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The E5 Oncoprotein of Human Papillomavirus Type 16 Inhibits the Acidification of Endosomes in Human Keratinocytes

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The human papillomavirus type 16 E5 oncoprotein possesses mitogenic activity that acts synergistically with epidermal growth factor (EGF) in human keratinocytes and inhibits the degradation of the EGF receptor in endosomal compartments after ligand-stimulated endocytosis. One potential explanation for these observations is that E5 inhibits the acidification of endosomes. This may be mediated through the 16-kDa component of the vacuolar proton-ATPase, since animal and human papillomavirus E5 proteins bind this subunit protein. Using a ratio-imaging technique to determine endosomal pH, we found that the acidification of endosomes in E5-expressing keratinocytes was delayed at least fourfold compared with normal human keratinocytes and endosomes in some cells never completely acidified. Furthermore, E5 expression increased the resistance of keratinocytes to protein synthesis inhibition by diphtheria toxin, a process dependent on efficient endosomal acidification. Finally, artificially inhibiting endosomal acidification with chloroquine during the endocytosis of EGF receptors in keratinocytes demonstrated many of the same effects as the expression of human papillomavirus type 16 E5, including prolonged retention of undegraded EGF receptors in intracellular vesicles.

Human papillomaviruses (HPVs) infect basal human keratinocytes and propagate in the differentiating layers of the epithelium (25). The E6, E7, and E5 proteins possess oncogenic activities that contribute to the pathogenesis associated with HPV infection (7, 19, 21, 23, 34, 42). Of particular interest are the genital HPV types with pathogenic effects ranging in severity from benign neoplasia, as with HPV type 6 (HPV-6) and HPV-11, to the malignant carcinomas associated with HPV-16 and -18 (26). How these proteins contribute to the life cycle of the virus is a matter of speculation, since the viruses have yet to be propagated in vitro, making their study difficult.

The HPV-16 E5 open reading frame encodes a small, highly hydrophobic protein (2) with activities that may contribute both to the pathogenicity of the virus and to its replication. Recent reports (19, 21, 42) have demonstrated the abilities of HPV-16 E5 to transform murine fibroblasts and stimulate mitogenesis in human keratinocytes. Furthermore, these activities were enhanced specifically in the presence of epidermal growth factor (EGF), the primary essential mitogen for keratinocytes in vitro and in vivo (36). EGF is the ligand for the EGF receptor (EGFR), a transmembrane receptor protein present on all epithelial cells (4, 5). Ligand binding to the extracellular domain of the receptor initiates receptor dimerization and stimulates the tyrosine kinase activity of the intracellular domain of the receptor (3, 4, 46), resulting in autophosphorylation and the initiation of a cascade of events leading to the stimulation of DNA synthesis (3, 6, 29). The down-regulation of the active receptor involves the rapid migration of the ligand-receptor complex to clathrin-coated pits, internalization via receptor-mediated endocytosis into endosomes, dissociation of receptor and ligand, and fusion of endosomes with lysosomes in which both ligand and receptor are degraded (3, 4, 46). Little EGFR recycles to the cell surface, and the reappearance of EGFR at the plasma membrane following down-regulation involves primarily de novo synthesis (4). We have shown (42) that the expression of HPV-16 E5 in human keratinocytes increases the number of EGFRs at the cell surface. In addition, HPV-16 E5 modifies the response of these receptors to ligand, and the EGFRs are hyperphosphorylated, receptor degradation is inhibited, and increased recycling of receptors to the cell surface occurs. However, the internalization and degradation of ligand occur normally in these cells.

Another reported property of HPV-16 E5 is the ability to bind a 16-kDa protein (9) identified as a component of a vacuolar proton-ATPase pump complex (H^+-ATPase). This protein complex is a member of a family of H^+-ATPases responsible for the acidification of a variety of compartments and organelles in the cell, including those involved in the receptor-mediated endocytosis of the EGFR (13, 24, 39). The 16-kDa subunit of the mammalian H^+-ATPase pump is homologous to the yeast 17-kDa subunit, an integral membrane protein forming all or part of the pore utilized for proton translocation (13, 20). Through an unknown mechanism the pump complex is either rapidly recruited to budding endosomes or activated through a regulatory event, since the plasma membrane of mammalian cells is devoid of active H^+-ATPase (13, 18, 39). The pump establishes and maintains the low internal pH 4.5 to 5.0 in endosomes and lysosomes relative to the cytoplasmic pH 7.0 to 7.2 by utilizing the energy from ATP hydrolysis to generate an influx of protons into the vesicle (13, 14, 24, 39). Acidification dissociates receptor-ligand interactions, such as the release of nutrients from their transport proteins, and also catalyzes the penetration of some viruses (15, 33, 35) and toxins. Diphtheria toxin, for example, consists of α and β subunits: the low pH of the endosome activates the β subunit to form a channel through the endosomal membrane that allows the active α subunit to enter the cytoplasm (22, 32).

On the basis of the previous finding that HPV-16 E5 binds

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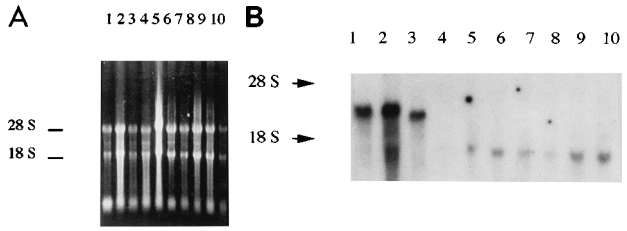


FIG. 1. HPV-16 E5 mRNA expression. (A) Ethidium bromide stain of total RNA isolated from keratinocytes separated on an agarose-formaldehyde gel; (B) autoradiograph of the Northern blot of the gel shown in panel A after hybridization with an in vitro-transcribed E5 RNA probe. Lanes: 1 to 3, separate isolates of mass cultures of pLXSN16E5 (which expresses E5 from the Harvey murine sarcoma virus LTR); 4, mass culture of pLXSN (a vector control); 5, pKV16E5 clone 5 (which expresses E5 from the hCMV promoter); 6 and 7, pAT16E5X clones 1 and 2 (full-length HPV-16 DNA containing a premature stop codon in E5); 8 and 9, pAT16 clones 4 and 5 (full-length HPV-16 DNA); 10, K1/16 clone 1 (full-length HPV-16 DNA).

the 16-kDa component of the H^+-ATPase pump, we sought to determine whether HPV-16 E5 expression resulted in alteration of endosomal acidification and whether this activity might account for the alterations in EGFR metabolism previously observed in HPV-16 E5-expressing cells. All experiments were performed with human foreskin keratinocytes, normal host cells for HPV. These were maintained in culture with serum-free KGM or E medium with EGF and feeder cells as described in reference 42. Keratinocytes were transfected via electroporation with HPV-containing plasmids and pSV2neo as a selectable marker, from which individual clones were isolated for analysis (42). The electroporated plasmids included pKV16E5 (HPV-16 E5 expressed alone from the human cytomegalovirus immediate-early gene promoter [hCMV]), pAT16E5X (full-length HPV-16 genome with a translation termination linker inserted at bp 3879, resulting in premature termination of the E5 protein), and pAT16 and K1/16 (full-length HPV-16 genome with E5 expressed from the endogenous promoter). Mass cultures of G418-selected keratinocytes transduced with defective retroviral particles (multiplicity of infection, 1 to 2; with 10 μg of Polybrene per ml) containing either pLXSN (a vector-only control) or pLXSN16E5 (which expresses HPV-16 E5 from the Harvey murine sarcoma virus long terminal repeat [LTR]) were used in some assays to determine the effect, if any, of vector DNA and the transfection procedure. The pLXSN vector and the preparation of defective amphotropic retrovirus using the packaging cell line PA317 are described in references 27 and 28. Human foreskin keratinocytes and mass cultures were used prior to the fifth passage. All cell lines were used at early passages (passage 25 or earlier), except for K1/16 cells, which were used at passage 240 or greater. Several clones of each transfectant, multiple keratinocyte isolates, and several mass cultures were tested at different passages, and the results were pooled for analysis and presentation.

Transfected clones were analyzed by Northern (RNA) blot hybridization to detect HPV-16 E5 in transcripts (Fig. 1). Total cellular RNA was prepared by an AGPC method adapted from Chomczynski and Sacchi (8) and separated on a 7% formaldehyde–0.8% agarose gel. The RNA was loaded in buffer containing ethidium bromide, and the resulting gel is shown in Fig. 1A, which shows the relatively even loading of the RNA between wells. HPV-16 E5 mRNA expression was determined after transfer of the RNA to nitrocellulose and Northern blot with a radiolabeled HPV-16 E5 RNA probe made from pGEM16E5 by using the in vitro transcription kit from Promega Corporation (Madison, Wis.). The E5-containing messages expressed from the vectors used in the subsequently described experiments are shown in Fig. 1B. The vector control plasmid pLXSN does not show any E5-specific message (lane 4), while the pLXSN16E5-containing cells express an E5-containing mRNA of approximately 3 kbp from the LTR (lanes 1 to 3). The pKV16E5 clone expresses an E5 message of approximately 1.5 kbp from the hCMV promoter (lane 5), while the messages from the full-length HPV DNA in pAT16E5X (lanes 6 and 7), pAT16 (lanes 8 and 9), and K1/16 (lane 10) clones are approximately 1.4 kbp. This 1.4-kbp message corresponds to the approximately 1.4-kbp E6*-E7-E1/E4-E5 polycistronic message detected by Doorbar et al. (10) and Rohlfs et al. (37) in HPV-16 containing keratinocyte cell lines. Different levels of E5 message are clearly seen in Fig. 1B compared with the total RNA loaded (Fig. 1A), particularly if the pLXSN16E5 mass cultures (lanes 1 to 3) are compared with the other E5-expressing clones (lanes 5 and 8 to 10). Furthermore, the expression of an E5 protein seems to have little effect on the level of the polycistronic message as shown by comparison of pAT16 clones (lanes 8 and 9) with pAT16E5X clones (lanes 6 and 7). Unfortunately, the level of HPV-16 E5 protein expression could not be determined, as there are no antibodies presently available that will detect this extremely hydrophobic protein. However, genetic proof that E5 is the causal agent of the effects described below comes from the combination of vectors used in this study.

Determination of endosomal pH. Initially, we wished to determine whether there was a difference between the endosomal pH of E5-expressing keratinocytes and that of normal cells. We used a ratio-imaging technique that takes advantage of the pH-dependent fluorescence of fluorescein molecules. Details of the procedure and the use of digitized video microscopy for these measurements can be found in references 16 and 51. At the excitation wavelength of 440 nm the intensity of fluorescein fluorescence monitored at 520 nm is independent of pH, but the fluorescence intensity at an excitation wavelength of 490 nm rapidly decreases as the pH becomes more acidic (24, 31, 45). The ratio of these emission intensities compared with a standard curve (pH 5 to 8) provides a pH measurement that is independent of dye concentration and optical path length and unaffected by photobleaching (16, 24, 45, 49, 51). We used fluorescein isothiocyanate-dextran of low molecular weight (average molecular weight, 9,400; Sigma Chemical Co., St. Louis, Mo.), which is nonspecifically incorporated into clathrin-coated and uncoated endocytic vesicles during a 5-min incubation at 37°C. During label incorporation, the cells were simultaneously treated with 100 ng of EGF per ml to stimulate EGFR activity and internalization.

Figure 2 shows the results of the experiments to determine the pH of endosomes in E5-containing cells. The pH values obtained by this assay ranged from ≥7.0, determined at the start of the incubation, to 5, the lowest value reportable by this method. The acidification is reported as percent change in pH rather than absolute pH values so that the measurements between experiments could be normalized to a common scale. In Fig. 2A the average endosomal pH fell rapidly in normal keratinocytes and in those transfected with pAT16E5X, which has a stop codon in E5, from ≥7.0 (0% change) to 5 (100% change) in 600 to 1,200 s. Cells expressing E5 from either an exogenous promoter or the HPV-16 genome incompletely acidified their endosomes and required more time to do so. pKV16E5 took four times as long as normal keratinocytes to experience a maximal 96.9% average endosomal pH change (at 4,800 s), and the endosomes in pAT16 and K1/16 experienced only an 82.1% change and a 55.3% change in pH, re-

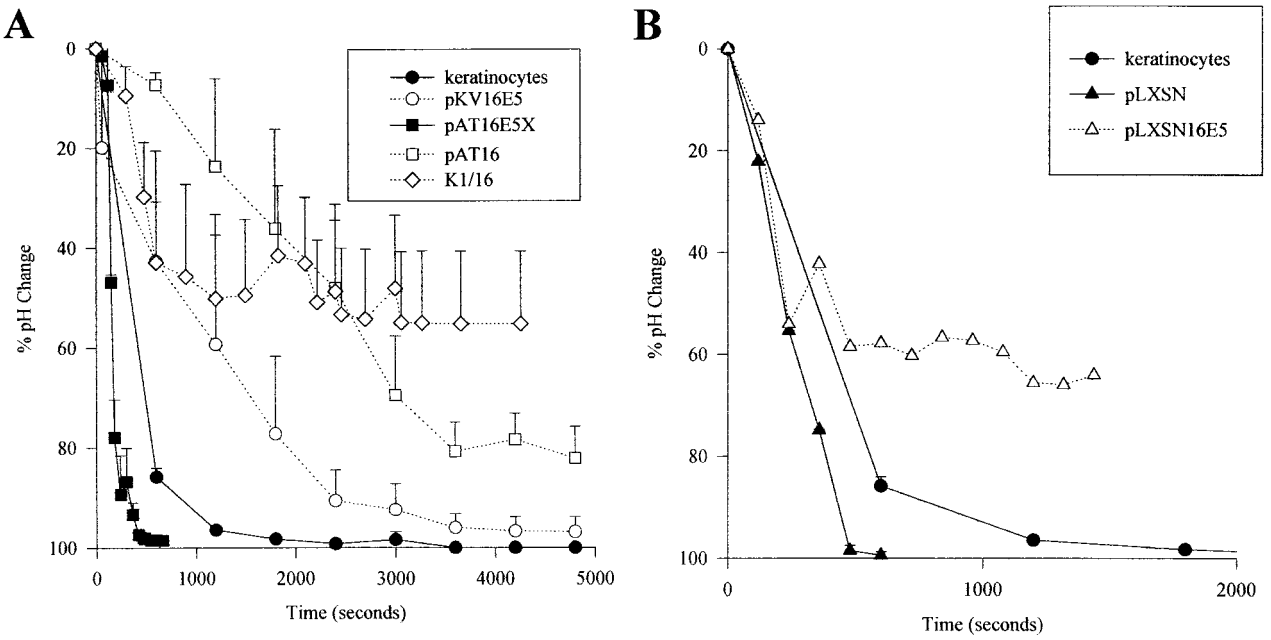


FIG. 2. Percent change in average endosomal pH over time. After the incorporation of fluoresceinated dextrans into endosomes, the pH was determined by the ratio-imaging technique described in the text. Percent pH change was calculated as $100 \times (pH_0 - pH_T/pH_0 - pH_{min})$, where pH_0 is the initial pH at time zero (corresponding to physiological pH 7.2 to 7.5), pH_T is the pH at time T of the time course, and pH_{min} is the minimum pH in normal keratinocytes (pH 5.0); therefore, 0% pH change represents pH ≥7.0, and 100% change represents pH 5.0. The data were pooled from four to seven experiments per cell type. Bars, standard errors of the means (n, 4 to 7). (A) Normal human keratinocytes and keratinocyte clones transfected with the following vectors: pKV16E5 (which expresses E5 from the hCMV promoter), pAT16E5X (full-length HPV-16 DNA containing a premature stop codon in E5), and pAT16 and K1/16 (separate isolates of keratinocytes transfected with full-length HPV-16 DNA). (B) Normal human keratinocytes and mass cultures of G418-selected transfectants using pLXSN (a vector control) and pLXSN16E5 (which expresses E5 from the Harvey murine sarcoma virus LTR).

spectively, even after 80 min. The reduced rate of pH change in E5-expressing cells was more evident at 1,200 s; at this time, normal keratinocytes and pAT16E5X had completely acidified (100% pH change), while pKV16E5, pAT16, and K1/16 had experienced pH changes of only 59.3, 23.7, and 50.1%, respectively. The ability of HPV-16 E5 to inhibit endosomal acidification in keratinocytes when expressed either alone or in an HPV-16 genomic background was consistent across several clones tested at a number of different passage levels.

Whereas Fig. 2a shows the results of this assay using G418-selected, clonally derived cell lines, Fig. 2b shows the results of using G418-selected mass cultures of cells at low passage so that the effect of the vector and transfection itself could be observed in these cells. This was necessary because the short-lived nature of keratinocytes in tissue culture precludes the isolation of clones without the inclusion of a transforming gene. Normal human keratinocytes were included in both panels as a baseline reference for the sake of comparison. The endosomes of pLXSN-containing cells rapidly acidified to pH 5 (100% pH change at 10 min) at a slightly faster rate than the normal keratinocytes, much as the pAT16E5X clones. In contrast, the endosomes in pLXSN16E5 cells were only 58% acidified at 10 min and became only gradually more acidic with time (66% pH change at 20 min).

These ratio-imaging studies showed a significant defect in both the rate and extent of average endosome acidification in E5-expressing cells compared with normal keratinocytes. The defect in acidification appears to be greater in some cells than in others, especially at later time points. This is apparently not due to the effects of other HPV-16 genes, present in some of the vectors, on the metabolism of the keratinocyte, since pLXSN16E5 showed nearly as large a defect as pAT16 and K1/16. The extent of the defect also seems not to correlate with the apparent E5 mRNA levels (Fig. 1), and instead, the differences in acidification may be due to differences in the amount of E5 protein expressed, which is currently unknown. Although relatively large standard errors were observed in some of the pH measurements of E5-expressing cells, the differences from the results for normal keratinocytes and pAT16E5X clones were still significant. Furthermore, the mass culture of pLXSN16E5 was significantly different from the control vector pLXSN and normal keratinocytes. Finally, in all cells examined the endosomes acidified to at least pH 6.3, which is sufficient for ligand dissociation from EGF-EGFR complexes (41). Furthermore, the apparent pK_a of the EGF-EGFR interaction is unaffected by the expression of E5 in keratinocytes (43), and therefore, separate fates for ligand and receptor, as reported in earlier work (42), are possible in the E5-expressing cells examined.

Diphtheria toxin sensitivity. To determine if the pH changes due to HPV-16 E5 would inhibit general endosomal activity, we examined the sensitivity of normal and transfected keratinocytes to the toxicity of diphtheria toxin. A lysogenic phage infecting *Corynebacterium diphtheriae*, a pathogen of the upper respiratory tract in humans, encodes the 61-kDa diphtheria exotoxin (32). This toxin potently inhibits protein synthesis in eukaryotes by ADP-ribosylating a diphthamide residue (a modified histidine) of elongation factor 2 (12, 32). A single molecule of toxin in the cytosol is enough to result in protein synthesis inhibition and cell death (12). The entry of diphtheria toxin into susceptible eukaryotic cells involves a receptor-mediated mechanism (32), and toxin activation and cytotoxicity are dependent on the low-pH environment of endocytic vesicles generated by the activity of the vacuolar H^+-ATPase (47). A pH of 5 or less is optimally required to activate the fusion of the β subunit with the endosomal vesicle membrane, forming a

channel for the passage of the catalytic α subunit to the cytosol (11, 22, 32, 38).

The diphtheria toxin sensitivity assay was adapted from references 11 and 38. Trypsin-nicked diphtheria toxin was prepared by a method adapted from references 12 and suspended at various concentrations in growth media buffered with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.2). For 30 min the toxin was bound to cells kept on ice to prevent internalization of the toxin. Cells treated only with buffered growth media were included as controls. After washing away unbound toxin with ice-cold phosphate-buffered saline, one of the pair of dishes was acid shocked with buffered growth medium (20 mM HEPES, pH <4.0) for 5 min at 37°C to measure receptor-dependent endosome-independent diphtheria toxin sensitivity. All dishes were then incubated at 37°C for 1 h in growth media buffered to pH 7.2. After thorough washing, the cells were labeled for 1 h with cysteine-free Dulbecco modified Eagle medium plus 2 μCi of [^{35}S]cysteine per ml (1,300 Ci/mmol; Amersham; Arlington Heights, Ill.). After unincorporated label was washed away, the cells were solubilized in 200 μl of 0.1 N NaOH, and 50 μl of the solute was blotted to 2.4-cm glass fiber disks (Whatman, Maidstone, England). Disks were washed with 10% trichloroacetic acid, rinsed with 95% ethanol, and air dried. The incorporation of label into the trichloroacetic acid-insoluble fraction was determined by a scintillation counter as a measure of protein synthesis. The experiment was repeated three to five times in quadruplicate for each toxin concentration.

Figure 3 shows the results of this assay, with the ability of diphtheria toxin to inhibit protein synthesis determined in keratinocytes with and without E5. The results for the toxin-treated cells are presented as the fraction of the incorporation of [^{35}S]cysteine in control cells against the toxin concentration, so the more sensitive a cell is to diphtheria toxin, the lower the fraction of incorporation. Normal keratinocytes (Fig. 3A) were more sensitive to protein synthesis inhibition by diphtheria toxin than the E5-expressing cell line pKV16E5 (Fig. 3A) at lower concentrations of diphtheria toxin at pH 7.2. For example, protein synthesis in normal keratinocytes was inhibited 19% by 0.01 μg of diphtheria toxin per ml, compared with only 3% inhibition in pKV16E5 cells. Similarly, pAT16E5X cells (Fig. 3B) are also more sensitive to diphtheria toxin (24% inhibition at 0.01 μg/ml) than pAT16 (Fig. 3B) (5% inhibition at 0.01 μg/ml). Therefore, the expression of HPV-16 E5, regardless of the promoter, interferes with the inhibition of protein synthesis by diphtheria toxin. Complete resistance to diphtheria toxin by E5 expression was not expected, because diphtheria toxin activation is still possible at a less than optimal pH. Toxin activity is known to be approximately 35% of normal at pH 6.5 compared with pH 5.0 (38), and, according to the ratio-imaging data, endocytic vesicles do not acidify on average below pH 6.3 in some E5-expressing cell lines, so suboptimal activation may explain the slight reduction in protein synthesis in these cells at higher toxin concentrations. Also, all the keratinocytes were equally sensitive to the highest toxin concentration used (35 to 40% inhibition at 10 μg/ml), most likely because of the amount of toxin present and the fact that only one toxin molecule needs to penetrate a cell to cause cell death (12).

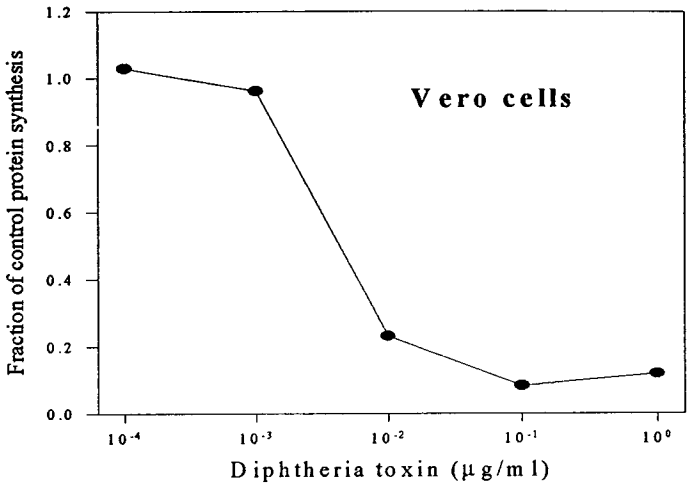
It is unlikely that the difference in sensitivity between E5-expressing and nonexpressing cells can be attributed to a difference in ability to bind diphtheria toxin. The low-pH environment of the endosome can be mimicked by acidifying the culture medium (11, 17, 30, 38). This acid shock allows bound toxin to enter the cytosol through direct penetration of the plasma membrane. When the requirement for endosome acidification was bypassed, there was no difference between cell types in their sensitivity to diphtheria toxin (data not shown). This indicates that the number of binding sites for the toxin on the cells with E5 is apparently similar to that on the cells without E5 and also demonstrates that the difference in toxin sensitivity exhibited under normal (pH 7.2) conditions is due to differences in endosome acidification. Furthermore, it is unlikely that the reduced sensitivity of keratinocytes is due to a sensitive subpopulation in a culture of resistant cells, because, with the exception of the untransfected keratinocytes, the cells used were clonally derived. The mass cultures of pLXSN and pLXSN16E5 were not tested in this assay.

The Vero cell line is derived from the green monkey kidney and is typically used to study diphtheria toxin because of the high sensitivity of the cells to low concentrations of the toxin (Fig. 3, inset). The inclusion of these cells as a control demonstrates that the assay was functioning as expected and that human keratinocytes are at least 1,000-fold less sensitive to diphtheria toxin than Vero cells.

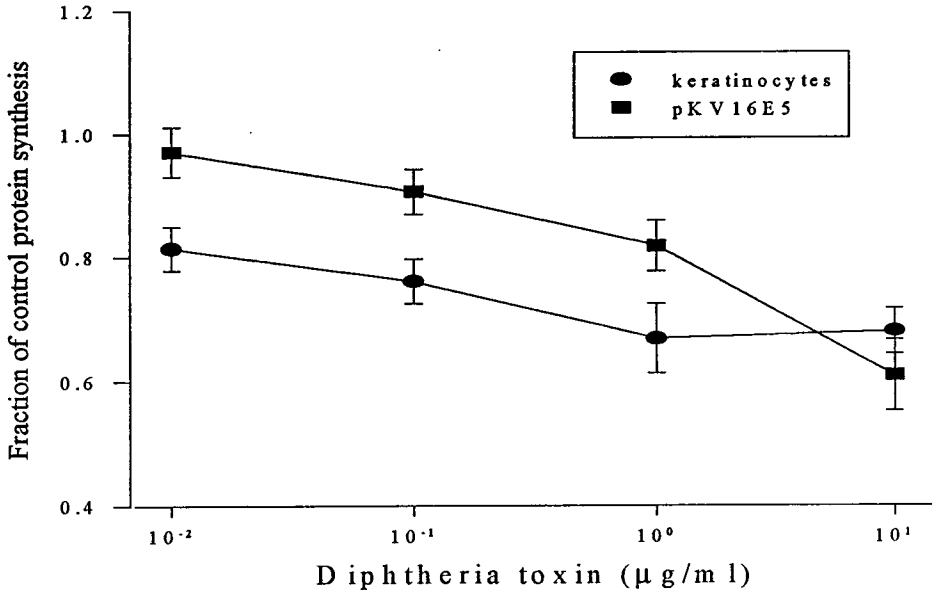
Effect of chloroquine on EGFR immunofluorescence. In order to investigate whether the inhibition of acidification could explain our previous observations of decreased degradation and the increased recycling the EGFR in E5-expressing cells (42), we examined the effect of artificial inhibition of endosomal acidification on EGFR endocytosis. Chloroquine, a weak acidotropic base, inhibits endosomal acidification by accumulating in acidic vesicles like endosomes and countering the influx of protons, thereby increasing vesicle pH toward cytoplasmic levels (24, 40). For example, treatment of cells with 0.1 mM chloroquine results in an increase of vesicular pH from 4.7 to 6.4 (31). The experiment was performed in the presence and absence of 25 μM chloroquine diphosphate (Sigma Chemical Co., St. Louis, Mo.). EGF-starved keratinocytes were exposed to saturating concentrations of EGF (100 ng/ml, or 16.5 nM) while on ice to synchronize receptor-ligand complex formation. Unbound EGF was removed, and the cells were warmed to 37°C for up to 3 h to allow the internalization of EGFRs. At various intervals the cells were fixed with 3.7% formaldehyde. Duplicate preparations were permeablized with 0.1% Triton X-100 to view internalized EGFR. This assay, without the use of chloroquine, is detailed in reference 42.

At various times after the addition of EGF, the location of EGFRs in the cell was visualized by immunofluorescence with monoclonal antibody R1 (48) and a secondary antibody conjugated to fluorescein isothiocyanate. Control staining was performed with secondary antibody only. Details of the staining protocol can be found in reference 42. Figure 4 shows representative results of this assay. At the start of the experiment the EGFRs were present at the surface of the pAT16E5X (Fig. 4A) and pAT16 (Fig. 4B) cells, and chloroquine treatment did not significantly alter this localization of receptor for either cell type (Fig. 4C and D, respectively). Both cell types internalized

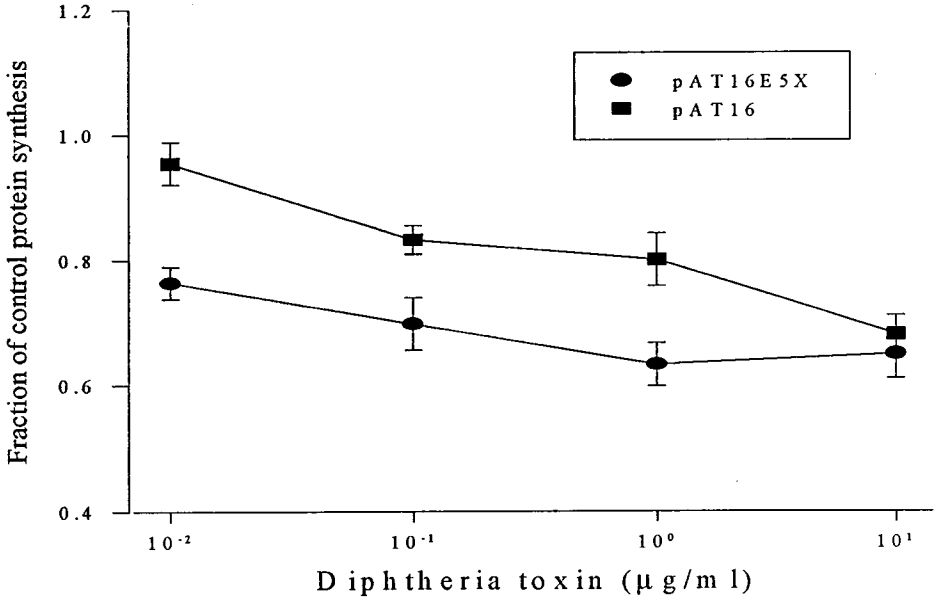
FIG. 3. E5-expressing cells are less sensitive to endosome-dependent diphtheria toxin-mediated protein synthesis inhibition. The ability of diphtheria toxin to inhibit protein synthesis in keratinocytes was determined by the techniques described in the text. Protein synthesis was measured as the fraction of trichloroacetic acid-insoluble [^{35}S]cysteine incorporated by toxin-treated cells compared with untreated cells. Bars, standard errors of the means (*n*, 12 to 20). (A) Normal human keratinocytes and keratinocyte clones transfected with pKV16E5 (expressing E5 from the hCMV promoter); (B) keratinocyte clones transfected with pAT16E5X (full-length HPV-16 DNA containing a premature stop codon in E5) or pAT16 (full-length HPV-16 DNA). The data for Vero cells (control for the efficacy of the assay) are given in the inset.


Vero cells
Fraction of control protein synthesis
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10⁻⁴
10⁻³
10⁻²
10⁻¹
10⁰
Diphtheria toxin (µg/ml)

A


keratinocytes
pKV16E5
Fraction of control protein synthesis
1.2
1.0
0.8
0.6
0.4
10⁻²
10⁻¹
10⁰
10¹
Diphtheria toxin (µg/ml)

B


pAT16E5X
pAT16
Fraction of control protein synthesis
1.2
1.0
0.8
0.6
0.4
10⁻²
10⁻¹
10⁰
10¹
Diphtheria toxin (µg/ml)

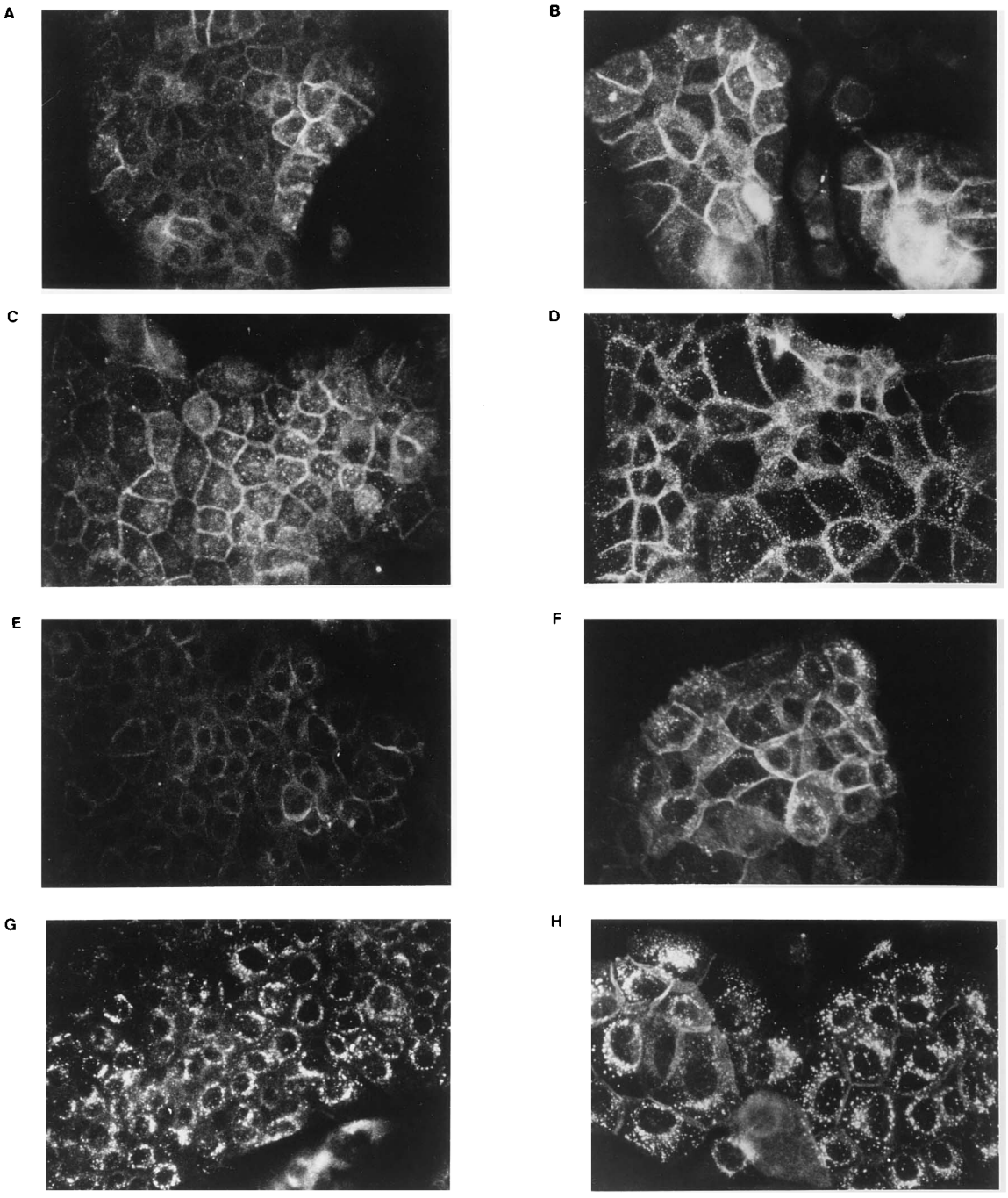


FIG. 4. Chloroquine mimics the effect of E5 expression on EGFR degradation in keratinocytes. Human keratinocytes stably transfected with pAT16E5X, a full-length HPV-16 DNA with a premature stop codon in E5 (A, C, E, and G), or pAT16, a full-length HPV-16 DNA (B, D, F, and H), were fixed, permeabilized, and stained for EGFRs as described in the text at time zero (A to D) or 180 min post-stimulation with EGF (E to H). Panels C, D, G, and H show cells treated with 25 μM chloroquine during the course of the experiment. Magnification, ×320.

EGFRs within 10 min in response to ligand stimulation regardless of the presence of chloroquine (data not shown). However, pAT16E5X cells rapidly degraded internalized EGFRs (within 90 min) (data not shown). By 180 min post-EGF stimulation, pAT16E5X had completely degraded internalized receptors and showed no EGFR immunofluorescence at the cell surface (Fig. 4E). However, pAT16 at 180 min had failed to degrade EGFRs, which remain in intracellular vesicles, and also exhibits apparent recycling of receptors to the cell surface (Fig. 4F). The recycling of receptor in pAT16 is apparent, though to a lesser extent, at 60 min post-EGF stimulation (data not shown). At 180 min the chloroquine-treated pAT16E5X (Fig. 4G) exhibited a pattern of EGFR retention in cytoplasmic vesicles and had failed to degrade the internalized receptor, a

TABLE 1. Similar effects of HPV-16 E5 and chloroquine on the EGFR immunofluorescence assay

Cell type[a]	EGFR immunofluorescence the following no. of min after addition of EGF[b]:							
	60				180			
	−CQ		+CQ		−CQ		+CQ	
	S	I	S	I	S	I	S	I
Keratinocytes	−	+	−	+	−	−	−	+
pLXSN	−	+	−	+	−	−	−	+
pAT16E5X	−	+	−	+	−	−	−	+
pLXSN16E5	+	+	+	+	+	+	+	+
pKV16E5	+	+	+	+	+	+	+	+
pAT16.5	+	+	+	+	+	+	+	+
K1/16	+	+	+	+	+	+	+	+

[a] See the text.
[b] 100 ng/ml. −CQ, without chloroquine; +CQ, with 25 μM chloroquine. S, surface immunofluorescence (unpermeabilized cells); I, intracellular immunofluorescence (permeabilized cells). + and −, presence and absence of immunofluorescence, respectively. At time zero, all cell types had surface fluorescence under both conditions, and none had intracellular fluorescence.

result much like the effect of E5 expression. However, from the lack of surface staining for EGFR in these cells at 180 min, chloroquine apparently did not induce recycling of internalized receptors to the cell surface. In pAT16 cells chloroquine did not alter recycling of EGFRs to the cell surface nor the cytoplasmic localization of undegraded EGFRs after 180 min (Fig. 4H), although vesicles containing EGFRs became more prominent with the passage of time.

Table 1 summarizes the results of this assay for all the cell types examined. In none of the cells examined did the addition of 25 μM chloroquine reorganize the distribution of EGFRs prior to stimulation with EGF. Normal keratinocytes, pLXSN mass cultures, and pAT16E5X clones rapidly internalized and degraded EGFRs in the absence of chloroquine, while E5-expressing cells (mass culture pLXSN16E5 and clones pKV16E5, pAT16, and K1/16) failed to degrade internalized EGFR up to 3 h after EGF stimulation and exhibited recycling of receptors to the cell surface within 60 min. Chloroquine did not alter recycling or the cytoplasmic localization of EGFRs in cells expressing E5 exogenously (pLXSN16E5 and pKV16E5) or within the context of the HPV-16 genome (pAT16 or K1/16), although EGFR-containing vesicles swelled noticeably by 180 min. At 60 and 180 min after EGF stimulation of chloroquine-treated normal keratinocytes, pLXSN and pAT16E5X retained EGFRs in cytoplasmic vesicles and failed to degrade the internalized receptor, much like the effects of E5 expression. However, from the lack of surface staining for EGFR at 60 to 180 min, it was apparent that chloroquine did not stimulate recycling of internalized receptors to the cell surface in the normal keratinocytes and pAT16E5X. In a similar experiment, keratinocytes were stimulated with EGF while treated with bafilomycin A1, a specific inhibitor of the vacuolar H^+-ATPase (1, 50). This resulted in the retention of apparently undegraded EGFRs in cytoplasmic vesicles and the absence of any apparent recycling (data not shown). The distribution of receptors at 180 min was identical to that in chloroquine-treated cells (Fig. 4G). Furthermore, there were no apparent differences in EGFR distribution in E5-expressing cells treated with bafilomycin A1 (data not shown).

We have shown that E5 expressed from the endogenous HPV-16 promoter, or two different exogenous promoters, inhibits acidification of endosomes. The ability of chloroquine to inhibit endosomal acidification and thereby prevent EGFR degradation in normal keratinocytes supports the hypothesis that the inhibition of EGFR degradation in E5-expressing cells is due to defective acidification of endosomes. However, artificially inhibiting endosomal acidification with chloroquine did not result in the recycling of EGFRs in normal keratinocytes as was seen with E5-expressing cells. This is consistent with the observation that chloroquine did not interfere with normal recycling of proteins like transferrin receptor (40). Furthermore, results similar to the effect of chloroquine were found after keratinocytes were treated with bafilomycin A1. From these experiments we conclude that the inhibition of endosome acidification can account for some, though not all, of the effects of E5 on internalized EGFR and that the defect in acidification in E5-expressing cells may be due to interference with the H^+-ATPase pump.

It is still unknown how the interaction of HPV-16 E5 with the 16-kDa component of the vacuolar H^+-ATPase inhibits endosomal acidification. The most straightforward possibility is that the function or assembly of the proton pump may be disrupted by the binding of the E5 protein to the pump complex through either hydrophobic interactions, steric hindrance, or blockage of the proton pore that is composed, at least in part, of 16-kDa subunits. Another potential mechanism is that E5 may exclude the pump complex from the maturing endosome. This has been demonstrated as the means by which *Mycobacterium* spp. prevent acidification of mycobacterium-containing vacuoles in infected macrophages (44).

The observations presented here reinforce the proposed role for E5 in the life cycle of HPV-16: by inhibiting endosomal acidification to delay EGFR degradation, in combination with being able to increase EGFR recycling and to heighten the mitogenic response to EGF, HPV-16 E5 may boost the mitogenic responses of host cells in the epithelium to provide the replication machinery required for viral propagation in otherwise differentiating keratinocytes.

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