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# Antibodies Specific for the Human Retinoblastoma Protein Identify a Family of Related Polypeptides

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**Even though the retinoblastoma gene is one of the best-studied tumor suppressor genes, little is known about its functional role. Like all tumor suppressor gene products, the retinoblastoma protein (pRB) is thought to inhibit some aspect of cell proliferation. It also appears to be a cellular target of several DNA tumor virus-transforming proteins, such as adenovirus E1A, human papillomavirus E7, or simian virus 40 large T antigen. To help in the analysis of pRB, we have prepared a new set of anti-human pRB monoclonal antibodies. In addition to being useful reagents for the study of human pRB, these antibodies display several unexpected properties. They can be used to distinguish different subsets of the pRBs on the basis of their phosphorylation states. Some are able to recognize pRB homologs in other species, including mice, chickens, and members of the genus *Xenopus*. In addition, some of these antibodies can bind directly to other cellular proteins that, like pRB, were originally identified through their association with adenovirus E1A. These immunologically cross-reactive proteins include the p107 and p300 proteins, and their recognition by antibodies raised against pRB suggests that several members of the E1A-targeted cellular proteins form a structurally and functionally related family.**

Studies of the retinoblastoma gene (*RB1*) have greatly enforced the notion that tumor development is advanced not only by the gain of dominantly acting mutations but also by the loss of proteins that normally restrict cell growth (1, 27, 37). Inactivation of both alleles of the *RB1* gene has been found in all retinoblastoma tumors that have been studied to date, as well as in many small-cell lung carcinomas, osteosarcomas, breast carcinomas, soft tissue sarcomas, bladder carcinomas, and prostate carcinomas (3, 5, 7, 11, 13, 18–20, 26, 31–36, 41, 43). Evidence to support the notion that retinoblastoma protein (pRB) acts as a tumor suppressor comes from studies that show that the reintroduction of a wild-type pRB cDNA into cells without a functional retinoblastoma gene suppresses or delays tumorigenesis (4, 22).

The *RB1* gene product (pRB) also forms a complex with several DNA tumor viral oncoproteins, including the adenovirus E1A proteins, the polyomavirus large T antigens, and human papillomavirus E7 proteins (6, 9, 30, 38). More important, genetic studies have shown that the sequences required for these oncoproteins to form complexes with pRB are also needed for oncogenesis (6, 10, 28, 29, 39, 40). These studies strongly suggest that binding to pRB is essential for the oncogenic properties of these viral proteins. Moreover, the sequences essential for pRB to interact with E1A or simian virus 40 (SV40) T antigen are frequently mutated in the naturally occurring pRB mutants (21, 23, 24). This implies that these oncoproteins have evolved to target one of the important functional domains of pRB. It is believed that viral proteins, such as E1A or large T antigen, bind to and inactivate an important function of pRB, thus mimicking the loss of retinoblastoma gene function seen in many human tumors. This concept is further supported by studies with transgenic mice that show that the expression of the SV40 large T antigen in the retinas of mice produces heritable mouse retinoblastoma. These tumors are histochemically indistinguishable from naturally occurring human tumors, which are thought to be caused by the loss of the retinoblastoma gene.

Why is it so important for E1A or large T antigen to form complexes with pRB? To answer this question, we need to know both the biochemical properties of pRB and the manner in which the viral proteins change these biochemical functions. To help in these studies, we have prepared a set of monoclonal antibodies specific for pRB. In addition to providing a useful set of reagents for pRB, these antibodies have several unexpected properties. Several of the new antibodies identify homologs for the human pRB in other species. In addition, some of the antibodies bind directly to other human proteins, most notably, other cellular proteins that interact with the E1A proteins. This suggests that, as well as binding to E1A, these cellular proteins share structural similarities with pRB.

## MATERIALS AND METHODS

**Hybridoma production.** Hybridomas were prepared by standard protocols (16). Briefly, the carboxy-terminal polypeptide representing approximately 60 kDa of the pRB coding region was overexpressed in bacteria and used to immunize female BALB/c mice. After high titers of antibodies specific for the p60 immunogen were detected, a representative mouse was sacrificed and its splenocytes were used for hybridoma production. By using pools of supernatants, positive tissue culture supernatants were detected by immunoprecipitation of in vitro-translated pRB polypeptides (21), and the correct cells were single cell cloned by using limiting dilution and single-cell picks (16). For the initial

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TABLE 1. Mapping of the XZ monoclonal antibody epitopes

| Monoclonal antibody | Results[a] of immunoprecipitations with: | | | | | | | | | Epitope location(s) |
|---|---|---|---|---|---|---|---|---|---|---|
| | pRBΔ1–393 | pRBΔ303–443 | pRBΔ536–619 | pRBΔ573–619 | pRBΔ622–645 | pRBΔ622–714 | pRBΔ666–928 | pRBΔ803–909 | pRBΔ911–928 | |
| XZ104 | + | − | − | + | + | ND | + | ND | ND | 393–572, 646–665 |
| XZ56, XZ61, XZ160, XZ161 | + | ND | ND | ND | ND | + | ND | + | + | 393–621, 715–802 |
| XZ75, XZ78, XZ156 | + | ND | ND | ND | ND | + | ND | ND | + | 393–621, 715–910 |
| XZ91 | + | + | + | + | − | − | + | ND | ND | 444–535 |
| XZ77, XZ133, XZ140, XZ150, XZ151 | + | + | + | + | − | ND | + | ND | ND | 444–535, 620–665 |
| XZ19, XZ105, XZ121 | + | + | − | − | + | + | + | + | + | 444–621 |
| XZ37, XZ55, XZ142 | + | + | − | − | + | ND | + | ND | ND | 444–621, 646–665 |

[a] ND, not determined.

pooling strategy, 10 μl of tissue culture supernatant from each well of a 96-well plate was pooled along either vertical columns (1, 2, . . . 12) or horizontal rows (A, B, . . . H) of a 96-well microtiter plate. Each pool was tested for anti-pRB antibodies, and the wells at the intersections of positive vertical and horizontal pools were retested individually to confirm the locations of positive clones.

**Cell labeling and immunoprecipitation.** 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 labeling, cells were incubated at 37°C in medium without methionine for 20 min prior to addition of any labeling materials. Except where noted, the cells were labeled for 4 h at 37°C with 500 to 1,000 μCi of Tran$^{35}$S-label (ICN) per plate in 2 ml of methionine-free medium. The labeled cells were collected 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). Cellular debris were removed by centrifugation at 10,000 × *g* for 1 min. The resulting lysate was precleared with 40 μl of normal rabbit serum and 100 μl of fixed, killed *Staphylococcus aureus* cells (Zymed), and the supernatant was used in immunoprecipitation. Fifty microliters of tissue culture supernatant from a hybridoma was added into each reaction as described by Harlow et al. (15). The proteins were resolved on sodium dodecyl sulfate (SDS)-polyacrylamide gels, and the gels were processed by fluorography and visualized by autoradiography (2, 16, 25).

**Western blotting (immunoblotting).** Six plates of 90% confluent 293 cells were lysed in 2 ml of lysis buffer. After preclearing with normal rabbit serum, half of the lysate was immunoprecipitated with 300 μl of anti-E1A monoclonal antibody M73 (15). The other half was precipitated with 300 μl of anti-SV40 large T antigen antibody PAb419 (14). Each of the immunoprecipitations was divided into two parts, and the proteins were resolved on an SDS–6% polyacrylamide gel and transferred to a nitrocellulose membrane (16). One half of the membrane was probed with XZ37 (1:5 dilution), and the other half was probed with XZ77 (1:5 dilution). The location of the antibodies was determined with $^{125}$I-labeled goat anti-mouse immunoglobulin G antibody (1:1,000 dilution; New England Nuclear). The proteins were visualized by autoradiography.

**Elution of p300 from M73 immunoprecipitations.** Two plates of 80% confluent 293 cells were labeled with 2.0 mCi of tran$^{35}$S-label (ICN). The cells were collected and lysed in 0.5 ml of lysis buffer as described above and precipitated with 0.5 ml of M73 antibody. The proteins were resolved on a 6% SDS gel and visualized by autoradiography without drying the gel. The 300-kDa protein band was cut out from the wet gel. The gel slice was passed through a 5-ml syringe several times in the presence of 3 ml of the lysis buffer and rocked in a cold room overnight. The supernatant was collected and replaced with 2 ml of fresh lysis buffer. After 4 h of rocking at 4°C, the supernatant was collected and combined with the previous one. The eluted p300 protein was concentrated by filtration with a Centricon 30 filter (Amicon) and then immunoprecipitated with XZ55, XZ91, XZ77, or M73.

## RESULTS

**Production of monoclonal antibodies for pRB.** The antigen used for immunizing mice was expressed in bacteria and represented the pRB polypeptide sequence from residues 387 to 928. This fragment encompasses the E1A- and large T antigen-binding regions of pRB, which have been mapped to residues 393 to 572 and 646 to 772 (21). After the second boost, the most responsive mouse was used to make hybridomas. In order to obtain antibodies that would recognize native forms of pRB, the screening procedure was conducted by using immunoprecipitations. The number of immunoprecipitations was reduced by pooling the hybridoma tissue culture supernatants. Labeled pRB polypeptides were prepared by in vitro transcription and translation by using the human pRB cDNA as a source of cRNA and rabbit reticulocyte lysates for the synthesis of [$^{35}$S]methionine-labeled proteins.

After positive hybridomas were single cell cloned, the locations of their epitopes on pRB were determined by immunoprecipitating pRB polypeptides synthesized from a set of deletion mutants that were prepared in our previous studies (21). Polypeptides were synthesized in vitro in the presence of [$^{35}$S]methionine and immunoprecipitated with each individual XZ monoclonal antibody. The results are shown in Table 1. Only positive immunoprecipitation data were used to determine the locations of the epitopes on pRB. If the epitope is formed by a linear series of amino acids, as would normally be seen if an antibody recognized a denatured antigen, negative results could be used to help narrow the location of an epitope. Since a preparation of denatured antigen may refold partially to produce a conformational epitope, deciding whether an antibody recognizes a linear or

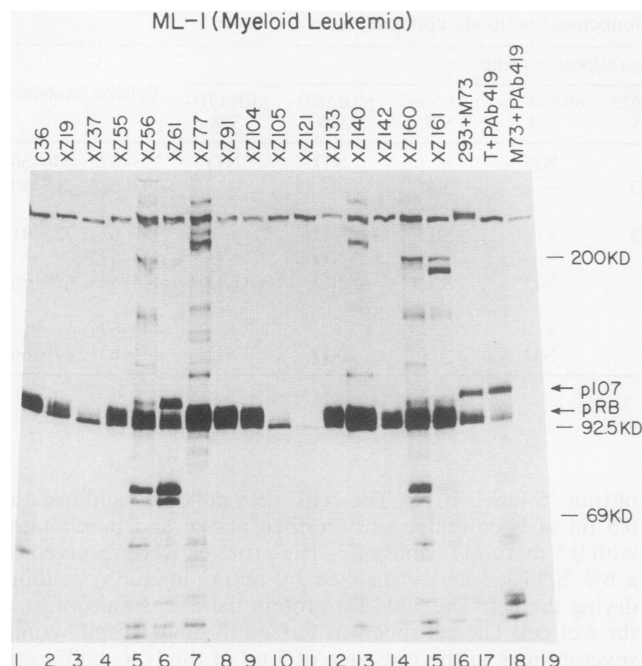



FIG. 1. Immunoprecipitations of pRB from ML-1 cells. $^{35}$S-labeled ML-1 cell lysates were precipitated with either C36 (anti-pRB; lane 1) or XZ antibodies (lanes 2 to 16). The labeled ML-1 lysate was also mixed with 293 cell lysate or purified SV40 large T antigen and precipitated with M73 (anti-E1A; lane 17) or PAb419 (anti-SV40 T antigen; lane 18), respectively. The proteins were resolved on a 6% polyacrylamide-SDS gel. The gel was processed by fluorography and visualized by autoradiography. The pRB and p107 bands are indicated.

conformational epitope has some unknown probability of failure. Consequently, using this distinction to classify antibodies for epitope mapping also has an unknown probability of misassigning the location. Therefore, we have not used any of the negative results to localize their epitopes further. Although a number of the antibodies map to the same regions of pRB, they often recognized different epitopes, as they have other distinguishing features. This is most noticeable when comparing the patterns of pRBs immunoprecipitated from cells (for examples, see XZ37, XZ77, and XZ91 in Fig. 1).

**Characterization of the anti-pRB antibodies.** To characterize the XZ antibodies further, each was tested for the ability to immunoprecipitate native pRB from human cells and for the ability to recognize the pRB on immunoblots. Human myeloid leukemia (ML-1) cells were chosen as the source of in vivo pRB because they have a high level of pRB. $^{35}$S-labeled ML-1 cell lysates were immunoprecipitated with the anti-pRB monoclonal antibodies (Fig. 1). The position of pRB on the gel was determined by comparison with authentic pRB detected through its interaction with E1A or SV40 large T antigen. E1A or large T antigen complexes were prepared by mixing lysates of [$^{35}$S]methionine-labeled ML-1 cells with lysates of unlabeled 293 cells as a source of E1A or by mixing ML-1 lysates with purified SV40 large T antigen (the kind gift of D. McVey and Y. Gluzman).

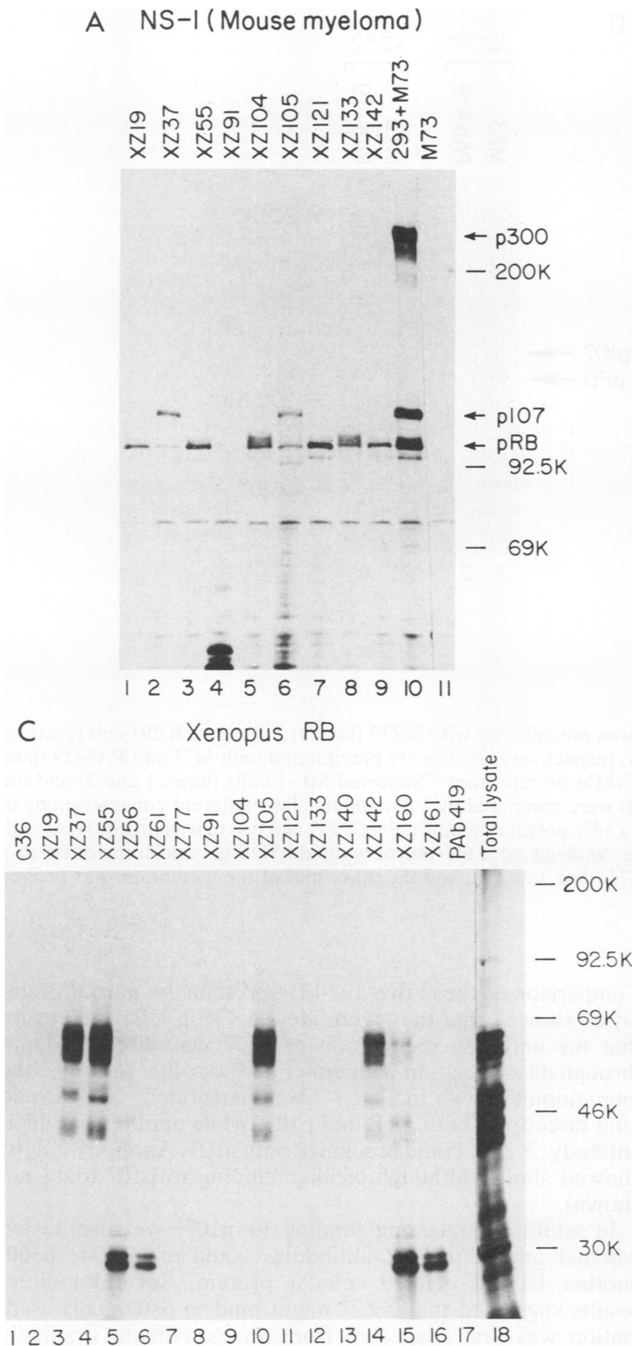
All of the XZ antibodies were able to recognize the native forms of pRB, although some apparently had preference for the status of pRB phosphorylation, as summarized in Table 2. pRB migrates as a broad series of bands on SDS-polyacrylamide gels because of phosphorylation. Some antibodies, such as XZ77 and XZ91, precipitated all the forms of pRB, whereas others, such as XZ19 and XZ55, preferred the underphosphorylated forms of pRB. In contrast, antibodies such as XZ121 were able to recognize only the unphosphorylated pRB. These preferences were also observed in immunoblot analysis (Table 2) and in the chicken pRB immunoprecipitation (Fig. 2B).

**Recognition of the pRB homologs in other species.** Analyzing pRB function in human cells has its limitations, particularly with studies of development and differentiation. Therefore, it was important to determine whether any of these

TABLE 2. Characterization of XZ antibodies

| Antibody | Binding of antibody to pRB[a] | | | | | Subclass |
|---|---|---|---|---|---|---|
| | Human pRB (IP) | Human pRB (WB) | Mouse pRB (IP) | Chicken pRB (IP) | *Xenopus* pRB (IP) | |
| XZ19 | ++ | ++ | ++ | ++ | + | IgG1 |
| XZ37 | ++ | ++ | + | + | + | IgG2a |
| XZ55 | ++ | ++ | ++ | ++ | + | IgG1 |
| XZ56 | +++ | ++++ | ++ | +++ | + | IgG2a |
| XZ61 | +++ | ++++ | ++ | +++ | + | IgG1 |
| XZ75 | ++ | ++++ | ND | ND | ND | IgG1 |
| XZ77 | ++++ | ++++ | − | ++++ | − | IgG2a |
| XZ78 | ++ | ++++ | ND | ND | ND | IgG1 |
| XZ91 | ++++ | ++++ | − | ++++ | − | IgG2a |
| XZ104 | ++++ | − | +++ | ++++ | − | IgG1 |
| XZ105 | ++ | ++ | + | + | + | IgG2a |
| XZ121 | + | + | + | + | + | IgG2a |
| XZ133 | ++++ | − | +++ | ++++ | − | IgG1 |
| XZ140 | ++++ | ++++ | − | ++++ | − | IgG2b |
| XZ142 | +++ | +++ | ++ | + | + | IgG1 |
| XZ150 | ++++ | ++++ | − | ++++ | − | IgG2a |
| XZ151 | ++++ | ++++ | − | ++++ | − | IgG1 |
| XZ156 | ++ | +++ | ND | ND | ND | IgG1 |
| XZ160 | +++ | +++ | ++ | +++ | + | IgG2a |
| XZ161 | +++ | +++ | ++ | ++ | + | IgG2a |

[a] Proteins were either immunoprecipitated (IP) or analyzed by Western blot (WB). Symbols: ++++, antibody precipitated all forms of pRB; +++ or ++, antibody preferred underphosphorylated pRB; +, antibody precipitated only underphosphorylated pRB. ND, not determined.



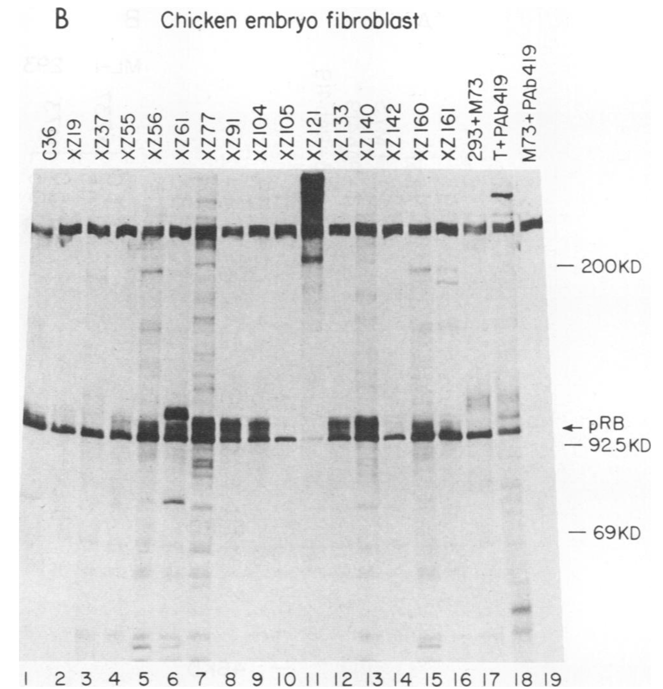




FIG. 2. Immunoprecipitations of pRB homologs from other species. (A) $^{35}$S-labeled mouse myeloma (NS-1) cell lysates were precipitated with XZ antibodies (lanes 1 to 9) or mixed with 293 cell lysates and precipitated with M73 (lane 10). The pRB, p107, and p300 bands are indicated. (B) $^{35}$S-labeled chicken embryo fibroblast cell lysate was precipitated with C36 (lane 1) or XZ antibodies (lanes 2 to 16) or mixed with 293 cell lysate or purified SV40 T antigen and precipitated with M73 or PAb419, respectively. pRB is indicated. (C) *Xenopus* pRB cDNA was in vitro transcribed and translated in rabbit reticulocyte lysates in the presence of [$^{35}$S]methionine. The labeled *Xenopus* pRB polypeptides were precipitated with C36 (lane 1), XZ antibodies (lanes 2 to 16), or PAb419 (lane 17, as a negative control). Lane 18 contained the total in vitro-translated *Xenopus* pRB polypeptides without precipitation.

anti-human pRB antibodies could recognize homologs in other species. Because authentic pRBs have not been identified in all species, we used a combination of criteria to identify potential pRB homologs. These criteria included similar molecular masses, the ability to bind to E1A or large T antigen, and, if available, pRB cDNA clones. Obviously, this information was immediately supplemented by the experimental results themselves; i.e., the ability to be recognized by multiple monoclonal antibodies lends strong support for the identity of a pRB homolog.

Figure 2A shows the immunoprecipitation of pRB from mouse myeloma cells (NS-1), in which several of the XZ antibodies were able to recognize a protein of around 105 kDa. The antibodies for which data are not shown failed to bind to the 105-kDa protein. On the basis of its molecular mass, recognition by multiple monoclonal antibodies, and association with either E1A or large T antigen, we feel confident that this protein is mouse pRB. Figure 2B presents the immunoprecipitation of pRB from chicken embryo fibroblasts. All the XZ antibodies, as well as C36 (38), were able to precipitate the chicken pRB on the basis of the criteria mentioned above. Why avian pRB is recognized by more of the XZ antibodies than the rodent pRB is not clear, although this result has been independently confirmed by others by using independent sources of chicken embryo fibroblast cells (10a).

We also checked the ability of the XZ antibodies to recognize *Xenopus* pRB. The cDNA for the *Xenopus* pRB has recently been cloned and characterized by Destrée and colleagues (6a). The *Xenopus* pRB cDNA shows homology to exons 2 through 27 of the human pRB gene, with several regions showing stretches of 15 or more identical residues. The overall homology is more than 50% identity at the amino acid level. This cDNA was kindly provided to us to test the ability of the XZ antibodies to recognize the *Xenopus* pRB. We tested whether in vitro translation products prepared from this construct were able to be recognized by the

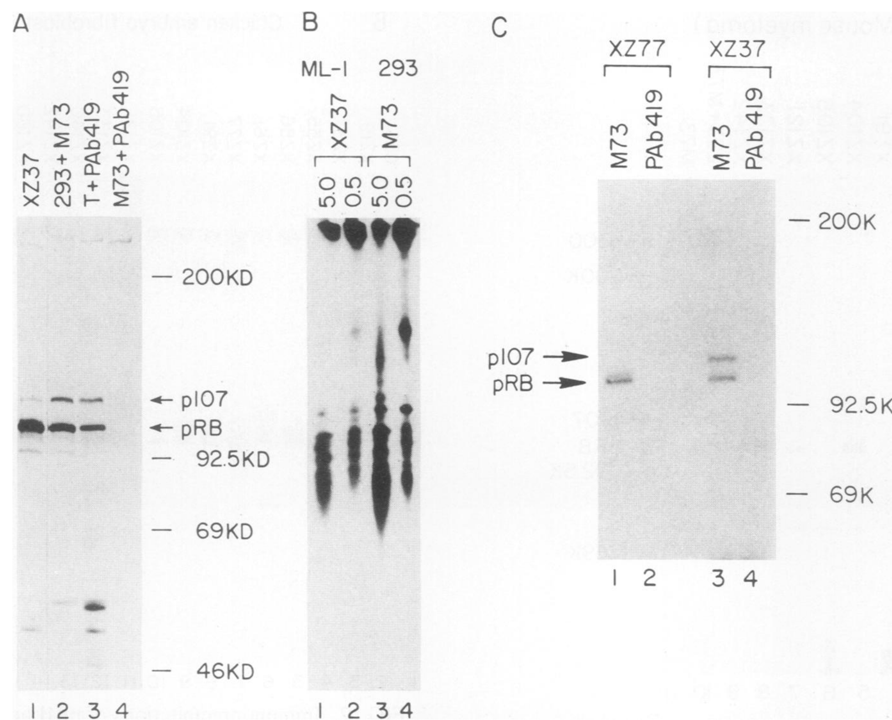



FIG. 3. XZ37 binds directly to p107. (A) $^{35}$S-labeled ML-1 cell lysate was precipitated with XZ37 (lane 1), mixed with 293 cell lysate or with SV40 T antigen and precipitated with M73 (lane 2) or PAb419 (lane 3), respectively, or directly precipitated with M73 and PAb419 (lane 4). The pRB and p107 bands are indicated. (B) The XZ37-precipitated 107-kDa protein from $^{35}$S-labeled ML-1 cells (lanes 1 and 2) and the M73-precipitated 107-kDa protein from $^{35}$S-labeled 293 cells (lanes 3 and 4) were compared by V8 mapping. Two different concentrations of V8 were used: 5.0 and 0.5 μg per lane. The polypeptides were resolved on a 15% polyacrylamide gel. (C) 293 cell lysate was precipitated with either M73 (lanes 1 and 3) or PAb419 (lanes 2 and 4). The proteins were resolved on a 6% polyacrylamide-SDS gel and transferred to a nitrocellulose membrane. One half of the membrane was probed with XZ77 (lane 1 and 2), and the other half of the membrane was probed with XZ37 (lanes 3 and 4). The pRB and p107 bands are indicated.

antibodies. The cDNA was cloned in an in vitro transcription vector and cRNA was prepared for in vitro translations as described previously (21). The particular cDNA used for this experiment was missing the amino-terminal approximately 100 nucleotides and therefore could not produce full-length *Xenopus* pRBs. As seen in Fig. 3C, the total translation products showed extensive initiations at internal methionines. This is common for long RNAs translated in vitro or for RNAs without efficient translational initiation sites. The most predominant of the *Xenopus* pRB products ran with a molecular mass of approximately 68 kDa. Many of the XZ antibodies were able to recognize these *Xenopus* RB polypeptides. Some of the XZ antibodies also recognized smaller proteins made from these clones. These appear to be authentic pRB translation products, but we do not understand the origin of these fragments, although internal initiations are the most likely source.

**Cross-reaction with other E1A-targeted cellular proteins.** From genetic studies, it is known that E1A uses similar sequences to target a number of cellular proteins, including pRB, p107, p300, p130, and cyclin A (12, 17, 40, 42). This observation suggests that these cellular proteins might share some sequence or structural similarity, particularly in the E1A-binding regions. To explore this possibility, we examined each XZ antibody carefully for cross-reactions with other cellular proteins. As shown in Fig. 3A, at least one of these antibodies, XZ37, was able to immunoprecipitate a 107-kDa protein in addition to pRB. This protein comigrated precisely with the E1A- and T antigen-associated p107. Comparison of these two 107-kDa proteins by partial proteolysis showed that they were identical (Fig. 3B). To ensure that the antibody recognition of p107 was direct and not through its association with either pRB or other proteins, the immunoblot shown in Fig. 3C was performed. XZ37 could bind directly to both pRB and p107, while another anti-pRB antibody, XZ77, could recognize only pRB. Antibody XZ105 showed similar although weaker binding to p107 (data not shown).

In addition to testing binding to p107, we also tested whether any of the XZ antibodies could precipitate p300, another E1A-associated cellular protein. Several indirect results suggested that XZ77 might bind to p300. This association was first suggested from an experiment originally performed for a different purpose. As shown in Fig. 4A, when lysates from 293 cells, which constitutively express the adenovirus E1A proteins, were precleared with a nonspecific antibody, such as the anti-SV40 large T antibody PAb419, and then immunoprecipitated with the anti-E1A antibody M73, both the E1A and associated proteins were readily detected (Fig. 4A, lane 2). However, when anti-pRB antibody XZ77 was used to preclear similar lysates, an unexpected result was obtained. The XZ77 antibody removed all of the pRB that was detectable in this reaction, but the antibodies also removed the p300 proteins. The p107 and cyclin A bands were unaffected. This indicated one of two possibilities: either the XZ77 antibody removed p300 through an interaction with pRB, or it bound to p300 directly. To test the first possibility, similar preclearing experiments

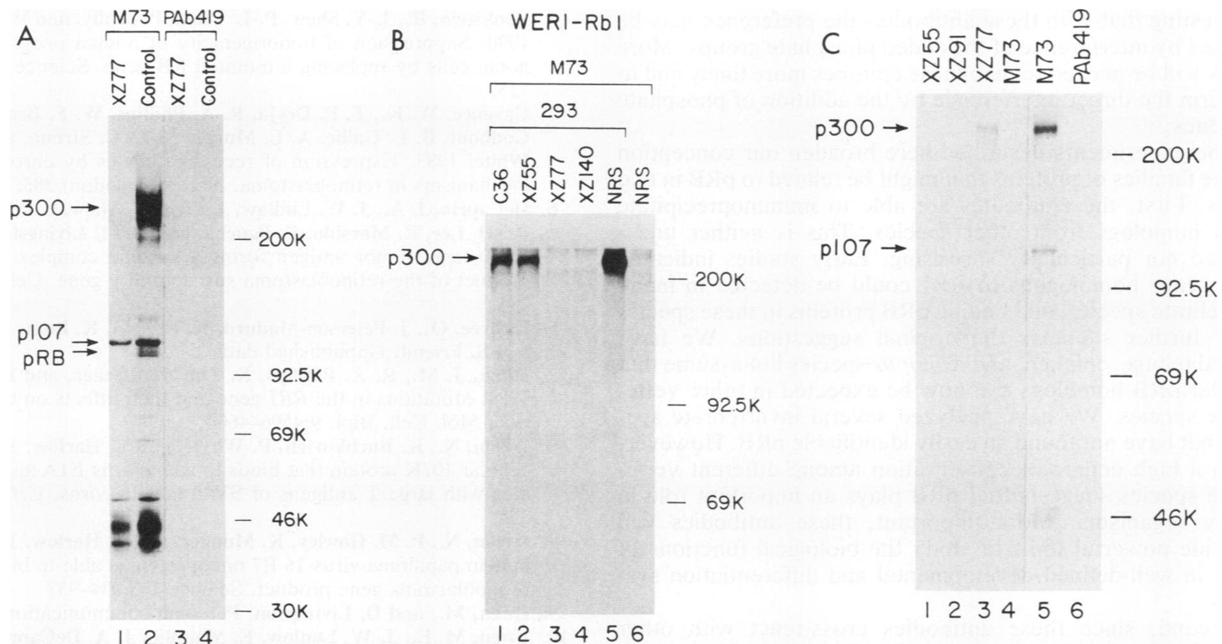



FIG. 4. XZ77 binds directly to p300. (A) [$^{35}$S]methionine-labeled cell lysates were precleared with XZ77 (lanes 1 and 3) or PAb419 (lanes 2 and 4). The supernatants were then immunoprecipitated with M73 (lanes 1 and 2) or PAb419 (lanes 3 and 4). (B) [$^{35}$S]methionine-labeled WERI-1 cell lysates were precleared with different antibodies as indicated. The supernatants were mixed with unlabeled 293 cell lysates and then immunoprecipitated with M73. (C) $^{35}$S-labeled 293 cell lysates were precipitated with M73. The p300 protein band was excised and eluted from the gel slice. Then the purified p300 was precipitated with XZ55 (lane 1), XZ91 (lane 2), XZ77 (lane 3), and M73 (lane 4). Lanes 5 and 6 are immunoprecipitations of $^{35}$S-labeled 293 lysates with M73 and PAb419, respectively. The pRB, p107, and p300 bands are indicated.

were performed with a cell line that lacked pRB (Fig. 4B). WERI-1 cell lysates were precleared with a series of anti-pRB antibodies, and the resulting lysates were mixed with a cold lysate of 293 as a source of E1A. The mixed lysates were then immunoprecipitated with anti-E1A antibodies. Most of the anti-pRB antibodies did not affect the pattern of bands that could associate with E1A. However, XZ77 and another antibody, XZ140, both removed the p300 band prior to binding to E1A.

To prove that the recognition was direct, we tested whether XZ77 could bind to purified p300 (Fig. 4C). Because of the technical difficulty of transferring large proteins to nitrocellulose for use in an immunoblot, we chose an alternative approach. Lysates from 293 cells, which constitutively express E1A, were immunoprecipitated with M73, an antibody specific for E1A. The immunoprecipitated proteins, including the p300 polypeptides, were resolved on SDS-polyacrylamide gel electrophoresis, and the p300 protein band was excised. This protein was eluted and then reprecipitated with different antibodies. XZ77 precipitated the eluted p300, indicating that XZ77 directly recognized p300 in the absence of other proteins. Similar results were also seen with XZ140 (data not shown).

These results show that several of the antibodies raised against pRB directly recognize epitopes on other E1A-associated proteins. These cross-reactions suggest that these proteins carry structural similarities that are recognized as common epitopes. Since the number of epitopes that have been used is limited, we do not know from these experiments the extent of similarity among the proteins. These antibodies appear not to be directly at the site of interaction with E1A, since they can precipitate pRB with associated E1A complexes (data not shown). Likewise, the addition of these antibodies to in vitro binding assays between E1A and pRB is not sufficient to block the interaction of these two proteins. However, these antibodies do appear to link pRB, p107, and p300 structurally. Recently, Ewen and colleagues have cloned the cDNA for p107 and have shown that it carries extensive homology with pRB (9a). Therefore, the ability of the antibodies raised against pRB to recognize p107 probably represents a legitimate structural relationship. Since the cDNA for p300 is not yet cloned, the ability of pRB antibodies to directly recognize p300 provides the first suggestion that these two proteins are structurally related. This finding also provides the first reagents for studying p300 in the absence of its association with E1A.

## DISCUSSION

We have obtained a new set of anti-human pRB monoclonal antibodies with epitopes located in the carboxy-terminal half of the pRB polypeptide. In contrast, the epitopes for C36 and other previously prepared anti-pRB monoclonal antibodies are located in the amino-terminal half of the protein. Although all the XZ antibodies can recognize the native forms of pRB, several have a clear preference for different phosphorylation states. Some of them, such as XZ77 and XZ91, can precipitate all forms of pRB; some, such as XZ19 and XZ55, prefer the underphosphorylated pRB; and a few, such as XZ121, recognize only unphosphorylated pRB. Since these antibodies see many different sites on pRB, their ability to distinguish between different phosphorylated forms suggests that pRB must undergo major conformational changes following phosphorylation. With some antibodies, such as XZ37 and XZ121, these preferences are also seen when pRB is detected by immunoblots,

suggesting that with these antibodies the preference may be caused by interference of the added phosphate groups. More work will be needed to map these epitopes more finely and to confirm the direct interference by the addition of phosphate residues.

The experiments described here broaden our conception of the families of proteins that might be related to pRB in two ways. First, the antibodies are able to immunoprecipitate pRB homologs from other species. This is neither unexpected nor particularly surprising. Early studies indicated that genes homologous to RB1 could be detected in many vertebrate species, and finding pRB proteins in these species only further supports the original suggestions. We have tested mouse, chicken, and *Xenopus* species but assume that similar pRB homologs can now be expected in other vertebrate species. We have analyzed several invertebrate species but have not found an easily identifiable pRB. However, such a high degree of conservation among different vertebrate species suggests that pRB plays an important role in many organisms. More important, these antibodies will provide powerful tools to study the biological functions of pRB in well-defined developmental and differentiation systems.

Second, since these antibodies cross-react with other cellular proteins, the family of proteins that are related to pRB is expanded by these studies. Several of the XZ antibodies were able to bind directly to other cellular proteins that were originally identified through their interaction with adenovirus E1A polypeptides. Genetic studies had indicated that E1A uses similar regions to target several cellular proteins, including p300, p130, p107, pRB, and cyclin A (12, 40). Therefore, it is possible that these cellular proteins share some structural similarities, at least in the E1A-targeted regions. Using the XZ antibodies, we have shown that at least one antibody, XZ37, can directly recognize both pRB and p107. More surprisingly, we also have identified a second antibody, XZ77, that can directly recognize both pRB and p300. While the properties of p107 and pRB had suggested that they might be structurally related (8, 10), there was little indication that p300 and pRB might be related. It is also somewhat surprising that the two cross-reactions that were detected here were seen with different monoclonal antibodies. Nevertheless, these results continue to suggest that pRB is one example of an extended family of cellular proteins that show structural homology, particularly in the E1A-large T antigen interaction domains.

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