Membrane Orientation of the Human Papillomavirus Type 16 E5 Oncoprotein

Ewa Krawczyk, Frank A. Suprynowicz, * Sawali R. Sudarshan, and Richard Schlegel

Department of Pathology, Georgetown University Medical School, 3900 Reservoir Road NW, Washington, DC 20057

Received 16 September 2009/Accepted 20 November 2009

The E5 protein of human papillomavirus type 16 is a small, hydrophobic protein that localizes predominantly to membranes of the endoplasmic reticulum (ER). To define the orientation of E5 in these membranes, we employed a differential, detergent permeabilization technique that makes use of the ability of low concentrations of digitonin to selectively permeabilize the plasma membrane and saponin to permeabilize all cellular membranes. We then generated a biologically active E5 protein that was epitope tagged at both its N and C termini and determined the accessibility of these termini to antibodies in the presence and absence of detergents. In both COS cells and human ectocervical cells, the C terminus of E5 was exposed to the cytoplasm, whereas the N terminus was restricted to the lumen of the ER. Finally, the deletion of the E5 third transmembrane domain (and terminal hydrophilic amino acids) resulted in a protein with its C terminus in the ER lumen. Taken together, these topology findings are compatible with a model of E5 being a 3-pass transmembrane protein and with studies demonstrating its C terminus interacting with cytoplasmic proteins.

Human papillomaviruses (HPVs) are small, nonenveloped, double-stranded DNA viruses (25) that are the causative agents of benign and malignant tumors in humans (43). Most cancers of the cervix, vagina, and anus are caused by HPVs, as are a fraction of oropharyngeal cancers (29, 44). HPV type 16 (HPV-16) is the type most frequently found in anogenital cancers (15, 29), including cervical cancer, the most common cancer of women worldwide (44).

Some of the biological activities of the HPV-16 E5 protein (16E5) include the augmentation of epidermal growth factor (EGF) signaling pathways (8), stimulation of anchorage-independent growth (38), alkalization of endosomal pH (11), and alteration of membrane lipid composition (39). 16E5 also exhibits weak transforming activity in vitro (12), induces epithelial tumors in transgenic mice (13), and plays an important role in koilocytosis (20). There are multiple documented intracellular binding targets for 16E5 such as the 16-kDa subunit of the vacuolar H^+-ATPase (7, 36), the heavy chain of HLA type I (1), EGF receptor family member ErbB4 (6), calnexin (16), the zinc transporter ZnT-1 (21), the EVER1 and EVER2 transmembrane channel-like proteins that modulate zinc homeostasis (21, 31), the nuclear import receptor family member karyopherin β3 (KpNB3) (19), and BAP31, which was previously reported to contribute to B-cell receptor activation (35).

16E5 is a small, hydrophobic protein that localizes to intracellular membranes. When overexpressed in COS cells, it is present in the endoplasmic reticulum (ER) and, to a lesser extent, in the Golgi apparatus (7). At a lower level of expression in human foreskin keratinocytes and human ectocervical cells (HECs), 16E5 is present predominantly in the ER (10, 39). 16E5 contains three hydrophobic regions (14, 16, 22, 30, 41), and it was reported previously that the first hydrophobic region determines various biological properties of the protein (16, 22). It was also shown previously that the 16E5 C terminus plays a role in binding to karyopherin β3 (19) and in the formation of koilocytes (20). While theoretical predictions have been made for the topology of E5 in membranes (16), no experimental data exist. However, a recent study suggested that some highly expressed 16E5 localizes to the plasma membrane, with its C terminus exposed externally (18).

The aim of the present study was to establish the orientation of 16E5 in the ER membrane. By using immunofluorescence microscopy coupled with differential membrane permeabilization (24, 34), we demonstrate the membrane orientation of an N- and C-terminally tagged, biologically active 16E5 protein. Our results indicate that the N terminus is intraluminal and that the C terminus is cytoplasmic, consistent with a model of E5 being a three-pass transmembrane protein and with current data on the interaction of its C terminus with cytoplasmic proteins.

MATERIALS AND METHODS

Cells and viruses. Retroviruses encoding HPV-16 E6 and E7 genes in vector pBabePuro (28) or encoding codon-optimized 16E5 (10) and HPV-16 E6 in vector pLXSN were generated by using the Phoenix cell system (33). The cloning of codon-optimized 16E5 into the pJS55 expression vector was described previously (10, 37). A C-terminal deletion mutant of codon-optimized 16E5 lacking the last 25 amino acids [16E5(–25)] was cloned into the EcoRI and BamHI restriction sites of pJS55. Both 16E5 and 16E5(–25) were N-terminally tagged with the AU1 epitope (DTYRYI) (23) and C-terminally tagged with a small antigenic peptide (YPYDVPDYASL) containing the influenza virus hemagglutinin (HA) epitope (32). These constructs [AU1-16E5-HA and AU1-16E5(–25)-HA] were confirmed by sequencing.

Primary HECs were derived from cervical tissue after hysterectomy for benign uterine disease, as described previously (3), and were immortalized by infection with HPV-16 E6/E7-encoding retrovirus and selection in the presence of puromycin (0.5 μg/ml). 16E5-expressing cell lines were generated from immortalized HECs by infection with retroviruses encoding 16E5 or the empty pLXSN expression vector and selection in the presence of Genetecin G418 (100 μg/ml).
Nonimmortalized HECs expressing HPV-16 E6 and/or 16E5 were generated by infection with E6- and E5-encoding retroviruses (with or without selection). HECs and HEC lines were grown at 37°C and 5% CO₂ in keratinocyte growth medium (KGM; Invitrogen, Carlsbad, CA) supplemented with gentamicin sulfate (10 μg/ml). To maintain constant levels of 16E5 expression, G418 was administered to cell cultures at alternating passages.

Retroviral infection. HECs and HEC lines were grown as described above in 25-cm² or 75-cm² tissue culture flasks. Prior to infection, the growth medium was aspirated, and retroviruses mixed with polybrene (1 μl/ml) were added to the cells for 1.5 h at 37°C while shaking. After infection, this mixture was removed, and growth medium was added to the cells.

Transfection. Prior to transfection, COS cells were grown to 80% confluence on 22- by 22-mm sterile glass coverslips in six-well tissue culture plates in antibiotic-free Opti-MEM (Invitrogen) containing 4% FBS. For each coverslip, 4 μg DNA (in pSS55 or pLXSN) was mixed with 0.5 ml serum-free Opti-MEM and 5 μl Lipofectamine 2000 transfection reagent (Invitrogen) and added to the cells for 4 h. Twenty-four hours later, the cells were fixed and processed for immunofluorescence microscopy or were lysed in radioimmunoprecipitation assay (RIPA) buffer for immunoprecipitation and immunoblot detection of 16E5 or the 16-kDa subunit of the vacuolar H⁺-ATPase (38). The following antibodies were used: a 1:5,000 dilution of mouse anti-AU1 ascites fluid (Covance, Princeton, NJ) or 12CA5 anti-HA mouse ascites fluid (a gift from J. Bolen, Millennium Pharmaceuticals, Cambridge, MA) (for immunoblotting) and 4 μg of rabbit anti-calnexin polyclonal antibody (Covance) or 10 μl of anti-16K rabbit serum (raised against the peptide CKSGTGIASSVMRPEQ conjugated to keyhole limpet hemocyanin) (for immunoprecipitation).

Cell cycle analysis. HECs were harvested by using trypsin, fixed with Vindalol citrate buffer (40), stained with propidium iodide (2), and analyzed by use of a FACStar Plus dual-laser system (Becton Dickinson, San Jose, CA). At least 40,000 events were collected and analyzed for each cell line.

Immunofluorescence microscopy. HECs and COS cells were grown on 22- by 22-mm sterile glass coverslips in six-well tissue culture plates. HECs were cultured in keratinocyte serum-free medium (KSF; Invitrogen) for approximately 24 h prior to fixation to increase flatness. HECs and COS cells were fixed in 4% (wt/vol) paraformaldehyde, and stained with hematoxylin and counterstaining with methyl green (5 μg/ml). To maintain constant levels of 16E5 expression, G418 was administered to cell cultures at alternating passages. COS cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate (Invitrogen).

RESULTS

In a recent study (20), we described the ability of codon-optimized and AU1 epitope-tagged 16E5 to induce koilocytosis in cooperation with the HPV-16 E6 oncoprotein. For the present study, we constructed a doubly epitope-tagged version of 16E5, which includes AU1 at the N terminus and HA at the C terminus (AU1-16E5-HA). To confirm the biological activity of this construct, we examined whether AU1-16E5-HA could induce koilocyte formation in HECs. HECs were coinfected with retroviruses encoding either AU1-16E5 or AU1-16E5-HA and with retroviruses encoding HPV-16 E6. As a control, we used cells expressing 16E5 only, since the expression of this

FIG. 1. The HPV-16 E5 protein, which is epitope tagged at both its N and C termini, retains biological activity. (A) The abilities of the indicated E5 constructs to cooperate with HPV-16 E6 in the induction of koilocytosis are indicated. HECs were transduced with the indicated HPV-16 E5- and HPV-16 E6-encoding retroviruses and were stained 4 days later (H&E) to determine the frequency of koilocytosis. The inductions of koilocytosis were similar for the singly tagged and doubly tagged E5 proteins. Nontagged E5 has been shown to have activity similar to that of these epitope-tagged constructs (20). Error bars represent standard deviations (SD). (B) Association of AU1-16E5-HA with the 16-kDa subunit of the vacuolar H⁺-ATPase (16K). COS cells were transfected with the indicated DNA and lysed 24 h later. Molar mass markers (in kDa) are shown on the left. Samples in the first and second lanes of the right panel were also diluted 5-fold to improve clarity (left). IP, immunoprecipitation; IB, immunoblotting.
oncoprotein alone is not sufficient to generate koilocytes (20). As shown in Fig. 1A, the presence of two epitope tags on 16E5 has little effect on the frequency of koilocyte formation (less than 20% inhibition) compared to AU1-16E5. Also, it was demonstrated previously that the presence of a single epitope tag on 16E5 has no effect on its ability to induce koilocytosis (less than 5% inhibition) compared to untagged 16E5 (20).

To further determine whether the double-epitope-tagged E5 protein retained the known biological activity of the single-tagged protein, we investigated whether AU1-16E5-HA bound to the 16-kDa subunit of the vacuolar H^+-ATPase (16K), a cellular target for wild-type and single-tagged E5 proteins. COS cells were transfected either with AU1-16E5-HA and the pJS55 expression vector or with AU1-16E5-HA and HA-16K.

FIG. 2. Digitonin and saponin differentially permeabilize cellular membrane compartments. The localization of calnexin and the EGFR in detergent-treated cells is shown. COS cells (A) and HEC cells (B) were fixed and permeabilized with saponin or digiton as described in Materials and Methods. Immunofluorescence microscopic detection of the calnexin ER luminal domain (green) and the EGFR extracellular domain (red) is shown. The domains of the indicated proteins were appropriately accessible to antibodies. Scale bars represent 10 μm.
The proteins were immunoprecipitated (using anti-AU1 or anti-16K antibodies) and detected by Western blotting (using 12CA5 anti-HA antibody). Our results demonstrate that AU1-16E5-HA binds the 16-kDa subunit (Fig. 1B). Thus, the double-tagged E5 protein retains at least two biological activities of the E5 protein.

The intracellular localizations of the N and C termini of AU1-16E5-HA were determined with a differential permeabilization technique. To demonstrate the validity of this assay, we first showed that digitonin selectively permeabilized the plasma membrane and that saponin permeabilized all cellular membranes. For this purpose, cells were labeled with an antibody recognizing the luminal domain of calnexin, an integral membrane protein of the ER (4), and an antibody specific for extracellular domain of the EGFR, which is present on the extracellular surface of the plasma membrane and in the lumen of the Golgi apparatus and perinuclear multivesicular endosomes (26, 27). While the calnexin antibody produced a typical ER staining pattern in saponin-permeabilized COS cells (Fig. 2A, middle) and HECs (Fig. 2B, middle), it did not label

FIG. 3. Membrane topology of 16E5. COS cells transfected with AU1-16E5-HA (A) and HECs stably expressing AU1-16E5-HA (B) were fixed and permeabilized with saponin or digitonin. AU1-16E-HA was detected by using mouse anti-HA (green) and rabbit anti-AU1 (red) antibodies. The N terminus of E5 was inaccessible to antibodies in digitonin-permeabilized cells, indicating its ER intraluminal location. The C terminus of E5, on the other hand, was accessible to antibodies in digitonin-permeabilized cells, thereby demonstrating that it is exposed to the cytoplasm. Scale bars represent 10 μm.
digitonin-permeabilized or nonpermeabilized cells (Fig. 2A and B, middle). These results are consistent with digitonin being incapable of permeabilizing the ER membrane at the stated concentrations. The EGFR antibody labeled both the plasma membrane and perinuclear area in saponin-permeabilized COS cells (Fig. 2A, left) and HECs (Fig. 2B, left), whereas the EGFR was detectable only at the plasma membrane in digitonin-permeabilized and nonpermeabilized cells (Fig. 2A and B, left). Combined, these results demonstrate that saponin permeabilizes both the plasma membrane and intracellular membranous structures, while digitonin permeabilizes only the plasma membrane.

We next determined the orientations of the N- and C-terminal ends of AU1-16E5-HA in HEC lines stably expressing this construct. Cells were fixed without subsequent permeabilization or were permeabilized with saponin or digitonin after fixation and were then doubly labeled with rabbit anti-AU1 and mouse anti-HA antibodies. As would be expected, staining typical of ER- and Golgi-localized proteins (COS cells) (Fig. 3A, top) and of ER-localized proteins (HECs) (Fig. 3B, top) was observed for both anti-AU1 and anti-HA antibodies following saponin permeabilization to allow antibody access to all intracellular compartments (7, 10, 19, 39). The AU1 and HA staining patterns merged completely (Fig. 3A and B, top), indicating that both epitope tags are accessible in the same locations. In contrast, only HA staining was visible in digitonin-permeabilized cells, where antibodies are unable to access the lumen of the ER and Golgi apparatus (Fig. 3A and B, middle). Without permeabilization, no staining of AU1-16E5-HA was observed (Fig. 3A and B, bottom). These results demonstrate that in both COS cells and HECs, the C terminus of 16E5 is exposed to the cytoplasm, whereas its N terminus is most likely present in the lumen of the ER.

To further refine the topological orientation of E5 in the membrane and to rule out the possibility that the HA epitope tag was responsible for the cytoplasmic localization of the 16E5 C terminus, COS cells were transfected with AU1-16E5(−25)-HA, a C-terminal deletion mutant of codon-optimized 16E5 lacking the last 25 amino acids. This deletion will remove the predicted third hydrophobic domain and C-terminal hydrophilic residues of 16E5 (16, 30). The cells were fixed without subsequent permeabilization or were permeabilized with saponin or digitonin after fixation and were doubly labeled with rabbit anti-AU1 and mouse anti-HA antibodies. Staining typical of ER- and Golgi-localized proteins was observed for anti-AU1 and anti-HA antibodies following saponin permeabilization, indicating that AU1-16E5(−25)-HA is properly targeted to the ER and Golgi apparatus. Both staining patterns merged completely (Fig. 4, top). In digitonin-permeabilized (Fig. 4, top) and nonpermeabilized (Fig. 4, bottom) cells, neither AU1 nor HA labeling was observed. This indicates, therefore, that both the N and C termini of the deletion mutant are localized in the lumen of the ER, a finding which is consistent with the postulated 3-pass model for E5 membrane orientation. In addition, these results demonstrate that the HA epitope tag is not responsible for the cytoplasmic localization of the 16E5 C terminus.

A recent study suggested that the 16E5 protein localizes to the plasma membrane and that its C terminus faces extracellularly where it induces cell-cell fusion and the formation of heterokaryons in HaCaT cells (17, 18). This orientation of 16E5 is opposite of our current experimental findings. Our immunofluorescence studies also did not detect the 16E5 protein in the plasma membrane in COS cells or in HECs. Importantly, propidium iodide staining of several E5-expressing HECs (Fig. 5A and B) demonstrated that there is no 16E5-dependent induction of cell fusion; that is, there is no increase in the percentage of cells with 4N DNA content (i.e., binucleated cells) or DNA content greater than 4N (i.e., multinucleated cells).
As shown in Fig. 6, disulfide-linked dimers of 16E5 were detected in immunoprecipitates when the protein was grossly overexpressed in COS cells, in agreement with data from previous reports (10, 38). However, this dimerization is highly concentration dependent, since only monomers were detected in immunoprecipitates prepared from 16E5-expressing HEC lines and from COS cells that do not grossly overexpress 16E5 (Fig. 6). Longer exposures of pLXSN-16E5-transfected COS cells
and HECs (Fig. 6, bottom) also failed to reveal the presence of E5 dimers.

**DISCUSSION**

16E5 is a small (83-amino-acid), hydrophobic protein that localizes to membranes of the endoplasmic reticulum and nuclear envelope (10, 39) and, if overexpressed in COS cells, to membranes of the Golgi apparatus as well (7). In the current study we determined the orientation of 16E5 in intracellular membranes. To date, there have been no data directly showing the intracellular membrane topology of 16E5, although many studies have proposed models based on hydrophobicity plots (14, 16, 22, 30, 41).

Our immunofluorescence results, which employed differential permeabilization and double-epitope tagging, demonstrate that the C terminus of 16E5 is located in the cytoplasm, while the N terminus is in the ER lumen, in both COS cells and HECs. The fact that anti-HA antibodies do not label the AU1-16E5 (–25)-HA deletion mutant in digitonin-permeabilized cells demonstrates that the HA epitope tag does not artificially pull the C terminus of 16E5 out of the membrane. We have shown previously that the interaction of 16E5 with karyopherin β3 (KNβ3) is mediated primarily by 10 amino acids at the C terminus of 16E5 (19). This finding is consistent with a clastomembrane localization of the 16E5 C terminus, since KNβ3 is an abundant cellular protein localized mainly in the cytoplasm (9, 42). During the course of the present study, we also attempted to determine the membrane topology of 16E5 using immuno-electron microscopy, but we were not able to obtain both a satisfactory preservation of membranous structures and robust immunostaining under the same fixation conditions (data not shown).

Two studies suggested that the 16E5 protein localizes to the plasma membrane and that its C terminus faces extracellularly, where it induces cell-cell fusion and the formation of binucleated HaCaT cells (17, 18). Our immunofluorescence studies did not detect the 16E5 protein in the plasma membrane in COS cells or in HECs. Moreover, cell cycle analysis of 16E5-expressing and control HEC lines and 16E6/E7-immortalized HECs infected with the pLXSN empty expression vector and untagged, single-tagged, and double-tagged 16E5 demonstrated no 16E5-dependent induction of cell fusion leading to the formation of binucleated cells (Fig. 5A and B). We also failed to detect an increased binucleation in 16E5-expressing cells during the induction of koilocytosis. Most likely, the presence of 16E5 in the plasma membrane is the consequence of the overexpression of 16E5 by the adenovirus system in HaCaT cells. For example, when the bovine papillomavirus E5 protein is expressed at very high levels, such as in baculovirus-infected cells, it can be detected on the plasma membrane rather than its usual location in the ER and Golgi apparatus (5).

Several studies have postulated that the 16E5 protein forms three membrane-spanning domains that lie entirely or almost entirely within the lipid bilayer (16, 30). Those models of 16E5 membrane topology predict an intramembrane localization for all six cysteine residues, making it unlikely that the protein forms disulfide-linked dimers in vivo. We have shown that 16E5 dimerization is highly concentration dependent, since dimers are detected only in immunoprecipitates prepared from COS cells that grossly overexpress 16E5 but not in immunoprecipitates from 16E5-expressing HEC lines or from COS cells that do not overexpress 16E5 (Fig. 6). Given that the membrane localization and orientation of 16E5 are the same in HECs and COS cells, the concentration dependence of dimerization suggests that it is an artifact that occurs after cell lysis.

In conclusion, our demonstration of the orientation of 16E5 in ER membranes is an important step toward predicting its potential cellular targets and the biological consequences of these interactions.

**ACKNOWLEDGMENT**

This work was supported by grant R01-CA053371 from the National Cancer Institute (to R.S.).

**REFERENCES**
