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The retinoblastoma protein binds to RIZ, a zinc-finger protein that shares an epitope with the adenovirus E1A protein

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ABSTRACT **The retinoblastoma protein (Rb) is a target of viral oncoproteins. To explore the hypothesis that viral proteins may be structural mimics of cellular proteins, we have searched cDNA libraries for Rb-binding proteins. We report here the cloning of a cDNA for the protein RIZ from rat and human cells. RIZ is a 250-kDa nuclear protein containing eight zinc-finger motifs. It contains an Rb-binding motif that is related to that of the adenovirus E1A oncoprotein; RIZ also shares an antigenic epitope with the C terminus of E1A. A domain is conserved between RIZ and the PRDI-BF1/Blimp-1 differentiation factor. Other motifs of RIZ include putative GTPase and SH3 (*src* homology domain 3) domains. RIZ is preferentially expressed in both adult and embryonic rat neuroendocrine tissues. It is also expressed in human retinoblastoma cells and at low levels in all other human cell lines examined. While the function of RIZ is not yet clear, its structure and pattern of expression suggest a role for RIZ in transcriptional regulation during neuronal differentiation and pathogenesis of retinoblastoma.**

The retinoblastoma protein (Rb) is a tumor suppressor that is thought to normally function in transcriptional control of the cell cycle and differentiation (1, 2). The elucidation of the molecular mechanisms of Rb action was greatly advanced by the finding that Rb is a target of the oncogenic products of DNA tumor viruses, as illustrated by the physical association of Rb with the adenovirus E1A protein, simian virus 40 large T antigen, and human papillomavirus E7 protein (3–5). These viral proteins use the same motif, known as cr2 and characterized by the core sequence LXCXE, to bind Rb, even though they do not share overall sequence similarity and are not evolutionarily related. This observation suggests that viral oncoproteins may be structural mimics of cellular Rb-binding proteins which normally mediate Rb function.

To explore this hypothesis, we screened cDNA libraries by using Rb as the probe. Others have used similar approaches to uncover several Rb-binding proteins, the best studied of which is the E2F-1 transcription factor (6–9). We report here the cloning of a cDNA for the Rb-binding protein RIZ. Different from other cellular Rb-binding proteins, RIZ has both extensive sequence homology and specific antigenic similarity to E1A.†

MATERIALS AND METHODS

cDNA Cloning of RIZ. A linker encoding the EE epitope, EEEEYMPME (10), was ligated into the plasmid pET8Rbc (11) to generate a bacterially expressed, epitope-tagged Rb. Bacterial lysates precipitated with 60% ammonium sulfate were used for screening a rat cardiac myocyte λgt11 cDNA library (gift from H. Zhu, University of California, Los Angeles) as described (12). Anti-EE monoclonal antibody (gift from G. Walter, University of California, San Diego) and alkaline phosphatase-conjugated rabbit anti-mouse antibody (Promega) were used to identify Rb-positive plaques. Full-length cDNA was cloned from a rat B49 tumor cell cDNA library (gift from W. Stallcup, La Jolla Cancer Research Foundation) and human fetal brain and retinoblastoma Y79 cDNA libraries (Clontech). Both strands of RIZ cDNA were sequenced using Sequenase (United States Biochemical).

Plasmid Construction. For glutathione *S*-transferase (GST)–RIZ fusion proteins, segments of rat RIZ cDNA encoding amino acids 245–573 and amino acids 215–412 were ligated into pGEX-KG (13) to generate pKG7.1S and pKGSE, respectively. The full-length rat RIZ cDNA was cloned into pRc-CMV vector (Invitrogen) to generate pCMVRIZ, which was used for T7 *in vitro* transcription/translation to produce full-length RIZ protein containing eight extra amino acid residues (MEYMPMEF) at the N terminus. Mutagenesis was performed by using the T7 GEN mutagenesis kit (United States Biochemical). The E1A 12S plasmids for *in vitro* transcription and GST fusion proteins were gifts of P. Raychaudhuri (University of Illinois, Chicago) and W. Kraus (Duke University, Durham, NC), respectively.

RNA Analysis, Antibody Production, and Immunoprecipitation. Standard procedures were followed for these experiments (14, 15). RNA samples of rat embryonic tissues were a gift from W. Stallcup. Two polyclonal antisera were produced: one by injecting a rabbit with the GST–RIZ fusion protein produced from pKG7.1S, the other by injecting mice with the fusion protein of pKGSE. Monoclonal antibody 2D7 was generated by using the pKGSE GST fusion protein as the immunizing antigen. 2D7 did not react with the pKG7.1S fusion protein, indicating that its epitope was located between amino acid residues 215 and 244.

Cells were lysed in EIA lysis buffer (ELB; 250 mM NaCl/0.1% Nonidet P-40/50 mM Tris·HCl, pH 7.0), and proteins bound to protein A-Sepharose were washed four times with ELB as described (3, 7). Immunoblotting was done using alkaline phosphatase-conjugated second antibodies. For ^{32}P-labeling of cells, rat B50 cells (a gift from W. Stallcup) were grown in RPMI 1640 medium containing 10% (vol/vol) fetal calf serum and 3 mCi of [^{32}P]orthophosphate (1 Ci = 37 GBq) for 3 hr before lysis. The *in vitro* Rb-binding assay was performed by mixing p56Rb with reticulocyte-lysate-translated RIZ protein in ELB buffer followed by immunoprecipitation with anti-Rb antibodies.

RESULTS

An epitope-tagged, 56-kDa, C-terminal fragment of Rb was generated to screen a rat cDNA library. Of the five specific clones obtained, one encodes the rat homolog of the previously reported human RBP1 protein (6). The other four clones were found to be identical, and sequence analysis of one of them, clone 7.1, revealed two motifs: the LXCXE motif and three

Abbreviations: Rb, retinoblastoma protein; GST, glutathione *S*-transferase; SH3, *src* homology domain 3.

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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U17837 and U17838).

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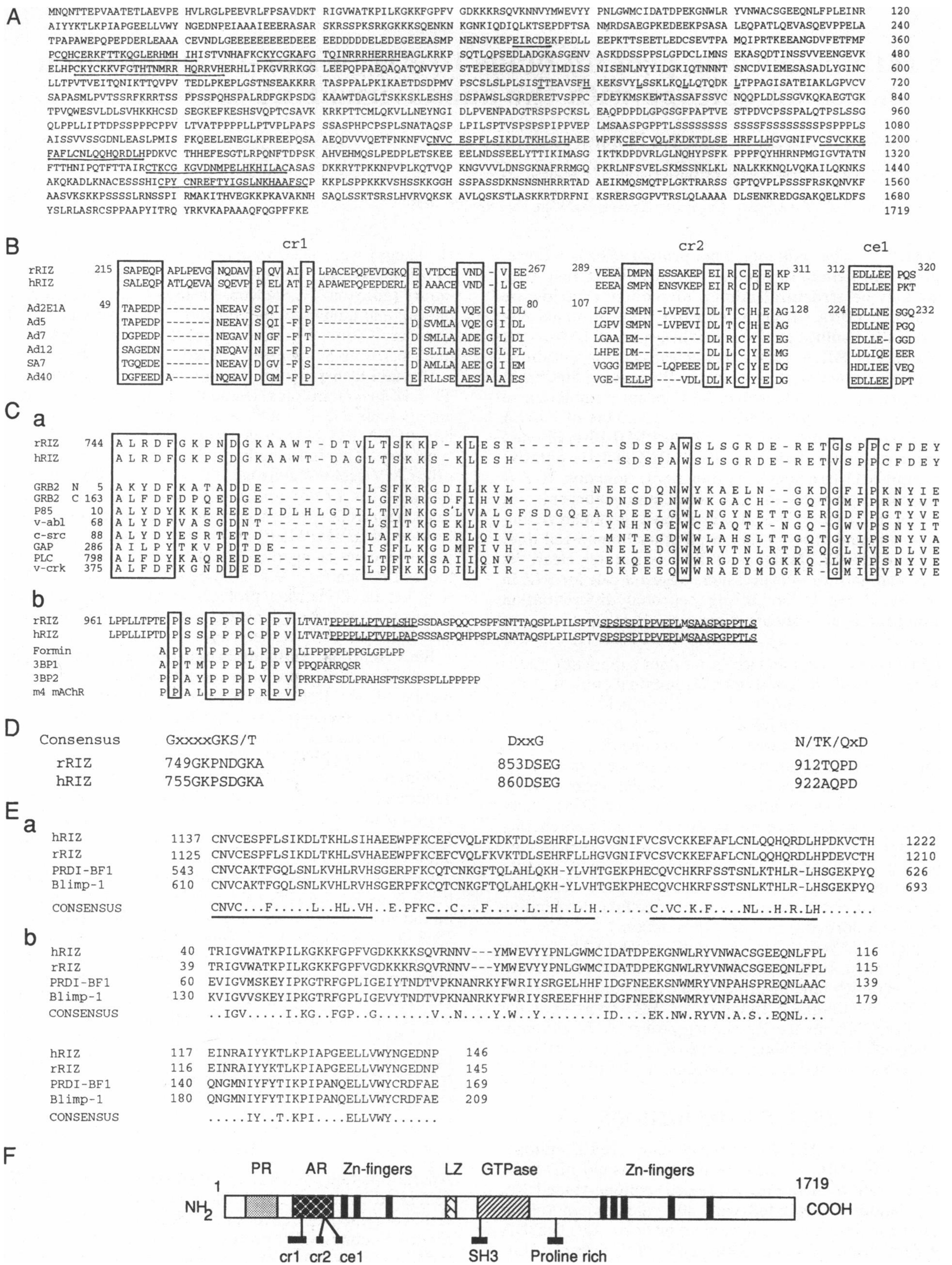

FIG. 1. Analysis of RIZ domains. (*A*) Full-length amino acid sequence of human RIZ protein (amino acids 1–1719). The cr2 motif, zinc-finger motifs, and a leucine zipper are underlined. (*B*) Alignment of human and rat RIZ sequence with E1A proteins of different strains of adenoviruses (16–18). ce1, Conserved epitope 1. (*C*) *src* homology domain 3 (SH3) and SH3-binding motifs (*a*). Comparison of rat and human RIZ with known SH3 domain proteins (*b*). One of the SH3-binding motifs is compared with known SH3-binding proteins and the rest are underlined. (*D*) Identification of possible GTPase motifs in rat and human RIZ. (*E*) RIZ is related to PRDI-BF1/Blimp-1. (*a*) Alignment of RIZ zinc fingers 4–6 with PRDI-BF1/Blimp-1 zinc fingers 1–3. The C2-H2 motifs are underlined in the consensus sequence. (*b*) PRDI-BF1 and RIZ homologous domain (PR) domain alignment for the same proteins as in *Ea*. (*F*) Schematic map of human RIZ domains. AR, acidic region or EIA-related region; LZ, leucine zipper.

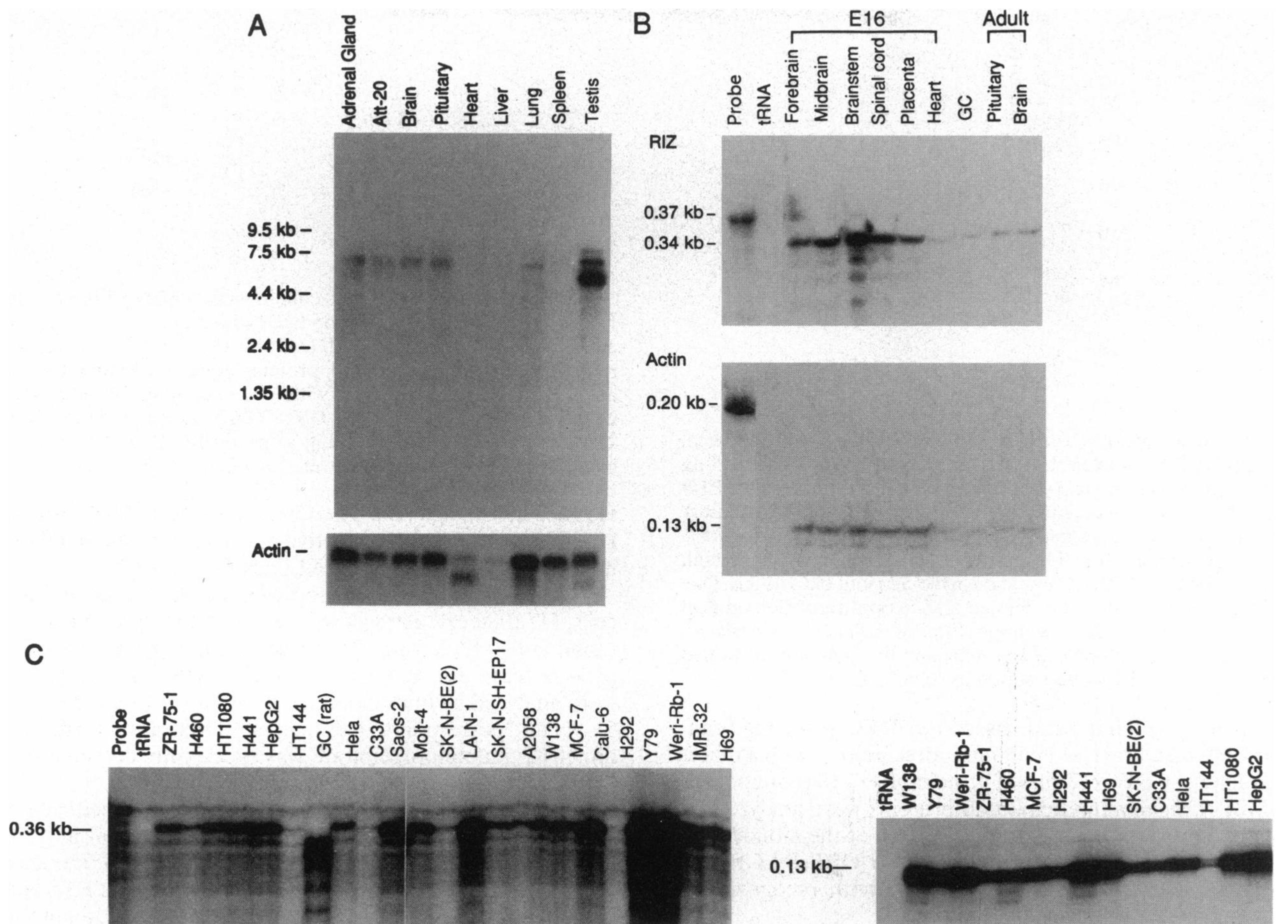

FIG. 2. RNA analysis. (*A*) Northern blot of RIZ mRNA in adult rat tissues [2 μg of poly(A)$^+$ mRNA in each lane; Att-20 is a murine cell line]. After hybridization with the RIZ probe (the 1.9-kb insert of clone 7.1), the blot was rehybridized with a rat β-actin probe. (*B*) RNase-protection experiment in which 20 μg of total RNA from embryonic day 16 (E16) or adult rat tissues was used to protect a rat RIZ cRNA probe (amino acids 463–574) or a rat β-actin probe as a control. (*C*) RNase-protection experiment in which 20 μg of total RNA from each listed human cell line was used to protect a human RIZ cRNA probe (amino acids 457–579) (*Upper*) or a rat β-actin probe as control (*Lower*).

zinc-finger motifs (see below). Hence, the protein encoded by clone 7.1 was designated RIZ for Rb-interacting zinc-finger protein. The 1.9-kb insert of clone 7.1 (amino acids 245–883) was used to screen several cDNA libraries to obtain full-length rat and human RIZ cDNAs. Analysis of the complete cDNA sequences revealed a large open reading frame of 1706 amino acids in rat and 1719 amino acids in human. The human protein sequence is shown in Fig. 1*A* and is 84% identical to the rat sequence.

The RIZ protein sequence contains a number of interesting motifs. A region of 20 amino acids near the N terminus of RIZ is about 45% similar to the cr2 motif of E1A (Fig. 1*B*). Nearby, an E1A cr1-related region was also found (16). In the middle part of RIZ, we noted a GTPase domain, an SH3 domain, and a proline-rich domain with several SH3-binding motifs (19–21) (Fig. 1 *C* and *D*). A data base search revealed 6 C2-H2 (fingers 1–6) and 2 C2-HC (fingers 7–8) types of zinc-finger motifs (22) (Fig. 1 *A* and *F*). The most significant homology was found for fingers 4–6, which are 39% identical to fingers 1–3 in the human PRDI-BF1 protein and its murine homolog Blimp-1 (23–25) (Fig. 1*Ea*). In addition, a region of ≈100 residues, designated the PR domain, is 42% identical between RIZ and PRDI-BF1/Blimp-1 (25) (Fig. 1*Eb*).

Northern blot analysis showed a 7.2-kb major RIZ mRNA species in rat neuroendocrine tissues; testis also contained a shorter form of the mRNA (Fig. 2*A*). RNase-protection experiments detected RIZ mRNA in day 16 rat embryonic neural tissues (Fig. 2*B*). Analysis of human cell lines revealed RIZ mRNA in retinoblastoma cells Y79 and Weri-Rb-1 and lower level RIZ expression in all other human cell lines examined (Fig. 2*C*).

To examine RIZ binding to Rb, *in vitro* translated RIZ protein was produced from the rat cDNA construct pCMVRIZ by using T7 RNA polymerase and reticulocyte lysate. The full-length product ≈250 kDa is larger than the predicted 190 kDa (Fig. 3*A*, lane 1). This difference may be due to anomalous migration of RIZ in SDS polyacrylamide gels. Upon incubation with Rb, the full-length and a series of truncated RIZ products of larger than ≈65 kDa were coimmunoprecipitated by anti-Rb antibody (Fig. 3*A*). The truncated products could be recognized by an anti-RIZ monoclonal antibody 2D7 specific for an N-terminal region (amino acids 215–244) of RIZ, and hence were probably products of premature termination. This indicated that the N-terminal ≈65-kDa segment may contain the Rb-binding domain; we estimated that this segment might be encoded by the N-terminal ≈350 amino acids on the basis of anomalous migration observed for several RIZ N-terminal fragments by SDS/PAGE (data not shown). Together, the data suggested that the Rb-binding site of RIZ may be located in a small region near residue 350.

To determine whether the nearby putative cr2 motif IR-CEE-309 is necessary for RIZ binding to Rb, single amino acid substitution was introduced into RIZ cDNA to change Cys-307 to Gly. *In vitro* translated, full-length, wild-type and mutant RIZ were compared for Rb binding. The wild-type RIZ bound to Rb, but the point mutant did not (Fig. 3*B*). We also found that the regions of Rb involved in binding RIZ overlap with the T antigen/E1A-binding domain (I.M.B. and S.H., unpublished data).

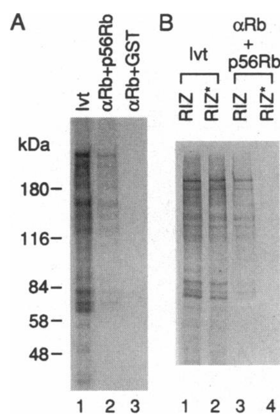

FIG. 3. RIZ interacts with Rb *in vitro*. (*A*) *In vitro* translated, full-length RIZ from pCMVRIZ (Ivt, lane 1) was incubated with 0.5 μg of either purified GST protein (lane 3) or p56Rb (lane 2) in 0.5 ml of ELB buffer. The mixture was immunoprecipitated with anti-Rb antisera (αRb), and the immunoprecipitated proteins were resolved by SDS/7.5% PAGE. (*B*) Rb binding of full-length, wild-type RIZ and a point mutant, RIZ*, with the Cys-307 to Gly mutation in the cr2 motif IRCEE-309. One microliter of either wild-type or mutant RIZ *in vitro* translation product was loaded in lane 1 or 2, respectively; 10 μl of each *in vitro* translation product was mixed with p56Rb and αRb, and the immunoprecipitated proteins (lanes 3 and 4) were resolved by SDS/7.5% PAGE.

To identify cellular products of the RIZ gene, rat brain tumor B50 cell extracts were immunoprecipitated with a rabbit serum against GST–RIZ fusion protein pKG7.1S (amino acids 245–573). The immunprecipitated proteins were analyzed by immunoblot with either a mouse antiserum or the monoclonal antibody 2D7, both raised against another GST–RIZ fusion protein pKGSE (amino acids 215–412); both recognized a 250-kDa protein (Fig. 4*A*).

Immunoprecipitation followed by immunoblot of nuclear or cytoplasmic fractions of cells showed that RIZ is located in the nucleus of the cell (Fig. 4*B*). Immunoprecipitation of extracts of [^{32}P]orthophosphate-labeled cells indicated that RIZ is a phosphoprotein (Fig. 4*C*). This ^{32}P-labeled, 250-kDa RIZ protein comigrated with the *in vitro* translated, full-length RIZ (Fig. 4*C*, lane 3). The anti-RIZ serum raised against the rat protein also recognized the human protein of the same size in extracts of retinoblastoma Weri-Rb-1 cells and fibrosarcoma

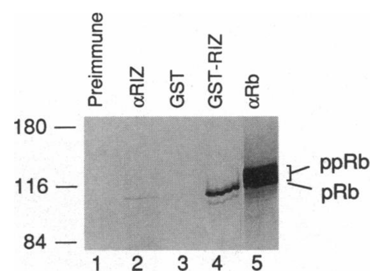

FIG. 5. Anti-RIZ antibodies coimmunoprecipitate Rb. Extracts of HT1080 cells were immunoprecipitated with preimmune serum, rabbit anti-RIZ serum, or anti-Rb PMG245 (PharMingen) (lanes 1, 2, and 5, respectively) and precipitated proteins were immunoblotted with anti-Rb PMG245. To compare with *in vitro* binding, HT1080 extracts were mixed with 4 μg of GST or GST–RIZ fusion protein (amino acids 215–412) pKGSE (lanes 3 and 4, respectively). Bound proteins collected on glutathione-agarose were analyzed on an immunoblot.

HT1080 cells (Fig. 4*D*). The lower amount of RIZ protein in HT1080 cells was representative of the level found in all other human nonretinoblastoma cell lines examined.

To obtain evidence for association between RIZ and Rb in cells, HT1080 cell extracts were immunoprecipitated with the rabbit anti-RIZ serum. This serum did not crossreact with Rb protein (data not shown). The immunoprecipitated products were analyzed by immunoblotting with anti-Rb antibody (Fig. 5). A band corresponding to the fastest migrating forms of the direct Rb immunoprecipitate was specifically detected in the anti-RIZ immunoprecipitate.

We found that the rabbit anti-RIZ serum specifically immunoprecipitated *in vitro* translated E1A protein, indicating antigenic similarity between RIZ and E1A (Fig. 6). The shared epitope was mapped to a position adjacent to cr2 in RIZ and to a motif at the C terminus of E1A (I.M.B. and S.H., unpublished data). This E1A C-terminal motif EDLLEE is conserved among E1A proteins of different adenoviral serotypes and hence is designated conserved epitope 1 or cel (Fig. 1*B*).

DISCUSSION

We have cloned a cDNA encoding an Rb-binding protein, RIZ. We show that RIZ is a cellular 250-kDa protein; this 250-kDa protein comigrated with RIZ cDNA-produced polypep-

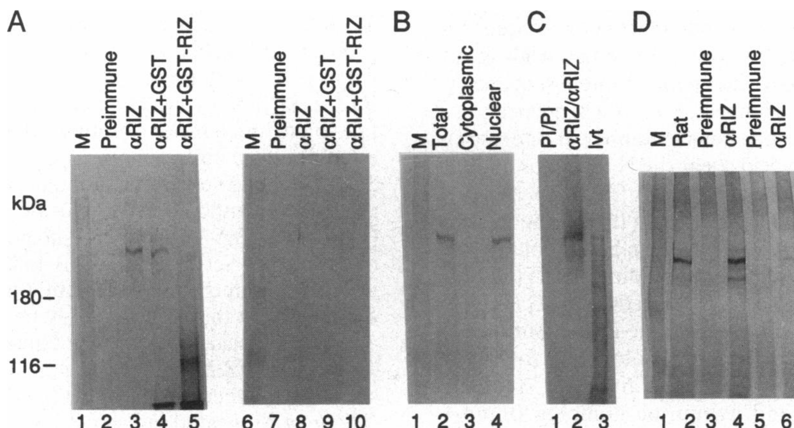

FIG. 4. RIZ is a 250-kDa nuclear phosphoprotein. (*A*) Extracts of rat brain tumor B50 cells were immunoprecipitated with the rabbit anti-RIZ serum. The immunoprecipitated products were analyzed by immunoblot with the mouse anti-RIZ serum (lanes 1–5) or the monoclonal antibody 2D7 (lanes 6–10). Ten micrograms of GST or GST–RIZ antigen was added during immunoprecipitation in the indicated lanes. Molecular weight markers (M) were run in lanes 1 and 6. (*B*) Nuclear and cytoplasmic fractions of B50 cells were immunoprecipitated with the rabbit anti-RIZ serum and immunoblotted with the mouse anti-RIZ serum. (*C*) Extracts of [^{32}P]orthophosphate-labeled B50 cells were immunoprecipitated with preimmune (PI) or rabbit anti-RIZ sera. The immunoprecipitated proteins were boiled in SDS buffer, and the released proteins were immunoprecipitated again with the same serum. (*D*) Similar amounts of cell extracts prepared from rat B50 (lane 2), Weri-Rb1 (lanes 3 and 4), or HT1080 cells (lanes 5 and 6) were immunoprecipitated with rabbit anti-RIZ serum or preimmune serum, and then the precipitated proteins were immunoblotted with the mouse anti-RIZ serum.

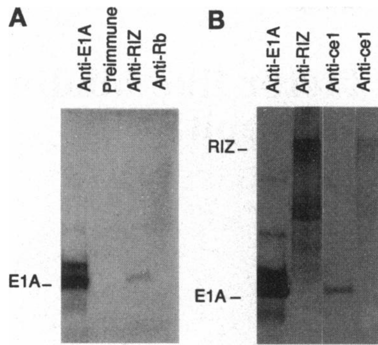

FIG. 6. Anti-RIZ antibodies crossreact with E1A. (*A*) *In vitro* translated E1A 12S was immunoprecipitated with the antibodies indicated on top of each lane. The rabbit serum was used as the anti-RIZ antibody. (*B*) *In vitro* translated E1A and a RIZ segment (amino acids 1–972 of rat RIZ) were immunoprecipitated with the indicated antibodies. The rabbit anti-RIZ serum was purified on a GST-E1A protein column by standard procedures (15). The high pH buffer eluate from the column was designated anti-ce1.

tide and could be recognized by three different antibodies raised against two different regions of RIZ. Several lines of evidence suggest that RIZ binds to Rb. RIZ contains the cr2 motif of E1A; this motif is required for RIZ binding to Rb. The C-terminal 56-kDa segment of Rb containing the T antigen/E1A-binding domain is sufficient for binding to RIZ. The RIZ-Rb interaction could be detected *in vitro* and in lysates of mammalian cells by coimmunoprecipitation. However, functional significance of this interaction requires further investigation.

RIZ is the fourth LXCXE-containing cellular protein that has been cloned by functional screening of libraries either *in vitro* or *in vivo* by using the yeast two-hybrid technique, the others being RBP1, RBP2, and BRG1 (6, 26); our screening also yielded a clone for RBP1. That Rb screening would reveal such a small number of proteins is unexpected, given that the LXCXE motif is found in more than 1000 known proteins in the data base (unpublished data). This indicates that the LXCXE sequence is unlikely to mediate Rb-binding in most of the proteins in which this motif occurs.

Many Rb-binding proteins identified by either cloning or guessing, including E2F-1, PP-1α2, c-Abl, MyoD, and others (7–9, 27–29) do not share sequence similarity with viral oncoproteins. Among the few that do, including Cyclin D and Elf-1 (30–32) and the above mentioned RBP1, RBP2, and BRG1, the relationship is limited to the Rb-binding motif LXCXE. Remarkably, the similarity between RIZ and E1A goes beyond the Rb-binding motif; we also show that RIZ is antigenically related to E1A. This observation may suggest an evolutionary relationship between RIZ and E1A.

RIZ shares homologous domains with PRDI-BF1/Blimp-1, a DNA-binding protein that is a repressor of interferon β gene expression but may be an inducer of other genes during B-lymphocyte maturation (23–25). This homology with a DNA-binding differentiation factor may suggest a role for RIZ in transcriptional regulation of cellular differentiation, perhaps in the nervous system, where RIZ is preferentially expressed and where Rb is known to play a role in neuronal differentiation (33, 34). We found high levels of RIZ mRNA and protein in retinoblastoma cells but low levels in all other human neural and nonneural cell lines examined; future studies will be required to find out whether RIZ might play a role in the pathogenesis of retinoblastoma.

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