Activity of the human papillomavirus E6 PDZ-binding motif correlates with an enhanced morphological transformation of immortalized human keratinocytes

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Summary

Human papillomavirus E6 oncoproteins induce the proteasomal degradation of several multi-PDZ (PSD95/Dlg/ZO-1) domain-containing proteins such as the human homologue of *Drosophila* discs large. Binding to PDZ domain-containing proteins is mediated by a PDZ-binding motif contained within the C-terminus of E6. The ability of E6 proteins to induce degradation of PDZ domain-containing proteins correlates with their oncogenic potential. Here we examined the biological effect of this region of the human papillomavirus type 18 E6 oncoprotein on keratinocyte morphology. Our results show that in simian virus 40-immortalized human keratinocytes, stable expression of E6 correlated with the induction of an exaggerated mesenchymal-like morphology and actin cytoskeleton disorganization compared with parental cells. The altered phenotype was accentuated in cells expressing an E6 protein containing a mutation (Arg153Leu) within a protein kinase A recognition motif that abrogates protein kinase A's negative regulation of the activity of the PDZ-binding domain. The E6-induced changes indicated an epithelial-mesenchymal transition and were supported by the finding that E6-expressing cells contained vimentin. Changes to the epithelial phenotype of cells expressing a mutant E6 protein (Thr156Glu) that is unable to degrade discs large was significantly less marked, although they did show evidence of epithelial-mesenchymal transition. These observations imply that the activity of the E6 PDZ-binding motif contributes only to a part of the transition. Further analysis of the E6 cell lines showed a decrease in adherens junction and desmosome formation. Cells expressing a functional PDZ-binding motif showed the greatest disruption of intercellular junction formation, but this did not correlate with a decrease in total cellular levels of the individual components of adhesion junctions. This suggests that the activity of the PDZ-binding motif may have influenced either the assembly or integrity of functional adhesion complexes. An E6-mediated decrease in peripheral membrane levels of PDZ proteins like discs large could be the basis for the enhanced morphological transformation of immortalized keratinocytes.

Key words: Human papillomavirus, E6, Discs large, Cell shape, Intercellular adhesion, Epithelial-mesenchymal transition

Introduction

A small subset of human papillomavirus (HPV) types has been shown to be the causative agents of several epithelial-based cancers, the most common being carcinoma of the uterine cervix (Walboomers et al., 1999). With regard to infections of the anogenital tract, those caused by HPV types such as 16 and 18 have a high risk of progressing to a malignant state, whereas infections associated with HPV types 6 and 11 are regarded as low risk. The malignant potential of the high-risk types is attributable largely to the transforming activities of the two early proteins E6 and E7. The activity of these two oncoproteins is essential and sufficient for oncogenic transformation of cells in vitro (Hawley-Nelson et al., 1989; Munger et al., 1989), and their expression is sustained in HPV-associated malignancies (Schwarz et al., 1985).

Studies in transgenic mice intended to dissect the individual roles of E6 and E7 in immortalization and transformation conclude that these oncoproteins have different contributions to these processes; mice expressing HPV16 E7 in the skin form multiple tumours that are predominantly benign, whereas E6 expression correlates with the formation of highly aggressive metastatic skin tumours (Herber et al., 1996; Song et al., 1999). It has been suggested that in the development of HPV-induced cancers, E7-associated functions may contribute primarily to the early stages of oncogenesis, whereas those of E6 contribute to the later stages (Song et al., 2000). Although an E6-p53 interaction is important in the ability of E6 to bring about cell transformation, there is growing evidence that many other targets of the oncoprotein play a vital role in E6-induced transformation and development of malignancy and that, like p53, E6 directs several of these targets for degradation through ubiquitin-proteasome pathways (for a review, see Thomas et al., 2002b). These include several proteins that contain multiple PSD95/Dlg/ZO-1 (PDZ) domains. E6 proteins of high-risk but not low-risk virus types contain a C-terminal motif that resembles a class 1 PDZ-binding motif (S/TXV), and this

facilitates the interaction of the E6 oncoprotein with PDZ domain-containing proteins. The first identified target of the PDZ-binding motif was the human homologue of the *Drosophila discs large-1* gene product Dlg/SAP97, a member of the membrane-associated guanylate kinase (MAGUK) family (Lee et al., 1997; Kiyono et al., 1997; Gardiol et al., 1999). Subsequently, three other MAGUKs – the highly homologous MAGI-1, -2 and -3 proteins – together with the human homologue of the leucine-rich repeat and PDZ domains (LAP) family member Scribble (hScrib/Vartul) and a tight junction PDZ protein, MUPP1, were identified as targets of high-risk E6 proteins (Glaunsinger et al., 2000; Lee et al., 2000; Nakagawa and Huibregtse, 2000; Thomas et al., 2002a).

On the basis of invertebrate studies, MAGUK and LAP proteins are thought to function as molecular scaffolds, organizing macromolecular complexes that consist of signalling and structural proteins at specific sites at the membrane (Dimitratos et al., 1999; Bryant and Huwe, 2000). In *Drosophila*, Dlg locates to septate junctions (equivalent to mammalian tight junctions), and disruption of *Dlg* results in loss of formation of these junctions, defective cell polarity and loss of differentiation (Bilder et al., 2000; Woods et al., 1996). Furthermore, recessive mutations in the *Dlg* locus results in neoplastic transformation of epithelial cells (Stewart et al., 1972; Woods and Bryant, 1989). It has therefore been proposed that Dlg functions to organize signalling complexes at cell junctions and thereby negatively regulates the growth of epithelial cells. Interestingly, mammalian forms of Dlg can successfully rescue the phenotype of the *Drosophila* Dlg mutants, implying that this protein is also involved in controlling adhesion junction formation and epithelial cell growth (Thomas et al., 1997). Growth control may occur at the G1-to-S-phase progression (Ishidate et al., 2000) and involve complex formation with the adenomatous polyposis coli (APC) protein (Matsumine et al., 1996; Ishidate et al., 2000). Interestingly, severe defects in both epithelial organization and polarity also occur in *Drosophila* embryos that lack Scribble (Bilder and Perrimon, 2000). In addition, Dlg is required for the maintenance of epithelial architecture in *Caenorhabditis elegans* (Bossinger et al., 2001; Firestein and Rongo, 2001). As many growth factor receptors are polarized to a specific membrane domain, mislocalization of such proteins may affect signalling pathways that maintain cells in a differentiated, nonproliferative state.

The preservation of the PDZ-binding motif in E6 proteins of the high-risk but not the low-risk HPV types suggests that inactivation of proteins such as Dlg may contribute to the oncogenic functions of E6. Studies investigating this have shown that although this C-terminal region may not be necessary for HPV16 E6-mediated immortalization of mammary cells (Kiyono et al., 1998), it is required for HPV16 E6 transformation of rodent cells, and this is independent of p53 inactivation (Kiyono et al., 1997). Furthermore, HPV is not unique in its ability to inactivate MAGUK proteins. Several other viral oncoproteins such as human T-cell leukaemia virus (HTLV)-1 Tax protein and adenovirus (Ad) type 9 E4-open reading frame (ORF) 1 contain a similar PDZ-binding motif at their C-termini and are able to bind to Dlg, membrane-associated guanylase kinase (MAGUK) with inverted orientation (MAGI)-1 and multi-PD2-domain-containing protein (MUPP-1) (Lee et al., 1997; Lee et al., 2000; Glaunsinger et al., 2000). The conservation of this C-terminal PDZ-binding motif between unrelated oncoproteins supports the hypothesis that its activities are involved in HPV-mediated carcinogenesis. However, despite the revelations about the molecular basis for the interaction between the C-terminal PDZ-binding domain and its protein targets, the biological function of this region in E6 is not well understood. To understand in more detail the biological effects of this E6 domain, we generated stable lines that expressed HPV18 E6 in SV40-immortalized human keratinocytes and compared the phenotype of these cells to those generated with mutant HPV18 E6 molecules that were impaired or enhanced in their ability to interact with PDZ domain-containing proteins.

Materials and Methods

Plasmids

The E6 expression plasmids used in this study contain the HPV18 E6 wild-type or mutant (Arg153Leu and Thr156Glu) cDNAs under the control of the cytomegalovirus early promoter in the plasmid vector pcDNA-3 (Invitrogen, Abingdon, UK). Their construction has been described elsewhere (Gardiol et al., 1999; Kühne et al., 2000).

Generation of stable cell lines

SVJD cells, an SV40-immortalized human skin keratinocyte line (Brown and Gallimore, 1987; Roberts et al., 1993) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 20 mM HEPES, glutamine and antibiotics. On reaching 70% of confluency, 2×10^6 cells were harvested by trypsinization, resuspended in 800 μl ice-cold phosphate-buffered saline (PBS) and placed in an electroporation cuvette (BioRad, Hemel Hempstead, UK) with an electrode spacing of 4.0 mm. Following the addition of 20 μg plasmid DNA, cells were electroporated using 960 μFD/0.22 kV, incubated on ice for 10 minutes and transferred to 10 ml normal growth medium before plating. Transfected cells were selected by the addition of 400 μg/ml G418 sulphate, beginning 24 to 48 hours after transfection. Colonies that formed were either pooled for immediate use or cryopreserved in the vapour phase of liquid nitrogen.

Isolation of messenger RNA and PCR amplification

Messenger RNA (mRNA) was isolated from cultured cells using an mRNA Isolation kit (Roche, Mannheim, Germany) following the manufacturer's instructions. Before polymerase chain reaction (PCR) amplification, contaminating DNA was removed from the mRNA preparations by incubating 1 μg of mRNA with 1 unit of RNase-free DNase I (Gibco BRL) at room temperature for 15 minutes. The reaction was terminated by heating at 65°C for 10 minutes in the presence of 25 mM EDTA, pH 7.5. Full-length cDNA was generated using the Reverse-iT 1st Strand Synthesis Kit (ABgene). To amplify E6 cDNA from the first strand synthesis reaction, the E6-specific primers 5′-ctatggcgcgctttgaggatc-3′ and 5′-tctgcgtcgttggagtcattcc-3′ (Gibco BRL) were used at a final concentration of 0.2 μM in the presence of 250 μM of each dNTP and 1.5 units of *Taq* DNA polymerase (Roche). An initial 2 minute incubation at 94°C was followed by 35 cycles of: 94°C for 20 seconds, 59°C for 45 seconds (55°C for Leu18E6), 72°C for 30 seconds. Finally, the reactions were incubated at 72°C for 7 minutes. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) amplifications were performed with GAPDH-specific primers (a kind gift from Aris Eliopoulos, Cancer Research UK Institute for Cancer Studies, Birmingham, UK) as previously described (Eliopoulos et al., 2000). The PCR-amplified products were electrophoresed in a 0.8% (w/v) agarose gel and viewed on a UV-transilluminator.

Antibodies, immunofluorescence microscopy and western blotting

For indirect immunofluorescence staining, monoclonal antibodies (MAb) against E-cadherin (1/200 dilution) and P-cadherin (1/200 dilution) were obtained from BD Transduction Laboratories (Oxford, UK). MAbs against α-, β- and γ-catenins (1/200, 1/300, 1/200 dilutions, respectively) and the 58K Golgi protein (1/100 dilution) were obtained from Sigma (Poole, UK). A Dlg MAb (2D11, 1/30 dilution) was obtained from Santa Cruz Biotechnology (California, USA). F-actin was visualized using rhodamine-conjugated phalloidin (Sigma).

Cells grown on glass slides were fixed in 4% (w/v) paraformaldehyde in PBS for 10 minutes and permeabilized in 0.2% (v/v) Triton X-100 for 5 minutes at room temperature. Immunofluorescence staining was performed as described elsewhere (Roberts et al., 2003). Immune complexes were visualized using Alexa 488- or Alexa 595-labelled anti-species specific antibody conjugates (Molecular Probes, Eugene, OR). Immunofluorescence was observed using a Nikon Eclipse E600 microscope with appropriate filters sets and images were captured using a Leica DC200 digital camera. Images were assembled in Adobe Photoshop 6.0.

For western blotting, cells were lysed in 8 M urea, 50 mM Tris pH 8.0, 0.15 M β-mercaptoethanol, and equal amounts (40 μg) of protein electrophoresed on 8-10% SDS-polyacrylamide gels. Separated proteins were transferred to a nitrocellulose membrane and western blotting performed with the antibodies listed above (Dlg used at 1/400 dilution, E- and P-cadherin used at dilutions of 1/5000 and 1/2000, respectively, and α- and β-catenin used at 1/2000 and 1/7500, respectively). For intermediate filament detection, anti-vimentin (Progen, 1/200 dilution) and anti-cytokeratin 8 (Cl. M20, Sigma, 1/1000 dilution) MAbs were used. To control for loading, actin levels were determined with an anti-β-actin MAb (Sigma, 1/40,000 dilution). Primary antibodies were detected with horseradish peroxidase-labelled anti-species immunoglobulins (DAKO) and membranes subjected to chemiluminescence (ECL, Amersham Pharmacia).

Results

The aim of this study was to address the biological effect of the C-terminal PDZ-binding domain of HPV18 E6 in the target cell type of HPVs – the human keratinocyte. We generated stable cell lines that expressed HPV18 E6 molecules containing an intact PDZ-binding domain (wild type) or HPV18 E6 proteins containing C-terminal Thr156Glu or Arg153Leu mutations (Fig. 1A). Threonine 156 lies within the consensus PDZ-binding motif and the hydroxyl oxygen of this residue is reported to be involved directly in the interaction with the PDZ domain (Doyle et al., 1996). It has been shown previously that the Thr156Glu mutation abrogates the ability of HPV18 E6 to bind to Dlg and induce its degradation in an in vivo degradation assay (Gardiol et al., 1999). Arginine 153 lies outside the PDZ-binding motif but is part of a consensus protein kinase A (PKA) recognition site ((RXX[S/T]), residues 153 to 156) and overlaps the PDZ-binding motif, the threonine at position 156 acting as the putative phosphoacceptor. Stimulation of PKA activity inhibits HPV18 E6-induced degradation of Dlg, but not of p53 (Kühne et al., 2000). Substituting arginine 153 in the PKA recognition site with leucine abrogates PKA's negative regulation of Dlg degradation, and consequently this mutant protein degrades Dlg in an E6-dependent, constitutive manner, which is independent of cellular PKA levels (Kühne et al., 2000).

An SV40-immortalized human epidermal keratinocyte line, SVJD, was used to generate stable cell lines expressing the HPV18 E6 proteins. This keratinocyte line retains many of the characteristics of primary keratinocytes, and cells grow as colonies with a 'cobblestone' appearance and display typical epithelial adhesion characteristics, e.g. formation of strong adherens junctions (AJ) and desmosomes (Fig. 3, Fig. 6A,B, and data not shown). In these experiments, SVJD cells were used at relatively low passage (between 30 and 35). At this passage level SVJD cells are immortal but not tumorigenic (P. H. Gallimore, personal communication), and will differentiate in the presence of high levels of calcium and serum, as shown by the expression of filaggrin and keratins K1 and K10 – late markers of terminal differentiation (J. C. Steele and S.R., unpublished).

The HPV18 E6 plasmids were introduced into SVJD cells by electroporation, and approximately similar numbers of G418-resistant colonies were pooled and used either immediately or expanded through no more than six passages. In all of the experiments described in this study, the different cell lines had either been through an equal number of passages, or were within one passage of each other. The cell lines are referred to as WT18E6 (wild type), Glu18E6 (Thr156Glu) and Leu18E6 (Arg153Leu). A control line that carried the empty expression cassette, and which was negative for E6 expression, is referred to as Neo. At least seven cell lines were generated for each plasmid.

Expression of an intact PDZ-binding motif correlates with a low level of Dlg protein

Historically the detection of HPV E6 proteins in cells has proved problematic, largely because of a lack of high avidity E6 antisera and low levels of E6 expression in cells. In addition, the use of N- or C-terminal epitope-tagged forms of E6 may not give a true reflection of E6's biology as the presence of tags can adversely affect the function of the oncoprotein, such as its ability to target and degrade the cellular PDZ proteins (L.B., unpublished). Therefore, to be confident that E6 was being expressed in the generated cell lines, we assayed for E6 mRNA transcripts by semi-quantitative reverse transcription (RT)-PCR. An equal amount of mRNA was isolated from each of the SVJD-E6 lines and the Neo control cell line, then converted into cDNA by reverse transcription and amplified using HPV18 E6-specific primers. Messenger RNA from HPV18 DNA-positive invasive cervical adenocarcinoma-derived HeLa cells was included as a positive control for E6 in the RT-PCR reactions. Gel analysis of the PCR products showed that several bands were produced from RNA isolated from each of the different E6 lines, and these were identical in size to those from HeLa RNA (Fig. 1B). Previous analysis of E6 transcripts in HeLa cells has shown that the upper band of approximately 450 bp corresponds to the full-length E6 protein, whereas the more abundant lower band of about 280 bp represents the spliced transcript for the E6* protein (Schneider-Gädicke and Schwarz, 1986); this was confirmed by DNA sequencing (data not shown). No E6-specific bands were detected in the control cell line, Neo. The E6* protein lacks the PDZ-binding motif present in the full-length protein and hence cannot interact with E6's PDZ domain-containing targets. Using the same pcDNA-derived expression plasmid in transient transfection studies the levels of E6 and E6* protein are approximately the same (data not

A

		Dlg Degradation		
		In Vitro	In Vivo	PKA Inhibition
	S			
PDZ Consensus	XTXV			
HPV18	CCNRARQERLQRRRETQV (141–158)	Yes	Yes	Yes
PKA Consensus	RXXT			
Thr156Glu	CCNRARQERLQRRREEQV	No	No	NT
Arg153Leu	CCNRARQERLQRLRETQV	Yes[a]	Yes	No

B

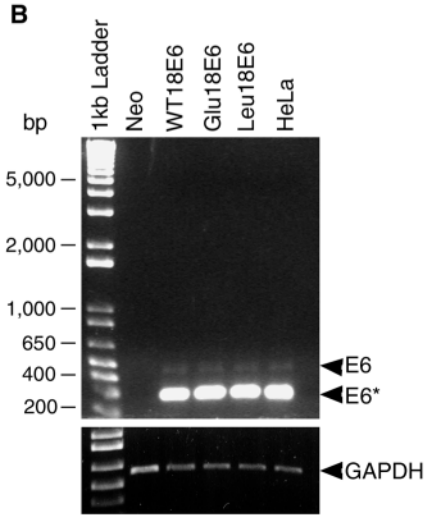

Fig. 1. (A) The C-terminal sequences of HPV18 E6 and two mutants, Thr156Glu and Arg153Leu, are shown, together with the class 1 PDZ-binding consensus sequence and PKA recognition motif. The activities of the wild-type and mutant proteins in Dlg degradation assays (in vitro and in vivo), and their response to PKA activity are shown (Gardiol et al., 1999; Kühne et al., 2000) (our unpublished results). (B) Expression of E6 mRNA in SVJD keratinocytes was analysed by semi-quantitative PCR using primers specific for HPV18 E6 or GAPDH. Equal volumes of the PCR reaction were separated on a 1% agarose-ethidium bromide gel. HPV18 DNA-positive cervical carcinoma cell line, HeLa, and all three E6 lines contained two amplified bands, one of ~450 bps corresponding to full-length E6 and a second of ~ 280 bps representing the spliced E6* product. The position of size markers is shown.

shown) and E6 can still readily target its PDZ domain-containing substrates for degradation (Gardiol et al., 1999; Thomas et al., 2002a). This finding suggests that E6* is not influencing E6 function with respect to the PDZ proteins. Amplification of the housekeeping gene that encodes GAPDH showed equal amounts of GAPDH product, indicating that the mRNA levels in the E6-PCR reactions had been equal (Fig. 1B). These data are a strong indication that the HPV18 E6 transcripts were produced in similar amounts in each of the SVJD-E6 lines. Significantly, E6 transcription in these lines was comparable qualitatively and quantitatively to E6 transcription in HPV18-postive HeLa tumour cells.

To assess the biological activity of the different E6 molecules, we examined the protein levels of Dlg – one of the cellular targets of the PDZ-binding motif. A western blot of total cell lysates was probed with the anti-Dlg MAb 2D11. We have previously shown that this MAb identifies Dlg as a doublet or triplet of bands of relative molecular mass (M_r) of 120 kDa (Watson et al., 2002). A typical western blot is shown in Fig. 2A. All of the cell lines contained detectable levels of Dlg, although the levels were variable between the different lines. Typically, WT18E6 and Leu18E6 lines expressed levels of Dlg that were consistently lower than those present in Glu18E6 or Neo cells, and these levels were comparable to those in HeLa cells (Fig. 2A). In some of the Leu18E6 lines generated, Dlg levels were significantly less than those in WT18E6 cells. Both Neo and Glu18E6 lines contained similar high levels of Dlg protein.

In epithelial cells, Dlg primarily locates to sites of intercellular contact (Lue et al., 1994), and therefore to assess whether the lower levels of Dlg in WT18E6 and Leu18E6 cells correlated with a reduction in peripheral localization of the protein, cells were stained with the 2D11 MAb and examined by immunofluorescence microscopy. The pattern of Dlg staining in the different E6 cells was consistent between each of the cell lines generated. Typical staining patterns are shown in Fig. 2B. In Neo cultures grown to high cell density, intense Dlg staining was observed at intercellular contact sites formed between cells. Cells expressing an active E6 PDZ-binding domain (WT18E6 and Leu18E6) showed a sharp reduction in Dlg levels at the peripheral membranes of cells, with Leu18E6 cultures showing the greatest loss (Fig. 2B). By contrast, Glu18E6 cells displayed strong peripheral Dlg staining, although this was less intense than the staining in Neo cells. In comparison to WT18E6, Leu18E6 cells and Neo cells, Glu18E6 cells also exhibited significant punctate cytoplasmic Dlg staining (Fig. 2B). These Dlg structures probably represent vesicular structures in which Dlg is thought to reside before recruitment to the cell periphery (Reuver and Garner, 1998).

Together, these results indicate that HPV18 E6 molecules with an intact PDZ-binding motif are biologically active in SVJD keratinocytes. Furthermore, the effect of the different mutants on Dlg levels in SVJD cells is consistent with the behaviour of these mutants in in vitro and in vivo Dlg degradation assays (Gardiol et al., 1999; Kühne et al., 2000) (Fig. 1A); Glu18E6 is defective in reducing the level of Dlg, whereas the putative constitutively active protein – Leu18E6 – exhibits an enhanced ability to reduce the levels of this cellular target. However, complete loss of Dlg in WT18E6 or even Leu18E6 cell lines was not observed. This is consistent with retention of low levels of Dlg in cervical carcinoma cells like HeLa (Fig. 2A) (Mantovani et al., 2001).

Cell shape change correlates with activity of E6 PDZ-binding domain

The E6 cells showed striking changes in both their morphology and their ability to form colonies. Examination of low-density cell cultures by phase contrast microscopy revealed that E6-expressing cells adopted a fibroblast-like appearance, becoming elongated and more spread out than Neo cells (Fig. 3a′-d′). WT18E6 cells, and especially Leu18E6 cells, had a more ‘stretched out’ morphology than Glu18E6 cells, with numerous membrane protrusions extending in different directions (Fig. 3b′-d′). These changes were accompanied by a loss of formation of colonies consisting of tightly adhered cells with a uniform cobblestone appearance, although the

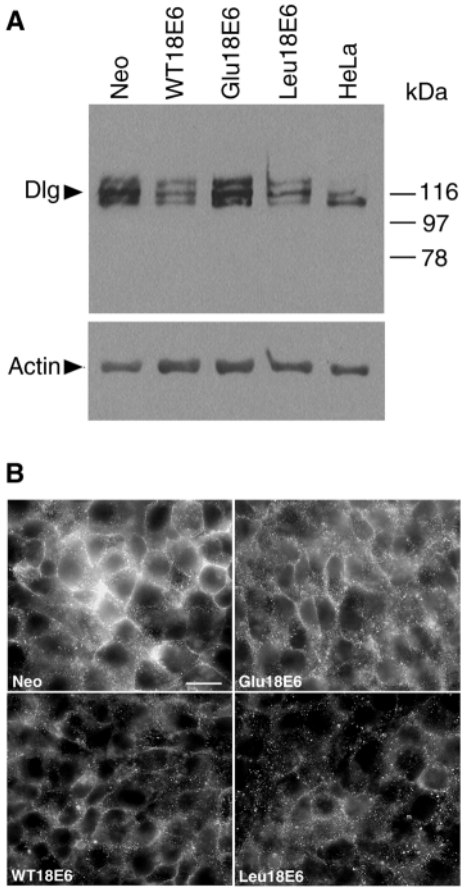

Fig. 2. Dlg expression in E6 cell lines. (A) Western blot analysis of Dlg in cell extracts of E6 cell lines, Neo and HeLa cells. A decrease in total cell levels of Dlg correlates with expression of E6 with an active PDZ-binding motif. Position of markers of M_r (kDa) are as indicated. Actin levels control for loading. (B) Immunofluorescence microscopy of cells at high density stained with the anti-Dlg MAb 2D11. In Neo cells Dlg is recruited to the cell periphery at sites of intercellular contact. Peripheral levels of Dlg are significantly decreased in E6 cells expressing an active PDZ-binding domain (WT18E6 and Leu18E6), but are only partially reduced in cells expressing an E6 unable to degrade Dlg (Glu18E6). Bar, 20 μm.

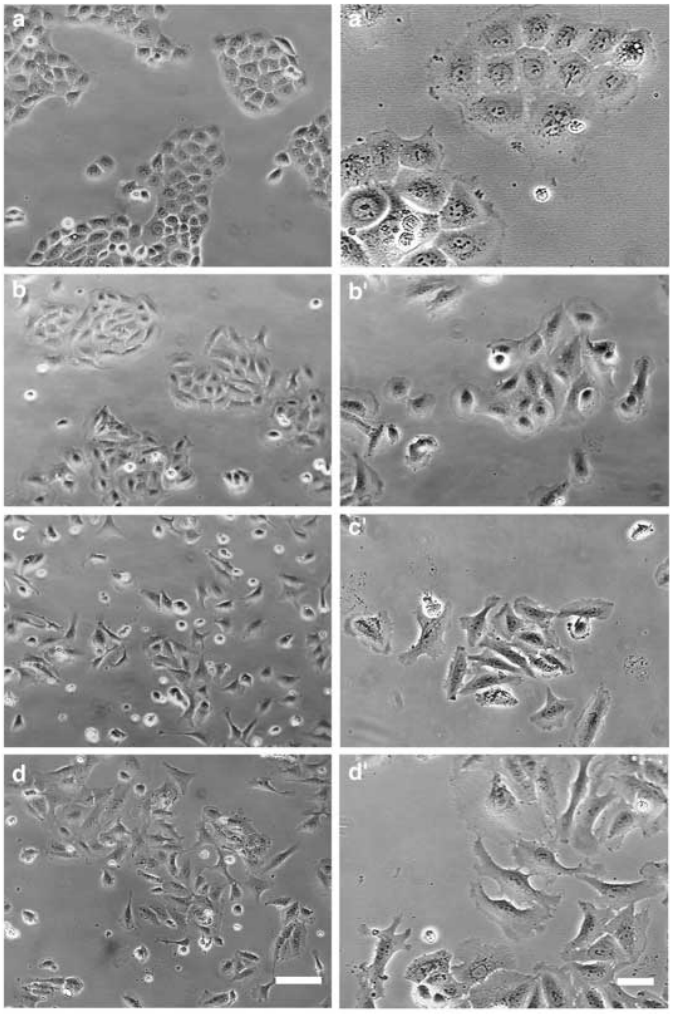

Fig. 3. Morphology of E6 cells. Cells seeded onto culture dishes at a density of ~5×10^5 cells/ml were allowed to adhere overnight before being photographed in a temperature-controlled stage chamber. a,a′, Neo; b,b′, Glu18E6; c,c′, WT18E6; d,d′, Leu18E6. (a-d) Light microscopy of cell cultures shows that cells expressing an active E6 PDZ-binding domain (WT18E6 and Leu18E6) were less likely to associate into organized colonies compared with control cells (Neo) or cells expressing an E6 with an inactive PDZ-binding motif (Glu18E6). Phase contrast microscopy (a′-d′) of cells at higher magnification shows that E6 cells have undergone shape change and increased spreading. These effects were more prominent in WT18E6 and Leu18E6 cell lines. Bars, 100 μm (a-d) and 50 μm (a′-d′).

degree of disruption was variable between the different E6 cell lines (Fig. 3a-d). Glu18E6 cells formed reasonably well-defined colonies, although the colonies were not as compact as those formed by Neo cells (Fig. 3a,b). Notably, WT18E6 and Leu18E6 cells grew as loose collections of cells (Fig. 3c,d), and it was evident that in the presence of a functional PDZ-binding motif cells were unable to engage in tight intercellular contact. This was most evident in Leu18E6 cells which, at best, formed intermittent point contacts with their neighbours rather than establishing contacts along the entire cell boundary (Fig. 3d′), as observed in cultures of Glu18E6 cells.

At high cell density, Glu18E6 cells closely associated with one another, forming a cell sheet with a defined edge and representative of the monolayer seen in the Neo control line. Few isolated cells and small colonies were observed, and these remained close to the main cell mass (data not shown). By contrast, high-density cultures of Wt18E6 and Leu18E6 cells formed poor monolayers with an ill-defined edge and many isolated cells distributed around the edge of the tissue culture dish (data not shown). This was especially evident in cultures of Leu18E6 cells. We therefore conclude that expression of HPV18 E6 molecules in immortalized keratinocytes induces changes in both cell shape and in colony formation. The degree of change in both of these characteristics correlates with the activity of the C-terminal PDZ-binding motif.

Activity of the E6 PDZ-binding domain correlates with disruption of actin stress fibre formation

The transition of E6 cells to a ‘fibroblast-like’ morphology, the loss of tight intercellular association and alterations of cell

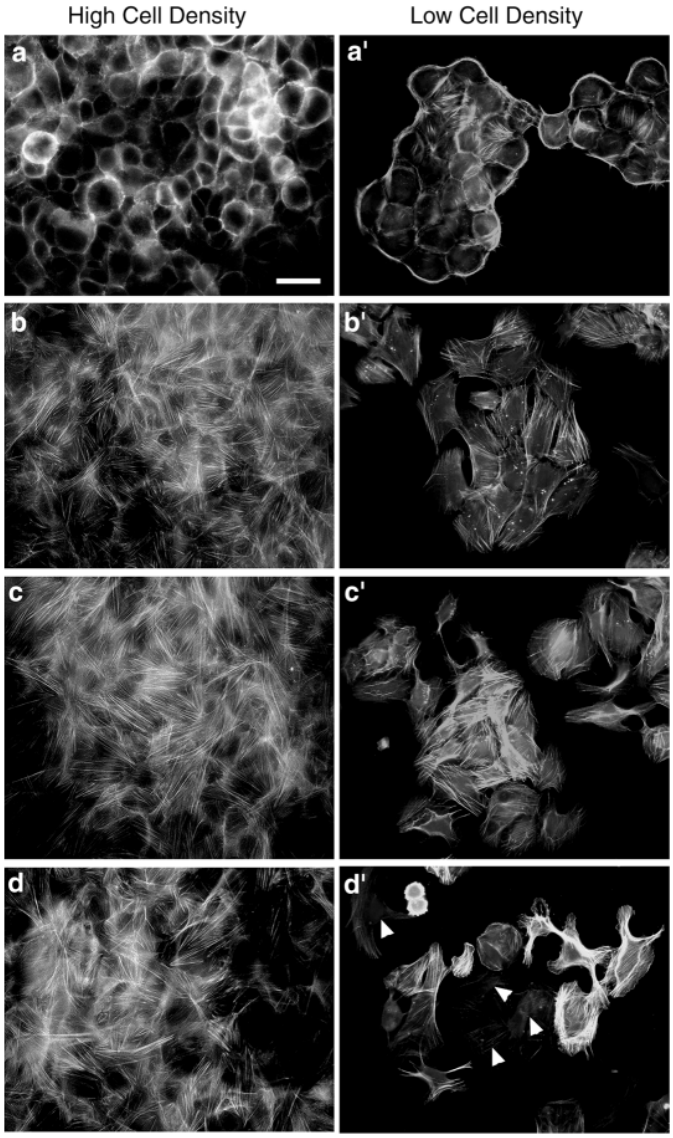

Fig. 4. Organization of F-actin cytoskeleton in E6 cells. Cells at high (a-d) and low (a′-d′) cell densities were stained with rhodamine-conjugated phalloidin. (a,a′) Neo cells; (b,b′) Glu18E6 cells; (c,c′) WT18E6 cells; (d,d′) Leu18E6 cells. Bar, 40 μm (a-d) and 20 μm (a′-d′). Arrowheads in d′ indicate cells that lack actin filaments.

shape led us to examine the F-actin cytoskeleton in these cells, as this cytoskeletal matrix is a determining factor of cell morphology (Ben Ze'ev, 1991). F-actin was visualized by staining cells with rhodamine-conjugated phalloidin (Fig. 4). Consistent with their rounded cell shape, Neo cells showed strong F-actin staining around the cortex of the cell, characteristic of the formation of a circumferential belt of actin (Fig. 4a). Cortical actin structures were lost in all E6 lines and instead were replaced by a network of actin stress fibres (Fig. 4b-d). The appearance of stress fibres and their organization was, however, different between the E6 cell lines, although this was most noticeable in low-density cultures (Fig. 4a′-d′). Glu18E6 cells had a well-defined stress fibre network, with short fibres bundled together to form organized cables, many of which were orientated along the length of the cell in the direction of spreading (Fig. 4b′). Longer stress fibres were observed in the WT18E6 and Leu18E6 cells, which is consistent with the elongated morphology of these cells (Fig. 4c′,d′). Notably, the intensity of phalloidin-staining was variable in low-density cultures of WT18E6 and Leu18E6 cells, with some cells showing intense fluorescence. In Leu18E6 cultures, a fraction of cells showed very little phalloidin staining and appeared to lack actin filaments altogether (examples are indicated by arrowheads in Fig. 4d′).

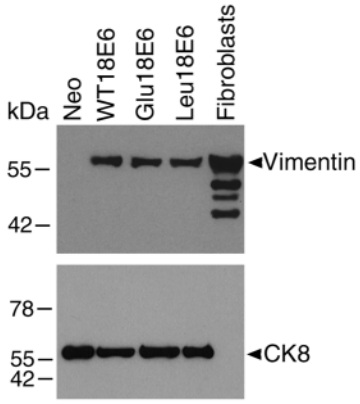

Fig. 5. Intracellular levels of vimentin and cytokeratin 8. Western blots of total cell extracts from confluent cultures of Neo, E6 cells and fibroblasts were probed with a vimentin MAb or a cytokeratin 8 (CK8) MAb. The positions of markers of M_r (kDa) are shown.

E6 cell lines showed evidence of epithelial-mesenchymal transition

The dissociation of cortical actin, formation of actin stress fibres and the fibroblast-like morphology of all three E6 cell lines suggested that expression of E6 in SV40-immortalized keratinocytes had induced an epithelial-to-mesenchymal transition (EMT). To confirm an EMT, cell lysates were analysed by western blotting for the expression of the intermediate filament protein vimentin – a biological marker of EMT (Fig. 5). All of the E6 cell lines expressed approximately equal levels of vimentin, whereas this mesenchymal maker was not present in Neo cells. However, all lines retained expression of cytokeratin 8, an epithelial marker, indicating that E6-expressing cells had converted to a mesenchymal state that retained elements of their epithelial origin.

Location of epithelial adhesion junction proteins at intercellular contacts is diminished in cells expressing a functional E6 PDZ-binding domain

The observation that cells expressing an active PDZ-binding motif exhibited a tendency to dissociate from their close neighbours suggests that these cells may have impaired intercellular adhesion. Strong intercellular contacts are mediated by the formation of membrane-associated specialized junction complexes, such as adherens junctions (AJs) and desmosomes. Two cellular targets of the E6 PDZ-binding domain – Dlg and Scribble – have both been shown to be necessary for proper formation of intercellular junctions in epithelial tissues of *Drosophila* and *C. elegans* (Bilder and Perrimon, 2000; Köppen et al., 2001; Bossinger et al., 2001; Firestein and Rongo, 2001). Because there is evidence that the mammalian homologues of these cellular proteins may encode

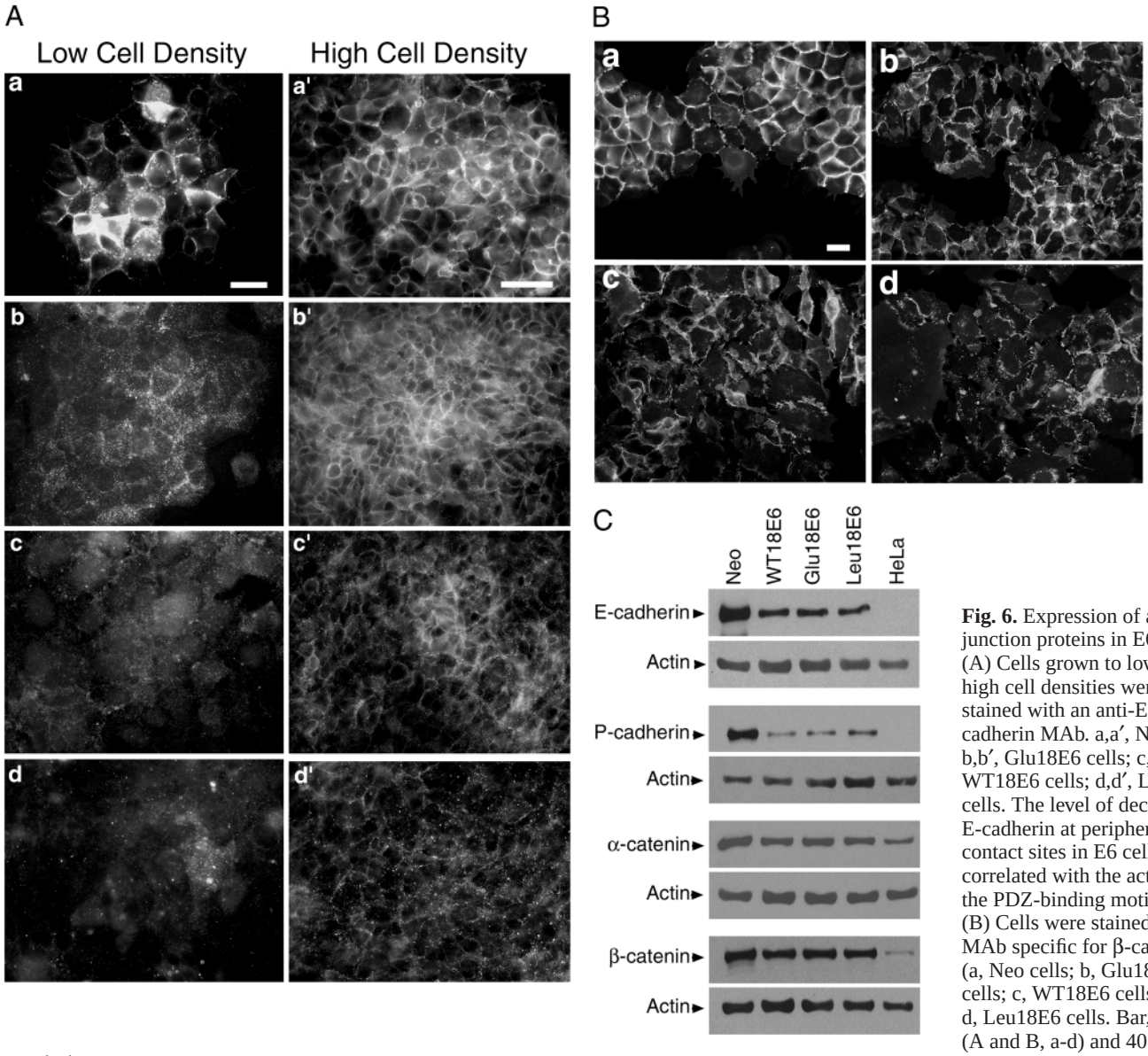

Fig. 6. Expression of adherens junction proteins in E6 cells. (A) Cells grown to low and high cell densities were stained with an anti-E-cadherin MAb. a,a′, Neo cells; b,b′, Glu18E6 cells; c,c′, WT18E6 cells; d,d′, Leu18E6 cells. The level of decrease of E-cadherin at peripheral contact sites in E6 cells correlated with the activity of the PDZ-binding motif. (B) Cells were stained with a MAb specific for β-catenin (a, Neo cells; b, Glu18E6 cells; c, WT18E6 cells; d, Leu18E6 cells. Bar, 20 μm (A and B, a-d) and 40 μm (A, a′-d′). (C) Total extracts of cells grown to 80% confluency were analysed for protein expression levels of adherens junction proteins (E-cadherin, P-cadherin, α-catenin and β-catenin). Note that HeLa cells are E- and P-cadherin negative. Actin levels control for loading.

similar functions to their invertebrate counterparts (Thomas et al., 1997), we examined whether intercellular adhesion structures are disturbed in the E6 cell lines.

Adherens junctions are composed of a Ca^{2+}-binding transmembrane cadherin bound to the actin cytoskeleton via cytoplasmic proteins known as the catenins (for a review, see Pokutta and Weis, 2002). Therefore, E6-expressing cells were stained for the epithelial cadherins E- and P-cadherin, and the cytoplasmic components α, β- and γ-catenin. Because both E- and P-cadherin showed similar distributions and patterns of expression, only E-cadherin is described (Fig. 6A). Similarly, the description of β-catenin is representative also of the subcellular distributions of both α- and γ-catenin (Fig. 6B). Control staining performed in the absence of the primary antibodies showed no evidence of nonspecific staining (data not shown).

Although E-cadherin and β-catenin were detected strongly and continuously around the plasma membrane at regions of intercellular contact in Neo cells, the level of detection of both junction proteins at the cell periphery was reduced in the E6 lines. Less peripheral E-cadherin (Fig. 6A) and β-catenin (Fig. 6B) was observed in WT18E6 and Leu18E6 cells compared with Glu18E6 cells, with Leu18E6 cells showing a particularly significant reduction in staining. Notably, the reduction in E-cadherin staining in the E6 cells was most pronounced at low cell density, with Leu18E6 cells showing no E-cadherin staining at cell-cell contacts (Fig. 6Aa-d). Both E-cadherin and β-catenin staining at the membranes of WT18E6 and Leu18E6 cells was more punctate and discontinuous than the staining in Glu18E6 or Neo cells. In Leu18E6 cells in particular, β-catenin localized to clusters of intercellular contacts positioned intermittently along the plasma membrane rather than labelling the entire cell-cell boundary as in Glu18E6 cells [Fig. 6B, compare b (Glu18E6) with d (Leu18E6)]. This observation is

consistent with the notion that Leu18E6 cells only formed intermittent point contacts with adjacent cells rather than continuous seals. In addition to its participation in intercellular adhesion, β-catenin also localizes to the nucleus (Henderson and Fagotto, 2002). Although a fraction of β-catenin was located in the nuclei of E6 and Neo cells, the intensity of nuclear staining did not differ between each of the lines (Fig. 6B). The decrease in detection of AJ proteins in the E6 lines was not an aberration of the immunofluorescence staining procedure or a general lack of protein detection, since staining with a Golgi-specific MAb showed equal detection of the Golgi protein in each of the E6 cell lines (data not shown).

The cell lines were also examined for the presence of desmosomes by staining for the desmosomal plaque protein, desmoplakin. In E6 cells, desmoplakin staining at the cell periphery was less intense than in Neo cells, and, consistent with AJs proteins, loss of staining was most evident in Leu18E6 cells, and least so in Glu18E6 cells (data not shown).

It was important that we determined whether the reduced peripheral location of adhesion junction proteins was due to a decrease in protein expression, or rather an inability of proteins to migrate and/or assemble to the plasma membrane. To begin to answer this, the levels of AJs molecules in E6 and Neo cells were examined by western blotting. In all three E6 lines the levels of E- and P-cadherin were significantly reduced compared with the Neo line, whereas α-catenin and β-catenin levels were only marginally reduced (Fig. 6C). However, the extent of reduction was the same in each of the E6 lines, suggesting that expression levels of these AJ molecules is not affected by the activity of the E6 PDZ-binding motif.

Collectively, these observations indicate that in the E6 lines there is a reduction in E- and P-cadherin-dependent AJs, and a reduction in the formation of desmosomes. This is most pronounced in E6 lines expressing an 'active' E6 PDZ-binding motif – that is, WT18E6 and Leu18E6. Although the level of adhesion junction proteins is likely to contribute to disassembly of intercellular junctions in the E6 lines, the activity of the PDZ-binding domain of E6 is also likely to have an effect, as loss of intercellular contact is most obvious in the WT18E6 and Leu18E6 cells. The inability of the junction components to assemble into adhesion complexes or failure of these proteins to traffic to the plasma membrane could account for our observations. Significantly, these effects correlate with a reduction in location of Dlg to sites of intercellular contact (see Fig. 2B).

Discussion

The stable expression of HPV18 E6 in SV40-immortalized human epidermal keratinocytes induced significant changes in cell morphology, organization of the microfilament network and in formation of intercellular adhesion junctions (Figs 3, 4 and 6). Collectively, these E6-induced changes indicate an EMT supported by the finding that E6-expressing cells contained vimentin – a biological marker of EMT (Fig. 5). However, compared with those cells expressing E6 proteins with functional PDZ-binding domains (WT18E6 and Leu18E6), the change to the epithelial phenotype of cells expressing an E6 with a nonfunctional C-terminal PDZ-binding motif (Glu18E6) was significantly less marked, suggesting that the function of the E6 PDZ-binding domain might contribute to the transition. Significantly, the differences in the behaviour of these E6 cell lines correlated with the level of Dlg protein, a cellular target of the E6 PDZ-binding motif. Leu18E6 cells expressed lower levels of Dlg than Glu18E6 cells, and WT18E6 expressed intermittent levels (Fig. 2). Although we were unable to assess directly the stability of the Thr156Glu protein expressed in Glu18E6 cells, it has been reported that this mutation does not compromise the stability of the E6 polypeptide expressed in vitro or in vivo (Gardiol et al., 1999). Furthermore, we showed that each of the E6 lines contained similar levels of the E6 mRNA transcripts (Fig. 1B). It is therefore most likely that the increase in Dlg levels in Glu18E6 cells reflects the inability of the mutant to support Dlg degradation (Gardiol et al., 1999).

Compared with WT18E6 and Leu18E6 cells, Glu18E6 cells had an increased tendency to associate with neighbouring cells (Fig. 3). The restoration of intercellular association in these cells was most probably largely attributable to an increase in the formation of epithelial cadherin-based adhesion junctions (AJs and desmosomes) at intercellular contact sites (Fig. 6A,B). This was not however, a result of increased protein levels of the individual cell adhesion proteins such as E-cadherin and β-catenin, as these remained unchanged between all of the E6 cell lines (Fig. 6C). These data suggest that Glu18E6 cells might have an increased ability to initiate assembly of junctions and/or maintain their integrity compared with WT18E6 cells. A decrease in the levels of PDZ proteins such as Dlg could be the basis for the loss of integrity of adhesion structures in WT18E6 and Leu18E6 cells. Studies of *dlg-1*(RNAi) embryos of *C. elegans*, in which AJs disassemble, suggest that Dlg is a probable candidate to participate in the organization of a protein scaffold that controls the assembly of junctions (Bossinger et al., 2001; Firestein and Rongo, 2001).

In E6 cells, F-actin was organized into stress fibres; however, the morphological appearance of stress fibres between Glu18E6 and WT18E6 differed, and they were noticeably more disorganized in Leu18E6 cells, suggesting that the activity of the C-terminal PDZ-binding motif may influence the organization and assembly of actin structures (Fig. 4). In *Drosophila*, loss of Dlg is associated with disorganization of actin cytoskeleton (Woods et al., 1996), and similarly in *C. elegans*, Dlg is required for the proper organization of the microfilaments during embryogenesis (Bossinger et al., 2001). Although the cortical belt of actin present in Neo cells is disassembled in E6 cells, this was not dependent on an intact C-terminal PDZ-binding motif as this structure did not reassemble in Glu18E6 cells. Other E6 activities might have also been involved in the reorganization of microfilaments. For instance, HPV16 E6 associates with two cellular proteins that are involved in remodelling the microfilament network, the focal adhesion protein paxillin (Vande Pol et al., 1998) and PKN, a protein kinase that binds to the Rho family of GTPases (Gao et al., 2000).

Destabilization and dissociation of intercellular adhesions structures is an integral part of EMT, and it is generally perceived that cell-cell dissociation occurs by a multistep process involving downregulation in the levels of the cell-cell adhesion structural components, as well as abrogation of the pathways that initiate and maintain these structures (for reviews, see Cavallaro and Christofori, 2001; Savagner, 2001). In immortalized keratinocytes, E6 disrupts cell-cell association, probably by

multiple mechanisms – downregulation of expression levels of cell junction components and by interfering with pathways that assemble or stabilize these structures at the cell membrane. It is the latter of the two mechanisms that may be dependent on the activities of the C-terminal PDZ-binding domain.

Glu18E6 cells express a nonfunctional PDZ-binding domain and show cellular levels of Dlg that are comparable with those of Neo control cells. However, these cells do show evidence of an EMT – vimentin expression and formation of stress fibres. These observations imply that the activity of the E6 PDZ domain contributes only to a part of EMT, and additional functions of E6 are likely to be involved in mesenchymal transition. Oncogenic E6 proteins are multifunctional proteins and interact with a large number of different cellular targets (for a review, see Thomas et al., 2002b). We have already suggested that E6 interactions with cellular targets such as paxillin and PKN may be involved in the remodelling of the actin cytoskeleton. It will be important to identify which other functions of E6 are involved in the changes we observe in the immortalized keratinocytes, as an EMT is associated with malignant progression and studies in mice have shown a strong association between E6 and malignant progression (Song et al., 2000).

Studies of the mammalian homologue of the *Drosophila* tumour suppressor Dlg indicate that it is a tumour suppressor in higher eukaryotes. Our studies suggest a correlation between a reduction in Dlg and increased morphological transformation of keratinocytes. Our observations closely resemble an observed loss of Dlg at sites of intercellular contact in high-risk HPV-associated high-grade dysplastic lesions of the uterine cervix (Watson et al., 2002). A further reduction in total Dlg levels was noted in uterine cancers (Watson et al., 2002). Additional experimentation is required to determine whether the E6 oncoprotein is responsible for the loss of adhesion junction-associated Dlg in vivo, and whether the actions of a functional E6 PDZ-binding motif contribute to the formation and progression of HPV-associated cancers.

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