In Vitro Analysis of the E1A-Homologous Sequences of RIZ

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The RIZ (G3B/MTB-Zf) gene was first isolated based on its ability to bind to the retinoblastoma protein (Rb). An acidic, ~100-amino-acid region around the Rb-binding motif of RIZ has structural and antigenic similarity to the conserved sequences of the E1A viral oncogene. We show here that this region interacts specifically with the E1A-binding domain of Rb. This interaction could be disrupted by E1A or by a peptide of RIZ homologous to the CR2 motif of E1A which is involved in binding to Rb family proteins. Also like E1A, RIZ can form a ternary complex with Rb and E2F1. Despite this similarity to E1A, however, RIZ could not bind to the Rb family proteins p107 and p130 in vitro. The data show that the RIZ CR2 motif can mediate differential binding to Rb family proteins. We also mapped the shared antigenic determinant between RIZ and E1A to a conserved sequence, designated CE1, which is located in the C terminus of E1A. Unlike that of E1A, the CE1 motif of RIZ is located next to the CR2 motif. Despite this proximity, CE1 and CR2 appear to act independently. The data show similarities as well as differences between the homologous sequences of RIZ and E1A and contribute to an understanding of the biochemistry of these proteins.

The retinoblastoma protein (Rb) forms complexes with viral oncoproteins (4, 7, 32). This binding of Rb is mediated by a region, designated CR2, that is conserved among these viral oncoproteins (20). CR2 contains the signature LXCXE core motif that is required for full oncogenic activity of viral oncoproteins (4, 22, 23, 29, 33). CR2 interacts with a domain of Rb, commonly referred to as the “pocket,” that is frequently mutated in human cancers (14, 18, 19). The conservation of CR2 among these evolutionarily unrelated viral proteins suggests that this motif may originate from cellular Rb-binding proteins. While most cellular Rb-binding proteins, including the best characterized, E2F1 (21, 25), do not have structural similarities to viral oncoproteins, several use the CR2 motif to bind Rb (for a review, see reference 15). Among these, the zinc finger protein RIZ is unique in that it shows more extensive sequence similarity, and even antigenic similarity, to E1A (Fig. 1) (2). RIZ may function as a DNA-binding transcription factor, as evidenced by its independent isolation as a GATA-3 factor binding protein (G3B) or as a DNA-binding protein (MTB-Zf) (24, 28). To further understand the sequence similarities between RIZ and E1A, we examined the biochemical and immunological properties of the E1A-related sequences of RIZ.

An acidic, ~100-amino-acid (aa) region of RIZ (aa 215 to 320) contains sequence similarities to the conserved motifs of E1A (Fig. 1). We have previously shown that RIZ can interact with Rb in vitro and in vivo; a single amino acid substitution of Cys to Gly in the RIZ CR2-like motif EIRCEE abolished RIZ binding to Rb in vitro (2). To further determine whether the CR2-like motif of RIZ is functional and sufficient in binding Rb, an 11-aa peptide, EIRCEEKPEDL, was synthesized and tested for its ability to inhibit RIZ binding to Rb. An in vitro-translated RIZ segment containing residues 245 to 883 was incubated with purified 56-kDa Rb protein and RIZ peptides; the binding mixture was immunoprecipitated with anti-Rb se-

rum as described previously (2). Coimmunoprecipitation of RIZ was inhibited by wild-type peptide but not by a point mutant peptide containing a single amino acid substitution of cysteine to glycine (Fig. 2A).

The fact that the CR2 motif can block RIZ binding to Rb suggests that E1A protein should also do so. To examine this, glutathione *S*-transferase (GST) pull-down assays were performed with the GST-RIZ fusion protein containing residues 215 to 462 (2) and extracts prepared from either the HT1080 cell line or a derivative of it, p2AHT2a, that stably expresses E1A protein (10). Anti-Rb immunoblotting of bound proteins revealed that the fastest-migrating forms representing the hypophosphorylated Rb were bound by RIZ (Fig. 2B, lane 2). No Rb binding was observed when we used p2AHT2a cell extracts (Fig. 2B, lane 4), suggesting that the presence of E1A prevented RIZ-Rb complex formation.

The data shown in Fig. 2 suggest that RIZ may bind to the Rb pocket domain targeted by E1A. The C-terminal 56-kDa Rb protein (p56Rb) is sufficient for binding RIZ (2) and contains both the pocket domain and a C-terminal domain that is needed for certain cellular proteins (E2F1 and c-Abl) to bind Rb (17, 26, 31). To determine which domain within the C-terminal half of Rb is required for RIZ binding, several Rb C-terminal mutant polypeptides were produced by in vitro translation (Fig. 3, lanes 1 to 5) (18) and mixed with GST-RIZ fusion protein, followed by immunoprecipitation with anti-RIZ serum (Fig. 3, lanes 6 to 10). RIZ binding was observed for wild-type Rb (A9) and for a mutant Rb (B3) lacking the C-terminal sequences proximal to the pocket but not for any of the pocket domain mutants, suggesting that the pocket domain, but not the distal C-terminal domain, is involved in binding RIZ.

Because E2F1 binding to Rb requires the C terminus of Rb and E2F1-bound Rb can still bind to E1A (9), we asked whether RIZ, RB, and E2F1 can also form a ternary complex in vitro. GST-RIZ fusion protein was mixed with in vitro-translated labeled E2F1 protein and purified p56Rb (1 µg) or GST protein. Binding of E2F1 to RIZ was observed only in the presence of Rb protein (Fig. 4). As a positive control, GST-E1A also bound to E2F1 in an Rb-dependent manner (Fig. 4). The results show that RIZ does not tightly interact with E2F1 nor compete with E2F1 for binding Rb but instead can coexist

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FIG. 1. Similarities between RIZ and the conserved motifs in E1A. A schematic map of human RIZ protein structure is shown above the E1A homology alignment. The rat (r) and human (h) RIZ sequences and the E1A 12S protein sequences of various adenovirus strains are aligned, and identical or related residues are boxed. Only the part of the CR1 region of E1A that shows homology to RIZ is shown. PEST, proline-, glutamic acid-, serine-, and threonine-rich region; PR, PRD1-BF1- and RIZ-homologous region.

with E2F1 and Rb in a ternary complex. This result provides additional evidence for the involvement of the Rb C terminus in binding E2F1 but not RIZ.

The CR2 motif of E1A and other viral oncoproteins is known to interact with the Rb family proteins p107 and p130 (5, 8, 13, 34). We next examined whether in vitro-translated p107 and p130 proteins could bind to GST-RIZ fusion protein in vitro in a GST pull-down assay. As shown in Fig. 5, RIZ binding was observed only for Rb but not for p107 and p130. In comparison, E1A bound to all three proteins. Although it remains possible for RIZ to interact with p107 or p130 in vivo, the result suggests that the CR2 motif of RIZ can mediate differential binding to Rb family proteins at least in vitro. This is reminiscent of the E2F1-like Rb-binding motif shared by the E2F family proteins; different members of this family differentially interact with Rb family proteins (1, 6, 12, 27, 30). The identification of cellular proteins that can bind to Rb but not to Rb-related proteins is important given the unique biological function of Rb.

To further explore the apparent sequence homology between RIZ and E1A, we examined whether antiserum raised against the E1A-related region of RIZ could cross-react with E1A. As described previously, anti-RIZ serum against RIZ

FIG. 2. Inhibition of RIZ binding to Rb by CR2 peptide and E1A protein. (A) A peptide consisting of the RIZ CR2 motif blocked RIZ binding to Rb. Mixtures of an in vitro-translated RIZ segment (aa 245 to 883), 33 nM p56Rb protein, and 0, 10, or 50 μ M indicated peptide (pep) were immunoprecipitated by anti-Rb serum. The sequence of RIZ-pep is EIRCEEKPEDL. RIZ-pep* represents the mutant peptide EIRGEEKPEDL. (B) E1A protein inhibition of RIZ binding to Rb. HT1080 cell extracts (500 μ l) were mixed with 4 μ g of GST (lane 1) or the GST-RIZ fusion protein (aa 215 to 462) (lane 2). Extracts of p2AHT2a cells, a derivative of HT1080 that expresses E1A, were similarly incubated with GST-RIZ fusion protein (lane 4). Bound proteins collected on glutathione-agarose were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with anti-Rb antibody PMG-245. Anti-Rb immunoprecipitation products of HT1080 cell extracts were loaded in lane 3.

FIG. 3. RIZ interacts with the E1A-binding pocket domain of Rb. (Top panel) In vitro-translated (IVT) wild-type (wt) and mutant Rb (lanes 1 to 5) were each precipitated with anti-RIZ serum after being mixed with purified GST-RIZ fusion protein (α RIZ) (aa 215 to 462) (lanes 6 to 10). (Bottom panel) Schematic map of Rb mutants. The blank box represents full-length wild-type Rb protein. The two subdomains of the Rb pocket are represented by black boxes. Gaps represent the deleted regions of Rb. p56Rb is the bacterium-expressed Rb protein (16); the other mutant proteins were made by in vitro translation (18).

FIG. 4. Ternary complex formation among RIZ, Rb, and E2F1 proteins. In vitro-translated radiolabeled E2F1 protein as shown in lane 1 (10% input loaded) was mixed with p56Rb protein (1 μ g), GST protein, GST-RIZ protein, or GST-E1A protein (2 μ g) as indicated at the top of each lane. Glutathione agarose-bound proteins were resolved on sodium dodecyl sulfate gel followed by autoradiography.

residues 245 to 575 can bind to E1A (2). To map the cross-reactive epitope, we purified this serum on an E1A affinity column and designated the purified antibody anti-CE1 (2). We generated several deletion mutants of RIZ and E1A and tested each one for binding to anti-CE1 antibody. The results mapped the epitope within residues 304 to 318 of RIZ and the E1A C-terminal 20-aa region (data not shown). An 11-aa peptide of RIZ consisting of the common sequence ³¹²EDLLEE (Fig. 1) was then synthesized; this peptide effectively blocked binding of anti-CE1 to both RIZ and E1A, whereas an overlapping peptide from residues 304 to 314 did not (Fig. 6A). The results show that CE1 is located in the sequence ³¹²EDLLEE in RIZ and in its homologous sequence, ²²⁴EDLLNE, in E1A.

CE1 and CR2 motifs in E1A are separated by a region of ~100 residues, whereas in RIZ they are located next to each other, raising the question whether these motifs are truly independent. To examine this, in vitro-translated Rb protein was mixed with purified recombinant RIZ protein containing residues 215 to 412 (the GST portion had been cleaved), followed

FIG. 5. RIZ did not bind to p107 and p130 in vitro. In vitro-translated (Inv) Rb, p107, and p130 (lane 1 of each gel) were incubated with 4 μ g of GST, GST-E1A, and GST-RIZ fusion proteins (lanes 2, 3, and 4, respectively, of each gel). Bound proteins collected on glutathione-agarose were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography.

FIG. 6. CE1 motif mapping and its independence from CR2. (A) In vitro-translated RIZ protein containing residues 1 to 318 (RIZ318) or E1A 12S protein (E1A243) was immunoprecipitated with anti-CE1 in the absence of peptides (lanes 1 and 4), in the presence of 200 μ M CR2 peptide EIRCEEKPEDL (lanes 2 and 5), and in the presence of 200 μ M CE1 peptide KPEDLLEEQS (lanes 3 and 6). (B) In vitro-translated full-length Rb was mixed with 1 μ g of GST protein (lane 1) or thrombin-cleaved GST-RIZ fusion protein (aa 215 to 462) (lanes 2 and 3). The GST portion of the fusion protein was cleaved off by thrombin as judged by fractionation on sodium dodecyl sulfate gel and immunoblotting with anti-RIZ (not shown); residual noncleaved fusion proteins were removed by absorption with glutathione-agarose. The mixture was immunoprecipitated with anti-RIZ serum (lanes 1 and 2) or anti-CE1 (lane 3). The smaller amount of Rb that was coprecipitated by anti-CE1 (lane 3) reflected the normal RIZ immunoreactivity of anti-CE1 relative to anti-RIZ serum. (C) In vitro-translated RIZ residues 1 to 575 were preincubated with 2 μ g of recombinant Rb protein for 1 h (lane 2) or without Rb (lane 1). The mixture was then immunoprecipitated by anti-CE1.

by anti-CE1 immunoprecipitation. As shown in Fig. 6B, Rb coimmunoprecipitated with anti-CE1, indicating ternary complex formation among anti-CE1, RIZ, and Rb. Conversely, we observed no inhibitory effect of Rb on the immunoprecipitation of RIZ by anti-CE1 (Fig. 6C). The results show that antibody binding to CE1 does not interfere with Rb binding to CR2, and vice versa, and suggest that CE1 and CR2 are independent motifs. The CR2-CE1 region of RIZ is predicted to form an α -helix by several algorithms (3, 11). One explanation for the lack of steric hindrance between proteins bound to this region may be that they bind to opposite faces of the α -helix. Although the conserved nature of the CE1 motif among E1A genes of different strains may indicate a biological function, the role of CE1 remains to be determined.

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