MOLECULAR AND CELLULAR BIOLOGY, May 1992, p. 1905–1914
0270-7306/92/051905-10$02.00/0

Vol. 12, No. 5

Human Papillomavirus Type 16 E7 Protein Inhibits DNA Binding by the Retinoblastoma Gene Product

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Received 26 August 1991/Accepted 7 February 1992

The human papillomavirus E7 gene can transform murine fibroblasts and cooperate with other viral oncogenes in transforming primary cell cultures. One biochemical property associated with the E7 protein is binding to the retinoblastoma tumor suppressor gene product (pRB). Biochemical properties associated with pRB include binding to viral transforming proteins (E1A, large T, and E7), binding to cellular proteins (E2F and Myc), and binding to DNA. The mechanism by which E7 stimulates cell growth is uncertain. However, E7 binding to pRB inhibits binding of cellular proteins to pRB and appears to block the growth-suppressive activity of pRB. We have found that E7 also inhibits binding of pRB to DNA. A 60-kDa version of pRB (pRB60) produced in reticulocyte translation reactions or in bacteria bound quantitatively to DNA-cellulose. Recombinant E7 protein used at a 1:1 or 10:1 molar ratio with pRB60 blocked 50 or >95% of pRB60 DNA-binding activity, respectively. A mutant E7 protein (E7–Ala-24) with reduced pRB60-binding activity exhibited a parallel reduction in its blocking of pRB60 binding to DNA. An E7(20-29) peptide that blocks binding of E7 protein to pRB60 restored the DNA-binding activity of pRB60 in the presence of E7. Peptide E7(2-32) did not block pRB60 binding to DNA, while peptide E7(20-57) and an E7 fragment containing residues 1 to 60 partially blocked DNA binding. E7 species containing residues 3 to 75 were fully effective at blocking pRB60 binding to DNA. These studies indicate that E7 protein specifically blocks pRB60 binding to DNA and suggest that the E7 region responsible for this property lies between residues 32 and 75. The functional significance of these observations is unclear. However, we have found that a point mutation in pRB60 that impairs DNA-binding activity also blocks the ability of pRB60 to inhibit cell growth. This correlation suggests that the DNA-binding activity of retinoblastoma proteins contributes to their biological properties.

Human papillomaviruses (HPV) are the etiologic agents responsible for warts and are believed to contribute to the pathogenesis of cervical cancer (16, 55). The most commonly studied isolates of HPV associated with cervical cancer include HPV-16 and HPV-18 (3, 54). The HPV E6 and E7 genes are believed to participate in cell growth regulation. These genes are generally found in cervical cancer cells and cell lines associated with HPV infections, while the remainder of the HPV genome is commonly absent (3, 41, 43). Moreover, the E6 and E7 genes together are capable of immortalizing primary human keratinocytes (37). The E7 gene by itself is capable of transforming NIH 3T3 cells and can cooperate with *ras* in the transformation of primary baby rat kidney cells (39, 46). E6 alone does not transform murine fibroblasts or cooperate with *ras* in transforming rat cells (5, 46). Since the E7 gene appears to be the most potent transforming activity in the HPV-16 genome, our laboratory has focused on the role of the E7 gene product in the pathogenesis of HPV-induced disease.

The HPV-16 E7 gene encodes a phosphoprotein of 98 amino acids (44). No enzymatic activity has been associated with the E7 gene product, but E7 protein has been shown to bind to the retinoblastoma (RB) gene product (pRB) (18, 26). The region of HPV-16 E7 which confers pRB-binding specificity has been mapped to amino acids 20 to 29 (19, 26, 36). This region contains an LXCXE motif (X = any amino acid). The viral transforming proteins of adenovirus and simian virus 40, E1A and large T, respectively, also bind to pRB (13, 52). Comparison of the amino acid sequences of these proteins with that of HPV-16 E7 reveals conserved amino acid sequences that also contain LXCXE motifs (13, 36, 53). Mutations in these regions dramatically reduce the pRB-binding and cellular transforming activities of the large-T, E1A, and E7 gene products (13, 35, 36, 39, 53). More recently, we and others have shown that peptides derived from these regions also inhibit the binding of large T, E1A, and E7 to pRB (14, 26, 52).

The RB suppressor gene product is a 105-kDa phosphoprotein (pRB105) (32, 34) which is thought to play a key role in regulating mammalian cell replication (8, 11, 14). The RB gene is frequently deleted or mutated in a variety of human tumors and tumor cell lines (9, 17, 21, 31, 47). The association of tumors with the loss of wild-type RB alleles has given rise to the hypothesis that the normal function of the RB gene is to inhibit cell proliferation. The precise mechanism employed by the RB gene to block cell proliferation is unknown. However, two general biochemical properties of RB proteins have been described. First, the RB gene product can form specific complexes with a variety of proteins, including the viral transforming proteins—large T, E1A, and E7. pRB also binds normal cellular proteins (15, 24, 28, 41) and has recently been shown to associate with the E2F-DRTF transcription factor complex (2, 4, 10, 12). The segment of pRB that binds to these proteins consists of two discontinuous regions encompassing amino acids 394 to 571 and 649 to 772 (22, 23, 27). These regions have been proposed to form a "binding pocket" that both viral onco-

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proteins and normal cellular proteins use to make contact with pRB. The second biochemical property associated with pRB is its ability to bind double-stranded DNA in a non-sequence-specific manner (21, 32, 48). Both full-length pRB105 and a smaller 60-kDa version of the RB protein (pRB60) contain this protein-binding pocket and exhibit DNA-binding activity.

HPV-16 E7 binding to RB protein has been shown to displace other proteins from the pRB-binding pocket. However, the effect of E7 on pRB DNA-binding activity has not been previously reported. In the present study, we examined the effect of full-length E7 protein and several shorter segments of E7 on the DNA-binding activity of pRB60.

MATERIALS AND METHODS

Plasmids. The human pRB-coding sequence was cloned from a human lung fibroblast library (Clonetech, Palo Alto, Calif.) by using oligonucleotide probes derived from a published RB nucleic acid sequence (6, 20). The cloning of the 60-kDa fragment of p105, which begins at Met-387, has been described previously (26). The single substitution mutation at Cys-706 was made by site-directed mutagenesis using an oligonucleotide which changed the coding sequence from cysteine to phenylalanine. Each of these constructs was placed in pGem4Z (Promega, Madison, Wis.) for in vitro transcription-translation of the pRB fragment encoded.

Cell culture and transfections. NIH 3T3 cells were obtained from Douglas Lowy (National Institutes of Health, Bethesda, Md.). Cell cultures were maintained in Dulbecco's modified Eagle's medium plus 10% fetal calf serum and antibiotics (GIBCO, Grand Island, N.Y.). Transfections were performed by using LIPOFECTIN (BRL, Gaithersburg, Md.) in accordance with the manufacturer's procedure. Transfected cells were identified by using G418 (GIBCO) selection at 800 μg/ml of culture medium.

Reticulocyte translated pRB. *Eco*RI-linearized pGem4Z-RB constructs were used to transcribe capped mRNAs by using T7 DNA polymerase in accordance with the protocols described by the manufacturer (Promega). The $m^7G(5')ppp(5')Gm$ cap analog was obtained from Pharmacia (Piscataway, N.J.). Rabbit reticulocyte lysate translations were performed as described by the manufacturer (Promega) by using [^{35}S]methionine (1,100 Ci/mmol; Amersham, Arlington Heights, Ill.).

***Escherichia coli*-expressed pRB60.** The 60-kDa RB protein was expressed by using a Tac promoter vector in *E. coli* DH5αI^q (GIBCO-BRL, Gaithersburg, Md.). The protein was purified from inclusion bodies by solubilization in guanidine-HCl, refolding at dilute concentrations, and chromatography on an E7 peptide affinity column. A detailed description of the purification procedure will appear elsewhere (19a). Protein concentrations were determined by a Bradford assay (Bio-Rad, Richmond, Calif.) standardized against an amino acid composition analysis.

HPV-16 E7 expression and purification. The HPV-16 E7 gene (P. M. Howley, National Cancer Institute, Bethesda, Md.) was subcloned into a Tac promoter bacterial expression plasmid (33) and termed pTAC-E7. This cloning resulted in the addition of 16 amino acids from the amino terminus of β-galactosidase placed at the amino terminus of E7. The E7 fusion protein was expressed in *E. coli* DH5αI^q (GIBCO-BRL) and purified by anion-exchange chromatography and gel filtration column chromatography. A description of the expression system and purification procedure will be published elsewhere (38). Protein concentration was determined by spectrophotometric reference to an amino acid composition standard.

Peptide synthesis. Peptides were prepared by solid-phase synthesis by using a double-coupling protocol on an Applied Biosystems 430A automated peptide synthesizer. Deprotection, purification, and identity testing were done as described previously (26). The E7(20-29) peptide sequence was TDLYCYEQLN-amide. The scrambled E7 peptide sequence was YNELCQYDL-amide. Lyophilized peptides were taken up in 0.1 M Tris-HCl (pH 7.5) and 1 mM dithiothreitol, and the pH was adjusted to neutrality with a final peptide concentration of 2 mM. The E7(2-32) peptide was a generous gift from E. Harlow (Massachusetts General Hospital Cancer Center, Charlestown, Mass.).

E7 proteolytic fragments. βgal-E7 fusion protein was purified as described above and subjected to proteolytic digestion. Cleavage with either trypsin (Promega), endoprotease lys-C (Promega), or cyanogen bromide yielded E7 fragment βgal-E7 1 to 60, βgal-E7 1 to 49, or E7 13 to 84, respectively. The desired fragments were isolated by high-performance liquid chromatography, quantitated by spectrophotometric determination of the A_{275}, and tested for pRB-binding activity in an enzyme-linked immunosorbent plate assay (25).

pGEX-E7 deletion mutants. C-terminal deletions of E7 were generated as glutathione *S*-transferase fusions in the pGex2T plasmid vector system (Pharmacia). The E7-coding sequence was inserted into pGex by blunt-end ligation of an *Nsi*I-*Pst*I E7 DNA fragment into the *Sma*I-*Eco*RI-cut and blunt-ended pGex2T vector. This construct expresses a fusion protein containing glutathione *S*-transferase and E7 residues 3 to 98. C-terminal deletions were constructed by using synthetic oligonucleotides to insert a termination codon at the *Ban*II or *Acc*I site of E7 (3-to-50 and 3-to-75 constructions, respectively). The 3-to-84 construction was generated by inserting an oligonucleotide coding for amino acids 74 to 84, followed by a termination codon, into the *Acc*I site. These fusion protein constructions were expressed in *E. coli* and purified by using a glutathione affinity column. The purified fusion proteins were shown to be of the expected sizes, and >95% homogeneous by analysis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Quantitation of the fusion proteins was performed by Bradford assays. For use in the DNA-binding competition assays, fusion proteins were cleaved with thrombin (Calbiochem, La Jolla, Calif.) to yield an E7 molecule beginning at residue 3. The thrombin reaction was terminated by using Thrombstop enzyme inhibitor (American Diagnostics Inc., Greenwich, Conn.). The thrombin-treated proteins were again analyzed by SDS-PAGE to validate complete enzyme cleavage. pRB-binding activity was determined for each species of E7 protein and found to be equivalent for similar-size fragments whether derived by peptide synthesis or proteolytically from fusion proteins.

DNA-binding assays. For reticulocyte lysate-translated material, a 30-μl reticulocyte translation reaction was diluted to 500 μl in load buffer (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]-NaOH, 100 mM NaCl, 5 mM $MgCl_2$, 0.1% Triton X-100 [pH 7.2]). It was determined empirically that the presence of $MgCl_2$ in the load buffer enhanced pRB binding to the DNA-cellulose columns. All binding reactions were performed at 4°C unless indicated otherwise. Preincubation of the diluted reticulocyte lysate was for 10 min on ice, after which the reaction was applied to the top of a 1.5-ml double-stranded calf thymus DNA-cellulose column (Pharmacia). The columns were polypropylene Econocolumns (Bio-Rad) with a packed

bed volume of 0.8 by 3 cm. The loaded columns were washed with 3 ml of the load buffer, and both the flow-through and wash fractions were collected. Bound material was eluted with load buffer containing 0.5 or 1.0 M NaCl. An SDS-PAGE reducing sample buffer was added to aliquots of the column fractions, and the samples were boiled for 10 min. Denaturing SDS-PAGE was performed in the manner of Laemmli (30). Sample loads for each fraction were adjusted relative to the volumes collected so as to obtain a direct semiquantitative comparison on the gels. After electrophoresis, gels were fixed, equilibrated in fluorescence enhancer, and dried. Autoradiography was performed to detect the pRB60 product. For *E. coli*-produced pRB60, DNA-binding experiments were run as described above, except that defined quantities of pRB60 were diluted to 500 μl in load buffer and analysis of column fractions was done by silver staining of SDS-PAGE gels.

When E7 protein was used in conjunction with pRB60, a stock solution of 1 mg of E7 per ml was diluted in load buffer and then added to the reaction mixture to obtain the desired final concentration. Reactions with E7 and pRB60 were allowed to incubate for 10 min on ice before being loaded onto the DNA-cellulose column. For reactions containing pRB60, E7, and a peptide, the peptide was added to the reticulocyte-translated pRB and allowed to incubate on ice for 10 min, after which the E7 was added and incubation was continued for 10 min on ice. The mixture was then loaded onto the DNA-cellulose column. Reactions in which E7 was added to pRB60 before the peptide gave the same result as when the peptide was added before E7. The E7 fragments and peptides were assayed for pRB60-binding activity. All of the E7 fragments were found to have binding affinities for pRB60 within 20-fold of one another. The final concentrations of E7 peptides and E7 fragments used in the DNA-binding reactions were selected to produce saturation of the E7-binding site or binding pocket on pRB60 (5.0 to 100 μM).

RESULTS

pRB60 binds DNA. To test the DNA-binding activity of RB protein, several versions of pRB were cloned into expression vectors (see Materials and Methods). The 60-kDa version of pRB, containing the binding pocket and the remaining C-terminal region of the RB gene, was used to produce pRB60 via in vitro transcription and reticulocyte lysate translation reactions. pRB60 was also produced in *E. coli* as a recombinant protein and affinity purified. Both preparations of pRB60 were passed over a DNA-cellulose column, and the bound material was eluted by using increasing concentrations of NaCl. As seen in Fig. 1, both pRB60 preparations bound to the DNA-cellulose column when a loading buffer containing 125 mM NaCl was used. Elution of both preparations of pRB60 from the column occurred between 0.15 and 0.2 M NaCl. Virtually all of the affinity-purified pRB60 made in bacteria bound to the initial DNA-cellulose column. However, a small fraction of the pRB60 made in reticulocyte lysates flowed through the first column (Fig. 1). The reticulocyte-made pRB60 in the flowthrough from the first column was loaded onto a second DNA-cellulose column, where the remaining pRB60 bound and was eluted at salt concentrations similar to those of the first column. The need for a second DNA-cellulose column to bind all of the pRB prepared in the reticulocyte lysate reactions may reflect the high concentration of other proteins in these preparations (0.6 mg of total protein per ml in the reticulocyte lysate reaction mixture). In control experiments, pRB60 made in either

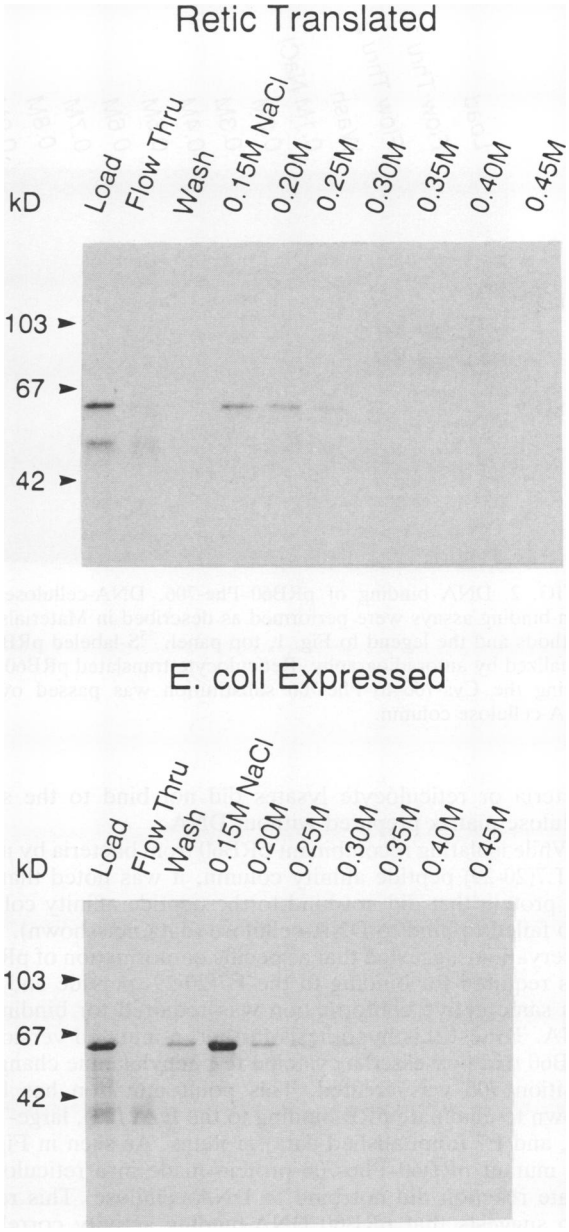

FIG. 1. DNA-binding and salt elution profiles of reticulocyte lysate (Retic)- and *E. coli*-produced pRB60. (Top) Reticulocyte-translated pRB60. A 30-μl volume of reticulocyte lysate containing translated ^{35}S-labeled pRB60 was diluted in 470 μl of column load buffer and passed through a 1.5-ml calf thymus DNA column. The column was washed with 2 column volumes of load buffer, and the bound protein was eluted with a step gradient of load buffer plus increasing concentrations of NaCl. Gel electrophoresis and autoradiography were performed as described in Materials and Methods. The upper band represents the complete pRB60 protein, while the lower band represents a protein generated from an internal methionine start site 77 amino acids from the pRB60 N terminus. (Bottom) *E. coli*-expressed pRB60. A 50-μl volume of 0.6-mg/ml affinity-purified pRB60 was diluted to 500 μl in column load buffer and passed through a calf thymus DNA-cellulose column as described above. Elution and gel electrophoresis were done as described in Materials and Methods. Gel proteins were visualized by silver staining. The upper band represents pRB60 protein, while the lower band represents a contaminating C-terminally truncated form of pRB60.

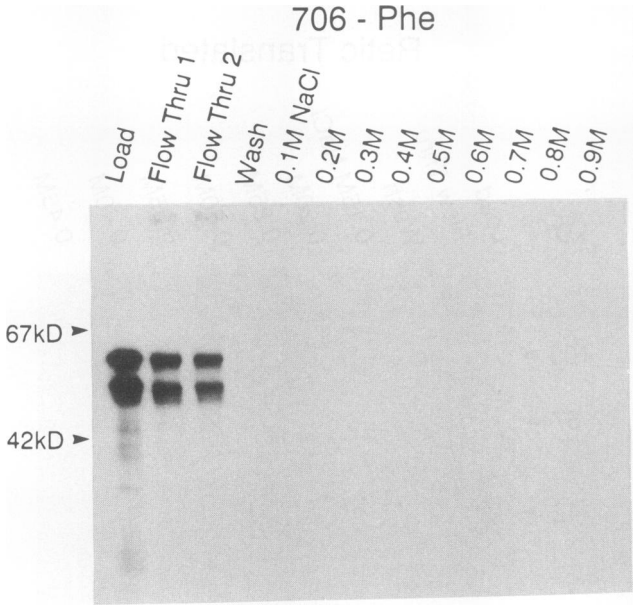

FIG. 2. DNA binding of pRB60–Phe-706. DNA-cellulose column-binding assays were performed as described in Materials and Methods and the legend to Fig. 1, top panel. ^{35}S-labeled pRB was visualized by autoradiography. Reticulocyte-translated pRB60 containing the Cys-706–to–Phe-706 substitution was passed over a DNA-cellulose column.

bacteria or reticulocyte lysates did not bind to the same cellulose matrix prepared without DNA.

While isolating recombinant pRB60 from bacteria by using an E7(20-29) peptide affinity column, it was noted that the RB protein that did not bind to the peptide affinity column also failed to bind to DNA-cellulose (data not shown). This observation suggested that a specific conformation of pRB60 was required for binding to the E7(20-29) peptide and that this same active conformation was required for binding to DNA. To test this hypothesis further, a mutated version of pRB60 that possessed a cysteine-to-phenylalanine change at position 706 was created. This point mutation has been shown to eliminate pRB binding to the E1A (29), large-T (7, 29), and E7 (unpublished data) proteins. As seen in Fig. 2, the mutant pRB60–Phe-706 protein made in a reticulocyte lysate reaction did not bind to DNA-cellulose. This result also suggests that pRB60 DNA-binding activity correlates with the ability of pRB60 to bind E7 protein.

pRB inhibits cell growth. The genes that encode the pRB105, pRB60, and pRB60–Phe-706 proteins were cloned into a mammalian expression vector under control of a murine retrovirus long terminal repeat promoter. These expression plasmids also contained a neomycin resistance gene to allow for selection of successfully transfected DNAs. In triplicate experiments, NIH 3T3 cells were transfected with 10 μg of each plasmid DNA and subjected to G418 selection. This procedure did not permit growth of colonies of NIH 3T3 cells in the absence of transfected DNAs. The LTR vector DNA alone yielded a mean of 206 ± 12 (standard error of the mean) colonies per 100-mm-diameter petri dish. Both the pRB105 and pRB60 DNAs dramatically inhibited colony formation, allowing only 7 ± 2 and 12 ± 3 colonies per dish, respectively. Interestingly, the pRB60–Phe-706 DNA caused a much smaller inhibition of colony formation, yielding 155 ± 14 colonies per plate. These experiments indicate that the Cys-706 mutation to Phe in pRB60 not only interferes with pRB binding to DNA (see above) and to viral oncoproteins (7, 29) but also affects the cell growth-inhibitory activity of pRB60. Taken together, these observations suggest that a correlation between the DNA-binding activity of pRB proteins and their functional properties in vivo exists.

HPV-16 E7 protein blocks DNA binding. To examine the effect of E7 protein on the DNA-binding activity of pRB60, RB protein prepared in reticulocyte lysates was mixed with recombinant E7 protein and passed through DNA-cellulose columns. As shown in Fig. 3, E7 protein at 30 nM reduced pRB60 binding >90%. This suggested that pRB60 binding to DNA was specifically inhibited by E7 protein. Studies of the ability of E7 to block pRB60 binding to DNA were also done by using recombinant pRB60 made in bacteria. Use of bacterially produced pRB60 allowed quantitation of the relative concentrations of pRB and E7 proteins in each reaction. Recombinant E7 protein was added to recombinant pRB60 at molar ratios of 1:10, 1:1, and 10:1 in standard load buffer. The concentration of pRB60 in each reaction was 0.4 μM. A silver-stained gel of the column fractions from these experiments is shown in Fig. 4. At an E7-pRB ratio of 1:10, negligible inhibition of DNA binding was observed, while at a 1:1 ratio, approximately half of the pRB60 was prevented from binding to the DNA column. At an E7-pRB ratio of 10:1, >95% of the pRB60 was blocked from binding to the column. These results confirmed that E7 protein was able to block purified pRB60 binding to DNA.

To determine the specificity of E7 inhibition of pRB60 binding to DNA, a mutant form of E7, in which the cysteine residue at position 24 was changed to an alanine, was created. Substitution mutants of HPV-16 E7 at position Cys-24 have previously been shown to lack cell-transforming activity (19, 51). The mutant E7–Ala-24 protein was purified to homogeneity and exhibited a chromatographic profile similar to that of the wild-type E7 protein. However, as expected, the E7–Ala-24 protein was approximately 28-fold less potent than wild-type E7 as a binder of RB protein in a competitive plate binding assay (26). A titration experiment using both the wild-type and mutant E7 proteins was performed to determine the inhibitory concentration at which 50% (IC_{50}) of the pRB60 DNA-binding activity was blocked by these proteins. Reactions were run with increasing concentrations of the E7 proteins added to identical aliquots of reticulocyte-translated pRB60. As seen in Fig. 5, the wild-type E7 protein exhibited an IC_{50} of approximately 0.3 nM for blocking of pRB60 binding to DNA. The E7–Ala-24 mutant protein was approximately 33-fold less potent than the wild-type E7 protein as a blocker of pRB60 binding to DNA, with an apparent IC_{50} of 10 nM.

E7(20-29) peptide does not block DNA binding. To determine what part of the E7 protein was responsible for inhibition of pRB60 binding to DNA, several fragments of E7 were examined. E7(20-29) peptide has been shown to compete with full-length E7 protein for binding to RB proteins via attachment to the pRB binding pocket (25, 26). The E7(20-29) peptide inhibited E7 binding to pRB in these assays with an IC_{50} of approximately 100 nM. However, as seen in Fig. 3C, the E7(20-29) peptide did not alter pRB60 DNA-binding activity at concentrations of up to 100 μM. Moreover, the E7(20-29) peptide actually blocked the inhibitory effect of recombinant E7 protein on pRB60 DNA-binding activity. When the E7(20-29) peptide (100 μM) was present in large molar excess over the E7 protein (30 nM), pRB60 bound to DNA as well as it did in the absence of E7 protein. As a control for this study, a scrambled version of

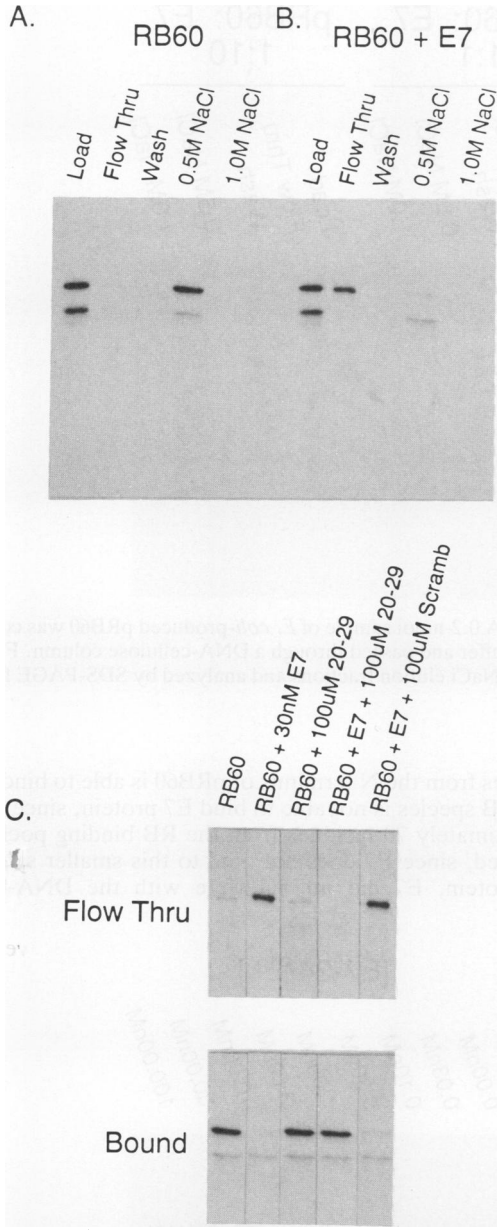

FIG. 3. Effect of E7 or E7(20-29) peptide on DNA binding of pRB60. DNA-cellulose column-binding assays were run as described in Materials and Methods. Reticulocyte lysate-produced pRB60 was analyzed for the ability to bind DNA in the presence of E7 protein, an E7(20-29) peptide, E7 protein plus the E7(20-29) peptide, or E7 protein plus a scrambled 20-to-29 peptide. In each assay, fractions of the load, column flowthrough, wash, 0.5 M NaCl eluate, and 1.0 M NaCl eluate were taken. Panels: A, pRB60 alone; B, pRB60 plus 30 nM E7 protein; C, composite of flowthrough and bound fractions from panels A and B and parallel experiments containing pRB60 plus 100 μM E7(20-29) peptide, pRB60 plus 30 nM E7 protein plus 100 μM E7(20-29) peptide, and pRB60 plus 30 nM E7 protein plus 100 μM scrambled 20-to-29 peptide (Scramb).

the E7(20-29) peptide, containing the same residues but out of sequence, was also tested. This scrambled peptide did not block E7 protein binding to pRB60. The scrambled peptide (100 μM) also did not block the inhibitory effect of E7 on pRB60 DNA-binding activity. The fact that only the E7(20-29) peptide reversed E7 inhibition of pRB60 DNA binding demonstrates that the effect of E7 on DNA binding is dependent upon attachment of E7 to the protein binding pocket in pRB60. These studies further demonstrate that a complex of pRB60 plus the E7(20-29) peptide can remain bound to DNA while a complex of pRB60 plus full-length E7 protein cannot.

Since the 10-residue E7(20-29) peptide did not mimic the effect of full-length E7 protein on pRB60 binding to DNA, we examined several larger fragments of the E7 protein. Four synthetic peptides, comprising E7 residues 2 to 32, 14 to 29, 20 to 29, and 20 to 57, were tested in the pRB60 DNA-binding assay. A CNBr cleavage fragment of the recombinant E7 protein containing residues 13 to 84, a trypsin digestion product containing residues 1 to 49, and a lys-C enzyme digestion product containing residues 1 to 60 were also isolated and tested. Additionally, four recombinant pGEX-E7 fusion proteins containing E7 residues 3 to 50, 3 to 75, 3 to 84, and 3 to 98 were produced in bacteria, purified, cleaved with thrombin to remove their glutathione *S*-transferase residues, and assayed as competitors of pRB60 binding to DNA. On the basis of these studies, each E7 fragment was used at a concentration known to saturate the available pRB60 E7-binding sites. As seen in Fig. 6, the E7(2-32), E7(14-29), and E7(20-29) peptides had no effect on pRB60 binding to DNA but the E7 3-to-75, 3-to-84, 13-to-84, and 3-to-94 protein fragments were fully effective blockers of DNA binding. The E7(20-57) peptide and the E7 1-to-49, 3-to-50, and 1-to-60 protein fragments showed an intermediate capacity to block pRB60 binding to DNA. The degree of binding inhibition exhibited by these intermediate-capacity fragments was not appreciably increased in separate experiments performed by using a 10-fold higher concentration of each fragment. These results indicate that the ability of E7 to block pRB60 binding to DNA does not require the entire E7 protein. The results further suggest that E7 residues 2 to 32 do not appreciably inhibit pRB60 binding to DNA while residues 33 to 60 contribute to but are not fully capable of blocking pRB60 DNA-binding activity. E7 residues 61 to 75, in conjunction with residues 33 to 60, appear to restore completely the ability of E7 to block pRB60 binding to DNA.

DISCUSSION

No sequence-specific DNA-binding site has been identified for the RB gene product, although pRB clearly does bind DNA in a non-sequence-specific manner. This lack of sequence specificity need not imply that the DNA-binding activity of pRB is not a legitimate and potentially significant biochemical property of this protein. Rather, several lines of evidence suggest that pRB60 DNA-binding activity is an important characteristic of biologically active RB protein. For example, the ability of pRB60 to bind DNA at a physiologically relevant ionic strength (125 mM NaCl) implies that pRB is a true DNA-binding protein (1). Another characteristic of true DNA-binding proteins is the requirement for proper protein conformation to bind DNA (45). Loss of DNA-binding activity associated with loss of proper tertiary structure can be an indication that specific domains within a protein are organized to mediate DNA binding (49, 50). The lack of DNA-binding activity associated with the pRB60 species that failed to bind to the E7 peptide column suggests that a particular conformation of pRB60 is required to bind both DNA and the E7(20-29) peptide. Similarly, the fact that a single cysteine-to-phenylalanine change in the pRB60–Phe-706 mutant protein abrogated both the E7 protein- and DNA-binding activities of pRB60 suggests that

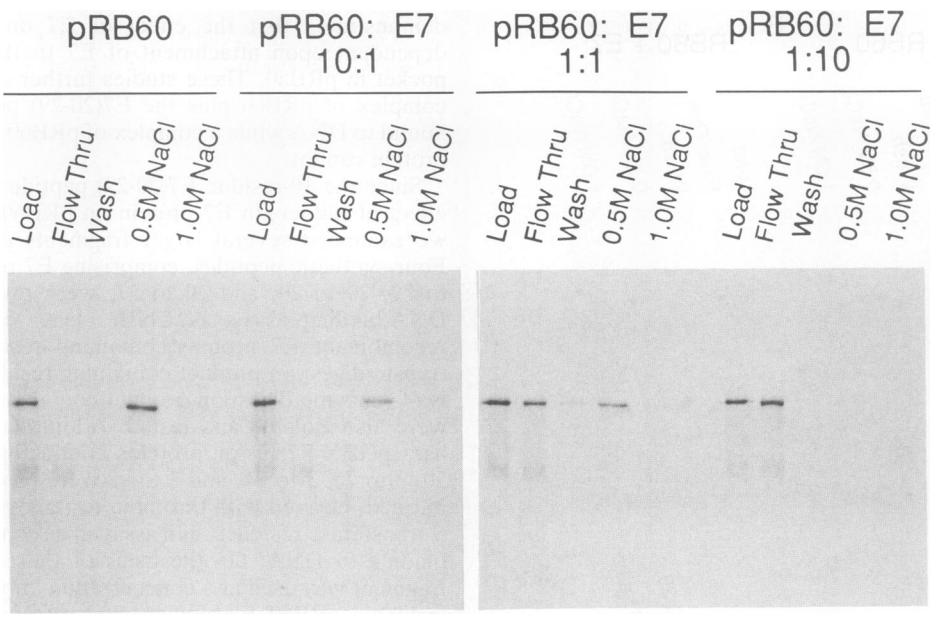

FIG. 4. Inhibition of *E. coli*-produced pRB60 binding to DNA by E7 protein. A 0.2-nmol sample of *E. coli*-produced pRB60 was combined with 0.0, 0.02, 0.2, or 2.0 nmol of *E. coli*-produced E7 protein in 0.5 ml of load buffer and passed through a DNA-cellulose column. Fractions were collected from the load, flowthrough, wash, 0.5 M NaCl elution, and 1.0 M NaCl elution fractions and analyzed by SDS-PAGE followed by silver staining.

these biochemical properties are linked and may be dependent upon maintenance of a normal pRB60 conformation. However, the RB protein properties of DNA binding and E7 binding are not absolutely linked. As seen in Fig. 1, a smaller species of the RB protein that initiates at a methionine 77 residues from the N terminus of pRB60 is able to bind DNA. This RB species is not able to bind E7 protein, since it lacks approximately 70 residues from the RB binding pocket. As expected, since E7 does not bind to this smaller species of RB protein, E7 did not interfere with the DNA-binding

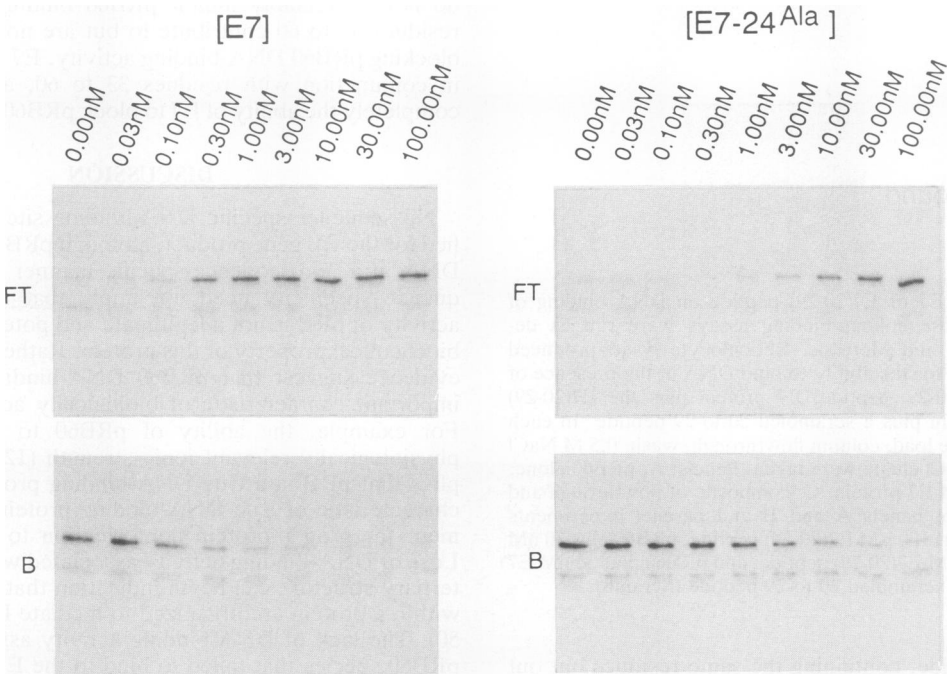

FIG. 5. Inhibition of pRB60 binding to DNA with E7 or E7–Ala-24 protein. Reticulocyte-translated pRB60 was passed through a DNA-cellulose column in the presence of increasing concentrations of the E7 protein or a mutant E7–Ala-24 protein. The flowthrough and 0.5 M NaCl elution fractions were electrophoresed on SDS–12% PAGE gels, and the ^{35}S-labeled pRB60 was visualized by autoradiography. The autoradiograph on the left was prepared by using the wild-type E7 protein. The autoradiograph on the right was prepared by using the E7–Ala-24 protein. FT, flowthrough fractions; B, bound fractions eluted at 0.5 M NaCl.

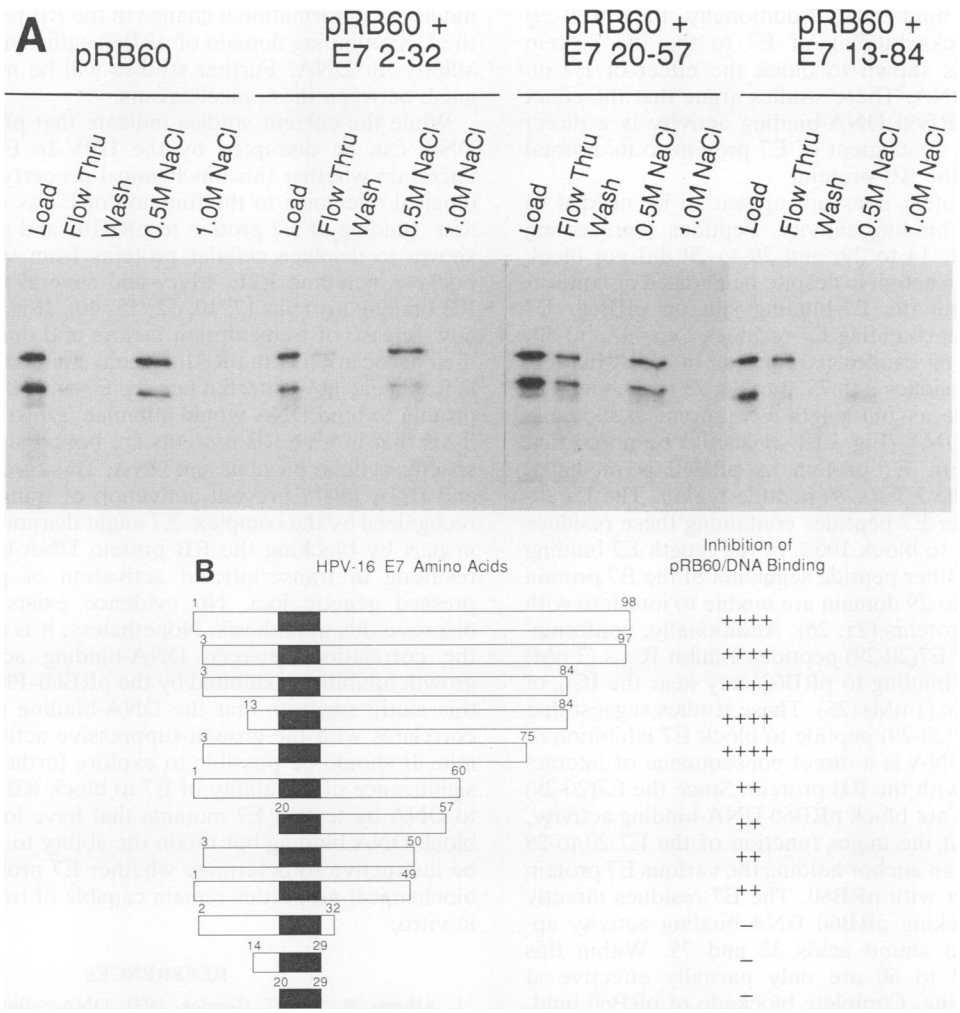

FIG. 6. Effects of E7 deletions on pRB60 DNA-binding competition. Protein or peptide fragments of E7 were added to the pRB60 DNA-binding assay at molar concentrations that saturate the pRB60 binding site for E7. The E7 fragments were graded for the ability to inhibit reticulocyte-translated pRB60 binding to DNA-cellulose. The three classes of inhibition are shown in panel A, using E7(2-32) peptide, E7(20-57) peptide, and E7 13 to 84 as representative examples of no inhibition (−), moderate inhibition (++), and full inhibition (++++), respectively, of DNA binding. Panel B displays the full set of E7 peptides and E7 protein fragments analyzed as inhibitors of pRB60 binding to DNA. The proteolytic E7 fragments that are listed as beginning at amino acid residue 1 contain a short β-galactosidase leader sequence (see Materials and Methods). The darkened region indicates the segment of E7 responsible for specific binding to RB proteins.

activity of this species (Fig. 3). It should also be noted that recombinant pRB60 obtained from bacteria bound DNA with approximately the same affinity as pRB60 made in reticulocyte lysates. This implies that there are no accessory proteins in the reticulocyte lysate that are needed to enable pRB60 to bind DNA. These findings also imply that no postsynthetic modifications of pRB60 that might occur solely in mammalian cells are required for DNA-binding activity. In this regard, it should be noted that Shew et al. (42) have described a mutant form of RB protein that has lost the capacity to be phosphorylated but retains its DNA-binding activity.

Additional evidence linking the DNA-binding activity of RB protein with its biologically relevant form comes from the observation in the present study that attachment of E7 protein to pRB60 disrupts pRB60 DNA-binding activity. While addition of 30 nM E7 protein (300 pg/ml) virtually eliminated pRB60 DNA-binding activity, the other proteins present in the rabbit reticulocyte lysates at 600 μg/ml did not significantly affect pRB60 binding to DNA. More quantitative titration experiments using bacterially produced pRB60 and E7 proteins indicated that nearly stoichiometric amounts of E7 protein blocked approximately 50% of pRB60 DNA-binding activity. The ability of E7 to block pRB60 binding to DNA is unlikely to be due to competition between pRB60 and E7 for protein-binding sites on the DNA-cellulose column, since the recombinant E7 protein used in these studies does not bind to the DNA column under our experimental conditions (38). Similarly, the E7 peptides and E7 protein fragments by themselves did not bind to the DNA-cellulose column. Rather, the ability of E7 to block pRB60 DNA-binding activity appears to require E7 binding to the RB protein. The cysteine residue at position 24 in E7 is critically important for E7 binding to the RB protein binding pocket (19, 26). As expected, the E7–Ala-24 mutant protein exhibited a dramatic reduction in binding to pRB60. Coincident with this loss of binding to pRB60, the mutant E7 protein showed a parallel loss of inhibitory activity against the

ability of pRB60 to bind DNA. Additionally, the E7(20-29) peptide, which blocks binding of E7 to the RB protein binding pocket, was shown to block the effect of E7 on pRB60 binding to DNA. These studies argue that the effect of E7 protein on pRB60 DNA-binding activity is a direct consequence of the attachment of E7 protein to its normal binding domain on the RB protein.

The entire E7 protein does not appear to be needed to block pRB60 DNA-binding activity. Peptides representing E7 residues 2 to 32, 14 to 29, and 20 to 29 did not block pRB60 DNA-binding activity, despite being used at concentrations that saturate the E7-binding site on pRB60. E7 protein fragments representing E7 residues 1 to 49, 3 to 50, 20 to 57, and 1 to 60 caused partial loss of DNA-binding activity, while E7 residues 3 to 75, 3 to 84, 13 to 84, and 3 to 97 were as effective as full-length E7 protein at blocking pRB60 binding to DNA (Fig. 6B). It should be noted that binding of full-length E7 protein to pRB60 is mediated predominantly by the E7(20-29) peptide region. The E7(20-29) peptide and other E7 peptides containing these residues are able specifically to block 100% of full-length E7 binding to the RB protein. Other peptide segments of the E7 protein that lack the E7 20-to-29 domain are unable to interfere with E7 binding to RB proteins (25, 26). Additionally, conformationally constrained E7(20-29) peptides exhibit IC_{50}s (2 nM) for inhibition of E7 binding to pRB60 very near the IC_{50} of full-length E7 protein (1 nM) (25). These studies suggest that the ability of the E7(20-29) peptide to block E7 inhibition of pRB60 binding to DNA is a direct consequence of interaction of the peptide with the RB protein. Since the E7(20-29) peptide by itself did not block pRB60 DNA-binding activity, it appears likely that the major function of the E7 20-to-29 region is to serve as an anchor holding the various E7 protein fragments in contact with pRB60. The E7 residues directly responsible for blocking pRB60 DNA-binding activity appear to lie between amino acids 32 and 75. Within this region, residues 33 to 60 are only partially effective at blocking DNA binding. Complete blockade of pRB60 binding to DNA requires the presence of residues 61 to 75 in addition to residues 33 to 60. It is unclear whether these regions represent functionally distinct domains or simply exhibit similar additive effects on the inhibition of DNA binding. However, it is noteworthy that despite possession of an additional 11 C-terminal residues, the E7 1-to-60 protein fragment appeared to be equivalent to the E7 1-to-49 fragment at blocking pRB60 binding to DNA. Similarly, fragments 3 to 75, 3 to 84, and 3 to 97 were approximately equal inhibitors of pRB60 binding to DNA. These results suggest that a critical C-terminal limit of the E7 domain responsible for blocking pRB60 binding to DNA lies between residues 60 and 75.

The precise mechanism by which E7 protein blocks pRB60 DNA-binding activity is unclear. Several possibilities are apparent: steric hindrance of pRB60 attachment to DNA, ionic changes resulting from E7 binding to pRB60, or an E7-induced conformational change in pRB60. If the E7-binding and DNA-binding domains of the RB protein overlap one another, then binding of E7 protein to pRB60 may physically block DNA from its binding site on the RB protein. A variation of this model could involve ionic interactions between the side groups of specific E7 and pRB60 residues that are juxtaposed by binding of E7 protein to pRB60. Such interactions might neutralize key charged residues on the pRB60 molecule required for interaction with the phosphate backbone of a DNA molecule. Alternatively, it is possible that binding of the E7 protein to pRB60 may induce a conformational change in the RB protein that alters the DNA-binding domain of pRB60 sufficiently to reduce its affinity for DNA. Further studies will be needed to distinguish between these mechanisms.

While the current studies indicate that pRB60 binding to DNA can be disrupted by the HPV-16 E7 protein, it is uncertain whether this biochemical property has any physiological relevance to the function of E7 as a viral oncoprotein. Binding of E7 protein to pRB105 and pRB60 has been shown to displace cellular proteins from the pRB binding pocket, including E2F, Myc, and several newly identified RB binding proteins (2, 10, 12, 15, 40). It is easy to imagine how release of transcription factors and oncoproteins from their association with pRB105 could stimulate cell growth. It is less clear how interference by E7 with the ability of RB protein to bind DNA would stimulate growth. One possibility is that in vivo RB proteins are bound simultaneously to specific cellular proteins and DNA. This complex of proteins and DNA might prevent activation of transcriptional units recognized by the complex. E7 might disrupt such a complex in part by blocking the RB protein DNA-binding activity, resulting in transcriptional activation of previously suppressed genetic loci. No evidence exists to confirm or disprove this hypothesis. Nonetheless, it is noteworthy that the correlation between DNA-binding activity and cell growth inhibition exhibited by the pRB60–Phe-706 mutant in this study suggests that the DNA-binding activity of pRB correlates with the growth-suppressive activity of this protein. It should be possible to explore further the functional significance of the ability of E7 to block RB protein binding to DNA by testing E7 mutants that have lost the ability to block DNA binding but retain the ability to bind RB. It will be instructive to determine whether E7 proteins with these biochemical properties remain capable of transforming cells in vitro.

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