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Protein Domains Governing Interactions between E2F, the Retinoblastoma Gene Product, and Human Papillomavirus Type 16 E7 Protein

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Human papillomaviruses (HPVs) are the etiological agents for genital warts and contribute to the development of cervical cancer in humans. The HPV E7 gene product is expressed in these diseases, and the E7 genes from HPV types 16 and 18 contribute to transformation in mammalian cells. Mutation and deletion analysis of this gene suggests that the transforming activity of the protein product resides in the same domain as that which is directly involved in complex formation with the retinoblastoma gene product (pRB). This domain is one of two conserved regions (designated CRI and CRII) shared by E7 and other viral oncoproteins which bind pRB, including adenovirus E1A protein. Binding of HPV type 16 E7 protein to pRB has previously been shown to affect pRB's ability to bind DNA and to form complexes with other cellular proteins. In the current study, we map the functional interaction between E7 protein and pRB by monitoring the association between a 60-kDa version of the pRB, pRB60, and the cellular transcription factor E2F. We observe that CRII of E7 (amino acids 20 to 29), which completely blocks binding of full-length E7 protein, is necessary but not sufficient to inhibit E2F/pRB60 complex formation. While CRI of E1A (amino acids 37 to 55) appears to be sufficient to compete with E2F for binding to pRB60, the equivalent region of E7 is neither necessary nor sufficient. Only E7 fragments that contained both CRII and at least a portion of the zinc-binding domain (amino acids 60 to 98) inhibited E2F/pRB60 complex formation. These results suggest that pRB60 associates with E7 and E2F through overlapping but distinct domains.

Human papillomaviruses (HPVs) constitute a large group (>60 types) of viruses, about half of which can infect the genital mucosa and cause both benign and malignant lesions (for a review, see reference 47). Only a few of the HPV subtypes are associated with most cervical cancers (75 to 100%). HPV-16 is the strain most commonly associated with this disease (3, 46). In these cases, two viral genes, E6 and E7, are transcriptionally active (3, 11). HPV-16 E6 and E7 are necessary and sufficient to transform primary human keratinocytes (30, 42), and the E7 gene alone can transform established rat embryo fibroblasts (25) or, in conjunction with *ras*, primary rat epithelial cells (12, 28).

The HPV-16 E7 gene encodes a protein of 98 amino acids which contains two regions of homology with adenovirus type 5 (Ad5) E1A and simian virus type 40 large T proteins (5, 15, 16, 35, 44). These two conserved regions, designated CRI and CRII, contribute to the transforming properties of these oncogenes and have been the subject of numerous mutagenesis studies. CRII of these oncoproteins has been shown to mediate binding to the retinoblastoma gene product, pRB (16, 17, 31, 41, 44). In the case of E7, CRII corresponds approximately to amino acids 20 to 30 and is required for the association of E7 with pRB (16). Alterations or deletions in this region which reduce or eliminate pRB binding also abrogate E7's transforming activity. Peptides from this region bind to pRB with high affinity and prevent formation of E7 protein/pRB complexes, verifying that these residues are necessary and sufficient to bind to pRB (13, 16, 26, 27).

The regions of pRB which are required for binding to the CRII-containing viral oncoproteins have also been delineated. These regions show similarity to a recently cloned homolog of pRB called p107 (19). The carboxy-terminal sequences of both proteins form a binding region commonly referred to as a pocket. In the case of pRB, analysis of mutations in this region suggests that only amino acids 379 to 772 are required for binding to the viral proteins (23). This domain has been incorporated into a truncated form of pRB, pRB60, which can be readily expressed and purified from *Escherichia coli* and retains full E7 protein-binding activity (18).

The cellular protein pRB appears to play a critical role in regulating the cell cycle (for a review, see reference 10). Besides binding HPV-16 E7, pRB also binds the cellular transcription factor E2F/DTRF (4, 8, 9). E2F's interaction with pRB has been verified via formation of E2F/DNA complexes whose mobility in gel retardation assays can be altered with antibodies to pRB. Formation and dissociation of these complexes is correlated with the progression of mammalian cells through the cell cycle (29, 37). These observations suggest that complex formation with E2F and/or other transcription factors may contribute to pRB's ability to regulate cellular proliferation (2, 22). The transforming properties of the pRB-binding viral oncogenes may therefore be explained by the ability of these proteins to directly compete with E2F for binding sites on pRB. The adenovirus E1A gene products and HPV-16 E7 protein have both been shown to disrupt pRB-E2F association (1, 7, 33,

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36). The conversion of the pRB/E2F complex to free E2F correlates with an increase in transcriptional activity, suggesting that one function of these transforming proteins is to increase transcription of genes dependent on E2F promoter sequences. Genes containing these promoter sequences encode proteins known to participate in cell growth regulation, such as c-Myc, n-Myc, c-Myb, dihydrofolate reductase, and the epidermal growth factor receptor (21).

To better understand the mechanism by which E7 disrupts pRB/E2F complex formation, HPV-16 E7, Ad5 12S E1A, and peptides derived from these proteins were assayed for the ability to compete with E2F for binding to pRB. Free E2F and E2F/pRB complexes were distinguished by a DNA-binding gel mobility assay. The results of these experiments allow delineation of different binding domains on pRB for the association of E7, E1A, and E2F.

MATERIALS AND METHODS

Purification of E2F. Human E2F was purified from HeLa cells by methods similar to those described previously (45). Cells were lysed by a modified Dignam procedure (14). Whole cell lysate was fractionated over heparin agarose, and peak E2F fractions were then applied to a high-performance anion-exchange column (Bio-Rad MA7Q). Detergent {Nonidet P-40 or 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfate} was added to a final concentration of 0.1% to the column fractions. The E2F-containing fractions were bound to a sequence-specific DNA affinity column and eluted essentially as described previously (45). The E2F pool obtained from the DNA affinity column contained multiple protein bands when analyzed by silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A dominant DNA-binding species was observed in the gel shift assay using an E2F probe (see Results). The major band was resistant to treatment with 0.6% deoxycholate, suggesting that this band represents free E2F. Two minor bands were also present.

Purification of recombinant proteins and E7 fragments. Recombinant pRB60 was isolated from *E. coli* by E7 peptide affinity chromatography essentially as described previously (18). This fusion protein begins with a short leader sequence and contains amino acids 387 to 928 of human pRB. This protein contains conserved domains A and B of pRB, including all sequences necessary to bind to E7 protein (23). The C-terminally modified form of this protein, pRB50, contains a deletion of amino acids 777 to 909 of pRB60 and was purified in an identical manner. pRB105 from a baculovirus expression system was the generous gift of Carol Prives (Columbia University, New York) and was purified by E7 peptide affinity chromatography.

For the binding competition experiments, wheat germ-translated E7 and E1a or recombinant E7 was used. HPV-16 E7 or Ad5 12S E1A (a generous gift from J. Schaack, University of Colorado, Denver) protein was translated from pGEM constructs containing the appropriate cDNA sequences. Recombinant HPV-16 E7 protein was isolated from *E. coli* as a fusion protein as previously described (32). This protein contains a 16-amino-acid leader sequence from pUC18 (MAMITNSSSVPPGIST). Thus, the full-length recombinant protein is designated E7(−*16*–98). Cyanogen bromide cleavage of recombinant E7 protein yielded E7(13–84), trypsin (Promega) digestion of E7 yielded E7(−*16*–49), and endopeptidase Lys C (Promega) digestion of recombinant E7 yielded E7(−*16*–60). These protein fragments were isolated by anion-exchange and gel filtration chromatography. The identity of each fragment was verified by amino acid composition analysis and/or N-terminal amino acid sequencing. Protein concentrations were determined spectrophotometrically, calibrated against a sample whose concentration was verified by amino acid composition analysis. [*N*-Methyl-Leu-22,28,*N*-methyl-Tyr-25] E7(20–29) peptide amide, referred to as the E7 CRII peptide, was synthesized as previously described (26). Scrambled E7 CRII peptide (YNELCQYDL-amide) and E7(1–29) amide were synthesized by standard solid-phase methodology. *N*-Acetyl-E1A(37–55) amide, referred to as E1A CRI peptide, and scrambled E1A CRI peptide (*N*-acetyl-LAHYEDPLFTHPD TEPELV-amide) were synthesized by Multiple Peptide Systems (San Diego, Calif.).

Gel shift assay and binding competitions. E2F was detected by a DNA-binding gel shift assay. pRB-E2F complexes were reconstructed by using DNA affinity-purified E2F and recombinant pRB60. Conditions for E2F DNA binding were as follows: 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9), 10 mM $MgCl_2$, 0.1 M EDTA, 40 mM KCl, 10% glycerol, 0.15% Triton X-100. In addition, each binding reaction mixture contained 60 μg of bovine serum albumin and 200 ng of single-stranded salmon sperm DNA. The protein-protein interactions in this assay were specifically dependent on carrier DNA and $MgCl_2$ concentrations and ionic strength. The specific E2F DNA probe was synthesized by Midland Certified Reagent Co. (Midland, Tex.). It contained a single E2F site and was based on the Ad5 E2 promoter (5'-TAGTTTTCGATATTAAATTTGAG AAAGG**GCGCGAAA**CTAG-3'). Double-stranded DNA was phosphorylated with (γ-^{32}P]ATP (Amersham) by T4 polynucleotide kinase (Boehringer) to a specific activity of approximately 10^5 dpm/ng of DNA. Cold competitor DNA contained either a wild-type E2F site or a mutated site (**TATCGAAA**).

Binding competitions for E2F were done in the following manner. Affinity-purified E2F was mixed with inhibitor (e.g., E7). pRB60 was then added at a concentration of 2.5 nM. This procedure allows E2F and the inhibitor to compete for binding to pRB60. In the absence of antagonist or pRB, binding buffer was added so that all reaction mixtures were in the same final volume. Complexes were then allowed to bind to 1 fmol of radioactively labeled DNA for 30 min at approximately 26°C and resolved on a nondenaturing polyacrylamide gel (Novex) in half-strength Tris-borate-EDTA (Novex) for approximately 1.5 h at 150 V at 4°C.

Competitive enzyme-linked immunosorbent assays (ELISAs) for binding to pRB were performed as previously described (27).

RESULTS

Identification of the E2F DNA-binding species. E2F was identified by binding to a specific DNA sequence (see Materials and Methods). DNA affinity-purified E2F fractions contained three species which bound to the E2F sequence (Fig. 1A). The major band and the two minor bands were resistant to treatment with 0.6% deoxycholate (data not shown) and were unaffected by competition with a 20-fold excess of a DNA probe containing a mutated E2F binding site (Fig. 1A). The minor bands could represent either breakdown products of E2F which retain DNA-binding activity or related proteins which bind to the E2F sequence.

Binding of E2F to PRBs. The cellular transcription factor E2F has previously been shown to form a complex with pRB obtained either from *E. coli* as a recombinant fusion with

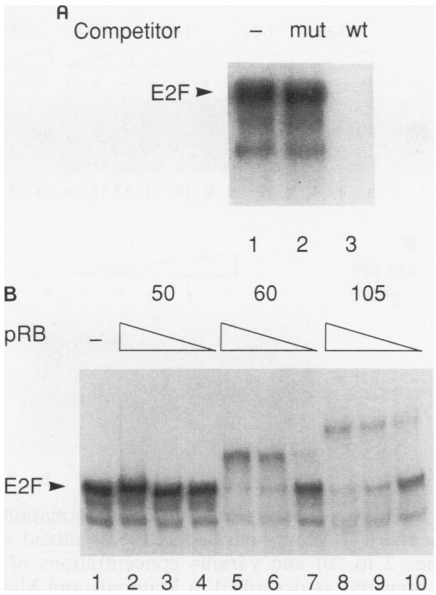

FIG. 1. Gel shift analysis of E2F complexes. DNA affinity-purified E2F was identified by binding to a DNA oligonucleotide containing a single E2F site (see Materials and Methods). (A) Gel shift assay performed in the absence of competitor (lane 1) or the presence of a 20-fold molar excess of mutant (lane 2) or wild-type (lane 3) oligonucleotide. (B) Titrations of affinity-purified E2F (lane 1) with recombinant pRB50 (lanes 2 to 4), pRB60 (lanes 5 to 7), and pRB105 (lanes 8 to 10). pRBs were tested at 10 nM, 2 nM, and 400 pM.

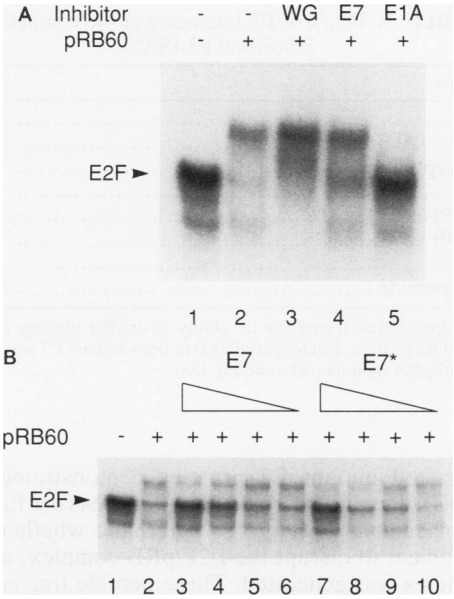

FIG. 2. Inhibition of E2F/pRB60 complex formation by E7 and E1A proteins. (A) Affinity-purified E2F was mixed with 2.5 nM pRB60 (lanes 2 to 5) and wheat germ extract (lane 3), wheat germ-translated E7 (lane 4), or wheat germ-translated E1A (lane 5). (B) Affinity-purified E2F was mixed with 2.5 nM pRB60 (lanes 2 to 10) and various concentrations of recombinant E7 protein (lanes 3 to 6) or Cys-24→Ala E7 protein (E7*) (lanes 7 to 10). The concentrations of each E7 protein were as follows: 3.5 μM, 350 nM, 35 nM, and 3.5 nM.

glutathione *S*-transferase or from mammalian cells (4, 7, 22). The ability of DNA affinity-purified E2F to form complexes with affinity-purified full-length and truncated pRBs was tested by using the gel shift assay. All three pRBs, pRB105 (full-length), pRB60, and pRB50, contain intact E7-binding pockets and have similar affinities for the E7 protein (data not shown). In contrast, only pRB105 and pRB60 readily form complexes with E2F, binding all of the E2F at a concentration of 2.5 nM (Fig. 1B). Treatment of these complexes with 0.6% deoxycholate disrupted the protein complex, regenerating free E2F as previously reported (1, 7). These results suggest that the C-terminal tail of pRB, which is not essential for E7 binding, is important for optimal E2F binding.

In the presence of excess salmon sperm DNA (see Materials and Methods), the pRB/E2F gel shift bands are not as intense as the untreated free E2F bands, presumably because of less efficient binding of the pRB/E2F complex to the E2F probe (Fig. 1B). Because of this observation, we assayed the binding of E2F to pRB by monitoring the disappearance of the free E2F band. This approach provides a more sensitive assay than monitoring the appearance of the less prominent E2F/pRB gel shift band.

E1a and E7 compete with E2F for pRB binding. To assay the abilities of the HPV-16 E7 and Ad5 E1A 12S gene products to compete with E2F for binding to the pRB pocket, cDNA clones encoding these genes were transcribed and translated in wheat germ extracts and mixed with E2F and 2.5 nM pRB60 (Fig. 2A). While wheat germ-translated 12S E1A protein (lane 5) completely inhibited formation of the pRB60/E2F complex, E7 protein (lane 4) was considerably less potent. At the concentration of E7 protein generated in the wheat germ translation system (which was estimated to be in the picomolar range by trichloroacetic acid precipitation and autoradiography), only slight inhibition of E2F/pRB60 complex formation was observed. From these studies, it appears that the E1A 12S gene product is more potent than the E7 protein in its ability to compete with E2F for binding sites on pRB60.

To quantitatively assess the effect of E7 protein on the E2F/pRB60 complex, recombinant E7 protein was mixed with partially purified E2F and allowed to compete for binding with pRB60. By monitoring the intensity of the free E2F band in the gel shift assay, it is apparent that recombinant E7 protein completely blocks binding of E2F to pRB60, with a 50% inhibitory concentration (IC_{50}) of approximately 350 nM (Fig. 2B, lanes 3 to 6). To verify the specificity of this competition, a mutated E7 protein was generated. Previous studies have demonstrated that a single substitution mutation at Cys-24 (in CRII) of E7 dramatically reduces the affinity of the mutant protein for pRB60 (5, 17). A mutant E7 in which Cys-24 was changed to alanine was constructed. This mutated protein was expressed in *E. coli* and purified, and its activity in the E7 and E2F competition assays was determined. As shown in Table 1, the mutant protein binds to pRB60 with approximately 20-fold-lower affinity than does the wild-type protein. Similarly, the mutant protein exhibits an IC_{50} of approximately 3.5 μM in the E2F competition assay, 10-fold higher than that of wild-type E7 (Fig. 2B, lanes 7 to 10). The similarity in the sensitivities of these two assays to a single point mutation suggests that both interactions are dominated by the interaction of CRII of E7 with pRB. These observations are consistent with earlier reports (7, 35).

Mapping of competitive epitopes of E1A and E7 for pRB binding by E2F. The titrations of the E2F/pRB complex with

TABLE 1. IC_{50}'s of E7 fragments in the competitive E7/pRB60 ELISA[a]

Peptide	IC_{50} (nM)
E7	1.2
E7 (Cys-24→Ala)	25
E7 CRII peptide	130
E7(1–29)	1,000
E7(−*16*–49)	100
E7(−*16*–60)	35
E7(13–84)	5
E1A CRI peptide	>100,000

[a] Each peptide was tested for its ability to inhibit binding of pRB60 to immobilized E7 protein. Binding of pRB60 to immobilized E7 was detected by using an anti-pRB monoclonal antibody (38).

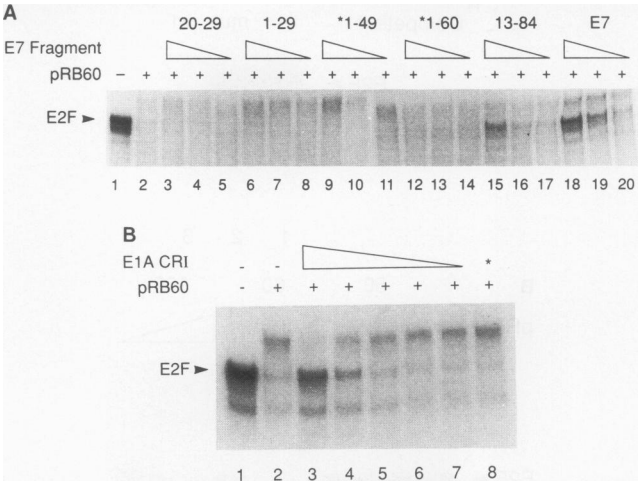

FIG. 3. Inhibition of E2F/pRB60 complex formation by E7 and E1A fragments. (A) Affinity-purified E2F was mixed with 2.5 nM pRB60 (lanes 2 to 20) and various concentrations of E7 protein fragments, prepared as described in Materials and Methods. Concentrations of each E7 fragment were normalized to the E7/pRB competitive ELISA IC_{50}s presented in Table 1. Concentrations tested were 100 × IC_{50}, 10 × IC_{50}, and 1 × IC_{50}. Fragments are as follows: 20–29, E7 CRII peptide; 1–29, E7(1–29); *1–49, E7(−*16*–49); *1–60, E7(−*16*–60); 13–84, E7(13–84); E7, wild-type E7. (B) Affinity-purified E2F was mixed with 2.5 nM pRB60 (lanes 2 to 8) and E1A CRI peptide (lanes 3 to 7) at concentrations ranging from 250 μM to 25 nM in log-dilution steps. In lane 8, the scrambled E1A CRI peptide was tested for activity at 250 μM.

wild-type and mutant E7 proteins demonstrated that an intact CRII domain in E7 protein is necessary for optimal E2F displacement activity. To determine whether CRII of E7 is sufficient to disrupt the E2F/pRB complex, a series of E7 fragments was generated. These peptide fragments were obtained by peptide synthesis or proteolytic digestion of recombinant E7 and were independently assayed for their abilities to compete with E7 or E2F for binding to pRB60. The relative affinities of these peptides for pRB60 were determined by a competitive ELISA against wild-type E7 protein (Table 1) and are expressed as IC_{50} values. As expected, the full-length E7 protein was approximately 100-fold more potent than the conformationally constrained CRII peptide, [*N*-methyl-Leu-22,28,*N*-methyl-Tyr-25] E7(20–29) peptide amide, in this assay (26). Larger E7 fragments displayed intermediate affinities for pRB60.

The effects of these same E7 fragments on E2F/pRB complex formation were determined in the gel shift assay at concentrations ranging from the corresponding ELISA IC_{50} to 100 times the ELISA IC_{50}. Selecting the concentration ranges in this manner permits testing of each peptide at comparable levels of pRB saturation. Inhibition of E2F/pRB60 complex formation was determined by monitoring the presence of free E2F in the gel shift assay (Fig. 3A). The E7 CRII peptide did not inhibit binding of E2F to pRB60 (lanes 3 to 5) even at concentrations 100-fold higher than the IC_{50} for inhibiting E7 binding to pRB60. A larger peptide encompassing the N-terminal CRI and CRII domains, E7(1–29) peptide amide, was also not able to block E2F binding to pRB60 (lanes 6 to 8). In the case of E1A, the CRI region has previously been shown to be necessary to compete with E2F for pRB binding (36). These results suggest that the CRI region of E7 is not sufficient to block E2F and pRB interactions.

Larger fragments of E7, which included increasing portions of the carboxy terminus, were also not able to successfully compete with E2F for pRB60. Fragments of E7 beginning at the amino terminus and ending at either amino acid 49 or amino acid 60 had no effect on binding between pRB60 and E2F (Fig. 3A, lanes 9 to 14). Each of these fragments contains an intact CRII domain and binds to pRB60. The only fragment of E7 which displayed any significant activity in the E2F/pRB displacement assay, however, included amino acids 13 to 84 (lanes 15 to 17). This fragment contains CRII as well as one of the two Cys-X-X-Cys motifs which have been shown to participate in zinc binding (6, 32) and exhibited slight, but reproducible, inhibition of E2F/pRB60 complex formation. Nevertheless, the full-length E7 protein was considerably more potent in this assay than was the E7(13–84) fragment (lanes 18 to 20). The full-length protein contains the second important Cys-X-X-Cys motif, thus completing the putative zinc-binding domain. Because the E7(1*–12) region does not appear to be important for this activity [see E7(1*–60), lanes 12 to 14], these data suggest that the zinc-binding domain is important for E7's inhibition of E2F binding to pRB.

The CRI region of the Ad5 12S E1A gene had previously been shown to be necessary to displace pRB from E2F (36). To determine whether this region was also sufficient to block E2F-pRB60 association in our assay, an E1A CRI peptide [*N*-acetyl-E1A(37–55) amide] was tested for its ability to block E2F binding to pRB60. The data presented in Fig. 3B clearly demonstrate that this peptide is sufficient to block association of E2F with pRB60 and does so with an IC_{50} of approximately 250 μM (lanes 3 to 7) under the conditions tested. A peptide containing the same amino acids but with a scrambled sequence had no effect (lane 8). These results confirm that, in contrast to the CRI of E7, the CRI of E1A is critical for disrupting E2F-pRB interactions, and they suggest that an important interaction site between E2F and pRB is occluded by the E1A CRI peptide.

Reversal of the E7 and E1A effect with the CRII peptide. The data in Table 1 and Fig. 3A demonstrate that the E7 CRII peptide, E7(20–29) amide, blocks binding of E7 to pRB60 but does not block binding of E2F to pRB60. Conversely, the E1A CR1 peptide blocks binding of E2F to pRB60 but does not block binding of E7 to pRB60. These results suggest that E2F binds to pRB at a site which is distinct from the E7 CRII contact site and that the CRII peptide and E2F can simultaneously bind pRB60. To verify this hypothesis, the E7 CRII peptide was titrated into a competition reaction containing excess E7, E2F, and pRB60

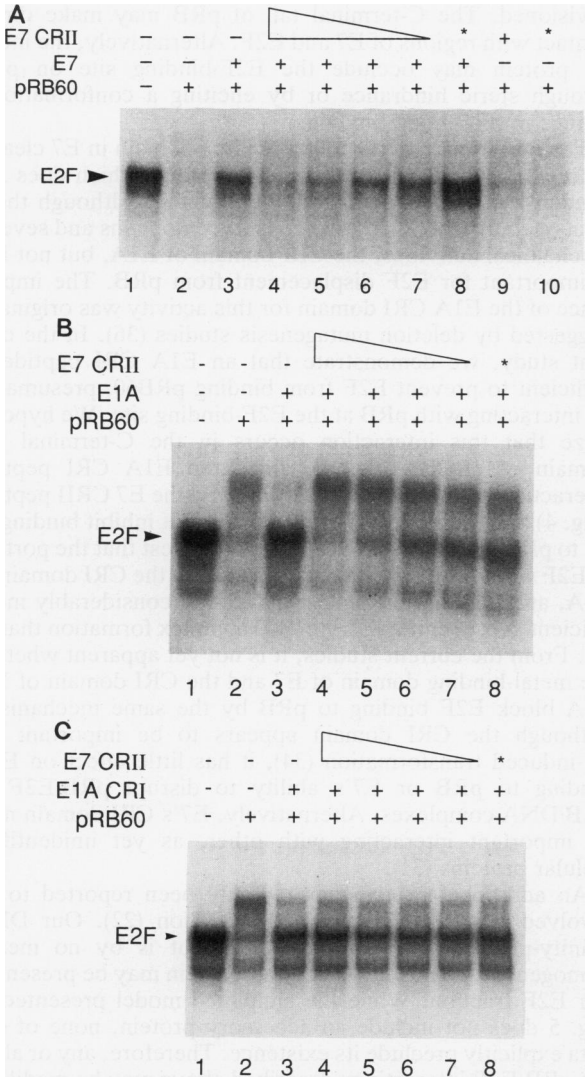

FIG. 4. Reversal of E7 and E1A inhibition of E2F/pRB60 complex formation by E7 CRII peptide. (A) Affinity-purified E2F was mixed with 2.5 nM pRB60 (lanes 2 to 10), 350 nM E7 (lanes 3 to 8), and various concentrations of E7 CRII peptide (lanes 4 to 7; 740, 320, 148, and 74 μM, respectively). Lane 8 shows that the scrambled E7 CRII peptide does not alter complex formation at 740 μM. Lanes 9 and 10 show that the wild-type (lane 9) and scrambled (lane 10) peptides have no effect at 740 μM in the absence of E7 protein. (B) An experiment identical to that presented in panel A was performed by using a 1:5 dilution of wheat germ-translated E1A protein instead of E7 protein. Peptide concentrations for lanes 4 to 7 range from 7.6 μM to 7.6 nM in 10-fold steps. The concentration of scrambled peptide in lane 8 is 7.6 μM. (C) An experiment identical to that presented in panel A was performed by using 250 μM E1A CRI peptide instead of E7 protein. E7 CRII peptide concentrations ranged from 760 μM (lane 4) to 760 nM (lane 7). The E7 CRII scrambled peptide was tested at 760 μM (lane 8).

(Fig. 4A). In the absence of peptide, E7 inhibits E2F binding to pRB60 (lane 3). As CRII peptide is added, however, the peptide displaces E7, but not E2F, from pRB60, allowing formation of a complex between pRB60, peptide, and E2F (lanes 4 to 7). Since the displacement of E7 by the CRII peptide occurs by a competitive mechanism (26), the peptide must be able to bind the CRII-binding site within the pRB60/E2F complex. This result verifies that E2F binds to pRB at a site which is distinct from the E7 CRII-binding site. This interpretation is further supported by similar results obtained in E1A 12S protein competition experiments (Fig. 4B), in which the E7 CRII peptide effectively blocked E1A-induced disruption of the E2F/pRB60 complex.

To verify that the E1A CRI peptide-induced inhibition of E2F/pRB60 complex formation occurred at a site which was distinct from the CRII-binding site, we attempted to reverse the disruption of the E2F/pRB complex by E1A CRI peptide, using the E7 CRII peptide. The CRII peptide had no observable effect on the distribution of E2F between the upper and lower complexes (Fig. 4C). The E1A CRI peptide also does not inhibit E7 binding to pRB60 at concentrations up to 100 μM (Table 1). These results are consistent with the hypothesis that E2F interacts with pRB through a region which is similar to the E1A CRI interaction site but is distinct from the E7 CRII interaction site.

DISCUSSION

Several lines of evidence suggest that transcription of genes dependent on the E2 promoter is at least partially regulated by pRB. E2F/pRB complexes are observed in cell lysates, and the presence of these complexes appears to correlate with a loss of E2F-mediated transcription (2, 22). It has been suggested that the transforming viral proteins, E7 and E1A, interfere with this regulatory process by binding pRB and releasing E2F. It is therefore important to understand how these viral oncoproteins interact with pRB and E2F.

Deletion mapping studies have demonstrated that two regions of pRB are essential for its binding to the viral E7 and E1A proteins. These regions, domains A and B, appear to generate a binding pocket for the viral proteins (23). Deletion of the region of pRB60 C terminal to the pocket has relatively little effect on E7-binding activity or thermal stability of the protein (data not shown). In this study, we verify that deletion of this same C-terminal domain virtually eliminates E2F-binding activity. This domain, consisting of amino acids 777 to 909, appears to be important for binding of pRB60 to E2F but not to E7. This observation suggests that the E7- and E2F-binding sites on pRB are not identical. Interestingly, this same C-terminal region has previously been shown to contribute to pRB's binding to double-stranded DNA (39, 40). It is not yet apparent whether these two activities are related.

Our data suggest that at least two parts of the E7 protein are required for optimal inhibition of E2F binding to pRB60: the CRII domain, which has previously been shown to be necessary and sufficient for E7's binding to pRB, and carboxy-terminal sequences which include the Cys-X-X-Cys zinc-binding motifs. The importance of the CRII domain is demonstrated by the observations that a single point mutation in this region reduces E7's binding to pRB60 and its ability to inhibit E2F binding to pRB60 to a comparable degree. Thus, the ability of E7 to inhibit E2F binding to pRB appears to be predicated on E7's ability to bind to pRB, which has previously been shown to depend on an intact CRII domain (16, 17). The CRII domain of E7 alone, however, is not sufficient to block E2F binding to pRB60. This conclusion is most vividly demonstrated by the CRII peptide/E7/E2F competition experiments (Fig. 4), in which the peptide was able to reverse E7's inhibition of E2F/pRB complex formation. This observation is consistent with saturation of the CRII binding site on pRB60 by the E7 CRII

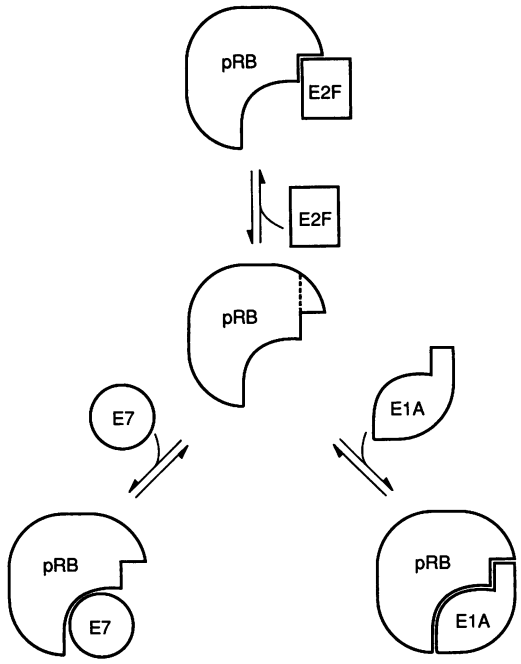

FIG. 5. Schematic model for the interactions of E7, E1A, and E2F with pRB60. pRB60 is shown in the center. The portion of the protein to the right of the dotted line in the central figure represents the C-terminal tail of pRB60 which is deleted in generating pRB50, which no longer binds E2F. The square binding site which incorporates this tail is envisioned as an important contact site for E2F and possibly E1A (through CRI) but not E7. The primary interaction between E7 (and possibly E1A) with pRB60 occurs in the round CRII binding site, which is conserved in pRB50 and pRB60. In this model, the inhibition of E2F binding by E7 is schematically represented as a steric blockade, although other models are possible (see Discussion).

peptide, resulting in the displacement of E7 from pRB and the binding of E2F to pRB. The upper complex observed under these conditions, then, is actually a ternary peptide/pRB60/E2F complex. The CRII peptide may provide a model for a potential anti-E7 therapeutic agent, since it specifically blocks E7's binding to pRB without disrupting the normal cellular complex of pRB and E2F.

In addition to the CRII domain, carboxyl-terminal sequences of E7 are required for displacement of E2F from pRB. A critical motif which is present in active fragments is the $(Cys\text{-}X\text{-}X\text{-}Cys)_2$ zinc-binding motif. These four cysteines have previously been shown to bind a zinc ion in the intact protein (6, 32). It is possible that the residual E2F displacement activity in the E7 fragment containing only one intact Cys-X-X-Cys motif [E7(13–84)] is due to dimerization mediated by metal ions in solution. The role of the metal-binding region on E7's biochemical activity is quite dramatic. Affinity for pRB60, ability to block pRB's E2F-binding activity, and ability to block pRB's DNA-binding activity are all affected by the presence of at least one Cys-X-X-Cys motif, with both domains required for optimal activity (39). Together with the observation that optimal E2F binding requires the C-terminal tail of pRB (amino acids 777 to 909), these results suggest that the metal-binding domain of E7 occludes an interaction of E2F with the C-terminal region of pRB (Fig. 5). The molecular mechanism of this occlusion is not apparent from this study, but several models can be envisioned. The C-terminal tail of pRB may make direct contact with regions of E7 and E2F. Alternatively, the intact E7 protein may occlude the E2F-binding site on pRB through steric hindrance or by eliciting a conformational change in pRB upon binding.

The importance of the metal-binding domain in E7 clearly differentiates it from the 12S E1A protein, which does not contain an apparent metal-binding domain. Although these viral oncoproteins share two conserved domains and several biochemical activities, the CRI domain of E1A, but not E7, is important for E2F displacement from pRB. The importance of the E1A CRI domain for this activity was originally suggested by deletion mutagenesis studies (36). In the current study, we demonstrate that an E1A CRI peptide is sufficient to prevent E2F from binding pRB60, presumably by interacting with pRB at the E2F-binding site. We hypothesize that this interaction occurs in the C-terminal tail domain of pRB60 (Fig. 5), since the E1A CRI peptide interaction with pRB is not disrupted by the E7 CRII peptide (Fig. 4) and the E1A CRI peptide does not inhibit binding of E7 to pRB60 (Table 1). These results suggest that the portion of E2F which contacts pRB may resemble the CRI domain of E1A, and they may explain why E1A is considerably more efficient at preventing E2F/pRB60 complex formation than is E7. From the current studies, it is not yet apparent whether the metal-binding domain of E7 and the CRI domain of 12S E1A block E2F binding to pRB by the same mechanism. Although the CRI domain appears to be important for E7-induced transformation (34), it has little effect on E7's binding to pRB or E7's ability to disrupt pRB/E2F or pRB/DNA complexes. Alternatively, E7's CRI domain may be important interacting with other, as yet unidentified cellular proteins (31).

An additional protein has recently been reported to be involved in pRB/E2F complex formation (22). Our DNA affinity-purified E2F binds to pRB but is by no means homogeneous. Thus, an accessory protein may be present in our E2F fraction. While the simplified model presented in Fig. 5 does not include an accessory protein, none of our data explicitly preclude its existence. Therefore, any or all of the pRB/E2F interactions described above may be mediated by one or more additional proteins.

After the completion of this study, the sequence of a pRB-binding protein with E2F activity, designated RBP3 or RBAP-1, was published (20, 24). Antibodies to this protein supershift some protein complexes in a human T-cell lysate which specifically bind to the E2 promoter sequence (24). From these studies, it is likely that this clone represents a component of E2F or is itself one of several E2Fs. Interestingly, the binding of the cloned protein to the pRB pocket protein was inhibited by an E7 peptide containing the CRII domain but lacking the zinc-binding region. This interaction occurred despite the fact that RBP3/RBAP-1 does not contain the conserved CRII LXCXE sequence found in E7 and E1A (20, 24). Several possible explanations for these results can be proposed. First, RBP3/RBAP-1 may represent a subset of E2F and may not contribute significantly to the E2F that we have purified from HeLa cells. Second, because the RBAP-1 experiment was performed with a glutathione *S*-transferase fusion protein which contains pRB sequences analogous to our pRB50, we would predict that this binding is a low-affinity interaction. The data presented in our report demonstrate that, as monitored by the gel shift assay, the E7 CRII peptide is not sufficient to disrupt the high-affinity interaction between HeLa cell-derived E2F and pRB60, which contains an intact C terminus.

Full-length E7 protein has been shown to transactivate the E2 promoter (33, 35). Our data are consistent with the finding that a deletion of CRII or mutations in the Cys-X-X-Cys motifs in E7 result in loss of transactivation activity (31, 33, 34, 41). The relevance of this biochemical activity of the E7 protein to the transforming and immortalizing properties of E7 is currently under investigation.

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