Golgi Alkalinization by the Papillomavirus E5 Oncoprotein

Florencia Schapiro,*‡ Jason Sparkowski,§ Alex Adduci,§ Frank Suprynowicz,§ Richard Schlegel,§ and Sergio Grinstein*‡

*Division of Cell Biology, Research Institute, Hospital for Sick Children, Toronto, Ontario, M5G 1X8 Canada; ‡Department of Biochemistry, University of Toronto, Toronto, Ontario, M5S 1A8 Canada; and §Department of Pathology, Georgetown University Medical Center, Washington, DC

Abstract. The E5 oncoprotein 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 oncoprotein that correlates with cellular transformation.

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

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 (Orth, 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 α-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 oncoprotein can bind and induce autophosphorylation of PDGF-R in several cell lines (Petti et al., 1991; Petti and

Address correspondence to Sergio Grinstein, Division of Cell Biology, Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, M5G 1X8 Canada. Tel.: (416) 813-5727. Fax: (416) 813-5028. E-mail: sga@sickkids.on.ca

[1]*Abbreviations used in this paper:* BPV, bovine papillomavirus; PDGF-R, PDGF receptor; pH_G, Golgi pH; PV, papillomavirus; V-ATPase, vacuolar H^+-ATPase.

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 (Adduci 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; Adduci 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 V_0 moiety of the V-ATPase (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-ATPase and, consequently, with organellar 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-ATPase function, measurements were made of the luminal pH of the Golgi complex (pH_G) 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 fluorescent H^+-sensitive probes, bound to the cell surface, and allowed to translocate to the Golgi complex. Spectroscopic measurements of pH_G were performed in living cells by fluorescence ratio imaging. Our results indicate that the E5 oncoprotein impairs acidification of the lumen of the Golgi complex, and that inhibition of the V-ATPase 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-ATPase 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 AU1 epitope was from the Berkeley Antibody Company. A mouse mAb to GM130 was from Transduction Laboratories. Cy3-labeled donkey anti–mouse and donkey anti–rabbit antibodies were from Jackson ImmunoResearch Laboratories, Inc. Nigericin was from Molecular Probes, Inc. and concanamycin A from Kamiya Biochemical Company. Lyophilized streptolysin O was provided by Dr. S. Bhakdi (Johannes-Gutenberg Universitat, Mainz, Germany) and dissolved in modified Dulbecco'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 KCl-rich solution contained (in mM): 140 KCl, 10 glucose, 10 Hepes, 10 MES, 1 $CaCl_2$, and 1 $MgCl_2$ (pH ranged from 6 to 7). The permeabilization buffer consisted of (in mM) 90 potassium glutamate, 50 KCl, 10 NaCl, 1 $MgCl_2$, 2 $CaCl_2$, 4 EGTA, 2 K_2HPO_4, 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 pJS55, 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 DME containing 10% heat-inactivated FBS (Cansera International, Inc.), 100 U/ml penicillin, and 100 μg/ml streptomycin under 5% CO_2. 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 Rothman (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 chloride. 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 0.1 M EDTA. Of this mixture, 5.6 ml were placed at the bottom of a 12-ml 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 SW41 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 sedimented before determination of protein content and electrophoresis.

For immunoblotting, the samples were analyzed by SDS-PAGE on 10% acrylamide gels, and blotted onto nitrocellulose. After blocking, the blot was incubated with either a 1:1,000 dilution of anti–V-ATPase antibody or 1:250 of anti-GM130. After 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 (Amersham).

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, and 0.5 mCi [^{35}S]methionine (Pro Mix; Amersham). 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 EDTA, 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, AU1 (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 MS 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–AU1 antibody (1:800 dilution in PBS containing 0.1% BSA and 0.1% Triton X-100) and anti–α-mannosidase II antibody (1:500 dilution in PBS containing 0.1% BSA and 0.1% Triton X-100) for 2 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. After 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).

Analysis of the samples was performed using the 100× objective of a Leica DM1RB 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 Adobe Photoshop (Adobe Systems, Inc.) and imported to Microsoft PowerPoint for assembly and labeling. All images are representative of at least three separate experiments.

Toxin Labeling and pH$_G$ Imaging

Cells were washed three times in cold PBS containing 1 mM CaCl$_2$ and 1 mM MgCl$_2$, 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. After washing twice with PBS, internalization was initiated by incubating the cells at 37°C for 2 h in DME.

For imaging experiments, FITC-CTB–labeled cells grown on 25-mm diam glass coverslips were inserted into a Leiden CoverSlip Dish (Medical Systems Corp.), which was in turn placed into a thermostatted perfusion chamber (Open Perfusion Micro-Incubator; Medical Systems Corp.) on the stage of an inverted microscope (Zeiss Axiovert 135; Oberkochen) equipped with a 63× oil immersion objective and epifluorescence optics. An electronically controlled shutter and filter-wheel (Sutter Instruments Co.) was used to alternately position two excitation filters (490BP10 and 440BP10 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 emitted fluorescence (>510 nm) and the transmitted red light (>620 nm) were separated by a 580-nm emission dichroic mirror. The red light was directed to a video camera for continuous visualization of the cells, whereas the fluorescent signals were processed through a 535BP25-nm band-pass filter. Digitized images were captured on a frame transfer air-cooled CCD camera (Princeton Instruments Inc.). Data were recorded every 30 or 60 s by irradiating the cells for 1 s at each wavelength using 4 × 4 pixel binning. Control of image acquisition was achieved using Metafluor software (Universal Imaging Corp.), operating on a Pentium Dell 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 media buffered to pH values ranging from 5.5 to 7.0 in the presence of 5 μM nigericin. Approximately 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 Metamorph/Metafluor 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-ATPase 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 NH$_2$ terminus with a 6–amino acid epitope (AU1), 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 AU1 antibody. As 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 (pH$_G$)

We next studied whether the interaction between E5 and the 16-kD subunit of the V-ATPase resulted in alterations of the pH of the Golgi complex (pH$_G$). To measure pH$_G$, 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. As shown in Fig. 1, C and D, the pH-sensitive toxin colocalizes precisely with E5. As 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.
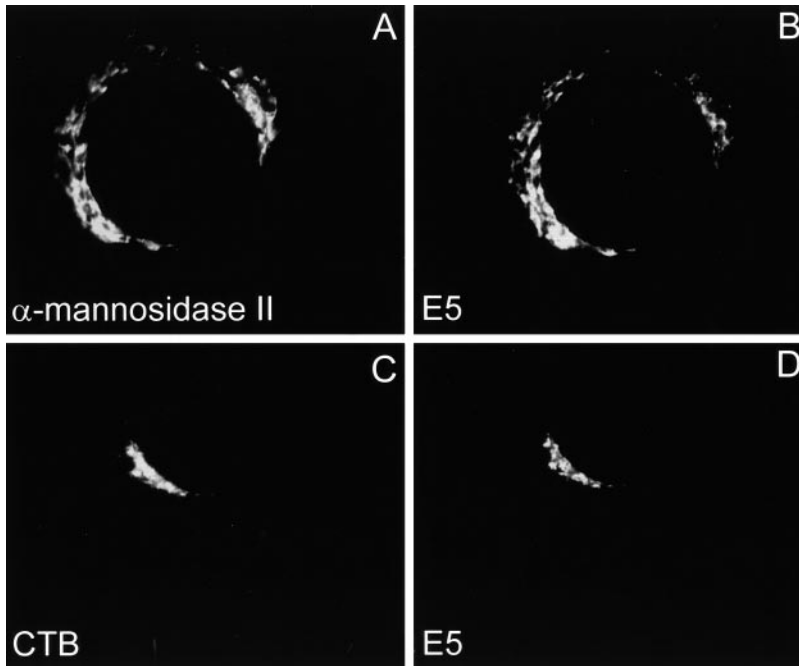
Figure 1. Subcellular localization of E5. (top) NIH-3T3 cells that were stably transfected with AU1-tagged E5 were fixed, permeabilized, and immunostained with anti–α-mannosidase II antibodies (1:500; A) and anti–AU1 antibodies (1:800; B). (bottom) E5-transfected cells were preincubated at 4°C for 1 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.

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 aposteriori in each individual experiment, as shown in Fig. 2. Calibrations were initially performed using the K^+/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. 2 A). 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. 2

B). Analysis 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
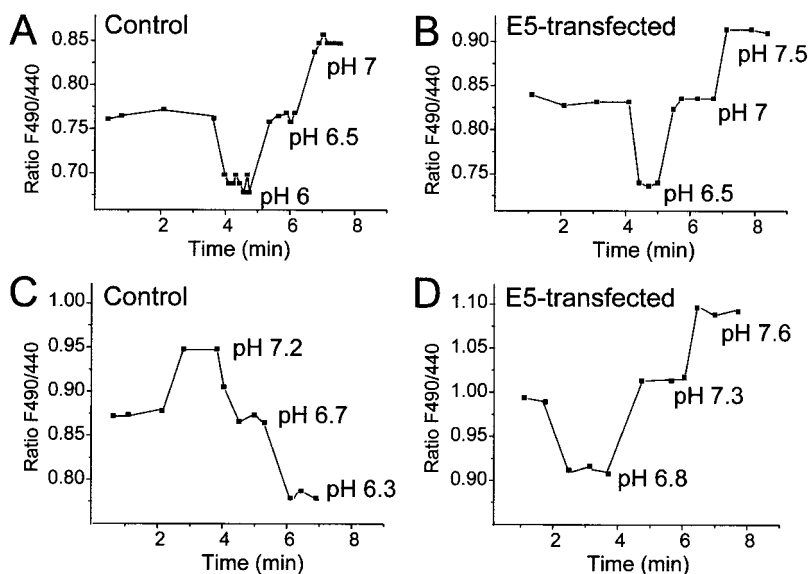

Figure 2. In situ measurements of pH_G. Control (A and C) and E5-transfected cells (B and D) were labeled with FITC-CTB as described in Materials and Methods. Fluorescence images were acquired sequentially with excitation at 490 and 440 nm and the ratio of the emission (ordinate) calculated after off-line 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_G at which each solution is predicted to equilibrate (i.e., produce no net change in pH) 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; 1.6:1 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_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_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_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^+ by V-ATPases (Lowe and Al-Awqati, 1988; Moriyama et al., 1989; Kim et al., 1996). As illustrated in Fig. 3, a similar mechanism is responsible for the acidification of pH_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_G in E5-transfected cells (Fig. 3).

The failure of concanamycin to alter pH_G in E5-transfectants suggests that H^+ 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^+ leak in the Golgi membrane; or (4) an inordinately large H^+ 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, can vary with pH. To appropriately compare the buffering power of con-
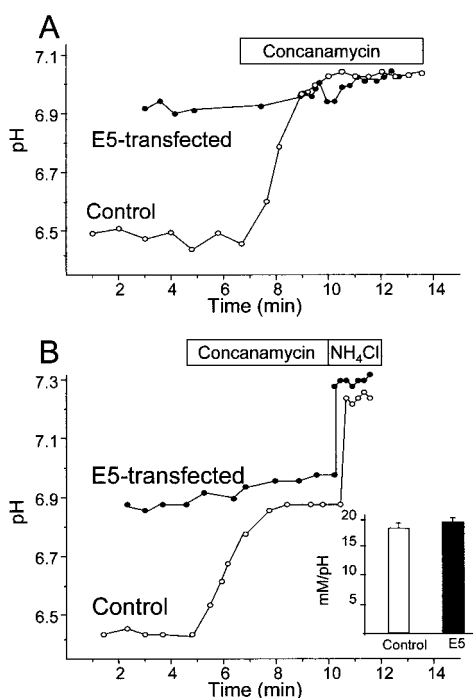

Figure 3. Effect of concanamycin on pH_G and measurement of buffering power. (A) Resting pH_G was measured in control and E5-transfected cells labeled with FITC-CTB, as described for Fig. 2. Where indicated, 100 nM concanamycin was added to both samples. (B) The resting pH_G and effect of concanamycin were measured as in A. Where indicated, the medium was supplemented with 10 mM NH_4Cl. Data in A and B are representative of four similar experiments. (Inset) Determinations of buffering power in control (open bar) and E5-transfected cells (solid bar) calculated by measuring the ΔpH induced by a 1-mM NH_4Cl pulse. Data are means $\pm$ SEM of four determinations.

trol and transfected cells, the measurements were performed at the same pH_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_3; Fig. 3 B) as described by Roos and Boron (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^+ (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 alkalinizes this compartment by inducing H^+ leakage in excess of H^+ pump activity (Sugrue and Hay, 1991; Pinto et al., 1992). To determine whether the E5 oncoprotein increases pH_G by a similar mechanism, we estimated the passive H^+ 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 pH gradient. As before, the measurements were performed at the same pH, by pretreating the cells with concanamycin. The inclusion of the inhibitor also ensured that the 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 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) pH was suddenly decreased from 7.3 to 5.0 as pH_G was monitored continuously (Fig. 4). This resulted in a rapid decrease in pH_G, which is indicative of H^+ permeability. The initial rate of pH_G change was measured and, because the buffering power is comparable in both cell types, was taken as a measure of the net H^+ flux. In three experiments, the rate of acidification averaged 0.53 ± 0.03 pH/min in control cells and 0.50 ± 0.05 pH/min in cells expressing E5 (Fig. 4 C). These measurements indicate that the passive permeability of the Golgi membrane to 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 H^+ pump. Because the rates of H^+ pumping and leakage are identical at steady state, V-ATPase activity could in principle be inferred from the net H^+ efflux recorded upon inhibition with concanamycin. As shown in Fig. 3, only the untransfected cells responded to concanamycin with a sizable alkalinization, consistent with the notion that V-ATPase activity is reduced in E5-expressing cells. However, the initial pH_G is different for both cell types. At the more alkaline pH noted in the E5 transfectants, the gradient driving the leakage of 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, H^+ pumping was assessed at a comparable pH, without using inhibitors. To unmask the activity of the V-ATPase, pH_G was elevated by pulsing the cells with NH_4Cl. To preclude a simultaneous cytosolic alkalinization that would alter the pH gradient across the Golgi membrane, the plasmalemma was initially permeabilized using streptolysin O. As shown in Fig. 5, A and B, permeabilization of the plasma membrane (established independently by several criteria) had little effect on pH_G, confirming the integrity of the Golgi membrane. Addition of NH_4Cl rapidly increased pH_G, as a result of preferential permeation of 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 NH_4Cl pulse.
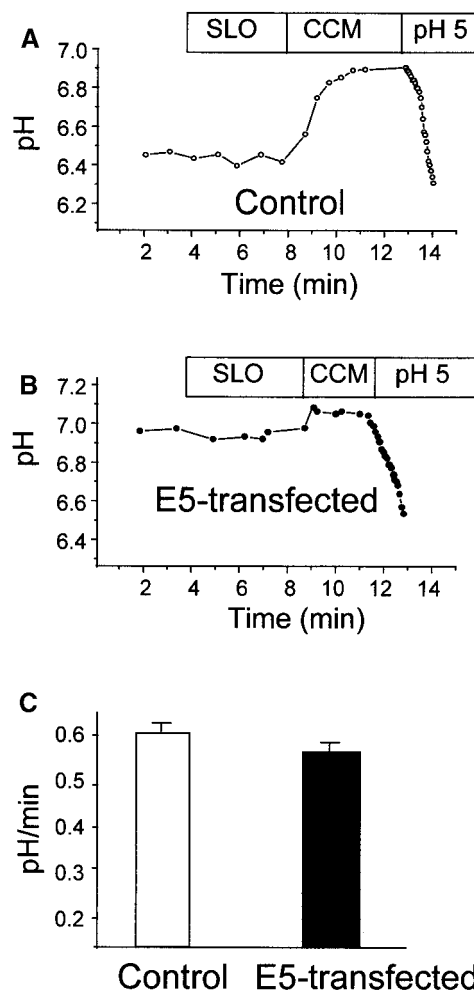
Figure 4. Estimation of the passive H^+ (equivalent) permeability of the Golgi complex. Control (A) and E5-transfected cells (B) were labeled with FITC-CTB and used for pH_G determinations as in Fig. 2. Where noted, the plasma membranes were permeabilized by addition of streptolysin O (SLO; 0.25 μg/ml) in permeabilization buffer. Next, concanamycin (100 nM) was added to the medium and finally the bathing solution was replaced for medium of pH 5.0, as pH_G was continuously monitored. Data are representative of four similar experiments. (C) The rates of acidification recorded upon acidification of the medium in experiments like those in A and B were recorded over the initial 20 s. The rates of control (open bar) and E5-transfected cells (solid bar) are summarized. Data are means ± SEM of four experiments.

In this case, the secondary acidification phase was lacking and a sustained alkalinization was recorded.

In contrast, no evidence of V-ATPase activity was found in the Golgi complex of E5 transfectants. As illustrated in Fig. 5 C, elevation of pH_G in these cells was not followed by the acute recovery described for the control cells, despite the fact that both cell types were alkalinized to a comparable degree (pH ~7.3). The sustained alkalosis not only confirms the notion that V-ATPase activity is minimal in the Golgi of cells expressing E5, but also indicates that the Golgi membrane is comparatively tight to NH_4^+. Rapid permeation of the cationic species would have re-
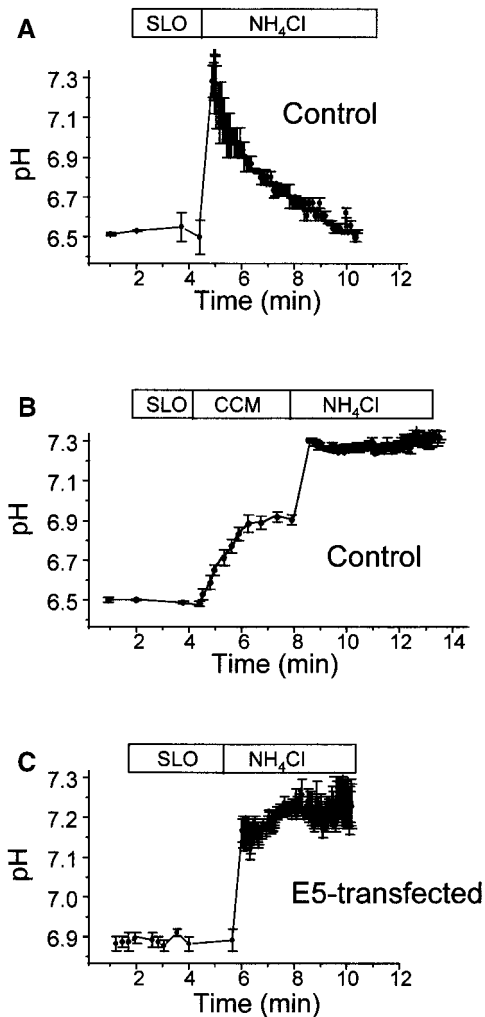
Figure 5. Estimation of the V-ATPase activity in the Golgi complex. (A) Control cells were labeled and permeabilized with streptolysin O (SLO) as in Fig. 4. Where indicated, NH$_4$Cl (10 mM) was added and the response was continuously monitored. (B) After permeabilization with SLO as in A, the cells were exposed to concanamycin (CCM; 100 nM). After a steady state was attained, the cells were pulsed with NH$_4$Cl as above. (C) E5-transfected cells were labeled and permeabilized with SLO as in A. Where indicated, NH$_4$Cl (10 mM) was added. Data are means ± SEM of four cells from a typical experiment out of four.

transfectants (not illustrated), suggesting that counterion conductance was not the factor limiting the activity of the V-ATPase.

E5 Does Not Alter the V-ATPase Content of the Golgi Complex

Interaction with E5 may result in mistargeting and/or instability and degradation of the V-ATPases of the Golgi complex. This could account for the reduced proton pumping activity described above. This possibility was tested by quantifying the V-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-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 GM130 (Fig. 6 B). Most importantly, the V-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-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 GM130 content.

Correlation between Alteration of pH$_G$ 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-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
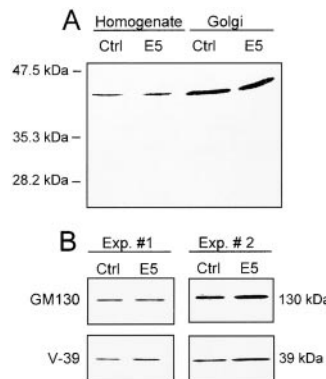
stored the original pH$_G$, albeit in a concanamycin-insensitive manner.

The reduced ability of the transfected cells to pump H$^+$ is likely the result of the inhibition of V-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$^+$. In this event, an active V-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$^+$ ionophore that readily reaches intracellular membranes. Treatment with this ionophore failed to reduce pH$_G$ in the

Figure 6. Quantitation of the V-ATPase content of the Golgi complex. Control (Ctrl) and E5-transfected cells were homogenized and used for purification of the Golgi complex. (A) Samples of the homogenate and purified Golgi fractions were analyzed by SDS-PAGE and blotted with antibody to the 39-kD subunit of the V-ATPase. Data are representative of three similar experiments. (B) Samples of purified Golgi fractions from control and E5-transfected cells were blotted with antibodies to the Golgi marker GM130 and to the 39-kD subunit of the V-ATPase (V-39). Two separate experiments are illustrated.
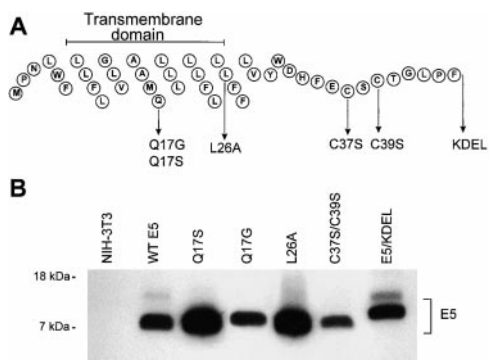
Figure 7. Expression of E5 mutants in NIH-3T3 transfected cells. (A) Structure of the E5 oncoprotein, indicating sites of mutations. The proposed transmembrane-spanning region of the α-helical domain is indicated. (B) Immunoprecipitation of E5 from metabolically labeled NIH-3T3 cells was transfected 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.
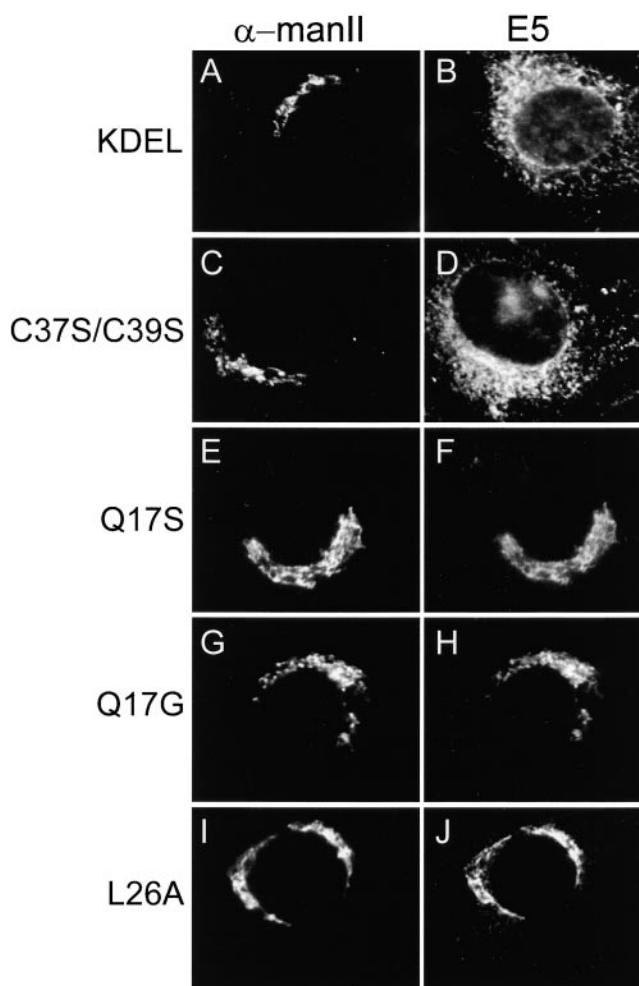

Figure 8. Subcellular distribution of mutant forms of E5 in transfected cells. NIH-3T3 cells were stably transfected with epitope-tagged E5/KDEL (A and B), C37S/C39S (C and D), Q17S (E and F), Q17G (G and H), and L26A (I and J). The distribution of the E5 mutants was revealed by labeling with anti–AU1 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.

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 ob-

served for the transformation-defective C37S/C39S E5 mutant. Interestingly, both of these transformation-defective E5 mutants failed to alkalinize 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-ATPase.

However, Golgi localization alone is not sufficient for E5-mediated transformation. Q17G is a transformation-defective E5 mutant that translocates to the Golgi complex (Fig. 8 and Table I). Despite being normally localized and expressed in amounts comparable to the wild-type E5 (88% of control), this mutant does not interfere with Golgi acidification. Rather, the failure of Q17G to interfere with V-ATPase function can be explained by its inability to bind the 16-kD subunit protein.

Table I.

Cell line	Golgi localization	PDGFR 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.

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 mutations, 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 (Adduci 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 $pH_G = 6.52 \pm 0.03$ and 6.60 ± 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 is known to be essential for normal pump activity (Noumi et al., 1991). Because this glutamate was also found to be required for interaction with E5, mutants lacking this residue had been tested earlier for transformation. Andresson et al. (1995) found that expression of a 16-kD subunit harboring the E143R mutation subunit converted NIH-3T3 cells to an anchorage-independent state. Therefore, we tested the effect of this mutation on pH_G using CTB and imaging, as detailed in Materials and Methods. The results are summarized at the bottom of Table I. In eight separate experiments, pH_G averaged 6.85 ± 0.062 in cells expressing the E143R form of the 16-kD subunit, whereas the pH of parallel controls, transfected with wild-type 16 kD, was 6.57 ± 0.07 ($n = 9$). Thus, as in E5-transfected cells, transformation correlated with increased Golgi pH.

Discussion

PDGF-R–independent Transformation

PDGF-R was the first growth factor receptor to be identified in a complex with E5 (Petti and DiMaio, 1992), although there was previous evidence that E5 could cooperate with the EGF receptor in the transformation of fibroblasts (Martin et al., 1989). The close correlation between PDGF-R activation and fibroblast transformation by E5 suggested that this association may represent the sole mechanism for E5-mediated cell transformation. Later studies suggested that the following alternative mechanisms may exist: (1) keratinocytes, which lack PDGF-R, can be converted to the tumorigenic phenotype by E5 (Leptak et al., 1991); (2) the E5/KDEL mutant protein induces PDGF-R autophosphorylation but is transformation-defective (Sparkowski et al., 1995), and (3) some variants of E5 with mutations in the transmembrane domain transform fibroblasts without inducing PDGF-R autophosphorylation (Sparkowski et al., 1996).

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. Although 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; Llopis et al., 1998). 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 globotriaosyl 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 ATPase, 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 electrogenic 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 ATPase 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

Alteration 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. 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 trans-

formation. 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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