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Role of the PDZ Domain-Binding Motif of the Oncoprotein E6 in the Pathogenesis of Human Papillomavirus Type 31

Choongho Lee and Laimonis A. Laimins*

Department of Microbiology-Immunology, Feinberg School of Medicine, Northwestern University, Chicago, Illinois

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A number of PDZ domain-containing proteins have been identified as binding partners for the oncoprotein E6 of the high-risk type human papillomaviruses (HPVs). These include hDlg, hScrib, MAGI-1, MAGI-2, MAGI-3, and MUPP1. The PDZ domain-binding motif (-X-T-X-V) at the carboxy terminus of E6 is essential for targeting PDZ proteins for proteasomal degradation. The presence of this motif only in the high-risk HPVs suggests its possible role in HPV-induced oncogenesis. To investigate the role of the PDZ domain-binding motif of E6 in the HPV life cycle, two mutant HPV31 genomes were constructed: E6ValΔ, with a deletion of the last amino acid residue of E6 (valine), and E6ETQVΔ, with a deletion of the entire PDZ domain-binding motif of E6 (ETQV). Three human foreskin keratinocyte (HFK) cell lines were established which maintained transfected wild-type HPV31 or either of two mutant genomes. Cells containing either of two mutant genomes were significantly retarded in their growth rates and reduced in their viral copy numbers compared to those transfected with wild-type genomes. Western analysis did not reveal any significant changes in the levels of PDZ proteins following stable transfection of any HPV31 genomes into HFKs. Although the E6ETQVΔ-transfected HFKs exhibited a pattern of morphological differentiation that appeared different from the HPV31 wild-type-transfected HFKs in organotypic raft cultures, immunohistochemical analysis failed to identify substantial changes in the differentiation-dependent membrane localization of hDlg proteins. These results suggest that binding of E6 to PDZ proteins modulates the early viral functions such as proliferation and maintenance of the viral copy number in undifferentiated cells.

Human papillomaviruses (HPVs) are small double-stranded DNA viruses that induce hyperproliferative lesions in epithelial tissues (42). A subset of HPV types infect epithelia in the anogenital region and are the etiological agents of cervical cancers. These HPV types are called "high-risk" and include HPV16, HPV18, HPV31, and HPV54 (37, 43, 72, 73). The oncogenic potential of these high-risk types is dependent on the cooperative action of the two early viral gene products, E6 and E7, which bind and alter the activity of cell cycle-regulatory proteins. E6 forms a ternary complex composed of the tumor suppressor protein p53 and E6AP (E6-associated protein), a member of E3 ubiquitin ligase family of proteins, resulting in the ubiquitination and subsequent degradation of p53 (28, 59, 60, 70). E7 binds to and inactivates the retinoblastoma (pRb) family of proteins, thereby alleviating the pRb-mediated repression of E2F transcription factors that are responsible for transactivating many genes involved in progression into S phase (6, 12, 44, 48). Selective retention and expression of these two viral oncoproteins is essential for HPV-induced oncogenesis (1, 2, 61, 62).

The productive life cycle of HPVs is closely associated with the differentiation program of the host epithelial tissue (25). Following infection of keratinocytes in the basal layer, viral genomes are established in the nucleus as extrachromosomal elements (episomes). These episomes are replicated in synchrony with host DNA synthesis through the action of the early viral proteins, E1 and E2, and are maintained at approximately 50 copies per cell (38). As infected keratinocytes migrate away from the basal layer and begin to differentiate, they remain active in the cell cycle through the action of the E7 protein. In differentiated suprabasal cells, the induction of the late viral functions is observed (6, 16, 24). The differentiation-dependent amplification of viral genomes coincides with activation of the late viral promoter, which in HPV31 is called p742 (29). The late viral transcripts encode late viral proteins such as L1, L2, E1^E4, and E5 (10, 17, 58). Progeny virions are assembled in highly differentiated cells and then released to the extracellular environment (17, 46).

The targeting of p53 for degradation by E6 is the most extensively studied function of E6. However, a large amount of evidence suggests the existence of the p53-independent functions of E6 that are also necessary for transformation. Support for this idea comes from the observation that transformation of cells by E6 does not always correlate with its ability to degrade p53. For instance, several p53 degradation-defective E6 mutants are still able to immortalize mammary epithelial cells and transform 3Y1 rat fibroblasts (32, 41). In addition, activation of the human telomerase reverse transcriptase (hTERT) by E6 is more important for immortalization of epithelial cells than is inactivation of p53 (32, 34, 51). These observations indicate that E6 contributes to malignant conversion of HPV-infected cells through mechanisms in addition to inactivation of p53.

Numerous cellular factors have been identified as binding partners for E6. These factors are involved in a variety of cellular processes such as calcium signaling (8), cell adhesion (68), transcriptional control (9, 36, 53, 57), DNA synthesis (35), apoptosis (15, 66), cell cycle control (18), DNA repair (30, 63), and small-G-protein signaling (19). Interestingly, one

* Corresponding author. Mailing address: Department of Microbiology-Immunology, Feinberg School of Medicine, Northwestern University, Mail Code S213, 320 E. Superior St., Chicago, IL 60611-3010. Phone: (312) 503-0648. Fax: (312) 503-0649. E-mail: l-laimins@northwestern.edu.

group of these E6-interacting proteins harbors multiple copies of a protein-protein interaction domain called PDZ (for "PSD-95/Discs Large/ZO-1"). These PDZ domain-containing proteins include hDlg, a human homolog of the *Drosophila* discs large tumor suppressor protein (33, 40), hScrib, a human homolog of the *Drosophila* scribble tumor suppressor protein (49), MAGI-1, MAGI-2, and MAGI-3 (for "Membrane-Associated Guanylate kinase homology proteins with an inverted domain structure") (21, 67), and MUPP1, a multi-PDZ protein (39). All these PDZ proteins are targeted for proteasome-mediated degradation by E6 in heterologous overexpression systems (20, 21, 39, 49, 56, 67). The high-risk HPV E6 proteins bind to these PDZ proteins through a motif [X-(T/S)-X-V, where X is any amino acid] located at their extreme carboxy terminus, which is conserved among all high-risk types. In contrast, none of the low-risk HPV E6 proteins possess such domains. Mutation of the second or fourth conserved amino acid residue [(T/S) or V] in this motif has been demonstrated to compromise the transforming activity of E6, suggesting that this PDZ domain-binding motif plays a critical role in E6-induced oncogenesis (33). Additional studies of transgenic mice have demonstrated an important role for this motif in E6-mediated alterations in cellular proliferation in the eye lens (50).

PDZ proteins are localized at the membrane-cytoskeleton interfaces of cell-cell contact and form multiprotein signaling complexes at the inner surface of the membrane to modulate cell growth, cell polarity, and cell adhesion in response to cell contact (7, 13). In *Drosophila* embryos, mutation of *dlg* or *scrib* causes cellular hyperproliferation and loss of cell polarity in epithelial tissues such as the imaginal discs (4, 5, 23, 54, 71). This indicates that structural as well as signaling functions of PDZ proteins are critical for their antitumor activities. Similar PDZ domain-binding activities have been shown for both adenovirus type 9 E4 open reading frame (ORF) 1 protein and human T-cell leukemia virus Tax proteins, which act to disrupt the functions of PDZ proteins through their carboxy terminal PDZ domain-binding motifs (21, 39, 40, 64).

Most studies investigating the interactions of PDZ proteins with E6 have been conducted using heterologous overexpression systems. The true physiological significance of the association of E6 with PDZ proteins in HPV pathogenesis is therefore still unclear. In this study, we examined the role that targeting of PDZ proteins by E6 plays in the context of the complete viral genome. We found that the PDZ domain-binding motif of E6 modulates the growth of cells, influences the viral copy number, and alters the morphological differentiation of cells.

MATERIALS AND METHODS

Cell culture. Human foreskin keratinocytes (HFKs) were isolated from circumcised neonatal foreskin and grown in serum-free keratinocyte growth medium (Clonetics, San Diego, Calif.) as previously described (24). The untransfected and HPV31 genome-transfected HFKs were maintained with mitomycin-treated NIH 3T3 fibroblast feeders in E-medium (3). E-medium also contains 5% fetal bovine serum and was supplemented with mouse epidermal growth factor (Collaborative Biomedical Products, Bedford, Mass.) to a final concentration of 5 ng/ml. NIH 3T3 fibroblast feeders were removed with 0.5 mM EDTA in phosphate-bufferred saline before HFKs were harvested to isolate DNA, RNA, and protein. To determine growth rates, the untransfected and HPV31 genome-transfected HFKs were first seeded onto 6-cm culture dishes at 3×10^5 cells per dish in the presence of mitomycin-treated feeders. At various time points, fibroblasts were removed, keratinocytes were trypsinized and stained with trypan blue, and viable cells were counted using a hemocytometer. SCC-13 and C33A cells were maintained in E-medium with fibroblast feeders and Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, respectively.

Differentiation of HFKs by methylcellulose and raft cultures. The HPV31 genome-transfected HFKs were suspended in 1.5% methylcellulose to induce differentiation as previously described (14). HFKs were grown in raft cultures as previously described (47). Briefly, cells were plated onto a solidified collagen matrix containing J2 3T3 fibroblasts, allowed to grow to confluency, and then transferred to a metal grid to provide an air-liquid interface for differentiation. Cultures were harvested at 14 days, fixed in 4% paraformaldehyde, paraffin embedded, sectioned, and stained with hematoxylin and eosin for visualization of differentiated raft tissue.

Plasmids. pBR322-HPV31 contains the complete HPV31 genome sequence inserted into the EcoRI site of pBR322. Deletions of the GTG and GAA-AC C-CAA-GTG of the E6 ORF, which encode the last one and last four amino acid residues upstream of the stop codon of HPV31 E6 protein, respectively, were performed using QuickChangeXL site-directed mutagenesis kit (Stratagene, La Jolla, Calif) as described by the manufacturer. To avoid introduction of possible second-site mutations by the DNA polymerization reaction used in the QuickChangeXL site-directed mutagenesis kit, the mutant pBR322-HPV31 plasmid derived from the initial mutagenesis was digested with SpeI and BanII to release the 1.2-kb DNA fragment encompassing the entire E6 sequence. This 1.2-kb DNA fragment was inserted into the 9.8-kb fragment derived from gel purification and restriction digestion of pBR322-HPV31 with SpeI and BanII. The 1.2-kb SpeI-BanII fragment region of the resultant plasmid was then sequenced to confirm the absence of any additional mutations and the presence of the desired E6 deletions. pSV2neo encodes the neomycin resistance gene. pGEX is an expression vector for a glutathione *S*-transferase (GST) gene. In pGEX-31E6, an HPV31 E6 PCR fragment was cloned in frame at the 3′ end of the GST coding sequence and used to produce a GST-E6 fusion protein in *E. coli.* The HA-tagged HPV18 E6 expression plasmid GW1-HA18E6 was previously described elsewhere (20, 21). A PCR fragment of HPV18 E6 with deletion of the last one or last four amino acid sequences was inserted into GW1-HA18E6 by using the HindIII and EcoRI restriction sites to generate GW1-HA18E6ValΔ and GW1-HA18E6ETQVΔ, respectively. Expression vectors for HA tagged hDlg, HPV18, HPV16, and HP31 E6 proteins were described previously (20).

Generation of stable cell lines. pBR322-HPV31 plasmid (10 μg) was digested with EcoRI to release the HPV31 DNA sequence. The restriction enzyme was heat inactivated by incubation at 65°C for 15 min, and the released viral genome was religated with 800 U of T4 DNA ligase at 15°C overnight. The religated DNA was then precipitated with isopropyl alcohol and washed with 70% ethanol. The DNA pellet was then resuspended in 10 mM Tris–1 mM EDTA (pH 7.5) and used for transfection. HFKs were grown in a 6-cm culture dish to 50 to 60% confluency in serum-free keratinocyte growth medium (Clonetics, San Diego, Calif.). A 1-μg sample of religated HPV31 genome was cotransfected with 1 μg of pSV2neo plasmid into HFKs by using Fugene (Roche Diagnostics, Mannheim, Germany) transfection reagents as described by the manufacturer. The transfected HFKs were transferred onto mitomycin-treated feeders the next day. Drug selection with G418 (Gibco BRL) started 2 days after transfection and was done by the following schedule: 200 μg of G418 per ml for 4 days and 100 μg of G418 per ml for 3 days. Drug-resistant colonies were pooled and expanded for analysis.

Southern and Northern blot analysis. For the transient-replication assay, transfections of circularized HPV31 genomes into SCC-13 cells and the following DNA isolations were carried out as described previously (26). For the stable-replication assay, total genomic DNA was prepared by phenol-chloroform extraction and analyzed by Southern analysis as previously described (14). Total RNA was isolated from the HPV31 genome-transfected HFKs with TRIzol reagent (Gibco BRL) as described by the manufacturer. For Northern analysis, 10 μg of RNA was separated on a 1.0% agarose–2.2 M formaldehyde gel in 1× MOPS buffer (10× MOPS buffer is 0.2 M morpholinepropanasulfonic acid [MOPS], 50 mM sodium acetate, 10 mM EDTA) and transferred onto DuPont GeneScreen Plus nylon membrane (NEN Research Products, Boston, Mass.) as described by the manufacturer. Hybridization with radioactive probes was done as previously described (14). Quantitative analysis of the Southern blot was done using a PhosphorImager (Molecular Dynamics).

GST pull-down assay. GST-E6 fusion proteins were expressed and purified from *Escherichia coli* DH-5α transformed with the pGEX-31E6 plasmid. Methods used to purify GST fusion proteins from the *E. coli* cell lysates were as previously described (45). To isolate bound proteins, 5 μg of GST fusion proteins

conjugated with glutathione-agarose beads was mixed with 300 μg of HFK lysates in RIPA buffer (150 mM NaCl, 50 mM Tri-HCl [pH 8.0], 5 mM EDTA [pH 8.0], 0.5 mM dithiothreitol, 100 mM sodium fluoride, 200 μM sodium orthovanadate, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]). This mixture was then incubated at 4°C overnight under constant rotation. The agarose beads were centrifuged and washed with RIPA buffer three times. The cellular proteins precipitated by GST-E6 fusion proteins bound to glutathione-agarose beads were eluted by adding sodium dodecyl sulfate (SDS)-protein sample buffer (150 mM Tri-HCl [pH 6.8], 300 mM dithiothreitol, 6% SDS, 0.3% bromophenol blue, 30% glycerol) and were separated on an SDS–10% polyacrylamide gel for Western blot analysis.

Western blot analysis. Whole-cell extracts were prepared in RIPA buffer containing a cocktail of protease inhibitors (Complete, Mini; Roche Diagnostic) and quantitated by the Bradford assay (Bio-Rad). Equal amounts of protein were electrophoresed on an SDS-polyacrylamide gel, subsequently transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, Mass.), and probed with the following primary antibodies as previously described (14): anti-p53 (Ab-6; Oncogene Research Products, Cambridge, Mass.), anti-hDlg (2D11; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.), anti-hSrib (C-20; Santa Cruz), anti-MUPP1 (Upstate Biotechnology, Lake Placid, NY), and anti-HA (12CA5; Boehringer Mannheim). Proteins were visualized via enhanced chemiluminescence (Amersham Pharmacia).

Immunofluorescence. The untransfected and HPV31 genome-transfected HFKs were grown on coverslips to 70% confluency in the presence of mitomycin-treated feeders. Coverslips were rinsed in phosphate-buffered saline (PBS) three times. The cells were fixed at room temperature for 10 min in 4% paraformaldehyde, permeabilized in ice-cold 0.2% Triton-X in PBS for 5 min, and rinsed three times in PBS. Anti-hDlg antibody (2D11; Santa Cruz Biotechnology, Inc.) was applied, and the mixture was incubated for 2 h. After three washes in PBS, coverslips were incubated with fluorescein isothiocyanate (Amersham Pharmacia, Piscataway, N.J.)-conjugated anti-mouse secondary antibody for 1 h. Following three washes with PBS, the coverslips were mounted onto slides using Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, Calif.) and sealed. Fluorescent signals were examined and captured by a Leica DMIRE2 inverted fluorescence microscope system. Captured images were processed with Openlab software (Improvison).

Immunohistochemistry. Thin sections of paraformaldehyde-fixed, paraffin-embedded raft culture tissue on silanized slides were heated to 50°C for 30 min and given three 5-min rinses in xylene to remove residual paraffin. The sections were rehydrated in absolute ethanol and incubated in 10 mM citrate (pH 6.0) at 95°C for 20 min and then for 20 min at room temperature. Incubation with the primary antibody mouse anti-hDlg (2D11; Santa Cruz Biotechnology, Inc.) was performed overnight at 4°C. The sections were subsequently incubated for 1 h at room temperature with the secondary anti-mouse antibody conjugated to fluorescein isothiocyanate. Following 5 min of DNA counterstaining with 4′,6-diamidino-2-phenylindole-2HCl (DAPI) (1 μg/ml; Serva, Heidelberg, Germany), the sections were mounted using Vectashield mounting medium and sealed. Fluorescent signals were examined with a Leica DMIRE2 inverted fluorescence microscope system. Captured images were processed with Openlab software.

RESULTS

Previous studies using HPV16 E6 demonstrated that the four amino acids at the extreme carboxy terminus of E6 were necessary for binding to PDZ proteins (21, 33, 39, 40,49, 67). While HPV16 and HPV31 E6 proteins are very homologous, it was necessary to confirm that HPV31 E6 is also able to bind to PDZ proteins through its PDZ domain-binding motif. We constructed GST-HPV31E6 fusion vectors in which the last one (GST-31E6ValΔ) or last four (GST-31E6ETQVΔ) amino acid residues of E6 were deleted (Fig. 1). GST fusions of wild-type HPV31 and two mutant E6 proteins were expressed in bacteria and used to examine the ability to bind to one of PDZ proteins, hDlg. As seen in Fig. 1C, GST-31E6 fusion proteins bound to hDlg proteins from the HFK whole-cell lysates whereas neither GST-31E6ValΔ nor GST-31E6ETQVΔ fusion proteins were able to pull down any hDlg proteins. Another E6-binding PDZ protein, hScrib, was also able to be precipitated only by the wild-type GST-31E6 fusion protein and failed to bind to either of the GST-31E6 mutant fusion proteins (data not shown).

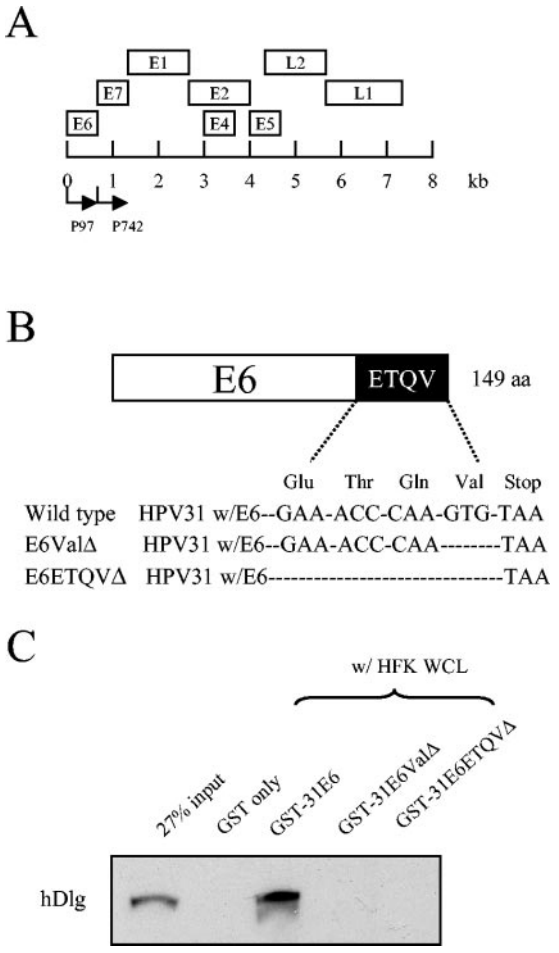

FIG. 1. Amino acids at the carboxy terminus of HPV31 E6 mediate binding to hDlg. (A) Diagram of a linearized HPV31 genome identifying viral ORFs and the two major promoters (P97 and P742). (B) Schematic depicting the amino acid residues and nucleotide sequences of the carboxy-terminal PDZ domain-binding motif of the HPV31 E6 gene. Two E6 PDZ mutant genomes were examined in this study; E6ValΔ contains deletion of one amino acid residue, while E6ETQVΔ contains deletion of four amino acid residues at the PDZ domain-binding motif of E6. (C) GST pull-down assay examining the interaction of HPV31 E6 with hDlg. Wild-type and two mutant GST-HPV31E6 fusion proteins conjugated with the glutathione-agarose beads were mixed with whole-cell lysates from HFKs. Bound cellular proteins were eluted and separated on an SDS-polyacrylamide gel for Western blot analysis using antibody to hDlg. As a positive control, 27% of the whole-cell lysates used above was run in the first lane. aa, amino acids.

To study the role of the PDZ domain-binding motif of E6 in the pathogenesis of HPV31, we constructed two mutant viral genomes in which the PDZ domain-binding motif of E6 was altered (Fig. 1B). The first, termed E6ValΔ, lacks the three nucleotides which encode the last amino acid residue, valine, of the E6 protein, and retains the natural stop codon. The second, termed E6ETQVΔ, has a deletion of the 12 nucleotides which encompass the entire PDZ domain-binding motif of E6 (ETQV). HPV16 E6 mutants with deletion of the last one or four residues of its PDZ domain-binding motif are

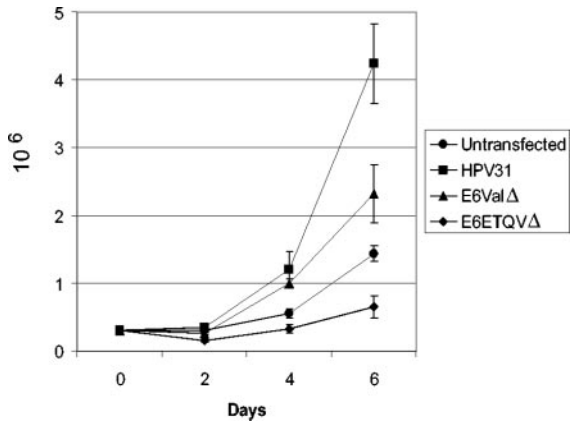

FIG. 2. Growth assay examining untransfected keratinocytes as well as keratinocytes transfected with wild-type HPV31 and two mutant genomes at early passage. Cells were seeded onto 6-cm culture dishes at 3×10^5 cells per dish. At the indicated time points, the cells were trypsinized and stained with trypan blue, and viable cells were counted using a hemocytometer. A representative analysis is shown. Three replicates were used for each time point, and standard deviations are shown as error bars at each time point. Two additional sets of cell lines showed comparable results when their growth rates were examined.

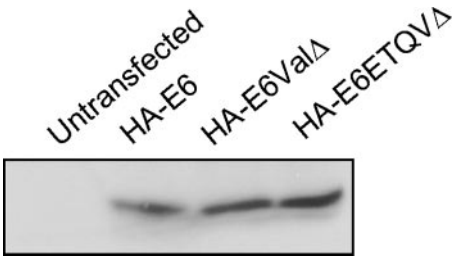

FIG. 3. Western analysis of HA-tagged wild-type, E6ValΔ, and E6ETQVΔ proteins expressed in C33A cells. Three different GW1-HA18E6 vectors encoding wild-type or either of two mutant HPV18 E6 proteins were transfected into C33A cells. After 48 h, equivalent amounts of whole-cell extracts from each of the transfected cells were isolated and separated on a SDS-polyacrylamide gels and transferred to a membrane. This membrane was probed with anti-HA antibody, and proteins were visualized by chemiluminescence as described in Materials and Methods.

defective for binding to PDZ proteins including hDlg and hScrib (33, 49) but still competent for other biological activities of E6 such as inactivation of p53 and induction of telomerase (32). To examine the effects of the E6 PDZ mutant proteins on viral replication, two mutant viral genomes as well as wild-type genomes were stably transfected into HFKs along with a drug resistance marker. Drug-resistant colonies were isolated, pooled, and expanded for further studies. We performed three independent transfections into different host keratinocytes and consistently observed the following results with all three sets of cell lines.

The PDZ domain-binding motif of E6 is necessary to induce efficient growth of the HPV31-transfected HFKs. We first investigated whether the PDZ domain-binding motif of E6 plays any role in the proliferation of the HPV31-transfected HFKs by determining growth rates at early passage following transfection. As can be seen in Fig. 2, cells containing either of two PDZ-binding mutant genomes were significantly retarded in their growth rates compared with those transfected with wild-type genomes. Typically, the HPV31 wild-type-transfected HFKs grew significantly faster than those containing either of two mutant genomes. Cells containing E6ETQVΔ mutant genomes grew at rates comparable to or even slightly reduced from those of the untransfected HFKs at early passage. Despite the negative effect of deletion of the PDZ domain-binding motif on cellular growth, cells transfected with either of two mutant genomes could still be passaged more than 20 times (approximately 100 population doublings) while the untransfected HFKs typically senescenced around passage 6 or 7. These data indicate that the PDZ domain-binding motif of E6 facilitates efficient growth of the HPV31-transfected HFKs but is not necessary for the extension of the life span seen with HPV-positive cells. At later passages, we also observed a higher frequency of integration of viral DNA in cells containing either of mutant genomes compared to that in cells with wild-type genomes. The integration of viral genomes into host DNA often provides host cells with a growth advantage due to the loss of E2-mediated inhibition of transcription of E6 and E7. At later passages, HFKs transfected with mutant genomes exhibited higher growth rates than at early passages, but this was still slower than that seen for cells transfected with wild-type genomes.

Deletion of the PDZ domain-binding motif does not affect the stability of HPV E6 protein. Since we observed a reduction in the growth rates of cells transfected with either of two mutant genomes, we wanted to see if this decrease was caused by any changes in the stabilities of E6 PDZ mutant proteins expressed from mutant genomes. Since it is not technically feasible to measure the levels of E6 proteins when expressed in the context of the whole-virus genomes, we used an HA-tagged HPV E6 expression system to examine stability. The HA-tagged HPV18 E6 protein has previously been used in several studies to study viral protein function (20, 21). For reasons that are not clear to us, we were unable to detect a tagged HPV31 protein with identical vectors and so relied on the tagged HPV18 proteins for analysis. We constructed the corresponding HA-tagged HPV18 E6 PDZ mutants and transfected them into C33A cells, which are HPV-negative cervical carcinoma cells. The transient-expression levels of wild-type and the two PDZ mutant E6 proteins were then compared. As shown in Fig. 3, no significant difference was detected in the amounts of E6 proteins expressed from wild-type or two mutant E6 expression vectors. These data indicate that deletion of the PDZ domain-binding motif does not affect the stability of E6 proteins.

The PDZ domain-binding motif of E6 is involved in maintenance of the viral copy number. We next examined the state of viral DNA in the transfected cells. Total DNA was isolated from each of the cell lines and analyzed by Southern analysis for the state of HPV genomes. As shown in Fig. 4A, the wild-type and the two mutant viral genomes were all maintained as episomes. However, significant differences were consistently detected in the viral copy numbers. Following linearization of viral genomes by XbaI digestion, the total viral copy numbers of the E6ValΔ- and E6ETQVΔ-transfected HFKs were approximately 60 and 20% of those of the cells containing

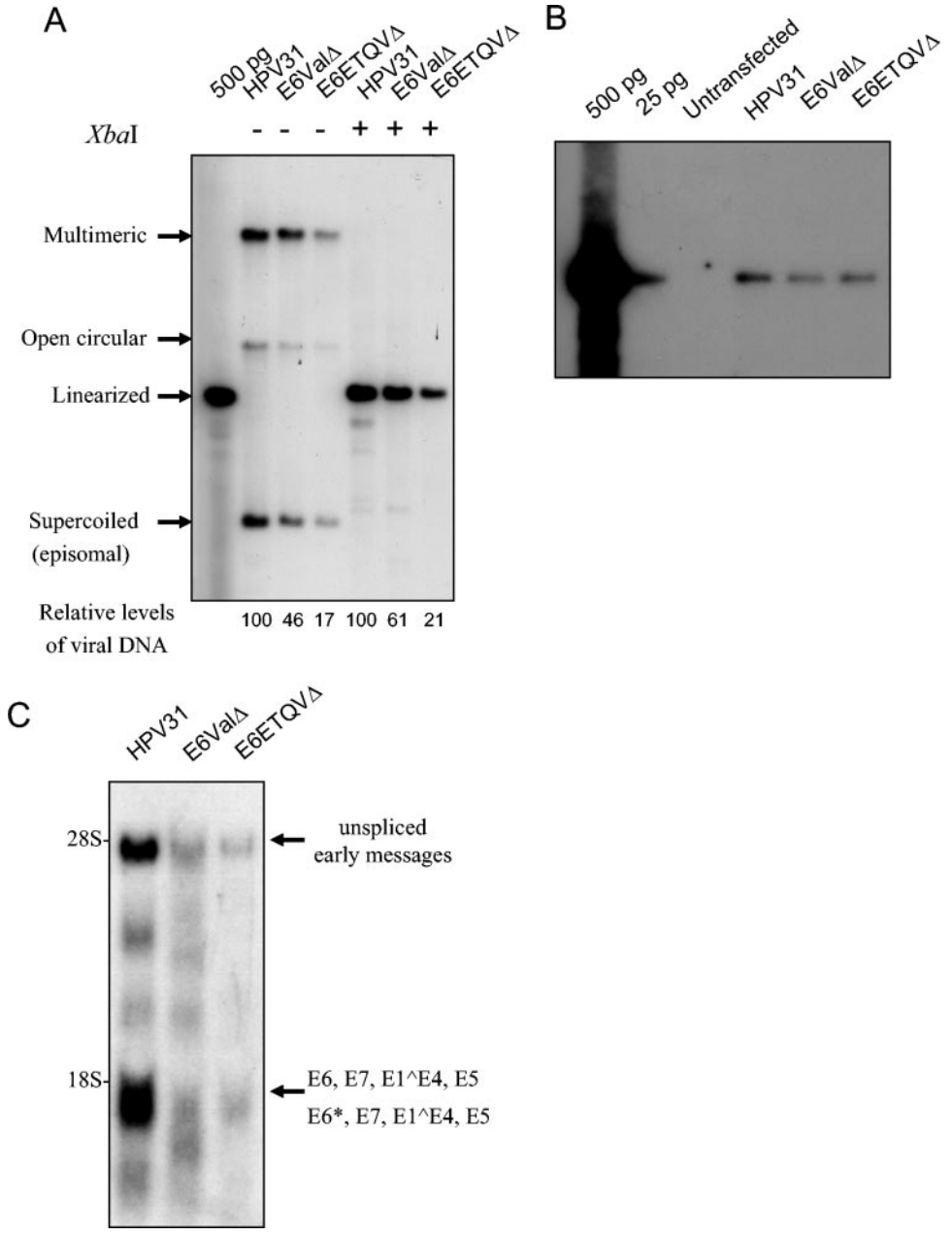

FIG. 4. Analysis of viral DNA in keratinocytes transfected with wild-type HPV31 and two mutant genomes (A) Southern blot analysis of HFKs stably transfected with HPV31 wild-type, E6ValΔ, and E6ETQVΔ genomes following selection and expansion in monolayer cultures. The first lane contains 500 pg of linearized HPV31 genome as the copy number control. The next three lanes contain 10 μg of uncut total DNA isolated from cells transfected with wild-type HPV31 and two mutant genomes. The adjoining three lanes contain 10 μg of DNA from same cells digested with XbaI. The multimeric as well as the episomal forms of the viral genomes are indicated on the left of the autoradiograph. Quantitations of the relative amounts of DNA in all HPV-positive bands in each lane are shown at the bottom of the autoradiograph. (B) Southern blot analysis of SCC-13 cells transiently transfected with HPV31 wild-type, E6ValΔ, and E6ETQVΔ genomes as well as an untransfected control. The first and second lanes contain 500 and 25 pg of linearized HPV31 genome, respectively, as the copy number control. (C) Northern blot analysis of the early viral transcripts in the HPV31 wild-type, E6ValΔ, and E6ETQVΔ genome-transfected HFKs following growth in monolayer culture. A 10-μg portion of total RNA was extracted from the HPV31 wild-type, E6ValΔ, and E6ETQVΔ genome-transfected HFKs, separated on an agarose-formaldehyde gel, and transferred to a membrane that was hybridized with a probe containing the complete HPV31 genome. The indicated 26S and 18S rRNAs are 4.7 and 1.8 kb, respectively. Ethidium bromide staining of these two rRNA markers confirmed the equal loading of the RNA samples.

wild-type genomes, respectively, in the representative experiment in Fig. 4A. Similar results were obtained in three independent transfection experiments using different primary HFKs isolates. In two additional experiments using different keratinocyte hosts, we observed only a slight reduction in copy number immediately after transfection, but on further passaging, the copy numbers in the cell lines with mutant genomes became significantly reduced (data not shown). When South-

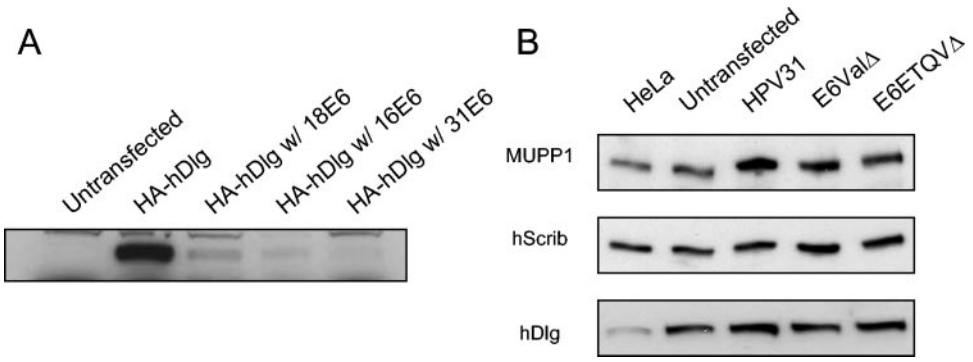

FIG. 5. (A) Western blot analysis of the HA-tagged hDlg protein in the presence or absence of high-risk HPV E6 protein in C33A cells. A 2-μg portion of each HA-tagged hDlg construct was transfected alone or together with 2 μg of high-risk E6 plasmid into C33A cells. At 48 h posttransfection, expression levels of hDlg protein were examined by Western blot analysis using an anti-HA antibody. (B) Western blot analysis of the PDZ proteins MUPP1, hScrib, and hDlg in untransfected keratinocytes as well as keratinocytes that contain HPV31 wild-type, E6ValΔ, and E6ETQVΔ genomes.

ern analysis was performed on cells at later passage, we observed that cells transfected with either of two mutant genomes had lost most of the episomal forms and contained primarily integrated DNAs. In contrast, cells transfected with wild-type genomes still maintained viral DNAs primarily as episomes (data not shown).

We have previously shown that in undifferentiated cells, transcripts encoding E1 and E2 initiate at the early promoter and include sequences in the E6 ORF. A transient-replication assay was used to show that *cis* mutations to the E6 splice donor or acceptor sites resulted in loss of replication ability due to reduced E1/E2 expression while insertion of the translation termination codon had no effect (27, 65). We used this assay to functionally test whether the reduction in the copy numbers of two mutant viral genomes is due to the inability of E6 to bind PDZ proteins or to a *cis* effect on E1 and E2 transcripts. As shown in Fig. 4B, 5 days after transfection of SCC-13 cells, both mutant viral genomes were able to replicate to the levels comparable to those of wild-type genomes. In one experiment we observed reduced levels of transient replication of E6ΔETQV mutant genomes and cannot exclude the possibility that there is a modest effect of this mutation on E1/E2 transcripts. However, we think the primary cause of the PDZ mutations on episomal copy number is that of the mutant protein rather than a *cis* effect.

The decreased viral copy numbers of two mutant HPV31 genomes correlate with the reduced expression of the early viral transcripts. Since we observed a reduction in the viral copy numbers in cells transfected with either of two mutant genomes, we next wanted to determine if there was a corresponding reduction in the expression of the early viral transcripts in cells maintaining either of two mutant genomes. To examine the early viral transcription levels, we performed a Northern blot analysis using RNAs extracted from the cells containing wild-type HPV31 as well as two mutant genomes (Fig. 4A). As can be seen in Fig. 4C, the HFKs transfected with wild-type genomes expressed the two sets of viral transcripts of approximately 1.5 and 4.3 kb. The larger transcript represents the unspliced mRNAs initiated at the early promoter P97, whereas the 1.5-kb mRNAs encode two of the most abundant spliced mRNAs: E6, E7, E1^E4, and E5, and E6*, E7, E1^E4, and E5. In contrast, in the E6ValΔ- and E6ETQVΔ-transfected HFKs, the levels of both the unspliced and spliced early viral mRNAs were significantly reduced. These data suggest that the decreased viral copy number by deletion of the PDZ domain-binding motif of E6 correlates with the reduced expression of the early viral transcripts. The 2.3-kb transcript seen in the wild-type lane in Fig. 4C was not observed in other experiments.

The PDZ proteins levels are not significantly changed in the HPV31-transfected HFKs. By using heterologous overexpression systems, high-risk HPV E6 proteins have been shown to target not only p53 but also PDZ proteins for proteasomal degradation (20, 21, 39, 49, 56, 67). To confirm the degradation of PDZ proteins by high-risk HPV E6 proteins, we transfected HPV18, HPV16, and HPV31 E6 expression plasmids as well as HA-tagged hDlg constructs into the HPV-negative cervical cancer line, C33A. As can be seen in Fig. 5A, we observed that HPV31 E6 was able to degrade hDlg similarly to the HPV18 and HPV16 E6 proteins. The ability of HPV31 E6 to degrade hDLG in similar assays has been previously reported (55). Based on these observations, we wanted to evaluate the effect of the expression of the E6 proteins from the whole HPV31 genomes on the levels of PDZ proteins. We compared the expression levels of PDZ proteins in the untransfected keratinocytes as well as in keratinocytes transfected with wild-type HPV31 and two HPV31 mutant genomes by Western blot analysis. As shown in Fig. 5B, no significant differences were observed in the levels of three PDZ proteins, hDlg, hScrib, and MUPP1, between the untransfected and HPV31 wild-type-transfected HFKs. Since HeLa cells express relatively high levels of HPV18 E6 (11), we also examined the levels of PDZ proteins in these cells. It was observed that HeLa cells maintained comparable levels of MUPP1 and hScrib proteins to those in the untransfected and HPV31-transfected cells while their hDlg levels were significantly reduced (Fig. 5B). We also examined the levels of p53 in the HPV31-positive cells and observed no differences between keratinocytes with wild-type genomes or either of the two mutant genomes (data not shown). The degree of p53 degradation by HPV31 E6 is not as extensive as that seen with HPV18 or HPV16. These results suggest that the HPV31 gene products, when expressed from

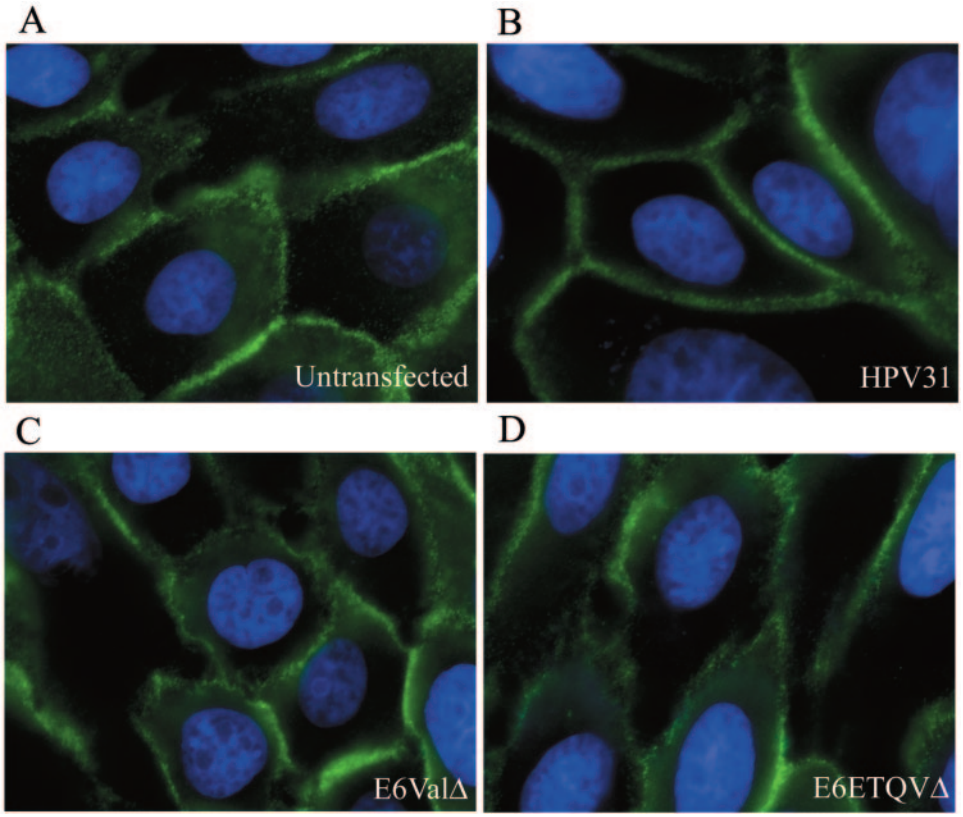

FIG. 6. Cellular localization of hDlg proteins in the untransfected keratinocytes as well as keratinocytes that contain HPV31 wild-type, E6ValΔ, and E6ETQVΔ genomes. Cells were grown on glass slides, fixed in paraformaldehyde, permeabilized in Triton-X, and probed with anti-hDlg antibody along with fluorescein isothiocyanate-conjugated anti-mouse secondary antibody to visualize the location of the hDlg proteins. The fluorescence signal was examined on a Nikkon inverted fluorescence microscope. (A) Untransfected HFKs; (B) HPV31 wild-type HFKs; (C) E6ValΔ HFKs; (D) E6ETQVΔ HFKs.

the whole viral genomes, do not significantly change the levels of PDZ proteins.

The stable transfection of the HPV31 genomes into HFKs minimally changes the localization of hDlg proteins. It has been shown that the association of adenovirus 9 E4ORF1 protein with PDZ proteins can lead to an aberrant localization of PDZ proteins (21, 22, 39, 40). It was important to determine if the expression of E6 in the context of the entire HPV31 genomes is also able to induce any changes in the localization of PDZ proteins. We therefore performed immunofluorescence analysis using the monolayer cell cultures of the untransfected HFKs as well as those transfected with wild-type, E6ValΔ, and E6ETQVΔ genomes. As can be seen in Fig. 6A, in the untransfected HFKs, a punctate pattern of localization of hDlg proteins was seen at the peripheral regions of the cell-cell contact. A minor population of hDlg proteins distinct from the membrane-bound form mentioned above was also observed in the cytoplasm of the untransfected HFKs. The hDlg proteins in the cytoplasm appeared more diffusely distributed than those in the membrane. In some wild-type HPV31-transfected cells, a reduction in the amounts of cytoplasmic forms of hDlg proteins was observed, with most hDLG proteins found at the intercellular membrane regions (Fig. 6B). However, in the majority of cells, no differences in the cytoplasmic and membrane-bound distribution of hDlg proteins were detected. These data suggest that in proliferating monolayer cultures, the expression of the HPV31 gene products from viral genomes does not induce dramatic changes in the subcellular distribution of hDlg proteins and only subtle effects are seen.

The PDZ domain-binding motif of E6 is not required for the differentiation-dependent amplification of the viral genomes. The studies described above suggest that the PDZ domain-binding motif of E6 is involved in modulating proliferation ability as well as impacting the early viral functions such as the maintenance of the viral copy number. Since the late phases of the viral life cycle are closely linked to the differentiation program of the host keratinocytes, we examined if there was any effect of the association of E6 with PDZ proteins on late viral functions following differentiation. Suspension of the HPV31 wild-type-positive HFKs in semisolid media such as methylcellulose has been used successfully to study the differentiation-dependent late viral functions, including amplification of the viral DNA and activation of the late viral promoter. We next examined whether cells containing either of two mutant genomes retained the ability to amplify upon epithelial differentiation induced by suspension in methylcellulose for 48 h. As can be seen in Fig. 7, cells transfected with either E6ValΔ or E6ETQVΔ genomes were able to amplify viral genomes approximately 3.3- and 2.7-fold upon differentiation,

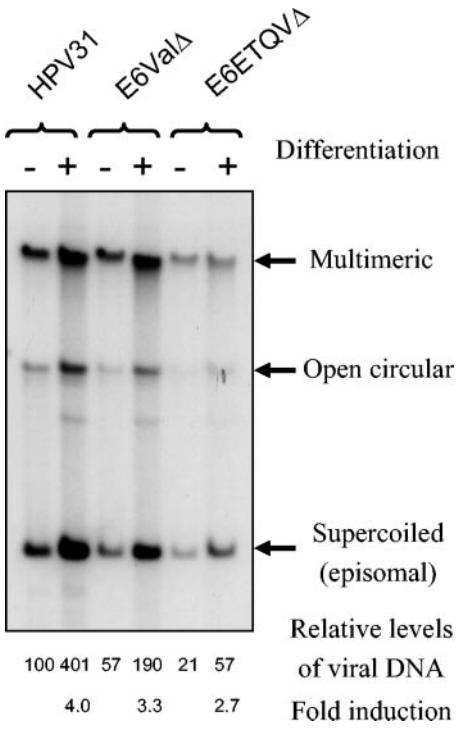

FIG. 7. Differentiation-dependent amplification of HPV31 wild-type, E6ValΔ, and E6ETQVΔ genomes. The HFKs that maintained the HPV31 wild-type, E6ValΔ, and E6ETQVΔ genomes were incubated in 1.5% methyl cellulose for 48 h to induce epithelial differentiation. Then 10 μg of total genomic DNA was extracted and examined by Southern analysis for HPV31 DNA. The multimeric as well as the episomal forms of the viral genomes are indicated on the right of the autoradiograph. Quantitations of the relative amounts of the HPV31 DNA in each lane are shown at the bottom of the autoradiograph.

respectively. Although cells containing wild-type genomes exhibited a slightly greater ability to amplify (4.0-fold) in this experiment, overall we observed minimal differences in the degree of amplification between wild-type-containing and either of two mutant genome-containing cells in two other experiments using pooled transfected cell lines (data not shown). These results suggest that the PDZ domain-binding motif of E6 is not required for the differentiation-dependent amplification of the viral genomes.

Comparisons of the differentiation-dependent morphological changes induced by HPV31 genomes with deletion of the PDZ domain-binding motif of E6. The next set of experiments examined whether cells containing either of two mutant genomes induced any changes in the patterns of differentiation in organotypic rafts that were distinct from those seen in cells containing wild-type HPV31. To examine the effect of loss of the PDZ domain-binding motif of E6 on epithelial morphology upon differentiation, we grew organotypic raft cultures by using E6ValΔ- and E6ETQVΔ-transfected HFKs. As can be seen in Fig. 8A, raft cultures of the untransfected HFKs exhibited a typical differentiation pattern, with nuclear staining found predominantly at the basal layer cells. In contrast, the HPV31 wild-type-transfected HFKs demonstrated an altered differentiation pattern with a thickening of the basal layer and extensive nuclear staining throughout all layers in their raft cultures (Fig. 8B). Normal keratinocytes rapidly lose nuclei upon differentiation, while cells that express high-risk HPVs maintain nuclei throughout the suprabasal layers. Histological cross sections of raft cultures from the E6ValΔ-transfected HFKs revealed a modest reduction in the overall thickness compared with those of the HPV31 wild-type-transfected HFKs. Nevertheless, their morphology was similar to that of cells containing wild-type genomes (Fig. 8C). More obvious changes in the morphological differentiation were observed in the raft cultures of cells transfected with E6ETQVΔ genomes. The overall thickness of these raft cultures was consistently reduced in multiple experiments compared with that of raft cultures using the HPV31 wild-type-transfected cells (Fig. 8D). In addition, the morphology more closely resembled that of normal keratinocytes than that of cells that maintained wild-type HPV 31 genomes (Fig. 8D). These data suggest that the PDZ domain-binding motif of E6 may play a role in the ability of HPV31-positive cells to stratify and differentiate.

Differentiation-dependent membrane localization of hDlg protein in the raft cultures. We next examined the cellular localization of hDlg protein in organotypic rafts by immunohistochemical analysis. As shown in Fig. 9A, the untransfected HFKs exhibited a weak diffusive immunoreactivity to anti-hDlg antibody in the cytoplasm of cells in the basal layer. However, cells in the more differentiated suprabasal layer showed a strong membrane localization of hDlg proteins. This differentiation-dependent membrane localization of hDlg proteins was also found in the raft cultures of cells containing wild-type genomes (Fig. 9B). The localization of hDlg in the basal cells was consistently observed to be more diffuse in the basal layer of the raft cultures of the HPV31 wild-type-transfected HFKs than that seen in the raft cultures of the untransfected cells. The raft cultures of cells containing either E6ValΔ or E6ETQVΔ mutant genomes also exhibited a diffuse pattern of hDlg localization in basal cells. In suprabasal cells, hDlg proteins were found predominantly at the cell peripheries in both normal and HPV31-positive raft cultures. Surprisingly, we did not observe pronounced differences in hDlg localization in differentiated layers between HFKs transfected with wild-type or mutant genomes. We suspect that deletion of the PDZ domain-binding motif of E6 may induce subtle changes in the distribution of hDlg protein in the suprabasal cells, but these were not readily discernible in our studies. Immunohistochemical analysis of another E6-binding PDZ protein, MUPP1, showed a differentiation-independent cytoplasmic localization in the raft cultures of both the untransfected and HPV31-transfected cells (data not shown). Again, we did not observe any dramatic alteration in the localizations of MUPP1 in cells containing either of two mutant genomes. These data suggest that the cellular localization of hDlg protein is not grossly altered by HPV31 gene products upon epithelial differentiation, but we cannot exclude the possibility that there are subtle changes not readily detected by our assays.

DISCUSSION

In the present study we demonstrate that the PDZ domain-binding motif of E6 proteins plays a significant role in the life cycle of high-risk HPVs. Mutation of the PDZ domain-binding motif of E6 in the context of the complete HPV31 genomes

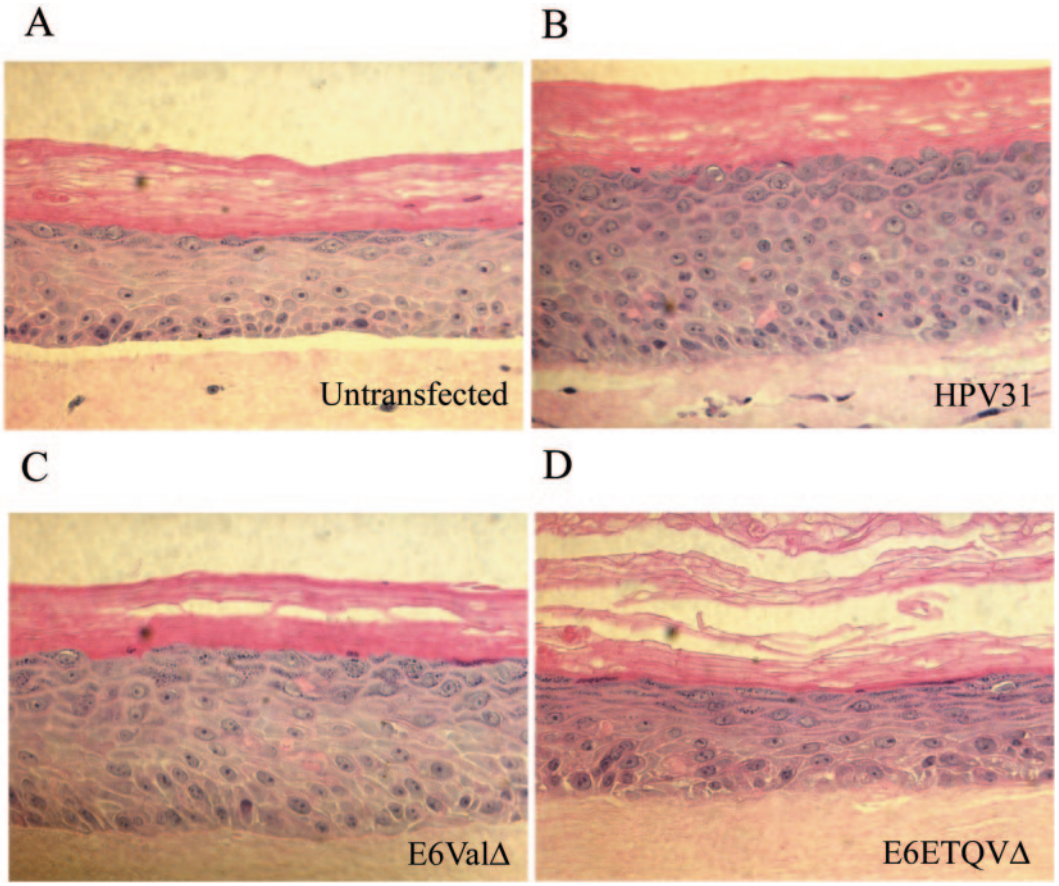

FIG. 8. Hemotoxylin-and -eosin-stained sections of the organotypic raft cultures of the untransfected HFKs as well as HFKs that maintain HPV31 wild-type, E6ValΔ, and E6ETQVΔ genomes. Keratinocytes were grown in raft cultures for 14 days, harvested, and paraformaldehyde fixed. Sections were stained with hemotoxylin and eosin. (A) Untransfected HFKs; (B) HPV31 wild-type HFKs; (C) E6ValΔ HFKs; (D) E6ETQVΔ HFKs.

was consistently found to induce a reduction in the rate of growth of cells harboring mutant genomes compared to those that maintained wild-type genomes. The growth rate of cells containing mutant genomes with deletion of the entire PDZ domain-binding motif of E6 was comparable to that of the untransfected normal human keratinocytes. Interestingly, this reduction in growth rate did not alter the ability to extend the life span of primary human keratinocytes. While we did not strictly measure the immortalization ability, we suspect that the loss of the PDZ domain-binding motif of E6 does not alter this capability. In three of five experiments, E6 PDZ mutant genomes were maintained at lower copy numbers than were wild-type genomes immediately following transfection. In the two sets of cells in which only a slight reduction was seen immediately after transfection, significantly reduced levels of E6 PDZ mutant genomes were observed after several additional passages. This reduction in episomal copy number may contribute to the reductions in growth rates we observed, although it is probably not the determining factor, given the variability seen in our studies. Previous studies have demonstrated that elimination of E6 protein by insertion of a translation stop codon in the context of the complete viral genome results in an inability to stably maintain viral episomes as well as a failure to extend the life span (65).

We also observed a much higher frequency of integration of viral DNA with extended passage in cells containing either of mutant genomes than in cells with wild-type genomes. The integration of viral genomes into host DNA often provides host cells with a growth advantage due to the loss of E2-mediated inhibition of transcription of E6 and E7. At late passages, HFKs transfected with mutant genomes grew faster but still slower than the wild-type-transfected cells. This may be the result of the selection for fastest-growing cells with passage. Furthermore, we did not observe any significant changes in protein stability on deletion of the PDZ domain-binding motif of high-risk E6 proteins. We therefore think that the effects we observed were due to the absence of the PDZ domain-binding motif.

The question arises how a loss of the PDZ domain-binding ability of E6 results in the reduction in growth rates of cells and lower levels of episomes. The maintenance of extrachromosomal DNAs is not well tolerated in normal cells and requires the action of viral proteins to overcome this block. We suspect that the alteration of cell cycle regulators such as p53, Rb, and PDZ proteins is required to facilitate the growth of cells and the maintenance of extrachromosomal DNAs. The activities of these cellular proteins are balanced, and loss of one function influences the action of other factors. For instance, it was

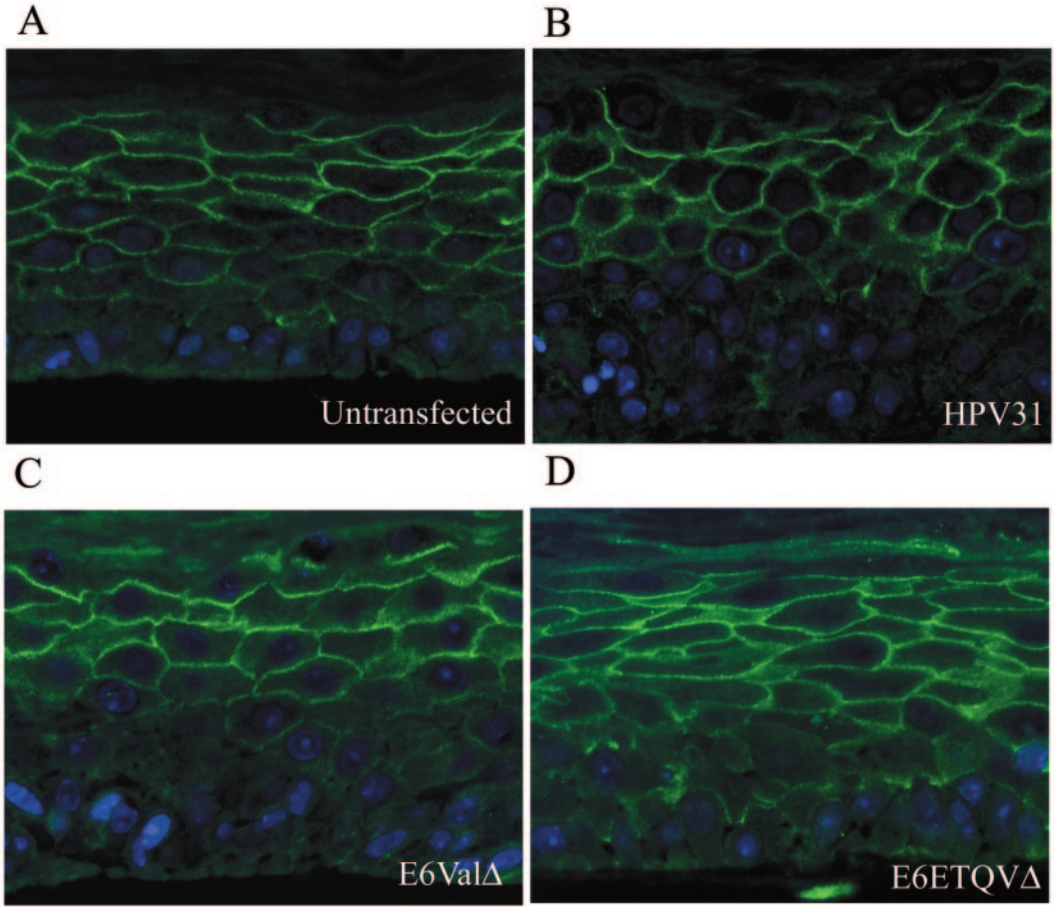

FIG. 9. Cellular localization of hDlg proteins in organotypic rafts of untransfected HFKs as well as HFKs that maintain HPV31 wild-type, E6ValΔ, and E6ETQVΔ genomes. Sections from raft cultures were stained with an anti-hDlg antibody and examined by immunohistochemical analysis. (A) Untransfected HFKs; (B) HPV31 wild-type HFKs; (C) E6ValΔ HFKs; (D) E6ETQVΔ HFKs.

recently demonstrated that abrogation of the ability of E6 to bind p53 in the context of complete HPV genomes led to a loss of episomal maintenance ability, but this occurred only if the corresponding E7 protein had high-affinity binding to Rb (52). When the Rb-binding domain of E7 was mutated to that found in low-risk E7 proteins, the ability of E6 to interact with p53 was no longer required for episomal maintenance. In a similar manner, the binding of E6 to PDZ proteins may act to create a balance with another activity of some other HPV proteins or activity of E6 itself.

Several PDZ domain proteins, including hDlg, hScrib, MAGI-1, MAGI-2, MAGI-3, and MUPP1, associate with E6 proteins in heterologous expression assays, but it is unclear which of these PDZ proteins is the primary physiological target of E6 in vivo. It is possible that the primary targets of E6 are all these factors, a subset, or as yet unidentified PDZ proteins. In heterologous overexpression systems, E6 induces the degradation of PDZ proteins (20, 21, 39, 49, 56, 67), and we observed similar effects of HPV31 E6 in this study. While we did not observe any reduction in hDlg levels in cells that maintained viral episomes, we did detect low levels of hDlg in the established HPV18-positive cervical cell line, HeLa. It is possible that the levels of expression of E6 from complete viral genomes are not sufficient to induce the degree of degradation seen in heterologous overexpression systems, where E6 is expressed at high levels. In addition, expression of E6 from integrated genomes in the absence of E2 repressive activity has been suggested to occur at higher levels than that seen in cells with episomal genomes (31). Therefore, degradation of hDlg may be more pronounced in HPV-positive tumors than in premalignant lesions that are actively producing virions. This could be one of the reasons why we failed to see any significant reduction of PDZ proteins in cells containing wild-type genomes. Nevertheless, our data suggest that binding of PDZ proteins alone is important to maintain high copy numbers of viral genomes.

An alternative explanation for our inability to observe a significant reduction in the hDlg level by E6 is that E6 induces the degradation of only a specific population of PDZ proteins such as the cytoplasmic forms as opposed to proteins found at cell-cell junctions. In recent studies, HPV18 E6 was shown to specifically induce the degradation of the cytoplasmic forms of hDLG (44a), and this would be consistent with this observation. When we examined the distribution of hDlg proteins in the proliferating monolayer cultures containing HPV31 wild-type genomes, we found that, in a subset of cells, wild-type E6 proteins induced a concentration of hDlg proteins at the cytoplasmic membranes and a reduction in the number of cyto-

plasmic forms. These changes were, however, observed in only a subset of cells. It is possible that the disappearance of the cytoplasmic forms of hDlg was the result of specific degradation, but our assays are not sufficiently sensitive to allow for such a conclusion.

In raft cultures of normal keratinocytes, a highly diffuse pattern of hDlg staining was observed throughout cells in the basal layers. In contrast, upon differentiation, hDlg proteins were localized to the cytoplasmic membranes in the suprabasal cells. Similar patterns of hDlg staining have been reported in biopsy samples of HPV16-positive lesions of the cervix (69). While we did not observe dramatic changes in cellular localization of hDlg in raft cultures, it is possible that E6 induces subtle changes in hDlg protein localization that are not easily detectable in our assays. It is interesting that the patterns of localization of PDZ proteins in monolayer cultures are most similar to that seen in cells in the suprabasal layers in organotypic raft cultures. This is not an effect due to the proliferation state of the cell since similar patterns of hDlg localization were seen in both normal and HPV-positive differentiating keratinocytes. Normal suprabasal keratinocytes have exited the cell cycle, while HPV31-positive cells continue to be active in the cell cycle. We also examined the distribution patterns of other PDZ proteins such as hScrib and MUPP1 but detected no significant differences. Similarly, we examined the expression of epithelial junction marker proteins such as ZO-1 and E-cadherin, as well as epithelial differentiation marker proteins such as keratin-10, in both monolayer and raft cultures of normal and HPV31-positive cells but detected no significant differences (C. Lee and L. A. Laimins, unpublished data). The reduced thickness we observed in raft cultures using E6ETQVΔ-transfected cells could be due to the combined effects of loss of viral copy number and expression of PDZ mutant E6 proteins.

Numerous binding partners of high-risk E6 proteins have been identified, including p53 and E6AP, and the ability of E6 to activate human telomerase reverse transcriptase expression is well documented (51). However, it is not clear which of the other E6-binding partners that have been identified using heterologous overexpression assays represent physiologically significant interactions. Our studies suggest that association of high-risk E6 proteins with PDZ proteins is physiologically important and that this interaction is significant for the pathogenesis of high-risk papillomaviruses. Future insights into these interactions will require the identification of the primary targets of E6 action on PDZ proteins in differentiating epithelial cells.

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