The region of the HPV E7 oncoprotein homologous to adenovirus E1a and SV40 large T antigen contains separate domains for Rb binding and casein kinase II phosphorylation

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Some genital human papillomavirus (HPV) types, such as 16 and 18, are highly associated with malignant cervical tumors while others, such as HPV 6, are only rarely found in these malignancies. The E7 oncoproteins of HPV 6, 16 and 18 each have a 17 amino acid region with striking homology to adenovirus E1a and SV40 LT. E1a, LT and the E7 oncoprotein of HPV16 all bind the cellular Rb protein *in vitro*, and for E1a and LT this region of homology contains sequences essential for interaction with Rb. We have now found that in HPV 16 E7 this region (amino acids 21–37) contains two separate biochemical activities, each of which contributes to E7-mediated transformation. Rb binding was localized to the N terminus of this region, while the C terminus was shown to serve as a substrate for casein kinase (CK) II, which phosphorylated serine-31 and serine-32. Replacement of the two serines by non-phosphorylatable amino acids led to a reduction in transforming activity and abolished phosphorylation but did not affect Rb binding. Rb binding and CK II phosphorylation were also examined for the E7 proteins of HPV 6 and HPV 18. HPV 16 and 18 E7 bound similar amounts of Rb, but HPV 6 E7 consistently bound less. Phosphorylation rates also varied, with HPV 18 E7 being 2-fold faster than HPV 16 E7, which in turn was 2-fold faster than HPV 6 E7. We conclude that Rb binding and phosphorylation of E7 by CKII are independent activities which are required for efficient transformation by E7 and that these activities correlate directly with the relative oncogenic potential of these viruses.

Key words: casein kinase II/HPV E7/Rb binding

Introduction

A sub-set of the >60 human papillomavirus (HPV) genotypes are found in the vast majority of genital infection by HPV, with types 6, 11, 16 and 18 being present most frequently. While the genital HPV types are more closely related to each other than to other HPVs, they appear to differ in their oncogenic potential. HPV 16 and 18 are found in a majority of cervical cancers (zur Hausen and Schneider, 1987), while HPV 6 and 11 are the predominant types in benign condylomas but are isolated only rarely from cervical cancers (zur Hausen, 1989). The association of HPV 16 and

18 with malignant tumors is correlated by their *in vitro* ability to transform established rodent cell lines and to immortalize primary rodent cells and primary human keratinocytes (Bedell *et al.*, 1987; Durst *et al.*, 1987; Matlashewski *et al.*, 1987; Pirisi *et al.*, 1987; Kaur and McDougall, 1988; Phelps *et al.*, 1988; Schlegel *et al.*, 1988; Vousden *et al.*, 1988; Woodworth *et al.*, 1989). In these assays the HPV types associated predominantly with benign lesions (HPV 6 and 11) were inactive or showed only very low activity compared to HPV 16 and 18. The basis for this difference in activity has not been studied.

In cervical cancers, the small region of the HPV genome that contains the E6 and E7 open reading frames (ORFs) is preferentially retained and expressed (Schneider-Gadicke *et al.*, 1986; Smotkin and Wettstein, 1986; Baker *et al.*, 1987), and genetic analysis of HPV 16 has localized its immortalizing and transforming functions to these two ORFs. While both E6 and E7 are required for full activity in some of these assays, E7 seems to account for most of the biological activity (Vousden *et al.*, 1988; Hawley-Nelson *et al.*, 1989; Munger *et al.*, 1989; Vousden and Jat, 1989).

The apparent importance of E7 to the development and maintenance of malignant tumors associated with HPV underscores the potential significance of elucidating its properties. Some insight has been gained through the recognition that HPV 16 E7 and adenovirus E1a share several characteristics. Both encode proteins which have transforming, immortalizing and *trans*-activating activities (Phelps *et al.*, 1988), are phosphorylated (Lucher *et al.*, 1985; Smotkin and Wettstein, 1989), and can form a complex with Rb, the protein product of a cellular tumor suppressor gene (Whyte *et al.*, 1988; Dyson *et al.*, 1989). Phosphorylation is known to be on serine in HPV 16 E7, but the kinase responsible for this modification, location of the affected residue(s) and the biological significance of this event have not been elucidated. The E7 sequences mediating Rb binding and the possible physiological relevance of this interaction are also unknown.

Comparison of the amino acids encoded by E1a, E7 and SV40 LT, a third viral oncogene whose transforming protein binds Rb (DeCaprio *et al.*, 1988) reveals a region of homology (Dyson *et al.*, 1989) (Figure 1). A stretch of 17 residues within this region has been found to be essential for the biological activity of all three proteins (Kalderon and Smith, 1984; Lillie *et al.*, 1986; Moran *et al.*, 1986; Edmonds and Vousden, 1989), and in E1a and LT the region has been shown to play a role in Rb binding (DeCaprio *et al.*, 1988; Moran, 1988). Another feature of this region of homology, which has received less attention, is an acidic stretch of amino acids which contains two serines in HPV 6, 16 and 18, SV40 LT, and one serine in E1a (Figure 2). This sequence has the characteristics of a potential casein kinase (CK) II phosphorylation site, which suggests the region of homology might possess a second biochemical function (Kuenzel *et al.*, 1987).

Fig. 1. Amino acid sequence of HPV 16 E7. Boxed amino acids are those conserved between E7 proteins from different papillomaviruses. The position of mutations used in this study are shown above the altered amino acid, the substituted residue and name of the mutation are indicated. Adenovirus E1a and SV 40 LT homologous sequences are also shown.

AdE1a	<u>121AspLeuThrCysHisGluAlaGlyPheProPro</u> <u>SerAspAspGluAspGlu</u> ₁₃₇
SV40 LT	<u>102AsnLeuPheCysSerGluGluMetPro</u> <u>SerSerAspAspGluAlaThrAla</u> ₁₁₈
HPV18E7	<u>24AspLeuLeuCysHisGluGlnLeu</u> <u>SerAspSerGluGluGluAsnAspGlu</u> ₄₀
HPV 16 E7	<u>21AspLeuTyrCysTyrGluGlnLeuAsn</u> <u>AspSerSerGluGluGluAspGlu</u> ₃₇
HPV 6b E7	<u>22GlyLeuHisCysTyrGluGlnLeuVal</u> <u>AspSerSerGluAspGluValAsp</u> ₃₈

Fig. 2. Sequence homology of peptides containing Rb binding and casein kinase II recognition sites among HPV types 6b, 16 and 18. Serine residues are underlined by thick lines. The acidic residue stretch is underlined by a thin line. The larger characters depict conserved amino acids essential for Rb binding and transforming activity in HPV 16 E7.

In the present study, we have determined that the adjacent serines in the region of homology in HPV 16 E7 are substrates for CKII, while other sequences within the region of homology mediate Rb binding. In addition to localizing these two biochemical functions to distinct sequences within this region, we show that mutations which selectively abolish either of these activities cause a defect in transforming activity. Furthermore, the E7 proteins of HPV 6 and 18 also possess both biochemical activities, but the relative efficiency of Rb binding and CKII phosphorylation is significantly lower in HPV 6 E7 than the E7 of HPV 16 and 18, which suggests that the lower oncogenic activity of HPV 6 E7 may result from additive, quantitative, rather than qualitative, differences.

Results

Serine-31 and serine-32 are phosphorylated by CKII

CKII is a serine-specific kinase, and HPV 16 E7 is known to be phosphorylated at serine residues (Smotkin and Wettstein, 1987). Given the potential CKII site in E7, we examined the possibility that E7 might be phosphorylated by CKII. The enzymatic activity of CKII can be distinguished from that of other kinases by its ability to utilize GTP and ATP with similar efficiency and to be inhibited at low concentrations of heparin (Hathaway and Traugh, 1982). In preliminary experiments, extracts from cervical carcinoma derived cell lines CaSki and HeLa (which expresses HPV 16 and 18 respectively) were found to phosphorylate a

Fig. 3. Phosphorylation of CNBr peptides of TrpE-E7 fusion proteins. Purified fusion proteins were CNBr digested. The resulting peptides were phosphorylated with purified casein kinase II as described in Materials and methods and separated by electrophoresis on a 17–27% SDS–polyacrylamide gel. CKII lane shows proteins in CKII preparation which are phosphorylated in the assay. Casein was used as positive control. Phosphorylated E7 CNBr peptides contain amino acids 12–82.

bacterial fusion protein composed of TrpE and the full-length HPV 16 E7; there was no detectable phosphorylation of TrpE under the same conditions. In addition, this phosphorylation of TrpE-E7 was inhibited by low concentrations of heparin and occurred equally well in the presence of GTP or ATP, suggesting that CKII was responsible for this activity (data not shown).

To establish directly that HPV 16 E7 was a substrate for CKII, the TrpE-E7 fusion protein was first cleaved with cyanogen bromide, which created a peptide composed of E7 amino acids 12–82, and then incubated with purified CKII in the presence of [γ - 32 P]ATP. The wild-type (wt) E7 peptide was efficiently phosphorylated under these conditions, confirming that E7 is a substrate for CKII (Figure 3).

We then examined the contribution of the potential CKII site located in the region of homology with E1a and SV40 LT to this phosphorylation. To study the importance of serine-31, a previously described single amino acid substitution mutant, which encodes arginine-31 (Edmonds and Vousden, 1989), was tested in the same assay and found to be phosphorylated, but much less than the wild-type protein (31 in Figure 3). The contribution of serine-32 was examined by making a substitution mutant that encoded tryptophan-32. As was true of the arginine-31 mutant, this mutant was also less efficiently phosphorylated. Since these results suggested that both serines might be subject to CKII dependent phosphorylation, a double mutant encoding arginine-31/proline-32 was constructed. It was not detectably phosphorylated by the purified CKII (Figure 3), by the HeLa cell extract that phosphorylated the wild-type protein or when cells were metabolically labeled with orthophosphate (data not shown).

Previous observations with CKII sites in other proteins have shown that acidic residues on the carboxy-terminal side of the serine, as found in E7, are absolutely required

Table I. Transformation of NIH3T3 cells^a

Constructs	Transforming activity ^b			
	Exp. 1	Exp. 2	Exp. 3	Mean
pMo (vector only)	0.00	0.00	0.00	0.00
pE7Mo (wt E7)	1.00	1.00	1.00	1.00
p24Gly ^c	0.00	0.00	0.00	0.00
p26Gly ^c	0.00	0.00	0.00	0.00
p31Arg ^c	0.46	0.89	0.66	0.67
p32Trp	0.54	1.02	0.73	0.76
p31/32Arg/Pro	0.03	0.24	0.30	0.19
p35/36Asp/His ^c	0.69	1.68	0.83	1.07

^aAnchorage-independent growth of NIH3T3 cells following transfection with wild-type E7 sequences (pE7Mo), vector sequences only (pMo) or each of the mutant E7 constructs.

^bValues representing transforming activity are normalized for the pE7Mo positive control for each experiment.

^cPreviously published results (Edmonds and Vousden, 1989).

Fig. 4. Immunoblot showing E7 protein in extracts of NIH3T3 cells transfected with a plasmid containing no E7 sequences (pMo) in lane C; pE7 in lane 1; p32Trp in lane 2; and p31/31Asp/Pro in lane 3.

(Kuenzel *et al.*, 1987). A run of three acidic amino acids is sufficient for CKII recognition but is less efficient than a stretch of five acidic residues as found in wild-type E7 (Marin *et al.*, 1986). To examine the influence of the last two acidic of these amino acids in E7, the ability of CKII to phosphorylate a previously described double mutant (encoding aspartate-35/histidine-36; Edmonds and Vousden, 1989) was studied for CKII phosphorylation. This mutant, which interrupted the stretch of five acidic residues by introducing the basic histidine at the fourth position, decreased the extent of phosphorylation, consistent with results for other CKII sites. In contrast to these results, mutations outside the CKII site did not alter the ability of CKII to phosphorylate E7. Data are shown for two single amino acid substitution mutants (glutamic acid-18 to lysine and serine-71 to isoleucine), but similar results were obtained for other point mutants, including glycine-24 and glycine-26.

We conclude that the region in E7 encompassing amino acids 31–37 can function as a substrate for CKII, and both serines (31 and 32) are phosphorylated. Since the double serine mutant was unable to incorporate 32 P in this assay, it is likely that these are the only two residues in E7 phosphorylated by CKII.

Fig. 5. *In vitro* translated Rb and E7 proteins. Lanes 1 and 2 show products of translation in rabbit reticulocyte lysates programmed with Rb RNA (lane 1) or water (lane 2). Lanes 3–10 show products of translations in wheat germ extracts programmed with water (lane 3), pE7 RNA (lane 4), p24Gly RNA (lane 5), p26Gly RNA (lane 6), p31 Arg (lane 7), p32Trp (lane 8), p31/32Arg/Pro (lane 9) and p35/36Asp/His (lane 10).

Mutation of both serines impairs biological activity

Having determined that serines 31 and 32 are phosphorylated by CKII, we examined the possible biological significance of this biochemical activity by testing the mutants in an E7 dependent assay of cellular transformation. Since E7 can induce anchorage-independent growth of NIH3T3 cells and we have previously tested some E7 mutants by this assay, the two newly constructed mutants with changed amino acids at the serine codons were also studied for this biological activity (Table I). The arginine-31 and the aspartate-35/histidine-36 mutants have been tested previously with this assay and found to possess transforming activity close to that of wild-type. The tryptophan-32 mutant described above also retained good biological activity. Therefore, eliminating either of the two serines individually had only a marginal effect on the biological activity of the gene. However, the arginine-31/proline-32 double mutant had a marked reduction in biological activity.

Analysis of E7 protein in NIH3T3 cells by immunoprecipitation followed by Western blotting showed that p32Trp and p31/32Arg/Pro both encoded stable E7 proteins which were present at levels comparable to wild-type E7 (Figure 4). The stability of the arginine-31 and aspartate-35/histidine-36 mutant proteins has been shown previously to be similar to that of wild-type protein (Edmonds and Vousden, 1989). We conclude that the hierarchy of biological activity obtained with mutants involving the serine codons reflects the intrinsic activity of the mutant proteins and correlates directly with their ability to be phosphorylated by CKII.

Rb binding by E7 mutant proteins

Recent studies have shown that two regions of E1a participate in Rb binding, one of which lies within the region of homology with E7 and LT (Whyte *et al.*, 1989) (see Figure 1). To determine whether amino acids in the region of homology mediate E7–Rb complex formation, we carried out *in vitro* Rb binding studies with E7 proteins carrying

Fig 6. Immunoprecipitation of HPV 16 E7/Rb complexes.

(A) Immunoprecipitations of complexes formed between radioactively labeled E7 and Rb proteins using anti-E7 antibody. Proteins were immunoprecipitated from complexes formed between *in vitro* translated water and water (lane 1), E7 and water (lane 2), E7 and Rb (lane 3), p24Gly and Rb (lane 4), p26Gly and Rb (lane 5), p31Arg and Rb (lane 6), p32Trp and Rb (lane 7), p31/32Arg/Pro and Rb (lane 8) and p35/36Asp/His and Rb (lane 9). (B) Immunoprecipitations of complexes formed between radioactively labeled E7 and cold Rb proteins using anti-Rb antibody. Lane 1 shows immunoprecipitation of radioactively labeled Rb protein. Proteins in the other lanes were immunoprecipitated from complexes formed between *in vitro* translations of water and E7 (lane 2), Rb and E7 (lane 3), Rb and p24Gly (lane 4), Rb and p31Arg (lane 5), Rb and p32Trp (lane 6), Rb and p31/32Arg/Pro (lane 7) and Rb and p35/36Asp/His (lane 8).

various mutations in the region of E1a/LT homology. E7 and Rb proteins were synthesized *in vitro*, mixed and immunoprecipitated with antibodies raised against E7 (Smotkin and Wettstein, 1987) or Rb (Whyte *et al.*, 1988).

In vitro translation of the RNA encoded by the Rb cDNA clone did not give rise to full-length Rb protein but rather to a ladder of Rb peptides thought to initiate from internal methionine residues (Figure 5) as previously observed (Whyte *et al.*, 1988; Dyson *et al.*, 1989). Interestingly, the E7 proteins migrated as 20 kD in size, which is the same migration rate as E7 isolated from cells. As noted previously (Smotkin and Wettstein, 1987), this is significantly slower than the 11 kD predicted from the amino acid sequence. While the basis of this phenomenon is not understood, our results make it extremely unlikely that the slower migration rate might be secondary to phosphorylation, since p31/32Arg/Pro is not phosphorylated but migrates at the same rate as wild-type E7. Furthermore, when E7, immunoprecipitated from CaSKi cells, was treated with either potato

Fig. 7. Immunoprecipitation of complexes formed between Rb and HPV 6b, 16 and 18 TrpE–E7 fusion proteins. Equal amounts of fusion protein were phosphorylated *in vitro* with purified CKII. These proteins were then allowed to associate with *in vitro* translated Rb and the complexes immunoprecipitated with monoclonal antibody against the TrpE protein. Proteins were resolved by electrophoresis on a 10% SDS–polyacrylamide gel.

acid or alkaline phosphatase, no change in mobility was seen (data not shown).

After radioactive Rb and E7 proteins were mixed and immunoprecipitated with E7 antibodies, the E7 proteins carrying mutations altering the cysteine at position 24 (p24Gly) or the glutamic acid at position 26 (p26Gly) were found to be severely impaired in their ability to complex with Rb, although levels of E7 protein comparable to wild-type E7 were used (Figure 6A). Similar mutations in E1a and LT have also been found to destroy the ability of these proteins to bind Rb (Moran, 1988; DeCaprio *et al.*, 1988). By contrast, none of the mutations in the CKII recognition sequence (p31Arg, p32Trp, p31/32Arg/Pro and p35/36Asp/His) seriously affected association with Rb. Deletions of the analogous sequences of E1a have been reported to retain Rb binding by that protein (Whyte *et al.*, 1989). The converse immunoprecipitation is shown in Figure 6(B), in which a complex between unlabeled Rb and radioactively labeled E7 has been analyzed using an anti-Rb antibody. As in the results shown Figure 6(A), the mutation in p24Gly abolishes binding, whereas mutations in the putative CKII recognition sequence have no effect on E7/Rb complex formation. These results indicate that the CKII site is not essential for Rb binding and that this binding can occur in the absence of phosphorylation of the protein. To test the possibility that phosphorylation might be able to modulate the efficiency of Rb binding, the wild-type and p31/32Arg/Pro TrpE–E7 fusion proteins were treated with CKII to be certain the wild-type protein was phosphorylated. No difference in the efficiency of Rb binding was detected (Figure 7).

The above results are therefore entirely analogous to those obtained for E1a mutants. They suggest that, as is true of E1a, Rb binding to E7 is mediated by residues in the region of homology that lies upstream from amino acid 31 (which corresponds to amino acid 131 in E1a). Since the mutations which abolish Rb binding (p24Gly and p26Gly) are also negative for transformation (and *trans*-activation) (Edmonds and Vousden, 1989), the results suggest that the ability to

Fig. 8. Phosphorylation of TrpE–E7 fusion proteins from HPV 6b, 16 and 18 by CKII. Five hundred nanograms of each of the fusion proteins were used in the kinase reactions as described in Materials and methods. (A) Coomassie stain of proteins used in kinase reactions; lane MWM has mol. wt standards of sizes, from top, 94, 67, 43 and 30 kd. (B) Autoradiogram of gel in (A).

Fig. 9. Rate of *in vitro* phosphorylation of HPV 6b, 16 and 18 E7 TrpE fusion proteins by CKII. Aliquots of kinase reactions described in Materials and methods were taken at indicated times. ^{32}P incorporation into the TrpE–E7 proteins was determined by counting the bands separated by SDS–PAGE. The slopes are indicated below each line. Under the described conditions the rates of phosphorylation were consistently linear within the first 20 min of the reaction.

bind Rb is important for biological activity. However, the data from the double serine mutant (p31/32Arg/Pro), which shows wild-type levels of binding to Rb but demonstrates reduced transforming activity, suggests that Rb binding, as measured in this *in vitro* assay, is not sufficient for full biological activity.

Rb binding and CKII activity of HPV 6 and 18

Despite their differences in biological activity, the E7 proteins of HPV 6 and 18 also contain a region of E1a/LT

homology similar to HPV 16 E7 (Figure 2). We therefore decided to compare their relative ability to bind Rb and be phosphorylated by CKII. When their ability to complex with *in vitro* translated Rb was studied, using TrpE–E7 fusion proteins that had been pre-treated with CKII, the HPV 16 and 18 proteins bound equal amounts of Rb, whereas the HPV 6b protein bound significantly less (Figure 7). Bradford assays and Coomassie-stained gels were used to ensure that the same amount of protein was used in each assay. Furthermore, the same monoclonal antibody (mouse anti-TrpE) was used to immunoprecipitate each of the complexes. Therefore, differences in binding are unlikely to be due to differences in antibody binding to the individual E7 proteins. Experimental variation in Rb binding to the same fusion protein in seven separate reactions was consistently far less than the differences observed between HPV 16 and 18 compared to 6b E7 fusion proteins (3.6- to 6.5-fold less Rb bound).

As already noted for the HPV 16 TrpE–E7 fusion protein, extracts of CaSki and HeLa cell had a strong E7-specific kinase activity for analogous HPV 6 and 18 TrpE–E7 fusion proteins; their relative efficiency was similar in the presence of ATP or GTP and was inhibited by low concentrations of heparin. Positive results were also obtained with extracts from normal keratinocytes or HPV negative tumor cell lines (data not shown). The HPV 6 and 18 fusion proteins could also serve as a substrate for purified CK-II when incubated with partially purified enzyme in the presence of [γ - 32 P]-ATP (Figure 8B). The specificity of the reaction was demonstrated by the failure of TrpE alone or a TrpE–E6 fusion protein to be phosphorylated under these conditions (data shown only for TrpE).

While Figure 8(B) shows that E7 from HPV 6, 16 and 18 can be phosphorylated by CKII, the incorporation of 32 P varied among the different E7 proteins, with HPV 18 being most highly phosphorylated and HPV 6 the least. These quantitative differences occurred although the levels of protein used in each kinase assay was identical (Figure 8A); they did not result from differences in the level of CKII activity, since the β subunit of CK-II was phosphorylated equally well in each of the reactions (Figure 8B). Furthermore, kinase reactions carried out in the presence of both TrpE–6bE7 and TrpE–16E7 or TrpE–18E7 still showed the same variation in phosphorylation, which ruled out the possibility that differences might have resulted from hypothetical contaminating inhibitors of kinase activity (data not shown).

The genetic basis for the observed differences in level of phosphorylation is obscure, since all three E7 proteins have two serines within the acidic residue stretch. We therefore investigated whether the differences were due to variation in total phosphorylatable amino acids or the rate of incorporation of 32 P into E7 by casein kinase II. A time curve analysis was done for each E7 polypeptide (Figure 9). It confirmed that the rates of phosphate incorporation differed, with that of TrpE–18E7 being 2-fold faster than TrpE–16E7, which was in turn 2-fold faster than TrpE–6bE7.

We conclude that the E7-containing polypeptides from the different HPV types are CKII substrates and bind Rb, with the TrpE–6E7 peptide being less active for each parameter than TrpE–16E7 or TrpE–18E7.

Discussion

It has recently been shown that the HPV 16 E7 transforming protein is phosphorylated on serine residues (Smotkin and Wettstein, 1987) and can bind the cellular Rb protein *in vitro* (Dyson *et al.*, 1989). The Rb gene was first identified as the locus deletion in retinoblastomas, where patients with a heritable disposition to the tumor were constitutively hemizygous for the deletion. Subsequently a deletion or alteration of expression of Rb has been implicated in a number of other tumors (Marx, 1988). This has led to the hypothesis that loss of the Rb protein may release the cell from normal growth control. The viral transforming proteins which bind Rb may therefore exert their effect by inactivating functional Rb.

The possibility that E7 might bind Rb was based upon the earlier findings that E1a and LT possessed this property, all three proteins have a stretch of ~20 homologous residues, and amino acids within this region of homology have been shown to mediate Rb binding by E1a. Our mutagenic analysis of the E1a/LT homology region in HPV 16 E7 has now documented that Rb binding is mediated by sequences in this region. The two point mutants we have found to be severely impaired in Rb binding (p24Gly and p26Gly) have been shown previously to be unable to transform NIH 3T3 cells, in contrast to wild-type E7 (Edmonds and Vousden, 1989). These results strongly suggest that the ability to bind Rb contributes significantly to the biological activity of E7.

Our experimental results have also elucidated the basis for the serine phosphorylation of HPV 16 E7. Using cell extracts and purified CKII, we have found that E7 is a substrate for phosphorylation by this serine specific kinase. Casein kinase II has been implicated in the regulation of RNA and protein synthesis as well as DNA metabolism by phosphorylating the enzymes and proteins mediating those processes (Sommercorn and Krebs, 1987). The recent finding that CKII mediates phosphorylation of *c-myc*-encoded proteins suggests that it may be involved in cell cycle regulation (Luscher *et al.*, 1989), although the biological significance of this activity has not been tested. Analysis of HPV 16 E7 mutants indicated that *in vivo* and *in vitro* phosphorylation by CKII is a second activity mediated by the E1a/LT region of homology, with serine-31 and serine-32 both capable of being phosphorylated in this process. It is likely that the homologous sequences in E1a and LT are also functional CKII sites. Our finding that mutation of either serine-31 alone or serine-32 alone results in a >2-fold reduction in the efficiency of CKII phosphorylation suggests that the two adjacent serines in HPV 16 E7 facilitate phosphorylation. However, it has not yet been determined for the wild-type protein whether both serines are phosphorylated on the same molecule, which makes it premature to speculate regarding the basis of this facilitation.

Our biological results with the double mutant that substitutes a non-phosphorylatable amino acid for each serine suggests that phosphorylation, like the ability to bind Rb, contributes to the biological activity of HPV 16 E7. An analogous double mutation within the LT had only a limited effect on its transforming activity (Schneider and Fanning, 1988). It may be that E7, which is a much smaller protein than LT and probably lacks some of the biological and

biochemical properties of LT, such as p53 binding, possesses more limited transforming potential than the SV40 protein. Our finding that Rb binding is not sufficient for efficient transformation is further supported by the observation that a mutation changing histidine-2 into proline-2 results in even lower activity than that of aspartate-31/proline-32 even though the mutant protein retained wild-type Rb binding (C. Edmonds and K.H. Vousden, unpublished observation).

Since we have identified mutants that retain wild-type levels of one biochemical activity despite severe impairment of the other, it is likely that distinct sequences within the E1a/LT homology region mediate the two activities, with N-terminal residues governing Rb binding and C-terminal ones responsible for CKII phosphorylation. The conclusion that the region of homology is composed of at least two distinct domains is also consistent with the sequences reported to mediate Rb binding by E1a and the sequence requirements for CKII-mediated phosphorylation in other proteins. Our biochemical evidence also supports this hypothesis, since we have no evidence that either activity influences the other, despite the close proximity of these two sites within E7, E1a and LT. Assuming that our *in vitro* assays reflect *in vivo* events, the apparent independence of RB binding and phosphorylation could be interpreted in at least two ways. It could indicate that, in addition to Rb binding, the phosphorylation dependent interaction with a second factor is required for E7-mediated transformation. Alternatively, it could suggest that a single factor, distinct from Rb, is the effector of E7 transformation and that its interaction with the Rb binding site is modulated by CKII phosphorylation. One potential effector is the 107 kd cellular protein which appears to associate with E1a via the sequences that mediate Rb binding.

Our examination of E7 proteins from three genital HPV types found a correlation between the oncogenic potential of the virus and both the rate of phosphorylation and Rb binding. The E7 proteins of all three viruses possess both activities, but differ with respect to their relative efficiency. E7 from HPV 18, the virus believed to have the highest oncogenic potential among the three (Kurman *et al.*, 1988; Schlegel *et al.*, 1988), is phosphorylated more rapidly than E7 from HPV 16, another papillomavirus with high oncogenic potential. In comparison, E7 from HPV 6, an HPV mostly associated with benign lesions, showed the slowest rate of phosphate incorporation. We presume that sequence divergence within their CKII recognition sequences (Figure 2) accounts for the differences in efficiency, although our inspection of these sequences has not immediately suggested which specific amino acid differences are most critical.

The E7 from oncogenic HPV types 16 and 18 were also found to bind more Rb than the E7 from HPV 6. The finding that Rb binding and CKII phosphorylation correlate with the relative oncogenic potential of the different viruses is consistent with previous conclusions that E7 is the major transforming protein of the genital HPVs. It also suggests that a combination of quantitative biochemical differences, rather than a complete lack of these properties, may account at least in part for the lower oncogenic activity of HPV 6. They may also contribute to the apparently qualitative differences between HPV 6 and 16/18 in certain *in vitro* assays, such as co-operation with *ras* to transform primary

rodent cells, which for HPV 16 has been shown to reside in E7.

Materials and methods

Generation of mutations in E7

Mutations in HPV 16 E7 sequences were generated as previously described (Edmonds and Vousden, 1989) using the uracil incorporation method of site-directed mutagenesis (Kunkel, 1985). Mutations were introduced into the construct pE7Mo which contained sequences cloned into pUC19 under the transcriptional control of the Moloney murine leukemia virus long terminal repeat. All the mutations generated were confirmed by sequence analysis and named according to the number of the residue altered and the amino acid which was substituted.

Cell culture and transformation assays

NIH3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% donor calf serum. Transfections were carried out as previously described (Edmonds and Vousden, 1989). Cells were seeded at 2.5×10^5 /60 mm dish 24 h before transfection. Two micrograms of the plasmid containing E7 sequences was co-transfected with 0.2 μ g pSV2Neo by calcium phosphate precipitation for 6 h. The cells from each transfection were then split into two 100 mm dishes 24 h later and selective medium containing 500 μ g/ml G418 added 24 h after this. After selecting for ~10 days, drug-resistant colonies were pooled and seeded into 0.4% agar at a density of 10^5 /60 mm dish. Each experiment was carried out in triplicate, and the dishes were scored for colony formation after 3 weeks.

Immunoblotting

The stability of mutant and wild-type E7 protein in transfected cells was compared by the immunoblotting procedure previously described (Crook *et al.*, 1989). Dishes (100 mm) of confluent cells were washed, then lysed in 200 μ l of E7 extraction buffer (250 mM NaCl, 0.1% Nonidet P-40, HEPES, pH 7.0, 1% aprotinin). The cells were left on ice for 30 min, then scraped and spun to remove debris. The E7 protein was precipitated using a polyclonal serum specific to E7 (Smotkin and Wettstein, 1987) on ice for 2 h. The proteins were then precipitated with protein A–Sephacrose beads, released by boiling and separated on a 15% polyacrylamide–SDS gel. The proteins were then electrophoretically transferred to nitrocellulose and E7 visualized with a mouse monoclonal antibody specific for HPV 16 E7 (Oltersdorf *et al.*, 1987) as described by Storey *et al.* (1988).

In vitro translation and association of E7 with Rb

E7 sequences were cloned into sp65 and transcribed *in vitro* using SP6 polymerase and translated in wheat germ extract including [35 S]cysteine. A cDNA Rb clone (kindly provided by J. Horowitz) was transcribed using T7 polymerase and translated in rabbit reticulocyte lysate, labeling with [35 S]methionine when necessary. The products of the *in vitro* translations were allowed to associate for 2 h on ice in ELB buffer (250 mM NaCl, 0.1% Nonidet P-40, 50 mM HEPES, pH 7.0, 0.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA). The complexes were then immunoprecipitated with either a polyclonal anti-E7 antibody (Smotkin and Wettstein, 1987) or a monoclonal anti-Rb antibody C36 (kindly provided by E. Harlow; Whyte *et al.*, 1988) by binding to protein A–Sephacrose beads. The pellet was washed five times in ELB, the proteins were dissociated by boiling in loading buffer and electrophoresed on 7.5–17.5% gradient polyacrylamide–SDS gels. The bands were visualized by autoradiography.

Fusion protein: construction, expression and purification

The fusion protein vectors for HPV 16 E7 wild-type and mutants were constructed by inserting the *NsiI*–*TaqI* fragment, nt 566 of HPV 16 to 430 of puc 19 (encoding amino acids 2–97), from each of the LTR activated E7 expression vectors into *PstI*–*Clal* sites of trpE vector pATH 11 (kindly provided by M. Crivallone and A. Tzagaloff). HPV 6b *NsiI* fragment, nt 534–1644 (encoding amino acids 2–98), was inserted at the *PstI* site of pATH 11 to give the trpE–6bE7 fusion vector. TrpE–18E7 fusion protein, encoding amino acids 1–105, has been described (Barbosa *et al.*, 1989). The fusion proteins were expressed and salt extracted as described by Kleid *et al.* (1981). Proteins were then separated on 10% SDS–polyacrylamide gels and transferred to Immobilon membranes by a standard Western procedure. Pure proteins were eluted as previously described (Szewczyk and Summers, 1988). Briefly, bands were cut out of the transfer membrane and proteins eluted with 50 mM Tris, pH 9, 1% Triton X-100, 2 mM DTT and 0.1 mM ZnCl₂ for 20 min at room temperature. Protein concentrations

were determined by the Bradford (BioRad) method and purity checked on Coomassie-stained SDS-polyacrylamide gels.

In vitro phosphorylation of E7 fusion proteins by casein kinase II

Casein kinase II reactions were carried out in a final volume of 30 μ l containing 500 ng of fusion protein in 20 mM HEPES, pH 7.5, 20 mM MgCl_2 , 56 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (~3000 Ci/mmol). Reactions were started with 5 μ l of 0.1 pmol/min/ μ l of partially purified CKII (kindly provided by M.Cobb) diluted in 80 mM β -glycerophosphate, 20 mM EGTA, 15 mM MgCl_2 pH 7.5. After incubations at room temperature for 15–30 min, reactions were stopped by the addition of an equal volume of 2 $\times$ sample buffer (0.25 M Tris, pH 6.8, 20% glycerol, 0.003% bromophenol blue, 6% SDS) and proteins analyzed by SDS-PAGE. Cervical carcinoma-derived cell extracts were tested for E7-specific phosphorylation activity by the method described by Sommercorn et al. (1987). Sensitivity of kinase activity to heparin, and its ability to use GTP was also tested as described by Sommercorn and Krebs (1987).

Analysis of complex formation between Rb and different HPV E7 proteins

One microgram of fusion protein was phosphorylated as described above, in a total volume of 100 μ l. Then 5 μ l of *in vitro* translated Rb was added and the mixture incubated at 0°C for 1 h. Four hundred microliters of NET-N (described by DeCaprio et al., 1988; 20 mM Tris, pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) containing 1 μ g of mouse monoclonal antibody directed against the TrpE protein (Oncogene Science) was added and the solution incubated at 0°C for another hour. To the immunocomplex mixture was then added 20 μ l of a 1:1 suspension of protein A-Sepharose in NET-N buffer and incubated overnight in the cold room on vertical rotator. Complexes were then washed four times in NET-N, proteins released in sample buffer and separated by SDS-PAGE.

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