Comparison of the binding of the human papillomavirus type 16 and cottontail rabbit papillomavirus E7 proteins to the retinoblastoma gene product

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Binding of the human papillomavirus type 16 (HPV-16) E7 oncoprotein to the retinoblastoma protein (pRb) is thought to be involved in the cellular transformation mediated by HPV-16. Here we show that the E7 protein of the cottontail rabbit papillomavirus (CRPV) binds to the same C-terminal portion of human pRb as HPV-16 E7, and that both the CRPV and HPV-16 E7 proteins bind specifically through similar domains to rabbit pRb.

Furthermore, a single amino acid substitution which reduces the binding of HPV-16 E7 to human pRb also abolishes binding of CRPV E7 to both human and rabbit pRb. The biochemical similarities observed between the HPV-16 and CRPV E7 proteins suggest that they are functionally conserved. These results further validate the use of CRPV as an animal model for the study of HPV-mediated disease.

A number of human papillomaviruses (HPVs) are known to be associated with cervical carcinomas (reviewed in zur Hausen, 1991a, b). HPV-16 DNA is found in the majority of cervical cancers, and has been shown to transform and immortalize cells in vitro (Chesters & McCance, 1989; Jewers et al., 1992; McCance et al., 1988; Pirisi et al., 1987; Yasumoto et al., 1986). Although both the E6 and E7 genes of HPV-16 are preferentially retained and expressed in cervical cancers (Baker et al., 1987; Schwarz et al., 1985; Smotkin & Wettstein, 1986), genetic analysis suggests that E7 is the more potent viral oncoprotein. Expression of E7 can immortalize primary human and rat cells (Halbert et al., 1991; Inoue et al., 1991; Kanda et al., 1988b), transform primary rat cells in cooperation with ras and independently transform established rodent cells (Kanda et al., 1988a; Phelps et al., 1988; Storey et al., 1988; Vousden et al., 1988). E7 can also immortalize primary human keratinocytes in cooperation with E6 (Hawley-Nelson et al., 1989; Munger et al., 1989a).

Mutational analysis (Barbosa et al., 1990; Chesters et al., 1990; Edmonds & Vousden, 1989; Munger et al., 1989b) and peptide competition experiments (Jones et al., 1990) have revealed that a critical region of E7 needed for transformation activity is also involved in the binding of E7 to the product of the retinoblastoma (pRb) tumour suppressor gene. Sequences homologous to this region of E7 are also present in the adenovirus E1A and simian virus 40 large T proteins, and these regions are known to be required for the transforming activity and pRb binding of these viral oncoproteins (Dyson et al., 1989b; Ewen et al., 1989; Moran, 1988).

pRb is an anti-oncogene which is believed to restrict cell proliferation (Marshall, 1991; Weinberg, 1991). In cervical carcinoma cell lines with no apparent HPV aetiology, pRb is found to be deleted or mutated in the region responsible for its association with E7 (Scheffner et al., 1991). These observations suggest that the binding of E7 to pRb may be a critical step in HPV-mediated cell transformation. The interaction of E7 with pRb may inactivate pRb, thus leading to enhanced cell proliferation. In this regard, recent evidence suggests that binding of E7 to pRb may contribute to its ability to trans-activate promoters via the transcription factor E2F (Phelps et al., 1991; Chellappan et al., 1992).

Our understanding of HPV-mediated cancer development has been hindered by the inability to develop a representative animal model using HPV. This has led to increasing interest in the use of the cottontail rabbit papillomavirus (CRPV), which induces papillomas and carcinomas in rabbits (Brandsma et al., 1991; Wettstein, 1987), as a model for HPV-induced pathology. Mutational analyses have shown that the transformation function of HPV-16 E7 is also manifested by that of CRPV (Brandsma et al., 1991; Meyers et al., 1992). However, the ability of CRPV E7 to bind pRb has not been directly demonstrated. Given the sequence divergence between the HPV-16 and CRPV E7 proteins (34% identity, see Fig. 1), it is not a foregone conclusion that CRPV E7 protein binds to pRb. In this study we
pRb60, containing amino acid residues 387 to 928 of a conserved region identified in the E7, E1A and large T antigens. The pRb or pRb60 complexes were immunoprecipitated (90 min) with a purified truncated form of recombinant pRb, which had been precleared with a non-specific mouse antibody. These experiments included a CRPV E7 mutant containing a single amino acid substitution within the conserved region identified in the E7, E1A and large T antigens as being involved in pRb binding and cellular transformation (Fig. 1). This region contains an LXCXE motif (where X is any amino acid; Dyson et al., 1989a), which is mutated in this study. Since mutation of the cysteine in this motif (amino acid 24) impairs binding to pRb (Barbosa et al., 1990; Munger et al., 1989b), we changed the analogues cysteine in CRPV E7 (amino acid 24) to an alanine to generate CRPV E7/C24A. To generate a mutant E7 containing the wild-type or mutant E7 open reading frames were used for in vitro translation followed by in vitro translation in rabbit reticulocyte lysates in the presence of [35S]methionine (Amersham) as described by the manufacturer (Promega). The E7 protein-containing lysates were precleared by incubation at 4 °C with 50 μl of each of the anti-pRb monoclonal antibodies (MAbs) XZ104 and XZ133 (generous gift from Dr Ed Harlow, Massachusetts General Hospital, Charleston, Mass., U.S.A.) followed by rabbit anti-mouse IgG (Cappel) and Zysorbin (Zymed). Binding reactions and immunoprecipitations were performed at 4 °C as described by Dyson et al. (1989a). Briefly, equivalent amounts of the precleared lysates were incubated (90 min) with either unlabelled extracts (Dyson et al., 1989a) from human T24 bladder carcinoma cells containing normal pRb, or with a purified truncated form of recombinant pRb, pRb60, containing amino acid residues 387 to 928 of human pRb (Edwards et al., 1992). After the binding reactions had been precleared with a non-specific mouse MAb, the pRb or pRb60 complexes were immunoprecipitated with anti-pRb MAb XZ104 and XZ133 (30 min) followed by rabbit anti-mouse IgG (30 min) and Protein A-Sepharose beads (Pharmacia). The beads were washed three times in cold LB buffer (Dyson et al., 1989a). Bound proteins were eluted in sample buffer (Dyson et al., 1989a), resolved on an 8 to 16% SDS–polyacrylamide gel (Novex) and detected by fluorography. In parallel experiments, the pRb60 protein was omitted to ensure that any precipitation of radiolabelled E7 protein was due to binding to pRb and not to any cross-reactivity with the anti-pRb antibodies used.

The results of the co-immunoprecipitation experiment are shown in Fig. 2. In (a) the anti-pRb antibodies coprecipitated the radiolabelled HPV-16 E7 in the presence of purified pRb60 (lane 2) or in the presence of T24 cell lysate containing full-length pRb (lane 3). HPV-16 E7 was not precipitated if pRb was omitted from the experiment (lane 1) or if a non-specific antibody was used instead of the anti-pRb antibodies for the precipitation step (data not shown). Similarly, the CRPV E7 was specifically coprecipitated (Fig. 2b) by binding to pRb60 (lane 2) or native pRb (lane 3), but not in the absence of pRb (lane 1). Thus, not only does CRPV E7 bind to human pRb, it interacts with the same C-terminal region of the Rb protein (pRb60) as HPV E7. The CRPV E7/C24A mutant (Fig. 2c) showed a reduced binding to both pRb60 (lane 2) and native pRb (lane 3). This demonstrates that the same cysteine residue (position 24) that has been shown to be critical for HPV-16 E7 binding to pRb and for transformation (Barbosa et al., 1990; Chesters et al., 1990; Edmonds & Vousden, 1989; Munger et al., 1989b) is also critical for CRPV E7 binding to human pRb.

We also wished to demonstrate the binding of CRPV E7 to rabbit pRb and to examine the effect of the C24A

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**Fig. 1.** Comparison of the amino acid sequences of the CRPV and HPV E7 proteins. Amino acid positions are indicated above (CRPV) or below (HPV-16) the sequence. Sequence alignment was performed using the Needleman and Wunsch algorithm taken from the Genetics Computer Group software (Devereux et al., 1984). Identical amino acid residues are connected by a bar. The conserved LXCXE motif involved in pRb binding and transformation activities is boxed. The cysteine residue that is mutated in this study is indicated (position 24).

**Fig. 2.** Co-immunoprecipitation of E7 proteins with pRb. Equivalent amounts of E7 proteins synthesized and radiolabelled in vitro were incubated with a source of pRb and immunoprecipitated with anti-pRb MAbs as described in the text. The source of pRb protein was as follows: lanes 1, no pRb; lanes 2, purified recombinant pRb60; lanes 3, native pRb from a T24 cell lysate. The in vitro translation reactions were programmed with RNA encoding HPV-16 E7 (a), CRPV E7 (b) or CRPV E7/C24A (c), or with no exogenous RNA (d). The positions of protein M, standards (Gibco BRL) are shown to the left.
immunoprecipitations as described above. A similar type of experiment demonstrating the binding of an HPV-16 E7 fusion protein to rabbit pRb from reticulocyte lysates has recently been reported (Scheffner et al., 1992). We reasoned that our polyclonal antibodies to the E7-binding domain of human pRb might also recognize rabbit pRb because the pRb amino acid sequence appears to be evolutionarily conserved (greater than 95% identity in the E7-binding domains of murine and human pRb). The E7 proteins for this experiment were synthesized and radiolabelled with [35S]cysteine in rabbit reticulocyte lysates as described above. Lysates containing equivalent amounts of TCA-precipitable counts were brought to equal volumes by adding unprogrammed rabbit reticulocyte lysate to ensure that the same amount of rabbit pRb was available for binding in each case. Samples were incubated for 90 min and precleared with a non-specific rabbit polyclonal antibody. The pRb-containing complexes were immunoprecipitated with either a rabbit polyclonal antiserum raised to human pRb60 or its corresponding preimmune serum. Immune complexes were captured on Protein A–Sepharose beads, washed, eluted and resolved as above.

The results of this experiment are shown in Fig. 3. In (a) the anti-pRb antibody coprecipitated the radiolabelled HPV-16 E7 (lane 1), but the preimmune serum did not (lane 2). Similarly, in (b) the CRPV E7 was coprecipitated by the anti-pRb antibody (lane 1), but not by the preimmune serum (lane 2). In addition, the binding of both HPV-16 and CRPV E7 proteins to rabbit pRb can be competed by a peptide corresponding to amino acids 20 to 29 (peptide 20–29) of HPV-16 E7 (a, b, lanes 3), but not by a control E7 peptide (a, b, lanes 4). Peptide 20–29 has previously been shown to be a potent inhibitor of HPV-16 E7 binding to human pRb (Jones et al., 1990). Thus binding of both E7 proteins to pRb is mediated through a similar, specific protein–protein interaction. In contrast to the wild-type CRPV E7, CRPV E7/C24A (c) shows minimal, if any, binding to rabbit pRb. These results demonstrate that both CRPV and HPV-16 E7 are capable of binding to rabbit pRb from reticulocyte lysates and that the CRPV E7/C24A mutant exhibits severely reduced binding to rabbit pRb, similar to that seen with human pRb (Fig. 2c).

A more quantitative comparison of the E7 proteins was performed by determining their ability to compete with recombinant HPV-16 E7 protein for binding to human pRb60. The competition experiment was performed using a plate binding assay as described (Jones et al., 1992). Purified recombinant HPV-16 E7 protein was bound to the plate and incubated with affinity-purified pRb60 in the presence or absence of various amounts of the E7 competitors. Purified glutathione-S-transferase (GST)–E7 fusion proteins were generated for use as competitors in this assay by subcloning the various E7 genes into the GST fusion vector pGEX-3X (Pharmacia). For this analysis, an HPV-16 E7/C24A mutant analogous to the CRPV E7/C24A mutant was also constructed by changing the cysteine at position 24 of the HPV E7 to an alanine using oligonucleotide-directed mutagenesis. The fusion proteins were expressed in Escherichia coli and purified using glutathione S-Sepharose as described by the manufacturer (Pharmacia). The results of the competition experiment are shown in Fig. 4. The GST–HPV E7 protein was five-fold better as a competitor than the GST–CRPV E7 protein. For both the HPV and CRPV E7 proteins, changing the cysteine at amino acid position 24 to an alanine caused a significant loss of binding activity (approximately five-fold for HPV 100-fold for CRPV). These results are in agreement with those of the immunoprecipitation experiments (Fig. 2 and 3), in which HPV and CRPV E7 proteins bound efficiently to both human and rabbit pRb, but CRPV E7/C24A did not. In addition, the data show that the CRPV E7 protein can completely inhibit pRb60 binding to the immobilized HPV E7 protein. This strongly
suggests that CRPV and HPV 16 E7 proteins interact with the same binding domain(s) in pRb.

Our results demonstrate that the CRPV E7 protein, like that of HPV-16, can bind specifically to human pRb through interactions with the C-terminal 60K region of the protein. In addition, both HPV-16 and CRPV E7 bind to a rabbit protein, presumably rabbit pRb, immunologically related to human pRb. Three additional lines of evidence suggest that the interaction of the HPV and CRPV E7 proteins with human and rabbit pRb has been evolutionarily conserved. First, an amino acid residue involved in HPV E7 binding to pRb is also critical for CRPV E7 binding to both human and rabbit pRb (cysteine at position 24). This residue may be a direct contact site for pRb or may be required for the conformational integrity of the E7 proteins. Second, peptide competition experiments suggest that binding of both E7 proteins to their cognate pRbs is mediated through a similar, specific protein–protein interaction involving the region encompassing the LXCXE motif of E7 (amino acids 20 to 29 of HPV-16 E7 protein). Third, the affinities of the HPV-16 and CRPV E7 proteins for pRb60 are fairly similar (Fig. 4). The slightly reduced binding affinity of CRPV E7 relative to that of HPV-16 E7 may result from our use of human pRb, which is the natural receptor for HPV E7 and not CRPV E7. Other evidence that addresses the relative abilities of HPV-16 and CRPV E7 proteins to bind pRb comes from an analysis of the sedimentation behaviour of the proteins expressed in COS-7 cells on non-denaturing glycerol gradients (Barbosa & Wettstein, 1988; Gage et al., 1990). Differences in the relative amounts of the E7 proteins sedimenting as a slower free form and a faster complexed form has led to the interpretation (Meyers et al., 1992) that CRPV E7 protein may not bind to pRb as strongly as that of HPV-16, in agreement with our data.

The similarity we have observed in our assays between the HPV and CRPV E7 proteins demonstrates that a crucial activity of HPV E7, pRb binding, is also manifested by the CRPV E7 protein. The conservation of the binding activity of the CRPV E7 protein argues for the conservation of functional activity as well. Thus, both of these viral E7 oncoproteins may cause cellular transformation through their ability to alter the function of a critical growth-suppressing protein, pRb. These results further support the use of CRPV as a model for HPV pathogenesis. Continued research with CRPV will be needed to determine the role of binding of the E7 protein to pRb in inducing fibroproliferative disorders and malignancies in vivo.

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References


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