RESEARCH ARTICLE

Vesicular Trafficking of Incoming Human Papillomavirus 16 to the Golgi Apparatus and Endoplasmic Reticulum Requires γ-Secretase Activity

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ABSTRACT **The route taken by papillomaviruses from the cell surface to the nucleus during infection is incompletely understood. Here, we developed a novel human papillomavirus 16 (HPV16) pseudovirus in which the carboxy terminus of the minor capsid protein L2 is exposed on the exterior of the intact capsid prior to cell binding. With this pseudovirus, we used the proximity ligation assay immune detection technique to demonstrate that during entry HPV16 L2 traffics into and out of the early endosome prior to Golgi localization, and we demonstrated that L2 enters the endoplasmic reticulum during entry. The cellular membrane-associated protease, γ-secretase, is required for infection by HPV16 pseudovirus and authentic HPV16. We also showed that inhibition of γ-secretase does not interfere substantively with virus internalization, initiation of capsid disassembly, entry into the early endosome, or exit from this compartment, but γ-secretase is required for localization of L2 and viral DNA to the Golgi apparatus and the endoplasmic reticulum. These results show that incoming HPV16 traffics sequentially from the cell surface to the endosome and then to the Golgi apparatus and the endoplasmic reticulum prior to nuclear entry.**

IMPORTANCE **The human papillomaviruses are small nonenveloped DNA viruses responsible for approximately 5% of all human cancer deaths, but little is known about the process by which these viruses transit from the cell surface to the nucleus. Here we show that incoming HPV16, the most common high-risk HPV, traffics though a series of vesicular compartments during infectious entry, including the endosome, Golgi apparatus, and endoplasmic reticulum. Furthermore, we show that γ-secretase, a cellular membrane-associated protease, is required for entry of the L2 minor capsid protein and viral DNA into the Golgi apparatus and endoplasmic reticulum. These studies reveal a new pathway of cell entry by DNA viruses and suggest that components of this pathway are candidate antiviral targets.**

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Papillomaviruses are important pathogens. The high-risk human papillomaviruses (HPVs), such as HPV16, play an etiologic role in essentially all cervical cancers and in a substantial fraction of other anogenital and oropharyngeal cancers (1, 2). Despite the importance of these viruses, relatively little is known about the crucial process of HPV entry.

Papillomaviruses are nonenveloped DNA viruses. HPV capsids are composed of 360 molecules of the major capsid protein L1 and up to 72 molecules of the minor capsid protein L2. Studies of HPV entry have been facilitated by the use of pseudoviruses (PsVs) comprised of L1 plus L2 encapsidating a reporter plasmid (3, 4). These PsVs, which can be readily prepared in cultured cells, mimic the behavior of authentic virus and allow infection efficiency to be easily monitored by reporter gene expression. The use of PsVs also allows the addition of epitope tags to the viral proteins to visualize the virus during entry. After L1-mediated binding of the capsid to heparan sulfate proteoglycans at the cell surface, the capsid proteins undergo conformational changes and L2 is cleaved by the cellular protease furin (5–14). HPV is then transferred to an internalization receptor and endocytosed (6, 7, 15–19). After endosome entry, capsid disassembly is initiated by acidification of the endosomal lumen (16, 19–21). HPV then travels to the nucleus, where virus replication occurs (22). Mitotic progression and nuclear envelope breakdown appear important for nuclear entry (23, 24). During virus entry, the L1 protein largely dissociates from the viral DNA, a step that requires cyclophilin B action, but the L2 protein remains associated with the viral genome and colocalizes with viral DNA at nuclear promyelocytic leukemia protein (PML) bodies (25, 26).

We conducted a genome-wide small interfering RNA (siRNA) screen for cellular genes that affect the efficiency of infection by HPV16 PsV. The screen showed that retrograde trafficking is required for HPV entry (27). Further studies showed that HPV16 L1 and L2 and viral DNA travel to the Golgi apparatus in both HeLa

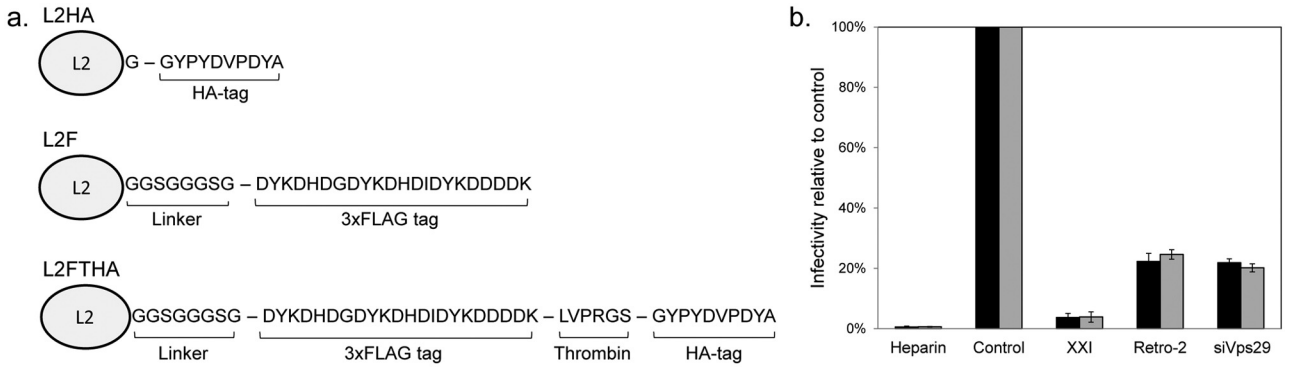

FIG 1 Recombinant HPV16 pseudoviruses with an L2-FLAG tag. (a) Schematic diagrams of the C terminus of the modified HPV16 L2 proteins used in this study. See text for details. (b) FLAG tag does not affect entry requirements. HeLa cells were transfected with control siRNA or siRNA targeting retromer subunit Vps29 or were pretreated with 250 nM γ-secretase inhibitor XXI or 100 nM Retro-2 for 1 h at 37° C. Alternatively, PsV was treated for 1 h with 100 μg/ml heparin prior to infection. Cells were infected with HPV16.L2F (black bars) or HPV16.L2HA (gray bars) expressing HcRed at a multiplicity of infection (MOI) of 0.5. Forty-eight hours postinfection, flow cytometry was used to determine the fraction of fluorescent cells. The results were normalized to the fraction of HcRed-positive cells in infected cells transfected with the control siRNA.

and HaCaT cells (23, 27–29) and that trafficking of HPV to the Golgi requires the retromer, a protein complex that initiates vesicular transport of cellular cargo from the endosome to the Golgi apparatus (27, 30, 31). The path taken by HPV from the endosome to the Golgi apparatus and from the Golgi apparatus to the nucleus is undefined. Endoplasmic reticulum (ER) components were also enriched in two independent HPV entry siRNA screens (23, 27), and knockdown of specific ER proteins and chemical inhibition of ER function inhibit infection by HPV (32, 33). Laniosz et al. reported that HPV16 L1 colocalizes with ER markers during entry (33), but Day et al. failed to observe ER entry (28).

The minor capsid protein L2 is required for proper HPV trafficking (13, 29, 32, 34–38). The L2 protein is largely buried inside the L1 shell in the intact capsid (38), but the amino-terminal segment of the L2 protein is exposed soon after cell binding (10, 11, 18, 39). A hemagglutinin (HA) epitope tag added to the carboxy terminus of the L2 protein is accessible to anti-HA antibody only after capsid disassembly has begun (26).

The membrane-associated cellular protease, γ-secretase, is required for HPV infection. γ-Secretase inhibitors severely inhibit infection by HPV PsV (40, 41), and all four γ-secretase subunits were strong hits in our siRNA screen (27). In fact, knockdown of the γ-secretase subunit PSENEN caused the most severe entry defect of the >18,000 genes tested. Karanam et al. showed that γ-secretase inhibition prevents the delivery of viral DNA and L2 protein to the nucleus without affecting virus internalization or capsid disassembly, but the HPV entry step that requires γ-secretase was not defined (41).

Dissection of the γ-secretase-dependent HPV entry pathway will provide important new insights into the cellular machinery involved in HPV entry and may identify additional factors and cellular processes that are potential new antiviral targets. Here, we develop novel reagents to show that HPV L2 undergoes sequential vesicular trafficking from the endosome to the Golgi apparatus and ER. Importantly, we demonstrate that γ-secretase inhibition blocks infection after exit from the early endosome but prior to arrival in the Golgi apparatus.

RESULTS

Construction of an HPV16 pseudovirus with constitutively exposed L2. In cells infected with HPV16 PsV containing an HA tag at the carboxy terminus of the L2 protein (designated here HPV16.L2HA), exposure of the HA epitope is a measure of capsid disassembly during entry (26). Here, we replaced the HA tag with a 3× FLAG tag to increase the sensitivity of L2 detection. A flexible serine-glycine spacer sequence followed by a 22-amino-acid 3× FLAG tag was added to the carboxy terminus of HPV16 L2 to construct HPV16.L2F (Fig. 1a). HPV16.L2F PsV was produced by cotransfection of 293TT cells with a plasmid expressing wild-type L1 and FLAG-tagged L2, and a reporter plasmid encoding green fluorescent protein (GFP) or HcRed, followed by density gradient centrifugation. As assessed by encapsidation of the reporter plasmid and electron microscopy (data not shown), HPV16.L2F assembled properly, and it efficiently induced GFP or red fluorescence in infected cells. When normalized for encapsidated reporter plasmids, HPV16.L2F was approximately twice as infectious as HPV16.L2HA in HeLa and HaCaT cells (see Fig. S1 in the supplemental material) (data not shown). As predicted, staining with anti-FLAG at late times during entry gave a stronger signal than staining with anti-HA (see Fig. S2a in the supplemental material). Furthermore, FLAG staining was eliminated by incubation of HPV16.L2F with soluble heparin prior to infection (Fig. S2b in the supplemental material). Because heparin blocks L1-mediated attachment of the capsid to cells (8), this result confirms that FLAG staining was due to PsV-mediated delivery of the L2 protein to cells and was not due to residual L2 present in the PsV preparation.

To determine if FLAG tag alters the entry pathway(s) used by HPV16 PsV, we tested the effect of various manipulations that inhibit entry of PsV lacking the FLAG tag. As shown in Fig. 1b, infectivity of HPV16.L2F and HPV16.L2HA in HeLa cells was inhibited by ~80% by an siRNA targeting the retromer subunit Vps29 or by treatment with Retro-2, a chemical inhibitor of retrograde transport. Treatment with soluble heparin or a chemical inhibitor of γ-secretase, compound XXI, caused near complete inhibition of both PsVs. Similarly, both PsVs were inhibited by furin inhibitors and bafilomycin A, which blocks endosome acidification (data not shown). Thus, addition of the FLAG tag does not appear to change the requirements for HPV entry.

We next examined the time course of capsid disassembly. To reduce kinetic heterogeneity in this experiment, we conducted a

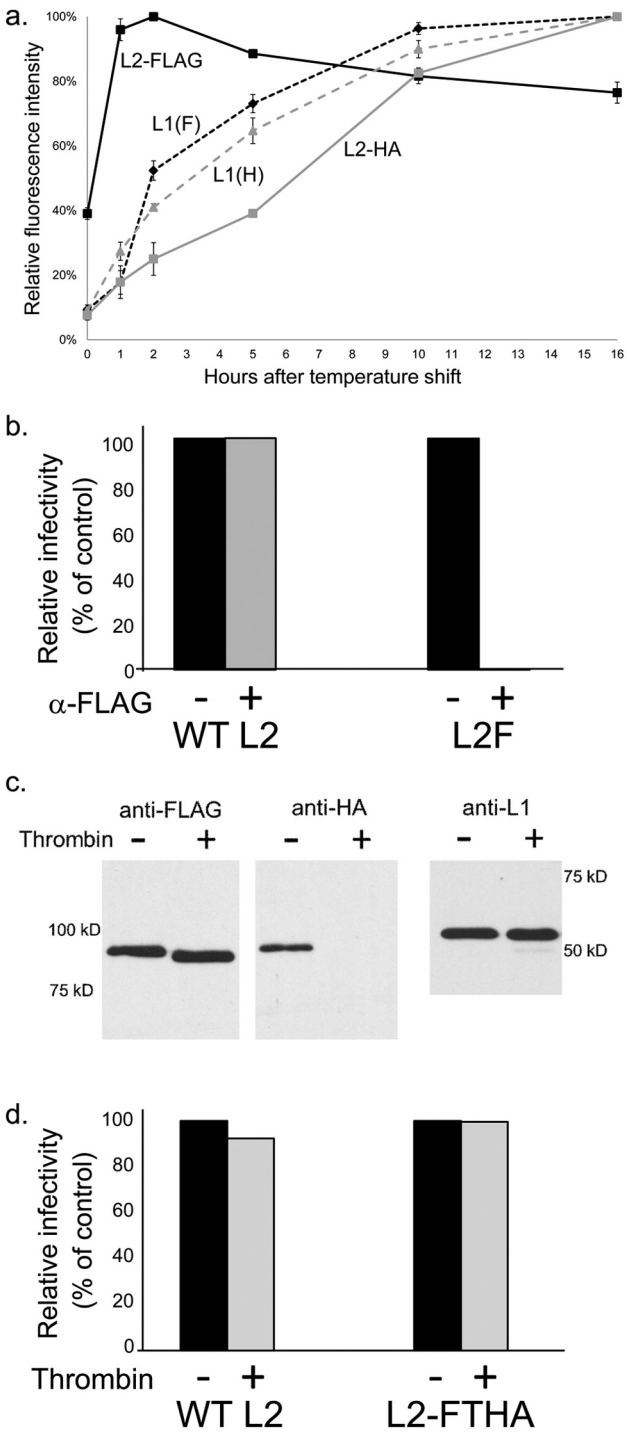

FIG 2 The FLAG tag at the C terminus of HPV16 L2 is constitutively exposed. (a) Time course of HPV16 capsid disassembly. HeLa cells were infected with HPV16.L2HA or HPV16.L2F at an MOI of 20 at 4°C. After being shifted to 37°C, the samples were fixed at various times and immunostained with 33L1-7 antibody to measure L1 exposure or with FLAG or HA antibody to measure L2 exposure in samples infected with HPV16.L2F or HPV16.L2HA, respectively. Mean fluorescent intensity (MFI) was measured by flow cytometry and is normalized to the maximum MFI for each treatment. Solid black line, anti-FLAG/L2F; dashed black line, anti-L1-7/L2F; solid gray line, anti-HA/L2HA; dashed gray line, anti-L1-7/L2HA. (b) Neutralization assay. The indicated PsVs were incubated with medium (black bars) or anti-FLAG antibody (dark gray bars) for 1 h and then used to infect HeLa cells at an MOI of 1. WT, wild

(Continued)

synchronized infection, in which cells were incubated with HPV16.L2HA or HPV16.L2F PsV at 4°C, a temperature at which virions bind cells but do not internalize, and then after removal of unbound virus, internalization was initiated by temperature shift to 37°C. At various times after the temperature shift, cells were fixed, stained with 33L1-7 to assess L1 exposure or with anti-HA or anti-FLAG to measure L2 exposure, and analyzed by flow cytometry. As shown in Fig. 2a, the L1 epitope was exposed with similar kinetics in cells infected with either PsV, with half-maximal exposure at 2 to 3 h after infection. As expected, exposure of the HA epitope occurred after the appearance of 33L1-7 reactivity, at approximately 6 h after infection. Surprisingly, there was substantial staining of the FLAG epitope at time zero, and staining was complete 1 to 2 h after infection. These results imply that the carboxy terminus of the L2 protein in HPV16.L2F is exposed in infected cells prior to capsid disassembly.

To determine if the L2 FLAG tag was constitutively exposed in intact capsids, we tested if HPV16.L2F was neutralized by anti-FLAG antibody. HPV16 PsV and HPV16.L2F were incubated with monoclonal anti-FLAG prior to addition to cells, and GFP fluorescence from the reporter plasmid was measured 48 h later. Although the anti-FLAG antibody had no effect on HPV16 PsV containing wild-type L2, it caused an approximately 100-fold inhibition of infection by HPV16.L2F, providing further evidence that the FLAG epitope was exposed on intact virions (Fig. 2b). As a biochemical test of constitutive exposure, we constructed a PsV in which a thrombin protease cleavage site followed by the HA epitope was appended to the carboxy terminus of HPV16.L2F to generate HPV16.L2FTHA (Fig. 1a). Incubation of purified HPV16.L2FTHA PsV with thrombin caused a slight increase in the electrophoretic mobility of the L2 fusion protein, consistent with removal of the HA tag, but no reduction in band intensity as detected by immunoblotting for FLAG (Fig. 2c). In striking contrast, reactivity with the HA antibody was totally abolished by thrombin treatment. Furthermore, thrombin cleavage did not inhibit the infectivity of HPV16.L2FTHA (Fig. 2d) or affect the L1 protein (Fig. 2c). Thus, thrombin cleaves the extreme carboxy terminus of the L2 fusion protein in intact capsids. These data show that the carboxy terminus of the FLAG-tagged L2 protein is constitutively exposed on the exterior of capsids. Because the HA tag was quantitatively removed from the L2FTHA fusion protein by thrombin cleavage, every L2 molecule in the capsid is exposed.

γ-Secretase function is required by multiple HPV types in multiple cell types. γ-Secretase inhibition or knockdown strongly inhibits HPV entry into HeLa cells. To determine whether γ-secretase was required for infection by FLAG-tagged PsV in additional cell types, we tested the effect of γ-secretase knockdown on infection of HPV16 E6/E7-immortalized human 2614 ectocervical keratinocytes and HaCaT cells, a spontaneously im-

Figure Legend Continued

type. Forty-eight hours postinfection, fluorescent cells were counted by flow cytometry. The results were normalized to the fraction of fluorescent cells in control cells infected with wild type. (c) Western blot analysis. HPV16.L2FTHA was digested with thrombin or incubated overnight with PBS. The samples were then subjected to SDS-PAGE and immunoblotted with the indicated antibody. Molecular mass markers are shown in kDa. (d) Thrombin cleavage assay. PsVs were incubated with PBS (black bars) or thrombin protease (gray bars) overnight and then used to infect HeLa cells at an MOI of 1. Infected cells were counted and normalized as in panel b.

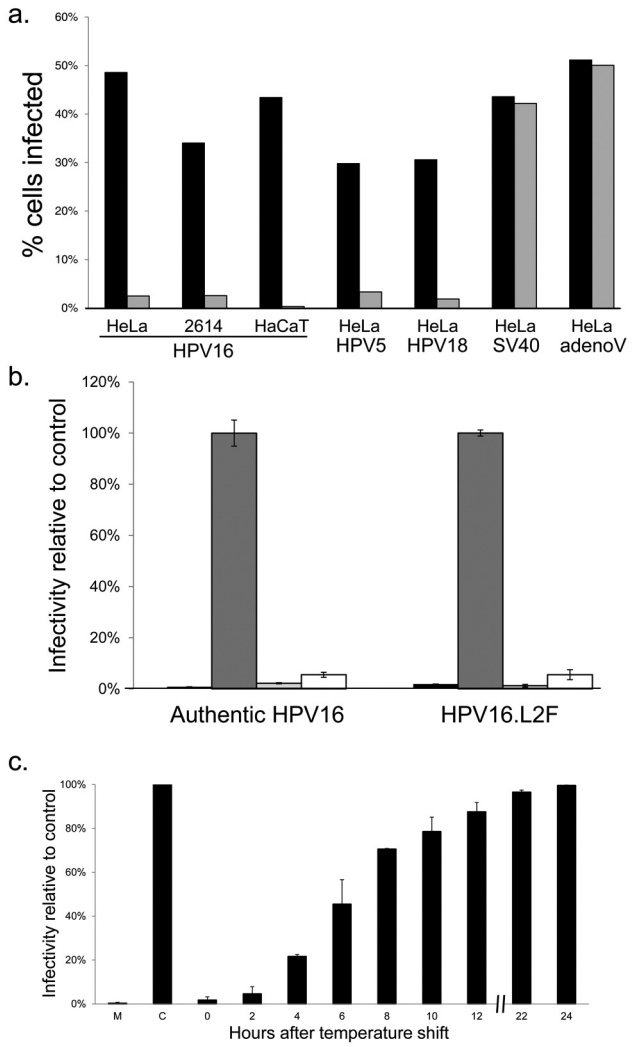

FIG 3 Characterization of γ-secretase sensitivity of HPV infection. (a) The indicated cell lines were transfected with control siRNA (black bars) or siRNA targeting the γ-secretase subunit, APH1A (gray bars). Forty-eight hours later, HeLa, HaCaT, or 2614 cells were infected with GFP-expressing HPV16.L2F, HPV5 or HPV18 PsV, or Ad5, as indicated, at an MOI of 0.5 (determined separately for each parental host cell type). SV40 was used to infect HeLa-Sen2 cells, and cells were immunostained for large T antigen. Flow cytometry was used to count fluorescent cells. (b) Authentic HPV16 requires γ-secretase activity. HeLa cells were transfected with control siRNA (dark gray bars) or siRNAs targeting APH1A (white bars) or were treated with 250 nM XXI (light gray bars). Forty-eight hours later, cells were mock infected (black bars) or infected with raft-derived HPV16 or HPV16.L2F PsV and assayed 2 days later by qRT-PCR for expression of viral early genes or GFP. mRNA levels relative to infected control cells are shown. (c) Time course of γ-secretase sensitivity. HeLa cells were infected at an MOI of 2 with HPV16.L2F at 4°C and treated with 250 nM XXI at the indicated time after temperature shift to 37°C or left untreated (control [C]). Forty-eight hours postinfection, flow cytometry was used to determine the fraction of GFP-positive cells. The results are normalized to control infected cells. M, mock infected.

mortalized, HPV-negative human skin keratinocyte cell line commonly used for HPV entry studies (42). Approximately 5- to 10-fold more HPV16.L2F or HPV16.L2HA is required to generate a similar number of infected HaCaT or 2614 cells compared to HeLa cells (data not shown). As shown in Fig. 3a, γ-secretase knockdown inhibited infection by HPV16.L2F by >90% in 2614 and HaCaT cells, as well as in HeLa cells. γ-Secretase knockdown also strongly inhibited infection by HPV5 and HPV18 PsV but had no effect on infection by two unrelated nonenveloped DNA viruses, simian virus 40 (SV40) and adenovirus type 5 (Ad5). These results are consistent with those from other laboratories, which showed that chemical inhibition of γ-secretase specifically inhibited infection by multiple HPV PsV types lacking the FLAG tag in various cells (40, 41).

To determine if γ-secretase was required for infection by authentic HPV16, we infected HeLa cells with HPV16 harvested from organotypic cultures of human keratinocytes or with HPV16.L2F produced in 293TT cells. Successful infection was assessed by quantitative reverse transcription-PCR (qRT-PCR) for expression of HPV early region mRNA or GFP mRNA from the HPV16 or the PsV genome, respectively. As shown in Fig. 3b, infection by both authentic HPV16 and HPV16.L2F PsV was inhibited by >90% by knockdown of the APH1A subunit of γ-secretase and by ~98% by compound XXI, demonstrating that γ-secretase is required for authentic HPV16 as well as HPV16 PsV.

The results presented above imply that γ-secretase is required for HPV infection in a variety of epithelial host cell types. We conducted most subsequent experiments in HeLa cells, because they are infected more efficiently than the other cell types. In addition, because HeLa cells were isolated from an HPV18-positive cervical carcinoma, they are derived from host cervical epithelial cells. To determine when in HPV infection γ-secretase activity is required, we conducted a synchronized infection with HPV16.L2F as described above. Compound XXI was added to the cultures at various times after temperature shift, and infectivity was assessed by GFP fluorescence 48 h postinfection. As shown in Fig. 3c, inhibition of infection by XXI was progressively attenuated by adding the inhibitor at later times after temperature shift, with half-maximal inhibition occurring when XXI was added approximately 6 h after infection. Thus, γ-secretase is required relatively early in the infectious process, at a similar time to exposure of the HA epitope. In contrast, Huang et al. reported that addition of the γ-secretase inhibitor GS1-X at 20 h postinfection caused ~50% inhibition of HPV16 PsV infectivity in HaCaT cells (40). Infection may proceed more slowly in HaCaT cells than HeLa cells, or GS1-X may act differently than XXI. Alternatively, the time course of entry may appear extended in the experiments of Huang et al. because a synchronized infection protocol was not used.

Internalization of HPV16 and initiation of capsid disassembly do not require γ-secretase. To identify the γ-secretase-sensitive step(s) during HPV16 entry, we used biochemical and imaging approaches. To examine internalization of HPV16.L2F, we incubated HeLa cells with PsV at 4°C, washed away unbound viruses, and then shifted the temperature to 37°C for 6 h to allow internalization to proceed. Cells were then treated with proteinase K to digest surface-bound virions, and internalized virus was detected by immunoblotting for L1 (Fig. 4a). In the absence of any additional treatment, a substantial proteinase K-resistant band of L1 was detected (lane 7). As expected, internalization was eliminated by pretreating the PsV with heparin (lane 5) or by incubating cells with PsV for 8 h at 4°C without temperature shift (data not shown). In contrast, internalization was not affected by pretreatment of the cells with compound XXI (lane 1) or APHA1 knockdown (lane 2), both of which eliminate γ-secretase activity; by inhibition or knockdown of furin (lanes 3 and 4); or by inhibi-

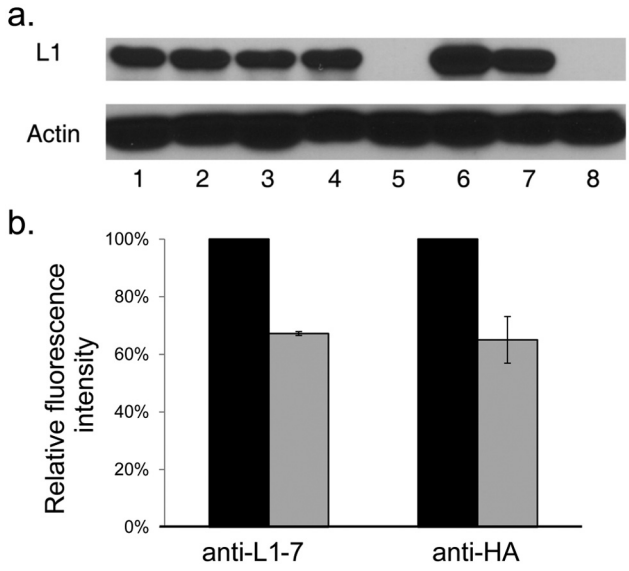

FIG 4 γ-Secretase function is not required for HPV16 internalization or capsid disassembly. (a) HeLa cells were incubated with HPV16.L2F at an MOI of 10 at 4°C for 2 h. After shifting to 37°C for 6 h, noninternalized virus was digested with proteinase K, and internalized virus was assessed by immunoblotting for L1 (top panel). Actin was used as a loading control (bottom panel). Prior to infection, cells were pretreated with inhibitor XXI (lane 1), APH1A siRNA (lane 2), furin inhibitor I (lane 3), furin siRNA (lane 4), balfilomycin A1 (lane 6), and control siRNA (lane 7). Lane 8 shows mock-infected cells. Lane 5 shows cells infected with PsV preincubated with heparin. (b) HeLa cells were transfected with control siRNA (black bars) or siRNA targeting APH1A (gray bars). Forty-eight hours later, cells were infected with HPV16.L2HA at an MOI of 20 at 4°C, shifted to 37°C, and harvested 16 h later. The cells were then immunostained with 33L1-7 or anti-HA antibody, as indicated. MFI was measured by flow cytometry and normalized to infected control cells.

tion of the vacuolar ATPase (lane 6). We also conducted immunofluorescence with anti-L1 polyclonal antibody in cells infected with HPV16.L2F. Uninfected cells were not stained, but infected cells showed punctate, intracellular staining at 16 h after infection, whether or not γ-secretase was inhibited (see Fig. S3a in the supplemental material). Thus, both experimental approaches demonstrated that internalization of HPV16 PsV does not require γ-secretase.

We used antibodies that detect conformational changes in L1 and exposure of the HA tag at the carboxy terminus of L2 to test whether γ-secretase was required for capsid disassembly. HeLa cells were synchronously infected with HPV16.L2HA, and 16 h after temperature shift, they were fixed and stained with the conformation-specific 33L1-7 antibody. The immunofluorescence images in Fig. S3b in the supplemental material show comparable perinuclear staining in the wild-type and APH1A knockdown cells, demonstrating that γ-secretase activity is not required for the conformational change in the major capsid protein. This conclusion was verified by flow cytometry for 33L1-7 staining at 10 h after infection (Fig. 4b), which demonstrated only a 35% reduction in staining by γ-secretase knockdown, even though knockdown essentially abolished infectivity, as assessed by GFP expression. Similarly, exposure of the carboxy terminus of L2, as assessed by flow cytometry for HA staining, was reduced by only 35% by γ-secretase knockdown. Thus, γ-secretase knockdown inhibits L1 and L2 exposure far less than it inhibits infectivity. In our earlier work, we reported that γ-secretase knockdown impaired capsid disassembly (27), but the more extensive studies reported here show that these steps are not the major targets of γ-secretase inhibition. Karanam et al. also reported that capsid internalization, 33L1-7 reactivity, and L2/HA exposure were not affected by γ-secretase inhibition (41).

Early endosome entry and exit do not require γ-secretase. To localize incoming HPV16 PsV in specific intracellular compartments, we employed the proximity ligation assay (PLA), an immune-based detection technique that generates a fluorescent signal only when two antigens of interest are within 40 nm of each other (43). To assess the localization of incoming HPV16 PsV, we analyzed HeLa cells by PLA after infection with HPV16.L2F. We tracked the endosomal location of L2 in these experiments by using one antibody that recognizes the FLAG epitope on the L2 protein and another antibody that recognizes EEA1, a marker of the early endosome. As shown in Fig. 5a, top panels, no signal was generated in uninfected cells. In infected cells, a clear punctate intracellular signal was generated 8 h after infection, which was indistinguishable in control cells and cells treated with the γ-secretase inhibitor. Thus, γ-secretase function is not required for entry of HPV PsV into the early endosome. By 16 h after infection, the EEA1/FLAG PLA signal was significantly diminished in the presence and absence of γ-secretase function. These results are quantitated in Fig. 5b. Similar results were obtained in HaCaT cells (see Fig. S4a in the supplemental material). The reduced signal at 16 h is not due to L2 degradation (see next section). Taken together, these results demonstrate that γ-secretase is not required for the entry of HPV16 into the early endosome or for its subsequent exit from this compartment.

Entry into the Golgi apparatus requires γ-secretase function. We next used PLA to assess the arrival of HPV16 in the Golgi apparatus. For this experiment, we incubated HPV16.L2F-infected cells with the anti-FLAG antibody and an antibody that recognizes TGN46, a marker of the *trans*-Golgi network and Golgi apparatus. As shown in Fig. 5a, bottom panels, no signal was generated in uninfected cells. Eight hours after infection, relatively little Golgi apparatus staining was evident in control cells or cells treated with compound XXI (Fig. 5a and b). However, by 16 h after infection, control cells displayed a clear TGN46/FLAG PLA signal in a perinuclear location characteristic of Golgi localization, coincident with the decrease in the endosomal signal. These results demonstrate that the virus traffics from the endosome to the Golgi apparatus between 8 and 16 h after infection. In striking contrast, there was little PLA signal in the Golgi apparatus at 16 h in cells treated with compound XXI (Fig. 5a and b). Similar results were obtained when a second Golgi marker, GM130, was used for PLA in conjunction with anti-FLAG (Fig. 5b; see Fig. S5 in the supplemental material), or if the 33L1-7 antibody instead of anti-FLAG was used for PLA with anti-TGN46 (see Fig. S6 in the supplemental material). γ-Secretase inhibition also blocked HPV entry into the Golgi apparatus in HaCaT cells (see Fig. S4b in the supplemental material). Compound XXI did not decrease L1 or L2 abundance, as shown by immunoblotting (Fig. 5c), demonstrating that the reduced PLA signal due to γ-secretase inhibition is not the result of degradation of L1 or L2. Taken together, these results demonstrate that in the presence of functional γ-secretase, incoming HPV PsV transits from the early endosome at early time points to the Golgi apparatus at later time points and that γ-secretase is required for Golgi localization.

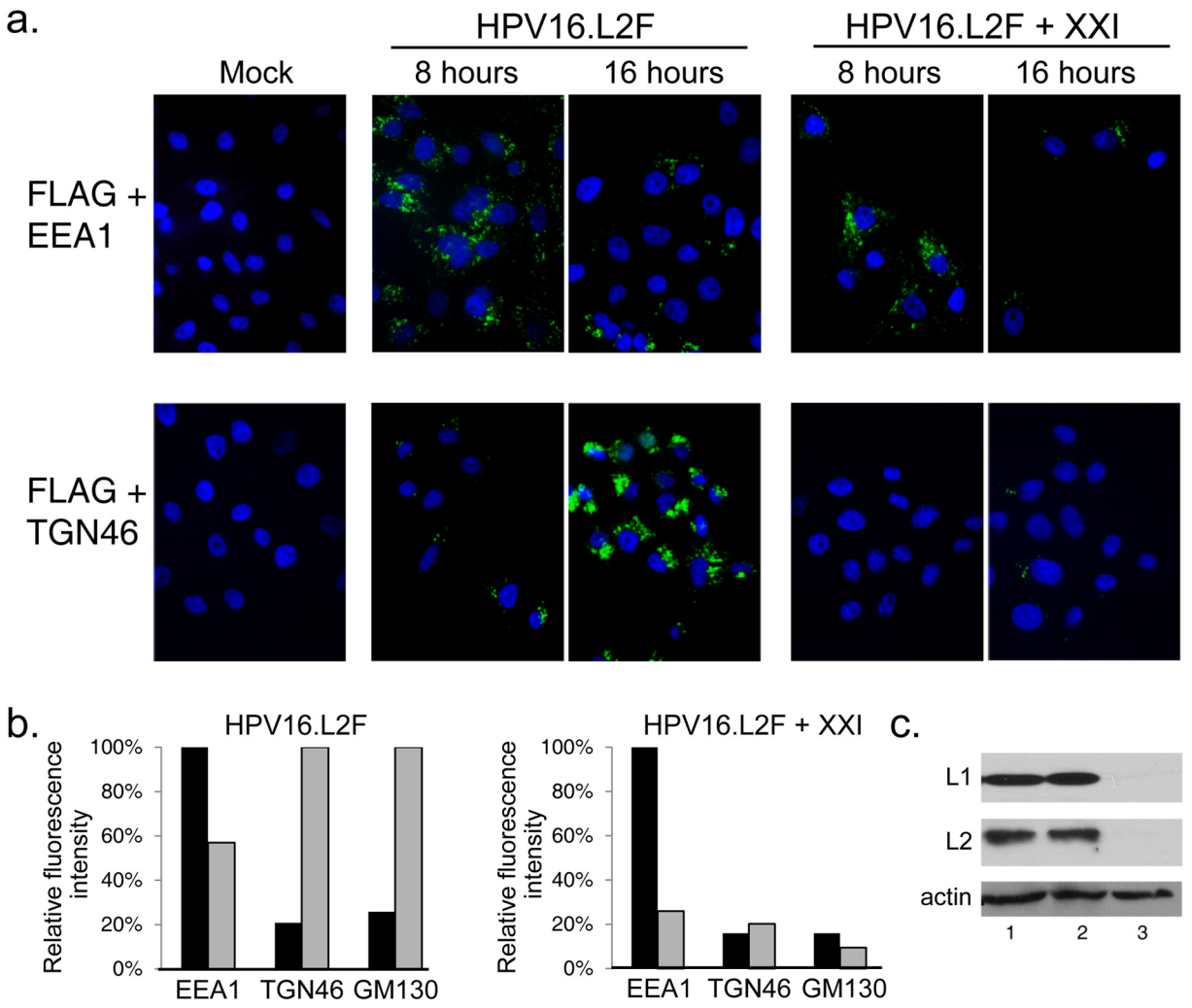

FIG 5 L2 entry into the Golgi apparatus, but not early endosome entry and exit, requires γ-secretase function. (a) HeLa-Sen2 cells were treated with 250 nM XXI or left untreated and then mock-infected (without XXI treatment) or infected with HPV16.L2F at an MOI of 100. At 8 and 16 h postinfection, the cells were incubated with anti-FLAG and an antibody recognizing EEA1 (early endosome marker) or TGN46 (Golgi marker). Cells were then processed for PLA, and the proximity of L2 with the indicated marker was visualized in green by fluorescence microscopy. Nuclei were stained with DAPI (blue). A single confocal slice is shown in each panel. (b) Images obtained as in panel a were processed by BlobFinder software to determine the PLA fluorescence intensity per cell in each sample. The average intensity for the EEA1/L2 samples was normalized to that of the control infected sample at 8 h postinfection, and the average intensities for TGN46/L2 or GM130/L2 (shown in Fig. S5 in the supplemental material) samples were normalized to that of the control infected sample at 16 h postinfection. Black bars, 8 h; gray bars, 16 h. The results show quantitation of a representative experiment. Similar results were obtained in two additional independent experiments. (c) HeLa-Sen2 cells were treated with 250 nM XXI or left untreated and then mock infected (without XXI treatment) or infected with HPV16.L2F PsV at an MOI of 10. Sixteen hours postinfection, cells were harvested, subjected to SDS-PAGE, and immunoblotted with anti-33L1-7 to visualize L1, anti-FLAG to visualize L2, and an actin antibody as a loading control. Lane 1, infected; lane 2, XXI treated and infected; lane 3, mock infected.

We next determined if γ-secretase is also required for entry of viral DNA into the Golgi apparatus. We infected control and XXI-treated HeLa cells with HPV16.L2F containing a luciferase reporter plasmid substituted with the nucleoside analogue EdU (25, 34). Eighteen hours postinfection, we detected reporter plasmids in infected cells by using the Click-iT chemical reaction to covalently link a fluorescent label to EdU, allowing *in situ* visualization of viral DNA. Cells were also stained with anti-TGN46, and colocalized viral DNA and TGN46 was pseudocolored white. As shown in Fig. 6, there was no EdU staining in mock-infected cells. Intracellular EdU staining was present in infected cells, whether or not they were treated with XXI, indicating that γ-secretase inhibition did not cause degradation of viral DNA. Colocalization of EdU and TGN46 was observed in the control infected cells, demonstrating passage of the incoming viral genome into the Golgi apparatus, as was reported by other laboratories (28, 29). Strikingly, Golgi localization was markedly inhibited by XXI treatment. BlobFinder image analysis showed an approximately 95% reduction in colocalization in response to XXI. These results demonstrate that γ-secretase is required for the entry of viral DNA as well as the L2 protein into the Golgi compartment.

Incoming HPV PsV traffics into the endoplasmic reticulum in a γ-secretase-dependent manner. As well as revealing that Golgi components are important for HPV entry, our genome-wide siRNA screen demonstrated enrichment of ER proteins, suggesting that HPV16 traffics through the ER during infectious en-

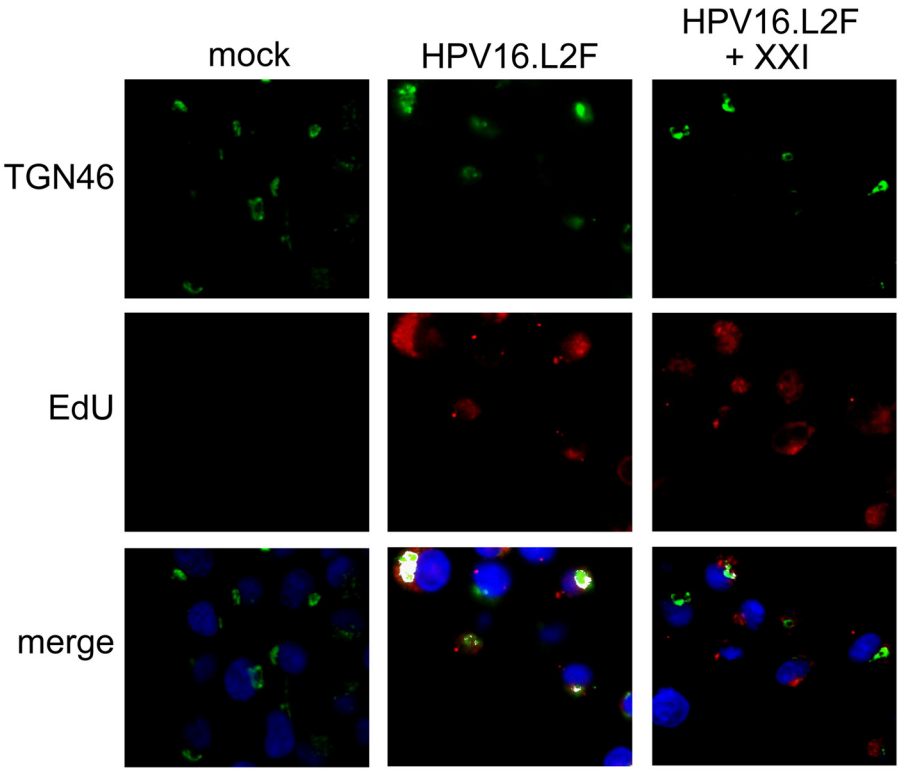

FIG 6 Entry of viral DNA into the Golgi apparatus requires γ-secretase. HeLa-Sen2 cells were treated with 250 nM XXI or left untreated for 1 h and then infected at an MOI of 10 with HPV16.L2F containing an EdU-substituted luciferase reporter plasmid. After 18 h, cells were incubated with the Click-iT reaction cocktail to label viral DNA (red). TGN46 was labeled green and nuclei blue. Cells were visualized by fluorescence microscopy, and the overlap between DNA and TGN46 signal was pseudocolored white. The same confocal slice is shown in each column. Similar results were obtained in two independent experiments.

try. In addition, there is a report that L1 colocalizes with ER markers during infection of HaCaT cells (33). We used PLA for FLAG and ER markers to determine if L2 reached the ER during entry. HeLa cells were infected with HPV16.L2F and subjected to PLA 20 h after infection with the anti-FLAG antibody and an antibody that recognizes either of two ER luminal markers, BiP and protein disulfide isomerase (PDI). As shown in Fig. 7a and b, there was relatively little staining in uninfected cells, but markedly increased ER staining was observed in infected cells 20 h after infection, when either ER marker was examined. ER staining was greatly reduced by γ-secretase inhibition. These results suggest that incoming HPV L2 is present in the ER at relatively late times

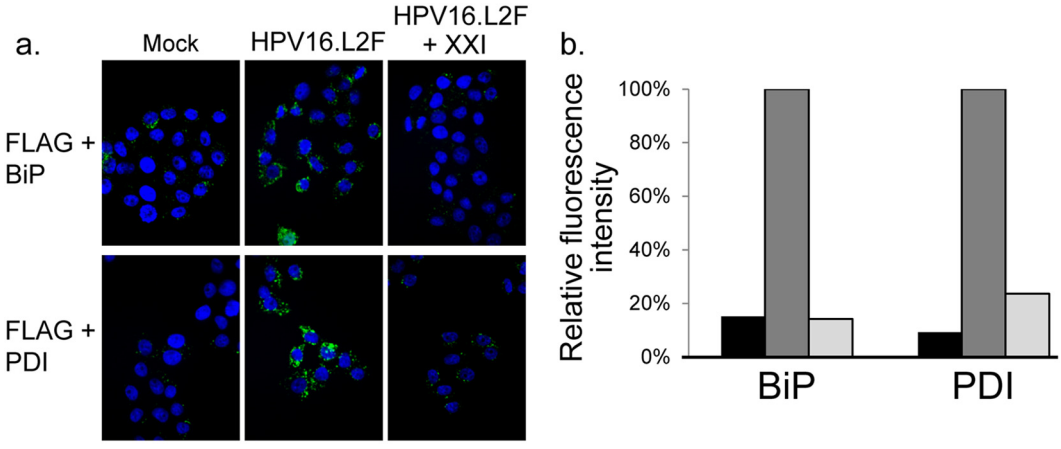

FIG 7 Incoming HPV PsV traffics into the endoplasmic reticulum in a γ-secretase-dependent fashion. (a) HeLa-Sen2 cells were treated and infected as in Fig. 5a. At 20 h postinfection, the cells were incubated with the anti-FLAG antibody and antibody recognizing BiP or PDI as indicated. Cells were then processed for PLA and visualized as in Fig. 5a. (b) Images obtained as in panel a were processed by BlobFinder software to determine the total fluorescence intensity per cell in each sample. The intensity for BiP/L2 or PDI/L2 was normalized to the control infected sample, respectively. Black bars, mock infected; dark gray bars, infected; and light gray bars, XXI treated and infected. The results show a representative experiment. Similar results were obtained in two additional independent experiments.

during the entry process and that γ-secretase is required for ER entry, presumably because it is required for prior delivery of the virus into the Golgi apparatus.

DISCUSSION

The experiments reported here demonstrate that the HPV16 L2 protein is sequentially transferred through a series of vesicular compartments—the endosome, the Golgi apparatus, and the ER—during the journey of HPV16 to the nucleus. We have temporally ordered these events based on the timing of the appearance of L2 at various locations and the effect of γ-secretase inhibition. We focused on L2 because of its essential role in infection and its association with viral DNA throughout the entry process (25, 26, 38). Previous work showed that HPV infection is inhibited by manipulations that block retrograde vesicular transport of HPV components to the Golgi apparatus (27, 28) and by L2 mutations that block transit of viral DNA through the Golgi apparatus (29). Here, we showed that γ-secretase inhibition or knockdown, which strongly inhibits infection, also interferes with vesicular transport and entry of L2 and viral DNA into the Golgi apparatus in HeLa and HaCaT cells. These findings establish the importance of this vesicular pathway for HPV entry. A primarily vesicular mode of entry may provide a number of benefits to HPV16. Virus inside a vesicle might be hidden from cytoplasmic innate immune sensors. The retrograde pathway may provide a preexisting route to an intracellular location close to the nucleus. Finally, the retrograde pathway may deliver the viral DNA to a downstream vesicle that contains a required cellular factor or is the location of a process essential for virus entry.

Our results demonstrate that the absence of γ-secretase inhibits infection by preventing transport of HPV from one vesicular compartment to another, not by blocking escape of the virus into the cytoplasm, as was previously suggested (41). The profound inhibition of infection by γ-secretase inhibitors and by γ-secretase knockdown implies that Golgi entry requires γ-secretase cleavage activity and not a nonenzymatic activity of γ-secretase. Attempts to detect γ-secretase-mediated cleavage of L1 or L2 have been unsuccessful (40, 41), implying that γ-secretase needs to cleave a cellular substrate for infection to proceed, either to generate a required factor or remove an inhibitory product. Because γ-secretase typically acts on transmembrane protein substrates (44), it may cleave a transmembrane protein of an endosome-to-Golgi transport vesicle or the target Golgi membrane. Viral components in infected cells lacking γ-secretase activity may provide markers for this putative transport vesicle. We also note that the as-yet-unidentified substrate involved in HPV entry may also play a role in the pathogenesis of γ-secretase-associated diseases.

We also showed that incoming L2 localizes to the ER in a γ-secretase-dependent manner. Polyomaviruses, another group of nonenveloped DNA viruses, also traffic through the ER, where important disassembly events occur (45, 46). However, trafficking of polyomaviruses through the Golgi apparatus has not been demonstrated, and polyomavirus entry does not require the retromer or γ-secretase activity. Thus, HPV16 utilizes a distinct trafficking pathway from other nonenveloped viruses.

The presence of incoming HPV in the ER may have important implications for the entry process. Because the ER lumen is continuous with the intermembrane space of the nuclear envelope, ER localization may provide passage for the virus to the nucleus. Alternatively, the viral genome may exit from the ER into the cytoplasm and later enter the nucleus. Indeed, Schelhaas and colleagues (23) proposed that viral DNA escapes from the endosomal system into the cytoplasm and that reformation of the nuclear envelope after mitosis traps viral DNA in the nucleus. However, another possibility is suggested by the presence of L2 in the ER. Components of the nuclear envelope redistribute into the ER upon nuclear envelope breakdown during mitosis (47, 48). The ER then serves as the source of membrane material for reassembly of the nuclear envelope when mitosis is complete. Thus, L2 and associated viral genomes might be directly transferred from the ER into the nucleus during reassembly of the nuclear envelope without first crossing the ER membrane into the cytoplasm. If this is the case, the viral genome and associated L2 might enter the cell and reach the interior of the nucleus without physically crossing any membranes.

The FLAG tag at the carboxy terminus of the L2 protein is constitutively exposed on every L2 molecule in the intact capsid, allowing immune detection of L2 throughout the entry process. Exposure of the FLAG tag on the exterior of the capsid implies that the carboxy terminus of wild-type L2 is also located near the surface of the infectious capsid, a position that may allow it to protrude through the L1 shell during entry. Because this segment of L2 contains a membrane-destabilizing sequence required for entry (35), its accessibility near the surface of the capsid is likely to be important for entry. Although the spacer-FLAG sequence is longer than the HA tag, it is unlikely that constitutive exposure of the FLAG tag is due solely to its length, because far longer carboxy-terminal extensions on L2 are located in the interior of HPV16 virus-like particles (49). Rather, the highly charged nature of the FLAG tag might contribute to its constitutive exposure. FLAG-tagged L2 might serve as a scaffold for constructing PsVs with even larger exposed moieties, which might allow the fusion of fluorescent proteins to infectious particles or improve the utility of PsVs as a gene delivery vehicle or a vaccine platform.

In summary, by employing a novel HPV PsV, we have demonstrated the vesicular mode of HPV16 entry to its passage to the ER and showed that a potent inhibitor of HPV infection acts by blocking transfer of L2 and the viral genome to the Golgi apparatus. Further analysis of HPV trafficking through various membrane-bound organelles is likely to provide additional insights into HPV entry, identify potential antiviral targets, and illuminate new features of cell biology.

MATERIALS AND METHODS

Detailed Materials and Methods are presented in the supplemental material.

Cell culture, plasmids, and virus. HeLa-S3 cells (here HeLa cells) and human 2614 ectocervical keratinocytes were obtained from the American Type Culture Collection. HeLa-Sen2 cells are a cloned strain of HeLa cells suitable for immunofluorescence experiments (50). 293TT cells were obtained from Christopher Buck (NIH). HPV pseudovirus production plasmids were gifts from Christopher Buck and Patricia Day (NIH). FLAG and FLAG-thrombin-HA tags were added to the C terminus of HPV16 L2 by standard cloning procedures. HPV PsVs were produced in 293TT cells and purified as described previously (27). Raft culture-derived HPV16 was a gift from Craig Meyers (Hershey Medical Center) (51, 52) and was handled in accordance with institutional biosafety requirements. Adenovirus type 5 expressing GFP (Ad5-GFP) was purchased from Vector Biolabs, and SV40 was produced in CV1 cells.

DNA and RNA quantification. Purified PsVs were pretreated with DNase to remove free DNA associated with capsids. Capsids were then digested with proteinase K, and DNA was isolated. Encapsidated reporter

plasmid was quantified by qPCR using primers for the reporter gene in comparison to a standard curve. Total RNA was reverse transcribed into cDNA and quantified in triplicate by single-color real-time PCR with specific DNA primers listed in Table S1 in the supplemental material. Actin mRNA was used for normalization.

Antibodies and chemicals. The anti-L1 rabbit polyclonal serum and the 33L1-7 mouse antibody were gifts from Patricia Day (NIH) and Martin Sapp (Louisiana State University), respectively. Other antibodies used are listed in Table S2 in the supplemental material. γ-Secretase inhibitor XXI and furin inhibitor I were purchased from Millipore. Retro-2 was purchased from Chembridge and heparin from Sigma. siRNAs (see Table S1 in the supplemental material) were purchased from Dharmacon.

Flow cytometry. To measure infectivity of HPV16 PsVs, cells were incubated with PsV at a multiplicity of infection (MOI) of 0.5 (determined by flow cytometry for reporter protein expression in untreated HeLa cells) or an equivalent number of packaged reporter plasmids. After 48 h, the fraction of cells expressing the reporter protein was assayed by flow cytometry on a BD Biosciences FACSCalibur flow cytometer. To measure capsid disassembly, HeLa cells were infected with HPV16.L2HA or HPV16.L2F at an MOI of 20 at 4°C for 2 h and then shifted to 37°C to initiate infection. At the indicated times postinfection, samples were stained with 33L1-7, anti-HA, or anti-FLAG and the appropriate Alexa Fluor secondary antibody and analyzed by flow cytometry to measure mean fluorescent intensity (MFI). To measure infection by authentic HPV, HeLa cells were infected with 5 μl raft-derived HPV or HPV16.L2F, and 48 h postinfection, RNA was isolated for qRT-PCR.

Neutralization assay and thrombin cleavage. Wild-type HPV16 PsV and HPV16.L2F containing the GFP reporter plasmid were incubated with anti-FLAG M2 antibody or medium and then used to infect HeLa cells at an MOI of 1. Forty-eight hours postinfection, GFP-positive cells were counted by flow cytometry. HPV16 PsVs and HPV16.L2FTHA were incubated with 10 NIH units of thrombin or phosphate-buffered saline (PBS) overnight and then used to infect HeLa cells at an MOI of 1. Infectivity was measured as described above. Thrombin- and PBS-treated samples were also subjected to SDS-PAGE and immunoblotting.

Internalization assay and immunoblotting. HeLa cells were transfected 48 hours prior to infection with Lipofectamine RNAiMAX regent and control siRNA or siRNAs targeting APH1A or furin or pretreated for 1 h with furin inhibitor I, compound XXI, or bafilomycin A. Alternatively, HPV16.L2F was preincubated with heparin. Cells were then infected at MOI of 10 with HPV16.L2F for 2 h at 4°C, washed, and shifted to 37°C for 6 h. Cells were harvested and treated with proteinase K to remove noninternalized virus particles, and internalized viruses were detected by Western blotting with the BD Biosciences anti-L1 antibody.

Fluorescence microscopy and PLA. HeLa-Sen2 cells grown on glass coverslips were infected with HPV16.L2HA or HPV16.L2F at an MOI of 20. At various times after infection, virus proteins in fixed and permeabilized cells were visualized by immunofluorescence with anti-FLAG mouse antibody, anti-HA rabbit antibody, anti-L1 rabbit polyclonal serum, or anti-33L1-7 and the appropriate fluorescently tagged secondary antibody. Images were acquired with a Zeiss Axiovert 200 inverted fluorescence microscope and processed with ImageJ.

For the proximity ligation assay, HeLa-Sen2 cells seeded on glass coverslips were transfected with siRNA targeting APH1A or control siRNA 48 h prior to infection or pretreated with 250 nM XXI for 1 h at 37°C. The cells were infected with HPV16.L2F containing HcRed plasmid at an MOI of 50 to 100, fixed, permeabilized at various times postinfection, and incubated with pairs of antibodies, one recognizing FLAG or 33L1-7 and the other recognizing a cellular marker. PLA was performed with Duolink reagents from Olink Biosciences according to the manufacturer's directions (27, 43). Briefly, cells were incubated with a pair of suitable PLA probes, which were then subjected to ligation and amplification with fluorescent substrate. Approximately 100 nuclei in each sample were imaged as described above. The images were processed by ImageJ and quantitatively analyzed by BlobFinder software to measure fluorescence intensity per cell. The average fluorescence intensity per cell was normalized to the appropriate control sample.

Visualization of viral DNA. EdU-substituted PsV genomes were visualized by using Click-iT EdU imaging kits from Invitrogen (kit C10339) as described previously (25, 34). 293TT cells transfected with p16sheLL.L2F and pMCS-*Gaussia* luciferase (Luc) were labeled 24 h later with medium containing with 10 μM 5-ethynyl-2′-deoxyuridine (EdU). At 72 h posttransfection, cells were collected and HPV16.L2F was purified. HeLa cells were infected with EdU-labeled HPV16L2.FL at an MOI of 100. At 18 h postinfection, the Click-iT reaction cocktail (Click-iT reaction buffer, $CuSO_4$, Alexa Fluor 594 azide, and reaction buffer additive) was added to fixed, permeabilized cells for 30 min at room temperature in the dark. Cells were then washed and stained with anti-TGN46 and DAPI (4′,6-diamidino-2-phenylindole) as described above, imaged with the Axiovert 200 microscope, and analyzed by imageJ.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01777-14/-/DCSupplemental.

Text S1, PDF file, 0.1 MB.
Figure S1, TIF file, 2.1 MB.
Figure S2, TIF file, 9 MB.
Figure S3, TIF file, 6.6 MB.
Figure S4, TIF file, 7.3 MB.
Figure S5, TIF file, 3.9 MB.
Figure S6, TIF file, 3.4 MB.
Table S1, PDF file, 0.04 MB.
Table S2, PDF file, 0.03 MB.

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