**Abstract.** The E5 oncprotein of bovine papillomavirus type I is a small, hydrophobic polypeptide localized predominantly in the Golgi complex. E5-mediated transformation is often associated with activation of the PDGF receptor (PDGF-R). However, some E5 mutants fail to induce PDGF-R phosphorylation yet retain transforming activity, suggesting an additional mechanism of action. Since E5 also interacts with the 16-kD pore-forming subunit of the vacuolar H^+^-ATPase (V-ATPase), the oncoprotein could conceivably interfere with the pH homeostasis of the Golgi complex. A pH-sensitive, fluorescent bacterial toxin was used to label this organelle and Golgi pH ($pH_G$) was measured by ratio imaging. Whereas $pH_G$ of untreated cells was acidic (6.5), no acidification was detected in E5-transfected cells ($pH \sim 7.0$). The Golgi buffering power and the rate of H^+^ leakage were found to be comparable in control and transfected cells. Instead, the E5-induced pH differential was attributed to impairment of V-ATPase activity, even though the amount of ATPase present in the Golgi complex was unaltered. Mutations that abolished binding of E5 to the 16-kD subunit or that targeted the oncoprotein to the endoplasmic reticulum abrogated Golgi alkalinization and cellular transformation. Moreover, transformation-competent E5 mutants that were defective for PDGF-R activation alkalinized the Golgi lumen. Neither transformation by sis nor src, two oncoproteins in the PDGF-R signaling pathway, affected $pH_G$. We conclude that alkalinization of the Golgi complex represents a new biological activity of the E5 oncprotein that correlates with cellular transformation.

**Key words:** vesicular traffic • pH regulation • cholera toxin • proton pump • V-ATPase

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**Introduction**

Papillomaviruses (PVs)^1^ are small, double-stranded DNA viruses that infect a wide range of vertebrate species and induce papillomas of squamous epithelia (zur Hausen, 1989; Band et al., 1990). In humans, a specific subset of PVs are associated with the progression of squamous lesions to dysplasia and carcinoma (Orrt, 1987; zur Hausen and Schneider, 1987). In most cases, the papillomaviruses contain three transforming genes: E5, E6, and E7.

The bovine papillomavirus (BPV-1) E5 protein was one of the first PV oncoproteins to be identified (Schlegel et al., 1986). To date, it is still the best characterized E5 oncoprotein since the E5 proteins of the human papillomaviruses have been notoriously difficult to detect and study in vitro. The E5 protein of BPV is a highly hydrophobic, type II membrane protein of 44 amino acids, which is predominantly expressed in the Golgi complex as a homodimer (Horwitz et al., 1988; Burkhardt et al., 1989). The COOH terminus of E5 consists of a 14-amino acid hydrophilic domain that extends into the Golgi lumen; this domain contains two highly conserved cysteine residues that form disulfide bonds and stabilize homodimer formation. The hydrophobic NH$_2$ terminus of E5 contains 30 amino acids that are predicted to be in $\alpha$-helical conformation and serve as a transmembrane domain (Horwitz et al., 1988). Interestingly, it is this transmembrane domain which mediates the physical interaction of E5 with two cellular proteins: the platelet-derived growth factor receptor (PDGF-R) and the 16-kD subunit of the vacuolar H^+^-ATPase (V-ATPase; Goldstein and Schlegel, 1990; Goldstein et al., 1991, 1992a; Petti and DiMaio, 1992, 1994).

The mechanism whereby E5 mediates transformation of the host cells has not been completely elucidated. Immunoprecipitation studies have demonstrated that the oncprotein can bind and induce autophosphorylation of PDGF-R in several cell lines (Petti et al., 1991; Petti and...
DiMaio, 1992; Goldstein et al., 1994; Drummond-Barbosa et al., 1995). Binding to PDGF-R appears to involve a hydrophilic glutamine residue in the E5 transmembrane domain (Sparkowski et al., 1996). A transformation mechanism has been proposed in which binding of the dimeric form of E5 to PDGF-R results in dimerization and transphosphorylation of the receptor, resulting in continuous mitogenic signaling and cell transformation. Indeed, recent results indicate that dimerization of E5 is critical for PDGF-R activation (A dducì and Schlegel, 1999). However, mutagenesis studies have also shown that the ability of E5 to activate the PDGF-R can be dissociated from its transforming ability. Several highly transforming mutants of E5 fail to induce autophosphorylation of PDGF-R in fibroblasts (Sparkowski et al., 1996; A dducì and Schlegel, 1999), suggesting that E5 has at least one alternative pathway to cell transformation. This possibility may also account for the finding that E5 is capable of transforming keratinocytes that lack detectable PDGF-R (Leptak et al., 1991).

In addition to the PDGF-R, the E5 oncoprotein has also been shown to associate with a 16-kD hydrophobic protein, subsequently identified as the 16-kD subunit of the V₅ moiety of the V-A TPase (Goldstein et al., 1991). Six copies of the 16-kD subunit assemble to form an H⁻ selective pore across the membrane bilayer (Sun et al., 1987; Mandel et al., 1988; Forgac, 1992), and the complete enzyme complex is responsible for acidifying the lumen of many endomembrane organelles, including the Golgi complex (Forgac, 1989). Therefore, it is conceivable that the association of E5 with this 16-kD subunit might interfere with H⁺ translocation through the V-A TPase and, consequently, with organelar acidification. Since E5 is predominantly localized in the Golgi complex, the pH of this compartment would be particularly affected by the oncoprotein, potentially altering the targeting, processing, and turnover of many regulatory proteins that are processed in this compartment.

To test the hypothesis that E5 might alter V-A TPase function, measurements were made of the luminal pH of the Golgi complex (pHₐ) in control and E5-transformed cells using ratio imaging of Golgi-targeted, H⁺-sensitive probes. These in situ measurements were accomplished by harnessing an endogenous retrograde pathway of the cells that delivers surface glycolipids to the Golgi complex (Schapiro et al., 1998). Nontoxic B subunits of cholera toxin, which bind avidly and specifically to cell surface glycolipids (monogangliosides), were conjugated to fluorescein isothiocyanate, which binds avidly and specifically to cell surface glycosphingolipids (colipids). The uptake of these probes was followed by confocal microscopy. Our results indicate that the E5 oncoprotein impairs acidification of the lumen of the Golgi complex, and that inhibition of the V-A TPase accounts for the observed effects.

Materials and Methods

Reagents and Antibodies

FITC-labeled CTB and ATP were purchased from Sigma Chemical Co. BSA (fraction V) was purchased from Boehringer Mannheim. Butyric acid was from J. T. Baker and trimethylamine from Aldrich Chemical Co. Polyclonal antibodies to the 39-kD subunit of the V-A TPase were raised in rabbits injected with a fusion protein encoding the entire bovine subunit. The polyclonal antibody to α-mannosidase II was a gift of Dr. M. Farquhar (University of California at San Diego, San Diego, CA) and the mAb to the A U 1 epitope was from the Berkeley A ntibody Company. A mouse mAb to GM 130 was from Transduction Laboratories. C34-labeled donkey anti–mouse and donkey anti–rabbit antibodies were from Jackson ImmunoResearch Laboratories, Inc. Nonspecific binding was from Molecular Probes, Inc. and concanamycin A from Kamiya Biochemical Company. Lymphopilized streptolysin O was provided by Dr. S. Bhakdi (Johannes-Gutenberg U niversit at, Mainz, Germany) and dissolved in modified Dubcco’s buffered saline solution (PBS; Pierce Chemical Co.) at 1 mg/ml and stored at -80°C. Immediately before use, streptolysin O was diluted in permeabilization buffer containing 2 mM DTT to a final concentration of 0.2 μg/ml. NIH-3T3 cells were from the American Tissue Culture Collection. Cell culture media and antibiotics were from GIBCO BRL. All other chemicals and reagents were of the highest purity available.

Solutions

The calibration of KCl-rich solution (in mM): 140 KCl, 10 glucose, 10 Hepes, 10 MES, 1 CaCl₂, and 1 MgCl₂ (pH ranged from 6 to 7). The permeabilization buffer consisted of (in mM) 90 potassium glutamate, 50 KCl, 10 NaCl, 1 M gCl₂, 2 CaCl₂, 4 EGTA, 2 K₂HPO₄, 20 Hepes, 4 ATP, 3 sodium pyruvate, and 1 mg/ml BSA, pH 7.2.

Plasmid Constructions, Cell Culture, and Cell Lines

Construction of the expression vector prs55, a modified pSG5 plasmid (Stratagene), and corresponding E5 mutants has been described previously (Sparkowski et al., 1994). These constructs were used for generating stable NIH-3T3 E5-expressing cell lines as described previously (Sparkowski et al., 1996). Stably transfected NIH-3T3 cells were cultured at 37°C in DM E containing 10% heat-inactivated FBS (Cansa Interna
tional, Inc.), 100 U/ml penicillin, and 100 μg/ml streptomycin under 5% CO₂. For immunofluorescence and ratio imaging, cells were plated onto 18- or 25-mm glass coverslips in 6-well plastic culture dishes 24 h before the experiments. Experiments were performed when cells reached 60–70% confluence.

Fractionation and Immunoblotting

Purification of the Golgi complex was performed by the method of Beckers and R othman (1992). In brief, cells grown to near confluence in seven 15-cm plates were detached, washed, and resuspended in 6 ml of 250 mM sucrose, 10 mM Tris-HCl, pH 7.4 with 1 mM phenylmethylsulfonyl fluoride. The cells were lysed by ~30 strokes of a Dounce homogenizer with a tight pestle, and the lysate was mixed with 5.5 ml of 62% sucrose in 10 mM Tris-HCl, pH 7.4 plus 230 μl of 1 M E DT A. Of this mixture, 5.6 ml were placed at the bottom of a 12-mm polycarbonate tube and overlaid with layers of 35 and 29% sucrose in Tris-HCl. The samples were subjected to centrifugation for 2.5 h at 25,700 rpm in a Beckman SW 41 rotor and Golgi membranes were collected from the 35-29% interface by puncturing the side of the tube. The sample was diluted into 10 ml of PBS and sedi
tented before determination of protein content and electrophoresis. For immunoblotting, the samples were analyzed by SDS-PAGE on 10% acrylamide gels, and blotted onto nitrocellulose. A filter blocking, the blot was incubated with either a 1:1,000 dilution of anti-V-A TPase antibody or 1:250 of anti-GM 130. A filter washing, the blots were exposed to a 1:2,000 dilution of HRP-conjugated secondary anti–rabbit or anti–mouse antibodies, respectively. Immunoreactive bands were visualized by enhanced chemiluminescence (A mersham).

Immunoprecipitation

Cell lines were plated on 100-mm dishes and allowed to grow to ~80% confluency in DME. Cells were washed once with PBS and incubated with 2 ml methionine- and cysteine-free DME, 0.5 mM L-[³⁵S]methionine (Pro I miX; A mersham). Plates were incubated at 37°C with rocking for 3 h. Labeled cells were placed on ice, aspirated of medium, and washed once with cold PBS. Cells were lysed in 1 ml of a modified RIPA buffer (20 mM morpholine propanesulfonic acid, 150 mM NaCl, 1 mM EDT A, 1% NP-40, 1% deoxycholate, and 0.1% SDS) by scraping into microcentrifuge tubes. Lysates were vortexed for 2 min and clarified by centrifugation. 4 μl of the mAb, A U 1 (Goldstein et al., 1992b), and 50 μl of protein
A-Sepharose beads (Pierce Chemical Co.) were added to supernatants followed by incubation at 4°C for 90 min. Beads were subjected to three washes with lysis buffer and one wash with PBS. Samples were eluted with sample buffer plus 10% β-mercaptoethanol, boiled for 4 min, and loaded onto 14% polyacrylamide gels for protein separation. Gels were fixed in 30% methanol, 10% acetic acid for 10 min, dried, and exposed to Kodak Biomax M S film.

**Immunocytochemistry and Fluorescence Microscopy**

Cells were fixed in 4% paraformaldehyde in PBS at room temperature for 30 min, washed with 100 mM glycine, and permeabilized with 0.1% Triton X-100 in PBS. Fixed and permeabilized cells were blocked with 5% donkey serum in PBS containing 0.1% BSA and 0.1% Triton X-100 for 20 min at room temperature, washed three times with PBS, and incubated with anti-A U1 antibody (1:100 dilution in PBS containing 0.1% BSA and 0.1% Triton X-100) for 1 h at room temperature. Samples were subsequently washed three times in PBS containing 0.1% BSA and incubated with Cy3-labeled donkey anti-mouse antibody and FITC-labeled donkey anti-rabbit antibody (1:1,000 dilution in PBS containing 0.1% BSA and 0.1% Triton X-100) for 1 h at room temperature. A flter washing three times in PBS with 0.1% BSA, samples were treated with Dako fluorescence mounting medium. Control experiments were performed omitting the primary antibody (not shown).

A nalysis of the samples was performed using the 100x objective of a Leica DM RB fluorescence microscope equipped with a Micromax cooled CCD camera (Princeton Instruments), operated from a Dell computer using Winview software (Princeton Instruments). Digitized images were cropped in A dob e Photoshop (A dob e Systems, Inc.) and imported to Micr osoft PowerPoint for assembly and labeling. All images are representative of at least three separate experiments.

**Toxin Labeling and pHG Imaging**

Cells were washed three times in cold PBS containing 1 mM CaCl₂ and 1 mM MgCl₂, pH 7.4, and then preincubated with 10 μg/ml of FITC-CTB in PBS for 1 h at 4°C to promote binding to the plasmalemmal receptors without endocytosis. A flter washing twice with PBS, internalization was initiated by incubating the cells at 37°C for 2 h in DM E.

For imaging experiments, FITC-CTB-labeled cells grown on 25-mm diam glass coverslips were inserted into a Leiden CoverSlip D Ish (Medical Systems Corp.), which was in turn placed into a thermostatted perfusion chamber (O pen Perfusion Micro-Incubator; M edical Systems Corp.) on the stage of an inverted microscope (Zeiss A xiovert 135; O berkochen) equipped with a 63x oil immersion objective and epifluorescence optics. An electronically controlled shutter and filter-wheel (Sutter Instruments Co.) was used to alternately position two excitation filters (490P10 and 440P10 nm) in front of a 75-W xenon lamp, whereas continuous 620-nm illumination was provided by a filtered incandescent light source. The excitation light was directed via a 510-nm dichroic mirror, whereas the emission was detected via a charge-coupled device (CCD) camera (Princeton Instruments Inc.). Data were recorded every 30 s by irradiating the cells for 1 s at each wavelength using 4 × 4 pixel binning. Control of image acquisition was achieved using Metatfluor software (Uni versal Imaging Corp.), operating on a Pentium D laptop computer. Regions of interest were selected for measurement by the imaging system. Background fluorescence was subtracted off line for each wavelength.

At the end of each experiment, a calibration curve of fluorescence versus pH was obtained in situ by two independent methods. Nigericin calibration was achieved by sequentially perfusing the cells with KCl-rich medium buffered to pH values ranging from 5.5 to 7.0 in the presence of 5 μM nigericin. A n approximate 3 min were allowed for equilibration at each pH. This calibration procedure was validated by the null point method (Eisner et al., 1989), using solutions containing varying ratios of butyric acid and trimethylamine, plus 50 mM NaCl and 20 mM Hepes, pH 7.2.

**Data Analysis**

Quantification of cell-associated fluorescence was performed using Meta morph/Imager software (Universal Imaging). Data were plotted using the Origin software (MicroCal Software Inc.), and are representative of at least four separate experiments of each type.

**Results**

**Expression and Subcellular Localization of E5 in NIH-3T3 Cells**

To evaluate the effect of E5 on Golgi V-A TPase activity, we generated cell lines that stably expressed the oncoprotein. Plasmid DNA encoding E5 was cotransfected into NIH-3T3 cells simultaneously with a plasmid conferring neomycin resistance, and cells were selected using G418 as described in Materials and Methods. Pooled colonies of G418-resistant cells were expanded and used for biochemical analysis. The E5 oncoprotein was tagged at its NH₂ terminus with a 6-amino acid epitope (A U1), to allow for efficient detection by immunofluorescence and immunoprecipitation (Sparkowski et al., 1994).

The expression of E5 in the stable transfectants and its subcellular localization were verified by immunoprecipitation (see below) and immunofluorescence microscopy using the A U1 antibody. A s shown in Fig. 1, the oncoprotein was found to concentrate in a compact juxtanuclear structure (a similar pattern was observed in two separate clonal cell lines). No staining was observed in NIH-3T3 cells stably transfected with the empty vector (not shown), validating the specificity of the immunolabeling reaction. The juxtanuclear structure where E5 was concentrated was found to colocalize with the Golgi marker α-mannosidase II (Fig. 1 A) confirming that, as reported earlier (Goldstein and Schlegel, 1990), the E5 oncoprotein accumulates in the Golgi cisternae. The distribution of α-mannosidase II in control and E5-transfected cells was similar, implying that the oncoprotein does not alter Golgi morphology.

**Measurement of Golgi pH (pHG)**

W e next studied whether the interaction between E5 and the 16-kD subunit of the V-A TPase resulted in alterations of the pH of the Golgi complex (pHG). To measure pHG, the lumen of the Golgi complex of intact cells was labeled with a fluorescent pH-sensitive probe. This was accomplished by incubating the cells with FITC-CTB, the nontoxic receptor subunit of cholera toxin, which binds to monogangliosides at the cell surface and is subsequently transported via a retrograde pathway to the Golgi (Schapiro et al., 1998). Cells were preincubated with the labeled toxin at 4°C for 1 h, washed, and incubated for two additional hours at 37°C to allow internalization and transport to the Golgi complex. The accumulation of CTB in the Golgi cisternae was verified by its colocalization with α-mannosidase II (not shown). To confirm that the oncoprotein and the probe were present in the same compartment, the distribution of the former was assessed by immunofluorescence using a Cy3-conjugated secondary antibody, whereas FITC-CTB could be visualized directly. A s shown in Fig. 1, C and D, the pH-sensitive toxin colocalizes precisely with E5. A s found for mannosidase, the subcellular distribution of CTB was not affected by the expression of E5. These observations demonstrate that FITC-CTB is appropriate for probing the pH of the compartment occupied by E5.
pH$_G$ was measured by fluorescence ratio imaging. Images of labeled cells were acquired sequentially with excitation at 490 and 440 nm, while measuring the emission at 535 nm. The ratio of the fluorescence recorded at the two excitation wavelengths was calibrated posteriori in each individual experiment, as shown in Fig. 2. Calibrations were initially performed using the K$^+/\text{H}^+$ ionophore nigericin while bathing the cells in KCl-rich media of varying pH. As described earlier for other cells (Kim et al., 1996; Kneen et al., 1998; Llopis et al., 1998), the resting pH$_G$ of untransfected NIH-3T3 cells was acidic (Fig. 2A). In four determinations, pH$_G$ averaged 6.48 ± 0.03. Strikingly, the basal pH$_G$ of E5-transfected cells was consistently more alkaline, averaging 6.97 ± 0.06 in four determinations (Fig. 2B). A nalysis by Student’s t test for unpaired samples indicated that the difference is highly significant (P < 0.01).

Calibration using exchange ionophores assumes that the monovalent cation activity in the Golgi complex and the surrounding medium are similar, and that the amount of K$^+$ transported during the transition between pH values does not affect its concentration significantly. Because the monovalent ion activity of the Golgi complex has not been reported, the absolute pH values estimated could potentially be inaccurate. More importantly, it is conceivable that the apparent difference in pH$_G$ between normal and E5-transfected cells may be artifactual, resulting from differences in their cationic content. For these reasons, a second calibration procedure was implemented. We chose the

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**Figure 1.** Subcellular localization of E5. (top) NIH-3T3 cells that were stably transfected with AU1-tagged E5 were fixed, permeabilized, and immunostained with anti-α-mannosidase II antibodies (1:500; A) and anti-AU1 antibodies (1:800; B). (bottom) E5-transfected cells were preincubated at 4°C for 1 h with 10 μg/ml FITC-CTB, followed by a 2-h chase at 37°C. The cells were fixed, permeabilized, and immunostained with anti-AU1 antibodies (1:800) followed by a Cy3-conjugated secondary antibody (D). The distribution of FITC-CTB is shown in C. Fluorescence images were captured as described in Materials and Methods and are representative of at least three experiments.

**Figure 2.** In situ measurements of pH$_G$. Control (A and C) and E5-transfected cells (B and D) were labeled with FITC-CTB as described in Materials and Methods. Fluorescence images were acquired sequentially with excitation at 490 and 440 nm and the ratio of the emission (ordinate) calculated after off-line background subtraction. (A and B) After acquiring basal readings, nigericin was added in KCl-rich solutions of the indicated pH. (C and D) After acquiring basal readings, pH$_G$ was estimated by the null-point method, by perfusing the sample with solutions of pH 7.3 containing varying concentrations of butyrate and trimethylamine. The pH at which each solution is predicted to equilibrate is indicated. The ratios of butyrate to trimethylamine used, calculated according to Eisner et al. (1989), were as follows: 0.25:1 for pH 7.6; 1:1 for pH 7.3; 1.61:1 for pH 7.2; 10:1 for pH 6.8; 16:1 for pH 6.7; and 100:1 for pH 6.3. Data are representative of four similar experiments.
null-point method, which can assign an absolute value of pH\textsubscript{G} without requiring assumptions regarding the monovalent ion composition. In this approach, various ratios of weak acids and bases are used to search for a null point where the rates of protonation/deprotonation of the permeable species of the electrolytes are identical (Eisner et al., 1989). For a given combination of acid and base, the null point is strictly a function of the luminal pH. Using this calibration method, we determined that pH\textsubscript{G} equals 6.51 ± 0.05 for control cells and 7.01 ± 0.03 for E5-transfected cells (Fig. 2, C and D, respectively), in good agreement with the determinations made using nigericin calibration. Similar results were obtained with both methods using a second clone of E5-transfected cells (not shown). Together, these results indicate that the expression of the E5 oncoprotein is associated with defective acidification of the Golgi complex.

**Effect of Concanamycin on pH\textsubscript{G} in Control and E5-transfected Cells**

In several cell types, acidification of the luminal fluid of the Golgi complex is accomplished by active pumping of H\textsuperscript{+} by V-ATPases (Lowe and Al-Awqati, 1988; Moriyama et al., 1989; Kim et al., 1996). A similar mechanism is responsible for the acidification of pH\textsubscript{G} in control NIH-3T3 cells. This conclusion was based upon the effects of concanamycin, which rapidly dissipated the acidification of the Golgi complex in these cells. At the concentration used (100 nM), concanamycin specifically inhibits V-ATPases, without any reported effects on other systems (Bowman et al., 1988). Importantly, addition of concanamycin had only a minute effect on pH\textsubscript{G} in E5-transfected cells (Fig. 3).

The failure of concanamycin to alter pH\textsubscript{G} in E5-transfected cells suggests that H\textsuperscript{+} pumping is greatly reduced in these cells. In principle, this could be attributed to the following: (1) a change in the number or intrinsic activity of V-ATPases; (2) a decreased counterion permeability, which in turn would limit the rate of electrogenic pumping; (3) a large H\textsuperscript{+} leak in the Golgi membrane; or (4) an inordinately large H\textsuperscript{+} buffering capacity of Golgi luminal contents. These possibilities were analyzed individually below.

**The Buffering Power of the Golgi Complex Is Not Affected by E5**

While the rate of acidification depends on Golgi buffering power, the final pH of a closed system at equilibrium would not be expected to vary. However, it must be considered that the Golgi complex is in a rapid state of flux, with vesicles being incorporated continuously on the cis side and budding off the trans side. The steady state pH of such an open system varies as a function of its buffering capacity. Therefore, we considered the possibility that expression of E5, which is greatly accumulated in the Golgi complex, might significantly increase luminal buffering power and minimize acidification by a limited number of proton pumps over a finite period of time.

The buffering power, which is a function of the concentration of titratable groups and of their pK\textsubscript{a}, can vary with pH. To appropriately compare the buffering power of control and transfected cells, the measurements were performed at the same pH\textsubscript{G}. This was accomplished by initially dissipating the acidification of the Golgi of control cells with concanamycin (Fig. 3 B). The drug was also added to the E5 cells to maintain identical conditions. The buffering power was assessed by pulsing with a permeable weak base (NH\textsubscript{3}; Fig. 3 B) as described by Roos and Born (1981). Data of three similar experiments, summarized in the inset to Fig. 3 B, demonstrated that the buffering power of the Golgi of control and E5-expressing NIH-3T3 cells, measured at pH 6.8–7.0, is not significantly different (17.8 ± 0.05 mM/pH and 18.79 ± 0.04 mM/pH, respectively).

**The Passive H\textsuperscript{+} (Equivalent) Permeability of the Golgi Complex Is Not Affected by E5**

M2, a small hydrophobic polypeptide of the influenza virus, forms a proton pore in the Golgi membrane and thereby alkalizes this compartment by inducing H\textsuperscript{+} leakage in excess of H\textsuperscript{+} pump activity (Sugrue and Hay, 1991; Pinto et al., 1992). To determine whether the E5 oncoprotein increases pH\textsubscript{G} by a similar mechanism, we estimated the passive H\textsuperscript{+} permeability of the Golgi membrane in both control and E5-expressing cell lines. This was accomplished by measuring the rate of pH change in response to...
an artificially imposed \( \text{pH} \) gradient. As before, the measurements were performed at the same \( \text{pH} \), by pretreating the cells with concanamycin. The inclusion of the inhibitor also ensured that the \( \text{pH} \) changes mediated by the passive permeability would not be counteracted by the V-ATPase. To rapidly expose the cytosolic face of the Golgi to media of varying \( \text{pH} \), the plasma membrane was selectively permeabilized using streptolysin O. This bacterial toxin binds to cholesterol and forms pores in the plasma membrane without altering the integrity of intracellular compartments, which have a considerably lower cholesterol content. After permeabilization, the external (and therefore also the cytosolic) \( \text{pH} \) was suddenly decreased from 7.3 to 5.0 as \( \text{pH}_c \) was monitored continuously (Fig. 4). This resulted in a rapid decrease in \( \text{pH}_c \), which is indicative of \( \text{H}^+ \) permeability. The initial rate of \( \text{pH}_c \) change was measured and, because the buffering power is comparable in both cell types, was taken as a measure of the net \( \text{H}^+ \) flux. In three experiments, the rate of acidification averaged 0.53 ± 0.03 \( \text{pH} \)/min in control cells and 0.50 ± 0.05 \( \text{pH} \)/min in cells expressing E5 (Fig. 4 C). These measurements indicate that the passive permeability of the Golgi membrane to \( \text{H}^+ \) equivalents is not affected by the E5 oncoprotein.

**E5 Impairs V-ATPase Activity in the Golgi Complex**

Since E5 interacts in vivo with the 16-kD channel-forming subunit of the V-ATPase (Goldstein and Schlegel, 1990; Goldstein et al., 1991), we next considered the possibility that this interaction reduces the activity of the \( \text{H}^+ \) pump. Because the rates of \( \text{H}^+ \) pumping and leakage are identical at steady state, V-ATPase activity could in principle be inferred from the net \( \text{H}^+ \) efflux recorded upon inhibition with concanamycin. As shown in Fig. 3, only the untransfected cells responded to concanamycin with a sizable alkalization, consistent with the notion that V-ATPase activity is reduced in E5-expressing cells. However, the initial \( \text{pH}_c \) is different for both cell types. At the more alkaline \( \text{pH} \) noted in the E5 transfectants, the gradient driving the leakage of \( \text{H}^+ \), as well as the proton motive force opposing the V-ATPase, are different. Moreover, inasmuch as concanamycin may not inhibit the pump instantaneously, the dissipation of the luminal acid can be influenced by the rate of association with the inhibitor.

To more adequately compare the activity of the control and E5 cell lines, \( \text{H}^+ \) pumping was assessed at a comparable \( \text{pH} \), without using inhibitors. To unmask the activity of the V-ATPase, \( \text{pH}_c \) was elevated by pulsing the cells with \( \text{NH}_4\text{Cl} \). To preclude a simultaneous cytosolic alkalization that would alter the \( \text{pH} \) gradient across the Golgi membrane, the plasmalemma was initially permeabilized using streptolysin O. As shown in Fig. 5, \( \text{pH}_c \) was different in control and E5 transfectants. The activity of the plasma membrane (established independently by several criteria) had little effect on \( \text{pH}_c \), confirming the integrity of the Golgi membrane. A dilution of \( \text{NH}_4\text{Cl} \) rapidly increased \( \text{pH}_c \), as a result of preferential permeation of \( \text{NH}_3 \) and its subsequent protonation. Despite the continued presence of the weak base, however, a rapid reacidification ensued in the case of control (untransfected) cells. That this reacidification is mediated by the V-ATPase is demonstrated in Fig. 5 B, where the cells were treated with concanamycin before the \( \text{NH}_4\text{Cl} \) pulse.
stored the original pH\textsubscript{G}, albeit in a concanamycin-insensitive manner.

The reduced ability of the transfected cells to pump H\textsuperscript{+} is likely the result of the inhibition of V\textsuperscript{-}ATPase by E5. Nevertheless, we considered the possibility that the oncoprotein may limit the permeability of the counterions required to compensate the electrogenic translocation of H\textsuperscript{+}. In this event, an active V\textsuperscript{-}ATPase would generate a sizable membrane potential, with little change in pH. This possibility was evaluated by providing an exogenous pathway for counterion permeation, which would not be anticipated to be affected by E5. Cells expressing the oncoprotein were incubated with valinomycin, a conductive K\textsuperscript{+} ionophore that readily reaches intracellular membranes. Treatment with this ionophore failed to reduce pH\textsubscript{G} in the transfectants (not illustrated), suggesting that counterion conductance was not the factor limiting the activity of the V\textsuperscript{-}ATPase.

**E5 Does Not Alter the V\textsuperscript{-}ATPase Content of the Golgi Complex**

Interaction with E5 may result in mistargeting and/or instability and degradation of the V\textsuperscript{-}ATPases of the Golgi complex. This could account for the reduced proton pumping activity described above. This possibility was tested by quantifying the V\textsuperscript{-}ATPase content of purified Golgi membranes by immunoblotting, by using an antibody to its 39-kD subunit, which is firmly membrane-bound and less likely to detach during purification. Typical results are shown in Fig. 6. The total cellular content of V\textsuperscript{-}ATPase was not altered by expression of E5 in NIH-3T3 cells (Fig. 6 A). Similarly, the degree of purification of the final Golgi preparation was also unaffected by E5, judged by the enrichment in the Golgi matrix protein GM 130 (Fig. 6 B). Most importantly, the V\textsuperscript{-}ATPase content of the Golgi complex was essentially identical in the control and E5-transfected cells (Fig. 6, A and B). In three experiments, V\textsuperscript{-}ATPases were ~15-fold enriched in the Golgi compared with the homogenate in both E5 and control cells, but the Golgi content was not different between them, whether normalized per protein loaded or per GM 130 content.

**Correlation between Alteration of pH\textsubscript{G} and Cell Transformation**

The relationship between Golgi alkalinization and cell transformation was analyzed using a series of E5 mutants (Fig. 7 A) that vary in their ability to localize to the Golgi complex and/or to interact with the V\textsuperscript{-}ATPase. The NIH-3T3 cell lines expressing the different E5 mutant proteins are summarized in Table I.

The expression of the mutant forms of E5 in the stable lines was verified by immunoprecipitation of metabolically labeled cells, using anti-AU1 antibody. Analysis of the immunoprecipitates by SDS-PAGE (Fig. 7 B) indicated that all mutant cell lines expressed the oncoprotein. The expression level of all the mutants was not identical, varying...
from a minimum of 65% of the control (wild-type E5) in the case of C37S/C39S, to a maximum of 191% for Q17S (average of three experiments; Fig. 7 B). The observed variation in E5 electrophoretic mobility is characteristic for the specific mutation present.

We anticipated that only those forms of E5 that concentrate in the Golgi complex would interfere with pH G, accounting for the reported correlation between the ability of the oncoprotein to localize to the Golgi complex and to transform cells (Goldstein and Schlegel, 1990; Sparkowski et al., 1995, 1996). Therefore, we studied the behavior of two E5 mutants, E5/KDEL and C37S/C39S, which have altered subcellular targeting. The SEKDEL sequence attached to the COOH terminus of the E5/KDEL protein contains an ER retention signal. When expressed transiently in COS cells, this mutant has been shown to be defective for cell transformation (Sparkowski et al., 1995). In stable cell lines, E5/KDEL localized to a reticular, cytoplasmic, and perinuclear compartment, consistent with the ER (Fig. 8). This pattern differed markedly from that of α-mannosidase II, which was used as a marker of the Golgi cisternae. The diffuse reticular pattern was also observed for the transformation-defective C37S/C39S E5 mutant. Interestingly, both of these transformation-defective E5 mutants failed to alkalinate the Golgi (Table I). This suggests that E5 must directly access the Golgi compartment to alter its luminal pH, presumably by binding to and inhibiting the resident V-A TPase.

Table I.

| Cell line | Golgi localization | PDGF activation | Transformed phenotype | pH\(_G\) |
|-----------|-------------------|----------------|----------------------|---------|
| NIH-3T3   | NA                | −              | −                    | 6.48 ± 0.03 |
| wt E5     | +                 | +              | +                    | 6.97 ± 0.06 |
| Q17S      | +                 | −              | +                    | 7.04 ± 0.05 |
| L26A      | +                 | −              | +                    | 6.96 ± 0.04 |
| Q17G      | +                 | −              | −                    | 6.47 ± 0.06 |
| C37S/C39S | −                 | −              | −                    | 6.52 ± 0.04 |
| E5/KDEL   | −                 | +              | −                    | 6.49 ± 0.02 |
| wt 16-V   | NA                | NA             | −                    | 6.57 ± 0.07 |
| E143R     | NA                | NA             | +                    | 6.85 ± 0.06 |

NA, not applicable.

Figure 7. Expression of E5 mutants in NIH-3T3 transfected cells. (A) Structure of the E5 oncoprotein, indicating sites of mutations. The proposed transmembrane-spanning region of the α-helical domain is indicated. (B) Immunoprecipitation of E5 from metabolically labeled NIH-3T3 cells was transfected with mutant forms of E5, illustrating the amounts of E5 expressed. The expression levels of the mutants, expressed relative to the wild-type E5 (100%) are shown below the radiogram. These numbers are averages of three similar immunoprecipitation experiments.

Figure 8. Subcellular distribution of mutant forms of E5 in transfected cells. NIH-3T3 cells were stably transfected with epitope-tagged E5/KDEL (A and B), C37S/C39S (C and D), Q17S (E and F), Q17G (G and H), and L26A (I and J). The distribution of the E5 mutants was revealed by labeling with anti-AU1 antibodies (1:800; B, D, F, H, and J), whereas the localization of the Golgi was assessed by staining with anti-α-mannosidase II antibody (1:500; A, C, E, G, and I) as described in Materials and Methods. Fluorescence images are representative of at least three experiments of each kind.
E5 Mutants that Are Defective for Induction of PDGF-R Autophosphorylation Alkalinize the Golgi Compartment and Transform Cells

By using two transformation-competent E5 mutants, Q17S and L26A, we were able to dissociate the ability of E5 to induce PDGF-R autophosphorylation and to alkalinize the Golgi. The Q17S and L26A mutants, which reside in the E5 transmembrane domain, do not alter the targeting of E5 to the Golgi complex (Fig. 8). It was previously shown that the Q17S mutant is unable to induce PDGF-R autophosphorylation yet retains transforming activity (Sparkowski et al., 1994). L26A is also defective for induction of PDGF-R autophosphorylation, but transforms NIH-3T3 cells with 225× the efficiency of wild-type E5 (A. ducu and Schlegel, 1999). In direct correlation with their retention of transforming activity, Q17S and L26A were able to alkalinize the Golgi (Table I). These results indicate that induction of PDGF-R tyrosine phosphorylation is not the only mechanism by which E5 can transform cells, and suggests that Golgi alkalinization may also initiate signals for cell proliferation/transformation.

Golgi Alkalinization Is Not a Consequence of Transformation

The close correlation between E5-induced transformation and the elevation of Golgi pH suggests that alkalinization contributed to the transformation process. However, it is equally possible that transformation is the cause, and not the consequence of Golgi alkalinization. This possibility was tested by measuring pH_G in cells transformed by oncogenes distinct from E5. Specifically, we measured pH_G in NIH-3T3 cells transfected with oncogenic forms of sis and src, both of which are components of the PDGF-R signaling pathway. In both cases, the Golgi pH was found to be acidic (6.5 ± 0.03 and 6.6 ± 0.05 for sis and src transformants, respectively, similar to that of nontransformed cells). These observations imply that alkalinization of the Golgi is not an obligatory consequence of cellular transformation. In addition, while both sis and E5 activate the PDGF-R, only E5 alkalinizes the Golgi lumen, demonstrating that receptor activation does not contribute to the observed changes in Golgi pH.

The Effect of Mutant Forms of V-ATPase on pH and Transformation

The glutamate residue at position 143 of the 16-kD subunit of the V-ATPase has also been identified in a complex with E5 (Goldstein and Schlegel, 1990; Goldstein et al., 1991), as well as in a ternary complex with E5 and PDGF-R (Goldstein et al., 1992a). Moreover, a good correlation exists between the ability of wild-type E5 to transform cells and to bind the 16-kD subunit of the V-ATPase (Goldstein et al., 1992b; Andresson et al., 1995). However, the functional significance of this interaction has never been shown. It was hypothesized that E5 might interfere with the acidification of intracellular compartments such as endosomes and the Golgi (Goldstein et al., 1991), early experiments indicated that the BPV E5 protein did not significantly alter endosomal pH (Schlegel, R., unpublished data) and techniques to directly measure Golgi pH were unavailable at that time.

Measurement of Golgi pH

Four methods have been used to measure pH_G in intact cells. The earliest method was based on the partition of weak bases, followed by fixation and detection by immunocytochemical means (Anderson et al., 1984). Subsequently, microinjection of pH-sensitive dyes trapped in liposomes of defined size was also used to estimate the pH of the Golgi (Seksek et al., 1995). More recently, an elegant approach using Golgi-targeted green fluorescent proteins was introduced to estimate pH_G (Kneen et al., 1998; Andresson et al., 1995). The method presented here takes advantage of the retrograde targeting of the B subunit of the cholera toxin to the Golgi complex. This method is an extension of the use of verotoxin B, another lipid-binding bacterial polypeptide that accumulates in the Golgi compartment and successfully estimates pH_G (Kim et al., 1996). CTB was the probe of choice in the present study because NIH-3T3 cells express little globothriaosyl ceramide, the receptor for verotoxin. Although quantitative differences exist, all available determinations of pH_G indicate that the Golgi lumen is considerably more acidic than...
the surrounding cytosol. Moreover, both Seksek et al. (1995) and Kim et al. (1996) found that the acidification was eliminated by inhibitors of the V-ATPase.

Because E5 has been shown to interact physically with the 16-kD subunit of the AT Pase, we hypothesized that V-ATPase might display abnormal activity in the Golgi. Indeed, functional alterations appeared likely, inasmuch as the site on the 16-kD subunit that interacts with E5, the glutamic acid residue at position 143, is thought to be essential for the activity of the H⁺ pump (Noumi et al., 1991). Our experimental data are consistent with this hypothesis. E5 expression resulted in a marked elevation of pH_G, which was attributable to the inhibition of H⁺ pumping; no alterations in the buffering capacity or passive H⁺ permeability of the Golgi were detected. We also ruled out the possibility that electronic pumping was limited by a lowered counterion conductance. Together, these findings are most simply explained by the direct inhibition of the V-ATPase via its physical interaction with E5, without significant change in the AT Pase content of the Golgi.

It is noteworthy that the human PV-16 E5 protein has also been demonstrated to bind to the 16-kD subunit of the V-ATPase (Conrad et al., 1993) and that keratinocytes expressing the human PV-16 E5 gene have endosomes with an alkalinized interior (Straight et al., 1995). These keratinocytes also display inhibition of EGF receptor turnover, suggesting that interference with receptor degradation may play an important role in the stimulation of keratinocyte cell growth (Straight et al., 1993). To date, we have not been able to demonstrate alkalinization of endosomes by BPV E5 or to observe any changes in EGF-R activation. Since the HPV-16 E5 protein also localizes to the Golgi complex, it will be important to determine whether it also interferes with the activity of the resident V-ATPase.

**Correlation between pH_G and Transforming Activity**

A iteration of Golgi pH may provide an alternative mechanism to account for cell transformation in cases where E5 fails to activate the PDGF-R. We have defined two classes of mutants that dissociate the transforming ability of E5 from PDGF-R activation. The first class is exemplified by E5/KDEL. This mutant protein induces phosphorylation of PDGF-R and yet is incapable of transforming cells.

A second class of mutants, including Q17S and L26A, is capable of transforming cells without phosphorylation of the PDGF-R. It is possible that E5 triggers signaling through the PDGF-R in a phosphorylation-independent manner. In this regard, mutation of the five critical tyrosines thought to become phosphorylated and, thereby, mediate signaling by the PDGF-R, failed to abrogate entirely the induction of immediate early genes (Fambrough et al., 1999). Alternatively, E5 may promote transformation by mechanisms that are independent of the PDGF-R. In this context, alkalinization of the Golgi is an attractive possibility. E elevating the luminal pH may alter the traffic, processing, or turnover of mitogenic proteins such as receptors or kinases, promoting transformation.

The hypothesis linking Golgi pH with transformation predicts that alkalinization of this organelle by means other than E5 will produce or at least contribute to transformation. We tested this hypothesis using V-ATPase inhibitors, ionophores, and weak electrolytes. Unfortunately, prolonged use of these agents, which is required to assess transformation, resulted in lethality. This likely reflects the fact that these treatments affect not only the Golgi, but all acidic organelles.

V-ATPase activity can alternatively be inhibited by the expression of competing inactive forms of one or more of its subunits. As discussed above, the glutamate residue at position 143 of the 16-kD subunit is essential for normal pump activity (Noumi et al., 1991). Accordingly, we found that expression of a mutated form of the 16-kD subunit (E143R) induced a significant alkalinization of the Golgi complex (Table I). Two separate clones expressing the mutant 16-kD subunit were able to transform NIH-3T3 cells, resembling the effects of E5. It is noteworthy that the transformants expressed only small amounts of mutant 16-kD protein, suggesting that only low-level interference with the V-ATPase was compatible with cell viability.

Together, these findings point to a correlation between pH_G and the induction of transformation. However, it is premature to conclude that a causal relationship exists between these events. Independent and more definitive establishment of the nature of the relationship will have to await the development of methods to more selectively manipulate pH_G.

While interaction with the V-ATPase may contribute to the oncogenic ability of E5, such association is insufficient to elicit transformation. Several mutants of the human papillomavirus (Chen et al., 1996), as well as some bovine papillomavirus mutants (Goldstein et al., 1992b) can bind to the 16-kD subunit, yet are transformation-defective. Other effects of E5, distinct from its ability to associate with the V-ATPase, are needed for effective transformation.

**Possible Effects of Golgi Alkalinization**

Perturbation of Golgi acidification could promote transformation and at the same time facilitate viral infection. For example, alkalinization of the Golgi might induce altered targeting, processing, or turnover of growth factor receptors or signaling intermediates (protein or lipid), thereby resulting in a sustained mitogenic stimulation. The ability of E5 and mutant 16 kD to transform cells is highly suggestive that Golgi-related events can regulate cell proliferation. In addition to its role in cell transformation, it is possible that E5 might interfere with antigenic presentation and help infected host cells evade immune recognition. Finally, it is conceivable that papillomaviruses disrupt Golgi function to facilitate the development of koilocytes. These abnormal vacuolated membranous structures in the Golgi region might facilitate viral release.

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