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Targeted degradation of the retinoblastoma protein by human papillomavirus E7-E6 fusion proteins

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The E6 and the E7 proteins of the oncogenic human papillomavirus types 16 and 18 can stably associate with p53 and the retinoblastoma protein, respectively. The E6-p53 interaction results in the accelerated degradation of p53 *in vitro* via the ubiquitin-dependent proteolysis system. In this study we demonstrate that a fusion protein consisting of the N-terminal half of the HPV-16 E7 protein and the full length HPV-16 E6 protein promotes the *in vitro* degradation of the retinoblastoma protein. This indicates that the property of the HPV-16 E6 protein to stimulate the degradation of p53 can be targeted to other proteins. Unlike the HPV-16 or HPV-18 E6 protein, the E6 proteins of HPV-6 and 11 do not bind to p53 and consequently do not target p53 for degradation. Analogous E7-E6 fusion proteins using the E6 proteins of HPV-6 and HPV-11, however, also have the ability to promote the degradation of the retinoblastoma protein, indicating that the property to target associated proteins for degradation is shared by the anogenital specific HPV E6 proteins.

Key words: human papillomaviruses/HPV E6/HPV E7/ retinoblastoma protein/ubiquitin

Introduction

The human papillomaviruses (HPVs) are etiologically linked to certain anogenital carcinomas, particularly cancer of the uterine cervix (zur Hausen, 1989). About 85% of human cervical carcinomas harbor HPV-DNA sequences (Riou *et al.*, 1990) and the viral E6 and E7 genes are regularly expressed in these cancers (Schwarz *et al.*, 1985; Yee *et al.*, 1985; Smotkin and Wettstein, 1986; Baker *et al.*, 1987). Only a subset of the >60 HPV types such as HPV-16 and HPV-18 are associated with anogenital carcinomas (DeVilliers, 1989) and these specific types are considered 'high risk' viruses. Another distinct group of HPVs which includes HPV-6 and HPV-11 is associated with benign anogenital lesions, such as condyloma accuminata, with little risk for malignant progression and these viruses are considered 'low risk' viruses.

The difference between the high risk and low risk groups of HPVs is also manifested in *in vitro* transformation systems. Transfection of cloned HPV-16 or HPV-18 DNA leads to transformation of rodent cells (Yasumoto *et al.*, 1986; Bedell *et al.*, 1987; Kanda *et al.*, 1987) and to immortalization of primary human cells (Dürst *et al.*, 1987; Pirisi *et al.*, 1987; Schlegel *et al.*, 1988), further supporting a causative role of these specific HPVs in carcinogenesis. In contrast, cloned DNAs of the low risk HPVs, such as HPV-6 or HPV-11, are either negative or only weakly transforming in the same assays. Genetic studies have revealed that the E6 and E7 genes encode the oncoproteins of the high risk HPVs. E7 alone is sufficient for transformation of established rodent cell lines (Kanda *et al.*, 1988; Phelps *et al.*, 1988; Vousden *et al.*, 1988; Bedell *et al.*, 1989; Tanaka *et al.*, 1989), and can transform primary rat kidney cells in cooperation with an activated *ras* gene (Phelps *et al.*, 1988; Storey *et al.*, 1988). Both E7 and E6 are necessary and sufficient for the efficient immortalization of the natural host cells of the HPVs, human squamous epithelial cells (Hawley-Nelson *et al.*, 1989; Münger *et al.*, 1989a; Watanabe *et al.*, 1989).

The E6 proteins of papillomaviruses are ~150 amino acids in length and contain four CXXC-motifs which may be involved in the zinc binding property of the proteins (Barbosa *et al.*, 1989; Grossman and Laimins, 1989). Thus far, little is known about the biochemical activities of the E6 proteins. The E6 proteins of HPV-18 and of bovine papillomavirus type 1 (BPV-1) have been detected in the nuclear matrix and non-nuclear membranes (Androphy *et al.*, 1987; Grossman *et al.*, 1989). BPV-1 E6, HPV-16 E6 and HPV-18 E6 have been reported to have transcriptional transactivation properties (Gius *et al.*, 1989; Lamberti *et al.*, 1990; Sedman *et al.*, 1991). It has been shown that the E6 proteins of the high risk HPVs are capable of binding to the tumor suppressor protein p53, whereas complex formation of p53 with the low risk HPV E6 proteins was not detected (Werness *et al.*, 1990). Complex formation of the high risk E6 proteins with p53 involves an additional cellular protein, designated as E6-AP (Huibregtse *et al.*, 1991).

The E7 proteins of the HPVs associated with anogenital lesions are acidic nuclear phosphoproteins of ~100 amino acids in length. The N-terminal 38 amino acids of the E7 proteins are strikingly similar to portions of conserved regions 1 and 2 of the adenovirus E1A proteins and to the homologous parts of SV40 large T antigen (Figge *et al.*, 1988; Phelps *et al.*, 1988). These regions of these oncoproteins are involved in binding of cellular proteins including the retinoblastoma gene product pRB and p107 (DeCaprio *et al.*, 1988; Whyte *et al.*, 1988, 1989; Dyson *et al.*, 1989a,b; Ewen *et al.*, 1989; Münger *et al.*, 1989b). The E7 proteins of the high risk HPVs have a greater transformation potential than the E7 proteins of the low risk HPVs, correlating with the higher binding affinity of the high risk E7 proteins for pRB (Münger *et al.*, 1989b, 1991; Gage *et al.*, 1990; Barbosa *et al.*, 1991).

Complex formation with products of tumor suppressor genes seems to be a common feature of oncogenes encoded by DNA tumor viruses. As indicated above, SV40 large T antigen, the adenovirus E1A proteins, and the HPV E7 proteins are capable of binding to pRB. The high risk HPV

E6 proteins (Werness *et al.*, 1990) as well as SV40 large T antigen (Lane and Crawford, 1979; Linzer and Levine, 1979) and the adenovirus 5 E1B 55 kDa protein (Sarnow *et al.*, 1982) associate with p53. It is thought that the complex formation with pRB and p53 prevents these proteins from performing their normal cellular function, and therefore accounts at least in part for the transforming properties of the various viral oncoproteins. In the case of p53, however, the consequence of these interactions seems to be different among the different oncoproteins. In SV40 or adenovirus 5 transformed cells, the steady state levels and the half life of p53 are increased (Oren *et al.*, 1981; Reich *et al.*, 1983) as a consequence of the interaction with large T antigen and E1B, respectively. However, in many HPV positive cervical carcinoma cell lines or in HPV immortalized cell lines the levels of p53 are generally quite low, in most cases even lower than in the untransformed parental cells (Scheffner *et al.*, 1991; Wrede *et al.*, 1991).

The binding of HPV-16 or HPV-18 E6 proteins stimulates the degradation of p53 *in vitro* via the ubiquitin-dependent proteolysis system (Scheffner *et al.*, 1990). This activity may account for the low level of p53 in HPV immortalized cells and in HPV positive cervical carcinoma cells (Scheffner *et al.*, 1991). The E6 promoted degradation was specific for p53 in that other proteins such as SV40 large T antigen, pRB, or the proteins of the brome mosaic virus, which do not associate with E6, were not degraded in the presence of E6. Furthermore, the low risk HPV E6 proteins, which do not detectably bind to p53, did not promote the degradation of p53. These findings raised several questions, including whether the high risk E6 proteins could target proteins other than p53 for degradation, and whether the low risk HPV E6 proteins also have the potential to facilitate the degradation of proteins with which they can associate. In this study we show that HPV-16 E6 does have the capacity to target other proteins for degradation brought into complex with itself, and that this property to promote the degradation of complexed proteins is shared by the E6 proteins of the low risk HPVs. Furthermore a factor interacting with both low risk and high risk E6 proteins is required for the E6 targeted degradation of associated proteins.

Results

Construction and characterization of HPV-16 E7–E6 fusion proteins

To investigate whether the HPV-16 E6 protein has a general ability to facilitate the degradation of associated proteins other than p53, an E6 fusion protein containing an unrelated protein binding domain was constructed. The N-terminal 50 amino acids of HPV-16 E7, which contain the binding domain for the retinoblastoma protein (Münger *et al.*, 1989b; Barbosa *et al.*, 1990), were fused to the N-terminal end of HPV-16 E6 to generate HPV-16 E7–E6 (Figure 1A). A construct deleted of four amino acids in the pRB binding domain and incapable of binding pRB (16 $E7^{\Delta}$–E6) (Münger *et al.*, 1989b) was similarly generated. The fusion proteins were synthesized in a combined *in vitro* transcription/rabbit reticulocyte lysate translation system and analyzed by SDS–PAGE and fluorography (Figure 1B). As shown previously, the HPV-16 E7 protein migrated with an aberrantly high mol. wt in SDS–PAGE (18 kDa instead of 12 kDa, as predicted) (Gate *et al.*, 1990; Münger *et al.*, 1991). This aberrant migration behavior is also reflected in the HPV-16 E7–E6 fusion proteins which migrate with a mol. wt of ~27 kDa, not at 22 kDa as predicted from its size.

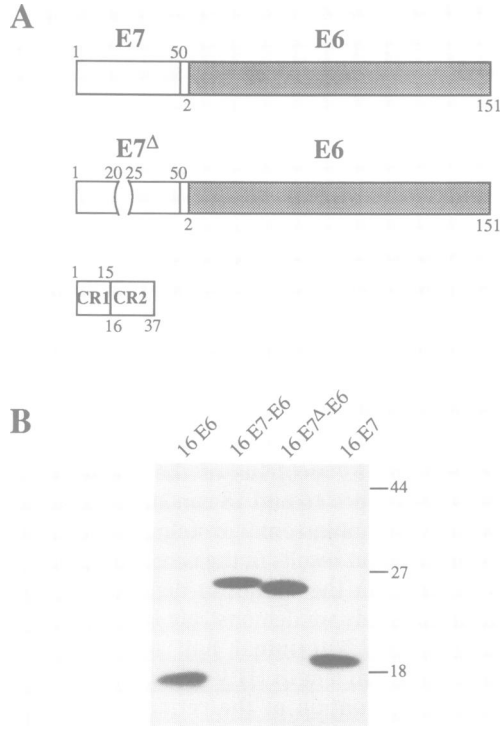

Fig. 1. The structure of the constructed fusion proteins is shown schematically in (**A**). The HPV-16 E7–E6 fusion protein consists of the 50 N-terminal amino acids of HPV-16 E7 linked by two amino acids (G and S) to the full length HPV-16 E6 protein beginning at its second amino acid. The HPV-16 $E7^{\Delta}$–E6 fusion protein is identical to HPV-16 E7–E6, but the E7 domain is deleted of four amino acids (DLYC, amino acids 21–24) in the pRB binding domain. The regions in E7 (CR1 and CR2) highly similar to regions in the adenovirus E1A proteins and SV40 large T antigen are shown below. (**B**) Synthetic RNAs encoding the proteins indicated above were translated in rabbit reticulocyte lysate in the presence of L-[^{35}S]cysteine. The apparent mol. wt of the generated fusion proteins was determined by electrophoresis on a 12.5% SDS–polyacrylamide gel followed by fluorography. The positions of mol. wt markers are indicated on the right.

To determine whether or not the HPV-16 E7–E6 fusion protein was capable of binding to pRB, a co-immunoprecipitation analysis was performed (Figure 2). First, *in vitro* synthesized mRNA encoding pRB was translated in rabbit reticulocyte (RRL) in the presence of ^{35}S-labeled methionine. The translation reaction yielded a variety of products with different mol. wts ranging from <27 kDa up to ~95 kDa (Figure 2, 'pRB'). The product with the highest mol. wt (~105 kDa) is not a pRB-related protein since it could not be precipitated with the monoclonal antibody C36 specific for pRB (data not shown). The pRB translate was then incubated at 4°C with unlabeled HPV-16 E7, E6 or E7–E6 fusion proteins. After 3 h potential pRB–HPV-16 protein complexes were assayed by immunoprecipitation using an HPV-16 E7 specific monoclonal antibody (αE7). As a negative control immunoprecipitations were also done with PAb 419, a monoclonal antibody against SV40 large T antigen. As expected the HPV-16 E7 protein co-

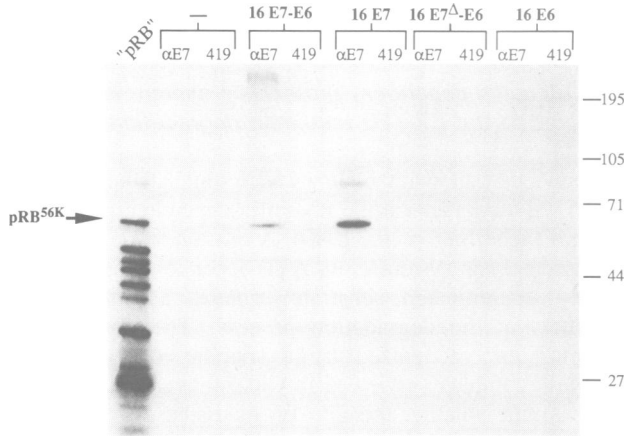

Fig. 2. Co-immunoprecipitation analysis of HPV-16 E7–E6 and pRB. *In vitro* translated, radioactively labeled pRB proteins were mixed at 4°C for 1 h with HPV-16 E6, HPV-16 E7 or the HPV-16 E7–E6 fusion proteins shown in Figure 1. Complexes were immunoprecipitated using an HPV-16 E7 specific monoclonal antibody (αE7) or an unrelated antibody (PAb 419) as a negative control. The original pRB translate is shown in the first lane ('pRB').

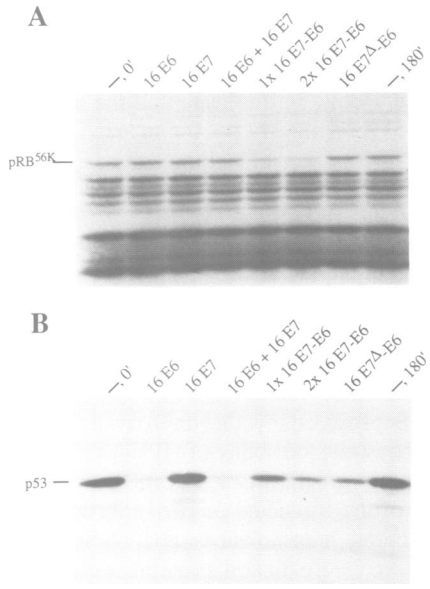

Fig. 3. HPV-16 E7–E6 fusion protein promotes the *in vitro* degradation of pRB. *In vitro* translated, radioactively labeled pRB (**A**) or p53 (**B**) was incubated either in the absence or presence of approximately equimolar amounts of the indicated HPV-16 proteins as described in Materials and methods. After 3 h incubation the total reaction mixtures were separated on 10% SDS–polyacrylamide gels and the proteins visualized by fluorography. The left and right hand lanes contain the *in vitro* translated proteins incubated without any added HPV-16 proteins for either 0 or 180 min, respectively. The positions of p53 or pRB^{56K}, the major RB translation product that binds to HPV-16 E7 or HPV-16 E7–E6, are indicated on the left.

precipitated RB proteins generated from internal initiations which had an apparent mol. wt of 56 kDa or more (Dyson *et al.*, 1989a). The same proteins were also co-precipitated with the HPV-16 E7–E6 fusion protein, demonstrating that the N-terminal 50 amino acid E7 domain of the fusion protein retained its ability to complex with pRB. The specificity of this binding reaction is established by the inability of HPV-16 $E7^{\Delta}$–E6 to co-immunoprecipitate any of these proteins. In addition, ^{35}S-labeled HPV-16 E7, E6 or E7–E6 fusion proteins were incubated with cell extracts prepared from ML-1 cells which contain normal pRB (Hu *et al.*, 1991) and potential pRB–HPV-16 protein complexes assayed by immunoprecipitation using the pRB specific monoclonal antibody C36. These experiments also showed that HPV-16 E7 and the E7–E6 fusion protein were each capable of binding to pRB and that HPV-16 E6 was not (data not shown).

In the presence of the HPV-16 E7–E6 fusion protein high molecular weight (HMW) forms of the radiolabeled pRB proteins were observed which were not detected in the original pRB translate or in the presence of HPV proteins other than the E7–E6 fusion protein. The HMW protein forms probably represent modified, presumably ubiquitinated RB proteins (see Figure 5, below). Quantitation of the autoradiogram shown in Figure 2 by densitometry revealed that the radioactivity contained in the sum of the protein bands coprecipitated with E7–E6 or E7 was about the same. Since equimolar amounts of proteins were used this indicates that both proteins have a similar affinity for pRB.

The HPV-16 E7–E6 fusion protein promotes the degradation of pRB

To determine if, by analogy with the interaction of HPV-16 E6 and p53, the HPV-16 E7–E6 fusion protein could facilitate the degradation of pRB, a radioactively labeled pRB translate was incubated with unlabeled fusion protein at 25°C as described in Materials and methods. After 3 h the total reaction mixtures were processed for SDS–PAGE and the total amount of labeled RB proteins determined. As shown in Figure 3A incubation at 25°C did not affect the stability of the pRB proteins in the absence of HPV proteins or in the presence of either HPV-16 E6, E7 or both proteins together. In contrast, in the presence of the HPV-16 E7–E6 fusion protein a marked decrease of the RB product indicated as pRB^{56K} was observed. A small decrease in the pRB species migrating with a higher mol. wt than pRB^{56K} was also seen whereas those pRB species which migrated faster than pRB^{56K} and did not bind to HPV-16 E7–E6 (see Figure 2) remained stable. The degradation of pRB by the HPV-16 E7–E6 fusion protein required the binding to pRB since the HPV-16 $E7^{\Delta}$–E6 fusion protein which did not bind pRB (Figure 2) did not promote the degradation of pRB. The incubation at 25°C for 3 h had no effect on the stability of E6, E7 or the fusion proteins.

In a parallel experiment the ability of the various E7–E6 constructs to degrade p53 was examined. As expected from our previous study (Scheffner *et al.*, 1990), p53 was efficiently degraded in the presence of HPV-16 E6 (Figure 3B). The HPV-16 E7–E6 and $E7^{\Delta}$–E6 fusion proteins were also capable of promoting the degradation of p53, although with a somewhat decreased efficiency compared with E6. This result indicates that the inability of HPV-16 $E7^{\Delta}$–E6 to degrade pRB is not due to the loss of its degradation property, but rather to its inability to bind to pRB.

Since the plasmid used to generate pRB *in vitro* did not yield any full length pRB, the ability of HPV-16 E7–E6 to degrade full length pRB which has an apparent mol. wt of ~105 kDa was next examined. RRL contains sufficient rabbit pRB to be readily detectable by Western blot analysis using the monoclonal antibody 245 specific for human pRB.

Unprogrammed RRL and E7−E6 mRNA programmed RRLs were mixed together in various ratios, with the total amount of RRL in each reaction mixture held constant. The mixings were done in duplicate and the amount of pRB in the RRL was determined after 0 min and 180 min incubation (Figure 4). The amount of pRB contained in HPV-16 E7−E6 mRNA programmed RRL was dramatically decreased compared with unprogrammed RRL. A time dependence of the decrease was not observed with the HPV-16 E7−E6 fusion protein, indicating that all the pRB that could be recognized by the E7−E6 fusion protein was already degraded during the translation reaction. However, the addition of HPV-16 E7−E6 programmed RRL to unprogrammed RRL resulted in a time-dependent degradation of the pRB provided by the unprogrammed RRL. These results demonstrate the ability of HPV-16 E7−E6 to promote the degradation of the full length pRB synthesized *in vivo* as well as of the truncated *in vitro* translated RB proteins.

As an initial step towards analyzing their properties *in vivo*, the E7−E6 fusion proteins were tested for their ability to co-operate with an activated *ras* oncogene in the transformation of baby rat kidney (BRK) cells (Phelps *et al.*, 1988). The construct containing the N-terminal 50 amino acids of E7 with an intact pRB binding domain was capable of co-operating with *ras* in the transformation of primary BRK cells, whereas the HPV-16 $E7^{\Delta}$−E6 plasmid was not (data not shown). This indicates that the 50 N-terminal amino acids of HPV-16 E7 are sufficient for transformation of BRK cells and confirms previous results showing that the integrity of the pRB binding domain is necessary for the transformation function of E7 (Barbosa *et al.*, 1990; Münger *et al.*, 1991). Although stable transformed cell lines were established, the pRB levels could not be evaluated since an antibody which quantitatively recognizes rat pRB is not yet available.

The pRB translated in RRL was used for the experiments described below, and as shown in Figure 3, pRB^{56K} which is efficiently bound by the E7−E6 fusion protein was the most abundant species targeted for degradation.

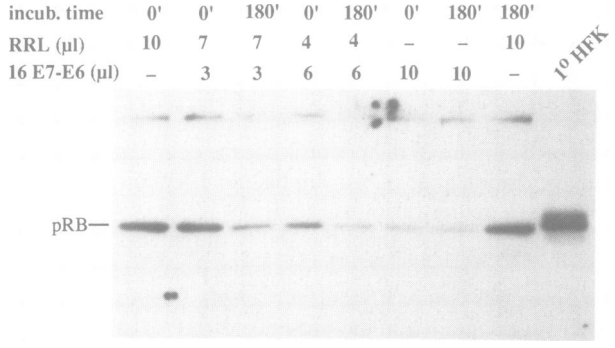

Fig. 4. HPV-16 E7−E6 promotes the degradation of full length *in vivo* synthesized pRB. Western blot analysis was used to determine the levels of pRB present in unprogrammed RRL, HPV-16 E7−E6 RNA programmed RRL, and in mixtures containing unprogrammed and programmed RRL. Following translation at 30°C for 1 h, reactions were incubated for either 0 min or 3 h at 25°C. The level of pRB present in 100 µg of a cell lysate from primary human foreskin keratinocytes (1° HFK) was also examined to determine the running position of hypophosphorylated and hyperphosphorylated forms of pRB. The hypophosphorylated form of pRB migrates faster in SDS−PAGE than the hyperphosphorylated forms (Ludlow *et al.*, 1989). The running position of the rabbit reticulocyte pRB is indicated to the left, and comparison with the running position of the human pRB suggests that pRB in rabbit reticulocytes is predominantly hypophosphorylated.

The ubiquitin-dependent proteolysis system is involved in the E7−E6 mediated degradation of pRB^{56K}

As previously shown, the HPV-16 E6 stimulated degradation of p53 is mediated via the ubiquitin-dependent proteolysis system (Scheffner *et al.*, 1990). A characteristic feature of this proteolysis system is its ATP-dependent formation of highly ubiquitinated substrate proteins as intermediate products in the degradation process. The ubiquitinated proteins are recognized by a specific ATP-dependent protease complex (reviewed in Ciechanover *et al.*, 1990). To examine whether the E7−E6 mediated degradation of pRB^{56K} was ATP dependent, pRB and HPV-16 E7−E6 translation reaction mixtures were passed through G25−Sephadex columns to remove nucleotides. The ATP-depleted translation mixtures were then incubated under standard degradation conditions in the presence or absence of ATP or ATP-γ-S, a non-hydrolyzable ATP analog.

As shown in Figure 5, depletion of ATP inhibited the ability of E7−E6 to promote the degradation of pRB^{56K}. When ATP was added back, pRB^{56K} was efficiently degraded demonstrating the ATP dependence of this degradation process. Upon addition of ATP-γ-S a similar decrease in the level of pRB^{56K} was observed; however, in contrast to the reaction with ATP, higher mol. wt protein forms appeared (marked with a star). These HMW forms correlated well in size with those observed in the co-immunoprecipitation analysis shown in Figure 2. Furthermore, HMW proteins of similar size were detected previously in a similar experiment studying the HPV-18 and HPV-16 E6 stimulated degradation of p53 (Scheffner *et al.*, 1990). In the case of p53 it was demonstrated that these HMW proteins were ubiquitinated forms of p53. Therefore it seems reasonable to conclude that the HMW proteins observed in Figure 5 reflect ubiquitinated pRB molecules.

The degradation property is not restricted to the high risk HPV E6 proteins

In contrast to the high risk HPV E6 proteins, the low risk HPV E6s such as HPV-6 or HPV-11 E6 are not capable of high affinity binding to p53 and consequently do not

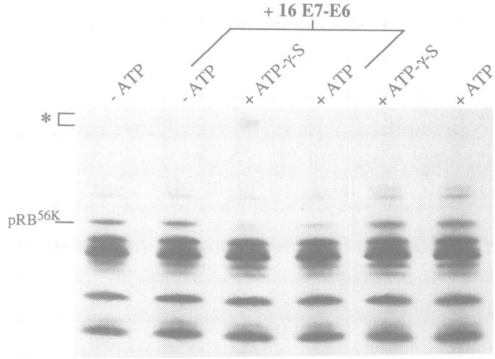

Fig. 5. ATP dependence of the HPV-16 E7−E6 induced degradation of pRB. ATP was removed from pRB and 16 E7−E6 translation mixtures using G25−Sephadex columns. The translation mixtures were then incubated under standard conditions (Materials and methods) in the absence or presence of 2 mM ATP or 2 mM ATP-γ-S. pRB^{56K} and the high mol. wt forms of pRB are indicated to the left.

facilitate the degradation of p53 (Werness *et al.*, 1990; Scheffner *et al.*, 1990). To determine if the low risk HPV E6 proteins share the ability to target associated proteins for degradation, analogous E7–E6 fusion proteins were generated, with the E6 proteins of the low risk viruses HPV-11 and HPV-6. These fusion proteins were incubated with radioactively labeled *in vitro* translated pRB for 3 h at 25°C and the amount of the various RB translation products determined. As shown in Figure 6, the low risk HPV E6 containing fusion proteins also facilitated the degradation of pRB^{56K}. The degradation was ATP dependent and HMW forms of pRB were observed in the presence of ATP-γ-S (data not shown). As with the HPV-16 E6 fusion protein, HPV-16 $E7^{\Delta}$–low risk HPV E6 constructs showed that the intact pRB binding domain was essential for pRB^{56K} degradation (not shown).

To examine if the degradation property was a feature common to the E6 proteins of papillomaviruses other than the anogenital specific HPVs, additional fusion proteins were created utilizing the E6 proteins of HPV-5, BPV-1 and cottontail rabbit papillomavirus (CRPV). The fusion proteins bound to pRB with affinities similar to HPV-16 E7–E6 (not shown). However, as shown in Figure 6 the fusion proteins containing either HPV-5, BPV-1 or CRPV E6 failed to promote the degradation of pRB^{56K}.

A cellular factor interacting with both high risk and low risk HPV E6 proteins is involved in E6 mediated degradation

The finding that the E6 proteins of the anogenital specific HPVs share the ability to target associated proteins for degradation suggested the possible involvement of a cellular factor that interacts with both low risk and high risk HPV E6 proteins in the degradation process. To test this hypothesis the following competition experiments were performed. HPV-16 or HPV-11 E6 proteins were added in excess to standard degradation reaction mixtures and analyzed as to whether or not their addition interfered with the E7–E6 fusion protein mediated degradation of pRB (Figure 7). In the presence of HPV-16 E6, the degradation of pRB^{56K} mediated by fusion proteins containing either high risk or low risk HPV E6 was almost completely inhibited. Since HPV-16 E6 does not bind and therefore does not compete for binding to pRB it was concluded that HPV-16 E6 is competing for a factor involved in degradation of pRB^{56K}. Surprisingly, addition of HPV-11 E6 had little or no effect suggesting that HPV-16 E6 binds more efficiently to this factor than HPV-11 E6 does (Figure 7). However, since the fusion protein containing the HPV-11 E6 protein could target pRB^{56K} for degradation, a less efficient interaction with this cellular factor would appear to be sufficient for the E7–E6 fusion proteins to promote the degradation of pRB^{56K}.

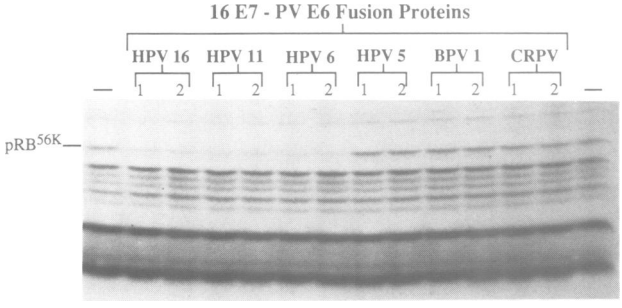

Fig. 6. The E6 proteins of the low risk anogenital specific human papillomaviruses share the ability to target associated proteins for degradation. E7–E6 fusion proteins analogous to the fusion protein shown in Figure 1 were generated with the E6 proteins of HPV-11, HPV-6, HPV-5, BPV-1 or CRPV. Approximately equimolar amounts of these fusion proteins were incubated with radioactively labeled *in vitro* translated pRB and the amount of the various pRB products was determined as described (see Materials and methods).

Discussion

Similar to SV40 large T antigen and the adenovirus 5 E1B 55 kDa protein, the E6 protein of HPV-16 and HPV-18 can bind the p53 tumor suppressor protein (Lane and Crawford, 1979; Linzer and Levine, 1979; Sarnow *et al.*, 1982; Werness *et al.*, 1990). The E6–p53 interaction results in the accelerated degradation of p53 *in vitro* (Scheffner *et al.*, 1990). In this study we have shown that fusion proteins composed of the pRB binding domain of HPV-16 E7 and the full length E6 protein of either the low risk or high risk HPVs, can facilitate the degradation of pRB *in vitro*. This indicates that the ability of the high risk HPV E6 proteins to stimulate the degradation of p53 can be targeted to other proteins, and furthermore that the ability to promote the degradation of associated proteins is a function common to the high risk and low risk anogenital specific HPV E6 proteins.

As has been demonstrated for the E6–p53 interaction, the E7–E6 facilitated degradation of pRB is probably mediated via the ubiquitin-dependent proteolysis system. This conclusion is based on the appearance of high molecular weight forms of pRB when incubated in the presence of HPV-16 E7–E6 and ATP-γ-S. The ubiquitin-dependent proteolysis contains two ATP-dependent processes (for a detailed description, see Ciechanover *et al.*, 1990). First, ubiquitin, a 7 kDa polypeptide, is activated by an E1 enzyme, with hydrolysis of ATP to AMP and PPi. Multiple activated ubiquitin molecules are then transferred and covalently linked to the substrate protein by ubiquitin-conjugating E2 enzymes in an ATP-independent manner. Finally, the highly ubiquitinated protein is recognized and

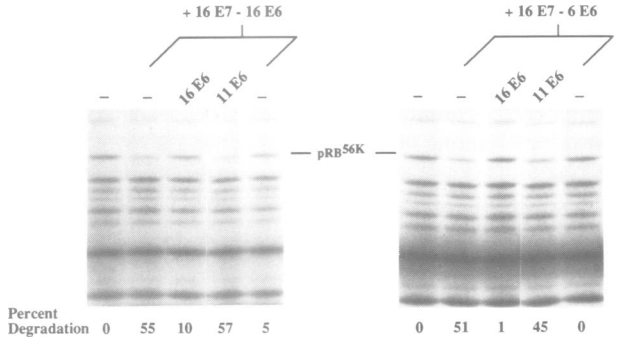

Fig. 7. Addition of HPV-16 E6 inhibits the E7–E6 mediated degradation of pRB. Purified HPV-16 E6 and HPV-11 E6 proteins expressed as glutathione-*S*-transferase fusion proteins in *E.coli* (Huibregtse *et al.*, 1991) were added to reaction mixtures under standard degradation conditions (see legend to Figure 3) in the absence of E7–E6 fusion proteins. After 30 min at 20°C E7–E6 fusion proteins were added as indicated and the mixtures further incubated for 2 h. Analysis of the various pRB products was performed as described (see Materials and methods). Quantitation was done by densitometry of the fluorograph.

degraded by a protease complex with hydrolysis of ATP to ADP and Pi. The differential hydrolysis of ATP in the ATP-dependent steps provides a likely explanation for the appearance of HMW protein forms in the presence of ATP-γ-S. ATP-γ-S, in which the oxygen between the β and γ phosphates is replaced by sulfur, can probably be used as an energy source in the E1 catalyzed activation of ubiquitin. ATP-γ-S thus does not interfere with ubiquitination of substrate proteins. In contrast, ATP-γ-S cannot be used as an energy source for the protease complex since it cannot be hydrolyzed between the β and γ phosphates. Thus, highly ubiquitinated substrates accumulate.

The degradation of both p53 and pRB requires complex formation of E6 with these proteins. Recently it has been reported that the domain of HPV-16 E6 involved in binding to p53 can be separated from the domain required for degradation of p53 (Crook *et al.*, 1991). However, this finding could not be reproduced in our laboratory. Moreover our data indicate that little if any manipulation of the amino acid sequence of HPV-16 E6 can be tolerated without greatly diminishing its ability to bind to p53 and therefore to promote the degradation of p53 (J.M.Huibregtse, M.Scheffner and P.M.Howley, in preparation). However, since binding of the E7–E6 fusion protein to pRB is mediated by the E7 domain and not by E6, this provides a system to identify the regions and the properties of E6 that are required to promote the degradation of p53 or other associated proteins. Using this system we have demonstrated that HPV-16 E6 binds stably to a cellular protein that is required for E6 mediated degradation. Although this factor is also involved in the low risk HPV E6 mediated degradation, a stable interaction with HPV-11 E6 could not be observed. This binding feature is similar to that of E6-AP, which is required for E6–p53 complex formation and binds detectably only to high risk HPV E6 proteins (Huibregtse *et al.*, 1991). However, further experiments will be necessary to determine whether or not the factor required for E6 mediated degradation is indeed E6-AP.

The demonstration that pRB can be targeted for degradation by the E7–E6 fusion protein raises the interesting possibility that E6 might facilitate the degradation of other proteins with which it can associate. E6 fusion proteins might therefore be useful tools in the study of protein–protein interactions *in vitro* and *in vivo*. However, since the mechanism by which E6 targets the degradation of associated proteins is not yet known, it is possible that there may be amino acid sequence or protein structure constraints limiting the recognition of the associated protein for ubiquitination and proteolysis. Furthermore, since our studies to date have used *in vitro* systems to demonstrate targeted degradation of proteins using E6 or fusion proteins containing E6, the general utility of this approach will depend upon the capacity of the E6 proteins to promote the degradation of a target protein *in vivo*.

This study suggests that the E6 proteins of both the high risk and low risk anogenital specific HPVs possess a common property: the ability to facilitate the degradation of cellular proteins with which they associate. Despite significant similarity in structure, this property may not be common to the E6 proteins of other papillomaviruses, at least under the experimental conditions used here. The reason for this difference remains unclear; however, it may prove useful in defining the domains which confer the degradation property of the anogenital HPV E6 proteins. Finally this common property of the anogenital HPV E6 proteins could indicate that the major functional difference between the E6 proteins of low risk and high risk HPVs are the specific cellular proteins they target for degradation. It will therefore be important to define the natural target proteins of the low risk and any additional cellular targets of the high risk HPV E6 proteins.

Materials and methods

Construction of plasmids encoding E7–E6 fusion proteins

To generate the various E7–E6 fusion proteins, the region of the HPV-16 genome encoding the first 50 amino acids of the E7 protein and the E6 genes of the different papillomaviruses (HPV-16, HPV-11, HPV-6b, HPV-5, BPV-1 and CRPV) beginning at codon 2 were amplified by PCR. The plasmids used for PCR have been described elsewhere (HPV types 16, 11, 6 and BPV-1, Werness *et al.*, 1990; HPV-5, Zachow *et al.*, 1987; CRPV, Giri *et al.*, 1985). The 5′ sense oligonucleotide primers used were: HPV-16 E7, GCGTCGACCACCATGCATGGAGATACA; HPV-16 E6, CGCGGATCCTTTCAGGACCCACAGGAG; HPV-11 E6, GGCGGATCCGAAAGTAAAGATGCCTCC; HPV-6b E6, CGCGGATCCGAAAGTGCAAATGCCTCCAC; HPV-5 E6, CGCGGATCCGCTGAGGGAG-CCG AACACCA; BPV-1 E6, CGCGGATCCGACCTGAAACCTTTTG-CAAG; CRPV E6, CGCGGATCCGAGAACTGCCTGCCACGCTC. The 3′ antisense oligonucleotide primers used were: HPV-16 E7, GCGGAT-CCGGCTCTGTCC-GGTTCTGCTTG; HPV-16 E6, CCAAGCTTGGAA-TTC TTACAGCTG GGTTTCTCT; HPV-11 E6, GCAAGCTTAGGGTAA CAAGTCTTC; HPV-6b E6, GCAAGCTTAGGGTAACATGTCT-TCCA; HPV-5 E6, GCAAGCTTGCACGGTGACCTCTTTACCA; BPV-1 E6, GCAAGCTT CTATGGGTATTTGGACCTTGA; CRPV E6, GCAAGCTTAGCCCTGCGCACAGGATAGC.

The HPV-16 E7 PCR product was cut with *Sal*I and *Bam*HI and the E6 PCR products with *Bam*HI and *Hin*dIII. The digested E7 PCR product was then ligated to each of the E6 PCR products and cloned into pGEM1 (Promega) precut with *Sal*I/*Hin*dIII. The E7 and the E6 domains of the fusion gene are linked by the *Bam*HI restriction site which encodes two additional amino acids, glycine and serine.

Proteins

Human pRB, human wild-type p53, HPV-16 E6 and E7, and the various fusion proteins were generated in a combined *in vitro* transcription–translation system. pGEM or pBSK$^+$ (Stratagene) clones containing sequences encoding these proteins were transcribed under the recommended conditions (Promega) using T7 or SP6 RNA polymerase. The construction of the pGEM and pBSK$^+$ clones has been described previously (pRB, Whyte *et al.*, 1988; p53 and HPV-16 E6, Werness *et al.*, 1990; HPV-16 E7, Dyson *et al.*, 1989a). mRNAs from 2 μg of template DNA was used in a 100 μl translation reaction containing 70 μl of pretreated rabbit reticulocyte lysate (RRL) (Promega). To generate radioactively labeled proteins, translations were performed in the presence of L-[^{35}S]methionine (Amersham) for pRB or in the presence of L-[^{35}S]cysteine (Amersham) for all the other proteins. Unlabeled proteins were generated in a parallel reaction in the presence of unlabeled cysteine and methionine. To determine approximate molar ratios of synthesized proteins, radioactively labeled proteins were separated by SDS–PAGE and the incorporated radioactivity determined using an Ambis Radioanalytic Imaging System.

The purification of HPV-16 and HPV-11 E6 proteins expressed as glutathione-*S*-transferase fusion proteins in *Escherichia coli* has been described previously (Huibregtse *et al.*, 1991).

Degradation assay

The degradation properties of the various fusion proteins were assayed as described previously for the HPV-16 or HPV-18 E6 promoted degradation of p53 (Scheffner *et al.*, 1990). Briefly, 4 μl of radioactively labeled pRB or p53 and 1–6 μl of unlabeled E7–E6 fusion proteins were incubated at 25°C in 25 mM Tris–Cl (pH 7.4), 100 mM NaCl and 3 mM DTT, unless stated otherwise. The total amount of RRL was adjusted in each reaction to 10 μl using RRL that was not programmed with exogenous RNA. The reactions were stopped after 3 h by the addition of 1 vol of 100 mM Tris–Cl (pH 6.8), 200 mM DTT, 4% SDS, 20% glycerol, followed by heating for 5–10 min at 97°C. Total reaction mixtures were then electrophoresed on SDS–polyacrylamide gels and the radioactively labeled proteins visualized by fluorography.

For the Western blot analysis of rabbit reticulocyte pRB (Figure 4), proteins were transferred from the SDS–polyacrylamide gel to a nitrocellulose membrane (Schleicher and Schuell) for 2 h at 6 V/cm. The proteins were then probed with the monoclonal pRB-specific antibody 245 [Mh-Rb-02 (PharMingen, San Diego, CA)] and bound antibodies detected with ^{125}I-labeled anti-mouse IgG (Amersham).

Co-immunoprecipitation

For co-immunoprecipitations, approximately equimolar amounts of HPV-16 E6, HPV-16 E7 or the various fusion proteins were mixed with radioactively labeled pRB in 25 mM HEPES (pH 7.0), 125 mM NaCl, 0.05% NP-40 (final concentrations) for 1 h at 4°C. The mixtures were precleared with whole rabbit serum and Staph A [Zysorbin (Zymed, San Francisco, CA)] and then immunoprecipitated with the HPV-16 E7 specific monoclonal antibody 100201 (Triton Biosciences, Alameda, CA) as described previously (Dyson *et al.*, 1989a). In control reactions, the SV40 large T antigen specific monoclonal pAb 419 was used. The immunoprecipitated proteins were separated on SDS–polyacrylamide gels and visualized by fluorography.

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