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Stabilization of HPV16 E6 protein by PDZ proteins, and potential implications for genome maintenance

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ABSTRACT

The E6 protein from high-risk human papillomaviruses appears necessary for persistence of viral episomes in cells but the underlying mechanism is unclear. E6 has many activities, including its ability to bind and degrade PDZ domain-containing proteins, such as hScrib. However little is known about the role of these interactions for E6 function and the viral life cycle. We now show that the levels of expression of wild-type E6 are increased in the presence of hScrib whilst a mutant E6 protein lacking the PDZ-binding motif is found at lower levels as it is turned over more rapidly by the proteasome. This correlates with an inability of genomes containing this mutation to be maintained as episomes. These results show that E6 association with certain PDZ domain-containing proteins can stabilize the levels of E6 expression and provides one explanation as to how the PDZ-binding capacity of E6 might contribute to genome episomal maintenance.

Introduction

Papillomaviruses (PV) are small DNA viruses that infect epithelial tissue and can give rise to hyper-proliferative lesions (reviewed in Howley and Lowy, 2001). In most cases these lesions are benign but a small number of papillomaviruses (high-risk types) cause lesions that may become malignant. Probably the most significant of these, from a clinical perspective is HPV16, which is responsible for the majority of cases of cervical cancer (Walboomers et al., 1999). The life-cycle of papillomaviruses is tightly linked to the differentiation of the host epithelium. The virus enters the basal cells, where multiple copies of the viral genome become established as persistent episomes. These cells divide, and as they move towards the surface of the epithelium they differentiate, triggering expression of the different viral proteins and viral DNA amplification (reviewed in Doorbar, 2006).

One of the early HPV genes expressed is E6, giving rise to the multi-functional E6 protein, and it has been observed that genomes that express some mutant E6 proteins fail to establish as episomes within cells (Park and Androphy, 2002; Thomas et al., 1999). The reasons for this are unclear; however the E6 protein is known to have a multitude of binding partners and activities, and its effects on the virus life-cycle and malignant progression are many and complex. Proposed roles of the E6 protein in the virus life-cycle include inhibition of apoptosis induced by the expression of the HPV E7 protein and also facilitation of viral DNA amplification in the upper layers (Wang et al., 2009). The most well-studied interaction of HPV E6 with a cellular protein is that of its association with p53, which results in the ubiquitin-mediated degradation of p53 by the proteasome (Scheffner et al., 1990). The E6-facilitated degradation of p53 appears to have a role in genome maintenance (Park and Androphy, 2002; Thomas et al., 1999), but whether this activity is strictly necessary and sufficient for establishment of episomes is uncertain. E6 mediates degradation of p53 by associating with E6AP, a cellular ubiquitin ligase, and changing its substrate specificity so that it now targets p53 (Huibregtse et al., 1991). E6AP is also implicated in the ability of E6 to activate the transcription of the catalytic subunit of telomerase, hTERT (Liu et al., 2005), and this activity is thought to be responsible for E6's ability to immortalize cells. Interestingly, it has recently been shown that E6AP also functions to protect the HPV16 and 18 E6 proteins from proteasomal degradation, thereby increasing their stability (Tomaic et al., 2009). Crucially this suggests that some of the activities of E6 that had previously been attributed to the catalytic activity of E6AP could instead be dependent on E6AP only to provide sufficient levels of E6.

The carboxy terminus of all the high-risk HPV type E6 proteins contains a motif (X—(S/T)—X—(V/I/L)—COOH) that is able to recognize and bind to PDZ domains (reviewed in Thomas et al., 2008). PDZ domains are structural domains composed of 80–90 amino acids that bind to short stretches on the C-terminus of other proteins. Again interaction of E6 with the PDZ domain-containing proteins can result in their degradation. In particular, the association between high-risk E6 proteins and a sub-category of PDZ domain-containing proteins that are

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plasma membrane-associated signal transducers appears to be of significance (reviewed in Pim and Banks, 2010). These proteins include Dlg, hScrib, MAGI and many others, and they are involved in activities such as tight junction assembly, the establishment of apicobasal polarity and cell cycle progression (Latorre et al., 2005; Nagasaka et al., 2006). The specificity for each of these PDZ domain-containing proteins depends on the particular E6 protein involved (Thomas et al., 2001), and on the localization and modification of the PDZ domain-containing proteins. Importantly, the ability to bind PDZ domain-containing proteins appears to be an activity characteristic of many high-risk HPV types. Most low-risk E6 proteins do not have a PDZ binding motif (PBM) and loss of PDZ domain-containing proteins appears to correlate with malignant progression. For these reasons the potential role of the association between high-risk E6 proteins and PDZ domains has been proposed as being important for the ability of high-risk viruses to transform cells. However the role of the interaction between PDZ domain-containing proteins and HPV E6 proteins in productive virus life-cycles is less well understood. In HPV type 31 it was found that mutation of the E6 PBM resulted in changes to the growth and morphological differentiation of cells and to the viral copy number (Lee and Laimins, 2004). However, the mechanism for how the mutation caused these effects was not investigated.

Here we show that loss of the capacity of HPV16 E6 to interact with PDZ domain-containing proteins results in enhanced proteasomal degradation of the E6 protein. Stabilization of E6 is dependent on its PBM and on the PDZ domains of, for example, hScrib. We also find that failure of a mutant E6 to be stabilized by PDZ domain-containing proteins correlates with the inability of genomes carrying this mutation to establish and maintain as episomes within cells.

Results

HPV16 E6 levels are increased by hScrib in a PDZ-dependent manner

Previous studies have shown that E6-PDZ targeting is important for several aspects of E6 biology (for review see Thomas et al., 2008). However little is known about its role in the viral life cycle or with respect to E6 function itself, the latter point being particularly relevant in the light of recent studies showing a role for E6AP in regulating E6 stability (Tomaic et al., 2009). Since a major PDZ interacting partner of HPV16 E6 is hScrib (Thomas et al., 2005) we decided to investigate whether the hScrib–E6 interaction could in any way affect E6 levels of expression. To do this, a plasmid that expresses wild-type E6 was co-transfected with either an HA-Scrib-expressing plasmid or control pcDNA plasmid into NIKS cells. We chose NIKS cells as these cells can support the HPV16 virus life-cycle (Lambert et al., 2005) and are therefore a good model system to work with when analyzing potential functions of HPV16 proteins during productive infection, rather than during cancer progression. The levels of E6 were analyzed by Western blot (Fig. 1A). The presence of hScrib significantly increased the levels of E6 protein, suggesting that binding of E6 to hScrib may increase the stability of E6. To assess whether the increase in levels of E6 was related to binding to hScrib, we repeated the experiment using an E6 mutant

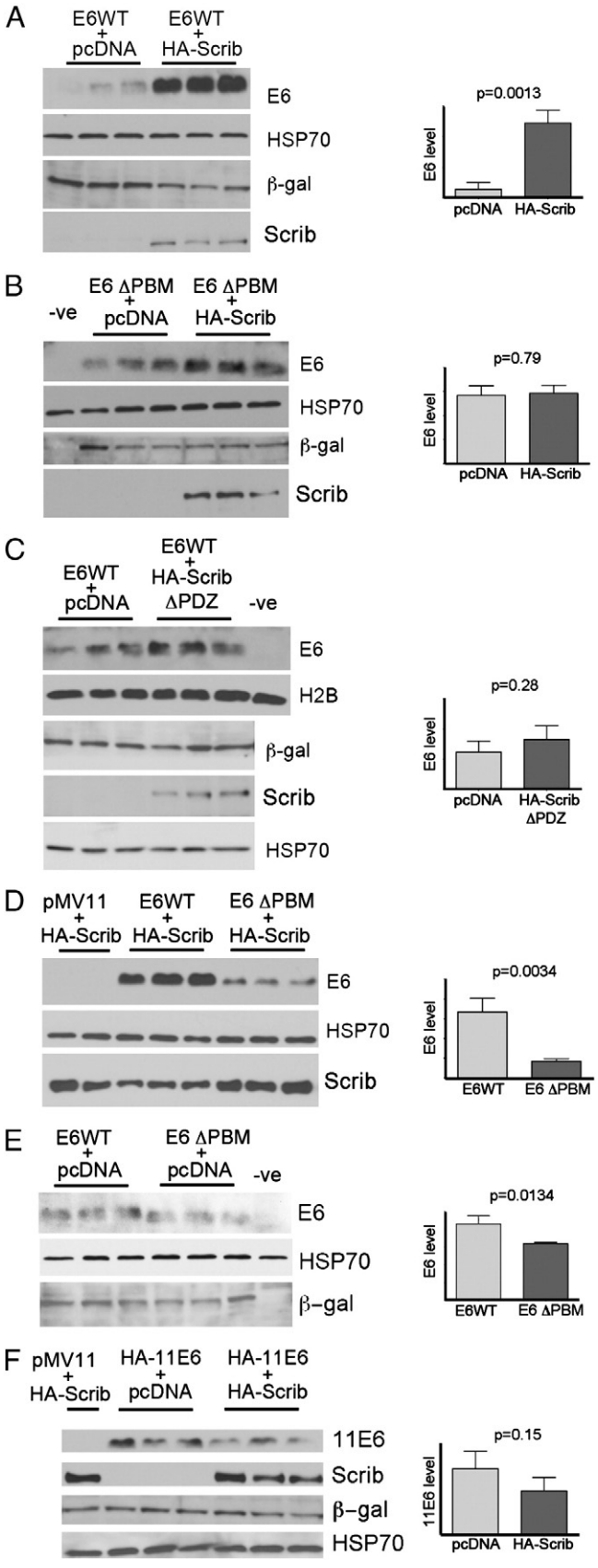

Fig. 1. E6 levels are increased by an interaction between E6 and hScrib. Normal immortalized human keratinocytes (NIKS) were transiently co-transfected with plasmids that express wild-type HPV16 E6 protein or a mutant lacking the PBM (E6WT and E6ΔPBM respectively), or HA-tagged HPV11 E6 protein (HA-11E6), and HA-tagged wild-type hScrib protein or a mutant lacking the PDZ region (HA-Scrib and HA-ScribΔPDZ respectively). A) E6WT co-transfected with either HA-Scrib or control pcDNA plasmid. B) E6ΔPBM co-transfected with either HA-Scrib or control pcDNA plasmid. C) E6WT co-transfected with either HA-ScribΔPDZ or control pcDNA plasmid. D) HA-Scrib co-transfected with E6WT, E6ΔPBM or control pMV11 plasmid. E) E6WT or E6ΔPBM co-transfected with control pcDNA plasmid. F) HA-11E6 or control pMV11 plasmid co-transfected with either HA-Scrib or control pcDNA plasmid. The transfections were spiked with a plasmid that expresses β-gal. Total cell extracts were obtained from cells 48 h post-transfection and Western blotted where appropriate for E6 proteins, HA-Scrib, β-gal and as loading controls HSP70 or histone 2 B (H2B). Levels of E6 were assessed using ImageJ software. Cells were transfected in triplicate and bar charts show the mean E6 protein levels (in arbitrary units) + the standard deviation, relative to the loading controls. Unpaired *T*-tests were used to compare the E6 levels and *p* values are given above the charts.

that lacks the PBM, ETQL (Fig. 1B) and an hScrib mutant that lacks any PDZ domains (Fig. 1C). Co-expression of wild-type HA-Scrib with ΔPBM mutant E6 did not result in a significant change in E6 levels compared to that in the absence of HA-Scrib. Similarly co-expression of wild-type E6 with the PDZ domain mutant of hScrib did not alter E6 levels. Together these data suggest that an interaction between the E6 PBM and the PDZ domains of hScrib increases the stability of E6. To test whether the level of E6ΔPBM protein was indeed lower than that of wild-type E6 protein in the presence of HA-Scrib, the co-transfection experiment was repeated and the levels of the wild-type and E6ΔPBM protein compared (Fig. 1D). As expected the levels of E6ΔPBM were significantly lower than wild-type E6 levels. We observed similar results when using other cell lines in addition to NIKS (data not shown). Of course, in addition to the HA-Scrib that we co-transfect, the NIKS cells also contain endogenous hScrib and we wanted to determine whether this would also have a similar effect on E6 levels. To test this we repeated the experiment but without co-transfecting HA-Scrib (Fig. 1E). Under these conditions we did not see a significant difference between the levels of wild-type and E6ΔPBM protein. However, since E6 is being expressed from a CMV promoter it seems likely that a potential role for the endogenous hScrib is masked by the very high levels of E6 expression. Indeed, we have found, using qPCR, that the level of E6 transcripts is 100× higher from our CMV promoter than from the HPV16 genome (data not shown). As the levels of E6 expressed from genomes are below the limits of easy detection, we decided to continue working with the CMV-derived E6 and co-transfection with HA-Scrib. Since the low risk HPV11 E6 lacks PDZ binding potential we proceeded to investigate whether hScrib could similarly affect the levels of HPV11 E6 expression. The results in Fig. 1F demonstrate that hScrib has no effect on HPV11 E6 expression levels, consistent with their inability to interact (Nakagawa and Huibregtse, 2000).

Differences in E6 levels are due to protein stability

To address what is causing the difference in levels between the wild-type and E6ΔPBM mutant proteins, we first checked the levels of β-galactosidase expressed from a co-transfected plasmid and these showed the transfection efficiencies to be the same (Fig. 2A). To further support this we checked that protein levels were not varying because of differences at the level of transcription. The wild-type and E6ΔPBM plasmids were co-transfected with HA-Scrib plasmid into NIKS cells. The cells were harvested at 48 h and RT-qPCR was used to assess the levels of E6 transcripts and these were normalized to the levels of β-actin transcripts (Fig. 2B). The levels of wild-type and E6ΔPBM mutant transcripts are not statistically significantly different and if anything, the E6ΔPBM mutant transcript levels appear higher. This indicates that the reduced levels of the E6ΔPBM protein are not due to reduced transcription of the E6ΔPBM mutant gene. To test whether using an antibody to the C-terminal half of E6 was causing the apparent differences in levels of the wild-type and E6ΔPBM proteins, we repeated the blots using an antibody that recognizes an epitope in the N-terminus of E6 (Fig. 2C). Again, the levels of wild-type E6 were significantly higher than the levels of the E6ΔPBM mutant, suggesting that the effect is not simply due to antibody affinities. To support that it was the presence of hScrib that was responsible for the elevated levels of wild-type E6, we decided to knock-down hScrib in cells that express endogenous E6. We were unable to achieve adequately high efficiency transfection of siRNA into NIKS cells (data not shown), but were able to substantially knock-down hScrib in HeLa cells. When these cells were transfected with siRNA to hScrib, the levels of E6 were greatly reduced (Fig. 2D), suggesting that again in this system, hScrib is promoting higher levels of E6.

To determine whether loss of E6 in the absence of an interaction with hScrib was due to increased protein turnover the wild-type and E6ΔPBM mutant plasmids were co-transfected with HA-Scrib plasmid into NIKS cells. After 46 h the cells were treated with the protein synthesis inhibitor cycloheximide (Fig. 3A). As can be seen, the rate of

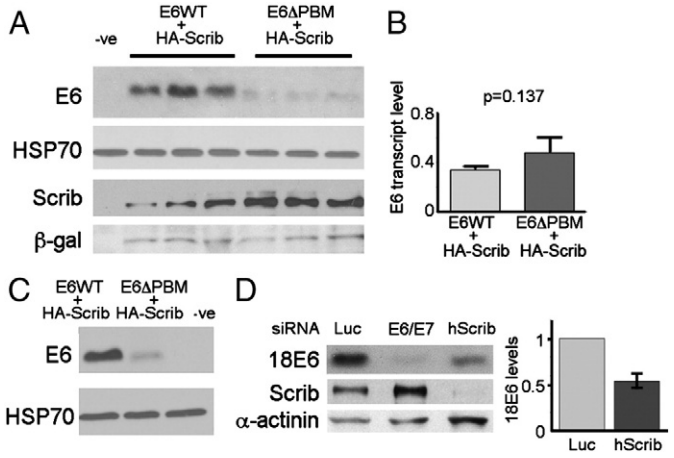

Fig. 2. The higher E6WT protein levels are dependent on hScrib, and do not result from increased levels of transcript. NIKS cells were transiently co-transfected with plasmids that express E6WT or E6ΔPBM, and HA-Scrib. A) At 48 h post-transfection, total cell lysates were obtained and Western blotted for E6, HA and β-gal, and as a loading control, HSP70. B) RNA was extracted from cells at 48 h post-transfection and converted to cDNA. Transcript levels were assessed using primers to E6 and cellular β-actin. Cells were transfected in triplicate and bar charts show the mean E6 transcript level (in arbitrary units) + the standard deviation, relative to the β-actin levels. An unpaired *T*-test was used to compare the E6 transcript levels and the *p* value is given above the chart. C) Extracts were Western blotted for E6 proteins using an antibody to the N-terminus of E6, and as a loading control, HSP70. D) HeLa cells were transfected with siRNA to hScrib or as controls E6/E7 or luciferase (Luc). At 72 h post-transfection total cell extracts were analyzed by Western blotting using antibodies to 18E6, hScrib and as a loading control, α-actinin. The cells were independently transfected twice and the bar chart shows the mean level of 18E6, normalized to the level present with control luciferase siRNA, ± the range.

loss of the E6ΔPBM protein is greater than that of the wild-type showing that the mutant is less stable than the wild-type in the presence of ectopically expressed hScrib. Given the structure of E6 (Nomine et al., 2006) it seems unlikely that the mutation itself is having any major effect on the structural integrity of the protein; therefore we hypothesize that the PBM on the wild-type protein is actively involved in stabilizing the protein. Since E6 is known to be degraded by the proteasome (Kehmeier et al., 2002; Stewart et al., 2004), the PBM may protect certain pools of E6 from proteasomal degradation. To test this the wild-type and E6ΔPBM plasmids were co-transfected with HA-Scrib plasmid into NIKS cells. At 46 h post-transfection, the cells were treated for 2 h with the proteasome inhibitor, MG132 (Fig. 3B). The levels of wild-type E6 protein in the presence and absence of inhibitor are not statistically significantly different. In contrast, the levels of the E6ΔPBM protein are significantly increased in the presence of MG132, with the majority of this protein being degraded by the proteasome in the absence of the inhibitor. Together these data suggest that wild-type E6 protein is protected from proteasomal degradation by virtue of its PBM. Given that the difference in protein levels between transiently transfected wild-type and E6ΔPBM is observed when HA-Scrib is co-transfected but not in its absence (Fig. 1E), it seems reasonable to conclude that the stabilization effect results from an interaction between E6 and hScrib, rather than indirect effects of loss of the PBM.

Other PDZ domain-containing proteins, in addition to hScrib, can stabilize E6

In addition to hScrib, other PDZ domain-containing proteins including Dlg (the human homologue of the *Drosophila* tumor suppressor protein, dlg, found localized with hScrib at adherens junctions) and MAGI-1 (localized at tight junctions) are also bound and directed for degradation by HPV E6 (Gardiol et al., 1999; Glaunsinger et al., 2000; Kiyono et al., 1997). The ability of E6 to bind and degrade these PDZ domain-containing proteins appears to

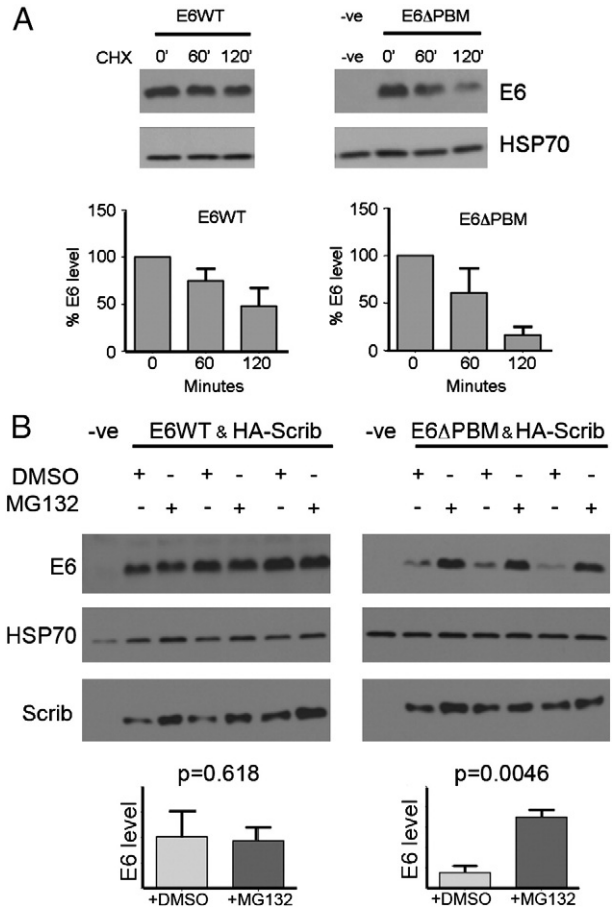

Fig. 3. The ΔPBM mutation reduces E6 levels by decreasing the protein's stability. NIKS cells were transiently co-transfected with plasmids that express E6WT or E6ΔPBM, and HA-Scrib. A) At 48 h post-transfection cells were treated with 50 μg ml^{-1} cycloheximide or DMSO for 60 or 120 min. Total cell extracts were analyzed by Western blot. Levels of E6 were assessed using ImageJ software. Cells were transfected in duplicate and bar charts show the mean E6 protein levels relative to the corresponding wild-type or E6ΔPBM level at time 0 + the standard deviation. An unpaired *T*-test was used to compare the E6 protein levels, at 120 min, $p = 0.032$. B) At 46 h post-transfection, cells were treated for a further 2 h with the proteasome inhibitor MG132 or DMSO. Total cell extracts were analyzed by Western blotting using antibodies to E6 and HA, and as a loading control, HSP70. Levels of E6 were assessed using ImageJ software. Cells were transfected in triplicate and bar charts show the mean E6 protein levels (in arbitrary units) + the standard deviation. Paired *T*-tests were used to compare the E6 protein levels and *p* values are given above the charts.

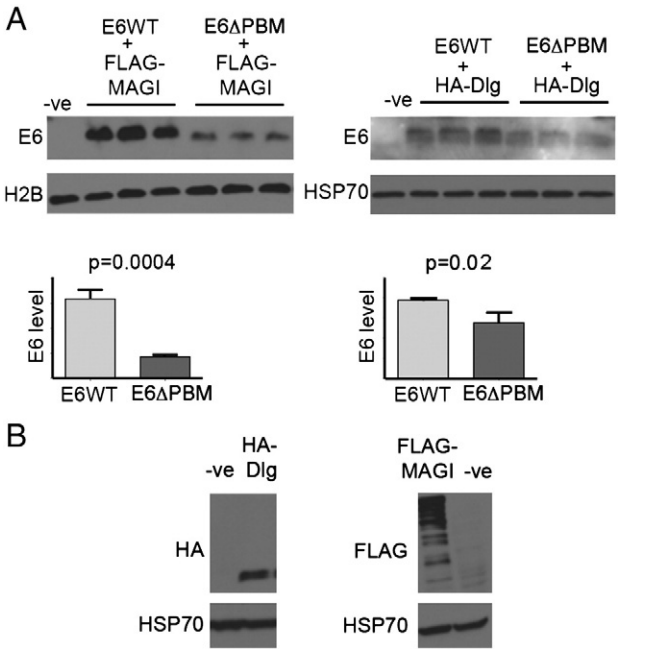

Fig. 4. Multiple PDZ domain-containing proteins can stabilize E6. A) NIKS or B) 293T cells were transiently transfected with plasmids that express HA-tagged Dlg (HA-Dlg) or FLAG-tagged MAGI (FLAG-MAGI), and E6WT or E6ΔPBM. Total cell extracts were obtained from cells 2 days post-transfection and Western blotted for E6, HA or FLAG, and as a loading control histone 2B (H2B) or HSP70. Levels of E6 were assessed using ImageJ software. Cells were transfected in triplicate and bar charts show the mean E6 levels (in arbitrary units) + the standard deviation, relative to loading controls. Unpaired *T*-tests were used to compare the E6 protein levels and *p* values are given above the charts.

vary, with HPV16 E6 showing a preference for hScrib, and 18 E6 preferring Dlg and MAGI-1 (Kranjec and Banks, 2010; Thomas et al., 2005). To determine whether other PDZ domain-containing proteins in addition to hScrib can affect E6 levels, the wild-type E6 and E6ΔPBM mutant plasmids were co-transfected with plasmids that express FLAG-MAGI or HA-Dlg into NIKS cells, and the levels of E6 proteins were assessed by Western blot (Fig. 4A). As with HA-Scrib, the level of wild-type E6 was higher than the ΔPBM mutant in the presence of both FLAG-MAGI and HA-Dlg, suggesting that these two PDZ domain containing proteins could similarly affect the levels of E6 expression in a PBM dependent manner. Since the FLAG-MAGI and HA-Dlg proteins could not actually be detected in the transfected NIKS cells, expression was verified by transfection into 293T cells (Fig. 4B).

Genomes lacking E6 proteins that can bind to PDZ domains show low-level E6 expression and a failure to maintain as episomes in NIKS cells

Having shown that hScrib can regulate E6 levels of expression, both in cervical cancer derived cell lines and under conditions of over-expression we were keen to assess whether the wild type and E6ΔPBM mutant proteins were similarly affected when expressed from the HPV16 genome and how this might impact upon the virus life cycle (Fig. 5). Initial attempts at detecting E6 protein expressed in the total cell populations from genomes were unsuccessful (data not shown), and this is not surprising given our previous observation that E6 transcript levels from genomes are over 100× lower than from the CMV promoter. This problem was further exacerbated by the relatively low efficiency of transfection into NIKS cells and the need to analyze the E6 levels very early after transfection, i.e. without prolonged selection, to prevent downstream effects indirectly affecting the E6 levels. To overcome this issue, the wild-type and E6ΔPBM genomes were co-transfected with a plasmid that expresses GFP into the NIKS cells. After 48 h FACS was used to identify GFP-expressing cells, thereby enriching for the cells containing HPV genomes. The levels of E6 proteins were compared in populations transfected with wild-type and E6ΔPBM mutant genomes, and again it was found that the levels of the E6ΔPBM protein were much lower than those of the wild-type (Fig. 5A). To confirm that the differences in protein were not due to differences in the levels of E6 transcripts these were assessed using RT-qPCR (Fig. 5B). The level of E6 transcripts is not significantly different between the wild-type and E6ΔPBM genomes, implying that the genomes are present at similar levels and that the difference in protein levels does not arise from differential transcription. This suggests that, when E6 is expressed from HPV genomes, the endogenous PDZ domain-containing proteins may stabilize the E6 protein via interaction with E6's PBM, as seen in the experiments carried out using recombinant expression plasmids.

Having confirmed that the E6 levels were lower in the E6ΔPBM genome populations than in the wild-type populations, we then assessed whether the difference was sufficient to disrupt aspects of the viral life-cycle. In order to have a productive life-cycle, it is first necessary for the viral genomes to become established as episomes in the host cell. It has already been shown for HPV31 E6, that mutations

in the PBM of E6 can affect viral copy numbers (Lee and Laimins, 2004), but in that study, it was not possible to directly assess the levels of the wild-type and mutant E6 proteins expressed from the genomes. We hypothesized that the lack of E6 stability we observe with the HPV type 16 E6ΔPBM mutant protein could also cause a reduction in viral copy number in cells transfected with the HPV16 genomes. First we confirmed using a transient replication assay that both the wild-type and E6ΔPBM genomes were able to be replicated within cells, i.e. that the ΔPBM mutation did not compromise other aspects of the plasmid's capacity to express and interact with the viral and cellular replication proteins. To do this NIKS cells were transfected with the genomes or a control plasmid that has the E4 open reading frame but no eukaryotic origin of replication or promoter. Four days post-transfection the proportion of replicated DNA (distinguishable from input DNA by virtue of its resistance to the restriction enzyme Dpn I) was quantified by qPCR using primers against E4 (Fig. 5C). Both genomes were found to replicate in this assay. To assess the ability of the wild-type and E6ΔPBM genomes to establish as episomes, the genomes were co-transfected with a blasticidin resistance plasmid into NIKS and populations were selected with blasticidin for 4 days. The cells were then passaged onto fresh feeders in the absence of blasticidin, and these cells were denoted passage one (P1). The DNA was extracted from the cells over a number of passages and the mean genome copy number per cell was assessed by qPCR (Fig. 5D). The copy number appears to be much lower in the 16E6ΔPBM populations than in the 16WT populations. Interestingly, the copy number of the 16E6ΔPBM mutant populations does seem to slightly increase at later passages. To further assess the populations, the status of their genomes was assessed by Southern blot with a view to determining whether they were episomal or integrated (Fig. 5E). The extracted DNA was cut with restriction enzymes that either do not cut, or cut once, the HPV16 genome. From this it was clear that the wild-type DNA was predominantly episomal, whilst the 16E6ΔPBM mutant DNA, although very difficult to detect, was predominantly integrated. To determine whether the inability to maintain episomes might be mediated by lower levels of E6 failing to degrade p53, the levels of p53 were assessed in the populations of transfected and FACS-sorted cells. It was found that the extent of p53 degradation was similar in the wild-type and E6ΔPBM mutant genome populations, suggesting that whatever the mechanism of loss of episomes, it does not involve p53 (Fig. 5F). Furthermore, the reduction in E6 levels is not sufficient to adversely affect p53 targeting, implying that only a subset of E6 is affected by loss of PDZ binding capacity.

To overcome the issue of the very low average copy number within the populations that made Southern blot analysis very difficult, the experiment was repeated but instead of analyzing populations of transfected cells, individual clones were isolated from the populations. Of course it was not possible to meaningfully assess the levels of the wild-type and E6ΔPBM proteins expressed from the genomes (as we already knew that the copy numbers would be different, even at the start of the cloning process). However, it was possible to assess the copy

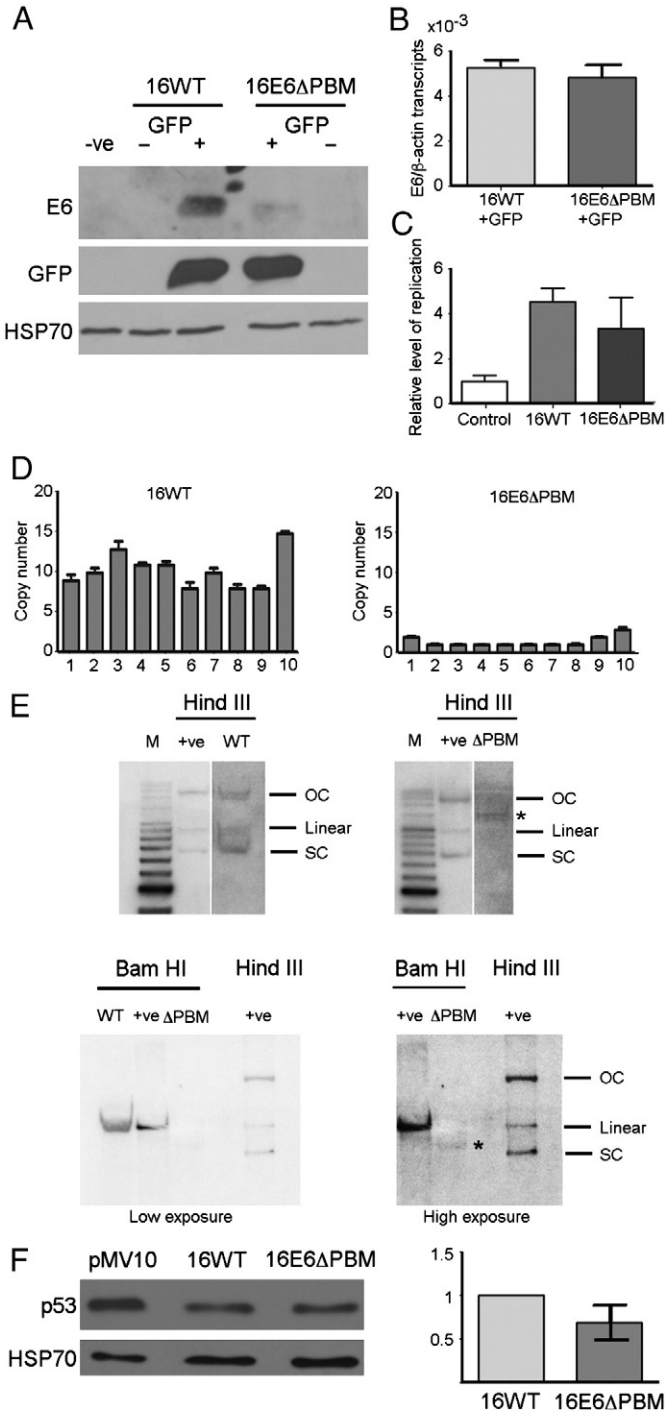

Fig. 5. Low E6 expression associated with the ΔPBM mutation correlates with the loss of episomes in HPV positive cell populations. NIKS cells were co-transfected with a plasmid that expresses GFP, and either wild-type HPV16 (16WT) or HPV16 genomes containing the E6ΔPBM mutation (16E6ΔPBM). A) At 48 h post-transfection the cells were harvested and the HPV genome-containing populations were enriched for using FACS based on their GFP signal. Total cell extracts were obtained from the GFP positive and negative populations, and Western blotted for E6, GFP, and as a loading control, HSP70. B) RNA was extracted from the total cell population, and the number of E6 transcripts, relative to β-actin transcripts was assessed using qPCR. The experiment was done in triplicate and the bar chart shows the mean level of transcripts + the standard deviation. An unpaired *T*-test was used to compare the E6 transcript levels, $p = 0.328$. C) NIKS cells were transfected with 16WT or 16E6ΔPBM genomes, or control plasmid (has E4 ORF but no eukaryotic origin of replication or promoter). At 4 days post-transfection episomal DNA was extracted and levels of replication quantified by qPCR. Four replicates were analyzed and the bar chart shows the mean level of relative replication (as a percentage value) + the standard deviation. D) NIKS cells were co-transfected with a blasticidin resistance plasmid and either the 16WT or 16E6ΔPBM genomes and HPV-positive populations were selected with blasticidin. DNA was extracted over the course of ten passages, and qPCR was used to assess the number of HPV copies per cell (copy number). Bar charts show the mean copy number per cell + the standard deviation of triplicate qPCR repeats and are representative of two repeat experiments. E) DNA was analyzed by Southern blot, using either restriction enzymes that do or do not cut HPV16 (Bam HI and Hind III respectively), and an HPV-positive episomal NIKS cell line DNA as a positive control. The positions of linear, open circular (OC) and supercoiled (SC) episomal genomes are indicated, as is the position of likely integrated DNA (*). F) NIKS cells were co-transfected with a plasmid that expresses GFP, and either 16WT or 16E6ΔPBM or a control plasmid pMV10. At 48 h post-transfection the cells were harvested and transfected populations were enriched for using FACS based on their GFP signal. Total cell extracts were obtained from the GFP positive populations, and Western blotted for p53, and as a control, HSP70. The cells were independently transfected twice and the bar chart shows the mean level of p53 in the16E6ΔPBM population, normalized to the level in the 16WT population, ± the range.

number of a large number of clones and monitor the status of the genomes over time (Fig. 6). After producing the clonal lines we found that whilst 70% of the 16WT clones were positive for HPV16 DNA, only 8% of the 16E6ΔPBM mutant clones contained genomes (Fig. 6A). This suggests that the 16E6ΔPBM mutant genomes are less able to be maintained in the cells than the wild-type genomes. In fact the 16E6ΔPBM clonal lines that were positive (2 out of 26), although having copy numbers similar to the 16WT clones tested, were both found to harbor integrated copies of HPV16 DNA (Fig. 6B). To confirm that the integration phenotype that we observed with the 16E6ΔPBM genome was specific to this mutant, and not a result of our methodology, we monitored the status of the wild-type genome over time (Fig. 6C). The wild-type genomes were observed to be episomal irrespective of the time-point analyzed. We have therefore no reason to believe that the loss of the episomal phenotype was a result of our methodology and rather is specific to the 16E6ΔPBM genomes.

Discussion

The interaction of high-risk E6 proteins with PDZ domain-containing proteins has been widely reported; however its role in the viral life-cycle has not been extensively analyzed. Here we show that, at the levels of E6 expressed from genomes, the interaction results in a significant stabilization of the E6 protein, which suggests that at least one consequence of the association may be to ensure that sufficient levels of E6 are present in the cells. Even in a cancer cell line in which the level of E6 is much higher, we observed a significant decrease in E6 levels when hScrib was knocked-down. It will be interesting to see whether the same change in levels is observed if the levels of Dlg or MAGI are knocked-down and whether the different HPV E6 types are stabilized to different extents by particular PDZ domain-containing proteins. The work also highlights the need for caution when interpreting the result of mutating the E6 PBM, where changes in phenotype may result from changes in E6 level rather than direct effects of E6 on PDZ domain-containing proteins. It is also worth noting that the particular mutation used is a 4 amino acid deletion of the E6 carboxy terminus; whether more subtle point mutations within the PBM will have a similar effect, remains to be determined.

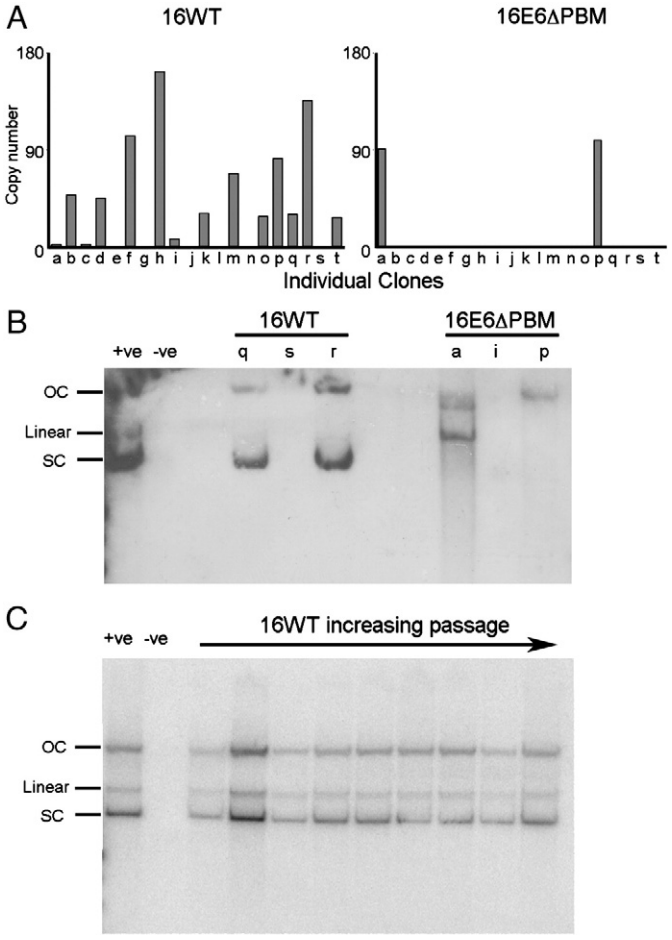

Fig. 6. The ΔPBM mutation is associated with a reduced ability to establish clonal HPV-positive episomal cell lines. A) NIKS cells were co-transfected with a blasticidin resistance plasmid and either the 16WT or 16E6ΔPBM genomes. The cells were grown for 2 days then seeded at low density with blasticidin treatment to obtain clonal lines of cells. DNA was extracted and qPCR was used to assess copy number. B) The DNA from three clones each of 16WT and 16E6ΔPBM was cut with Hind III restriction enzyme (does not cut HPV16 DNA) and Southern blotted. The positions of linear, open circular (OC) and supercoiled (SC) episomal genomes are indicated. HPV-positive and -negative NIKS DNAs were included as positive and negative controls respectively. C) DNA from one of the 16WT clones was extracted over multiple passages, cut with Hind III and analyzed by Southern blot.

It is interesting that a protein that E6 targets for degradation, hScrib, (Nakagawa and Huibregtse, 2000), itself appears to stabilize E6. This is however also the case for another of E6's targets, the ubiquitin ligase E6AP (Kao et al., 2000; Tomaic et al., 2009). Thus the interactions between E6 and its binding partners are complex and we hypothesize that the exact outcomes depend on the levels, as well as the modifications and localizations of the proteins, with different cellular pools behaving in different ways. E6 is already known to interact differently with PDZ proteins depending on which cellular site they are located at and how they are modified (Massimi et al., 2004; Massimi et al., 2006; Narayan et al., 2009). When expressed at high level, e.g. in cancer cell lines, E6 appears to induce degradation of PDZ domain-containing proteins, but this has not been reported for E6 expressed at levels typical of productive infections. Indeed in our own experiments, the levels of hScrib appear the same in the GFP-sorted populations that express wild-type or E6ΔPBM proteins as those in control populations (data not shown). How a given PDZ domain-interaction might stabilize E6 itself is currently unclear; however the E6 PBM is a highly unstructured region, but this obtains a defined structure once in complex with a specific PDZ domain (Charbonnier et al., 2011; Nomine et al., 2006; Zhang et al., 2007), suggesting that this may in turn contribute indirectly towards E6 stability. Now that current Western blotting protocols can detect E6 protein expressed from high-risk HPV genomes, future studies will be able to monitor the level of E6 protein expressed. Ensuring adequate expression of any E6 mutants may help elucidate the role of the different E6 activities, localizations, and binding partners in E6 protein function.

One known function of E6 is that it is necessary for viral episomal maintenance, as mutants lacking a functional E6 protein are unable to persist within the cell (Park and Androphy 2002; Thomas, et al. 1999). The precise activity of E6 that is necessary for this function is not yet clear. Here, we have shown that mutant HPV16 genomes that lack the E6 PBM are unable to persist episomally in NIKS cells and are found integrated into the cellular DNA. In agreement with these results, a study of HPV31 genomes containing E6 mutants that do not bind PDZ domains showed that these genomes do not maintain at wild-type levels and are often found integrated at later passages (Lee and Laimins 2004). Together these results suggest that loss of the PBM of E6 has a dramatic effect on genome maintenance. The reduction in E6 levels following loss of the E6 PBM might be expected to have additional effects, and although HPV31 E6 protein levels were not established in the study of Lee and Laimins (2004), the observed effects on cell growth and morphological differentiation might be explained by the lack of PDZ-mediated E6 stabilization. Lower levels of E6 might also explain the reduced epithelial hyperplasia observed in transgenic mice that express a PDZ-binding mutant rather than wild-type E6 (Nguyen et al., 2003).

Of primary interest remains as to why E6 is required for the episomal maintenance of viral genomes. Our maintenance experiments do not distinguish between direct effects of the E6ΔPBM mutation in preventing

binding to PDZ domain-containing proteins, and effects mediated by changing the E6 levels and therefore its effects on other cellular proteins. Other activities of E6 including the degradation of p53 (Oh et al., 2004; Park and Androphy, 2002; Thomas et al., 1999) and the degradation of E6TP1 (Lee et al., 2007) have previously been implicated in episomal maintenance. How these activities support maintenance is not understood, although it has been suggested that p53 may be a suppressor of the amplificational replication of HPV following infection (Lepik et al., 1998). However it seems reasonable to hypothesize that a tight regulation of the levels of E6 post-infection may be crucial for the establishment of a replication-competent environment in the basal cells. Our results suggest that in the absence of a PBM, the levels of E6 are not stabilized, and this may have implications for the ability of E6 to affect other cellular proteins. However, the capacity of the mutant genomes to target p53 seems to be unaffected, suggesting that only E6 found in conjunction with hScrib, or in that particular cellular location is susceptible to enhanced degradation when the PBM is deleted. This also suggests that the pool of E6 that is stabilized by hScrib may not be that which is involved in p53 degradation and this is supported by previous observations showing that the levels of expression of an E6 mutant defective in PDZ recognition were still sufficient to degrade p53 (Foster et al., 1994). We have also observed that genomes defective for p53 degradation do not maintain in NIKS cells; however, their phenotype appears different from that of the 16E6ΔPBM mutant genomes (data not shown). Taken together, these observations suggest that E6 activities in addition to those on p53 are necessary to facilitate episomal maintenance.

In addition to stabilizing E6, the interaction with some PDZ domain-containing proteins and their resulting degradation may also be directly involved in genome maintenance of the high-risk viruses. How this may come about is not clear. PDZ domain-containing proteins are signal transducers, involved in cell-cycle progression and regulation of cell polarity (Pim and Banks, 2010), and the disruption of these functions may be necessary for creating a cellular environment that is conducive to genome maintenance. A systematic knockdown of expression of selected E6 PDZ targets in the context of the wild-type and mutant genomes should help clarify which PDZ domain-containing proteins are relevant for the in vivo stabilization and any further involvement of these proteins in genome maintenance. It is interesting to note that low-risk HPV, whose E6 proteins are not capable of binding to PDZ domain-containing proteins, is not well maintained as episomes in cell culture (our own observations and Oh et al., 2004). This may suggest that the interaction between E6 proteins and PDZ domain-containing proteins is important in allowing maintenance of HPV genomes under particular conditions, such as in the proliferating basal cells present in lesions induced by high-risk papillomaviruses.

Materials and methods

Routine cell culture

NIKS, an HPV-negative, spontaneously immortalized keratinocyte cell line (Allen-Hoffmann et al., 2000), and an HPV16-positive episomal NIKS line (Laurson et al., 2010) were maintained at sub-confluent levels on γ-irradiated J2-3T3 feeder cells, as previously described (Lambert et al., 2005). When required, cells were treated with 40 μM MG132, 50 μg ml^{-1} cycloheximide or an equivalent volume of DMSO. HeLa cells were maintained as previously described (Kranjec and Banks, 2010). 293T cells were maintained in DMEM supplemented with 10% fetal calf serum and 1% penicillin and streptomycin.

Plasmids

The pSPW12 plasmid (a kind gift from Prof. Margaret Stanley), containing the HPV16 genome was mutated to pSPW12E6ΔPBM using PfuUltra (Stratagene), and the forward and reverse primers, gcagatcatcaagaacacgtagataaacccagctgtaatcatgcatgg and gcagctctgtgcataactgtggtaactttgtggcgctctcctgtgggtcctgaaacattgc. The 16WT and 16E6ΔPBM genomes were excised from the Bam HI site in these plasmids and recircularized as previously described (Lambert et al., 2005). The pMV11E6 and pMV11E6ΔPBM plasmids were created by sub-cloning the E6 ORFs from pSPW12 and pSPW12E6ΔPBM plasmids into the Bam HI and Eco RI sites of pMV11 (using the forward primer gctgggatccatgcaccaaaagagaactgcaatg, reverse primer aggcgaattcttacagctgggtttctctacgtgttc for wild-type E6 and aggcgaattcttacagctgggttatctacgtgttc for the E6ΔPBM mutant). The pMV10 plasmid that expresses β-galactosidase and the plasmid that expresses HPV11 E6 have been previously described (Forrester et al., 1992; Glaunsinger et al., 2000). Plasmids that express wild-type and mutant hScrib (pCDNA:HA-hScrib and pCDNA:hScribΔPDZ), HA-Dlg (pGWI:HA-Dlg) and FLAG-MAGI (pcDNA:FLAG-MAGI-1c) have been described previously (Gardiol et al., 1999; Glaunsinger et al., 2000; Nagasaka et al., 2010). The prokaryotic pET-28 plasmid containing 16E1^E4 has been described before (McIntosh et al., 2008).

Transfections

For transient transfections of NIKS cells, 1 μg of DNA was transfected into the cells using Effectene (Qiagen), according to the manufacturer's instructions. For stable transfections of NIKS cells, 5×10^5 cells were seeded over 1×10^5 J2-3T3 cells per well of a six well plate the day before the transfection. The cells were transfected with 800 ng of recircularized HPV16 DNA and 200 ng of the blasticidin-resistant plasmid pcDNA6 (Invitrogen) using Effectene. The next day the cells were seeded onto 10 cm plates over blasticidin-resistant feeders and the day after were treated with 8 μg ml^{-1} blasticidin. The blasticidin treatment was carried out for 4 days, at which point the surviving cells were transferred to new feeders in the absence of blasticidin and designated passage one (P1). For the cloning experiments, the cells were transfected as above, grown for 2 days and then plated at low density on blasticidin-resistant feeders. The cells were treated with blasticidin as above and were then grown to the stage of visible colonies that were transferred first to a 6 well plate, then to a 10 cm plate, at which point their DNA was analyzed for the presence of HPV16 episomes. For siRNA delivery, cells were transfected as previously described (Kranjec and Banks, 2010) but using SCRIB ON-TARGETplus SMARTpool siRNA, and harvested at 72 h post transfection.

Southern blots, qPCR and RT-qPCR

Total DNA was extracted using the QIAamp DNA Mini Kit (Qiagen) and quantified on a NanoDrop ND-1000 spectrophotometer. Total RNA was extracted using the QIAshredder and RNeasy Mini kits (Qiagen), and the DNA-free kit (Ambion). The SuperScript II Reverse Transcription Kit (Invitrogen) and random primers were used to generate cDNA. Absolute QPCR SYBR Green ROX mix was used to quantify the numbers of HPV genomes (using E4 primers, and as a control, GAPDH primers), or the levels of E6 transcript cDNA (using E6 primers, and as a control, β-actin primers) on a Prism 7000 Sequence Detection System (Applied Biosystems). The genomic DNA was also separated on 1% agarose gels, transferred to Immobilon-NY+ Transfer Membrane (Millipore) and Southern blotted using 32P-dCTP labeled pSPW12 plasmid as a probe. Southern blots were adjusted for brightness and contrast using Photoshop. Regions of gels that have been adjusted for brightness and contrast to different levels are separated by a white band and show only relative position, not relative intensity of bands.

Transient replication assays

The method for the transient replication assays was modified from a previously described protocol (Taylor and Morgan, 2003). Briefly,

NIKS cells (5.5×10^5) were seeded onto J2-3T3 cells (1×10^5) and the following day transfected with equimolar amounts of either recircularized wild-type or mutant HPV16 DNA (1 μg) or pET-28 plasmid carrying the E1^E4 cDNA (700 ng, plus 300 ng of pMV11 plasmid). Four days post-transfection the cells were trypsinized and resuspended in 0.5 ml PBS at a concentration of 1.8×10^6 cells/ml. Episomal DNA was extracted as previously described (Hoffmann et al., 2006). Following ethanol precipitation, 3 μl of DNA was digested with Dpn I for 3 h, and then exonuclease III for 30 min, followed by enzyme inactivation at 70 °C for 30 min. Another 3 μl of DNA was treated in the same way, but without any enzyme, and these represent the undigested samples. Amounts of HPV16 DNA were measured in both Dpn I-digested and undigested samples by qPCR, using primers against the E4 ORF of HPV16, and the former was divided by the latter to give a relative value for replication.

SDS–PAGE and Western blotting

To obtain total cell extracts from NIKS cells, 25 U of benzonase was added to the cell pellet, which was then lysed at 95 °C for 7 min in 150 μl of protein extraction buffer (6% SDS, 1% triton, 0.5% sodium deoxycholate, 150 mM NaCl, 0.005 mM EDTA, 50 mM Tris–HCl pH 8.0, supplemented with Protease Inhibitor Cocktail (Sigma)). The soluble protein concentration was assessed using the DC Protein Assay Kit (Bio-Rad), then 100 mM DTT and loading dye were added. HeLa cells were lysed as previously described (Kranjec and Banks, 2010). Equivalent amounts of protein were separated on SDS–polyacrylamide gels, and transferred to 0.2 or 0.4 μm PVDF membrane (Millipore). 16E6 was detected with antibodies 2E-3F8 or 1E-6F4 (Euromedex), 18E6 with an anti-18E6 antibody (kindly provided by Arbor Vita Corporation), HSP70 with W-27 (Santa Cruz), β-gal with ab616 (Abcam), hScrib with C-20 (Santa Cruz Biotechnology), HA-tag with H6908 (Sigma), FLAG-tag with 2368 (Cell Signalling), histone 2B with 07-371 (Upstate), α-actinin with H-2 (Santa Cruz Biotechnology), GFP with sc-9996 (Santa Cruz) then anti-mouse IgG-HRP NA931V (GE Healthcare), or anti-rabbit IgG-HRP NA934V (GE Healthcare) or anti-goat HRP P0160 (DAKO), followed by detection using either the ECL Western Blotting Detection kit (GE Healthcare), the Immobilon Western Chemiluminescent HRP kit (Millipore) or the ECL Advance kit (GE Healthcare). Western blots were quantified using ImageJ and adjusted for brightness and contrast in Photoshop.

Fluorescent activated cell sorting (FACS)

The day before transfection, 5.5×10^5 NIKS were seeded over 1×10^5 J2-3T3 cells per well of a six well plate. These were transfected with 800 ng of recircularized HPV16 DNA and 200 ng of pcieGFP plasmid. 25 wells were transfected with each recircularized DNA, 16WT and 16E6ΔPBM or with pMV10 plasmid as a control. Two days post-transfection, the cells were washed with 1× DPBS (PAA) and then detached from the plate using Accutase (Millipore). The cells were pelleted and resuspended in 1.5 ml of Dulbecco's PBS containing 0.5% FBS and 5 mM EDTA. Immediately before sorting, the cells were passed through a 40 μm cell strainer (BD Biosciences). The cells were sorted on a Becton Dickinson FACS ARIA II Cell Sorter.

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