In most cervical cancers, DNAs of high-risk mucosotropic human papillomaviruses (HPVs), such as types 16 and 18, are maintained so as to express two viral proteins, E6 and E7, suggesting that they play important roles in carcinogenesis. The carboxy-terminal PDZ domain-binding motif of the E6 proteins is in fact essential for transformation of rodent cells and induction of hyperplasia in E6-transgenic mouse skin. To date, seven PDZ domain-containing proteins, including DLG1/hDLG, which is a human homologue of the Drosophila discs large tumor suppressor (Dlg), have been identified as targets of high-risk HPV E6 proteins. Here, we describe DLG4/PSD95, another human homologue of Dlg, as a novel E6 target. DLG4 was found to be expressed in normal human cells, including cervical keratinocytes, but only to a limited extent in both HPV-positive and HPV-negative cervical cancer cell lines. Expression of HPV18 E6 in HCK1T decreased DLG4 levels more strongly than did HPV16 E6, the carboxy-terminal motif of the proteins being critical for binding and degradation of DLG4 in vitro. DLG4 levels were restored by expression of either E6AP-specific short hairpin RNA or bovine papillomavirus type 1 E2 in HeLa but not CaSkri or SiHa cells, reflecting downregulation of DLG4 mRNA as opposed to protein by an HPV-independent mechanism in HPV16-positive cancer lines. The tumorigenicity of CaSki cells was strongly inhibited by forced expression of DLG4, while growth in culture was not inhibited at all. These results suggest that DLG4 may function as a tumor suppressor in the development of HPV-associated cancers.

Infection with specific human papillomaviruses (HPVs), such as HPV type 16 (HPV-16) and HPV-18, is a major risk factor for human cancer of the uterine cervix. From the facts that E6 and E7 genes are expressed almost exclusively in cervical cancer cells and that they can inactivate the tumor suppressors p53 and Rb, respectively, they are believed to play key roles in carcinogenesis in the cervix. Accumulating lines of evidence suggest that the PDZ domain-binding motif is particularly important for transformation and tumorigenesis in cultured cells (16, 42) and hyperplasia and carcinogenesis in E6-transgenic mice (30). To date, several PDZ domain-containing proteins have been identified as E6 targets, namely, mammalian homologues of Dlg (DLG1/hDLG) and Scribble (Scrib/Vartul), MUPP1, MAGI-1, -2, and -3, and GIPC (5, 9,16, 20, 21, 27). Both Dlg and Scribble function as cortical tumor suppressors in Drosophila and are essential for cortical localization of Lgl, which is required for asymmetric cortical localization of basal determinants, such as Prospero and Numb, in mitotic neuroblasts (32, 33). In mammalian cells, their functions are less well characterized, although some reports suggest that DLG1 may possess tumor suppressor activity (13, 23). Dlg-related proteins are associated with the cortical actin cytoskeleton and appear to have both structural and functional roles. Several homologues of Dlg have been identified in humans. Among them, four are closely related to Dlg, namely, DLG1 (hDLG, SAP97), DLG2 (PSD93, Chapsyn-110), DLG3 (NEDLG, SAP102), and DLG4 (PSD95, SAP90). DLG1, -2, -3, and -4 have 56 to 60% amino acid identity to Dlg and have 70 to 75% amino acid identity to each other, while DLG5 (P-DLG), which is highly expressed in placenta and prostate, has 40%, and DLG6 (MPP4), which is predominantly expressed in retina, has 33% identity to Dlg and belongs to the p55 (MPP1) MAGUK family. DLG1, -2, -3, and -4 are highly expressed in neuronal tissues, especially in postsynaptic densities. However, at least some of them are rather widely expressed in other tissues, though at lower levels, and seem to have functions in nonneuronal tissues, including colorectal epithelium for DLG1 (24) and esophageal epithelium for DLG3 (22). In Drosophila, Dlg is expressed in both neuronal and epithelial cells. Clearly, DLG2, -3, and -4 as well as DLG1 could be E6 targets in cervical epithelial cells. Here, we focused on DLG4 first, as it is the closest homologue of DLG1 and Dlg, and we isolated it as an E6-interacting protein in a yeast two-hybrid system (8).
expressed in normal keratinocytes of both uterine cervix and skin and can be targeted for degradation by E6-E6AP complexes. Though DLG4 protein is thus down-regulated in HPV18 E6-positive HeLa cells, Dlg4 mRNA is down-regulated by an HPV-independent mechanism in the HPV16-positive cervical cancer cell lines SiHa and CaSkii. In addition, expression of DLG4 in CaSkii cells suppresses tumorigenicity to a remarkable degree, suggesting a tumor suppressor function in the development of HPV-associated cancers.

MATERIALS AND METHODS

Cell culture. The cervical cancer cell lines HeLa, CaSkii, SiHa, C33A, OM4C, and Yumoto were maintained in Dulbecco’s modified Eagle medium (DMEM; Sigma) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS). The HPV16-positive cervical cancer cell lines OG-H and SKG1116 were provided by Hiroshi Shirasawa and maintained in RPMI 1640 supplemented with 10% (vol/vol) FBS. HBE1C (human bronchial epithelial cells) and HDK1 (human dermal keratinocytes) were purchased from Cell Applications Inc. (San Diego, CA), and HFT2 cells (human foreskin fibroblasts) were from Cambrex (Walkersville, MD). Normal human keratinocytes (HCK1) were obtained with written consent from a patient who underwent a hysterectomy for a gynecological disease other than cervical cancer. They were infected with LXSH-hTERT retroviruses (28) encoding the catalytic subunit of human telomerase reverse transcriptase (hTERT), and the infected cells were selected in the presence of 20 μg/ml of bolemin B. The resulting HCK1T cells were maintained in keratinocyte serum-free medium (KSFM; Invitrogen) supplemented with 5 ng/ml epidermal growth factor (EGF) (Sigma) and 50 μg/ml of bovine pituitary extract (Hammond Techn, Windsor, CA). HCK1T cells had been cultivated for more than 150 population doublings (PD), and the majority of the cells maintained almost normal diploid chromosomes at 10, 40, and 120 PD. All cells were incubated in a humidified atmosphere containing 5% CO2 at 37°C.

Plasmids. Wild-type and mutant HPV16 E6 (16E6) and/or p53 mutants from the full-length DLG4 cDNA were produced by PCR with various primer sets specific to DLG4 cDNA (sequences specific to DLG4 cDNA are capitalized), and attB adaptor primers, 5′-gggccctaaagcttagcctaaagc-3′ and 5′-aagaagcttcgaagatctgaag-3′, were used as probes with horseradish peroxidase-conjugated anti-mouse, anti-rabbit (Jackson Immunnarchie Laborataries), or anti-goat (sc-2033; Santa Cruz) immunoglobulins as the secondary antibodies. All the antibodies were used at a dilution of 1:1,000 for Western blotting except E7 antibodies, which were used at a dilution of 1:1,000. A LAS3000 charge-coupled device imaging system (Fujiﬁlm Co. Ltd., Japan) was employed for detection and quantiﬁcation of proteins visualized by Lumi-light plus Western blotting substrate (Roche).

Quantiﬁcation RT-PCR analysis. Total RNA was obtained from cultured cervical cancer cell lines using an RNaseq kit (QIAGEN) according to the manufacture’s protocol. Single-stranded cDNAs were made from 500 ng of total RNA using a reverse transcription system (Promega) with random hexamers. Fifty nanograms of cDNA template was subjected to PCR ampliﬁcation with primer sets speciﬁc to Dlg4 or acidic ribosomal phosphoprotein P0 (36B4) using a SYBR Green PCR core reagents kit (Applied Biosystems; Foster City, CA). The expression level of the Dlg4 gene was then normalized to RNA content for each sample using 36B4 as a control. PCR analysis was performed under the following conditions: denaturation at 95°C for 10 min followed by 40 cycles (95°C for 15 s, 58°C or 60°C for 10 s for Dlg4 and 36B4 genes, respectively, and 72°C for 10 s). Ampliﬁed products were detected with a iCycler iQ real-time PCR detection system (Bio-Rad). Sequences of primer pairs used were as follows: 5′-GCTGATGCAACAGTGCTATAGA-3′ and 5′-GAAATCTAGTGATGATGG-3′ for Dlg4, 5′-TGCGAATGGCTGAGGACATTC-3′ and 5′-GGACACCTTGAGGAAGAGGTTG-3′ for 36B4.

Protein interaction assays. Various E6 and Dlg4 proteins were synthesized using the in vitro transcription-coupled reticulocyte lysate (TNT) system (Promega) in the presence of [35S]methionine and [35S]cysteine. Preparation and purification of maltose-binding protein (MBP) and GST fusion proteins were as described previously (16). The proteins immobilized to amylose- and glutathione-Sepharose (New England Biolabs and Amersham) were reincubated in TNT300 buffer (1% NP-40, 50 mM Tris-HCl [pH 7.5], and 300 mM NaCl containing 2% bovine serum albumin for at least 10 min, mixed with 4 to 20 μl of the programmed reticulocyte lysates containing in vitro-translated products or cell lysates, and incubated at 4°C for 2 h under constant rotation. The Sepharose beads were then washed with TNT300 buffer ﬁve times. The captured proteins were eluted by adding sodium dodecyl sulfate (SDS) sample buffer, separated by SDS-polyacrylamide gel electrophoresis (PAGE), and detected with a BAS2500 image analyzer (Fujiﬁlm Co. Ltd.) or by immunoblotting.

Material and methods

Full-length human DLG4 cDNA was isolated from HeLa cell RNA by reverse transcription-PCR (RT-PCR). After ampliﬁcation of the open reading frame with speciﬁc primers containing attB1 and attB2 sequences, 5′-aaaaaaggctgctcctgctgtcgtctctgcccagccacattcataagcagagataagagcattagtgattgtgctttggtcaatggagaggaggaagagag-3′ and 5′-aagaagcttgcaagtgctataagacaggtgtaagcgagggcctttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt ---- Downloaded from http://jvi.asm.org/ by guest on October 2, 2017 ----
Anchorage-independent cell growth assay. Cells were seeded at $1 \times 10^5$ per 35-mm plate in 70% (vol/vol) DMEM–30% (vol/vol) FBS with 0.4% agarose and a 0.7% agarose overlay. Fresh medium was overlaid once a week.

Tumorigenicity in nude mice. Tumorigenicity was tested by injecting $5 \times 10^6$ cells subcutaneously into BALB/cAJcl-nu female mice (Nihon Clea, Japan) at 6 weeks of age and measuring the sizes of the resulting tumors at least once a week for 2 months. Animal protocols were approved by the committee for Ethics of Animal Experimentation and were in accordance with the Guideline for Animal Experiments in the National Cancer Center.

Statistical analysis. The data were analyzed using Student's $t$ test, with a $P$ value of <0.05 considered significant.

RESULTS

Expression of DLG4 in cervical cancer cell lines and normal cervical keratinocytes. We first examined the expression levels of DLG4 as well as some other known E6 targets in cervical cancer cell lines and normal human cell strains, including hTERT-transduced cervical keratinocytes (HCK1T) by immunoblotting (Fig. 1A). HPV-positive cervical cell lines examined expressed very little DLG4, while HCK1T as well as normal dermal keratinocytes (HDK1) expressed easily detectable levels of DLG4. The expression level of DLG4 in HCK1T was about 30 and 50% of those in a neuroblastoma cell line, IMR32, and a glioblastoma cell line, U87, respectively (data not shown), suggesting a significant function of DLG4 in epithelial cells. We confirmed the result with another antibody (Calbiochem clone 6G6-1C9), though it was less sensitive (data not shown). Among HPV-negative cervical cancer cell lines, C33A and OMC4 (but not the Yumoto line) expressed low levels of DLG4. It is noteworthy that all the normal human cell strains, including nonepithelial fibroblasts (HFF2), expressed DLG4. Unexpectedly, some HPV-positive cervical cancer cell lines expressed rather higher levels of DLG1 and Scrib than HCK1T. Expression levels of ZO1, a MAGUK family protein similar to DLG1 and DLG4 but not an E6 target, were quite variable.

DLG4 expression is down-regulated by E6 proteins in HCK1T. We next examined the effects of 16E6, 18E6, and their mutants on the expression of DLG4 as well as p53 as a control in HCK1T cells (Fig. 1B). The E6 mutants included the 8S9A10T substitution mutant of 16E6 (16E6SAT), which is unable to target p53 for degradation but is still able to bind E6AP (15), and carboxy-terminal deletion mutants (16E6Δ151 and 18E6Δ158), which are unable to bind DLG1 (16). Expression levels of DLG4 were decreased by wild-type E6 proteins of both HPV16 and -18 but not by their carboxy-terminal deletion mutants. However, 18E6 was more potent than 16E6 in its activity. The difference seemed largely dependent on the difference in the last amino acid of the two proteins, as 16E6-151V, which had the same C terminus as 18E6, showed activity similar to that of 18E6, and 18E6-158L showed activity similar to that of 16E6. As expected, p53 levels were unchanged by the 16E6SAT mutants, but the DLG4 levels again clearly depended on the difference in their last amino acids. As the 16E6SAT mutants showed slightly weaker activities than their counterparts, degradation of p53 might be indirectly involved in the regulation of DLG4 expression. Unexpectedly, down-regulation of DLG1 and Scrib was not sufficiently clear to allow an unequivocal conclusion, probably because their basal levels were relatively low in subconfluent cultures (Fig. 2B).

Cell density-dependent regulation of DLG4. Based on the notion that PDZ domain-containing proteins play roles in signal transduction through cell-cell contact and/or in regulation of cell polarity, DLG4 levels at different cell densities were examined. DLG1 was remarkably up-regulated in postconfluent cultures of HCK1T, CaSi, and SiHa but not HeLa cells (Fig. 2A). A similar increase of DLG4 was seen in postconfluent cultures of C33A and less clearly in HeLa and SiHa, while a decrease was seen in HCK1T cells. Likewise, a significant decrease of Scrib was observed in postconfluent cultures of HeLa, SiHa, and C33A cells, while a significant increase was observed in HCK1T cells, suggesting different regulatory mechanisms in DLG1, DLG4, and Scrib. Next, we examined

FIG. 1. Expression of DLG4 in cervical cancer cell lines and primary human cells and the effects of various E6 proteins in HCK1T cells. (A) Expression levels of DLG4, DLG1, ZO1, Scrib, and p53 analyzed by immunoblotting. All cell proteins were extracted from subconfluent cultures. The same amounts of each cell extract (20 μg protein) were loaded per lane (β-actin levels were not used as loading controls, as they were different in each cell line) (data not shown). HPV18-positive (HeLa), HPV16-positive (CaSi, SiHa, OMC4, SKGIIb), and HPV-negative (OMC4, Yumoto, C33A) cervical cancer cell lines, an immortalized cervical keratinocyte cell line (HCK1T), normal human dermal keratinocytes (HDK1), human bronchial epithelial cells (HBEC1), and normal human foreskin fibroblasts (HFF2) were used. (B) HCK1T cells were transduced with the indicated genes by retroviral gene transfer. Cell proteins were extracted from subconfluent cultures, and expression levels of the indicated proteins were analyzed by immunoblotting. “Δ151” indicates deletion of the carboxy-terminal 151st amino acid; “V” indicates replacement of the carboxy-terminal leucine with valine; “L” indicates replacement of the carboxy-terminal valine with leucine; 16E6SAT is an E6 mutant with an N-terminal three-amino-acid substitution which is defective in p53 inactivation; LXSN is the vector control.
the effects of E6 on their expression at different cell densities. Maximum levels of DLG4 were observed at confluence in HCK1T cells, whereas in the DLG1, Scrib, and MAGI-1 cells they were observed in postconfluent cultures (Fig. 2B). E-cadherin and involucrin, which is an early differentiation marker protein of keratinocytes, were also up-regulated in confluent cultures of HCK1T but very little in the cervical cell lines (Fig. 2A), and levels remained low in HCK1T cells expressing 16E6 and 18E6 (Fig. 2B). Down-regulation of DLG4 by both E6 proteins was observed at different cell densities (Fig. 2B). Interestingly, upregulation of DLG1 and MAGI-1 was inhibited by both E6 proteins and that of Scrib was inhibited by 16E6 only under postconfluent conditions. Surprisingly, down-regulation of p53 by 18E6 was alleviated at confluence and almost cancelled postconfluence (Fig. 2B). Taking into account that 18E6 is less efficient in promoting degradation of p53 than 16E6 (Fig. 1B and 2B) (37), the result implies the possibility that PDZ domain-containing proteins are better substrates for 18E6-E6AP ubiquitin ligase complexes than p53 and that accumulated proteins after confluence sequester the enzyme complexes from p53, though the idea remains to be validated.

Our findings suggest that DLG4 as well as DLG1 has a specific role in normal epithelial cells, even though the expression levels are lower than those in neuronal tissues. Though calcium was reported to enhance differentiation of keratinocytes, the levels of DLG1 and DLG4 were unaffected by calcium concentration in the medium between 0.035 and 1 mM (data not shown), supporting a more critical role of cell-cell contact in keratinocyte differentiation (17).

**Restoration of DLG4 by repression of E6 and E7 in HeLa cells expressing BPV1 E2.** Previous studies showed that BPV1 E2 proteins can inhibit the E6/E7 promoters in the LCRs of HPV16 and -18 in HPV-positive cervical cancer cell lines so as to repress their expression and induce growth arrest (2, 3, 35). E6 can target DLG1, Scrib, MUPP1, and MAGIs for degradation (7, 9, 20, 27, 40). Therefore, we tested whether repression of high-risk E6 and E7 expression by BPV1 E2 can restore the expression of DLG4 and other E6 target proteins. In HeLa cells, expression levels of DLG4 and DLG1 proteins as well as p53 were restored by BPV1 E2 expression while the ZO1 level was rather reduced (Fig. 3, lanes 3 and 4). HeLa cells exogenously expressing 16E6 also demonstrated restored expression of DLG4 to an extent similar to that observed in parental HeLa cells with E2 expression, whereas the expression level of p53 was not restored (Fig. 3, lanes 1 and 2). DLG4 expression levels were very low in the 16E6-positive cervical cancer cell lines CaSki and SiHa and were not elevated by E2 expression, whereas p53 was clearly up-regulated (Fig. 3, lanes 5 to 8). DLG1 levels were relatively high in these cells and were increased very little by E2 expression. No change in expression levels of these proteins was observed in the control (the HPV-negative cervical cancer cell line C33A). These data together with those depicted in Fig. 1 suggest that DLG4 and DLG1 are efficient targets for degradation by 18E6 but not by 16E6 in vivo and that DLG4 down-regulation in HPV16-positive cervical cancer cell lines (CaSki and SiHa) depends not on either 16E6 or 16E7 function but on other mechanisms.

**Different Dlg4 mRNA levels in cervical cancer cell lines.** To address the mechanisms whereby DLG4 is down-regulated in the HPV16-positive cervical cancer cell lines SiHa and CaSki, mRNA levels were analyzed by quantitative RT-PCR. As expected, in CaSki and SiHa cells they were relatively low. In contrast, Dlg4 mRNA levels in HeLa cells were higher than those in HCK1T, C33A, and OMC4 cells (Table 1), all of which showed higher levels of DLG4 protein than HeLa cells (Fig. 1A). This discrepancy between mRNA levels and protein levels of DLG4 supports the notion that down-regulation of

[FIG. 2. Cell density-dependent regulation of DLG4 and other proteins. (A) Cells were harvested under subconfluent conditions (SC) or 7 days postconfluence (PC). Expression levels of the indicated proteins were analyzed by immunoblotting. The same amounts of each cell extract (20 μg of protein) were loaded in each lane, with β-actin being used as a loading control. Only a 170-kDa form of MAGI-1 detected in C33A and HCK1T is shown. (B) HCK1T cells transduced with 16E6, 18E6, and backbone vector (LXSN) were harvested under subconfluent (SC) or confluent (Conf) conditions or 9 days postconfluence (PC), and expression levels of the indicated proteins were analyzed by immunoblotting.]
FIG. 3. E6-dependent and -independent down-regulation of DLG4. HPV18-positive (HeLa), HPV16-positive (CaSki, SiHa), and HPV-negative (C33A) cervical cancer cell lines, as well as HeLa cells infected with LXSN-16E6SD, were harvested 7 days after infection with MSCVpuro-BPV1E2 (E2) or the control vector MSCVpuro and selected in medium supplemented with 1 μg/ml of puromycin (2 μg/ml for SiHa cells) from day 2. Mock-infected cells died before harvesting (data not shown). Expression levels were analyzed by immunoblotting. The same amounts of cell extracts (20 μg protein) were loaded in each lane.

DLG4 in HeLa cells is posttranscriptional and caused by enhanced degradation of the protein by 18E6. The mRNA results also suggested that the down-regulation of Dlg4 in SiHa and CaSki is due mainly to reduced mRNA levels by an HPV-independent mechanism.

Specific interactions between E6 proteins of high-risk mucosotropic HPVs and DLG4. To further clarify the mechanisms whereby E6 down-regulates expression of DLG4, we examined whether E6 proteins can physically interact with DLG4 in vitro. Various MBP-fused E6 proteins immobilized on amylase-Sepharose beads were mixed with lysates from DLG4-overexpressing C33A cells. DLG4 was efficiently bound by E6 proteins of HPV16 and -18 but not by those of HPV11, -5, or -1 or by HPV16 E7 (Fig. 4A). To delimit the segment of DLG4 necessary for binding to 16E6 or 18E6, deletion mutants of DLG4 were prepared by in vitro transcription and translation in reticulocyte lysates. DLG1 was used as a positive control for binding to E6. As expected, DLG4 as well as DLG1 was efficiently bound by E6 proteins of both HPV16 and -18 but not by β-galactosidase (β-Gal). Deletion mutants containing PDZ do- 

TABLE 1. Dlg4 mRNA levels in cervical cancer cell lines determined by quantitative real-time RT-PCR

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Relative Dlg4 mRNA level ( \times 10^{-3} )</th>
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<tbody>
<tr>
<td>HeLa</td>
<td>2.60 ± 0.95**</td>
</tr>
<tr>
<td>CaSki</td>
<td>0.49 ± 0.45*</td>
</tr>
<tr>
<td>SiHa</td>
<td>0.36 ± 0.20**</td>
</tr>
<tr>
<td>C33A</td>
<td>1.17 ± 0.08</td>
</tr>
<tr>
<td>OMC4</td>
<td>0.13 ± 0.06**</td>
</tr>
<tr>
<td>HCK1T</td>
<td>2.13 ± 0.24**</td>
</tr>
</tbody>
</table>

* Relative ratio of Dlg4 mRNA to 36B4 ribosomal protein mRNA (means ± standard errors for triplicate samples in a representative experiment; the mean value for HCK1T was set at 1). **, \( P < 0.05 \) compared to HCK1T; *, \( P < 0.05 \) compared to HeLa.
of the last amino acid, leucine, of HPV16 E6 with valine (16E6-151V) greatly increased the binding affinity for DLG4, and a change to isoleucine (16E6-151I) did so to a lesser extent. Conversely, replacement of the last amino acid, valine, of HPV18 E6 with leucine (18E6-158L) decreased the affinity. These results indicated that the PDZ domain-binding motif in the carboxy-terminal motif of E6 proteins is critical for interaction with DLG4 and that differences in the last amino acid, whether leucine or valine, are critical for the affinity, accounting for the variation between 16E6 and 18E6 activities shown in Fig. 1B. The importance of the last amino acid residue on 16E6 and 18E6 has also been shown for other PDZ targets (7, 39, 41).

**E6-induced degradation of DLG4 in vivo and in vitro.** We next examined E6-induced degradation of DLG4 on transient transfection. Expression of 16E6 and 18E6 reduced the steady-state levels of DLG4 in 293FT cells, and 18E6 was more potent than 16E6. Again the activity proved dependent on the last amino acids of both E6 proteins (Fig. 5A). The half life of DLG4 was less than 15 min in 18E6-expressing cells, whereas it was more than 120 min without the E6 protein (Fig. 5B). These results clearly indicate that E6 can strongly enhance degradation of DLG4 in vivo. We could also confirm E6-dependent degradation of DLG4 in vitro (Fig. 5C). However, surprisingly, 16E6 was more potent than 18E6 in degradation of DLG4 in a reticulocyte lysate system, and the difference in the last amino acid was rather trivial in this system. The observed difference is not due to expression levels of E6 proteins, as 18E6 (Fig. 5C, lane 2), which has 10 cysteines, was rather more abundant than 16E6 (lane 5), which has 14 cysteines. The stability of these E6 proteins was similar, as we observed similar amounts of E6 proteins to remain after 3 h of incubation (data not shown).

**E6-induced degradation of DLG4 depends on E6AP in vivo.** E6AP can recognize p53 as a substrate for ubiquitination only when making complexes with E6 proteins. Likewise, E6AP is reported to be required for degradation of another E6 target, Scrib (27), although another ubiquitin ligase was suggested to be necessary for degradation of DLG1 (10, 34). In order to examine whether E6AP is involved in the degradation of DLG4, E6AP-specific shRNAs were used to reduce the E6AP expression in the transient-transfection assay. Although the apparent reduction of E6AP levels was not very obvious, probably owing to imperfect transfection efficiency and the stability of E6AP (15), both E6AP-specific shRNAs inhibited the E6-dependent degradation of DLG4, and the additional expression of E6AP but not the dominant negative mutant of E6AP reversed the effect (Fig. 6A). E6AP-specific shRNA was stably expressed in three HPV-positive cell lines, HeLa, CaSki, and SiHa, and an HPV-negative cell line, C33A, and the steady-state levels of the E6 targets were measured by immunoblotting (Fig. 6B). As expected, steady-state levels of p53 were increased in the HPV-positive cell lines but not in C33A harboring mutant p53, indicating a specific effect of E6AP.
shRNA. Levels of DLG4, DLG1, and Scrib in HeLa were increased by the E6AP shRNA expression. In CaSki and SiHa cells, the levels of Scrib and DLG1 were significantly increased, while those of DLG4 were not. In C33A cells, no stabilization of DLG4, DLG1, or Scrib by E6AP shRNA was observed. These results thus suggest that E6AP is involved in degradation of DLG4, DLG1, and Scrib in HeLa cells and in that of DLG1 and Scrib in CaSki and SiHa cells.
To further investigate the potential role of E6 and E6AP in degradation of DLG4, we examined E6- and E6AP-dependent ubiquitination of DLG4 in vivo. Polyubiquitinated forms of FLAG-tagged DLG4 were increased by 18E6 coexpression, concomitantly with the reduced steady-state level of DLG4, and they were further increased by E6AP coexpression but decreased by coexpression of E6AP shRNA4 or a dominant negative mutant of E6AP (Fig. 6C). We next examined E6-dependent ubiquitination of DLG4 in vitro using proteins translated in rabbit reticulocyte lysates and wild-type and mutant E6 proteins of HPV16 and HPV18. As expected, formation of high-molecular-weight forms of p53, which could be considered to be ubiquitination, was enhanced by addition of 18E6Δ158 as well as the wild-type E6 proteins but not by 16E6SAT and 16E6SATΔ151. In a parallel experiment, formation of high-molecular-weight forms of DLG4 was enhanced by 16E6ΔAT but as wild-type E6 proteins but not by the carboxy-terminal deletion mutants (Fig. 6D). We did not observe significant difference in the activities of 16E6 and 18E6 in the reticulocyte lysate system. In the wheat germ extract system, where the functional ubiquitin ligase E6AP is absent (11), both E6 and E6AP were required for the formation of high-molecular-weight forms of DLG4 (Fig. 6E). 18E6 was clearly more potent than 16E6 in putative ubiquitination, concordant with the in vivo degradation assay results. Our findings as a whole indicate that E6AP is involved in E6-mediated ubiquitination and degradation of DLG4 both in vivo and in vitro.

**Tumor-suppressive effects of DLG4 in CaSki cells.** To address the biological significance of the observed down-regulation of DLG4 in cervical cancer cell lines, we decided to examine the biological effects of exogenous DLG4 expression in CaSki cells. As carboxy-terminal PDZ domain-binding motif-dependent transformation of 3Y1 cells is evident only after cells become confluent (16), we hypothesized that close cell-cell contact is required for any negative growth signal(s) involved with PDZ-domain containing proteins. We chose CaSki cells as the targets, as they express more E-cadherin than SiHa cells (Fig. 2A) and retain morphological features of epithelial cells. We prepared pooled populations of DLG4-expressing CaSki cells (CaSki/DLG4) as well as the control cells (CaSki/LXSN) by retroviral gene transfer. As expected, exogenously expressed DLG4 was not rigorously degraded by HPV16 E6 expressed in the cells and was easily detected by immunoblotting, though the expression levels were decreased at confluence (Fig. 7A). We first analyzed exponential growth of these pooled cells on plastic dishes, but no significant differences were observed (Fig. 7B). Then, we compared anchorage-independent growth of these pooled cells in soft agarose medium and found that CaSki/DLG4 formed smaller and fewer colonies than CaSki/LXSN (Fig. 7C). Finally, tumorigenicity in nude mice was examined. Surprisingly, DLG4 markedly suppressed tumor growth of CaSki cells in a pilot experiment. The result was confirmed in repeated experiments using the same pooled cells (Fig. 7D; Table 2), and four more sets of independently established pooled cells (Table 2); the tumor-suppressive effect was not detected with the second set of pooled cells, but here the control cells (CaSki/LXSN) formed no or small tumors, in contrast to the other experiments. These results together with the findings for anchorage-independent growth in soft agarose medium indicate that overexpressed DLG4 exerts tumor-suppressive effects on CaSki cells.
DISCUSSION

Identification of DLG4 as a novel E6 target. In this study, we identified the Dlg4 gene product, DLG4/PSD95, as a novel E6 target and showed that it was down-regulated in five out of five HPV-positive cervical cancer cell lines, whereas the known E6 targets DLG1 and Scrib were not (Fig. 1A). The result led us to consider that DLG4 might be an important E6 target in the development of HPV-positive cervical cancers. We found that E6 proteins of both HPV16 and -18 were able to bind DLG4 through the carboxy-terminal PDZ domain-binding motif in vitro (Fig. 4A and D) and down-regulate expression of DLG4 (Fig. 1B) through enhanced ubiquitination (Fig. 6C, D, and E) and degradation (Fig. 5A, B, and C) of DLG4 both in vivo and in vitro. Actually, accumulation of DLG4 as well as p53 in HeLa cells was observed with expression of both BPV1 E2 (Fig. 3) and E6AP-specific shRNA (Fig. 6A and B). Our results clearly indicate that the E6 proteins of HPV16 and -18 have the potential to induce proteolytic degradation of DLG4 as well as DLG1. Recently, Grm and Banks reported that degradation of DLG1 and MAGI1 by human papillomavirus E6 is E6AP independent (10). However, our results clearly indicate that degradation of DLG4 and DLG1 by E6 in HeLa cells is, at least in part, E6AP dependent, consistent with a recent report by others (25), though we cannot rule out the possibility that a ubiquitin ligase(s) other than E6AP is also involved. Low expression levels of DLG4 in the HPV16-positive cell lines CaSki and SiHa as well as in one of three HPV-negative cervical cancer cell lines tested, Yamoto, were due mainly to an HPV-independent reduction in DLG4 mRNA levels (Table 1).

Differential effects of HPV16 and HPV18 E6 proteins on DLG1, DLG4, and Scrib. We and others have described potential differences between 16E6 and 18E6 in affinity and effects on DLG1 (16, 23). In this report, we show the second PDZ domain of DLG4 to be critical for binding to both E6 proteins, similar to DLG1 (16, 23), but, unlike with DLG1, the third domain was not found to be involved in the binding to 18E6 (16, 23). We also show 18E6 to be more potent, at least in vivo, for targeting DLG4 as well as DLG1 than 16E6, which in contrast has a stronger influence on Scrib. This difference appeared to be primarily dependent on the last amino acids of these two proteins, leucine for 16E6 and valine for 18E6, because swapping of these last amino acids alone can change the targeting specificity (Fig. 1B and 4D). The same conclusion about DLG1 and Scrib was drawn by others recently (41). E6 proteins of most high-risk mucosotropic types end with valine like 18E6, and only that of HPV33, which has close homology to that of HPV16, ends with leucine. It is likely that these two groups of E6 proteins target overlapping PDZ domain-containing proteins with different specificities so as to lead to similar overall consequences. This notion again underlines the importance of functions dependent on the carboxy termini of E6 proteins. 16E6 appeared to have a weaker capacity for binding and inducing degradation of DLG4 both in vivo and in vitro (Fig. 1B, 4C and D, 5A, and 6A). However, in a reticulocyte lysate system, 16E6 showed a higher activity for inducing degradation of DLG4 (Fig. 5C) together with similar activity for inducing its ubiquitination (Fig. 6D), and surprisingly, the difference in the last amino acid (leucine or valine) had little effect on degradation (Fig. 5C). Although we have no data to explain the apparent discrepancy, one possibility is that the rabbit ortholog of E6AP or a putative ubiquitin ligase suggested for DLG1 degradation (10) favors 16E6 in the degradation of DLG4.

Tumor-suppressive effects of DLG4 in CaSki cells. If DLG4 functions as a tumor suppressor in the development of cervical cancer, restoration of its expression might be expected to change the carcinogenic phenotype(s) of cervical cancer cell lines. Based on this notion, we introduced DLG4 into CaSki cells (Fig. 7A). This resulted in reduced colony forming ability in soft agarose medium (Fig. 7C). More strikingly, CaSki/DLG4 cells were much less tumorigenic than control CaSki/LXSN cells (Fig. 7D and Table 2). However, CaSki/DLG4 cells did not show any difference in morphology and exponential growth in plastic dishes (Fig. 7B). This indicates that DLG4 would have no toxic effect on normal cell growth, as inferred from its expression in normal epithelial cells (Fig. 1), and that the effect on CaSki cells is specific to anchorage-independent growth and tumorigenicity in nude mice. One may argue that the reduced tumorigenicity by overexpression of DLG4 would depend on sequestering 16E6 from p53. However, the fact that the levels of p53 in the CaSki/DLG4 cells were as low as those in CaSki/LXSN cells (Fig. 7A) suggests this to be unlikely. Another possibility is that endogenous DLG4 has transforming/oncogenic activity like DLG1 (6) and that overexpressed DLG4 functions as a dominant-negative (DN) inhibitor of this activity, though this interpretation does not explain the reduced RNA levels of DLG4 in CaSki and SiHa cells. As DLG4 is expressed in not only cervical keratinocytes but also other epithelial cells, such as dermal keratinocytes and bronchial epithelial cells, we speculate that it might also function as a tumor suppressor in other tissues. Indeed, with immunoblotting of lung cancer cell lines, we found that three out of nine lung cancer cell lines examined expressed undetectable levels of DLG4 (K. Handa, unpublished data).

Though enhanced long-term potentiation and impaired learning are reported for mice lacking DLG4 (26), no association with cancer has been reported. However, as DLG1 to -4 are close homologues of Dlg, they might compensate for each other in epithelial tissues. Alternatively, it is possible that DLG4 has more functions in humans than in mice, as human DLG4 is also expressed in a wide range of nonneural tissues (38), possibly because of a unique genome organization. In humans, but not in mice, the DLG4 and VLCAD (very-long-chain acyl coenzyme A dehydrogenase) genes are arranged in a head-to-head orientation and share a 245-bp overlapping region that contains part of DLG4 exon 1 and the entire exon 1 of VLCAD, including 62 bp of the protein-coding sequence.

### TABLE 2. Tumorigenicity of CaSki cells expressing DLG4

<table>
<thead>
<tr>
<th>Expt</th>
<th>No. of mice</th>
<th>Tumor vol (mm$^3$)$^a$</th>
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<tr>
<td></td>
<td></td>
<td>LXSN</td>
</tr>
<tr>
<td>1</td>
<td>15</td>
<td>461 ± 351</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>18 ± 26</td>
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<tr>
<td>3</td>
<td>3</td>
<td>431 ± 417</td>
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<tr>
<td>4</td>
<td>3</td>
<td>365 ± 341</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>402 ± 260</td>
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</tbody>
</table>

$^a$ Volume at 8 weeks after injection (mean ± standard error). *, P < 0.0005 compared to vector-transduced cells.
Despite the overlap of their 5’ ends,Dlg4 and VLCAD exhibit peak mRNA expression in different tissues, suggesting that they are independently regulated at the transcriptional level (43). In CaSki, SiHa, and Yumoto cells (Table 1), Dlg4 mRNA was downregulated. However, as the promoter region is not a CpG island, promoter methylation is not a likely cause of the downregulation. Indeed, Dlg4 mRNA levels were down-regulated in these cell lines but easily detected by RT-PCR.

Possible roles of DLG4 in epithelial cells. We hypothesized that Dlg4, as well as DLG1, Scrib, and MAGI-1, might accumulate when HCK1T cultures reached confluence and that this might be altered in cervical cancer cell lines and E6-expressing HCK1T cells. Indeed, Dlg4 did accumulate in HCK1T cells at confluence, but unlike DLG1 and others, it was not evident 7 days thereafter (Fig. 2B). Dlg4 might thus have a specific function different from that of other PDZ domain-containing target proteins. PDZ domains act as protein-binding domains to make complexes with other PDZ domain-containing proteins in certain combinations (1, 12, 14, 31). It is likely that Dlg4 contributes to complex formation. Indeed, in Drosophila, both Dlg and Scribble are required for cortical translocation of another cortical tumor suppressor, Lgl. We first examined the effect of Dlg4 expression in HeLa as well as CaSki cells, but only mild repression of tumorigenicity was observed (Handa, unpublished), although the steady-state level of the ectopic Dlg4 in HeLa cells was about one-third of that in CaSki or C33A cells, probably because of 18E6-mediated degradation. Unlike CaSki cells, HeLa cells do not express E-cadherin and do not form a pavement-like sheet at confluence. It is possible that PDZ domain-containing proteins like Dlg4 can form functional complexes only when cell-cell contact is sufficiently tight and that a certain threshold level of other PDZ domain-containing proteins is also required for Dlg4 to function. Recently, DLG4, as well as DLG1, Scrib, and MAGI-1, might act as tumor suppressors in the development of cervical cancer. Clearly, further investigations are required to clarify the underlying mechanism(s) whereby these PDZ domain-containing E6 targets could function as tumor suppressors.

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REFERENCES


