Identification of a Novel Retinoblastoma Gene Product Binding Site on Human Papillomavirus Type 16 E7 Protein* 

(Received for publication, August 30, 1993, and in revised form, November 12, 1993)

Denis R. Patrick, Allen Oliff, and David C. Heimbrook†

From the Department of Cancer Research, Merck Research Laboratories, West Point, Pennsylvania 19486

Transformation of mammalian cells by human papillomavirus type 16 appears to require binding of the viral E7 protein to the cellular retinoblastoma growth suppressor gene product (pRB). Binding of E7 protein to pRB inhibits several of pRB's biochemical properties, including association with the transcription factor E2F. Fragments of E7 protein derived from its conserved region 2 (CR2) domain bind to pRB and are sufficient to inhibit binding of full-length E7 protein to pRB. However, these CR2 fragments exhibit reduced affinity for pRB compared to the full-length protein and do not inhibit formation of the pRB-E2F complex. These observations suggest the existence of additional contact sites between the E7 protein and pRB. In the current study we have identified a region of E7, distinct from the CR2 domain, which is sufficient to bind pRB. This new pRB binding motif encompasses the zinc-binding conserved region 3 (CR3) domain of E7. Studies with a series of pRB deletion mutants suggest that pRB residues between amino acids 803 and 841 are necessary for binding to the E7 CR3 domain. An E7 CR3 peptide inhibits binding of E2F to pRB, indicating that E2F and E7 (31–98) bind to pRB at the same or overlapping sites. These results are consistent with a model in which optimal binding of E7 to pRB requires at least two distinct contact sites: the previously identified high affinity interaction between the E7 CR2 domain and the pRB "pocket" region, and a second interaction between the E7 CR3 domain and the C(2)OH-terminal region of pRB. The latter interaction is sufficient for E7's inhibition of E2F binding to pRB.

Nearly half of the over 60 strains of human papillomaviruses (HPVs) can infect the genital mucosa, producing benign epithelial lesions (1, 2). Several HPV strains, including HPV-16, previously identified high affinity interaction between the E7 CR2 domain and the pRB "pocket" region, and a pRB at the same or overlapping sites. These results are consistent with a model in which optimal binding of E7 to pRB requires at least two distinct contact sites: the previously identified high affinity interaction between the E7 CR2 domain and the pRB "pocket" region, and a second interaction between the E7 CR3 domain and the C(2)OH-terminal region of pRB. The latter interaction is sufficient for E7's inhibition of E2F binding to pRB.

The binding of E7 protein to pRB appears to be essential to E7's ability to transform cells. The transforming potential of different HPVs has been shown to correlate with the affinity of their respective E7 proteins for pRB (20, 21). Furthermore, mutations within the CR2 domain of E7 which reduce E7's affinity for pRB also abolish its transforming activity (14, 22–24). Mutational analysis of pRB has demonstrated that amino acids 394–571 and 649–772 (referred to as the binding "pocket") comprise the CR2 binding site (25–27). Mutations in this region are observed in many human cancers. In cervical cancer cell lines which do not contain HPV, the retinoblastoma gene is invariably mutated (28). Thus, binding of E7 to pRB appears to "inactivate" pRB's cell growth regulatory function, and this interaction appears to be necessary for the full manifestation of E7's transforming activity.

The exact mechanism of E7's "inactivation" of pRB is not yet clear. The retinoblastoma gene product binds to several critical cell components, including the transcription factors E2F and myc, the d-cyclins, several nuclear proteins with unknown function, and double-stranded DNA (29–42). Binding of E7 protein to pRB inhibits pRB's binding to E2F, DNA, and other proteins. It has not yet been conclusively demonstrated which of these interactions is most important for eliciting E7's biological activity. However, the interaction between E2F and pRB has been the subject of much recent study (for a recent review, see Ref. 43). E2F's DNA recognition element is observed in the promoter regions of several genes which are important in cellular proliferation, including c-myc, c-myc, and dihydrofolate reductase (44). Furthermore, formation and dissociation of particular complexes between E2F, pRB, and other cellular proteins correlates with progression through the cell cycle (45, 46). These studies suggest that the transforming activity of E7 may be based on its ability to elicit the unregulated release of free E2F. While an E7 CR2 peptide containing fewer than 9 amino acids is sufficient to inhibit binding of E7 protein to pRB, this peptide does not mimic the complete activity profile of the full-length E7 protein (see Fig. 1). In competitive binding inhi-
tion assays, E7 peptides containing only the CR1 and CR2 domains are approximately 100-fold less potent than the full-length protein at inhibiting pRB binding to immobilized E7 (17, 47). In addition, E7 peptides which lack the CR3 domain do not inhibit binding of pRB to double-stranded DNA or E2F, while the full-length protein inhibits both interactions (41, 48). These results suggest that full-length E7 protein provides additional interactions with pRB which are not present in E7 fragments lacking the CR3 domain. In the current study, we identify a novel interaction between E7 and pRB outside of the canonical E7 CR2/pRB pocket contact site. The E7 CR3 zinc binding domain binds directly to a COOH-terminal region of pRB outside of the CR2 binding pocket, and this interaction is sufficient to inhibit binding of E2F to pRB. The regions of E7 necessary for this novel binding interaction are also required for high affinity binding of E7 to pRB in the context of the full-length protein, suggesting that this interaction may play an important role in regulating E7's biological activity.

MATERIALS AND METHODS

Plasmid Constructions

All PCR products were sequenced using Sequenase V2.0 sequencing kit (U. S. Biochemical Corp.) per the manufacturer's instructions. Plasmid constructs containing a tubulin epitope tag (Glu-Glu-Phe) are denoted with "t" suffix. This sequence is recognized by a monoclonal antibody to α-tubulin, YL1/2, and was used for immunofluorescence immunohistochemistry purification of the tagged proteins (49).

pGST-E7(31-98)t—PCR primers DP12 and DP13 (see below) were used to generate a DNA fragment coding for amino acids 31–98 of HPV-16 E7 with the addition of COOH-terminal codons for the Glu-Glu-Phe tubulin epitope. The PCR product was cleaved with BamHI and EcoRI and purified by agarose gel electrophoresis. Ligation of this DNA fragment into similarly cleaved pGEX-KT (50) resulted in a gene for E7 amino acids 31–98, using the TACSDE7202 expression plasmid (19) as a template. The 16 amino acids at the amino terminus are derived from the multiple cloning site of pUC18 and are present in the full-length E7 construct used to express and purify the recombinant E7 protein, suggesting that this interaction may play an important role in regulating E7's biological activity.

pGATCt—The ATC(84-153)EEF fusion coding for glutathione S-transferase (GST) followed by E7 amino acids 31-98 and EEF at the COOH terminus. The PCR product  was cleaved with XbaI and HindIII and purified by agarose gel electrophoresis. Ligation of this DNA fragment into similarly cleaved pGEX-KT resulted in a gene for E7 amino acids 31–98 and EEF at the COOH terminus.

pGATCt—This construct replaces the COOH-terminal 68 amino acids of E7 with a similar zinc finger structure from the Escherichia coli aspartate transcarbamoylase regulatory chain (ATC) (51). PCR primers DP7 and DP8 (see below) were used to generate a DNA fragment coding for E7 amino acids 16-35 using the TACSDE7202 expression plasmid (19) as a template. The 16 amino acids at the amino terminus are derived from the multiple cloning site of pUC18 and are present in the full-length E7 construct used to express and purify the recombinant E7 protein (19). The PCR product was cleaved with BamHI and XbaI and purified from an agarose gel. PCR primers DP9 and DP10 (see below) were used to generate a DNA fragment coding for amino acids 31–98 of HPV-16 E7 with the addition of COOH-terminal codons for the Glu-Glu-Phe tubulin epitope. The PCR product was cleaved with BamHI and EcoRI and purified by agarose gel electrophoresis. Ligation of this DNA fragment into similarly cleaved pGEX-KT (50) resulted in a gene for E7 amino acids 31–98 and EEF at the COOH terminus. The PCR product was cleaved with XbaI and HindIII and purified from an agarose gel. These two DNA fragments were ligated into the pTST expression vector (52) which had been cleaved with BamHI and HindIII.

pGATCt—The ATC(84-153)EEF XbaI to HindIII fragment described above was also ligated into pGEM-3Z which had been cut with XbaI and HindIII.

pGATCt—The ATC(84-153)EEF XbaI to HindIII fragment described above was also ligated into pGEM-3Z which had been cut with XbaI and HindIII.

pGATCt—The ATC(84-153)EEF XbaI to HindIII fragment described above was also ligated into pGEM-3Z which had been cut with XbaI and HindIII.

pGATCt—The ATC(84-153)EEF XbaI to HindIII fragment described above was also ligated into pGEM-3Z which had been cut with XbaI and HindIII.

PCR Primers

DP7—5’ CGG GGG GAT CCA TTG GAG GAT TAA ACC ATG GCC ACC ATT ACA AAT TGG 3’.

DP8—5’ GCC GCC CGC TCT AGA GTG ATT TAA TGG CTC ATCA AAG TGA GTT 3’.

DP9—5’ GCC GCC TCT AGA AAC CGT ATC GAC AAC TAT GAA GTG 3’.

DP10—5’ GCG CCA ACC TTA AAA CTC CTC ATT GCC GAG CAC CAC ATT ATG G 3’.

DP11—5’ GCG GGG GAT CCA TTG GAG GAT TAA ATG AAC GTC GAC AAC TAT GAA GTG 3’.

DP12—5’ GCG GGA TCC TCA GAG GAG GAT GAA AAT A 3’.

DP13—5’ GCG CCG AAT TCC TTA AAA CTC TCT TGG TTT CTG AGA ACA GAT G 3’.

Protein Expression and Purification

The E7-ATC and ATC(84-153) proteins were expressed from the pTST-based plasmids in B.l.E. coli cells (Novo Biochem). The GST-E7(31-98) fusion protein was expressed in DH5α E. coli cells (Life Technologies, Inc.). Overnight cultures grown at 37 °C were diluted 1:50 into 25 °C Luria broth and grown until mid log phase (A660 = 0.5–0.9). Isopropyl-1-thio-β-D-galactopyranoside was added to a 1 mg/ml final concentration, and the incubation was continued at 25 °C for 6–18 h. Cell pellets were resuspended (at approximately A660 = 25) in phosphate-buffered saline containing 1 μM protease inhibitor mixture (10 mg/ml benzamidine, 5 mg/ml each: leupeptin, pepstatin A, and apro tinin), 1 μM dithiothreitol, and 0.04% sodium azide and sonicated four times for 1 min each with cooling on ice between bursts. The lysate was clarified by centrifugation at 40,000 x g for 20 min at 4 °C and then filtered through a 0.2-mm filter. Purification of the tubulin-epitope-tagged proteins was as described elsewhere (49) from cell lysates using an immobilized YL1/2 monoclonal antibody column. GST fusion proteins were purified using glutathione Sepharose 4B (Pharmacia LKB Biotechnology Inc.). The immobilized GST-E7(31-98) pro tein was cleaved using thrombin (Calbiochem) to generate the E7(31–98) peptide. The thrombin reaction was terminated with Thrombatestop (American Diagnostics Inc.). Fractions containing recombinant protein were pooled, and protein concentrations were quantitated by amino acid analysis. Purity of the recombinant proteins was estimated by SDS-PAGE to be >90%.

The pGEM4Z-RB60 and pGEM4Z-RB50 vectors have been described (17, 41). These plasmids were used in Promega’s TNT rabbit reticulocyte lysate transcription and translation system to produce pRB60 and pRB50 proteins radiolabeled with [35S]methionine. The pRB105 deletion mutants were also transcribed and translated in the TNT system from plasmids kindly provided by Ed Harrow (Massachusetts General Hospital, Charleston MA) (25).

E2F from HeLa cells was purified essentially as described (53). This affinity purified E2F yielded a single dominan band in the E2F gel shift assay, which was not affected by treatment with 0.6% deoxycholate, and was competed with wild type oligonucleotide containing the E2F consensus binding site but not by a mutated oligonucleotides (48, 53).

The E7(20-29) CR2 peptide, [N-methyl-Leu22,N-methyl-Tyr24]-E7(20-29) amide, and E7 scrambled peptide (YNELQYDYL-amide) were synthesized as previously described (47).

Recombinant pRB60 was solubilized from E. coli inclusion bodies and affinity purified using an E7(20-29) peptide column as previously described (54). Baculovirus expressed pRB105 was a gift from Carol Prives (Columbia University) and was affinity purified as described for pRB60 (54).

Direct Binding Experiments

E. coli lysate containing GST fusion protein was incubated with GST-S-Sepharose beads in NETN buffer (100 mM NaCl, 20 mM Tris, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, and 0.1% Nonidet P-40) for 30–60 min at 4 °C and then washed twice with 1 ml of NETN buffer. The amount of lysate mixed with the beads was titrated so that approximately equivalent amounts of each different fusion protein was immobilized on the beads. The 1 x loaded beads contained ~50 μg of immobilized fusion protein, whereas 100 x loaded beads contained ~5 μg of fusion protein per binding reaction. A typical binding reaction consisted of 2 μl of rabbit reticulocyte translated pRB60, 30 μl of a 30% suspension of GST beads with 50 ng (1 x) or 5 μg (100 x) bound fusion protein, in 200 μl of NETN. The reaction was gently mixed at 4 °C for 1 h and then centrifuged to separate the unbound fraction from the bound proteins. The supernatant was removed and mixed with 0.33 volume of 4 x SDS gel loading buffer. The beads were washed once with 1 ml of NETN and resuspended in 286 μl of 1 x SDS gel loading buffer. Samples were boiled 5 min, separated on 12% SDS-PAGE gels, and transferred to a nitrocellulose membrane. The membrane was blocked with 3% methanol and 20% acetic acid, soaked for 30 min in Enlightning (Du Pont), dried onto No. 3MM (Whatman) paper, and exposed to XAR-2 autoradiography film (Kodak).
**FIG. 1. Biochemical properties of E7 protein fragments.** The ability of the indicated E7 fragment to inhibit pRB binding to full-length E7 protein, double-stranded DNA, or E2F was qualitatively evaluated based on the current study and published data (E7 data, current study and Refs. 44, 45; DNA data, Refs. 44, 45; E2F data, current study and Ref. 48). For E7 binding to pRB, these values represent the IC50 values for competitive binding inhibition; for E2F and DNA binding activity, these values represent inhibition of complex formation at saturating concentrations of the E7 fragment. The evaluation of activity ranges from "++"+" (same activity as recombinant full-length E7 protein) to "-" (no activity). The E7 CR2 domain is indicated by a box black. Entries associated with E7(20–29) peptide refer to N-acetyl-(N-methyl-L-Leu)23,28,N-methyl-Tyr28,E7(20–29) peptide amide, which binds to pRB with approximately 30-fold higher affinity than does unmodified E7(20–29) peptide amide (47). All other peptides described contain unmodified amino acid sequences. The E7 CR3 domain contains 4 cysteines (indicated by vertical lines) involved in binding a zinc ion (19). **E7/pRB Competitive Binding Inhibition Assay** Quantitative analysis of a peptide's ability to inhibit E7 protein binding to pRB was assessed in a competitive binding inhibition assay essentially as described (17, 47). Briefly, 96-well plates were coated with recombinant E7 protein. Solutions containing 50 ng of recombinant pRB80 and various concentrations of the inhibitors were then added to the plate and gently mixed. The plates were then washed, and the amount of pRB bound to the plate was quantitated using a mixture of α-pRB monoclonal antibodies. **Gel Shift Assays** E2F was detected by a DNA gel shift assay as previously described (48, 49). Affinity purified E2F was mixed with a ^32P-labeled oligonucleotide corresponding to the adenosine virus E2 promoter containing only one of the two E2F binding sites (5^-TAGTTTCGAGTATAATT-TAGAAGGGCCCCGAACTAG-3') (E2F binding site underlined) in 20 mM HepES, pH 7.9, 1 mM MgCl2, 0.1 mM EDTA, 40 mM KCl, 10% glycerol, 0.15% Triton X-100, 1 mM dithiothreitol, 5 mg/ml bovine serum albumin, and 1 μg/ml sheared salmon sperm DNA for 60 min at 4 °C. Protein-DNA complexes were separated on 6% non-denaturing polyacrylamide gels (Nvex), dried onto No. 3MM (Whatman) paper, and autoradiographed. Other proteins and peptides were added to the standard reaction at the final concentrations indicated in the figure legends. **RESULTS** The observation that the full-length E7 protein and E7 protein fragments lacking the CR3 domain do not exhibit the same biochemical activities in various assays with pRB suggests that the truncated proteins provide only part of the interactions provided by the full-length protein (Fig. 1). A likely explanation for these different activities is that the full-length E7 protein provides an additional contact site with pRB which does not occur with the CR2 domain of E7 alone. This possibility was explored by constructing a GST-E7(31–98) fusion protein containing the CR3 zinc binding domain of E7 but lacking the E7 CR1 and CR2 regions. This immobilized fusion protein was tested for its ability to bind reticulocyte-translated pRB60, an NH2-terminally truncated version of pRB which contains an intact E7 binding "pocket" and maintains full E7 binding activity. The results of these studies are presented in Fig. 2. In binding reactions containing low levels (approximately 50 ng) of GSH-Sepharose-bound fusion protein, pRB60 bound to immobilized E7(3–98) protein, but did not bind to beads containing the E7 CR3 fusion or control beads containing only GST (Fig. 2, left). Under these conditions, the shorter pRB fragments generated in the translation reaction did not bind to any of the GST constructs. Dramatically different results were obtained when higher E7 protein concentrations were used, however. In binding reactions containing 5 μg of immobilized fusion protein, both full-length pRB60 as well as the two major truncated translation products bound to both full-length E7 and to the CR3 domain fusion protein (Fig. 2, center). This interaction appears to be specific since the pRB translation products did not bind to GSH-Sepharose beads containing GST alone. The requirement for higher levels of immobilized protein on the resin to detect this interaction suggests that the E7 CR3 interaction with pRB is significantly weaker than that of the full-length E7 protein.

Complete binding of the translated pRB products to immobilized E7 was not observed (compare the "bound" and "not bound" lanes in Fig. 2, center). When the E7(3–98) "not-bound" fraction (Fig. 2, center, lane 11) was reapplied to fresh immobilized E7 protein, very little additional binding of the pRB translation products was observed (data not shown). This result suggests that much of the unbound translation product in the translation reaction mixture is denatured, phosphorylated, or otherwise incapable of binding to E7.

The two dominant truncated proteins in the pRB60 translation mixtures contain an intact COOH terminus (data not shown) and therefore appear to result from translation initiation at internal methionine codons (55) or NH2-terminal proteolysis. The fact that these proteins, which lack the pRB pocket that is essential for E7 CR2 domain binding, bind to E7 fusion proteins suggests that this binding activity arises from interactions lying outside of the pocket domain of pRB. This hypothesis was explicitly tested by assaying for binding of translated pRB60 to the GST fusion proteins. The pRB50 construct retains an intact CR2 binding pocket but lacks amino acids 777–909 in the COOH-terminal region. While this truncated protein retains E7 binding activity, it is deficient in binding to HeLa cell E2F (48). As is shown in the right panel of Fig. 2, reticulocyte-translated pRB50 binds to full-length E7 protein but not to the E7 CR3 fusion protein under conditions where pRB60 bound to both proteins. None of the truncated versions of pRB50 present in the translation mixture bound to full-length E7 protein. These results suggest that the COOH terminus of pRB, rather than an intact CR2 binding pocket, is essential for binding the CR3 domain of E7.

The possibility that pRB60 was binding to the E7 CR3 beads via an interaction mediated by contaminating DNA was ruled out by several observations (data not shown). First, no detectable DNA was present in the binding reactions, as assayed by ethidium bromide staining on an agarose gel. Second, addition of neither DNA nor DNAase I had any effect on pRB60's ability to bind to immobilized E7 CR3 fusion protein. Finally, a Cys^1060 residue mutation in pRB60's B domain of the pocket region inactivates pRB60's DNA binding activity (41), yet the mutant protein remains fully competent for binding to immobilized E7 CR2 fusion protein. The interaction between pRB60 and the E7 CR3 domain therefore does not appear to be mediated by DNA.

The existence of distinct binding sites on pRB for the E7 CR2 and CR3 domains was verified in E7 binding competition experiments (Fig. 3). An immobilized GST-E7(3–50) fusion protein, which contains intact E7 CR1 and CR2 domains, bound to
Novel E7/pRB Binding Interaction

Fig. 2. pRB binds to the E7 CR3 domain. Glutathione-S-Sepharose beads containing 50 ng (1x) or 5 μg (100x) of the indicated GST fusion protein were incubated with rabbit reticulocyte-translated pRB60 (left and center panels) or pRB50 (right panel) at 4 °C for 1 hr. The samples were centrifuged and the supernatants (not bound (NB) samples) were removed and mixed with SDS sample buffer. The pelleted beads were washed, and the beads were boiled in SDS sample buffer to remove the bound (B) protein. The volumes of each sample were normalized prior to analysis by SDS-PAGE and autoradiography. Full-length pRB60 and pRB50 are indicated by arrowheads to the left of each panel, and the positions of molecular mass markers (in kilodaltons) are indicated to the right. Lanes 1, 8, and 15 represent the total protein from the translation reaction.

Fig. 3. The E7 CR2 and CR3 domains bind to non-overlapping sites on pRB. Binding competition experiments were performed using 5 μg of immobilized GST-E7(3-50) (left panel) or GST-E7(31-98) (right panel). Rabbit reticulocyte-translated pRB60 was incubated with the indicated competitors at the following concentrations: E7, 14 μM; E7(31-98), 66 μM; E7(20-29); and scrambled E7(20-29), 200 μM. Samples were analyzed as described under “Materials and Methods.” NB, not bound; B, bound. The position of full-length pRB60 is indicated by the arrowhead to the left of each panel, and the positions of the molecular mass markers (in kilodaltons) are indicated to the right.

Reticulocyte-translated pRB60, but did not bind to the truncated proteins in the translation mixture (Fig. 3, lanes 2 and 3). This binding was inhibited by peptides containing an intact E7 CR2 domain (E7 (lanes 3 and 4)) or E7 CR2 peptide (lanes 8 and 9), but was not inhibited by a scrambled E7 CR2 peptide (lanes 10 and 11) or the intact E7 CR3 protein (lanes 6 and 7). Conversely, immobilized GST-E7(31-98), which contains an intact CR3 domain, bound both full-length pRB60 as well as the truncated translation products (Fig. 3, lanes 13 and 14). Binding of these proteins was inhibited by constructs containing an intact CR3 domain (E7 (lanes 15 and 16) and E7(31-98) (lanes 17 and 18)) but not by the E7 CR2 peptide (lanes 19 and 20) or by the scrambled E7 CR2 peptide (lanes 21 and 22). These results demonstrate that the E7 CR2 and E7 CR3 domains interact with pRB at independent and non-overlapping sites.

To determine whether other proteins present in the reticulocyte translation mixture are necessary for the binding of pRB60 to the E7 CR3 domain fusion protein, recombinant affinity-purified pRB60 isolated from E. coli was assayed for binding to the GST fusion proteins. As a control, a GST fusion protein containing amino acids 84–153 of the E. coli ATC was constructed. The ATC domain also forms a zinc binding motif, and contains approximately the same number of amino acids as the E7(31–98) zinc binding motif (51). Fig. 4 shows that recombinant pRB60 is completely bound to the immobilized GST-E7 CR3 fusion protein, but binds only very slightly to the immobilized GST-ATC control protein. Therefore, neither accessory eukaryotic proteins nor eukaryotic post-translational processing are necessary for binding of pRB60 to the E7 CR3 domain. These results, in conjunction with the experiments described above, demonstrate the existence of a second direct contact site between E7 protein and pRB outside of the canonical E7 CR2/ pRB pocket binding site. This new interaction appears to occur between the E7 CR3 domain and regions contained in the COOH terminus of pRB.

The affinity of the E7 CR3 domain for pRB was assessed in a quantitative competitive binding inhibition enzyme-linked immunosorbent assay (Fig. 5). In this assay, recombinant E7 protein displayed an IC50 of approximately 0.5 nM, while the CR2 peptide exhibited an IC50 of approximately 30 nM. E7(31–98) exhibited only weak activity in this assay, and failed to reach 50% inhibition of E7 protein binding to pRB60 at concentrations up to 6 μM. As expected, the ATC zinc binding domain exhibited no significant activity in this assay. These results demonstrate that the ability of the E7(31–98) domain to inhibit E7 binding to pRB is much lower than that of the E7 CR2 domain peptide, and suggest that the CR2/pRB pocket interaction is the primary site controlling E7 binding to pRB. An E7-ATC chimera consisting of the CR1 and CR2 domains of E7 fused to the zinc binding domain of ATC displayed binding inhibition activity similar to the E7 CR2 peptide alone (Fig. 5). This observation suggests that the increased affinity for pRB of full-length E7 protein compared to the CR2 peptide is not caused by steric occlusion of the pRB binding site due to the presence of a generic zinc binding domain, but is instead due to an additional specific interaction between the E7 CR3 domain and pRB. This interaction therefore appears to play a significant role in high affinity binding of full-length E7 protein to pRB.

A series of pRB deletion mutants were tested in the direct binding assay to map the E7 CR3 binding site in more detail. Reticulocyte-translated pRB105 constructs containing deletions in the pocket and COOH-terminal domains were tested for their ability to bind the GST-E7(31–98) fusion protein immobilized on GSH-Sepharose, as performed in Fig. 2, center. Under these conditions, pRB60 bound the E7 CR3 protein but pRB50 (lacking amino acids 777–909) did not (Fig. 6). The deletion construct lacking pRB amino acids 841–909 bound to the E7 CR3 domain protein, while the 803–909 deletion protein did not bind. This result suggests that amino acids between
Novel E7/pRB Binding Interaction

FIG. 4. Direct binding of recombinant pRB60 to recombinant E7 protein. Glutathione-S-Sepharose beads containing 5 μg of the indicated GST fusion protein were incubated with 1 μg of affinity-purified recombinant pRB60 as described above. The pRB60 in the bound (B) and supernatant (NB) fractions was resolved by SDS-PAGE and analyzed by a Western blot with an anti-pRB monoclonal antibody. The position of pRB60 is indicated by an arrowhead to the left of the panel, and the position of molecular mass (in kilodaltons) are indicated to the right.

FIG. 5. Quantitative inhibition of pRB60 binding to E7 protein by E7 protein fragments. Binding of recombinant pRB60 to immobilized recombinant E7 protein in the presence of the indicated proteins and peptides was assessed with a mixture of α-pRB monoclonal antibodies in an enzyme-linked immunosorbent assay (see “Materials and Methods”). The ATC and E7(31–98) proteins were obtained from the appropriate GST fusion proteins by cleavage with thrombin, as described under “Materials and Methods.”

FIG. 6. Mapping of the E7 CR3 domain binding site on pRB. Retinoblastoma proteins containing the indicated deletions were generated in a rabbit reticulocyte transcription/translation system. Binding of the translation products to 5 μg immobilized GST-E7(31–98) was determined as described (see “Materials and Methods”). Binding of the dominant pRB translation product (generally approximately 60 kDa) was scored as positive (+) or negative (–).

Fig. 7. Mapping of the pRB binding sites on E7. Binding of reticulocyte-translated pRB60 and truncated translation products to 5 μg of the indicated immobilized GST-E7 fusion proteins was assessed essentially as described above (see “Materials and Methods”). NB, not bound; B, bound. The location of full-length pRB60 is indicated by an arrow to the left of the panel. The positions of molecular mass markers is shown to the right of the panel, in kilodaltons.

residues 803 and 841 are necessary for pRB binding to the E7 CR3 domain protein. Internal pRB pocket deletions between amino acids 536 and 714 had no effect on E7 CR3 binding activity, indicating that these amino acids are not necessary for this interaction. Furthermore, a 20 kDa COOH-terminal pRB fragment generated in the rabbit reticulocyte translation reaction is sufficient to bind to immobilized E7 CR3 domain protein (data not shown). This fragment of pRB may be generated by internal translation initiation at Met761 or by proteolysis near this site. These results verify that an intact pRB pocket is not necessary for binding to the E7 CR3 domain, but amino acids COOH-terminal to the pRB pocket region are absolutely necessary and appear to be sufficient.

A similar analysis was conducted to map out the regions of E7 which comprise the CR3 pRB binding domain. A series of GST-E7 fusion proteins containing COOH-terminal deletions were assayed for their ability to bind reticulocyte-translated pRB60 and the NH2-terminally truncated products resulting from translation initiation at internal methionine codons (Fig. 7). These truncated pRB proteins lack the CR2 binding pocket but contain an intact COOH terminus (data not shown), and therefore bind E7 via the E7 CR3 domain. As expected, none of the pRB60 translation products bound to control beads containing the GST-ATC(84–153) fusion protein. Both the full-length and truncated pRB proteins bound to the E7(31–98), E7(3–98), E7(3–84), and E7(3–75) fusion proteins. Consistent with the data presented above (Fig. 3), only full-length pRB60 bound to GST-E7(3–50), through the E7 CR2/pRB pocket interaction. The truncated pRBs, which lack a pocket and can no longer bind via the E7 CR2 domain, do not bind GST-E7(3–50) because
it does not contain an intact CR3 domain. These results suggest that amino acids between residues 50 and 75 of E7 are necessary for constituting a functional E7 CR3 domain. This region of E7 contains two of the cysteine residues (Cys<sup>68</sup>, Cys<sup>61</sup>) involved in binding a zinc ion.

The regions of both E7 and pRB which are important for the novel binding activity described above are coincident with the regions of these proteins involved in interactions between E7, pRB, and the transcription factor E2F (Figs. 1, 6, and 7) (48). This functional overlap suggests that E2F might bind to pRB at the same site as the E7 CR3 domain. To test this hypothesis, the E7(31–98) peptide was assayed in an E2F gel shift assay. Binding of recombinant pRB60 to E2F resulted in a characteristic and reproducible reduction in the rate of migration of the complex (Fig. 8A, lanes 1 and 2), reflective of the formation of an E2F-pRB60 complex (Fig. 8C, lanes 1 and 2). Formation of this complex was inhibited by full-length E7 protein (Fig. 8A, lane 4), thus regenerating free E2F (Fig. 8C, lane 3), but not by the E7-ATC chimeric fusion protein. In binding reactions containing E7, pRB60, E2F, and excess E7 CR2 peptide (Fig. 8A, lane 5) or the E7-ATC chimera (Fig. 8A, lane 6), E7 binding to pRB60 was inhibited. Both of these CR2-containing E7 peptides allowed formation of ternary (E2F/pRB60/E7 CR2) complexes, which co-migrated with the E2F-pRB60 complex (Fig. 8C, lane 4). Different results were observed with the CR3-containing E7 constructs, however (Fig. 8B, lanes 3–7). E7(31–98) inhibited formation of the E2F-pRB60 complex in a dose-dependent fashion, generating free E2F (Fig. 8C, lane 5) with an IC<sub>50</sub> of approximately 1 µM. Unlike full-length E7 protein, this inhibition was not affected by the presence of excess E7 CR2 peptide or the E7-ATC chimera (Fig. 8B, lanes 9 and 10), again yielding free E2F (Fig. 8C, Panel 6). These results demonstrate that the E7 CR3 domain binding site overlaps the E2F binding site on pRB and is consistent with the data presented above (Fig. 3) showing that the E7 CR2 and CR3 peptides interact at independent and non-overlapping sites on pRB.

**DISCUSSION**

The binding of HPV E7 or adenovirus E1A proteins to pRB inhibits many of pRB’s normal interactions, including binding to E2F, DNA, and p-cyclins (40–42, 48). The p-cyclins appear to bind to pRB via an LXCXE motif, which is also contained in the CR2 domain of E7 and other viral oncoproteins. Recently a number of pRB-binding proteins, including E2F, have been identified which lack the LXCXE motif (37, 56, 57). Although binding of E2F to pRB is inhibited by E7 protein, E7 CR2 peptides containing the LXCXE motif are not sufficient to inhibit E2F binding. The additional regions of E7 which are required for inhibition of E2F binding to pRB are identical to those required for high affinity binding of E7 protein to pRB and for inhibition of DNA binding to pRB (Fig. 1) (41, 48). These observations suggest that a common interaction between full-length E7 protein and pRB, outside of the CR2/pocket interaction, underlies all of these biochemical phenomena.

We initially investigated the possibility that binding of E7 protein, but not the CR2 peptide, elicited a conformational change in pRB which might be linked to E7’s biochemical activities. In circular dichroism, tryptophan fluorescence, and thermal stability studies, however, the E7 CR2 peptide and E7 protein were virtually indistinguishable in their interaction with pRB (data not shown). From these observations we conclude that the different results observed with the CR2 peptide and full-length E7 protein in the pRB binding inhibition and E2F binding assays are not accounted for by induction of different conformational changes in pRB.

An alternative hypothesis to explain the dissimilar activities of the E7 CR2 peptide and the full-length protein is that the full-length protein contains an additional pRB binding domain, and that this binding domain occludes the E2F binding site on pRB. The E7 CR1 domain makes no significant contribution to the pRB binding interaction (48). Previous E7 deletion mutagenesis studies suggest that this hypothesized interaction must instead involve the E7 CR3 domain, which contains E7’s zinc binding site (Fig. 1). The data presented in the current study support this hypothesis. The interaction between E7’s CR3 domain and the COOH terminus of pRB does not require any additional eukaryotic proteins or post-translational processing, since it is observed in reactions containing only recombinant proteins purified from E. coli as well as in reactions containing reticulocyte lysate-translated protein. The E7 CR2 and CR3 binding interactions with pRB appear to be independent and non-overlapping. While full-length E7 protein inhibits the binding of either E7 domain to pRB, the CR2 peptide does not inhibit binding of the CR3 fragment, and vice versa. Consistent with this observation, mapping of the CR3 binding site on pRB indicates that amino acids outside of the CR2 binding pocket are important for binding the CR3 construct. The COOH-terminal residues of pRB necessary for binding the E7 CR3 domain (residues between amino acids 803 and 841) are similar to those reported to be necessary for optimal pRB binding to E2F (residues between amino acids 773–909 (48), double-stranded DNA (residues between amino acids 612 and 928) (41, 58), and cyclins D2 and D3 (residues between amino acids 793–928) (39). The COOH terminus of pRB therefore appears to mediate multiple biochemical binding interactions.

The affinity of the E7 CR3 domain for pRB is considerably lower than that of the CR2 peptide or the full-length E7 protein, as demonstrated by both the competitive enzyme-linked immunosorbent assay and by direct binding studies. While the E7 CR2 peptide is sufficient to inhibit binding of the full-length E7 protein to pRB, the results with the CR3 protein fragment are less compelling, since complete inhibition of E7 binding was not observed at the highest concentration tested. The weak inhibition of E7 binding by the CR3 protein suggests that the CR2/pRB interaction contributes most of the binding energy in the intact E7 protein. The CR2/pRB contact may serve a “docking” function for the E7 protein, stabilizing the weaker CR3/pRB interaction. This observation is consistent with numerous CR2 mutagenesis studies in E7 and other viral pRB-binding proteins which suggest that the LXCXE motif within the CR2 domain is the dominant mediator of pRB binding. Nevertheless, the full-length E7 protein containing the CR3 domain binds to pRB with approximately 100-fold higher affinity than E7 fragments lacking the CR3 region. This finding suggests the existence of a synergistic bivalent interaction between E7 and pRB that governs the high affinity binding of these two proteins (Fig. 8C, lane 3).

The fact that the CR3/pRB interaction is relatively weak compared to the CR2/pRB interaction belies its importance to the functional activity of the full-length protein. The residues of pRB involved in binding the E7 CR3 domain overlap the E2F binding site (48). Consistent with this observation, the E7 CR3 domain by itself inhibits binding of E2F to pRB. These results support the bifunctional model of E7 binding to pRB, in which the CR2 domain provides the bulk of the binding energy to form a high affinity complex with pRB and inhibits binding of cellular pocket-binding proteins, while the CR3 domain provides an additional, weaker binding domain which specifically disrupts the complexes of pRB with other cellular proteins outside of the pocket. It is interesting to note that the adenovirus E1A protein has evolved a similar bifunctional interaction with pRB (59). Like E7, E1A is a viral transforming protein which contains an LXCXE pRB pocket binding domain. Although this protein disrupts pRB/E2F complexes, the 12S E1A protein does...
Fig. 8. The E7 CR3 domain inhibits binding of E2F to pRB60. Binding of E2F to pRB60 was assessed in a gel shift assay as described (see “Materials and Methods”). Panel A, all lanes contain 1 µl of a 1:500 dilution of affinity-purified HeLa cell E2F. Samples analyzed in lanes 2–7 also contain 0.5 nM recombinant pRB60. Samples analyzed in lanes 4–7 also contain 10 nM recombinant E7 protein. The concentrations of the inhibitors at the top of each lane were as follows: E7-ATC, 800 nM; E7(20–29), 1 µM; scrambled E7(20–29), 10 µM. The location of the E2F and the pRB60/E2F complexes are labeled to the left of the panel. Panel B, the concentrations of E2F and pRB60 were the same as for Panel A. The concentration of E7(31–98) peptide was titrated from 10 µM (lane 3) to 1 nM (lane 7) in 10-fold dilutions. The concentration of E7(31–98) in lanes 8–11 was 10 µM. The concentrations of E7-ATC, E7(20–29), and scrambled E7(20–29) were the same as for Panel A. The locations of the E2F and E2F/pRB60 complexes are indicated by arrowheads to the left and right of the panel. Panel C, schematic model for E2F protein complexes observed in gel shift assay. All binding reactions were run in the presence of excess oligonucleotide. See “Results” for details.

not contain a zinc binding domain analogous to the E7 CR3 domain. Instead, E1A contains an NH3-terminal region, denoted the E1A CR1 domain, which is sufficient to inhibit E2F binding to pRB even in the absence of the CR2 domain (48, 59, 60). Although the E1A CR1 domain and the E7 CR3 domain display no sequence homology, these two domains appear to
perform similar functions with respect to their interaction with pRB. This observation highlights the importance of disrupting the pRB-E2F complex (or another pRB complex formed at the same site) in eliciting the biological phenotype of these oncoproteins, and appears to represent an example of convergent evolution.

The regions of E7 protein which are necessary for binding the COOH terminus of pRB includes several cysteines which have previously been demonstrated to be involved in zinc binding (19). An intact CR3 domain is not necessary for this interaction, since the E7(3-75) construct, which lacks the COOH-terminal Cys-X-X-Cys motif, displays pRB binding activity. Eliminating both of these Cys-X-X-Cys motifs, as in the E7(3-50) construct, completely abrogates the ability of the protein to interact with the COOH terminus of pRB, however (Fig. 7). This result is consistent with a zinc binding model proposed for HPV-18 E7 domains (40).

If an intact zinc binding domain, whether monomeric or dimeric, is essential to the biological activity of E7 protein, then one would predict that mutation of the cysteines involved in zinc binding would abrogate the transformation and E2 promoter-transactivating activities of E7. While several studies have addressed this subject, the results are often difficult to interpret because these mutations generate unstable proteins in mammalian cells. For studies in which the protein stability of the mutant was assessed, however, mutation of the cysteines involved in zinc binding either completely reduced or abolished both the trans-activating and transforming activities of E7 (14, 16, 22, 61, 62). In HPV-16 E7, mutation of one cysteine in each Cys-X-X-Cys motif abrogated the zinc binding and transforming activities of the mutated protein, but had no discernable effect on its pRB binding activity (61). Other mutations and deletions within the CR3 domain of E7, independent of the Cys-X-X-Cys motifs, also reduce these activities, suggesting the possibility that other amino acids within the CR3 domain are also necessary for E7's biological activities. The effect of these changes on the overall structure and zinc binding properties of the mutated HPV-16 E7 proteins are unclear, however. Eludication of the specific role of the zinc binding activity of E7 within the context of the biochemical function of the entire CR3 domain will require additional study.

Although an intact CR3 domain appears to be necessary for the full manifestation of E7's transforming activity, it is not sufficient for this activity. This conclusion is supported by both the CR2 domain mutagenesis studies cited above, as well as by peptide microinjection studies (63). Microinjection of an intact E7 CR3 domain is sufficient to trans-activate the E2 promoter in HeLa cells, but does not elicit a mitogenic response in NIH 3T3 cells. The full expression of E7's biological activity appears to require both intact CR2 and CR3 domains. Together, the E7 CR2 and CR3 domains provide a high affinity bivalent interaction with pRB which inhibits binding of cellular proteins to pRB at both sites.

Acknowledgments—We acknowledge Paula Goodhart, Ians Huber, and Lynette Miles for assistance in the purification of E2F and pRB60. Steven Stidravanti for several E7 constructs, and Jerry Vuocolo for many helpful discussions.

REFERENCES
1. DeVilliers, D.-M. (1993) J. Virol. 63, 4908-4913
2. zur Hausen, H., and Schneider, A. (1987) in The Papillomaviruses (Howe}

To require both intact CR2 and CR3 domains. Together, the E7

helpful discussions.

and Lynette Miles for assistance in the purification of E2F and pRB60. Steven Stidravanti for several E7 constructs, and Jerry Vuocolo for many helpful discussions.

REFERENCES
1. DeVilliers, D.-M. (1993) J. Virol. 63, 4908-4913
2. zur Hausen, H., and Schneider, A. (1987) in The Papillomaviruses (Howe, P. M., and Saltman, N. P., ed) Vol. 2, pp. 345-363, Plenum Press, New York.
3. Yue, C., Krishnan-Hewlett, I., Baker, C., Schlegel, R., and Howe, P. M. (1988) Am. J. Pathol. 139, 363-366
Novel E7/pRB Binding Interaction

6850

Ivey-Hayle, M., Barnett, S. F., Oliff, A., and Heimbrook, D. C. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3525–3529
54. Edwards, G. M., Huber, H. E., Defeo-Jones, D., Vuocolo, G., Goodhart, P. J., Maigetter, R. Z., Sanyal, G., Oliff, A., and Heimbrook, D. C. (1992) J. Biol. Chem. 267, 7971–7974
55. Whyte, P., Buchkovich, K. J., Horowitz, J. M., Friend, S. H., Raybuck, M., Weinberg, R. A., and Harlow, E. (1986) Nature 324, 124–128
56. Helin, K., Lee, J. A., Vidal, M., Dyon, N., Harlow, E., and Fattaey, A. (1992) Cell 70, 337–350
57. Kaelin, W. G., Jr., Kreid, W., Sellers, W. R., DeCaprio, J. A., Ajchenbaum, F., Fuchs, C. S., Chittenden, T., Li, Y., Farnham, P. J., Blanar, M. A., Livingston, D. M., and Flemington, E. K. (1992) Cell 70, 351–364
58. Wang, N. P., Chen, P. L., Huang, S., Donoso, L. A., Lee, W. H., and Lee, E. Y. (1990) Cell Growth Differentiation 1, 233–239
59. Dyson, N., Guide, P., McCall, C., and Harlow, E. (1992) J. Virol. 66, 4606–4611
60. Raychaudhuri, P., Bagchi, S., Devoto, S. H., Kraus, V. B., Moran, E., and Nevins, J. R. (1991) Genes & Dev. 5, 1200–1211
61. McIntyre, M. C., Prattini, M. G., Grossman, S. T., and Laimins, L. A. (1993) J. Virol. 67, 3142–3150
62. Storey, A., Almond, N., Osborn, K., and Crawford, L. (1990) J. Gen. Virol. 71, 965–970
63. Rawls, J. A., Pusztai, R., and Green, M. (1990) J. Virol. 64, 6121–6129