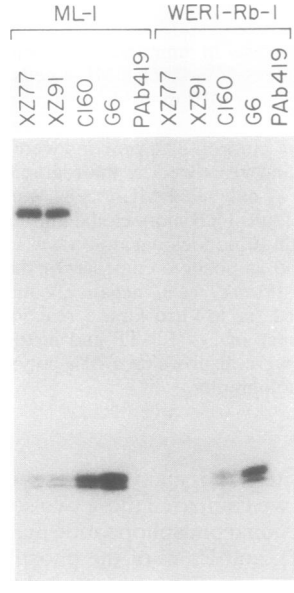

FIG. 4. Immunoprecipitation and in vitro kinase reactions with different cell lines. Lysates from ML-1 and WERI-1 cells were normalized to the same protein concentration and precipitated with an anti-pRB monoclonal antibody (XZ77 or XZ91), an anti-cyclin A monoclonal antibody (C160), an anti-$p34^{cdc2}$ peptide antiserum (G6), or an anti-SV40 large-T-antigen monoclonal antibody (PAb419). Kinase reactions were carried out in the presence of [γ-^{32}P]ATP and histone H1. The proteins were resolved on a 6% polyacrylamide gel and visualized by autoradiography.

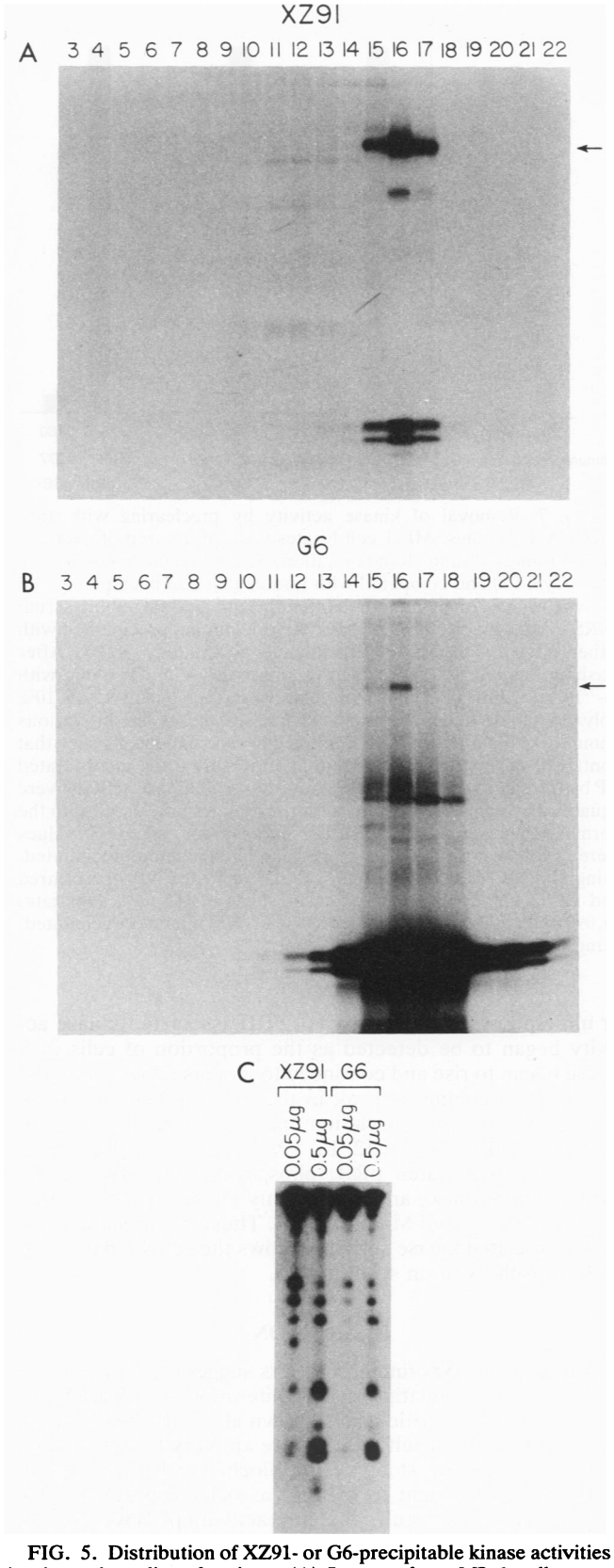

FIG. 5. Distribution of XZ91- or G6-precipitable kinase activities in glycerol gradient fractions. (A) Lysates from ML-1 cells were loaded on a 15 to 35% (vol/vol) glycerol gradient. After 24 h of centrifugation at 40,000 rpm at 4°C, the gradient was collected into 22 fractions. One half of each fraction was precipitated with monoclonal antibody XZ91 and then tested in the in vitro kinase assay in the presence of [γ-^{32}P]ATP and histone H1. (B) The other half of each fraction was precipitated with anti-p34^{cdc2} antisera (G6) and then assayed for in vitro kinase activity. (C) The ^{32}P-labeled proteins in fraction 15 to 17 of both panels A and B (marked by the arrow) were excised and compared by V8 proteolytic mapping, using 0.05 and 0.5 μg of V8 per lane, as indicated.

described above was immunoprecipitated with the G6 antiserum raised against the carboxy-terminal peptide of human p34^{cdc2} (Fig. 5B). As expected, the G6 antibody immunoprecipitated a potent histone H1 kinase from a large number of the fractions (11 to 22), but fractions 15 to 17 also contained a phosphorylated 105-kDa protein. When compared by V8 peptide mapping with pRB that was immunoprecipitated from the same fractions with XZ91 (Fig. 5A), this p34^{cdc2}-associated phosphoprotein was confirmed to be pRB (Fig. 5C). These results indicate that it is possible to detect an association between pRB and p34^{cdc2} by immunoprecipitation with antibodies specific for either component of this complex.

To confirm that p34^{cdc2} was associated with pRB by an independent, nonenzymatic assay, an immunoblot was performed (Fig. 6). ML-1 cell lysates were immunoprecipitated with M73, XZ55, XZ77, XZ91, G6, or normal rabbit serum. After separation by SDS-PAGE, the polypeptides were transferred to nitrocellulose filters, and the filter was probed with the anti-p34^{cdc2} antibody, G6. The G6 antibody easily detected the p34^{cdc2} protein in the G6 precipitation, as expected. The same or a closely related protein was also detected in the XZ77 and XZ91 immunoprecipitations, but at substantially lower levels. No p34^{cdc2} was found in association with XZ55, an anti-pRB monoclonal antibody that had no detectable kinase activity (Fig. 1B), or with any of the negative control antibodies. Therefore, the pRB complex contained not only kinase activity but also either p34^{cdc2} itself or a protein that was immunochemically related. This experiment also allowed us to estimate that approximately 1 to 5% of the total p34^{cdc2} protein found in ML-1 cells was associated with the retinoblastoma protein.

To determine whether other kinases in addition to p34^{cdc2} were found in the pRB complex, we compared the two-dimensional tryptic maps of pRB phosphorylated by the associated kinase with that of pRB phosphorylated by purified p34^{cdc2} kinase (Fig. 3). Since XZ142 had no detectable kinase activity in these experiments (Fig. 1), we used XZ142-precipitated pRB as a substrate for the purified p34^{cdc2} kinase (kindly provided by J. Bischoff and D. Beach). This p34^{cdc2}-phosphorylated pRB was then compared with the XZ77/XZ91-precipitated pRB that was phosphorylated in the in vitro kinase reaction. Since the patterns from the purified p34^{cdc2}-phosphorylated pRB and the XZ77/XZ91-phosphorylated pRB showed no difference (Fig. 3A and B), we suspect that p34^{cdc2}, or a p34^{cdc2}-related kinase, is likely to be the only kinase that is associated tightly with pRB. Furthermore, since the phosphotryptic maps of these in vitro-phosphorylated pRB samples comigrate with a subset of the spots from the in vivo-phosphorylated pRB (Fig. 3), these data suggest that p34^{cdc2} or a closely related enzyme is a major pRB kinase.

Characterization of the pRB-associated kinase. One of the properties of the p34^{cdc2} kinases is that part of their regulation involves interaction with polypeptide subunits known as cyclins. Many different cyclins have been identified, with

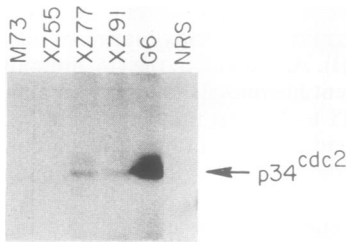

FIG. 6. Western immunoblotting analysis. Lysates from ML-1 cells were immunoprecipitated with an anti-E1A monoclonal antibody (M73), an anti-pRB monoclonal antibody (XZ55, XZ77, or XZ91), an anti-$p34^{cdc2}$ antiserum (G6), or normal rabbit serum (NRS). The proteins were resolved on an SDS–10% polyacrylamide gel and blotted on a nitrocellulose membrane. The membrane was probed with G6 antibody diluted 1:1,000 and then by ^{125}I-labeled goat anti-rabbit antibody. The bands were visualized by fluorography at −70°C.

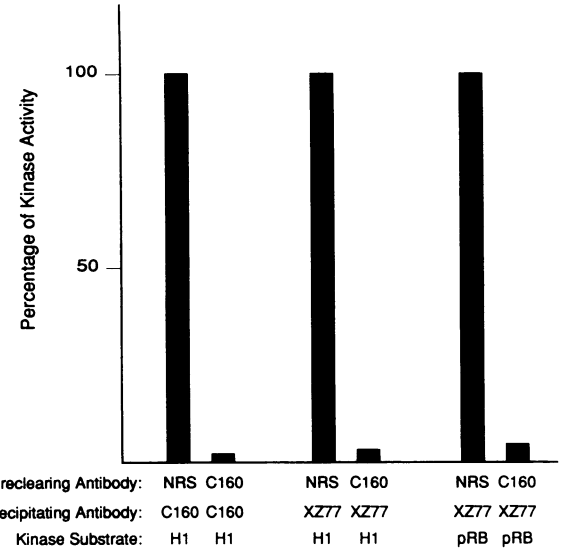

FIG. 7. Removal of kinase activity by preclearing with anti-cyclin A antibodies. ML-1 cell lysates were precleared of cyclin A by two rounds of immunoprecipitation with the cyclin A monoclonal antibody C160 and compared with lysates that had been precleared by two rounds of immunoprecipitation with normal rabbit serum (NRS). After preclearing, the lysates were immunoprecipitated with either C160 or the anti-pRB monoclonal antibody XZ77. After washing, histone H1 was added in kinase assay buffer along with [γ-^{32}P]ATP. Phosphorylated proteins were then resolved on 10% polyacrylamide gels. The levels of kinase activity in the various immunoprecipitations were determined by excising the gel slice that contained the particular substrate and measuring the incorporated ^{32}P by Cerenkov counting. The relative levels of kinase activity were equalized by adjusting the values for the samples precleared with the normal rabbit serum to 100%. For comparison, the 100% values were as follows: for NRS precleared and C160 immunoprecipitated, using histone H1 as a substrate, 108,633 cpm; for NRS precleared and XZ77 immunoprecipitated, using histone H1 as a substrate, 74,090 cpm; and for NRS precleared and XZ77 immunoprecipitated, using pRB as a substrate 104,440 cpm.

many new members of this class of proteins being identified in the last year (14, 31, 52, 56, 58, 59, 61, 62, 67, 70, 80, 81). Two of the best characterized are cyclins A and B. Preliminary experiments using immunoblots similar to the one shown in Fig. 6 suggested that immunoprecipitations of pRB contained detectable levels of human cyclin A (data not shown). However, while these experiments demonstrated that cyclin A was associated with pRB, they were unable to determine whether cyclin A and the pRB-associated kinase activity were present in the same or different pRB-containing complexes. To test whether cyclin A was found in the same complex that displayed the pRB kinase activity, a preclearing experiment was performed with monoclonal antibody C160 (Fig. 7). This antibody recognizes cyclin A but not any of the other human cyclins (B1, B2, C, D, or E) identified to date (49a). C160 was used to remove the immunoreactive material from lysates of ML-1 cells. Then an anti-pRB antibody, XZ77, was used to test for the presence of any remaining pRB-associated kinase activity. As a negative control, these same immunoprecipitations were also done from lysates that had been precleared with normal rabbit serum. To show that the preclearing worked as expected, we showed that the anti-cyclin A antibody could remove all of the cyclin A-associated histone H1 kinase activity detected by immunoprecipitation with C160. In these experiments, C160 antibodies removed greater than 95% of the pRB-associated kinase activity. This was true both for phosphorylation of pRB itself and for the added histone H1 substrate. Since it is not clear that all of the mammalian cyclins have been identified, we cannot preclude the possibility that C160 cross-reacts with another, as yet unidentified cyclin. However, this experiment confirms that the pRB-associated kinase contains a cyclin component and strongly suggests that this partner is cyclin A itself.

We next tested for changes in the appearance of the pRB-associated kinase activity during the cell cycle. Populations of CEM leukemic T cells representing different stages of the cell cycle were prepared by centrifugal elutriation (Fig. 8). Each population was analyzed by flow cytometry to determine the percentage of cells in the G_1, S, or G_2/M phase. After normalization for cell number, immunoprecipitations with XZ77 were performed and tested for pRB-associated kinase activity. As expected, each fraction had approximately the same level of pRB protein, as determined by immunoblot (data not shown). No associated kinase activity was seen in G_1 cells, where pRB is known to be under- or underphosphorylated in vivo. pRB-associated kinase activity began to be detected as the proportion of cells in S phase began to rise and continued to increase throughout the cell cycle, reaching its peak in the G_2- and M-phase cells. This profile of phosphorylation mirrors the phosphorylation of pRB found in vivo (8, 12, 16, 57). G_0 or G_1 cells have little or no phosphorylated pRB, phosphorylated pRB can be detected in S phase, and more highly phosphorylated forms are found in G_2- and M-phase cells. Thus, the appearance of pRB-associated kinase activity follows the general pattern of pRB phosphorylation seen in vivo.

DISCUSSION

A number of experimental results suggest that pRB plays a key role in regulating cell proliferation in a variety of tissues. However, little is yet known about the biochemical properties of pRB itself. Since there are very few clues with which to begin the study of the biochemical properties of pRB, we have sought to identify associated proteins. Our hope is that the molecules that interact with pRB will give us important clues as to the function of pRB. In essence, this strategy hopes to delineate the pathway in which pRB acts, identifying upstream regulators and downstream targets. In recent months, analogous studies have uncovered a group of

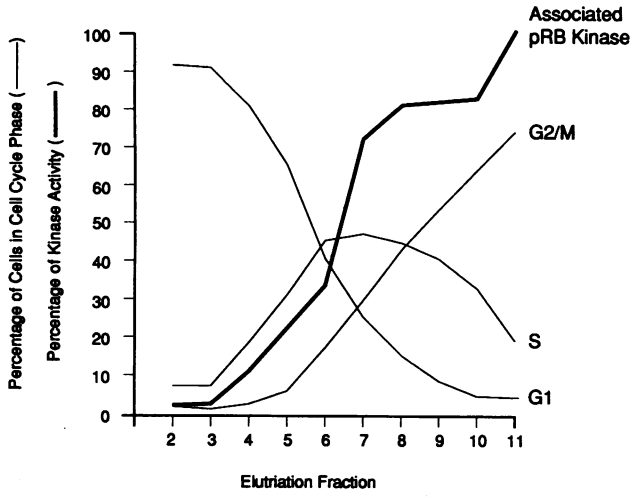

FIG. 8. Cell cycle regulation of the associated pRB kinase activity. Human CEM leukemic T cells were separated by centrifugal elutriation into populations that were enriched for cells from different stages of the cell cycle. The percentage of cells in the various stages of the cell cycle was determined by flow cytometry. Equal numbers of cells from each fraction were lysed, immunoprecipitated with the anti-pRB monoclonal antibody XZ77, and tested for associated kinase activity. Kinase reactions were carried out in the presence of [γ-^{32}P]ATP and histone H1. The levels of associated pRB kinase activity in the various immunoprecipitations were determined by excising the pRB gel slice and measuring the incorporated ^{32}P by Cerenkov counting.

proteins that are candidates for downstream targets for pRB. These include the E2F transcription factor (2, 3, 11), c-Myc (68), and proteins of unknown function that were identified or cloned by interaction with pRB (17, 45). In contrast to these interactions, the association between pRB and p34^{cdc2} described here most likely represents an element of the upstream regulation of pRB.

Our initial attempts to detect proteins that bind directly to pRB with sufficient avidity and at high enough stoichiometry to be detected by [^{35}S]methionine labeling have been unsuccessful. However, when immune complexes prepared by precipitating pRB with specific antibodies were examined for associated enzymatic assays, a potent kinase was detected. This associated kinase activity was able to phosphorylate both pRB and an exogenously added substrate, histone H1. The kinase activity was coprecipitated through its association with pRB, and immunoblotting and immunoprecipitation experiments indicated that the kinase is p34^{cdc2} or a closely related kinase. In addition, preclearing experiments confirm that this kinase has a cyclin partner, and they strongly suggest that this is cyclin A. At present, the possibility that the pRB-associated kinase is the product of a gene distinct from, but closely related to, p34^{cdc2} cannot be excluded. Such homologs have been found in other species (43, 51), and recent work from several laboratories, including our own, suggests that similar kinases will be found in human cells (23, 60, 74).

How closely does the in vitro kinase reaction reflect in vivo phosphorylation of pRB? It is always difficult to identify unequivocally the correct in vivo kinase for a particular protein, but the combined observations of several laboratories now argue strongly that pRB is phosphorylated in vivo by the p34^{cdc2} kinase or a related kinase. First, pRB's phosphorylation undergoes a cyclic oscillation during the cell cycle, and hence a kinase that is temporally regulated is the best candidate (8, 12, 16, 57). Second, purified pRB or synthetic peptides representing smaller regions of pRB can be efficiently phosphorylated in vitro by purified p34^{cdc2}, and the sites that are phosphorylated are the same sites that are modified in vivo (50, 53, 72). Third, the first five of the in vivo-phosphorylated residues that have been determined are surrounded by amino acids that fit the p34^{cdc2} consensus sequence (50). Fourth, in this report we show that pRB can be isolated from in vivo sources in a physical association with p34^{cdc2} or a related kinase. Together, these data argue that p34^{cdc2} or a related kinase is likely to be a genuine pRB kinase. Since phosphorylation appears to be a major mechanism of pRB regulation, it will be important to determine how this phosphorylation alters the activity of pRB.

Although our data argue strongly that this cdc2-like/cyclin A complex is a genuine pRB kinase, a number of observations suggest that some other kinase(s) will contribute to pRB phosphorylation in vivo. First, the pRB-associated kinase phosphorylates most but not all of the sites that are modified in vivo. Second, the initial phosphorylation of pRB occurs prior to the G_1/S transition, several hours before cyclin A is known to be expressed (57a). Third, this pRB-associated kinase appears to be present at very low levels. This conclusion is based on our inability to detect either cyclin A or p34^{cdc2} in anti-pRB immunoprecipitations from [^{35}S]methionine-labeled cells, even though cyclin A is found in association with the pRB-related protein, p107, under similar conditions (24). Together, these data argue strongly for the existence of at least one other in vivo pRB kinase.

What are the biological consequences of the association of cdc2 and pRB? A simple explanation for the demonstration of this complex is that it is an unusually stable enzyme/substrate intermediate. If this is true, then one would expect to find the pRB/cdc2 complex only at points in the cell cycle where pRB is normally phosphorylated, a prediction that agrees with our findings. What makes the stable enzyme/substrate model unattractive is that it removes an element of the dogma for enzyme reactions, i.e., that enzymes provide amplification, one molecule catalyzing a number of reactions. Obviously, binding to a substrate diminishes or delays the amplification of an enzyme. However, this interaction might add other important features. For example, binding to a substrate might help localize an enzyme until it was time to trigger its activity. This suggestion is particularly attractive for cell cycle-regulating enzymes. The p34^{cdc2} kinase would then be poised, bound to an important substrate, when the correct activating signal was received. Alternatively, pRB might act as a chaperon to bring p34^{cdc2} and other important molecules together. Other stable enzyme/substrate interactions have been reported. Perhaps the best characterized of the recent work on stable enzyme/substrate interactions involves the association of the platelet-derived growth factor receptor with a number of its substrates (recently reviewed in reference 9).

A different view is seen if the stable enzyme/substrate hypothesis is incorrect. Then one must suggest that pRB's association acts to control p34^{cdc2} activity. Whichever model is correct, either that pRB is a substrate and acts downstream of p34^{cdc2} or that pRB acts upstream and is a substrate through a feedback loop, one is forced to position pRB in direct apposition to the cell cycle control machinery. This provides physical evidence for a signal transduction pathway linked through pRB and suggests that key proteins in the control of the cell cycle will be important elements in this pathway.

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