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# Adenovirus E1A Makes Two Distinct Contacts with the Retinoblastoma Protein

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**Two regions near the amino terminus of the adenovirus E1A protein, which were first identified by sequence conservation among various adenovirus serotypes, have been shown by genetic studies to be essential for E1A-mediated transformation. These same regions are also required for interaction with a number of cellular proteins, including the retinoblastoma protein (pRB). Using synthetic peptides corresponding to portions of these conserved regions, we show that each region can bind independently to pRB. These interactions were observed in both competition and binding assays. In both types of assay, region 2 peptides (E1A amino acids 115 to 132) bound pRB with higher affinity than did region 1 peptides (E1A amino acids 37 to 54), while a peptide combining region 1 and 2 sequences consistently provided the highest-affinity interaction. Cross-blocking experiments using region 1 peptides and region 2 peptides suggested that these two regions of E1A make distinct contacts with pRB. These data support the notion that the pRB-binding domain of E1A contains at least two functional elements.**

Adenovirus E1A polypeptides are multifunctional molecules that are synthesized early in viral infection (31, 37, 46). One of the activities exhibited by E1A is the ability to cooperate with other oncogenes to produce fully transformed cells (18, 24, 29, 41, 48, 54). The molecular changes that cause E1A-mediated transformation are not well understood, but it appears that the formation of stable protein-protein complexes between E1A and several cellular proteins plays a crucial role (17, 22, 50, 52, 53). Genetic analysis has shown that amino acid sequences in two discrete regions of E1A are required for transformation activity (19, 33, 34, 42, 45, 49, 51, 54). These sequences are also essential for formation of stable complexes between E1A and several host cell proteins with sizes estimated at 300, 130, 107, 105, 60, and 33 kDa (17, 47, 50, 52). This correlation between transformation and binding suggests that these cellular proteins represent at least some of the targets for E1A-mediated transformation. The identities of four of these putative targets are known; the 105-kDa polypeptide is the product of the retinoblastoma susceptibility gene (pRB) (50), p107 is a pRB-related protein (15), the 60-kDa polypeptide has been identified as human cyclin A (39), and p33 has recently been shown to be $p33^{cdk2}$ (47), a cdc2-related kinase (13, 47).

All of the published evidence is consistent with the notion that E1A binds directly to pRB. E1A amino acid sequences from residues 30 to 60 and 120 to 127 are important for high-affinity association with pRB (11, 12, 52). These regions contain part of the blocks of amino acid sequence homology found in E1A of different serotypes that have been called conserved regions 1 and 2 (29). For simplicity, and to remain consistent with this nomenclature, in this report the two regions of E1A required for pRB binding will be referred to as region 1 (E1A amino acids 30 to 60) and region 2 (amino acids 120 to 127). Region 1 and region 2 do not contribute equally to pRB binding by E1A (12). E1A mutants in which region 2 sequences are deleted fail to coimmunoprecipitate pRB from infected cells (12, 52). However, E1A mutants in which the entire region from amino acids 30 to 60 is deleted still coimmunoprecipitate pRB, albeit at a greatly reduced level, either from virus-infected cells or in in vitro mixing assays (8, 12).

These observations suggest that region 2 sequences are sufficient for binding to pRB and imply that this region contains a contact site for the interaction. The mechanism by which region 1 sequences contribute to the E1A-pRB association is less clear. Several models are possible to explain the data. First, region 1 sequences may have no direct role in pRB association, but deletion of this region may alter E1A conformation so that pRB binding through region 2 is less stable. Second, both regions may contain contact sites, with only the interaction at region 2 being sufficiently strong to remain bound during immunoprecipitation. These sites may make distinct contacts with pRB or may be a duplicated binding domain. A third and perhaps less likely possibility is that region 1 of E1A contains the primary contact site for interaction with pRB. In this model, region 2 sequences that also provide a second but weaker binding site are required for correct conformation of region 1.

Amino acid sequences that are homologous to these two regions of E1A have been identified in other pRB-binding proteins, including human papillomavirus type 16 (HPV-16) E7 protein and simian virus 40 (SV40) large T antigen (1, 5, 9, 16, 38, 44). This sequence similarity suggests that both regions have functions that are conserved between viruses. Large T antigen and E7 protein have been shown to bind to pRB, and mutations in the sequences homologous to E1A region 2 inhibit their interaction with pRB (1, 5, 14, 27, 36). To date, however, no pRB-binding activity has been described for the sequences of SV40 large T antigen or HPV-16 E7 that are homologous to E1A region 1.

The E1A-pRB complexes described previously (11, 12, 22, 25, 53) were detected after immunoprecipitation of E1A from [$^{35}$S]methionine-labeled, virus-infected cells. To examine in more detail the contributions of region 1 and region 2 sequences to pRB binding, we have examined the properties of synthetic peptides that represent these regions in highly sensitive in vitro binding assays. The synthetic peptides used

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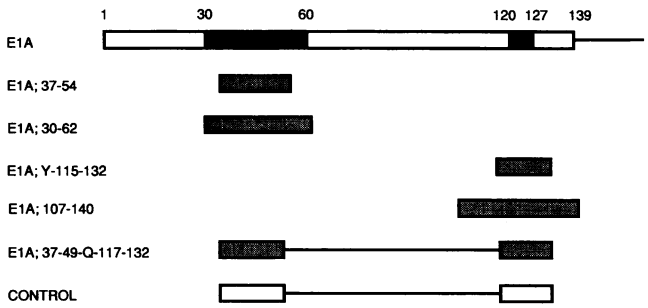


FIG. 1. The regions of E1A represented by synthetic peptides. The bar at the top shows the regions from the amino terminus of adenovirus type 5 E1A that have been shown to be important for association with pRB (52). Positions of the amino acid sequences of the synthetic peptides used in this study are indicated by the filled boxes. Open boxes indicate that the control peptide contained a mutated sequence. Joined boxes indicates that two regions of E1A amino acid sequence were joined together through a glutamine residue. The peptides used were HFEPPTLHELYDLDVTAP (E1A;37-54), DNLPPPSHFEPPTLHELYDLDVTAPEDPNEEAV (E1A;30-62), YLVPEVIDLTCH EAGFPPS (E1A;Y-115-132), LG PVSMPNLVPEVIDLTCHE AGFPPSDDEDEEGE (E1A;107-140), HFEPPTLHELYDLQPEVIDLTCHEAGFPPS (E1A;37-49-Q-117-132), and HFEPPTVHEVYD VQPEVIEVTSHDAGFPPS (CONTROL). Synthetic peptides were synthesized and purified by the core service facility under the direction of D. R. Marshak at Cold Spring Harbor Laboratory. Following synthesis on an Applied Biosystems 430A instrument, the peptides were cleaved from the resin, deprotected, and purified by high-performance liquid chromatography as described by Marshak and Carroll (32). Peptide purity was >95%, as determined by plasma desorption mass spectrometry.

in these experiments are shown in Fig. 1. Two peptides that contained amino acid sequences from E1A region 1 were prepared. The longer of these, comprising E1A amino acids 30 to 62, contained the entire upstream region reported previously to be essential for stable E1A-pRB association (52). A shorter peptide which consisted of E1A amino acids 37 to 54 was also prepared. This peptide was chosen to contain the leucine-rich motif that shows weak homology with sequences at the amino termini of SV40 large T antigen and HPV-16 E7 protein (9, 38). Two peptides were also synthesized to test the pRB-binding potential of E1A region 2. These peptides contained E1A amino acids 115 to 132 and 107 to 140. The shorter of these two peptides also contained a tyrosine residue at its amino terminus to facilitate iodination.

To compare the separate activities of the two regions with their activity in *cis*, we also synthesized and assayed a peptide containing amino acid sequences from both regions. This peptide contained E1A amino acids 37 to 49 and 117 to 132 which were separated by a glutamine. This configuration was chosen so that the linkage between the region 1 motif and the region 2 motif resembled the sequence of HPV-16 E7 protein, which has also been shown to bind to pRB. A negative control peptide based on this configuration was also synthesized. In this peptide, the amino acids that are highly conserved between adenovirus E1A polypeptides, HPV E7 proteins, and polyomavirus-type large T antigens were mutated to amino acids of similar characteristics, so that although the sequence was altered, the length and charge of the peptide were maintained. Leu-43, Leu-46, Leu-49, and Leu-122 were each replaced by Val, Cys-124 was replaced by Ser, Asp-121 was replaced by Glu, and Glu-126 was replaced by Asp (Fig. 1). Several peptide preparations of unrelated sequences were also used to provide negative controls.

The pRB-binding activities of these peptides were assessed in two complementary experimental approaches. In the first approach, peptides were covalently coupled to beads and incubated in lysates containing radiolabeled pRB polypeptides. A positive result in this type of experiment proves that the peptide contains a contact site for interaction. In the second approach, the peptides were assessed for the ability to compete with E1A for binding to pRB. This second assay also was used to estimate the relative binding affinities of the peptides.

**E1A peptides attached to beads bind efficiently to pRB polypeptides.** Peptides were coupled via amino groups to cyanogen bromide-activated Sepharose beads. Aliquots of a 50% bead slurry were added to 10 μl of [$^{35}$S]methionine-labeled pRB polypeptides diluted in 100 μl of ELB buffer (250 mM NaCl, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.5], 0.1% Nonidet P-40). Retinoblastoma polypeptides that were labeled with [$^{35}$S]methionine were synthesized in rabbit reticulocyte lysates programmed with cRNA prepared from the human cDNA clone (50). The mixture was rocked for 2 h at 4°C to allow binding of the pRB polypeptides. Beads were washed four times with 1 ml of ELB buffer. Peptide coupling was carried out as follows. A 0.15-mg sample of peptide was diluted in coupling buffer (0.1 M $Na_2SO_4$, 0.5 M NaCl [pH 8.3]). Cyanogen bromide-activated Sepharose 4B beads (Pharmacia) were washed in 4 mM HCl and rinsed in coupling buffer; 125 μl of slurry, containing 100 μl of beads, was added to the peptide, and the mixture was rocked overnight at 4°C. Peptide coupling efficiency was monitored by measuring $A_{205}$, using aliquots taken before addition of the beads and after the overnight incubation. Routine coupling efficiencies of 80 to 90% were obtained for each peptide. The beads were washed twice with 0.5 M $Na_2PO_4$ (pH 7.5) and once with 50 mM $Na_2PO_4$–1 M NaCl (pH 7.5) and then rocked at room temperature for 6 h in 1 M ethanolamine to block any remaining active groups. Nonspecific protein-binding sites were blocked by overnight incubation at 4°C in 137 mM NaCl–10 mM $Na_2HPO_4$–2.7 mM KCl–1.8 mM $KH_2PO_4$ containing 3% bovine serum albumin. Beads were stored on ice as 50% slurry in the same solution containing 10 mM NaF, 5 mM EDTA, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM $Na_3VO_4$, 1 mg of aprotinin per ml, and 1 mg of leupeptin per ml. Bound proteins were analyzed on sodium dodecyl sulfate (SDS)-polyacrylamide gels. As has previously been reported (50), these translation reactions produced a nested set of pRB polypeptides that share a common carboxyl terminus as a result of initiation at internal methionine codons.

Figure 2 shows the result of incubation of the various peptide-beads with pRB polypeptides. Labeled polypeptides failed to bind to beads that lacked peptide or carried control peptides. However, pRB polypeptides bound specifically to beads coupled with peptides that represented either region 1 or region 2 of E1A. In these experiments, as seen in experiments performed with full-length E1A or large T antigens (9, 26, 28), only pRB polypeptides greater than approximately 60 kDa were bound by the beads. This finding suggests that the same region of pRB is targeted by the peptides as by full-length protein.

In initial experiments, beads carrying peptide with sequences from both region 1 and region 2 (E1A;37-49,Q,117-132) appeared to bind pRB polypeptides more strongly than did other beads. This observation was tested more carefully

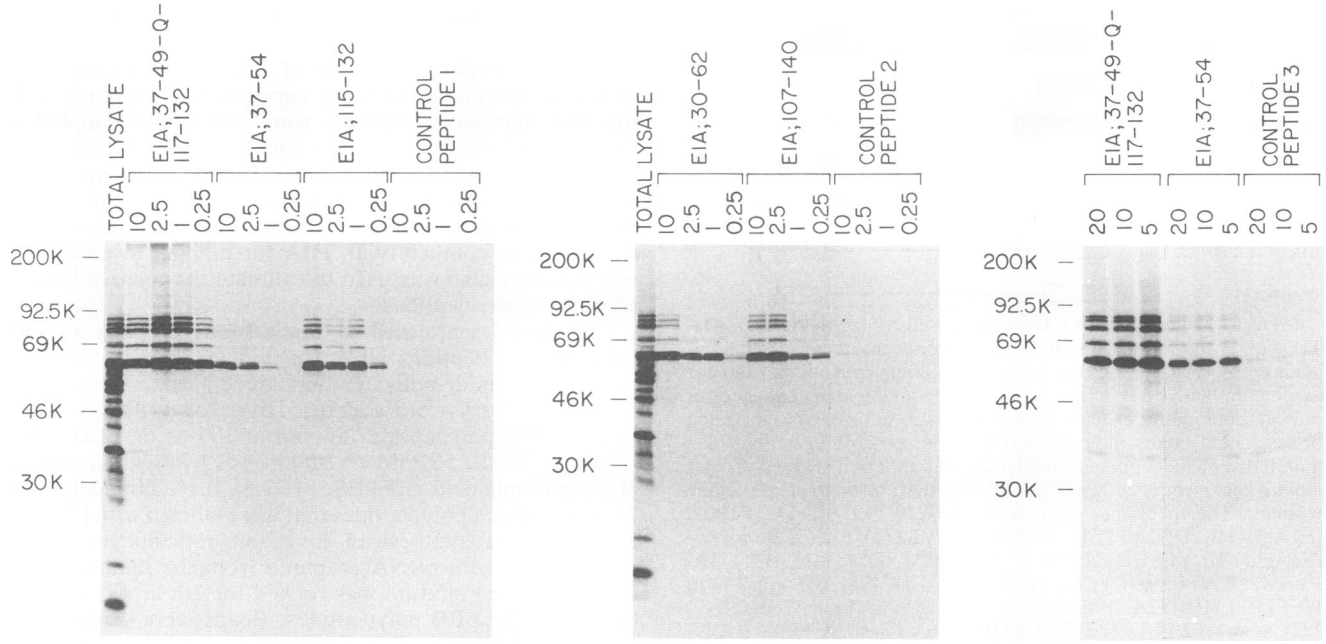


FIG. 2. Evidence that E1A peptides coupled to beads bind efficiently to pRB polypeptides. Peptides were bound to Sepharose beads though a single terminal amino group. Peptide beads were incubated with labeled pRB polypeptides and washed to remove unbound protein. Labeled proteins were separated on SDS–10% polyacrylamide gels (30) and detected by fluorography (2). The bead slurries were titrated into the binding reaction to reveal different binding avidities. The quantity of bead slurry added to the mix is indicated above each lane; 1 μl of total in vitro translation product was also separated on the gel (total lysate). The positions of size markers (in kilodaltons) are shown. In these experiments, two peptides containing non-E1A sequences (CYHQLEQNRRLTNEKLKLALNED and CASGAASTTTTASKAQ) were used as additional negative controls (control peptides 1 and 2) as well as the mutated E1A peptide (control peptide 3).

by titrating the amount of beads added to each mix (Fig. 2). Using beads carrying region 1 peptide sequences alone (E1A;37-54 or E1A;30-62), addition of fewer beads caused a pronounced decrease in binding signal. Surprisingly, a 40-fold reduction in the peptide beads carrying peptide E1A;37-49,Q,117-132 had little effect on the amount of pRB polypeptides bound, suggesting that these beads have the greatest binding capacity. This assay is not a good measure of binding affinity because of the uncertain effects both of immobilization of the peptide and of high local concentrations of the peptide on the bead. Nevertheless, these results suggest that the peptides bind to the pRB polypeptides with different potencies.

**Region 1 or region 2 peptides compete with E1A for binding to pRB.** To compare more accurately the pRB-binding affinities of the E1A sequences, peptides were used to compete with E1A for binding to pRB polypeptides in solution. Complex formation between retinoblastoma polypeptides synthesized by in vitro translation and E1A supplied in 293 cell lysate was performed as previously described (7, 9). Eight to 10 μl of reticulocyte lysate was diluted to a final volume of 135 μl of ELB buffer containing a variable concentration of synthetic peptide and put on ice; 3 μl (Fig. 3A) or 10 μl (Fig. 3B) of 293 cell lysate was added, and the mix was rocked for 2 h. The solution was precleared, divided into aliquots, and immunoprecipitated as described previously (20, 21) with a monoclonal antibody specific for E1A (M73) or with a control antibody (PAb416). Immune complexes were separated by electrophoresis through SDS-polyacrylamide gels (30), and labeled proteins were detected by fluorography (2).

As shown in Fig. 3, all of the E1A peptides were able to inhibit E1A association with pRB polypeptides, although with different effectiveness. The mutant E1A peptide consistently failed to compete for binding in this assay. The degree of competition seen with E1A peptides in these type of experiment is directly proportional to the amount of E1A added in the lysate and allows comparison of blocking activities of several peptides within a single experiment. Figure 3 shows two representative experiments carried out by using fivefold differences in 293 cell lysate. In Fig. 3A, in which 3 μl of 293 cell lysate was added, region 1 peptides (E1A;30-62 and E1A;37-54) gave 50% blocking activity at approximately 250 μM, whereas peptides containing region 2 gave greater than 50% blocking at the lowest concentrations assayed (7 to 9 μM). In Fig. 3B, in which 10 μl of 293 cell lysate was added, differences were apparent between the peptide containing regions 1 and 2 (E1A;37-49,Q,117-132) and a region 2 peptide (E1A;Y-115-132). The region 1 and 2 peptide (E1A;37-49,Q,117-132) completely inhibited the interaction at 1.4 μM, whereas 21 μM region 2 peptide (E1A;Y-115-132) was required to gave an equivalent level of inhibition; 230 μM region 1 peptide (E1A;37-54) gave only partial inhibition of the interaction. These differences were found to be reproducible in multiple repeat experiments. In summary, the greatest blocking activity was seen with the peptide containing sequences from both regions of E1A. Peptides containing region 2 sequences alone had approximately 5- to 10-fold-reduced blocking activity. The weakest blocking was found with region 1 sequences alone.

**Regions 1 and 2 make discrete contacts with pRB.** Since both region 1 and region 2 peptides were able to bind independently to pRB, it was surprising that peptides containing either region alone were able to compete for pRB's

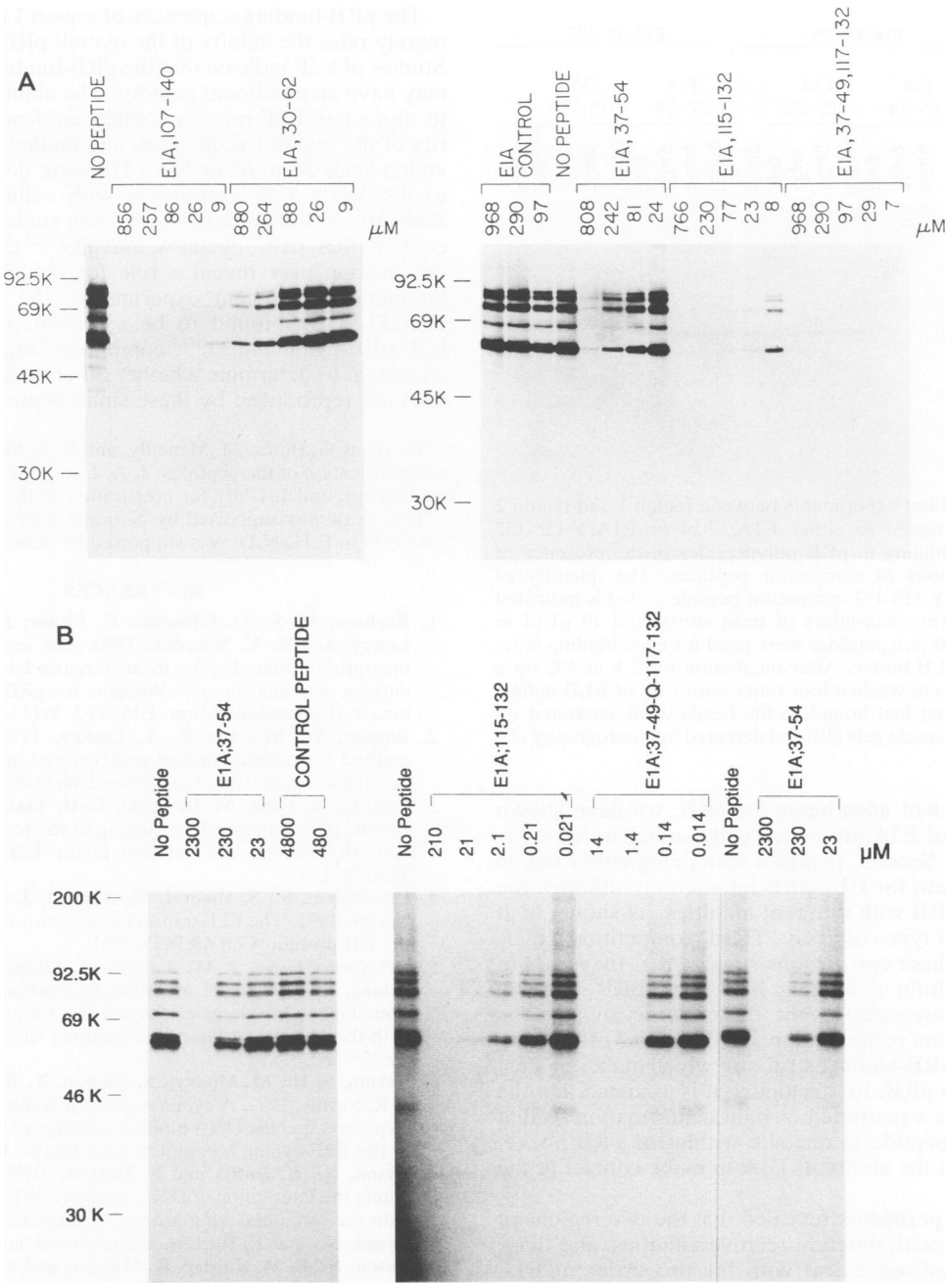


FIG. 3. Evidence that E1A peptides compete with E1A for binding to pRB polypeptides. Panels A and B show the comparable competition affinities of E1A peptides in two separate experiments. Synthetic peptides were titrated into an in vitro binding reaction between E1A and labeled pRB polypeptides. The concentration of peptide present in the mix is indicated above each lane. Positions of size markers (in kilodaltons) are shown.

interaction with full-length E1A containing both regions. This paradox suggests either that both regions bind to the same sequences on pRB or that the two regions bind separately to pRB but in a tightly linked manner. To test a model in which these two regions of E1A form structurally similar domains, we assayed the ability of a region 2 peptide to block pRB polypeptides from binding to region 1 peptide beads. The results of this and the converse experiment using region 2 peptide beads are shown in Fig. 4. Region 1 peptide was able to compete with beads coupled to region 1 peptide for binding to pRB polypeptides but failed to block binding to region 2 peptide beads. Conversely, region 2 peptide blocked only binding to region 2 peptide beads. Thus, the region 1 and 2 peptides make discrete contacts with pRB.

**Architecture of the E1A-pRB interaction.** These experiments provide three new pieces of information which add to our understanding of the architecture of the E1A-pRB interaction. First, using the observation that peptides attached to

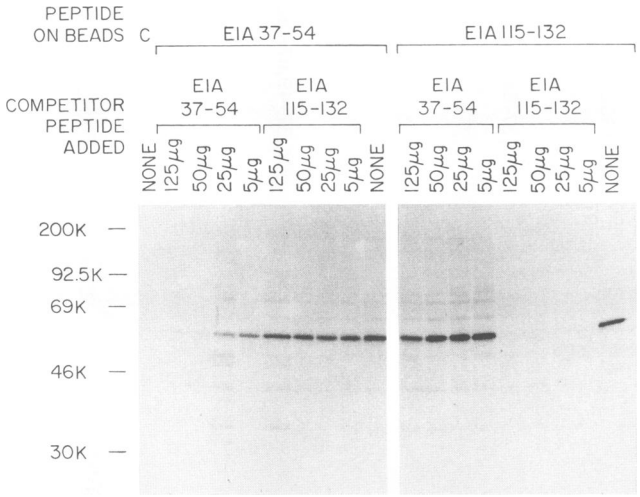


FIG. 4. Competition experiments between region 1 and region 2 peptides. Beads coupled to either E1A;37-54 or E1A;Y-115-132 were assayed for binding to pRB polypeptides in the presence of various concentrations of competitor peptides. The quantity of E1A;37-54 or E1A;Y-115-132 competitor peptide added is indicated above each lane. Ten microliters of bead slurry and 10 μl of in vitro-translated pRB polypeptides were used for each binding reaction in 135 μl of ELB buffer. After incubation for 2 h at 4°C on a rocker, the beads were washed four times with 1 ml of ELB buffer. Labeled proteins that had bound to the beads were separated on 10% SDS-polyacrylamide gels (30) and detected by fluorography (2).

beads act as efficient adsorbents for pRB, we have shown that two regions of E1A are individually sufficient for interaction with pRB. Second, peptides containing either region are able to compete for E1A-pRB interaction, although the regions bind to pRB with different affinities, as shown in at least two different types of assay. Third, competition experiments between these two regions suggest that they bind to different sites within a binding pocket of pRB. Several models can be suggested to fit these observations. The simplest explanation is that region 1 and region 2 of E1A are folded to form a pRB-binding structure which makes at least two contacts with pRB. In this model, it is assumed that the E1A structure has a restricted or rigid conformation so that the binding of a peptide to one site within the pRB pocket sterically restricts the ability of E1A to make contact at the other site.

Competition experiments revealed that the two regions of E1A bind to pRB with different relative affinities, and these results are in good agreement with the properties of E1A mutants in vivo. E1A mutants with region 1 deletions have a severely reduced level of pRB binding activity, but one that is still detectable in immunoprecipitations of proteins from infected cells. However, experiments with E1A mutants carrying region 2 deletions failed to detect coprecipitating pRB (12, 50). The identification of two contact sites on E1A suggests that the high affinity of E1A for pRB, compared with that of the E1A mutants, may be due to the cooperative effects of having two binding sites on a single molecule. The marked difference in pRB binding by peptides containing region 1 and 2 sequences compared with peptides containing region 2 alone (Fig. 3B) also suggests that contact at the two sites produces a stronger or more stable interaction. However, we cannot rule out the possibility that the presence of either region induces a conformational alteration that promotes higher-affinity interaction at the other site.

The pRB-binding sequences of region 1 may do more than merely raise the affinity of the overall pRB-E1A interaction. Studies of E2F indicate that the pRB-binding region 1 of E1A may have an additional activity. The ability of E1A mutants to dissociate E2F protein complexes depends on the integrity of the region 1 sequences, and mutants of E1A in which amino acids 38 to 67 or 51 to 116 were deleted were unable to dissociate E2F complexes with cellular proteins (40). Since the E2F complexes have been subsequently shown to contain pRB, p107, cyclin A, and $p33^{cdk2}$ (3, 4, 6, 35, 43), this observation may reveal a role for the E1A region 1-pRB interaction. In recent experiments, the synthetic peptide E1A;37-54 was found to be sufficient for dissociation of E2F-p107-cyclin A-$p33^{cdk2}$ complexes (23). Clearly, it will be important to determine whether other biological activities of E1A are reproduced by these small peptides.

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