Human Papillomavirus Type 16 Infection of Human Keratinocytes Requires Clathrin and Caveolin-1 and Is Brefeldin A Sensitive[†]

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Human papillomavirus type 16 (HPV16) has been identified as being the most common etiological agent leading to cervical cancer. Despite having a clear understanding of the role of HPV16 in oncogenesis, details of how HPV16 traffics during infection are poorly understood. HPV16 has been determined to enter via clathrin-mediated endocytosis, but the subsequent steps of HPV16 infection remain unclear. There is emerging evidence that several viruses take advantage of cross talk between routes of endocytosis. Specifically, JCV and bovine papillomavirus type 1 have been shown to enter cells by clathrin-dependent endocytosis and then require caveolin-1-mediated trafficking for infection. In this paper, we show that HPV16 is dependent on caveolin-1 after clathrin-mediated endocytosis. We provide evidence for the first time that HPV16 infection is dependent on trafficking to the endoplasmic reticulum (ER). This novel trafficking may explain the requirement for the caveolar pathway in HPV16 infection because clathrin-mediated endocytosis typically does not lead to the ER. Our data indicate that the infectious route for HPV16 following clathrin-mediated entry is caveolin-1 and COPI dependent. An understanding of the steps involved in HPV16 sorting and trafficking opens up the possibility of developing novel approaches to interfere with HPV16 infection and reduce the burden of papillomavirus diseases including cervical cancer.

Human papillomavirus (PV) type 16 (HPV16) is a member of the family *Papillomaviridae*, a group of double-stranded DNA (dsDNA) viruses with a tropism for squamous epithelia (70). Most PV infections result in benign lesions, although a subset of high-risk HPVs are capable of malignant transformation, resulting in various cancers including cervical carcinoma (21, 38). Infection with HPV16 is responsible for causing approximately half of the cases of invasive cervical cancer (7). In spite of the link between HPV16 and cervical cancer, the intracellular movement of HPV16 through target keratinocyte cells during infection has not been defined in detail.

Viruses can enter into target cells by taking advantage of the cell's natural endocytosis machinery (60). One of the best-characterized modes of internalization is by receptor-mediated, clathrin-dependent endocytosis. In this mode of entry, clathrin-coated pits internalize cargo into clathrin-coated vesicles, which are pinched from the plasma membrane by dynamin-2 in order to internalize (68). The process of clathrin-mediated endocytosis occurs rapidly, resulting in the delivery of cargo to early/sorting endosomes within seconds to minutes (23, 31). From the sorting endosome, most clathrin-dependent ligands are trafficked back to the plasma membrane in recycling endosomes or to lysosomes for degradation (35, 56). Another well-studied model of ligand entry is caveolin-1-mediated endocytosis. The caveolar pathway typically involves

entry via cholesterol-rich caveolae at the plasma membrane, which deliver their contents to pH-neutral organelles known as caveosomes (44, 65). The delivery of cargo from caveosomes to the Golgi apparatus and the endoplasmic reticulum (ER) was demonstrated previously (44, 46, 50). The traffickings of cargo internalized via clathrin- and caveolin-1-mediated endocytosis were once thought to be separate; however, it is becoming evident that viruses including bovine PV type 1 (BPV1), JCV, HPV31, and BKV rely on both pathways depending on the stage of infection (29, 32, 50, 63).

PV internalization is preceded by virion attachment to the extracellular matrix, followed by binding to heparan sulfate (14, 15, 25). The involvement of a secondary receptor has been suggested, putatively an α -6 integrin (24, 37). Postbinding, a conformational change in the PV capsid results in a furin cleavage event at the N terminus of the minor capsid protein L2, which has been suggested to play a role in the endosomal escape of the viral genome (19, 30, 52). An increasing body of evidence supports the entry of HPV16 by clathrin-mediated endocytosis (9, 27, 62). Electron microscopy of HPV16 infection in COS-7 cells demonstrated HPV16 pseudovirions in clathrin-coated vesicles 20 min after entry and within structures resembling endosomes by 1 h postentry (9). HPV16 infection of HaCaT keratinocyte, COS-7, and 293TT cells has been blocked by chlorpromazine, an inhibitor of the formation of clathrin-coated pits (9, 27, 62, 67). Importantly, those studies showed that two inhibitors of caveolin-1-mediated internalization, filipin and nystatin, did not interfere with HPV16 infection (9, 27, 62). Our laboratory demonstrated the importance of dynamin in HPV16 infection, presumably in the scission of clathrin-coated vesicles from the plasma membrane (1). Recently, a clathrin-, caveolin-, and dynamin-independent endocytosis of HPV16 was suggested, although the use of the

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HPV18-positive, heteroploid HeLa cell line calls into question the relevance of this finding to natural infection (64).

In a previous study, we described the postentry trafficking of BPV1 from endosomes to caveolin-1-positive vesicles, similarly to a related nonenveloped dsDNA virus, JCV (32, 50). Our data demonstrated that the infectious route of BPV1 involved entry by clathrin-mediated endocytosis followed by transport to the caveolar pathway in order to traffic to the ER (32). We found that BPV1 infection was neutralized by an antibody that prevented viral particle transport to the ER (33). The movement of BPV1 from the endosome to the caveosome provides a possible explanation for why BPV1 trafficking is so slow compared to those of other ligands of clathrin-mediated endocytosis (20, 26). The kinetics of BPV1 and HPV16 entry were previously reported to be identical, and the coincident internalization of HPV16 and BPV1 virus-like particles (VLPs) showed colocalization between the VLPs during infection (20, 62). These data suggest that HPV16 and BPV1 infection may be occurring by a similar mechanism.

Our goal in the present study was to determine the intracellular trafficking events leading to HPV16 infection. The use of reporter virion technology has allowed the production of high-titer HPV16 virions by a method previously shown to yield virions that are infectious *in vivo* (16). In this study, we used HPV16 reporter virions to study HPV16 infection in the spontaneously immortalized human HaCaT keratinocyte cell line. Our data show that the infectious route of HPV16 is from early endosomes to caveolin-1-positive vesicles and then to the ER. Using immunofluorescence and short hairpin RNA (shRNA) against caveolin-1, we demonstrate the importance of the caveolar pathway after HPV16 has been internalized. We show that HPV16 infection was blocked by inhibiting the formation of COPI transport vesicles, which function in trafficking between the ER and the Golgi apparatus and from caveosomes to the ER (5, 39). We provide evidence that after reaching the caveosome, HPV16 requires passage to the ER for successful infection, a trafficking event made possible by COPI vesicle-mediated movement from the caveosome to the ER.

MATERIALS AND METHODS

Reagents. HPV16 reporter virions were generated and titers were determined as described previously (11; <http://home.ccr.cancer.gov/lco/default.asp>). The 293TT cell line, p16LLWCHA, and DsRed reporter 8rwb plasmids were gifts from P. Day and D. Schiller (NCI, NIH, Bethesda, MD). Caveolin-1 shRNAs (shRNA A and shRNA B) and the luciferase control shRNA were kindly provided by W. Atwood and W. Querbes (Brown University, Providence, RI) and were previously described (50). Lentiviral vector pCSP-SP-PW containing small interfering RNA (siRNA) against luciferase (siRL) was a gift from V. Bottero (Rosalind Franklin University of Medicine and Science [RFUMS], North Chicago, IL). Experiments were performed using HaCaT cells, a spontaneously immortalized human keratinocyte cell line. The HaCaT cells were generously provided by M. Ozbun (University of New Mexico School of Medicine, Albuquerque, NM). The tetraploid cervical carcinoma HeLa cell line used for data reported in the supplemental material was a gift from P. Howley (Harvard Medical School, Boston, MA). Cells were grown in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (Mediatech, Manassas, VA) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA) (DMEM-10).

Chemical inhibitors. Brefeldin A (BFA) is an interfacial, noncompetitive inhibitor of the GTPase ADP-ribosylation factor 1 (ARF1). BFA forms a stable complex between ARF1-GDP and its guanine exchange factor, thereby leading to the inactivation of ARF1 and the subsequent failure to recruit coat protein I onto COPI vesicles (47, 69). For immunofluorescence studies and the time course of inhibition with BFA (Epicycle Biotechnologies, Madison, WI), 25

ng/ml was added to HaCaT cells. BFA was added along with virions/ligands or at the indicated time points after entry of the virus. Chlorpromazine is a cationic amphiphilic drug which disrupts clathrin-coated pit formation by dispersing an adaptor protein required for its configuration (67). The ability of chlorpromazine to block clathrin-dependent endocytosis was tested using fluorescently labeled Alexa Fluor 594-transferrin (Invitrogen). Briefly, HaCaT cells grown on glass coverslips (catalog no. 12-545-80; Fisher Scientific, Piscataway, NJ) were preincubated with 6 μ M chlorpromazine (Sigma, St. Louis, MO) in DMEM-10 for 1 h at 37°C. A total of 10 μ g/ml transferrin was added to chilled, pretreated HaCaT cells for 1 h on ice. Cells were shifted to 37°C to allow transferrin to internalize. After 30 min, HaCaT cells were acid treated prior to fixation to remove noninternalized transferrin, as described previously (58). Chlorpromazine was present at 6 μ M for the duration of the experiment. Immunofluorescence was performed to assess if transferrin had internalized into the HaCaT cells. In the time course experiment, 6 μ M chlorpromazine was added to HaCaT cells at the indicated time points before or after the addition of HPV16 reporter virions, where 0 h corresponds to the time when cells with bound virions were shifted to 37°C to allow for virus internalization.

Filipin is a sterol binding biochemical inhibitor which disrupts lipid rafts, preventing caveola-mediated entry (42, 55). The inhibitory effect of filipin on caveola-mediated internalization was confirmed using Alexa Fluor 594-cholera toxin B (Invitrogen). HaCaT cells grown on glass coverslips were preincubated with 2 μ M filipin (Sigma) in DMEM-10 for 1 h at 37°C. A total of 1 μ g/ml cholera toxin B was then incubated with pretreated HaCaT cells for 1 h on ice. HaCaT cells were gently washed with medium to remove unbound cholera toxin B, and fresh DMEM-10 with filipin was added. HaCaT cells were warmed to 37°C. After 30 min, cells were washed with phosphate-buffered saline and fixed, and immunofluorescence was performed. To test the effect of filipin treatment on HPV16 infection, filipin was added to HaCaT cells for 1 h at 37°C prior to the addition of virions. Filipin was used at 0.5 μ M, 1.0 μ M, and 2.0 μ M. Once added, the inhibitor was present for the duration of the experiment.

Cell viability assay. The toxicity of inhibitors used in experiments was determined using the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI) according to the manufacturer's protocol. Inhibitors were diluted in DMEM-10 to the indicated concentrations and added to HaCaT cells for 48 h. Cells were harvested 48 h after the addition of inhibitors and added to a 96-well microplate (Greiner Bio-One, Monroe, NC) along with CellTiter-Glo reagent. Luminescence was used as an indicator for the amount of ATP in the sample. Each concentration was tested three times, and error bars represent standard deviations. Cytotoxicity was determined by a statistically significant drop in ATP levels relative to that of untreated cells at an α value of 0.05.

Western blot analysis. HaCaT or HeLa cells were transfected using GeneJet according to the manufacturer's instructions (SignaGen Laboratories, Gaithersburg, MD). Forty-eight hours after transfection of HaCaT cells or 72 h after transfection of HeLa cells, lysates were harvested using cold 0.5% NP-40 buffer (0.5% NP-40 substitute, 150 mM NaCl, 5 mM EDTA, 10 mM Tris [pH 7.5]) containing protease inhibitors (GE Healthcare, Piscataway, NJ). Samples were run on a 12% sodium dodecyl sulfate-polyacrylamide gel as described previously (8). Primary antibodies mouse anti-caveolin-1 (Abcam, Cambridge, MA), or rabbit anti-caveolin-1 (Cell Signaling Technology, Danvers, MA), and mouse anti-actin (Sigma) were used at a 1:1,000 dilution overnight at 4°C. Alexa Fluor 800-nm anti-mouse and 680-nm anti-rabbit secondary antibodies were used at a 1:20,000 dilution for 30 min at room temperature (Invitrogen). The Odyssey imaging system was used to view blots, and band intensities were determined using Odyssey-provided software (Li-Cor Inc., Lincoln, NE).

Immunofluorescence. HaCaT cells were grown on glass coverslips (Fisher Scientific). Immunofluorescence was performed as previously described (8), with the exception that cells were fixed using 4% paraformaldehyde for 10 min at 4°C. Coverslips were mounted using ProLong Gold Antifade reagent (Invitrogen). A working dilution of 1:100 was used for the following primary antibodies: the capsid conformation-dependent mouse anti-HPV16 L1 hybridoma H16.V5 (gift from N. Christensen, Pennsylvania State University College of Medicine, Hershey, PA), the early endosome marker goat anti-EEA1 (Santa Cruz, Santa Cruz, CA), the caveolar marker rabbit anti-caveolin-1 (Cell Signaling Technology), the ER marker rabbit anti-calnexin (Abcam), the ER marker rabbit anti-ERp29 (Affinity BioReagents, Rockford, IL), and the Golgi apparatus marker rabbit anti-giantin (Covance, Princeton, NJ). Donkey anti-mouse 594, chicken anti-goat 488, and donkey anti-rabbit 488 fluorescent Alexa Fluor secondary antibodies were used at a 1:2,000 dilution (Invitrogen). Topro-3 was used for nuclear visualization at a 1:1,000 dilution in the secondary-antibody dilution (Invitrogen). Antibodies were diluted in blocking buffer (0.2% fish skin gelatin, 0.2% Triton X-100, phosphate-buffered saline) except that when studies involved transferrin,

FIG. 1. HPV16 traffics to early endosomes and overlaps with caveolin-1 by 20 min postentry. HaCaT cells infected with HPV16 reporter virions were stained with anti-L1 antibody H16.V5 (red) and either anti-EEA1 (A to C) (green) or anti-caveolin-1 (D to F) (green). HPV16 overlap with EEA1 was seen at 5 min (A), 20 min (B), and 2 h (C) (yellow arrows). HPV16 did not colocalize with caveolin-1 at 5 min (D) (red and green arrows). Colocalization of reporter virions and caveolin-1 was observed 20 min (E) and 2 h (F) postinfection (yellow arrows). The nucleus was stained using Topro-3 (blue). Images represent z stacks, showing the pattern of staining in the *x*, *y*, and *z* planes.

the blocking buffer was made using BD Perm/Wash buffer (BD Biosciences, San Jose, CA) and 0.2% fish skin gelatin. The Olympus Fluoview 300 inverted confocal microscope at the microscopy core of RFUMS was used for data collection, and analysis was performed using Fluoview software (Olympus, Melville, NY). z-stacked images were used to show nine 0.5- μ m sections of the cell in the *x*, *y*, and *z* planes.

Quantification of immunofluorescence data. Immunofluorescence images were analyzed using Stereo Investigator (MicroBrightField Bioscience, Williston, VT). Images were imported from Fluoview and enlarged, and pixels were counted using 10-pixel markers. A limitation of the analysis is that the lateral resolution of the confocal microscope (approximately 150 nm) precludes the ability to identify individual 50- to 60-nm virions (6). Quantification was performed as follows: the amounts of colocalized virions (represented by yellow pixels) and noncolocalized virions (represented by red pixels) were counted, and the number of yellow pixels was divided by the total number of yellow and red pixels to obtain the percentage of colocalization. A representative slice from the center of the cells was analyzed for at least three separate experiments per time point/treatment condition.

Transferrin, cholera toxin B, and HPV16 internalization in the presence of BFA. A total of 10 μ g/ml of the transferrin conjugate, 1 μ g/ml of the cholera toxin B conjugate, or HPV16 reporter virions were added to chilled HaCaT cells seeded onto glass coverslips and incubated for 2 h on ice in the presence of 25 ng/ml of BFA. After replacing the medium with fresh DMEM-10 containing BFA, cells were shifted to 37°C in 5% CO₂ for the indicated time points. BFA was present throughout the infection/ligand internalization.

Infection of cells expressing GFP shRNA against caveolin-1 or luciferase or siRNA control against luciferase. Cells were transfected with 2 μ g of the indicated construct or infected with luciferase control siRNA in lentiviral vector pCSC-SP-PW as previously described (32). All plasmids contained the green fluorescent protein (GFP) transgene. Cells were infected with HPV16 containing the DsRed reporter and harvested for analysis by flow cytometry at 48 h postinfection. The LSRII flow cytometer and FACS Diva software (BD Biosciences) at the flow cytometry core at RFUMS were used to detect the percentage of GFP-expressing cells that were also DsRed positive (i.e., double positive).

Statistical analysis. Bars from graphs represent the averages of data from three separate trials in which 10,000 cells from each were counted. Error bars show the standard deviations from the means of data from the three trials. Two-tailed unpaired *t* tests were used to compare experimental conditions to those of the respective controls. The significance level was set at an α value of 0.05.

RESULTS

HPV16 reporter virions colocalize with the early endosome marker EEA1 and then with the caveolar marker caveolin-1 as infection progresses in time. We wanted to identify the localization of HPV16 reporter virions after clathrin-mediated endocytosis (Fig. 1). Using the early endosome marker EEA1 (Fig. 1A to C, green), the caveolar marker caveolin-1 (Fig. 1D to F, green), and the conformation-dependent anti-HPV16 L1 H16.V5 antibody (Fig. 1, red), we showed that 5 min after internalization is induced (by returning cells to 37°C), reporter virions can be found in early endosomes (Fig. 1A, yellow). We did not observe an overlap of reporter virions with caveolin-1 after 5 min of internalization (Fig. 1D, red and green arrows). After 20 min, reporter virions could be visualized to be overlapping with endosomes and caveolin-1-positive vesicles (Fig. 1B and E, respectively, yellow). This pattern of staining was also seen at 2 h, although we observed a time-dependent decrease in costaining with EEA1 and an increase in costaining with caveolin-1 (Fig. 1C and F, respectively, yellow). These data suggest that reporter virions move from early endosomes into caveolar vesicles or that the reporter virions were able to enter the HaCaT cells by caveola-mediated endocytosis.

HPV16 infection in HaCaT cells is decreased by chlorpromazine in a time-dependent manner and is unaffected by filipin. Having observed the colocalization of HPV16 reporter virions with an early endosome marker and a caveolar marker, we wondered if virus entry into HaCaT cells was performed by both clathrin- and caveolin-1-mediated endocytosis (see Fig. S1 in the supplemental material). HPV16 was previously demonstrated to enter cells by clathrin-mediated endocytosis, a process that can be interfered with using chlorpromazine (9, 27, 62, 67). Two inhibitors of caveola-mediated entry, filipin

and nystatin, were shown to have no effect on HPV16 infection (9, 27, 62). We first determined that the addition of chlorpromazine at concentrations of 9 μ M or more was toxic to HaCaT cells (see Fig. S1A in the supplemental material) ($P < 0.001$). We tested the ability of the clathrin-mediated ligand transferrin to internalize into untreated cells (see Fig. S1B in the supplemental material) or into cells which were treated for 1 h with 6 μ M chlorpromazine prior to the addition of transferrin (see Fig. S1C in the supplemental material). HaCaT cells were fixed 30 min after the entry of transferrin (see Fig. S1B and S1C, red, in the supplemental material), and the early endosomes were labeled using an anti-EEA1 antibody (see Fig. S1B and S1C, green, in the supplemental material). In untreated HaCaT cells, transferrin internalized and colocalized with the early endosome marker EEA1 (see Fig. S1B, overlap in yellow, in the supplemental material). The nontoxic concentration of 6 μ M chlorpromazine prevented the entry of transferrin (see Fig. S1C in the supplemental material). The addition of chlorpromazine to the cells at 6 μ M 1 h prior to reporter virions decreased infection by more than 70%, as measured by the expression of the DsRed reporter gene after 48 h (see Fig. S1D in the supplemental material) (-1 h; $P < 0.001$). The decrease in infection was reduced the later chlorpromazine was added, and we did not observe a statistically significant decrease in infection when the inhibitor was added at or later than 4 h. The time course of chlorpromazine sensitivity suggested that 50% of infections could not be blocked after virions were allowed to internalize for 1 h, and over 75% of infections could not be blocked after 4 h (i.e., less than 25% of infectious virions were susceptible to chlorpromazine after 4 h). These data support the clathrin-mediated endocytosis of HPV16.

Because we observed a colocalization of HPV16 and caveolin-1 (Fig. 1), we wanted to rule out the caveola-mediated entry of HPV16. We found that 0.5 μ M, 1.0 μ M, and 2.0 μ M filipin were not toxic to HaCaT cells (see Fig. S1E in the supplemental material). We tested whether 2.0 μ M filipin was sufficient to prevent the entry of the caveolar ligand cholera toxin B. In untreated HaCaT cells, cholera toxin B (see Fig. S1F, red, in the supplemental material) colocalized with caveolin-1 (see Fig. S1F, green, in the supplemental material) 30 min postentry (see Fig. S1F, overlap in yellow, in the supplemental material). When HaCaT cells were pretreated for 1 h with 2.0 μ M filipin prior to the addition of cholera toxin B, the caveolar ligand did not enter cells and therefore did not colocalize with caveolin-1 (see Fig. S1G, green, in the supplemental material). HaCaT cells were pretreated with 0.5 μ M, 1.0 μ M, and 2.0 μ M filipin for 1 h prior to the addition of HPV16 reporter virions (see Fig. S1H in the supplemental material). Filipin did not decrease reporter virion infection at any of the concentrations tested compared to untreated HaCaT cells (see Fig. S1H in the supplemental material). The filipin concentrations used are comparable to doses that were previously shown to block HPV31 infection in HaCaT cells (62). Our data are in agreement with previous findings from the Ozburn laboratory showing that HPV16 infection in HaCaT cells is initiated by clathrin-mediated endocytosis (62).

HPV16 infection is decreased in HaCaT cells with reduced caveolin-1 protein expression levels. The block of HPV16 infection by chlorpromazine confirms the clathrin-mediated entry of HPV16 but does not explain the colocalization between

FIG. 2. Caveolin-1 shRNA expression in HaCaT cells reduces HPV16 infection levels. (A) HaCaT cells were transfected with shRNA against caveolin-1 (Cav-1) in a plasmid containing a GFP reporter gene (lanes 1 and 2, shRNA A and shRNA B, respectively) or infected with a lentiviral vector containing GFP and siRNA against luciferase (lane 3). Lane 4 shows the lysates from HaCaT cells alone. Cell lysates were harvested for analysis by Western blotting after 48 h. The amount of caveolin-1 protein expression was determined by blotting with an anti-caveolin-1 antibody. Actin was used as a loading control. (B) Infection in HaCaT cells expressing shRNA A, shRNA B, or siRL was assessed after 48 h. Cells were harvested, and the percentage of cells that were GFP positive (expressing shRNA or siRL) and also DsRed positive (infected) was measured by flow cytometry as the percentage of double-positive cells. The drop in infection in caveolin-1 knockdown cells was determined to be significant (*, $P < 0.002$). Each bar represents the average of data from three trials for which the standard deviations are shown by error bars.

the virions and caveolin-1. In order to determine if there is a biological role for caveolin-1 in HPV16 infection, caveolin-1 protein expression in HaCaT cells was knocked down using shRNA prior to the addition of reporter virions (Fig. 2). HaCaT cells were transfected with shRNA constructs (shRNA A or shRNA B) targeting two different regions of caveolin-1. Both caveolin-1 shRNA constructs effectively knocked down caveolin-1 protein expression by more than 60% (Fig. 2A, lanes 1 and 2) compared to control cells expressing luciferase siRNA (Fig. 2A, lane 3) or untransfected cells (Fig. 2A, lane 4). Caveolin-1 protein levels were normalized to actin levels. shRNA A, shRNA B, and siRL constructs were engineered to coexpress GFP to allow us to identify cells expressing these constructs. HaCaT cells were infected with HPV16 reporter virions containing DsRed cDNA, permitting us to quantify infection in shRNA- or siRNA-expressing cells, i.e., GFP-expressing cells that were also expressing the DsRed reporter (percent double positive). The percentage of HPV16 reporter virion infection in HaCaT cells expressing caveolin-1 shRNA A or shRNA B (Fig. 2B) (10.2% and 11%, respectively) was 60% less than that in cells expressing the siRL control (30.6%; $P < 0.002$) (Fig. 2B). The decrease in the rate of viral infection in HaCaT cells with caveolin-1 knockdown strongly suggests that caveolin-1 is essential for HPV16 infection in natural target keratinocyte cells.

HPV16 colocalizes with the ER markers ERp29 and calnexin by 4 h postentry. After verifying that reduced caveolin-1 protein expression levels resulted in decreased HPV16 infection rates, we wanted to determine if the virus was trafficking

FIG. 3. Colocalization of HPV16 and ER markers by 4 h after internalization. HaCaT cells infected with HPV16 reporter virions (red) were stained for ERp29 (A to D) (green) or calnexin (E to H) (green). (A) Overlap between HPV16 and ERp29 was not seen at 30 min (A3 and 4) (red and green arrows). (B to D) Colocalization of HPV16 and ERp29 was observed at 4 h (B3 and 4), 6 h (C3 and 4), and 12 h (D3 and 4) (yellow arrows). (E) HPV16 and calnexin did not overlap at 30 min postentry (E3 and 4, red and green arrows). (F to H) At 4 h (F3 and 4), 6 h (G3 and 4), and 12 h (H3 and 4) after HPV16 internalization, virions colocalized with calnexin (yellow arrows). Topro-3 was used to identify the nucleus (blue). z-stacked images show nine 0.5- μ m slices in the x, y, and z planes. Enlarged sections of the z-stacked images are shown to the right.

to the ER from the caveosome, as was shown previously for JCV and BPV1 (32, 33, 50) (Fig. 3). Because ER trafficking has not been described for HPV16, we wanted to use two markers to identify the ER: the luminal chaperone ERp29 (Fig. 3A to D, green) and the ER membrane-bound chaperone calnexin (Fig. 3E to H, green) (4, 54). The images shown are primarily from the center section of the cell, but slight variations from

this result in the visualization of the ER somewhat overlaying the nucleus, i.e., from slightly above the center plane. We show with these same ER markers, ERp29 and calnexin, that the ER is cytoplasmic (for example, see Fig. 3A1 and H1). HPV16 reporter virions were allowed to internalize into HaCaT cells and fixed at various time points postentry. At 30 min after HPV16 internalization, colocalization between HPV16 re-

porter virions (stained with anti-L1 antibody H16.V5) (Fig. 3, red) and the ER marker ERp29 or calnexin was not observed (Fig. 3A4 and E4, respectively, red and green arrows). By 4 h after viral entry, we saw that HPV16 began to colocalize with ERp29 (Fig. 3B4, overlap in yellow) and calnexin (Fig. 3F4, overlap in yellow). This signal overlap increased in the perinuclear region at 6 h postentry (Fig. 3C4 and G4, overlap in yellow) and was still present after 12 h (Fig. 3D4 and H4, overlap in yellow). The pattern of the observed overlap between HPV16 reporter virions and two ER markers suggests that the virus is reaching the ER at between 4 and 6 h.

Quantification of colocalization between HPV16 and EEA1, HPV16 and caveolin-1, and HPV16 and ERp29. To evaluate the percentage of colocalization observed between HPV16 reporter virions and markers for the early endosome (Fig. 1A to C), caveolar vesicles (Fig. 1D to F), and the ER (Fig. 3), confocal images were analyzed quantitatively (Fig. 4). At 5 min after virus entry, 13.1% of HPV16 virions colocalized with the early endosome marker EEA1, and 2.3% of virions colocalized with caveolin-1 (Fig. 4A and B, respectively). At 20 min postentry, 58.3% of HPV16 virions overlapped with EEA1, and 32% of the virions overlapped with caveolin-1 (Fig. 4A and B, respectively). At 2 h after entering HaCaT cells, less of the HPV16 virions colocalized with EEA1 (28.5%) and a greater percentage of the virions colocalized with caveolin-1 (48.1%) (Fig. 4A and B, respectively). The colocalization analysis of HPV16 and EEA1, and HPV16 and caveolin-1, supports a model whereby HPV16 virions first traffic to the endosome and then move to the caveolar pathway.

The colocalization of HPV16 and the ER marker ERp29 was quantified at 30 min, 4 h, 6 h, and 12 h after the entry of HPV16 reporter virions into HaCaT cells (Fig. 4C). The amounts of colocalization between HPV16 and calnexin at these time points were similar (data not shown). At 30 min postentry, 12.6% of virions overlapped with ERp29. At 4 h postentry, the relative amount of HPV16 virions colocalized with ERp29 increased approximately threefold (35.6%) (Fig. 4C). By 6 h after virion internalization, the percentage of HPV16 overlapping with ERp29 increased to 67.9%. The percentage of colocalization between HPV16 and ERp29 at 12 h was similar to that observed at 6 h (70.7%) (Fig. 4C). The levels of HPV16 and ERp29 colocalization at the time points analyzed suggest that HPV16 virions are accumulating in the ER with time.

HPV16 intracellular trafficking involves a BFA-sensitive step. In order to address how HPV16 reporter virions might be trafficking to the ER, we turned to the literature of related viruses: the movement of JCV, BKV, and simian virus 40 (SV40) to the ER is BFA sensitive (29, 39, 50). BFA prevents the formation of COPI vesicles, which have been implicated in the movement of cargo to the ER (5, 69). We first determined that BFA concentrations of 35 ng/ml or greater were cytotoxic to HaCaT cells ($P < 0.005$) (Fig. 5A).

We then incubated HaCaT cells with BFA at the nontoxic dose of 25 ng/ml and addressed whether BFA could interfere with HPV16 infection (Fig. 5B). The addition of BFA to HaCaT cells prior to HPV16 internalization resulted in a 45% decrease in the rate of HPV16 infection compared to infection of untreated cells ($P < 0.03$ for untreated versus 0 h) (Fig. 5B). A higher dose of BFA was able to block more than

FIG. 4. Quantification of overlap between HPV16 reporter virions and intracellular markers. Immunofluorescence images were analyzed to quantify the colocalization of HPV16 and intracellular markers. (A) Percentage of HPV16 virions that colocalized with the early endosome marker EEA1 at 5 min, 20 min, and 2 h. (B) Percentage of HPV16 virions that colocalized with caveolin-1, a marker for vesicles of the caveolar pathway, at 5 min, 20 min, and 2 h. (C) The colocalization of HPV16 reporter virions and the ER marker ERp29 was quantified. The percent overlap at 30 min, 4 h, 6 h, and 12 h shows a time-dependent increase in overlap between reporter virions and the ER. Analysis was performed with a minimum of three separate images per time point. Error bars represent the standard deviations from the means.

80% of HPV16 infection; however, a 16% increase in cytotoxicity was observed. While the increase in cytotoxicity probably does not account for the greater inhibition of HPV16 infection, we nonetheless used BFA at the less toxic dose. The addition of BFA even up to 4 h after HPV16 internalization had begun (initiated by shifting cells to 37°C) reduced infection by nearly 50% (Fig. 5B) (27% in untreated cells versus 14.1% with addition at 4 h for total infection). The drops in infection rates were statistically significant when BFA was added within the first 12 h postentry ($P < 0.04$). Statistical significance was determined using two-tailed unpaired *t* tests, comparing HPV16 reporter virion infection in the presence of BFA to infection in the untreated control cells ($\alpha = 0.05$). These data provide evidence that HPV16 reporter virion infection is BFA

FIG. 5. BFA treatment inhibits HPV16 infection in a time-dependent manner. (A) BFA was added to HaCaT cells at increasing concentrations. Forty-eight hours later, cell viability was assessed as a function of the level of ATP. Doses of BFA of 35 and 50 ng/ml were found to be toxic to HaCaT cells (*, $P < 0.005$). A total of 25 ng/ml of BFA (red arrow) was used in time course and immunofluorescence studies. Error bars show the standard deviations from the means of data from a minimum of three experiments. (B) BFA was added to HaCaT cells at various time points in relation to HPV16 internalization. HPV16 infection was reduced to a statistically significant level in cells treated with BFA until the addition of BFA 24 h after HPV16 entry (*, $P < 0.04$). Error bars show the standard deviations of data from three experiments in which 10,000 cells were analyzed by flow cytometry for reporter gene expression to obtain the percentage of infected cells.

sensitive, thus implicating COPI vesicles in the transport of HPV16 to the ER.

BFA treatment results in a loss of HPV16 colocalization with the ER marker calnexin. To determine if BFA treatment decreased the rate of HPV16 infection by blocking ER trafficking, we wanted to visualize viral trafficking to the ER in the presence of BFA. We showed that BFA treatment of HaCaT cells did not prevent the entry of the clathrin-dependent ligand transferrin or its transport to the EEA1-positive early endosome (see Fig. S2A and S2B, yellow arrows, in the supplemental material). This indicated that the loss of HPV16 infection in the presence of BFA was not due to an inhibition of clathrin-mediated endocytosis or trafficking to the early endosome.

Cholera toxin B moves via COPI-dependent trafficking from the Golgi apparatus to the ER (34, 42, 51). We confirmed that BFA was effectively shutting down COPI-mediated transport by analyzing the movement of cholera toxin B (Fig. 6A and B, red) in BFA-treated HaCaT cells. Cholera toxin B colocalized with the ER marker calnexin by 4 h postentry in the absence of BFA (Fig. 6A, overlap in yellow), but the overlap was not observed in the presence of BFA (Fig. 6B, red and green arrows).

After confirming that BFA was blocking COPI-mediated transport, we wanted to address if HPV16 reporter virions could traffic to the ER in the presence of BFA (Fig. 6D). HPV16 was detected using the capsid conformation-dependent H16.V5 antibody (Fig. 6C and D, red), and the ER was identified using a calnexin antibody (Fig. 6C and D, green). In untreated cells, HPV16 particles colocalized with calnexin in

the perinuclear region at 6 h postentry (Fig. 6C, yellow arrow). BFA treatment resulted in a near-complete loss of the overlap of HPV16 and calnexin 6 h after virion internalization (Fig. 6D, red and green arrows). Quantification of the overlap between HPV16 virions and calnexin at 6 h postentry in the absence and in the presence of BFA was performed (Fig. 6E). In untreated HaCaT cells, 67.7% of HPV16 virions colocalized with the ER marker calnexin (Fig. 6E). BFA treatment of HaCaT cells reduced the colocalization of HPV16 and calnexin to 16.6% overlap (Fig. 6E). These results demonstrate that BFA treatment significantly decreases HPV16 transport to the ER ($P < 0.001$).

Cholera toxin entry by caveola-mediated endocytosis is required for its toxicity (42). After entry, cholera toxin B is trafficked to endosomes and then the Golgi apparatus before its COPI-mediated retrograde transport to the ER (34). The movement of cholera toxin B to the Golgi apparatus is independent of COPI vesicle trafficking (42, 53); correspondingly, we observed an overlap between cholera toxin B and the Golgi apparatus in the absence and in the presence of BFA (see Fig. S2C and S2D, respectively, overlap in yellow, in the supplemental material). We did not observe a colocalization of HPV16 and the Golgi apparatus marker giantin from 30 min to 24 h (data not shown). This suggested that HPV16 was reaching the ER directly from caveosomes, bypassing the Golgi apparatus. This COPI-dependent pathway to the ER is also utilized by JCV and SV40 (39, 40, 50, 51). We propose that BFA treatment is reducing HPV16 infection due to a loss of

FIG. 6. BFA blocks cholera toxin B and HPV16 trafficking to the ER. (A and B) Cholera toxin B (CTB) (red) overlap with the ER marker calnexin (green) at 4 h was assessed. (A) Colocalization was seen between cholera toxin B and calnexin in untreated HaCaT cells (yellow arrow). (B) The amount of cholera toxin B trafficking to the ER was greatly reduced in the presence of BFA (red and green arrows). (C and D) HPV16 was internalized for 6 h, and cells were stained with anti-L1 antibody H16.V5 (red) and anti-calnexin (green). (C) Untreated HaCaT cells showed colocalization between HPV16 and calnexin and prominent perinuclear accumulation (yellow arrow). (D) BFA treatment resulted in a loss of this overlap (red and green arrows). Topro-3 (blue) was used for the detection of the nucleus. z stack pictures are shown, depicting the image in the x, y, and z planes. (E) The percentage of HPV16 reporter virions overlapping with calnexin in untreated (none) or BFA-treated (25 ng/ml) HaCaT cells was quantified. The reduction in the overlap between HPV16 and calnexin in BFA-treated cells was statistically significant compared to the overlap in untreated cells (*, $P < 0.001$). At least three separate images were quantified, and the error bars represent the standard deviations from means.

viral trafficking to the ER, presumably from caveosomes by COPI vesicles.

Blocking of COPI-mediated transport results in an accumulation of HPV16 in caveolin-1-positive vesicles. Having observed that HPV16 trafficking to the ER can be blocked with BFA, we wanted to use immunofluorescence to determine if the inhibitor was leading to a retention of HPV16 in caveolar vesicles (Fig. 7). HPV16 reporter virions were allowed to internalize into HaCaT cells in the absence (Fig. 7A to C) or in the presence (Fig. 7D to F) of 25 ng/ml BFA. At 4 h postentry, infected cells were fixed and stained for HPV16 (Fig. 7, red)

and caveolin-1 (Fig. 7, green). In the absence of BFA, an overlap of HPV16 and caveolin-1 was observed to various extents, suggesting that the virions were beginning to move out of caveolin-1-positive vesicles (Fig. 7A to C). In contrast, BFA treatment of HaCaT cells resulted in a striking overlap between HPV16 reporter virions and caveolin-1 at 4 h (Fig. 7D to F, overlap in yellow). Quantification of the immunofluorescence data revealed that the increase in the overlap between HPV16 and caveolin-1 as a result of BFA treatment was statistically significant (55.4% overlap for 25 ng/ml BFA versus 16.2% overlap for none; $P < 0.002$) (Fig. 7G). We conclude

FIG. 7. Postcaveolar vesicle trafficking of HPV16 is BFA sensitive. HPV16 reporter virions internalized into HaCaT cells for 4 h in the absence (A to C) or in the presence (D to F) of BFA were detected with H16.V5 antibody (red). Anti-caveolin-1 antibody was used to identify caveolar vesicles (green). (A to C) In untreated cells, HPV16 overlap with caveolin-1 was minimal. (D to F) Compared to untreated cells, BFA treatment of HaCaT cells led to increased colocalization between HPV16 and caveolin-1 (yellow). Images represent z stacks showing the cells in all three planes. (G) Overlap of HPV16 reporter virions and caveolin-1 in BFA-treated (25 ng/ml) and untreated (none) HaCaT cells was quantified. The increased colocalization of HPV16 and caveolin-1 in BFA-treated cells compared to untreated cells was statistically significant (*, $P < 0.002$). Error bars represent the standard deviations from the means after analysis of at least three separate images.

that BFA treatment leads to a backup of HPV16 in caveolar vesicles, supporting our hypothesis that the transport of HPV16 from caveosomes to the ER is COPI mediated.

DISCUSSION

Receptor-mediated, clathrin-dependent endocytosis is a common pathway by which nonenveloped viruses enter and traffic through cells. Exceptions to this include SV40, BKV, and HPV31, which enter cells by the caveolar pathway, although caveola-independent entry of SV40 and clathrin-mediated entry of HPV31 were also previously demonstrated (3, 17, 22, 27, 62). Classical clathrin-mediated endocytosis is typically followed by trafficking through the endolysosomal pathway; however, there is evidence that some viruses deviate from this pathway (45, 60, 61). Viruses with divergent trafficking include BPV1 and JCV, which enter cells by clathrin-mediated endocytosis and are then routed to the caveolar pathway (32, 50). Alternatively, HPV31 and BKV were previously shown to be sorted to endosomal vesicles after entering host cells via caveolae (29, 63). We became interested in the intracellular movement of HPV16 based on observations by our laboratory and

others suggesting that HPV16 trafficking may be similar to that of BPV1. Analogous to BPV1, the initial internalization of HPV16 into COS-7, 293TT, and HaCaT cells has been blocked by chlorpromazine, a chemical inhibitor of clathrin-mediated endocytosis (9, 27, 62). Preventing caveola-mediated internalization in these same cell lines with filipin and nystatin did not inhibit HPV16 infection (9, 27, 62). When BPV1 and HPV16 VLPs were coinfecting C127 cells and fixed at 4 and 8 h, extensive overlap was observed (20).

The goal of our study was to understand the infectious pathway that HPV16 travels in its natural host cell, the human HaCaT keratinocyte. We confirmed previous reports that HPV16 internalization into HaCaT cells is clathrin dependent and does not involve caveola-mediated entry (62). Specifically, we used the caveolar entry inhibitor filipin, which binds to cholesterol in the plasma membrane (10, 41, 49, 66). Filipin inhibits the initial entry of caveolae but does not seem to affect the downstream vesicles of the caveolar pathway. Indeed, JCV infection was unaffected by the treatment of cells with nystatin, an entry inhibitor that, like filipin, sequesters cholesterol from lipid rafts at the plasma membrane (48). Despite the lack of an effect of nystatin treatment on JCV infection, JCV utilizes the

caveolar pathway postentry (50). Immunofluorescence studies revealed that HPV16 reporter virions colocalized with both the early endosome marker EEA1 and the caveolar pathway marker caveolin-1 in the first 2 h after viral entry. Quantification of the colocalization in these immunofluorescence studies demonstrated that the overlap between HPV16 and EEA1 is greater at 20 min than the overlap between HPV16 and caveolin-1. In contrast, at 2 h postentry, a larger proportion of virions colocalized with caveolin-1 than with EEA1. This pattern of colocalization suggested that after clathrin-mediated endocytosis, HPV16 virions were trafficking to early endosomes before being transported to the caveolar pathway.

To explore the importance of the colocalization between caveolin-1 and HPV16, we used shRNA against caveolin-1 to examine the effect of caveolin-1 protein knockdown on HPV16 infection. We previously showed that the knockdown of caveolin-1 protein expression interfered with cholera toxin B binding and/or entry (32). After confirming the knockdown of caveolin-1 in HaCaT cells, we demonstrated that HPV16 infection was drastically reduced in HaCaT cells expressing caveolin-1 shRNA. These data led us to theorize that, similarly to BPV1, HPV16 entered HaCaT cells via clathrin-mediated endocytosis and was sorted to the caveolar pathway and then onto the ER.

We previously described that after colocalization with caveolin-1, BPV1 overlapped with the ER marker calnexin and ER-resident syntaxin 18 (32, 33). Because an involvement of the ER in HPV16 infection is a novel concept, we analyzed HPV16 trafficking in HaCaT cells in relation to two ER markers, ERp29 and calnexin. ERp29, a resident of the ER lumen, and the integral membrane protein calnexin function as protein chaperones and are exclusive to the ER (2, 18, 28, 59). We observed that HPV16 reporter virions overlapped with both of these ER markers by 4 h postentry. After 4 h, HPV16 continued to accumulate in the perinuclear region, showing increasing overlap with both markers. By 12 h after entry, the majority of HPV16 reporter virions colocalized with the ER markers ERp29 and calnexin.

To examine if the postcaveolar trafficking of HPV16 to the ER was biologically relevant, we used the inhibitor BFA, which prevents the recruitment of the COPI coat onto vesicles responsible for moving cargo toward the ER (5, 47). BFA has been shown to block the COPI-mediated movement of SV40 from caveosomes to the ER as well as the transport of cholera toxin B from the Golgi apparatus to the ER (39, 51). We observed a decrease in rates of HPV16 reporter virion infection in the presence of BFA even when the inhibitor was added up to 12 h postentry, suggesting a role for COPI vesicles in the intracellular trafficking of infectious HPV16. Using immunofluorescence, we showed that BFA was decreasing HPV16 infection by preventing the trafficking of the virus to the ER without interfering with clathrin-mediated endocytosis or the movement of viral particles to caveolin-1-positive vesicles. In fact, the addition of BFA to infected HaCaT cells resulted in an increased overlap of the virions with caveolin-1, a finding which we surmise is due to the retention of viral particles in caveosomes. While this did not tell us whether HPV16 was moving from caveolin-1-positive vesicles to the Golgi apparatus before reaching the ER, we failed to observe an overlap of HPV16 with the Golgi marker giantin in an analysis from 30 min to 24 h (data not shown). Although we cannot rule out that

HPV16 may transiently pass through the Golgi apparatus, our immunofluorescence studies indicate that HPV16 is trafficking from caveolin-1-positive vesicles to the ER. Viral trafficking from caveosomes directly to the ER in a BFA-sensitive manner was previously shown for other naked dsDNA viruses, namely, SV40, JCV, and BKV (29, 39, 50, 51). Our observations strongly suggested that the loss of HPV16 infection seen in the presence of BFA was due to a failure of the virus to traffic to the ER.

Data in this paper may seem to be inconsistent with the proposed endosomal uncoating/escape of PVs after a furin cleavage event and the requirement for lysosomal acidification during PV infection (20, 30, 52, 57, 63). Although we do not dismiss the role of furin or lysosome acidification in infection, we propose that our data are in line with these findings based on the following: (i) we have evidence that the pretreatment of mature particles with a cysteine protease can overcome the block in PV infection in the presence of a furin inhibitor (data not shown); (ii) lysosomotropic agents have been shown to block vesicle fusion (13), and in particular, caveola-to-endosome fusion was blocked using NH_4Cl and led to a loss of JCV infection (50); and (iii) our trafficking studies were performed using an L1 antibody that detects a conformation-dependent epitope in the HPV16 capsid. Our laboratory has observed an overlap between HPV16 and the late endosome/early lysosome using the LAMP-1 marker. We have also tested HPV16 infection after raising the cellular pH with NH_4Cl . We have found that NH_4Cl treatment reduces HPV16 infection (data not shown). Transport between endosomes and caveolin-1-positive vesicles has been shown to be pH dependent; therefore, we believe that NH_4Cl treatment is preventing the trafficking of HPV16 to caveosomes. Thus, the colocalization of HPV16 and LAMP-1 may represent virions being transported by a noninfectious route, i.e., to the lysosome for degradation.

Recently, endocytosis of HPV16 pseudovirions (reporter virions) into HeLa and 293TT cells in a clathrin-, caveolin-, and dynamin-independent manner was described (64). Spoden and colleagues recently showed that HPV16 utilizes tetraspanin-enriched microdomains on the plasma membrane for internalization by a poorly defined pathway (64). While those data contradict work from our laboratory and other laboratories, their use of HeLa and 293TT cells may account for these differences since there is increasing evidence that viral trafficking is cell type specific, relying on complex signals between the virus and the host cell (36, 45). We did test the effect of caveolin-1 knockdown on HPV16 infection in HeLa cells, and after decreasing caveolin-1 protein expression levels in HeLa cells, we again observed a loss of infection compared to control cells with normal caveolin-1 levels. We do not rule out the possibility that tetraspanin-enriched microdomains may play a role in HPV16 infection in HeLa and 293TT cells; however, our studies indicate that caveolin-1 is important for HPV16 infection in multiple cell lines, including HeLa cells.

We conclude that HPV16 enters the target HaCaT keratinocyte cell by clathrin-mediated endocytosis and subsequently traffics to a caveolar vesicle. The transport of HPV16 from the early endosome to the caveosome is an example of the regular cross talk between trafficking pathways within cells. The bidirectional movement between endosomes and caveosomes was previously shown to be mediated by the small GTPase Rab5

(43, 44). We have evidence that Rab5 may be mediating the transport of HPV16 from the endosome to the caveosome (our unpublished observations). Postcaveosome, HPV16 appears to reach the ER in a process that is at least in part COPI mediated. The role of the ER in HPV16 infection is currently unclear. Like the related polyomaviruses SV40 and BKV, it is possible that the ER is serving as the site of viral disassembly (29, 39). A recent report indicated that inhibiting the enzymatic activity of protein disulfide isomerase, a protein found primarily within the ER, interferes with HPV16 infection (12). The possible involvement of protein disulfide isomerase in HPV16 capsid disassembly provides an exciting avenue for future work.

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