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Homologous Sequences in Adenovirus E1A and Human Papillomavirus E7 Proteins Mediate Interaction with the Same Set of Cellular Proteins

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Studies of adenovirus E1A oncoprotein mutants suggest that the association of E1A with the retinoblastoma protein (pRB) is necessary for E1A-mediated transformation. Mutational analysis of E1A indicates that two regions of pRB are required for E1A to form stable complexes with the retinoblastoma protein. In addition to pRB binding, these regions are necessary for E1A association with several other cellular proteins, including p130, p107, cyclin A, and p33^{cdk2}. Here we show that short synthetic peptides containing the pRB-binding sequences of E1A are sufficient for interaction with p107, cyclin A, and p130. The E7 protein of human papillomavirus type 16 contains an element that binds to pRB and appears to be functionally homologous to the E1A sequences. Peptides containing this region of the E7 protein were able to interact with p107, cyclin A, and p130 in addition to pRB. These findings suggest that the common mechanism of transformation used by these viral oncogenes involves their association with a set of polypeptides.

The adenovirus E1A oncoprotein has pleiotropic effects on cell proliferation. These changes appear to be mediated, at least in part, by the association of E1A with several cellular proteins. Stable protein complexes are readily detected in adenovirus-infected or -transformed cells (29, 58), and several genetic studies have correlated the ability of E1A mutants to bind to cellular proteins with their activities in transformation assays, cell immortalization, or the stimulation of host cell DNA synthesis after viral infection (19, 33, 36, 53, 57). To date the binding sites of six cellular proteins have been mapped to the region of E1A with transforming activity. These are the retinoblastoma protein, pRB, and a related protein, p107 (22, 56); cyclin A and a cyclin A-associated kinase, p33^{cdk2} (24–26, 55); and two additional proteins, p130 and p300 (26, 57). Analysis of the protein-binding properties of various E1A mutants has identified short segments of E1A that are essential for stable association with several of these cellular proteins. Deletion of amino acids 120 to 127 largely inhibits the formation of E1A complexes with at least five polypeptides, pRB, p107, p130, cyclin A, and p33^{cdk2} (25, 26, 57). Similarly, E1A deletions that include amino acids 30 to 60 produce E1A mutants that have severely impaired binding to pRB, p130, cyclin A, and p33^{cdk2} (25, 26, 57). The binding site of p300 appears to be distinct from that of these other proteins (57); it lies within the amino-terminal 49 amino acids of E1A (42).

The early proteins of other small DNA tumor viruses also bind to pRB (11, 14, 17, 46). For the large T antigens of simian virus 40 (SV40) and polyomavirus and the E7 proteins of human papillomavirus type 16 (HPV-16), mutagenesis of the pRB-binding sequences reveals a correlation between the pRB-binding properties and the cell immortalization or transforming properties of these oncoproteins (11, 30, 41, 46–48). The similarities between these viral proteins suggests that these oncogenes may be using common mechanisms for their effects on cell proliferation. This common mechanism may also include association with the pRB-related protein p107, since it shares overlapping binding sites with pRB on E1A and also on the large T antigen of SV40 (15, 23).

Studies of E1A mutants have pinpointed regions of E1A that are important for protein binding. However, these studies share the limitations of any deletion-mapping strategy. In a deletion mutagenesis, only mutants that retain function can be interpreted, and as a result only the sequences that are essential for binding are revealed. If additional regions of E1A also contribute to the stability of its protein complexes but are not essential for the interaction, they may have been missed by the mutagenesis strategy. As a result, the small regions that are essential for binding to cellular proteins may not be sufficient for the interaction. A further limitation of the deletion-mapping approach is that it cannot distinguish between elements that determine protein conformation and those that are true binding sites. For example, deletion mutants showed that two regions of E1A are essential for its association with pRB, p130, cyclin A, and p33^{cdk2}, but the respective contributions of these regions were not clear.

In order to investigate further the roles of these regions in the association of E1A with pRB and other cellular proteins, we have assayed the protein-binding properties of short synthetic peptides that contain and represent these sequences. It has been shown that small peptides bearing regions of adenovirus type 5 E1A, HPV-16 E7, and SV40 large T antigen are able to inhibit the association between viral proteins and pRB (12, 16, 37, 39, 50). Jones et al. demonstrated that a peptide containing only amino acids 21 to 29 of the HPV-16 E7 sequence was sufficient for competition (37). DeCaprio et al. found that a peptide containing amino acids 102 to 115 of the SV40 large T antigen sequence could inhibit both the large T-p107 and large T-pRB interactions (12). Using synthetic peptides in both binding and competition studies, we have shown that both regions of E1A that are important for association with pRB contain

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contact sites for the interaction (16). A low-affinity binding site is located between amino acids 37 and 54 of E1A, and a second contact site that binds to pRB with higher affinity lies between amino acids 117 and 132. For simplicity and to avoid confusion, these sequences will be referred to as regions 1 and 2, respectively, throughout this paper.

Since the pRB-binding sequences have been implicated in the association of E1A with several cellular proteins, we have investigated further the binding properties of these regions. Here we show that the small peptides of E1A that contain the pRB-binding sequences are also sufficient for interaction with several E1A-associated proteins. These binding characteristics are also seen with peptides from the amino terminus of HPV-16 E7, indicating that these properties are conserved between the oncoproteins of at least two different DNA tumor viruses. These observations suggest that the transforming domains of E1A and E7 contain similar structures that target a set of cellular proteins.

MATERIALS AND METHODS

Peptide synthesis and coupling. The peptides used in this work were HFEPPTLHELYDLDVTAP (E1A;37–54), DN LPPPSHFEPPTLHELYDLDVTAPEDPNEEAV (E1A;30–62), YLVPEVIDLTCHEAGFPPS (E1A;Y-115–132), LGPV SMPNLVPEVIDLTCHE AGFPPSDDEDEEGE (E1A;107–140), HFEPPTLHELYDLQPEVIDLTCHEAGFPPS (E1A; 37–49-Q-117–132), HFEPPTVHEVYDVQPEVIEVTSHDA GFPPS (control), HGDTPTLHEYMLDLQPETTDLYCYE QLNDSS (E7;2–32), HGDTPTLHEYMLDLQPETT (E7;2–20), and QPETTDLYCYEQLNDSS (E7;16–32). The control peptide is a mutated version of the peptide E1A;37–49-Q-117–132 that has previously been shown to be unable to bind to pRB. Peptides were synthesized and purified by the core service facility under the direction of D. R. Marshak, 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. Peptide purity was >95% as determined by plasma desorption mass spectrometry.

Peptides were bound to cyanogen bromide-activated Sepharose 4B beads through a single terminal amino group as described previously (16). Peptide-coupling efficiency was monitored by measuring the A_{205}, and routine coupling efficiencies of 80 to 90% were obtained for each peptide.

Peptide competition of E1A complexes formed in vitro. ML-1, HeLa, and 293 cell lines were lysed in 250 mM NaCl–50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5)–0.1% Nonidet P-40 as previously described. Mixing reactions were carried out with lysate from approximately 2×10^6 unlabeled 293 cells and 2×10^6 labeled HeLa cells. Mixtures were incubated on ice for 2 h, precleared, divided into aliquots, and immunoprecipitated with anti-E1A (M73) or control (PAb416) antibodies as previously described. Competitor peptides were added to the in vitro binding reactions from stock solutions at 4 or 10 mg/ml. To allow comparisons of peptide competition over a wide range of concentrations, we set up mixing reactions with lysate from 4×10^5 unlabeled 293 cells and 2×10^6 labeled HeLa cells.

Peptide competition of complexes between E1A and pRB polypeptides synthesized by in vitro transcription and translation of the human cDNA clone was carried out as described previously (16).

Binding of proteins to peptide beads. ML-1 cells, a human myeloid leukemia cell line, were labeled with [^{35}S]methionine and lysed in lysis buffer (250 mM NaCl, 50 mM HEPES [pH 7.5], 0.1% Nonidet P-40, 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) as described by Harlow et al. (28). The lysate was adjusted to 750 mM NaCl with 5 M NaCl and precleared by incubation with 100 μl of fixed *Staphylococcus aureus* Cowan 1 cells and 100 μl of Sepharose beads. After incubation on ice for 1 h the mixture was centrifuged for 15 min at 4°C, and the lysate was divided into portions. Peptide coupled to beads was added as 50 μl of a 50% slurry, and the mixture was rocked for 4 hours at 4°C. Peptide beads were washed four times with 1 ml of lysis buffer. Proteins that had bound to the peptide beads were separated by electrophoresis through sodium dodecyl sulfate (SDS)-polyacrylamide gels, and labeled proteins were detected by fluorography.

The binding of unlabeled proteins to peptide beads was carried out as described above, except that lysate was prepared from larger numbers of cells. Lysate prepared from approximately 5×10^7 cells was used for each incubation with beads. Proteins specifically bound to the beads were separated by electrophoresis through SDS-polyacrylamide gels and transferred to nitrocellulose (54). Proteins were detected by using monoclonal antibodies to pRB (XZ77 [34]), cyclin A (C160 [27]), and p107 (XZ37 [34]), followed by incubation with alkaline phosphatase-conjugated rabbit anti-mouse antibody (Bethesda Research Laboratories, Inc.).

Assays measuring binding to peptide beads by pRB polypeptides synthesized by in vitro transcription and translation of the human cDNA clone were carried out as described previously (16).

Partial proteolytic mapping. V8 partial proteolysis was carried out by using the procedure described by Cleveland et al. (10). Slices containing the appropriate proteins were excised from a 6% gel and digested with *S. aureus* V8 protease. Digested proteins were separated on an SDS–15% polyacrylamide gel (40) and visualized by fluorography (4).

Immunoprecipitation and Western blotting analysis. The HFK1321 and HFK40 cell lines were described previously (45, 49) and were obtained by immortalization of primary human foreskin keratinocytes either with the HPV-16 E6/E7 genes expressed from the human β-actin promoter or with SV40. E7 immunoprecipitations were performed with a monoclonal antibody from Triton Diagnostics (no. 10021) as described previously (48). pRB was detected after Western immunoblotting by anti-pRB monoclonal antibody PMG3-245.11 (Pharmingen) followed by ^{125}I-labeled sheep anti-mouse antibody (Amersham) as previously described (51).

RESULTS

Synthetic peptides were used to examine the interactions between E1A and cellular proteins. The goals of these studies were twofold: first, to determine whether the small regions of E1A that contain its pRB-binding sites were also sufficient for stable binding to other cellular proteins that are putative targets for E1A-mediated transformation, and second, to determine whether the pRB-binding domain of the HPV-16 E7 protein interacts with the same set of cellular proteins.

Two small regions of E1A that contain pRB-binding sequences were synthesized as peptides and were assayed for binding to E1A-associated proteins. These peptides were used in two types of assay: competition assays and protein-binding assays. In the first type, synthetic peptides were

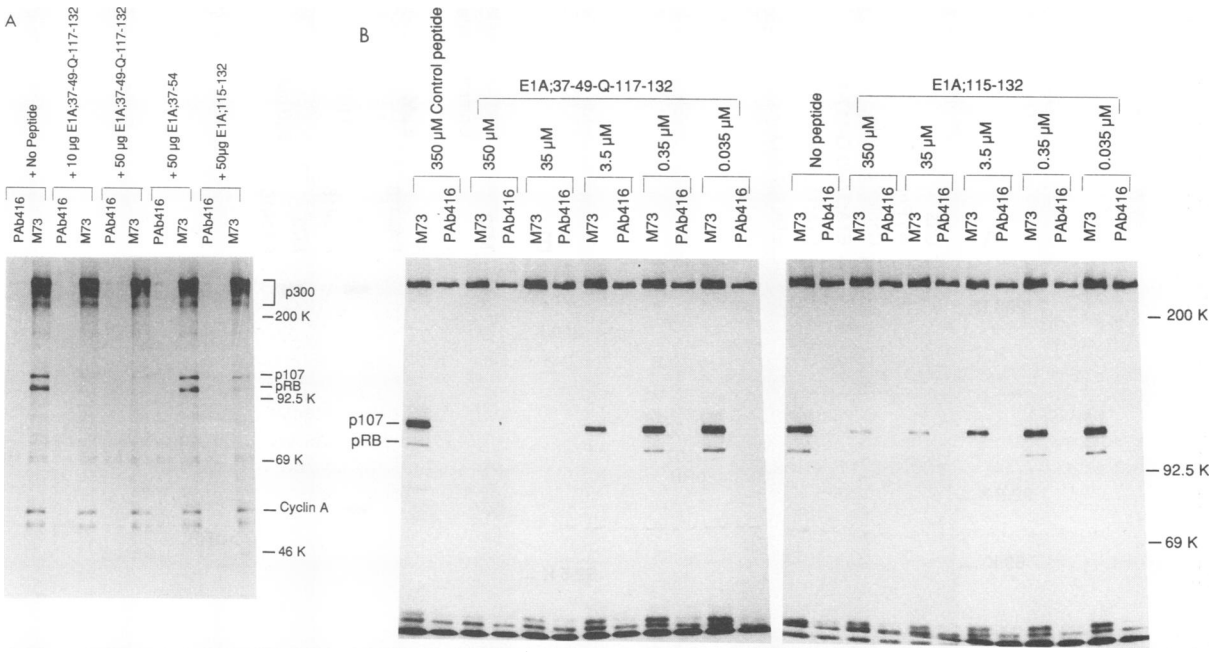


FIG. 1. E1A peptides compete with E1A for binding to ^{35}S-labeled cellular proteins. (A) In vitro association between E1A and cellular proteins was detected by immunoprecipitation of protein complexes formed between unlabeled E1A (in 293 cell extract) and methionine-labeled HeLa cell extracts. M73 is an E1A-specific antibody, and PAb416 is a control antibody. Addition of peptides containing region 2 of E1A specifically inhibited E1A association with p107 and pRB. (B) Titration of E1A peptides into the binding reaction. Extracts containing ^{35}S-labeled proteins were prepared from human ML-1 cells. Peptides were added at the concentrations shown.

added as competitors to in vitro binding reactions between E1A and cellular proteins. In the second type, peptides were coupled to beads and then assayed for specific immobilization of E1A-associated proteins. These two assays were used since each approach complements the limitations of the other. Potential pitfalls of peptide competition assays include the possibility that competition results from nonspecific interactions and the possibility that competition does not reflect true contact sites. By immobilizing the peptide on beads and incubating the peptide in a labeled cell lysate, we sought to determine the specificity of the peptide interaction. Furthermore, a positive result in this type of assay demonstrates that the peptide sequences are sufficient for formation of stable complexes and must therefore contain a contact site. We note that neither of these approaches nor the mutagenesis of E1A can distinguish proteins that are directly bound to E1A from those that are indirectly associated.

E1A peptides inhibit E1A association with pRB, p107, and p130 polypeptides. Many of the cellular proteins that bind to E1A in vivo form similar complexes with E1A in vitro. This is illustrated in Fig. 1A. Cell lysates prepared from HeLa cells metabolically labeled with [^{35}S]methionine were mixed with unlabeled cell extracts containing E1A (prepared from unlabeled 293 cells). After incubation, radiolabeled proteins that became bound to E1A were immunoprecipitated with an E1A-specific monoclonal antibody. This assay has been described in detail before (15, 29), and E1A-associated proteins that are readily detected are p300, pRB, p107, and p60. The identities of these proteins have been determined by comparisons of partial proteolytic digestion patterns.

We investigated the effects of adding synthetic peptides that represented small regions of E1A on the in vitro association of E1A with cellular proteins. Initially three peptides were tested. Two of these were peptides that contained the individual pRB-binding sites of E1A: a region 1 peptide (containing amino acids 37 to 54) and a region 2 peptide (containing amino acids 115 to 132). In addition, a peptide that linked sequences from both regions (region 1+2 peptide) through a glutamine (E1A;37–49-Q-117–132) was assayed. None of these peptides affected E1A association with p300. However, E1A binding to pRB and p107 was reduced in the presence of either the region 2 peptide or the region 1+2 peptide, but differences in the degrees of competition were apparent. In this experiment, no competition was seen when the region 1 peptide was used. In other experiments, high concentrations of this peptide marginally reduced E1A association with all of the major proteins (data not shown).

To quantitate the differences between the region 2 peptide and the region 1+2 peptide, we titrated these two competitor peptides into the binding reaction (Fig. 1B). The assay conditions were optimized to enable a wide range of peptide concentrations to be tested (see Materials and Methods for details), and under these conditions, no competition was seen with a negative control peptide at concentrations of 350 μM and higher. Region 2 peptide and region 1+2 peptide were efficient competitors of E1A association with pRB and, at 35 μM, inhibited E1A-pRB interaction. Surprisingly, these two peptides differed in their ability to inhibit the E1A-p107 association. Region 1+2 peptide at 35 μM inhibited E1A binding to p107; region 2 peptide at 35 μM significantly inhibited E1A-p107 binding but, even when added at a 10-fold-higher concentration, was unable to completely inhibit association with p107. This result suggests two possibilities. Either E1A sequences in addition to amino acids 115 to 132 contribute to the E1A-p107 interaction, or the region 2 peptide is unable to assume the correct conformation needed to bind efficiently to p107.

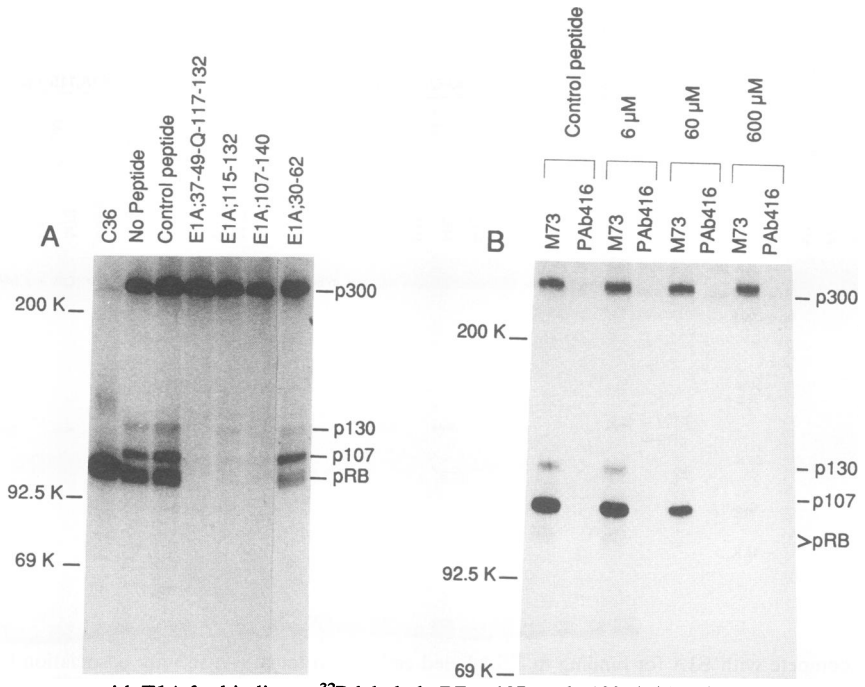


FIG. 2. E1A peptides compete with E1A for binding to ^{32}P-labeled pRB, p107, and p130. (A) In vitro association between E1A and cellular proteins was detected by immunoprecipation of protein complexes formed between unlabeled E1A (in 293 cell extract) and proteins extracted from $^{32}P_i$-labeled HeLa cells. Addition of 120 μM E1A peptide that contained region 2 of E1A specifically inhibited the association of E1A with pRB, p107, and p130. (B) Titration of peptide E1A;37–49-Q-117–132 in the competition assay. M73 is an E1A-specific antibody; PAb416 is a control antibody.

E1A-associated p130 is often difficult to detect in extracts of cells labeled with [^{35}S]methionine; however, longer exposure of the gels indicated that association of E1A with p130 may also be inhibited by E1A peptide. Earlier, we had noted that p130 was heavily phosphorylated, and we repeated the experiment with $^{32}P_i$-labeled cells to give a more sensitive detection of the E1A-p130 complex. As shown in Fig. 2, E1A association with $^{32}P_i$-labeled p130 was inhibited by the region 1+2 peptide (E1A;37–49-Q-117–132) and also partially by region 2 peptides (E1A;115–132 and E1A;107–140). Thus the peptide containing the pRB-binding sequences of E1A inhibited the association of E1A with at least three cellular proteins: pRB, p107, and p130.

E1A peptides coupled to beads act as efficient and specific adsorbents for cellular proteins including pRB, p107, and p60. To determine whether these small regions of E1A are sufficient for stable interaction and whether the peptides are binding specifically to E1A-associated proteins, we coupled the peptides to Sepharose beads and incubated them in cell lysates. Peptides immobilized in this way have previously been shown to bind efficiently to pRB produced by in vitro translation of the human cDNA clone (16). Initially the region 1+2 peptide (E1A;37–49-Q-117–132) was used since it was the most potent competitor peptide. This peptide was coupled to beads and incubated in cell lysate prepared from ML-1 cells that were metabolically labeled with [^{35}S]methionine. Very few proteins bound to the beads (Fig. 3), and their association was inhibited by incubation with uncoupled peptide but not with control peptide. Since the peptide bead-associated proteins comigrated with authentic E1A-associated proteins, it appeared that the binding properties of the peptides were highly specific. We wished to be certain that these proteins were the same proteins that associate

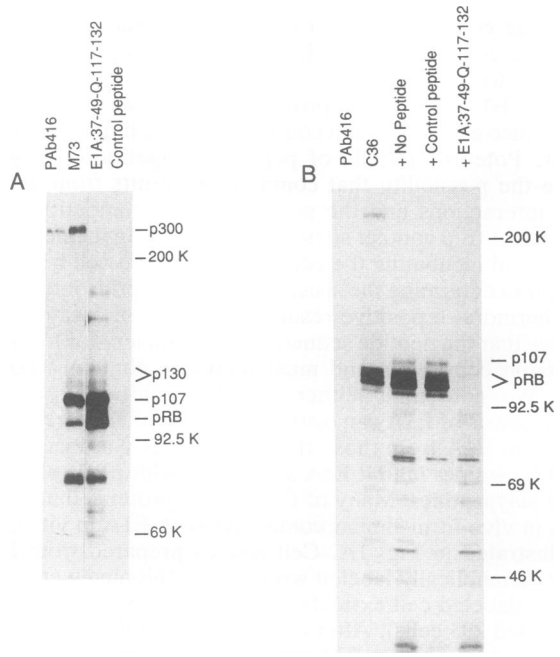


FIG. 3. pRB, p107, and p130 bind to immobilized E1A peptide. (A) Control and E1A peptides were covalently coupled to Sepharose beads and incubated in cell lysates prepared from [^{35}S]methionine-labeled HeLa cells. Peptide-associated proteins were separated on an SDS–6% polyacrylamide gel. E1A-associated proteins were prepared as for Fig. 1 and used to provide markers for pRB, p107, and p130. (B) Peptide-associated proteins isolated from [^{35}S]methionine-labeled ML-1 cells. C36 is an anti-pRB monoclonal antibody. Beads were incubated in cell lysate in either the presence or absence of 120 μg of peptide.

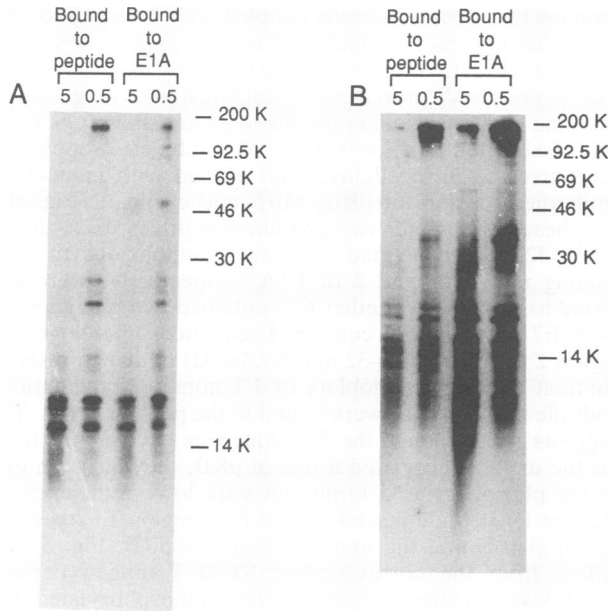


FIG. 4. Identification of pRB and p107 proteins by partial proteolytic mapping. (A) Comparison of E1A-associated pRB and the comigrating peptide-associated protein. E1A or peptide-associated proteins were prepared from [^{35}S]methionine-labeled ML-1 cell extracts and separated by SDS-polyacrylamide gel electrophoresis. Labeled proteins were located by autoradiography, excised from the gel, and digested with *S. aureus* V8 protease by the method of Cleveland et al. (10). The quantity of protease added is indicated above each lane. (B) Comparison of E1A-associated p107 and the comigrating peptide-associated protein.

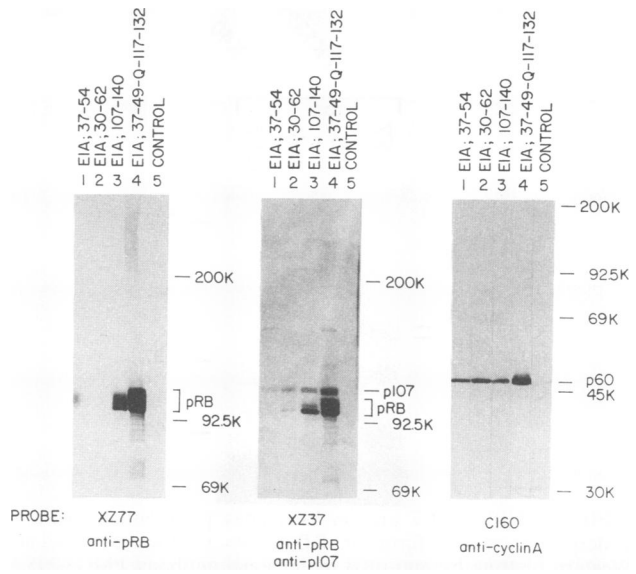


FIG. 5. pRB, p107, and cyclin A bind to immobilized E1A peptides. Control and E1A peptides were covalently coupled to Sepharose beads and incubated in cell lysates prepared from ML-1 cells. Peptide-associated proteins were separated on an SDS–6% polyacrylamide gel and transferred to nitrocellulose. pRB, p107, and cyclin A were detected by using the monoclonal antibodies XZ77, XZ37 (34), and C160 (27) as indicated.

with E1A in vivo, and we therefore tested the identities of three of these proteins by partial proteolytic mapping and on Western blots probed with specific monoclonal antibodies. Figure 4 shows a comparison of the products of partial proteolytic digestion of bead-associated proteins with the E1A-associated pRB and p107 proteins; the products of partial digestion with *S. aureus* V8 protease generated from bead-associated proteins gave patterns identical to those generated from authentic pRB and p107.

Western blots of proteins from ML-1 cell lysate that bound to E1A peptide beads are shown in Fig. 5. Nitrocellulose strips were probed separately with the anti-pRB monoclonal antibodies XZ77 and XZ37 and with C160, a monoclonal antibody that recognizes human cyclin A. XZ77 is an anti-pRB monoclonal antibody that cross-reacts weakly with the E1A-associated protein p300 (34). XZ37 is from the same series of RB antibodies but recognizes an epitope that is also found on p107. As shown in Fig. 5, multiple forms of pRB bound to the peptide containing regions 1 and 2 and were detected by both RB antibodies. The multiple bands represent different phosphorylated forms of pRB. The fastest-migrating forms of pRB have previously been shown to be largely unphosphorylated, present primarily in G_0- and G_1-phase cells, and selectively targeted by SV40 large T antigen. Lower levels of pRB, and apparently fewer of the phosphorylated forms, were bound by the region 2 peptide (E1A;107–140). pRB binding to the region 1 peptides (E1A; 30–62 and E1A;37–54) was barely detectable in these experiments, and a low level of binding was apparent only on the strip probed with XZ37.

XZ37 detected p107 bound to beads carrying the region 1+2 peptide. A lower level of p107 was also associated with beads carrying region 1 peptides or region 2 peptide but not with beads coupled with control peptide. The pattern of cyclin A binding seen after probing with C160 closely resembled that of p107 binding. These results show that two regions of E1A, when assayed separately, are sufficient for association with p107 and cyclin A. When taken together, the results of competition experiments and binding experiments indicate that the small regions of E1A that contain the binding sites for pRB are also sufficient for interaction with at least three other proteins: p107, p130, and cyclin A.

E7 peptides capable of binding to several E1A-associated proteins. Other oncoproteins of small DNA tumor viruses also contain pRB-binding sequences that are homologous to regions 1 and 2 of E1A. One of the best-studied examples is the E7 protein of HPV-16. Sequences near the amino terminus of E7 have been shown to be essential both for the association with pRB and for E7 transformation activity (3, 18, 30, 46, 48). Since the pRB-binding regions of E1A mediate association with several proteins, we wished to determine whether E7 sequences had the same properties. Initially we looked for evidence of E7 association with p107 and cyclin A. Immunoprecipitations prepared with anti-E7 antibodies were transferred to nitrocellulose and probed with antibodies that recognize p107, cyclin A, or pRB. pRB was detected in anti-E7 immunoprecipitations prepared from a human keratinocyte cell line immortalized by HPV E6/E7 (HFK1321) but not from a control keratinocyte cell line immortalized by SV40 (HFK40) nor in immunoprecipitations with control antibodies (Fig. 6). The E7 protein preferentially associated with the faster-migrating forms of pRB, although a lower level of slower-migrating, phosphorylated pRB was also seen bound to E7. This observation of complexes formed in vivo is consistent with results of in vitro binding studies that reported that E7 protein binds preferentially to underphosphorylated pRB (35).

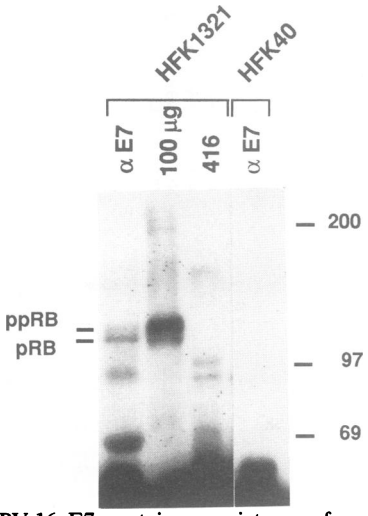


FIG. 6. HPV-16 E7 protein associates preferentially with the underphosphorylated forms of pRB in vivo. pRB was detected after Western blotting by anti-pRB monoclonal antibody PMG3-245.11. Proteins were immunoprecipitated with anti-E7 or control (PAb416) antibodies from extracts of human keratinocyte cell lines immortalized by either HPV E6/E7 (HFK1321) or SV40 (HFK40). The multiple bands of pRB present in 100 μg of total cell extract are the result of differential phosphorylation (5, 9, 12, 43).

Antibodies to p107 or cyclin A failed to reveal either of these proteins in E7 immunoprecipitations. However, neither of these antibodies are high-affinity reagents, and since the pRB association was barely detectable with better antibodies, it is probable that the detection of E7-associated p107 and cyclin A was beyond the limits of the sensitivity of the experimental system. We therefore tested synthetic peptides containing the pRB-binding sequences of HPV-16 E7 in binding and competition assays to investigate whether these sequences can interact with additional E1A associated proteins.

The pRB-binding site of HPV-16 E7 has been mapped to the amino terminus of the viral protein. A peptide containing amino acids 2 to 32 of E7 that was homologous in length and composition with the E1A region 1+2 peptide was synthesized. The E7 peptide was first assayed for pRB-binding activity as previously described for E1A peptides (16). E7 peptide, when coupled to Sepharose beads, bound to pRB polypeptides; both the level of binding and the pattern of polypeptides retained by the E7 peptide were identical to those seen with the E1A region 1+2 peptide (Fig. 7). In competition assays, the E7 peptide was a more potent competitor of E1A association with pRB polypeptides in vitro than any of the E1A peptides were.

E7 peptide was added as a competitor to an in vitro binding reaction between E1A and labeled cellular proteins (Fig. 7B). Both E7 and E1A peptides had similar effects on the binding reaction and inhibited E1A binding to pRB, p107, and p130. In this experiment it is also apparent that E1A binding to cyclin A was also significantly reduced in the presence of these peptides. Neither peptide had any effect on E1A association with p300, and no competition was seen with the control peptide. We investigated the specificity of the E7 peptide by coupling it to beads and incubating the beads in a labeled cell lysate. A small number of proteins were specifically bound to beads carrying the E7;2–32 peptide (Fig. 8). The most intense of these comigrated with proteins that bound to beads coupled with E1A peptide and were identified as pRB and p107 by two approaches. First, the products generated from these bands by partial digestion with *S. aureus* V8 protease were identical to those generated from the E1A-associated proteins (data not shown). Second, bead-associated proteins were separated by electrophoresis, transferred to nitrocellulose, and probed with monoclonal antibodies specific for pRB, p107, and cyclin A (Fig. 8C). For these experiments two additional peptides, E7;2–20 and E7;16–32, that contained sequences homologous to pRB-binding regions 1 and 2 of E1A, respectively, were also tested to determine whether E7 contained two binding sites. Both E7 peptides that contained sequences homologous to region 2 of E1A (E7;2–32 and E7;16–32) bound to pRB. In contrast to the immunoblots of E7 immunoprecipitations, multiple forms of pRB were bound to the peptide beads. This suggests that although the E7 sequences have a preference for the unphosphorylated forms of pRB, they also can bind to the phosphorylated forms but with lower efficiency. In this the E7 peptide resembles the E1A region 1+2 peptide, which also bound the multiple forms of pRB (Fig. 5), but differs from the corresponding SV40 T-antigen peptide, which shows a strong preference for unphosphorylated pRB when coupled to Sepharose beads (data not shown).

E7 peptides that contained sequences homologous to region 2 of E1A (E7;2–32 and E7;16–32) also bound to p107 and to cyclin A. Two additional proteins of 45 and 32 kDa bound to the E7;2–32 peptide and were detected by the cyclin A antibody. These proteins were not seen with E1A peptides, and their significance is unclear. Surprisingly, beads coupled to the E7 region 1 peptide (E7;2–20) failed to bind to any of the E1A-associated proteins. This suggests that the amino terminus of E7 lacks binding sites analogous to E1A region 1. However, because this E7 peptide showed no significant activity in any of the assays described above, it is possible that the peptide fails to reproduce binding properties of the full-length protein. Nevertheless, these experiments show that the pRB-binding sequences of HPV-16 E7 are able to interact with a set of cellular proteins, comprising pRB, p107, p130, and cyclin A, that share overlapping binding sites on E1A.

DISCUSSION

Genetic studies reveal a tight correlation between the association of E1A with pRB and its transformation activities. Analysis of E1A mutants have suggested that the regions of E1A that are necessary for pRB binding are also important for association with additional cellular proteins, including p130, p107, cyclin A, and $p33^{cdk2}$. Using synthetic peptides to represent these regions of E1A, we have found that these short sequences are sufficient for stable association with pRB, p130, p107, and cyclin A. The binding of the cyclin A-associated protein $p33^{cdk2}$ was not measured in these experiments but is likely to mirror the cyclin A binding pattern. In general, the properties of the synthetic peptides were consistent with the predictions of genetic studies and supported their conclusions. In several cases the peptide-binding properties revealed new pieces of information that complement the genetic analysis, and these results are summarized in Table 1.

A new piece of information is the finding that peptides from two regions of E1A were sufficient for binding to p107. This result was unexpected since the analysis of E1A mutants has indicated that only amino acids 120 to 127 were essential for association with p107. However, in previous

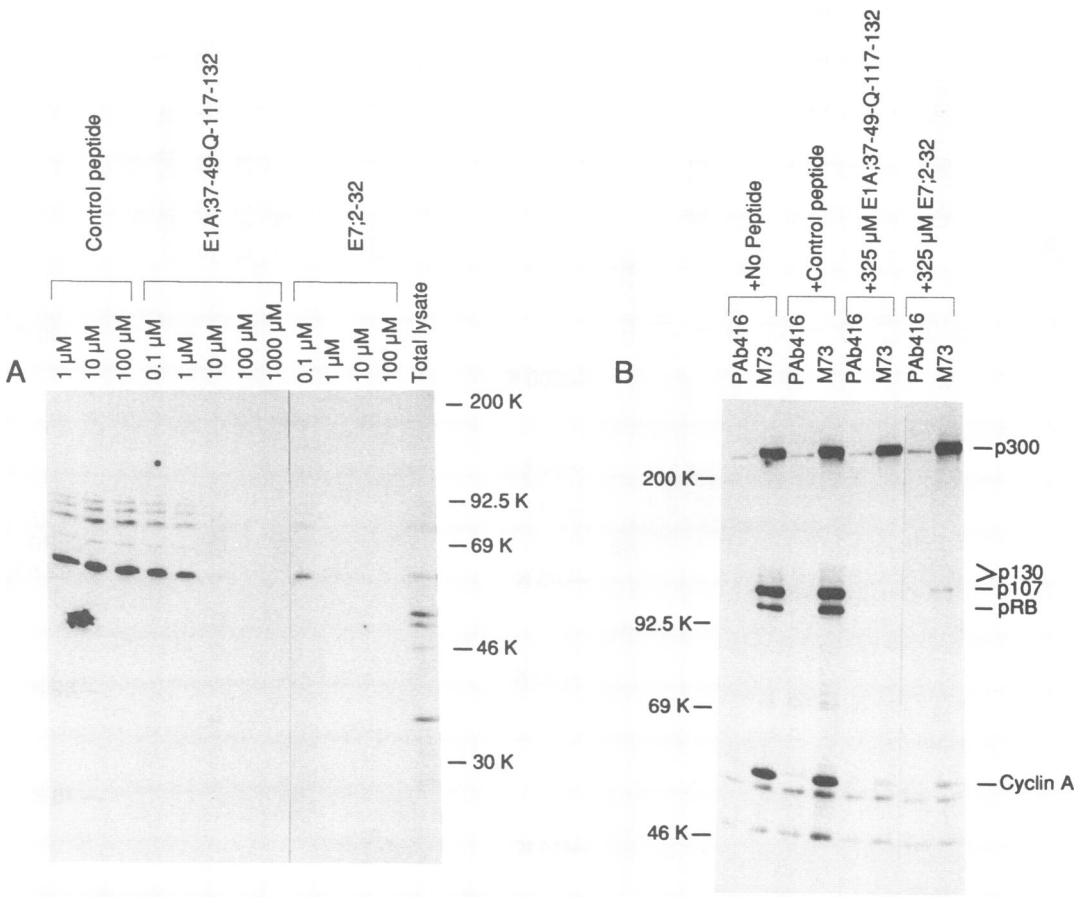


FIG. 7. E7 peptides compete with E1A for binding to pRB, p107, and p130. (A) E1A, E7, or control peptides were added at the concentrations shown to mixtures of E1A containing cell extracts (from 293 cells) and [^{35}S]methionine-labeled RB polypeptides (synthesized by in vitro transcription and translation of the cDNA clone). (B) E1A, E7, or control peptides were added at the concentrations shown to mixtures of E1A containing cell extracts (from 293 cells) and [^{35}S]methionine-labeled ML-1 cell extracts. The markers indicate the position of cellular proteins that specifically bind to E1A and are immunoprecipitated by the E1A-specific monoclonal antibody (M73). PAb416 is a control antibody.

studies it has been noted that mutants that disrupt this region still show a low level of binding to proteins that comigrate with p107 (deletion of amino acids 122 to 129 [57]; deletion of amino acids 26 to 35 and 124 to 127 [33]; deletion of amino acids 111 to 123 [20]). From the binding properties of the E1A peptides, it seems likely that the E1A-associated 107-kDa proteins observed in earlier studies may be due to a second p107-binding site that lies between amino acids 37 and 54 of E1A.

Genetic studies indicated that these two regions of E1A are necessary for E1A to associate with pRB and cyclin A. The peptide experiments suggest that these two regions make different contributions to these interactions. E1A amino acids 115 to 132 contain a pRB-binding site that is approximately 100-fold stronger than that between amino acids 30 and 60. Several assays indicate that the peptide-containing sequences from both of these regions have the highest affinity for pRB. In contrast, the pattern of cyclin A binding to peptides on beads shows a different pattern. Unlike pRB, cyclin A bound equally well to peptides containing either of the two regions of E1A and bound only slightly better to the peptide containing both regions 1 and 2. Surprisingly, in competition assays the ability of these peptides to inhibit E1A-cyclin A association proved to be variable between experiments. In Fig. 7B both the E1A region 1+2 peptide and the E7 peptide strongly inhibited cyclin A association; however, in other experiments the competition was less evident.

Mutational studies suggested that the same two regions of E1A are important for the E1A-p130 interaction. Consistent with this, the region 1+2 peptide that combines both regions inhibited p130 association with E1A. However, the relative contributions of regions 1 and 2 to E1A association with p130 were not clear in the peptide experiments. Proteins that comigrated with p130 were bound to region 1+2 peptide beads, but the level of association was too low to allow partial proteolytic mapping. p130 is one of the E1A-associated proteins that are difficult to detect, and more detailed studies of the E1A-p130 interaction are impossible until specific reagents for p130 are available.

The observation that small regions of E1A that are essential for its oncogenic activities provide association with multiple cellular proteins raises a number of issues. The most important of these is the question of whether the oncogenic activities of E1A are dependent on its association with all of these factors or only a few. To date, studies of E1A mutants have failed to separate the binding of any of these proteins from transforming activity. To address this

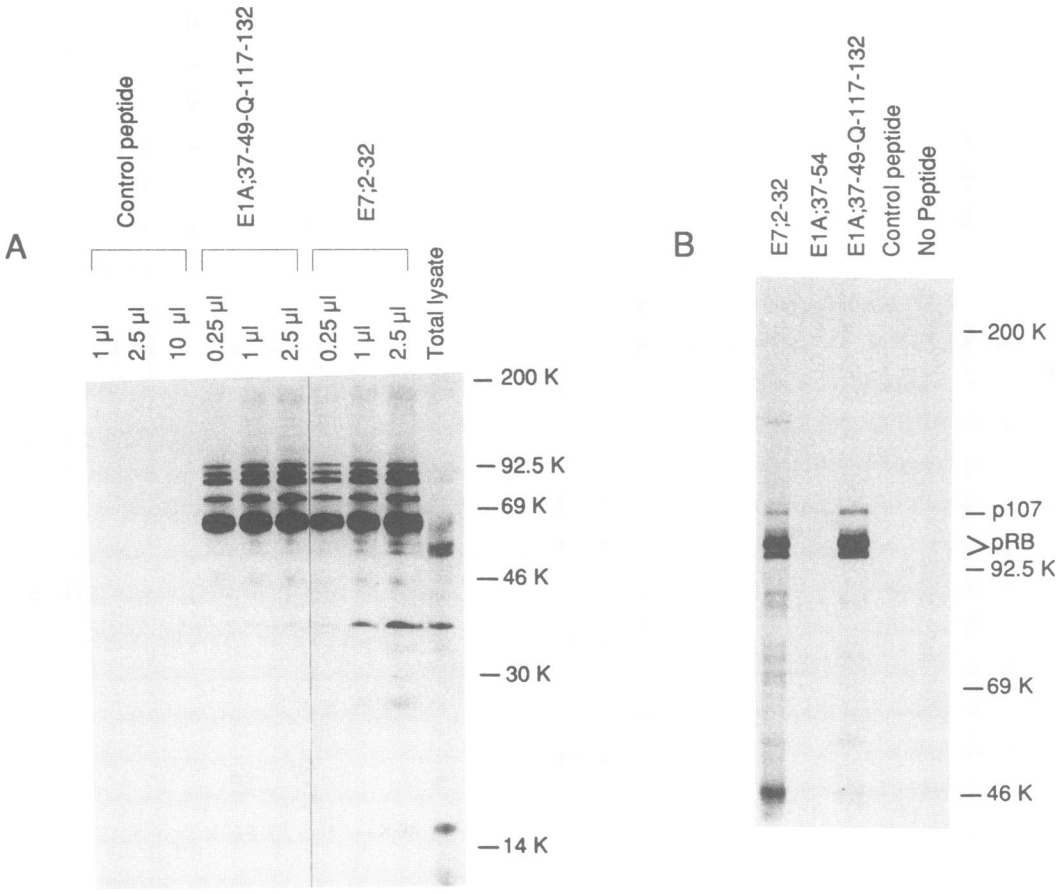
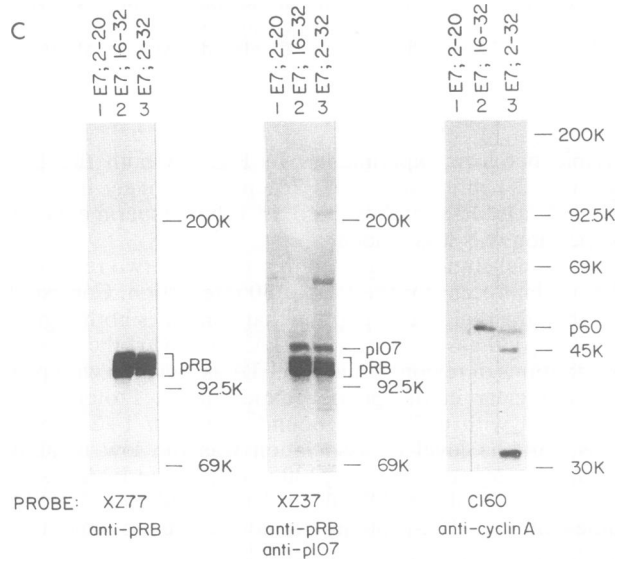


FIG. 8. pRB, p107, and cyclin A bind to immobilized E7 peptides. (A) E1A, E7, or control peptides were bound to Sepharose beads through a single terminal amino group. Various volumes of peptide beads were incubated with labeled pRB polypeptides, and bound proteins were separated by electrophoresis and detected by fluorography. Total lysate (1 µl) was run on the gel as a marker. (B) Sepharose beads carrying E1A, E7, or control peptides were rocked in extracts prepared from [^{35}S]methionine-labeled ML-1 cells. Specific peptide-associated proteins were separated by electrophoresis and detected by fluorography. (C) E7 peptides were covalently coupled to Sepharose beads and incubated in cell lysates prepared from ML-1 cells. Peptide-associated proteins were separated on an SDS–6% polyacrylamide gel and transferred to nitrocellulose. pRB, p107, and cyclin A were detected by using the monoclonal antibodies XZ77, XZ37 (34), and C160 (27) as indicated.



issue, we have investigated whether a second viral oncoprotein, HPV-16 E7, that also binds to pRB interacts with the same set of cellular proteins. By using synthetic peptides, it is apparent that E7 is able to interact with the same set of cellular proteins that are apparently targeted by E1A. Although it remains to be proven that E7 associates with these proteins in vivo, these results strongly suggest that the ability to target a set of cellular proteins is a functionally important property that has been conserved during the evolution of these viruses. At present it appears that pRB, p107, and p130 are all individually targeted by E1A, because there is no evidence of complexes that contain two of these proteins. Thus, the common mechanisms for transformation used by E1A and E7 seem to involve the targeting of a common set of proteins or protein complexes or both.

Four of the cellular proteins that associate with E1A, i.e., pRB, p107, cyclin A, and p33^{cdk2}, have been found more recently in protein complexes with the transcription factor E2F (1, 2, 6, 7, 13, 44, 52). Addition of E1A polypeptides causes the dissociation of E2F-pRB and E2F-p107-cyclin A-cdk2 complexes in vitro. Furthermore, the expression of E1A in vivo correlates with the failure to detect E2F-pRB and E2F-p107-cyclin A-cdk2 complexes, the appearance of unbound E2F, and an increase in transcription from E2F-

TABLE 1. Summary of the interactions detected between E1A or E7 peptide and E1A-associated proteins.

Peptide	Interaction with E1A-associated protein[a]:							
	Binding to immobilized peptide				Binding inhibited by peptide			
	pRB	Cyclin A	p107	p130	pRB	Cyclin A	p107	p130
E1A region 1	+	++	++	−	?	−	?	−
E1A region 2	+++	++	++	−	+++	?	++	+++
E1A regions 1+2	+++++	+++	++++	×	+++++	+	++++	+++++
E7	+++++	++	++++	−	+++++	+	+++	++++
Control	−	−	−	−	−	−	−	−

[a] The activities of the peptides in the two types of assay are represented as follows: −, no activity detected; ?, competition activity not consistently reproducible; ×, comigrating protein detected but identity of protein bound to beads not confirmed; +, very weak; ++, weak; +++, moderate; ++++, strong; +++++, very strong.

dependent promoters (7, 31, 32, 44). In similar experiments with HPV E7 proteins, GST-E7 fusion proteins were shown to dissociate the E2F-pRB complex in vitro, and the E2F-pRB complex was found to be reduced or absent in human cervical carcinoma cell lines in which E7 proteins were expressed (8). In each of these studies, the effects of E1A and E7 were dependent on their pRB-binding sequences, leading to the suggestion that the ability of E1A and E7 proteins to dissociate E2F complexes is one function of these homologous sequences.

Although E2F complexes may provide one target for these conserved elements, several lines of evidence indicate that these regions of E1A and E7 have more functions than simply the regulation of E2F. First, many cellular proteins bind to both pRB and p107 in vitro, and these interactions are inhibited by E7 and E1A peptides (21, 38). Second, E7 and large T antigen have a reduced ability to dissociate E2F-p107 complexes, although both proteins bind to p107 with high affinity (8). Third, as illustrated here, the pRB and p107 binding sequences also provide interaction with p130, a protein that has not been shown to associate with E2F.

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