

Chimaeric HPV E6 proteins allow dissection of the proteolytic pathways regulating different E6 cellular target proteins

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The ability of HPV E6 oncoproteins to induce the degradation of PDZ domain-containing MAGUK proteins correlates with their malignant potential. We previously showed that the HPV-6 E6 protein, when provided with the PDZ-binding domain from HPV-18 E6, acquires the ability to bind the Discs Large (Dlg) tumour suppressor and target it for degradation. Based on this finding we have extended this analysis to E6 proteins from a variety of different papillomavirus types. Cloning a PDZ-binding sequence onto the C-terminus of E6 proteins derived from low-risk mucosal, and low and high-risk cutaneous papillomavirus types, enables them to bind Dlg and a second MAGUK family member, MAGI-1. This renders the mucosally-derived low-risk chimaeric HPV E6 proteins capable of targeting Dlg for degradation, but they are unable to induce significant levels of degradation of MAGI-1. In contrast, none of the E6 proteins derived from cutaneous papillomavirus types induce significant degradation of either MAGI-1 or Dlg when provided with a PDZ-binding domain. These results demonstrate significant differences, both between mucosal and cutaneous HPV E6 proteins and in the pathways required for Dlg and MAGI-1 degradation.

Oncogene (2002) 21, 8140–8148. doi:10.1038/sj.onc.1206026

Keywords: HPV; E6; PDZ; Dlg; MAGI-1

Introduction

Papillomaviruses that are associated with *in situ* carcinoma are termed ‘high-risk’, and their E6 proteins, whether they are derived from human or other animal papillomaviruses, have been shown to be oncogenic in a variety of cell systems. However the routes by which these E6 proteins transform cells are clearly different, and depend on the host species and epithelial cell type. For example, the E6 proteins derived from the high-risk group of mucosal human papillomaviruses are considered to function, in part, by inducing the proteasome-mediated degradation of a number of cellular proteins, but their interaction with the cellular

tumour suppressor p53 has been considered central to the malignant potential of this subset of viruses. In this interaction, oncogenic E6 binds to p53 together with a cellular protein, E6-AP (Huibregtse *et al.*, 1991, 1993; Scheffner *et al.*, 1993), which functions as a ubiquitin ligase, and p53 is subsequently polyubiquitinated and degraded at the 26S proteasome (Scheffner *et al.*, 1990; Werness *et al.*, 1990). In contrast, the E6 proteins from the high-risk cutaneous group of human papillomaviruses neither interact with p53 nor induce its proteasome-mediated degradation (Elbel *et al.*, 1997) and only recent data showing the ability of these E6 proteins to induce the degradation of the pro-apoptotic Bak protein shed any light on the mechanism of their transforming ability (Jackson *et al.*, 2000). The E6 protein from Bovine papillomavirus type 1 (BPV-1) has been shown to possess two transforming functions; one correlating with its interaction with Paxillin, the other independent of binding to either Paxillin or any of its other charged leucine motif-containing targets, such as E6BP and E6-AP (Das *et al.*, 2000). Indeed, although BPV-1 E6 has been shown to bind to E6-AP, no cellular target has so far been shown to be degraded by it along a proteasome-mediated pathway.

The number of identified cellular targets for the oncogenic E6s derived from mucosal HPV types continues to grow, and there are now several studies which suggest that functions of E6 unrelated to p53 degradation may play an important role in the transformation process (Pim *et al.*, 1994; Ishiwatari *et al.*, 1994; Nakagawa *et al.*, 1995; Elbel *et al.*, 1997; Inoue *et al.*, 1998; Liu *et al.*, 1999). In support of this, it has been demonstrated that, the E6 oncoproteins bind to several other cellular targets that might contribute to their oncogenic potential (for reviews see: Thomas *et al.*, 1999; Mantovani and Banks, 2001) and, in some cases, have been shown to induce their ubiquitin-mediated degradation in an E6-AP dependent manner (Nakagawa and Huibregtse, 2000; Gao *et al.*, 2002). The discovery that, amongst these cellular proteins, the HPV E6 proteins target membrane associated guanylate kinase homologues (MAGUKs) for ubiquitin-mediated degradation at the 26S proteasome (Gardioli *et al.*, 1999; Glaunsinger *et al.*, 2000; Nakagawa and Huibregtse, 2000) was particularly interesting, considering the multiple roles these proteins play in mediating membrane-associated functions. MAGUKs are multifunctional proteins located at the membrane-cytoskeletal interfaces

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Received 5 April 2002; revised 4 September 2002; accepted 4 September 2002

of cell-cell junctions, where they mediate various functions such as receptor clustering, signal transduction, and cell polarity (Woods *et al.*, 1996; Goode and Perrimon, 1997; Craven and Brecht, 1998; Fanning and Anderson, 1999; Bilder *et al.*, 2000). Structurally, MAGUKs have a guanylate kinase homology domain (GuK) and a variable number of PDZ domains which function as specific protein binding motifs (Lue *et al.*, 1994; Saras and Heldin, 1996; Ponting *et al.*, 1997). The human homologue of the *Drosophila* tumour suppressor, Dlg, was the first member of this family demonstrated to be a target of the oncogenic HPV E6 proteins. This interaction takes place through the PDZ-2 domain of Dlg via a PDZ-binding motif at the extreme carboxy-terminus of E6 (Kiyono *et al.*, 1997; Lee *et al.*, 1997) and results in the degradation of Dlg along the ubiquitin pathway (Gardioli *et al.*, 1999). Initial studies showed that the efficiency of degradation mediated by oncogenic E6 proteins was related, in part, to the strength of interaction between their C-terminal residues and the PDZ domains of their targets; thus HPV-18 E6, which has a perfect consensus PDZ-binding domain, binds and induces degradation of Dlg more efficiently than does HPV-16 E6, which has a degenerate PDZ-binding domain (Gardioli *et al.*, 1999; Pim *et al.*, 2000; Thomas *et al.*, 2001). This was an important finding since there is evidence to suggest that HPV-18 produces more aggressive tumours than does HPV-16 (Barnes *et al.*, 1988; Kurman *et al.*, 1988; Burnett *et al.*, 1992; Zhang *et al.*, 1995) even though HPV-16 E6 induces the degradation of the p53 tumour suppressor more efficiently than does HPV-18 E6 (Scheffner *et al.*, 1990). Several recent studies have also shown that the high-risk E6 proteins can target several other PDZ domain-containing proteins for proteasome-mediated degradation. These include hScrib (Nakagawa and Huigbregste, 2000), whose function complements that of Dlg (Bilder *et al.*, 2000), as well as MAGI-1 family members (Glaunsinger *et al.*, 2000; Thomas *et al.*, 2002) and MUPP-1 (Lee *et al.*, 2000) all of which are expressed at sites of cell-cell contact and are believed to be involved in the regulation of cellular signal transduction pathways. Although the degradation of all these proteins can be directed by E6, the mechanisms by which it occurs are unclear. We have previously demonstrated that HPV-6 E6, which lacks a PDZ-binding consensus, can nonetheless interact with Dlg when provided with the PDZ domain-binding motif of HPV-18 E6. Most importantly, this chimaeric E6 protein is also capable of directing the proteasome-mediated degradation of Dlg, suggesting that HPV-6 E6 can also interact with components of the cellular proteolytic machinery, (Pim *et al.*, 2000). This latter observation supports previous studies, in which HPV-6 and 11 E6 proteins fused to the Rb-binding domain of HPV-16 E7 could also target pRb for degradation (Scheffner *et al.*, 1992).

The present study was initiated in order to investigate the pathways involved in E6-directed degradation of the different MAGUK proteins, and to try to differentiate between the different pathways used by other oncogenic E6 proteins in cell transforma-

tion. By making use of the novel approach of generating chimaeric E6 proteins we have undertaken an analysis of the E6 proteins from other high and low-risk mucosal HPV types, as well as the E6s from high and low-risk cutaneous HPVs plus high-risk animal papillomaviruses. This has allowed us to determine which of these PV E6 proteins is capable of interacting with the cellular proteolytic machinery and whether different MAGUK family members can be targeted for degradation with equal efficiency by these chimaeric E6 proteins. The results demonstrate major differences between the mechanisms by which mucosal and cutaneous HPV E6 proteins function, significant differences in how different MAGUKs are degraded, and provide additional evidence for the role of ubiquitin ligases other than E6-AP.

Results

Binding of wild type and chimaeric E6 proteins to PDZ domain-containing targets

We cloned the carboxy terminal seven amino acids from HPV-18 E6, which constitutes the PDZ-binding motif (Lee *et al.*, 1997; Pim *et al.*, 2000), onto the E6 proteins from low-risk mucosal types HPV-6 and -11, from low and high-risk cutaneous types HPV-1 and -8, respectively; and from the high-risk cutaneous BPV-1 and the long form of E6 from CRPV. The C-terminal sequences of all the E6 proteins used in this study are shown in Figure 1.

MAGI-1 has 5 PDZ domains (Dobrosotskaya *et al.*, 1997), yet only the PDZ-1 domain is bound by HPV-18 E6 (Thomas *et al.*, 2001). Therefore this domain of MAGI-1 and full-length Dlg were expressed as GST fusion proteins. The abilities of the wild type and chimaeric E6 proteins to bind to these cellular proteins were then assessed in GST pull-down assays. The results of a typical binding assay are shown in Figure 2. Because we observed differences in both translation efficiency and translated protein stability between the different E6 constructs, we considered it reasonable to carry out these assays a number of times to produce statistically significant data. The cumulative results of a series of assays are therefore represented graphically in Figure 3. As can be seen, of the wild type E6 proteins used, only those derived from the high-risk mucosal HPVs can intrinsically bind to a PDZ domain-containing target. Binding levels for the remaining E6 proteins are similar to the background binding seen with GST alone. This is consistent with previous studies showing that the interaction of HPV E6 with PDZ domain-containing proteins is high-risk, mucosal-specific (Lee *et al.*, 1997; Kiyono *et al.*, 1997; Gardioli *et al.*, 1999). However, when the last seven amino acids from HPV-18 E6 are added to the C-termini of the remaining E6 proteins, they are all, as expected, rendered capable of binding the two PDZ domain-containing targets. Most importantly, the wild type high-risk and chimaeric E6s all bind Dlg with approximately equal affinity. In general, the binding

		PDZ DOMAIN-BINDING CONSENSUS SEQUENCE		X ^T S ^S X ^V	
WILD-TYPE E6s	HPV-18	CCNRARQERLQRRRE	ETQV		High-Risk, Mucosal, Human
	HPV-16	CC.....RSSRTRR	ETQL		
	HPV-31	CW.....R.RPRT	ETQV		
	HPV-6	CLHCWTTCMEDMLP			Low-Risk, Mucosal, Human
	HPV-11	CLHCWTTCMEDLLP			
	HPV-8	KGVCRLCKHLYHDW			High-Risk, Cutaneous, Human
	HPV-1	KAKCSLC.RLY.AI			Low-Risk, Cutaneous, Human
	BPV-1	CYDCCRHGSRSKYP			High-Risk, Cutaneous, Non-Human
	CRPV	VCFCENCINFTEFR			
	HYBRID E6s	HPV-6.18	CLHCWTTCMEDMLP	RRRETQV	C-Terminal Sequences from HPV-18 E6
HPV-11.18		CLHCWTTCMEDLLP	RRRETQV		
HPV-8.18		KGVCRLCKHLYHDW	RRRETQV		
HPV-1.18		KAKCSLC.RLY.AI	RRRETQV		
BPV-1.18		CYDCCRHGSRSKYP	RRRETQV		
CRPV.18	VCFCENCINFTEFR	RRRETQV			

Figure 1 Alignment of the C-termini of the wild type and mutant E6 proteins used, compared with the consensus PDZ domain-binding motif

of all the E6s with either a natural or an added chimaeric C-terminal PDZ-binding domain was stronger to Dlg than to the PDZ-1 domain of MAGI-1, which may reflect the use of the PDZ-1 domain as opposed to the full-length protein. Although some differences in binding affinity are observed with the MAGI-1 PDZ-1, the chimaeric mucosal E6 proteins are approximately equivalent to each other and to HPV-31 E6.

The degradation of target proteins by wild type and chimaeric E6 proteins in vitro

Since we had previously demonstrated that cloning a PDZ-binding domain onto the C-terminus of low-risk HPV-6 E6 allows the chimaeric protein to both bind and degrade Dlg (Pim *et al.*, 2000), we then asked whether the other chimaeric E6 proteins described above would have the same phenotype. To answer this, we translated and radiolabelled the different E6 proteins *in vitro*, then incubated approximately equal amounts of each with *in vitro* translated, radiolabelled p53, Dlg and MAGI-1. Remaining target proteins were then assessed following immunoprecipitation with specific antibodies followed by SDS-PAGE and autoradiography. The results from a typical degradation assay are shown in Figure 4. As can be seen, under conditions where HPV-16 E6 degrades all

of the available p53, only the other high-risk derived E6, HPV-31 E6, reduces p53 levels and this is consistent with previous studies (Werness *et al.*, 1990; Scheffner *et al.*, 1990). In the case of Dlg, both HPV-16 E6 and HPV-31 E6 induce its degradation with similar efficiencies over the period of the assay. As expected, none of the other wild type E6 proteins affect Dlg stability, which is consistent with the data from the *in vitro* binding assays. However, the chimaeric E6 proteins display a very interesting phenotype. Although all are capable of binding Dlg with approximately equal affinity, due to the presence of the HPV-18 E6 PDZ-binding domain, only the mucosal HPV-6 and HPV-11 E6 proteins can induce the degradation of Dlg in this assay system; none of the cutaneous HPV E6 proteins appear to do so. This suggests that the mucosal HPV E6 proteins, whether derived from high or low-risk HPV types, can all interact with a cellular proteolytic system that is capable of degradation of this particular target. In contrast, in the case of MAGI-1, only HPV-16 E6 and HPV-31 E6 and none of the chimaeric E6 proteins are able to induce its degradation in this assay system. This was a very surprising result, and argues strongly that the precise protein complexes that are involved in E6 mediated degradation of Dlg are different from those involved in E6 mediated degradation of MAGI-1 and p53.

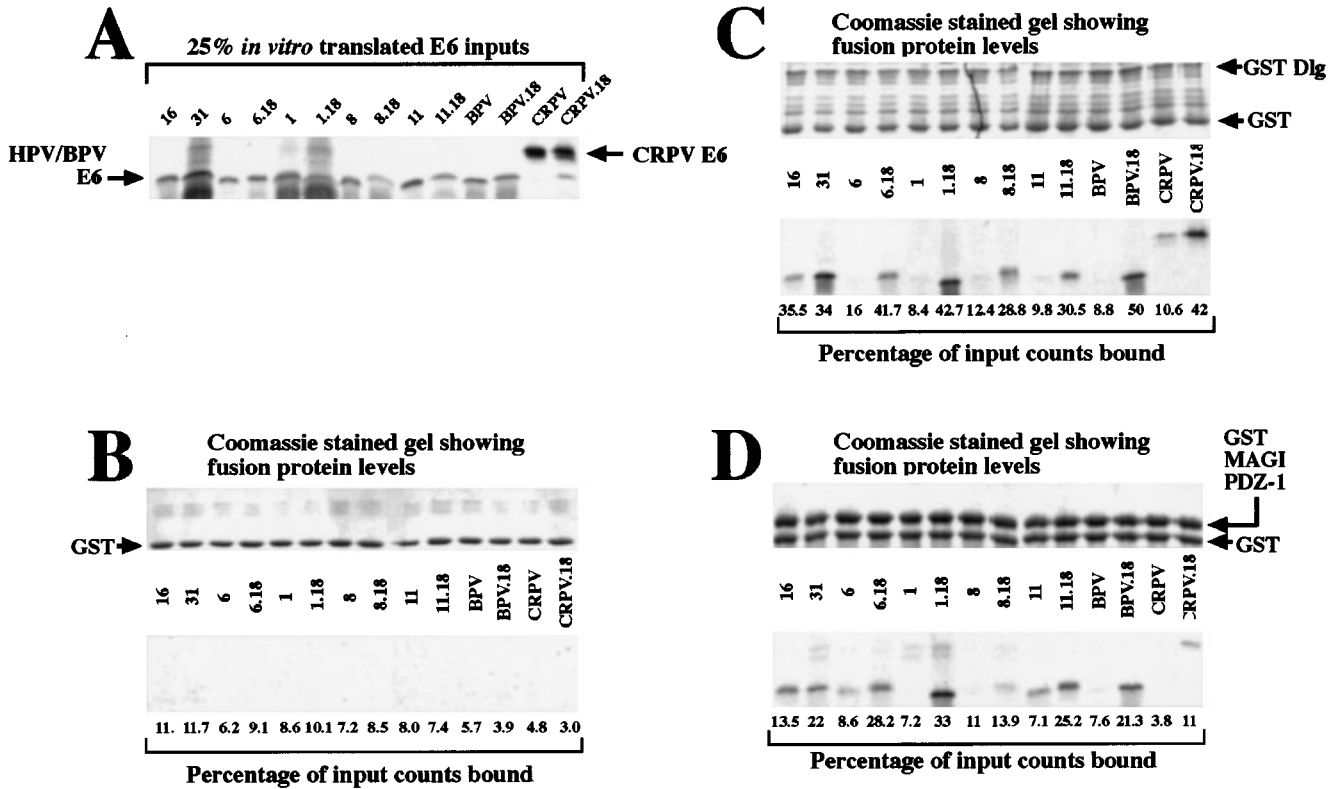


Figure 2 GST Dlg and GST MAGI-1 PDZ-1 binding to wild type and mutant E6s *in vitro*. (a) Shows 25% of the *in vitro* translated protein inputs: the arrow on the left of the Figure marks the position of the *in vitro* translated HPV and BPV E6s, the arrow on the right indicates the CRPV E6s. (b) Binding of *in vitro* translated E6 to GST alone. (c) Binding of *in vitro* translated E6 to GST Dlg. (d) Binding of *in vitro* translated E6 to GST MAGI-1 PDZ-1. For (b), (c), and (d), the Coomassie stained gels showing the inputs of GST and GST fusion proteins are shown above, and the percentage of bound protein, calculated by scanning the input and binding gels by Cyclone phosphorimager (Packard), is shown below

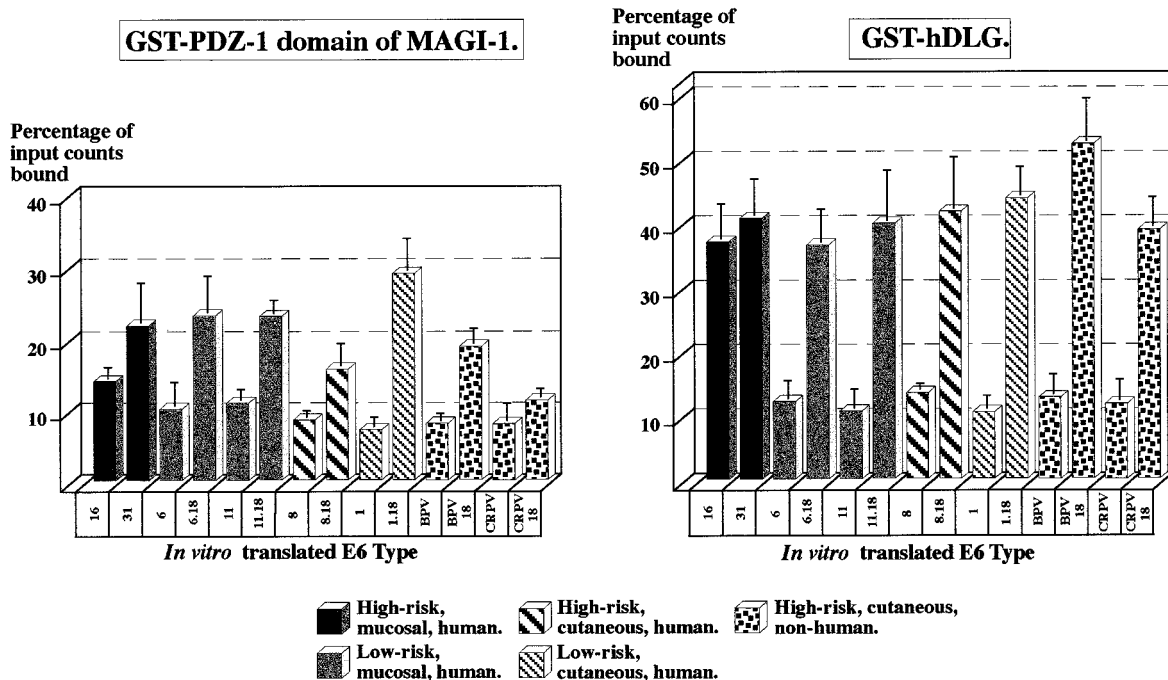


Figure 3 The cumulative data from a series of binding assays showing standard deviations

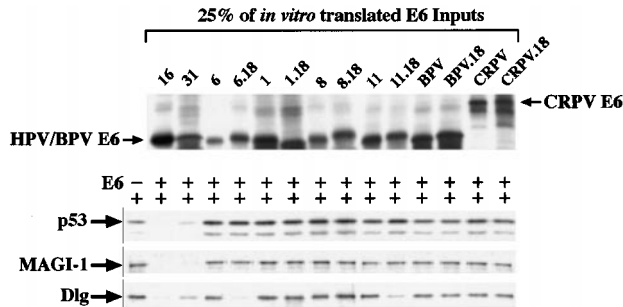


Figure 4 *In vitro* degradation of p53, Dlg and MAGI-1. The top panel shows 25% of the E6 protein inputs: the arrow on the left of the Figure marks the position of the *in vitro* translated E6s and BPV E6s, the arrow on the right indicates the CRPV E6s

The degradation of target proteins by wild type and chimaeric E6 proteins *in vivo*

The advantage of using an *in vitro* degradation system is that the inputs of the different *in vitro* translated proteins can be verified and balanced. However, we have previously noted that some HPV E6 mutants which appeared negative for E6-directed degradation of some cellular targets *in vitro*, were nevertheless able to induce degradation of their targets *in vivo* (Gardiol and Banks, 1998) arguing that the two assays are not absolutely reflective of each other. Therefore, to confirm that the general pattern of degradation of these target proteins *in vitro* also held true *in vivo*, we transfected 293 cells with constructs expressing HA-tagged Dlg or an HA-tagged, N-terminal construct of MAGI-1, together with constructs expressing various chimaeric E6 proteins. We used the N-terminus of MAGI-1 since we have observed that expression levels of the full-length protein are considerably higher than those obtained for Dlg, whereas the N-terminal MAGI-1 construct expressed equivalent levels; we observed no differences between full-length and N-terminal MAGI-1 in terms of E6-directed degradation (Thomas *et al.*, 2001). After harvesting the cells, separating whole cell extracts by PAGE, and Western transfer, the membranes were probed with antibodies to the respective target proteins. The results are shown in Figure 5, where it can be seen that there are indeed some differences between the *in vitro* and *in vivo* assay systems. Thus although both the 6.18 and 11.18 chimaeras can direct Dlg degradation with equal efficiency *in vitro*, the 11.18 chimaera is somewhat more efficient in the *in vivo* assay. We also reproducibly observe a slight decrease in Dlg levels with the 8.18 E6 chimaera in the *in vivo* assay, which contrasts with the lack of degradation *in vitro*. The 1.18 E6 chimaera exhibits no activity in this assay. This suggests that high-risk cutaneous HPV E6s may interact with cellular degradatory machinery; a model previously suggested by their ability to direct the degradation of the Bak protein (Jackson *et al.*, 2000). For MAGI-1, we observe no decrease in protein levels with the *in vivo* assay with the cutaneous chimaeric E6 proteins, but do see a modest reduction with the low-risk mucosal

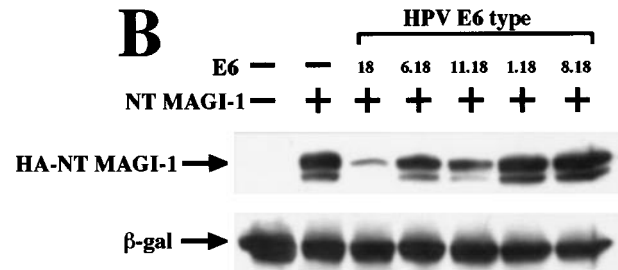
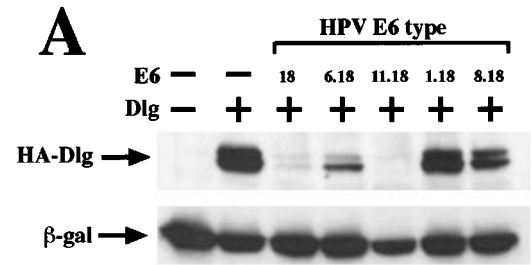


Figure 5 *In vivo* degradation of: (a) Dlg and (b) NT MAGI-1 by wild type and chimaeric HPV E6 proteins overexpressed in 293 cells. The lower portions of each panel show the same blot stripped and reprobed for β -gal, to confirm equal transfection efficiency and equal loading

chimaeras, 6.18 and 11.18, which was not observed in the *in vitro* assay.

Domains of HPV-6 E6 required for target protein degradation

Previous studies have shown that sequences in the carboxy terminal loop of high-risk E6 are essential for its ability to target several cellular proteins for degradation. Small deletion mutations within this region of E6 disrupt its ability to interact with protein partners possessing α -helical motifs, in particular E6-AP (Liu *et al.*, 1999; Be *et al.*, 2001). Certainly, mutational analysis of HPV-18 E6 around residues 126–130 shows that deletion of these residues abolishes the ability of the protein to direct the degradation of Dlg and p53 (Pim *et al.*, 1994, 2000). In order to investigate whether the same region of HPV-6 E6 was involved in its ability to direct the degradation of cellular targets, we made two deletion mutants within the 6.18 chimaera, analogous to HPV-18 E6 mutants Δ C and Δ G (Pim *et al.*, 1994), and analysed their ability to direct the degradation of Dlg *in vitro*. The results from an *in vitro* degradation assay of Dlg are shown in Figure 6. It can be seen that while HPV-6.18 E6 Δ C and Δ G are slightly reduced for Dlg degradation, both are functional. Identical results were also obtained when the assay was performed *in vivo* (data not shown). This result suggests that the ability of the HPV-6.18 chimaeric E6 to direct the degradation of Dlg does not require the same structural features as are required in HPV-18 E6. This further suggests that

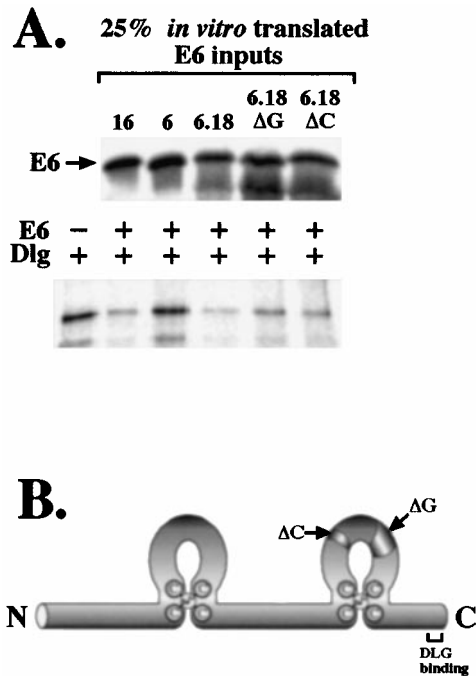


Figure 6 *In vitro* degradation of Dlg by wild type and mutant HPV E6 proteins. As previously demonstrated, HPV-18 E6 is positive for directing Dlg degradation, wild type HPV-6 E6 negative, but, when provided with a C-terminal PDZ-domain binding sequence, is positive. Mutants ΔC and ΔG of the 6.18 hybrid E6 are still capable of inducing Dlg degradation

there are different routes by which these viral E6 proteins interact with the cellular proteolytic machinery.

Degradation of Dlg by HPV-18 E6 does not require an interaction with charged leucine motif-containing proteins

Previous studies on HPV-16 E6 have shown that amino acid residues in the C-terminal cysteine loop are required for binding with cellular proteins that contain charged leucine α -helical domains, such as E6-AP, E6BP and Paxillin (Liu *et al.*, 1999). An HPV-16 E6 mutant containing an amino acid substitution within this domain, I128T, has been shown to be strongly reduced for binding to E6-AP, as was its ability to direct p53 degradation (Liu *et al.*, 1999). In order to investigate the role of this region of HPV-18 E6 in the degradation of PDZ domain-containing substrates, we constructed the homologous mutant in HPV-18 E6, termed I130T, and examined its ability to induce the degradation of p53, Dlg and MAGI-1 *in vivo*. 293 cells were transfected with plasmid constructs expressing HA-Dlg or HA- N-terminal MAGI-1, or Saos-2 cells with p53, in the presence of pcDNA3 constructs expressing wild type HPV-18 E6 or HPV-18 E6 I130T, along with plasmid LacZ as transfection control. Cells were harvested after 24 h, the lysates separated by PAGE and then transferred to nitrocellulose membranes by Western blot. The membranes were probed with antibodies against the expressed targets

and β -gal. The results, shown in Figure 7, show unequivocally that the I130T mutant is strongly reduced for its ability to direct the degradation of p53 and MAGI-1, whereas, under identical transfection conditions, it is almost as efficient as wild type HPV-18 E6 in its ability to direct the degradation of Dlg. These results provide further evidence that E6-AP is not the ubiquitin ligase involved in the degradation of Dlg.

Discussion

In this study we have demonstrated that only the E6 proteins derived from the high-risk mucosal HPVs have an intrinsic ability to bind and induce the degradation of PDZ domain-containing proteins.

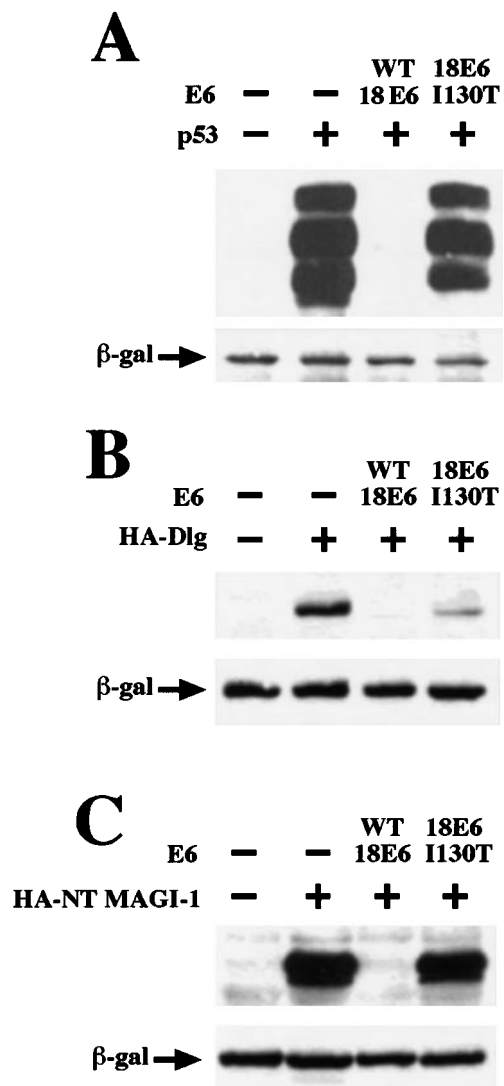


Figure 7 *In vivo* degradation by wild type HPV-18 E6 and 18 E6 I130T of: (a) p53 overexpressed in Saos-2 cells; (b) Dlg, overexpressed in 293 cells, and (c) NT MAGI-1 overexpressed in 293 cells. The bottom portion of the panel in each case is of the same blot stripped and reprobbed for β -gal to confirm equal transfection efficiency and equal loading

However, when the E6 proteins derived from low-risk mucosal HPV types are provided with a C-terminal PDZ-binding domain, they acquire the ability to both bind Dlg and induce its degradation *in vitro* and *in vivo*: the observation that *in vivo*, the 11.18 chimaera is somewhat more efficient than the 6.18 chimaera may be significant since HPV-11 is considered more aggressive than HPV-6 in terms of recurrent respiratory papillomatosis and its progression to malignancy (Rabah *et al.*, 2001; Rady *et al.*, 1998; Petersen *et al.*, 1998). In contrast, although these chimaeric E6 proteins gain the ability to bind MAGI-1, they can, at best, induce its degradation only weakly *in vivo*, and not at all *in vitro*. Most significantly, the chimaeric E6 proteins derived from cutaneous HPV types were unable to target MAGI-1 for degradation either *in vivo* or *in vitro*, while the HPV 8.18 chimaera induces weak degradation of Dlg *in vivo*, an effect we were unable to detect *in vitro*. These results highlight significant differences in how the mucosal and cutaneous derived E6 proteins interact with the cellular proteolytic machinery as well as highlighting differences in how the PDZ domain-containing substrates are themselves regulated. The differences observed between the *in vivo* and *in vitro* degradation assays, such that the chimaeric HPV-8.18 E6 protein appears to reduce Dlg levels *in vivo* but not *in vitro*, has two possible explanations. First, that the *in vitro* degradation system may lack sufficient sensitivity to observe the degradation of some cellular targets; we have observed that Dlg is relatively resistant to E6-directed degradation *in vitro*, but as sensitive as p53 for degradation *in vivo*. Secondly, that elements of the complex required for degradation may be lacking or limiting in rabbit reticulocyte lysate. This is not surprising, given that a recent study showed that degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs through a Cullin complex, and that this complex could lead to ubiquitination of p53 *in vitro*, but not its degradation, since this required NEDylation of Cullins (Querido *et al.*, 2001). It is possible that a similar situation may hold true for some aspects of E6's degradative capacity.

We had originally reasoned that the ability of E6 to interact with components of the cellular proteolytic machinery would most likely correlate with the risk status of a given papillomavirus type; high-risk types, whether mucosal or cutaneous, being able to interact, low-risk types not. In support of this idea, previous studies on the degradation of the pro-apoptotic protein Bak in human keratinocytes have shown that the E6 proteins from both high-risk mucosal and cutaneous HPVs can induce its degradation (Jackson *et al.*, 2000). Surprisingly, in the case of MAGI-1, we find that it is the tissue tropism rather than risk status that defines whether an E6 protein can recruit the appropriate cellular degradatory mechanism to target this particular substrate. Similarly, even low-risk mucosal HPV E6 proteins can induce the degradation of Dlg protein when provided with a clearly defined PDZ-binding domain. It also seems likely that the HPV-18 and the chimaeric HPV-6 E6 proteins have different structural

constraints on their ability to target substrates for degradation. Thus mutant ΔG of the 6.18 chimaera lacks a stretch of five amino acids, (125–129), equivalent to the HPV-18 E6 ΔG mutant, which is defective for p53 degradation (Pim *et al.*, 1994) and strongly reduced for Dlg degradation (Pim *et al.*, 2000). In contrast this equivalent mutation within the HPV-6.18 chimaeric protein has only minimal effects on its ability to target Dlg for degradation. Differences between the ways in which E6 targets p53 and Dlg for degradation were also provided by studies with the HPV-18 I130T E6 mutant. This is almost as efficient as wild type 18 E6 in its ability to induce Dlg degradation, while it is almost completely defective for inducing p53 and MAGI-1 degradation *in vivo*. Given that the equivalent HPV-16 I128T E6 mutant is also defective for p53 degradation, due to its reduced ability to bind E6-AP (Liu *et al.*, 1999), this result suggests strongly that E6-induced Dlg degradation does not involve E6-AP. Taken together, these studies suggest a complex pattern of protein interactions which govern target specificity, both with respect to different cellular targets as well as to different papillomavirus E6 proteins. Work is currently in progress to identify the different protein complexes involved in the regulation of PDZ domain-containing protein levels in the cell, both under normal conditions and during HPV E6-directed degradation.

Materials and methods

Construction of plasmids for in vitro expression and expression as GST fusions in bacteria

The mutant E6 proteins were constructed by PCR; the 3' oligonucleotide encoded the C-terminal seven amino acids derived from HPV-18 E6. The resulting constructs are designated 6.18, 1.18, 8.18, 11.18, BPV.18 and CRPV.18. The wild type and mutant E6s were cloned into either pSP64 or pcDNA3 (Invitrogen) for *in vitro* translation. The open reading frames for Dlg and the PDZ-1 domain of MAGI-1 were cloned into the bacterial expression vector pGEX 2T and expressed as GST fusion proteins in *E.coli*. These were extracted and purified as described previously (Smith and Johnson, 1988).

In vitro translations and binding reactions

Wild type and mutant E6 proteins were *in vitro* translated in rabbit reticulocyte lysate using the Promega TNT system and radiolabelled with [³⁵S]-cysteine (Amersham), using T7 RNA polymerase for constructs cloned in pcDNA3, or SP6 RNA polymerase for constructs cloned in pSP64. *In vitro* translated proteins were run on PAGE and their relative translation efficiencies estimated by Cyclone phosphorimager (Packard). Approximately equal levels of *in vitro* translated protein were added to GST, GST-Dlg, or GST-MAGI-1 PDZ-1 bound to glutathione resin, then incubated for 1 h at room temperature. The glutathione resin columns were washed twice with PBS containing Triton X-100 at 1%, twice in PBS containing NP40 at 2% and once in PBS containing Tween 20 at 0.5%. After washing, the bound proteins were visualized by PAGE, and quantitated by Cyclone phosphorimager.

In vitro degradation reactions

Wild type or mutant E6 proteins were translated and radiolabelled *in vitro* and their relative levels assessed, as described above. They were then incubated in the presence of *in vitro* translated, radiolabelled p53, Dlg, or MAGI-1. p53 and MAGI-1 degradation reactions were typically carried out for 1 h, Dlg incubations for 3 h. After incubation the amounts of residual target proteins were assessed by immunoprecipitation with specific polyclonal antibodies, followed by isolation of immune complexes with protein A sepharose and their analysis by SDS-PAGE and autoradiography.

In vivo degradation assays

Human Saos-2 and 293 cells were grown and maintained in Dulbecco's modified Eagle's medium, supplemented with foetal bovine serum to 10% concentration. Cells were transfected typically with 10 µg of pcDNA3 plasmid constructs expressing wild type, or chimaeric HPV E6s or HPV-18 E6 mutant I130T, plus 4 µg of pcDNA p53, of GW1 Dlg (Gardiol *et al.*, 1999) or of a GW1 construct expressing the first 734 amino acids of MAGI-1 with an N-terminal HA tag (Thomas *et al.*, 2001) termed NT MAGI-1. After 24 h, cells were harvested by lysing in a buffer containing, 50 mM HEPES pH 7.0, 250 mM NaCl, 0.1% NP40, and 20 mg ml⁻¹ aprotinin, and after separation by PAGE were transferred by

Western blot to nitrocellulose membranes (Schleicher and Schuell). After transfer the membranes were blocked with 10% milk powder in PBS, then probed with commercial anti-HA antibodies (Roche), or a pool of the anti-p53 antibodies pAbs 1801, 1802 and 1803. Amplification of the signal was carried out using biotinylated secondary rabbit anti-mouse antibodies (Dako), then visualized by enhanced chemiluminescence (Amersham), according to the manufacturers instructions. To confirm equal transfection efficiencies and equal gel loading, the blots were stripped by incubation for 1 h at 65°C in a buffer containing 2% SDS, 62.5 mM Tris pH 6.8, and 100 mM β-mercaptoethanol. After copious washing with distilled water, and blocking with PBS containing 10% milk powder, the β-gal expression was confirmed by incubation with anti-β-gal antibodies (Promega), diluted in PBS plus 10% milk powder, followed by biotinylated anti-mouse antibodies, (Dako) and visualized by enhanced chemiluminescence.

Acknowledgements

We thank the following for their kind gifts of papillomavirus E6 open reading frames: Janet Brandsma for CRPV, Elliot Androphy for BPV-1, John Doorbar for HPV-1, Herbert Pfister for HPV-8 and Lou Laimins for HPV-31. This work was supported in part by a research grant from the Associazione Italiana per la Ricerca sul Cancro.

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