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Papillomaviruses infect cells via a clathrin-dependent pathway

Patricia M. Day, Douglas R. Lowy, and John T. Schiller*

Laboratory of Cellular Oncology, Division of Basic Sciences, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

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Abstract

In this study we have examined the pathway by which papillomaviruses infect cells, using bovine papillomavirus (BPV) virions and mouse C127 cells as the model system. By confocal microscopy, the entry of BPV virions, BPV virus-like particles (VLPs), and HPV16 VLPs were very similar. In dually exposed cells, HPV-16 VLPs and BPV virions colocalized intracellularly. BPV VLPs colocalized with AP-2, a clathrin adapter molecular and a marker of the clathrin-dependent endocytic pathway; and also with transferrin receptor, a marker of early endosomes; and Lamp-2, a marker of late endosomes and lysosomes. BPV infection was detected within 12 h of virion cell-surface binding, as measured by an RT-PCR assay. Infection was prevented by several pharmacologic inhibitors, including chlorpromazine, which blocks clathrin-dependent endocytosis and the lysosomotropic agent, bafilomycin A. By contrast, two inhibitors of caveolae-dependent uptake, filipin and nystatin, did not prevent BPV infection. We conclude that papillomaviruses infect cells via clathrin-dependent receptor-mediated endocytosis. Surprisingly, the kinetics of internalization were unusually slow for this mechanism, with the $t_{1/2}$ of entry of BPV-1 being approximately 4 h versus 5–15 min for a typical ligand.

Introduction

Papillomaviruses (PVs) are nonenveloped, icosahedral DNA viruses that persistently infect stratified squamous epithelia from a wide spectrum of animal species. These viruses are implicated etiologically in a number of epithelial lesions, most notably human cervical carcinoma (Zur Hausen, 1991).

The completion of the infectious pathway of PVs is difficult to achieve in vitro, as the late events in the cycle are tied to keratinocyte differentiation (Taichman et al., 1984). This experimental difficulty has resulted in limited experimental analysis of the pathway by which PVs infect cells, with no systematic analysis of the trafficking pathways for PV virions. However, the early events in viral infectivity can be studied with PV virions and appropriate monolayer cultures. Aspects of this process can also be examined with PV virus-like particles (VLPs), providing that VLPs and virions can be shown to interact similarly with the cell. Previous analyses of the PV entry pathway have demonstrated a dependence upon an intact actin cytoskeleton. However, electron microscopic analysis has led to conflicting results as to whether PVs enter cells via coated or uncoated vesicles (Volpers et al., 1995; Zhou et al., 1995).

Following binding to cell-surface receptors, viruses are internalized into the intracellular milieu via one of several possible pathways. Nonenveloped viruses generally enter through clathrin-coated endocytic vesicles or the clathrin independent caveolae system, which produces uncoated vesicles. In the former system, a carrier vesicle intermediate is used to deliver virions into endosomes and lysosomes (reviewed in Schmid, 1997). This vesicular microenvironment may facilitate viral infection, possibly through a weakening of the previously resistant capsid as occurs for members of the reovirus family (Dryden et al., 1993; Ludert et al., 1987; Virgin et al., 1994) or through a pH-dependent release of virions into the cytoplasm as described for adenoviruses (Fujimoto et al., 2000; Greber et al., 1993). In the other system, virions entering the cell via caveolar endocytosis are transported into the smooth ER through a pathway that is independent of endosomes and lysosomes

* Corresponding author. Laboratory of Cellular Oncology, Division of Basic Sciences, National Cancer Institute, National Institutes of Health, Building 36, Room 1D-32, Bethesda, Maryland 20892. Fax: +1-301-480-5322.

E-mail address: schillej@dc37a.nci.nih.gov (J.T. Schiller).

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(Anderson et al., 1992; Pelkmans et al., 2001). The distinction between these two pathways can be achieved through analysis of the pattern of biochemical inhibition of ligand uptake and by colocalization of input virus with defined components of the cellular trafficking machinery.

It is difficult to predict a priori which pathway is utilized by PVs. For example, among polyomaviruses, whose capsid structure is similar to that of PVs, some members, such as JC, use the clathrin pathway (Pho et al., 2000), while others, such as SV40, enter cells via caveolae (Anderson et al., 1992; Chen and Norkin, 1999; Stang et al., 1997). To identify the pathway experimentally, we have here examined the entry of bovine papillomavirus type 1 (BPV) into C127 cells, a standard virus-cell system for the analysis of PV infection, which results in stable nonproductive cellular transformation (Dvoretzky et al., 1980; Law et al., 1981; Watts et al., 1984). We have combined microscopic evaluation of cell-associated virus with an RT-PCR assay that detects the expression of early spliced BPV-1 mRNA. By using these assays in conjunction with a battery of compounds that inhibit various trafficking pathways, we have determined that PVs enter cells via clathrin-dependent receptor-mediated endocytosis.

Results

VLPs and virions utilize the same intracellular pathway

It has recently been suggested that virions (or DNA-containing VLPs) and empty VLPs may differ in their stability and their sensitivity to protease digestion, as the result of additional disulfide bond formation in capsid proteins following DNA packaging (Fligge et al., 2001). It was hypothesized that this increased stability in DNA-containing particles may result in differential association with cellular receptors. This is a critical issue, as most studies of PV adsorption and penetration have utilized VLPs rather than virions.

To compare our virion and VLP preparations for possible differences in their rate of internalization or subcellular localization, we examined BPV virions, purified from bovine warts, and BPV VLPs in parallel microscopy time-course experiments. No obvious differences were detected between BPV virions and BPV VLPs (data not shown, though comparable to Fig. 2B). It was not feasible to perform the cytometric analysis, which requires larger amounts of particles, with the virions due to limited availability of this material.

Therefore, to address this question even more stringently, we simultaneously incubated cells with BPV virions and HPV-16 VLPs. Although evolutionarily divergent, it is likely that these viruses share a common receptor as HPV 16 VLPs can compete with BPV for cell-surface binding (Roden et al., 1994a). Since there are monoclonal anti-capsid antibodies that specifically recognize BPV or HPV16 L1 epitopes, double staining with these antibodies enabled us to compare the localization of virions and VLPs within the same cells. Using this approach, we found that the pattern of staining overlapped substantially (Fig. 1 shows double staining against HPV-16 L1 and BPV-1 L1 at the 4 and 8 h time points), although some vesicles contained only one particle type. The use of monoclonal antibodies that exhibit different profiles of reactivity (Booy et al., 1998; Roden et al., 1994b; Slupetzky et al., 2001) could explain the apparent lack of absolute colocalization.

We conclude that major differences do not exist in the uptake pathways of our preparations of VLPs and virions due to the evidence that they share a common cell-surface receptor and colocalize in intracellular compartments. Therefore, examining VLP trafficking and localization should give an adequate representation of the authentic viral pathway.

BPV-1 VLPs are slowly internalized

Before examining the post-binding events in detail, we sought to determine the rate at which BPV is internalized. As preparative amounts of VLPs are more readily available than virions and, as we had previously established their utility, we characterized this process with BPV VLPs.

To determine the $t_{1/2}$ of VLP internalization, VLPs were bound to the surface of C127 cells at 0°C and allowed to be internalized at 37°C. At defined times cells were removed and stained with monoclonal antibody 5B6, which recognizes a conformational determinant on the BPV capsid. 5B6 reactivity was quantified by flow cytometric analysis. Triplicate stainings were averaged for each time point. The standard error was below 3% for each series. The mean channel fluorescence at the 0-min time point was designated 100%. There was an apparent lag in initiation of endocytosis of VLPs, with no significant internalization occurring during the initial 60 min (Fig. 2A). After this time, the loss of cell-surface VLPs was linear, with approximately 50% of the VLPs internalized by approximately 4 h. In parallel incubations analyzed microscopically, capping of surface VLPs was evident during the first hour of incubation at 37°C with significant cytoplasmic vesicles evident by 2 h at 37°C (Fig. 2B).

To rule out the possibility that some of the time-dependent loss of 5B6 reactivity from the cell surface might have resulted in part from VLPs that dissociated from the cell surface, rather than from VLP internalization, cell supernatants from each time point were analyzed for the presence of 5B6-reactive material by ELISA. However, no VLPs were found to be released from the cell surface after initiation of internalization (data not shown), although the 5B6 ELISA was sensitive enough to detect as little as 1% of the VLPs bound to the cells. These results strongly suggest that the time-dependent decrease in cell-surface 5B6 staining has resulted primarily from the entry of VLPs into the cell.

Colocalization with markers of endocytic uptake

To study the internalization pathway followed by BPV VLPs, we first examined their possible colocalization with established markers of endocytic pathways. The markers studied included the following: AP-2, which is a clathrin adaptor protein; transferrin receptor, which is a marker for the early endosomal compartment; and Lamp-2, which is a marker of late endosomes and lysosomes.

Cells were allowed to internalize VLPs for defined time periods. They were then fixed and stained with the various marker proteins and the 5B6 antibody against BPV L1 protein. After 2 h at 37°C, colocalization of BPV L1 and the clathrin adaptor protein, AP-2 (Fig. 3,A–C), was clearly evident. By 3 h of internalization colocalization of BPV L1 and transferrin receptor was readily apparent (Fig. 3D–F), indicating further passage of the VLPs to the early endocytic compartment. By contrast, no clear colocalization with anti-Lamp-2 staining was evident with the anti-L1 antibody 5B6 at any time point (data not shown).

Many viruses escape the endocytic pathway prior to this point. However, an alternate possibility was that the BPV L1 VLPs might have been transported to the late endosomal and lysosomal compartments, but that transit to these compartments might have been associated with a conformational change in L1 leading to the loss of reactivity to the 5B6 antibody. To evaluate this possibility, the fixed cells were stained with another BPV L1 antibody, no. 9, which recognizes a conformational epitope that is distinct from that of 5B6. In contrast to the results obtained with 5B6, antibody no. 9 did display strong localization within Lamp-positive compartment (late endosomes and lysosomes) beginning approximately 4 h after initiation of internalization (Fig. 3G–I show the localization at 7 h). The staining of the membrane-associated Lamp-2 protein appears to encircle the anti-VLP staining, indicating their presence within these vesicles. These positive results indicate BPV L1 protein that has retained some of its conformational epitopes can be found in late endosomes and/or lysosomes. The use of antibodies raised against linear L1 epitopes yielded no further information due to extensive background staining (data not shown).

Biochemical inhibition of BPV-1 infection

The above results localizing the VLPs within AP-2-postive vesicles and later vesicles of the endocytic compartment are consistent with the particles having been internalized via clathrin-coated pits. However, this conclusion would seem to be at odds with the observed kinetics of PV internalization, as most ligands that are endocytosed via the clathrin-dependent pathway do so with a $t_{1/2}$ of 5 to 15 min (Goldenthal et al., 1988; Schmid et al., 1988), while the $t_{1/2}$ of BPV-1 internalization was approximately 4 h (Fig. 2). Therefore, to strengthen the conclusion that BPV internalization is mediated via this pathway, we examined the sensitivity of BPV infection to a variety of biochemical inhibitors known to inhibit distinct cellular processes.

To identify successful virus entry and uncoating, we measured the appearance of an early spliced mRNA in C127 cells after the addition of infectious BPV virions. We reasoned that this type of assay would be a sensitive and rapid way to definitively monitor the completion of the early events in viral entry and uncoating. BPV virions were added to C127 cells; cells were harvested at set time points postinfection, and RNA was prepared. RT-PCR was performed using primers across a splice site of the early genes E6^E7 of BPV, and as a control for mRNA integrity, across a spliced junction in the cellular γ-actin gene. A second round of PCR was run using primers internally nested to the first set. The amplimers from this reaction were analyzed electrophoretically. Fig. 4A shows that the specific RNA band indicating transcription of the BPV-1 early gene was first evident 12 h postinfection. The actin transcripts demonstrate RNA integrity at all time points.

To determine the sensitivity of the assay, we added decreasing amounts of virions to C127 cells and allowed the infection to continue for 14 h. We found that a strong signal was detectable down to 80 ng/10^6 cells; a weaker signal was obvious at 32 ng/10^6 cells, and no signal was observed below this input level (Fig. 4B). One microgram of virions is equal to approximately 3×10^{10} particles, so the detectable limit of 32 ng/10^6 cells is equivalent to approximately 1000 virions per cell. Given the high particle-to-infectivity ratio described for papillomavirus (Roden et al., 1996; Smith et al., 1995), this is not a high m.o.i. In a focus formation assay, a particle-to-infectivity ratio of 2×10^4 was determined for BPV virions, purified from warts in an identical manner to our material (Roden et al., 1996). Additionally, we evaluated the assay by processing RNA obtained from BPHE-1 cells. These cells contain an estimated 50 copies of autonomously replicating genome (Zhang et al., 1987). Material from approximately 10 BPHE-1 cells yielded an obvious signal in the RT-PCR assay (data not shown), indicating the utility of this method for sensitive detection of BPV-infected cells.

This sensitive PCR assay was then used to determine if BPV infection could be prevented with inhibitors of endocytic pathways (see Table 1). Virions were bound to C127 cells for 60 min at 0°C; unbound virions were removed, and infection was allowed to proceed by addition of 37°C medium. For initial analysis, the inhibitor was present during the entire infection, and two levels of input virions were assayed to evaluate possible intermediate effects (Fig. 5). The drugs that prevented infection were chlorpromazine, bafilomycin A, NH_4Cl, nocodazole, latrunculin B, and sodium azide. The first three inhibitors act on the clathrin-endosomal pathway at various points (Bayer et al., 1998; Clague et al., 1994; Subtil et al., 1994). Latrunculins prevent polymerization of actin monomers (Spector et al., 1989). Actin has been found to be nonessential for the internalization of some clathrin-dependent ligands (Fujimoto et al.,

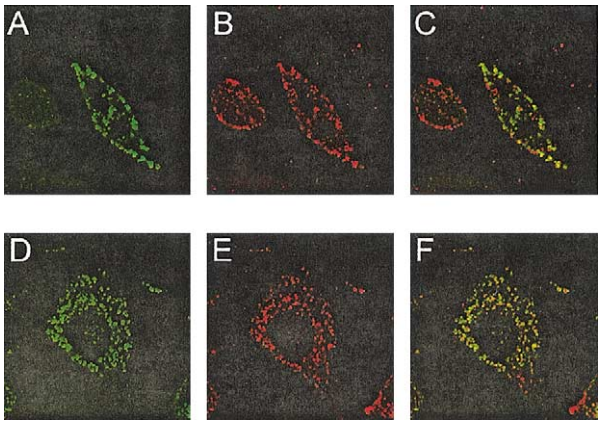

Fig. 1. Colocalization of HPV-16 VLPs and BPV-1 virions. C127 cells were coincubated with the two particle preparations and fixed after incubation for 4 h (A–C) or 8 h (D–F). A and D show the distribution of HPV 16 L1 VLPs as detected with antibody V5 and goat anti-mouse IgG-FITC. B and D show the pattern of BPV-1 virions, detected with 5B6-biotin and a streptavidin Texas red conjugate. The merged images, C and F, illustrate the extensive colocalization of VLPs and virions.

2000; Salisbury et al., 1980; Sandvig and van Deurs, 1990). Sodium azide causes the inhibition of oxidative phosphorylation. Each of these inhibitors prevented infection at both amounts of input virus. In contrast, treatment with filipin and nystatin (Rothberg et al., 1992; Schnitzer et al, 1994), which are two inhibitors of the caveolae-mediated uptake pathway, had no effect on papillomavirus infection. However, infection with SV40, used as a positive control for this pathway, was inhibited under these conditions (data not shown). Additionally, the caveolar pathway bypasses the acidified vesicular compartments so the effect of bafilomycin A and NH_4Cl on papillomavirus infection would be inconsistent with caveolar uptake (Pelkmans et al., 2001).

We further analyzed a subset of the active inhibitors in a time-course experiment to determine when each of the inhibitory drugs exerted its influence on infection. To determine at which point in the infectious entry pathway the various inhibitors exerted their effects, inhibitors were added 2, 4, 6, or 8 h after the initiation of the infection, or for the entire infection as a positive control. The infection was continued for a total of 12 h, at which point RNA was prepared and analyzed for the presence of the BPV transcript (Fig. 6).

Treatment with chlorpromazine or bafilomycin A resulted in a similar time course of inhibition. Each of these drugs was inhibitory when added 2 h after infection, but did not affect infection when added at the 4-h time point or later, suggesting that they inhibit early aspects of infection. This inference is also consistent with the known sites of action of the drugs. Chlorpromazine blocks clathrin-dependent endocytosis by causing the redistribution of AP-2 (Subtil et al., 1994; Wang et al., Anderson, 1993); bafilomycin A is a vacuolar ATPase inhibitor (Lamaze et al., 1997; Spector et al., 1989) that has also been shown to affect transit of ligands from early endosomes into endosomal carrier vesicles. Inhibition of infectivity at 2 h by these agents is consistent with the above finding that the internalization of VLPs is a relatively slow process. In contrast to the above inhibitors, nocodazole is a microtubule depolymerizing agent that acts at a later point, by blocking the transport from early to late endosomes in some cell types (Bayer et al., 1998; Bomsel et al., 1990; Parton et al., 1991). Consistent with a later site of inhibition, nocodazole prevented infection when added through the initial 6 h of infection. When it was added for the final 4 h of the infection, there was no effect. Additionally we found that treatment with latrunculin B inhibited infection with a profile indicating a relatively early action. While it has been described that latrunculin B can prevent receptor-mediated endocytosis at the stage of coated vesicle budding (Durrbach et al., 1996; Lamaze et al., 1997), other reports indicate no role for actin in clathrin-dependent endocytosis (Fujimoto et al., 2000; Salisbury et al., Sandvig and van Deurs, 1990). This early role of actin could provide a valuable clue as to the identity of the PV receptor.

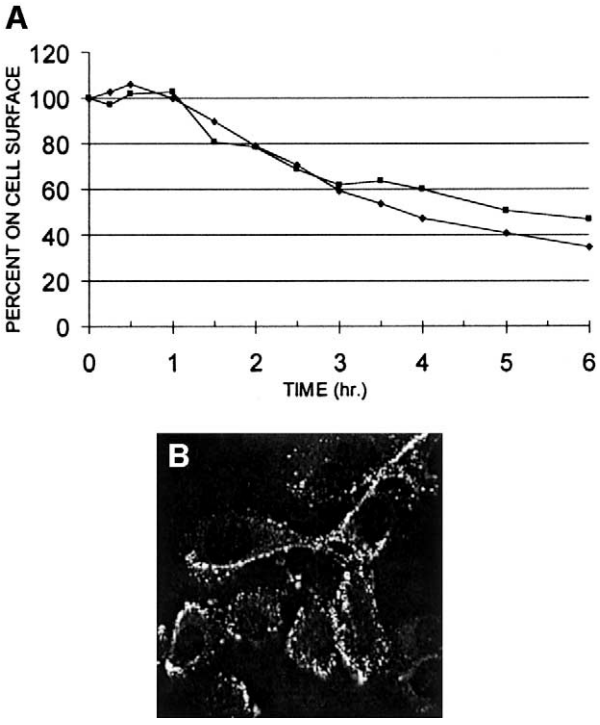

Fig. 2. BPV VLPs are slowly internalized. A shows the $t_{1/2}$ of BPV VLPs on the surface of C127 cells. Cells were incubated with VLPs at 0°C for 1 h. Excess VLPs were removed by extensive washing and cells were incubated at 37°C for the described times. Shown are two independent experiments. The plot for each experiment shows the average mean channel fluorescence, which was calculated from triplicate samples for each time point. The value at the beginning of the 37°C incubation was considered to be 100%, and each subsequent value is based on that value. The dissociation of VLPs from the cell surface was examined by ELISA (not shown) and determined to insignificant (<1% of bound). B shows an example of the cell distribution of VLPs after 2 h incubation at 37°C. Significant surface staining is evident and capping is obvious. Numerous cytoplasmic vesicles are also becoming evident at this point.

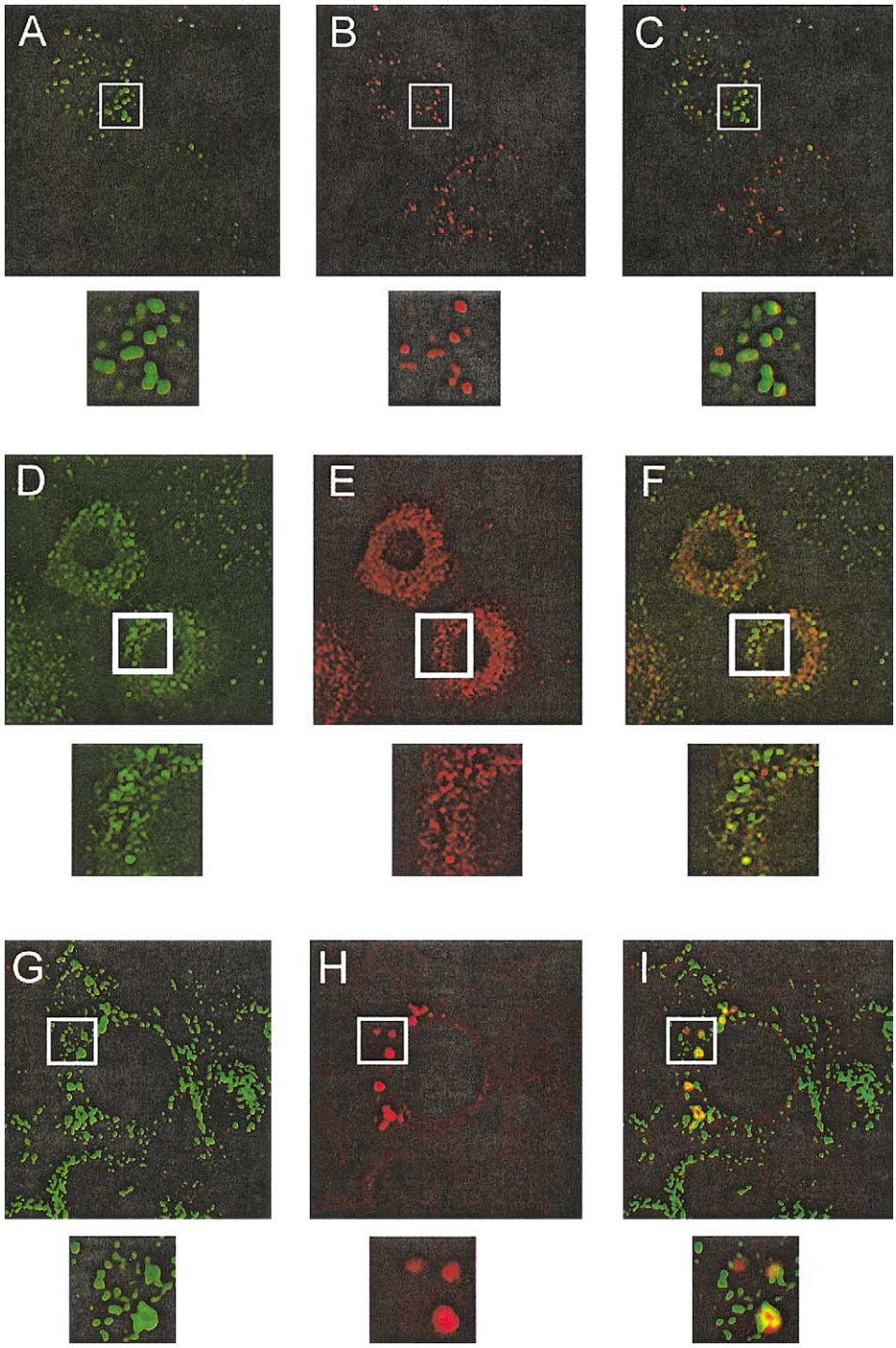

Fig. 3. Colocalization of VLPs with endocytic markers. VLPs were internalized in C127 cells for 2 h (A–C). 3 h (D–F), or 4 h (G–I). Cells were fixed and double stained for endocytic markers and VLPs. Staining against BPV L1 is shown in B, E (antibody 5B6), and H (antibody no. 9). Anti-AP-2 staining, shown in A, was detected with goat anti-mouse IgG-FITC. In this instance biotinylated 5B6 and streptavidin–Texas red were used to detect VLPs (B). The merge of the two images is shown in C. Antitransferrin receptor staining is shown in D. This antiserum was detected with goat anti-rabbit IgG-FITC. 5B6 binding was detected with goat anti-mouse IgG–Texas red. The merged image is shown in F. Localization of the Lamp-2 protein is shown in G. Binding was detected with goat anti-rat IgG-FITC. Monoclonal antibody no. 9 was detected with goat anti-mouse IgG–Texas red. The merged image is in I. For all antibodies, control images were evaluated to ensure nonoverlapping binding of secondary antibodies and specific detection for each excitation channel.

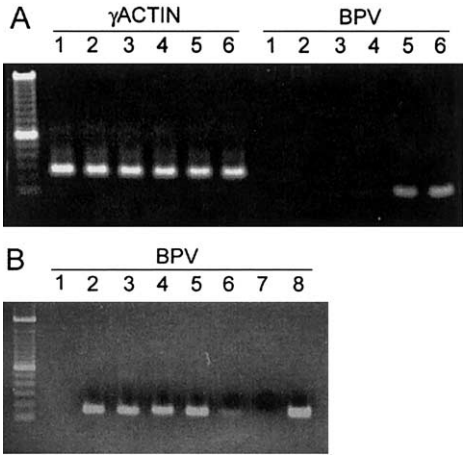

Fig. 4. RT-PCR of BPV-infected C127 cells. (A) A BPV-specific transcript is evident in infected cells after a 12-h infection. Purified BPV virions were added to C127 cells at a dose of 500 ng/1 $\times$ 10^6 cells. Infection was continued for the described times. RNA was extracted and RT-PCR reactions for both BPV and γ-actin were performed. γ-Actin amplimers are shown in the left half of the panel. BPV amplimers are shown on the right. Lanes 1–6 indicate harvest at 6, 7, 8, 10, 12, and 14 h of infection, respectively. The BPV specific band is evident at the 12- and 14-h time points. (B) Titration of input virus. Different amounts of virions were used to infect C127 monolayers. The BPV amplimers are shown. Lane 1 is the uninfected control. Lane 8 shows the positive control of amplification from a BPV-1+cell line. Lanes 2–7 show the titration of virions from 1250, 500, 200, 80, 32, 13 ng/1 $\times$ 10^6 cells, respectively. The BPV amplimer is clearly visible down to 80 ng/1 $\times$ 10^6 cells and weakly visible at 32 ng/1 $\times$ 10^6 cells.

Discussion

The early events of viral infection include virion attachment to cellular receptors, penetration, and uncoating, which is followed by viral gene transcription. As noted above, nonenveloped DNA viruses generally enter cells via one of two pathways, the clathrin-dependent endosomal vesicular pathway or caveolae. In the current studies, we have used three different approaches to identify the cellular pathway used for early infection by PVs: morphology, subcellular localization, and inhibition of viral transcription by drugs with distinct sites of action. The evidence developed by each approach supports the conclusion that PVs enter cells via the clathrin-dependent pathway, rather than caveolae, although with much slower kinetics than usually seen with clathrin-dependent processes.

Morphologically, PVs were found in vesicles that are characteristic of the clathrin-dependent pathway of internalization. By subcellular localization, the PVs colocalized with markers of this pathway. Biochemically, PV mRNA synthesis, used as an index of viral entry and uncoating, was inhibited by several drugs that interfere with the clathrin-dependent pathway, but not by drugs that inhibit the caveolae-dependent pathway.

Coated pits contain two major structural proteins, clathrin and the adaptor protein AP-2 (Pearse, 1976, 1978, 1989). AP-2 complexes are localized to the plasma membrane-derived coated pits. Adaptors play a critical role in the attachment of clathrin to membranes and the recruitment of membrane proteins that localize to clathrin-rich membrane regions (Beck et al., 1992; Chang et al., 1993; Glickman et al., 1989; Pearse, 1988; Pearse and Bretscher, 1981). The observed colocalization of PV VLPs with AP-2 in cytoplasmic vesicles strongly implicates the clathrin-dependent pathway in viral penetration. This conclusion was further supported by colocalization, in a vesicular compartment, of VLPs with the transferrin receptor, which is a marker of early endosomes (Harding et al., 1983; Yamashiro and Maxfield, 1984), a later compartment of the endocytic lineage than that identified by AP-2. In these compartments, the VLPs reacted with anti-L1 antibody 5B6, but not strongly with antibody no. 9 (data not shown). Both antibodies are neutralizing and recognize a conformational surface epitope. However, no. 9 reacts with the top of the capsomers and can interfere with virion binding to the cell surface, while 5B6 reacts with the sides of the capsomers, at a site just above intercapsomere cross-bridges, and does not inhibit cell-surface binding (Booy et al., 1998; Roden et al., 1994b). Therefore, one interpretation of the negative results with antibody no. 9 is that when the virions or VLPs are associated with the clathrin or early endosomal compartments, their interaction with receptors is so extensive that detectable no. 9 binding is prevented.

Although some viruses may escape from, or undergo uncoating in, early endosomes (Bartlett et al., 2000; Bayer et al., 1999; Greber et al., 1993), many continue to be passaged, via endosomal carrier vesicles, to late endosomes and then to lysosomes (Zeichhardt et al., 1985). Using the Lamp-2 protein as a marker for late endosomes and lysosomes (Chen et al., 1985), we found that it colocalized with BPV L1, at a later time than colocalization was observed with the transferrin receptor. In contrast to the L1 antibody

Table 1
Panel of biochemical inhibitors that was examined for an effect on PV infection

Inhibitor	Site/Mode of Action	PV Infx
Bafilomycin A	Inhibits vacuolar ATPase Inhibits endosomal trafficking	No
Chlorpromazine	Blocks entry of clathrin-coated vesicles	No
Filipin	Prevents caveolar-mediated uptake	Yes
Latrunculin B	Depolymerizes actin	No
NH_4Cl	Raises endosomal pH	No
Nocodazole	Depolymerizes microtubules Blocks trafficking into Les	No
Nystatin	Prevents caveolar-mediated uptake	Yes
Sodium azide	Inhibits oxidative phosphorylation	No

Note. Inhibitors were used at the following concentrations: bafilomycin A, 100 nM; chlorpromazine, 25 μM; filipin, 400 nM; latrunculin B, 63 μm; NH_4Cl 20 mM; nocodazole, 10 μm; nystatin, 27 μM; sodium azide, 63 mM. The described mode of action of these inhibitors is listed. The observed effect on BPV infection is also noted in the final column.

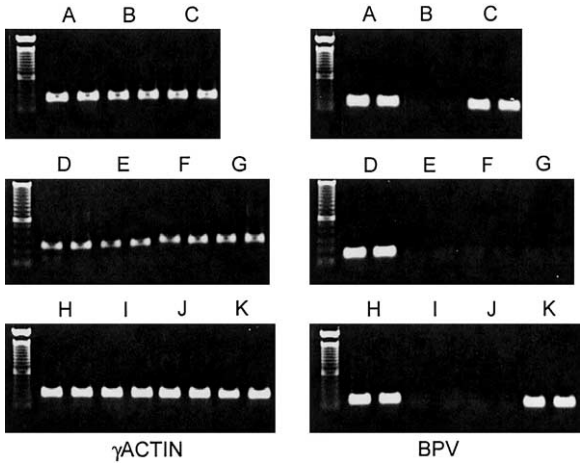

Fig. 5. Pharmacalogic inhibition of BPV infection. C127 cells were infected with either 500 ng/1 × 10^6 cells (leftmost lane in each pair) or 50 ng/1 × 10^6 cells (rightmost lane). Cells were either untreated (lanes A, D, and H) or treated with various inhibitors throughout the entire infection. Inhibitors are as follows: sodium azide, lanes B; filipin, lanes C; bafilomycin A, lanes E; chlorpromazine, lanes F; latrunculin B, lanes G; nocodazole, lanes I; NH_4Cl, lanes J; nystatin, lanes K. The γ-actin amplimers are shown in the gels in the right column and the corresponding BPV amplimer is in the left column.

results seen in association with AP-2 or the transferrin receptor, L1 antibody no. 9 staining was positive in the compartments associated with Lamp-2, while antibody 5B6 was largely negative. The positive reactivity to no. 9 suggests that this epitope is no longer occupied by receptor binding, a dissociation often seen with other viruses in the Lamp-associated compartments. The negative reactivity to 5B6 suggests either that the viral particles have undergone some relatively subtle conformational change or that they have become partially disassembled. Substantial disassembly seems unlikely, as it has been reported that when BPV VLPs are dissociated to pentamers in vitro, they retain their ability to bind 5B6 but lose their binding to no. 9, and chimeric BPV VLPs have been identified that bind no. 9 but not 5B6 (Slupetzky et al., 2001). The observation that some no. 9 reactive material does not localize within the Lamp + compartments may indicate the appearance of this epitope coincides only with passage into the lysosome, leaving the Lamp + late endosomes unreactive.

The above "bulk flow" analysis is useful in studying viral entry. However, the particle-to-infectivity ratio raises the possibility that nonproductive events could dominate results obtained with this approach and thereby obscure visualization of the authentic infective pathway. Therefore, we also analyzed the production of an early viral mRNA transcript as a measure of true infection and the ability of drugs that inhibit various endocytic processes to interfere with production of this transcription. By adding the inhibitors at various times after the start of infection, it was also possible to infer when during infection each drug was likely to be active.

We found that chlorpromazine, latrunculin B, and bafilomycin A each inhibited accumulation of the viral transcript if they were added 2 h after infection, but not 4 h after infection, while nocodazole blocked even when added 4 h after infection, although not when added at 6 h or later. These results indicate PVs likely use the clathrin endocytic pathway and that nocodazole probably inhibits a later step than the other three drugs. A summary of these experiments is illustrated in Fig. 7.

Latrunculins specifically affect the actin cytoskeleton by sequestering actin monomers (Lamaze and Schmid, 1995; Spector et al., 1989), and actin filaments have been shown to be required at early steps in receptor-mediated endocytosis in some cell systems (Durrbach et al., 1996; Lamaze et al., 1997). However, despite the well-established requirement for actin in endocytosis in yeast (reviewed in Geli and Riezman, 1998), in mammalian cells a connection between the cytoskeleton and endocytic mechanisms has not been clearly demonstrated (Fujimoto et al., 2000; Salisbury et al., 1980; Sandvig and van Deurs, 1990). Actin has been shown to be essential for the internalization of some heparan-sulfated proteoglycans (HSPGs) (Fuki et al., 1997, 2000). Cell-surface heparan sulfate is a requirement for PV infec-

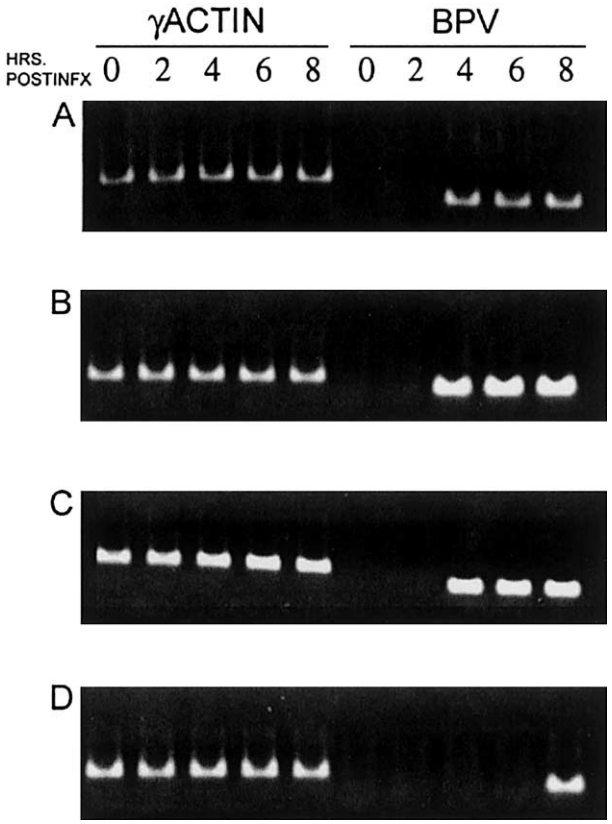

Fig. 6. Time course of pharmacologic inhibition. Virions were added to C127 cells at 500 ng/1 × 10^6 cells. Infection was initiated by addition of 37°C medium. Inhibitors were present for either the entire infection or added at 2-h intervals as indicated above the lanes. Infection was continued for 12 h total whereupon the cells were processed for RNA extraction. The γ-actin amplimers are shown on the right-hand side of the gel and the BPV amplimers are on the left. A, Treatment with chlorpromazine; B, with latrunculin B; C, with bafilomycin A; D, with nocodazole.

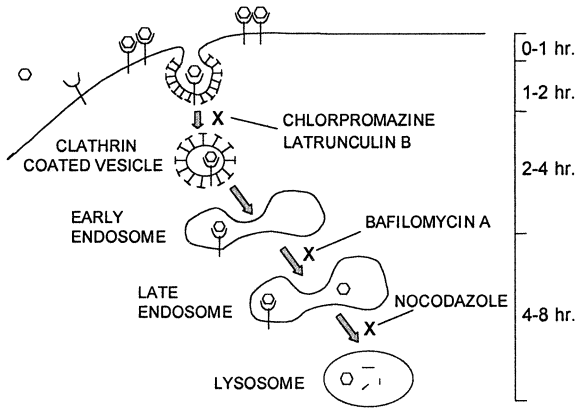

Fig. 7. PVs enter the cell via a clathrin-dependent pathway. This illustration shows the well-established endocytic pathway via clathrin-coated vesicles. The points at which the various inhibitors act in the internalization scheme are shown. The approximate time that it takes PVs to reach each compartment of the pathway is indicated on the far right. This determination was based on microscopic colocalization with markers specific for the compartments and by inhibition of infection with the designated compounds.

tion (Giroglou et al, 2001). This could indicate a link between the poorly defined HSPG internalization pathway and the well-defined clathrin pathway. Our future studies will explore this possibility.

Chlorpromazine specifically blocks clathrin-mediated endocytosis by causing a redistribution of AP-2 away from clathrin-containing pits (Subtil et al., 1994; Wang et al., 1993). The ability of chlorpromazine and latrunculin B to inhibit early during infection suggests that early events in PV infection are mediated by a clathrin-dependent process and require an intact actin cytoskeleton. The actin requirement is also consistent with the results of a previous study of PV VLP internalization (Zhou et al., 1995).

Bafilomycin A has been shown to affect early endosomal trafficking (Bayer et al., 1998; Clague et al., 1994), in addition to its well-established inhibition of vacuolar ATPases (Bowman et al., 1988; Yoshimori et al., 1991). Therefore, the inhibition with bafilomycin A can be interpreted as implying that PV uncoating depends on exposure to low pH and/or that productive uncoating occurs in late endosomal compartments. The latter possibility must be seriously considered, since nocodazole prevents the trafficking of early endosomes to late endosomes by depolymerizing microtubules. However, infection was found to be inhibited by other lysosomotropic drugs, NH_4Cl (Fig. 5) and chloroquine (data not shown), implying a likely requirement for low pH. It should also be noted that nocodazole interfered with infection even if added at a time when infection was resistant to treatment with chlorpromazine, latrunculin B, or bafilomycin A. As the infective process is beyond the early-to-late endosome transition by then, this result implies that microtubule function may also be required at a relatively late step, possibly the transition from the vesicular compartments to the nucleus. An association between papillomavirus L1 and microtubules has recently been described (Liu et al., 2001).

The inhibitor profiles and internalization kinetics indicate that PV infection occurs much more slowly than conventional endocytosis, where typical ligand–receptor complexes transit to peripheral early endosomes in fewer than 10 min (Hanover et al., 1984, 1985; Schmid et al., 1988). Our observations suggest that virions do not exit early endosomes within the first 2 h of internalization, with their transit into late endosomes occurring by approximately 4 h, and their exit from late endosomes in the 6- to 8-h window. We do not have a clear explanation for this slow rate. JC virus uses the clathrin-dependent pathway, but it enters cells with much faster kinetics (Pho et al., 2000). By contrast, the long period at the cell surface prior to entry is also seen with SV40 (Pelkmans et al., 2001). However, SV40 internalization is well established to occur via caveolar endocytosis (Anderson et al., 1996; Stang et al., 1997).

Given that the polyomaviruses SV40 and JC enter cells via distinct pathways, it is theoretically possible that not all PVs follow the clathrin-dependent pathway. However, previously published evidence indicates that HPV 16 VLPs can compete with BPV for cell-surface binding (Roden et al., 1994a), and here we have found that HPV 16 VLPs, BPV VLPs, and BPV virions enter cells with the same kinetics and via the same compartments. Since HPV 16 and BPV are evolutionarily divergent (Chan et al., 1995; Myers et al., 1996), it therefore seems likely that all PVs enter cells by the same process.

The kinetics, subcellular localization, and biochemical inhibition profiles of PV do not completely align with any defined internalization schemes. The clathrin-dependent pathway utilized by interleukin-2 receptors does intersect with the endosomal compartment. However, it exhibits rapid kinetics and would not show colocalization with preendosomal markers such as AP-2 (Lamaze et al., 2001). Several bacterial toxins enter cells via a clathrin-independent, noncaveolar pathway. However, these toxins, cholera toxin, shiga toxin, and verotoxin, do not intersect with the later endosomal compartments and are not affected by bafilomycin, nocodazole, and NH_4Cl. They are directly transported from early endosomes into the Golgi apparatus (Falnes and Sandvig, 2000; Mallard et al., 1998; Nichols and Lippincott-Schwartz, 2001; Orlandi and Fishman, 1998). Therefore, although the kinetics are atypical for ligands internalized via clathrin-dependent pathways, the preponderance of evidence, including colocalization of PV with markers of this pathway and inhibition with biochemical agents consistent with clathrin-mediated uptake, supports the conclusion that PV infection occurs via this pathway. The explanation for the slow rate of internalization and actin dependence may lie in the identification of the PV receptor(s). Although the definitive receptor remains undefined, a dependence upon the presence of cell-surface heparan sulfate has been well established. HSPGs have been shown to be internalized slowly and this pathway of uptake

is inhibited by biochemical agents that affect actin polymerization (Fuki et al., 1997, 2000). The pathway utilized by most HSPGs has not been elucidated. Perhaps our observations indicate that some ligands internalized by association with HSPGs enter into a classical endocytic route.

Materials and methods

Reagents

C127 cells were grown in Dulbecco's modified Eagle's medium supplemented with antibiotics and 10% fetal calf serum. Antibodies 5B6 and no. 9, which recognize the native BPV L1 protein, and antibody V5, which recognizes native HPV16 L1, have all been described previously (Booy et al., 1998; Roden et al., 1994b). For some experiments biotinylated 5B6 was used. This conjugate was prepared using a succinimidyl ester of biotin according to standard techniques. Mouse monoclonal antibody recognizing AP-2 was purchased from Affinity Bioreagents. Rabbit polyclonal antiserum raised to the transferrin receptor was purchased from Santa Cruz Biotechnology. Rat monoclonal antibody ABL-93, against Lamp-2, was provided by Dr. Jonathan Yewdell. HPV L1 and BPV-1 L1/L2 VLPs were prepared from a baculovirus expression system in SF-9 cells as previously described (Kirnbauer et al., 1993). BPV-1 virions were purified from bovine warts according to previously described methods (Roden et al., 1996).

Infection with BPV-1 virions

Unless otherwise stated, purified virions were added to cultures of adherent cultures of C127 cells at a dose of 500 ng/10^6 cells. Virions were bound to cells in PBS, 2% FBS for 60 min on ice. Unbound virions were removed by three PBS washes. Infection was initiated by addition of 37°C medium and continued for 12 h, whereupon the cells were trypsinized, washed twice with PBS, and processed for RNA extraction. For inhibitor studies, the 37°C medium containing the specified drug was added to the infection at the indicated times. Inhibitors were used at the following concentrations: bafilomycin A, 100 nM (Alexis Biochemical); chlorpromazine, 25 μM (Calbiochem); filipin, 400 nM (Sigma); latrunculin B, 63 μm (Molecular Probes); NH_4Cl, 20 mM (Sigma); nocodazole, 10 μm (Calbiochem); nystatin, 27 μM (Sigma), sodium azide, 63 mM (Sigma). The sodium azide treated cells were incubated in glucose-free medium. All other incubations were performed in the standard growth medium.

RT-PCR and PCR

PCR primers to detect BPV transcripts are as follows: forward outside primer; 5′ gcgcgcaccagctcgacgtccctgct 3′ (BPV nt 761–780); reverse outside primer; 5′ cacacagctctgatgggaccgcaggc 3′ (BPV nt 3350–3331); forward inside primer; 5′ gcgcgcactcagatttagacctcttg 3′ (BPV nt 801–820); reverse inside primer: 5′ cacacaggctgggctggctcggcttc 3′ (BPV nt 3305–3286). PCR primers to detect γ-actin transcripts are as follows: forward outside: 5′ gcgcgcgtcatggtgggcatgggcca 3′ (actin nt 145–165); reverse outside: 5′ cacacaccggccagccaggtccagac 3′ (actin nt 587–567); forward inside; 5′ gcgcgctgggacgacatggagaagatc 3′ (actin nt 277–297); reverse inside; 5′ cacacaagagtcgatgacaatgccagt 3′ (actin nt 484–504). RNA was prepared using the kit RNAqueous (Ambion) according to the manufacturer's directions with the additional step of DNase 1 digestion (30 U/ml for 30 min at 37°C). One microgram of RNA was used as the template for RT-PCR using an RT-PCR kit (Roche) according to the manufacturer's directions. One microliter, equivalent to 2% of the completed reaction, was used as a template for a second round of PCR. The products of the second reaction were analyzed by agarose gel electrophoresis.

Immunofluorescent staining

For microscopy C127 cells were seeded onto acid-washed no. 01 coverslips in 24-well plates at a density of 10^5 cells/well and cultured overnight. VLPs were attached to cells at a concentration of 5 μg/ml in PBS, 2% FBS for 60 min on ice. Unbound VLPs were removed by washing and cells were incubated in complete medium at 37°C for the specified time. Cells were fixed and processed for microscopy as previously described (Day et al., 1998). For double immunofluorescent staining involving primary antibodies from different host species, the antibodies were incubated in unison. For double staining using 5B6 and a second mouse antibody, biotinylated 5B6 was used and detected with Texas red conjugated streptavidin after completion of the staining procedure for the other antibody. Fluorescence was examined with a Bio-Rad MRC 1024 laser scanning confocal system attached to a Zeiss Axioplan microscope. All the images were acquired with a Zeiss 63× N.A. 1.4 Planapo objective. The use of control coverslips established that fluorescence in the green and red channels was not overlapping and that antibody binding was specific for the intended antigen. The images were collaged and subjected to scale adjustment with Adobe Photoshop software. For flow cytometric analysis VLPs, 5 μg/ml in PBS, 2% FBS, were bound to 1×10^6 C127 cells in suspension with rocking at 4°C for 60 min. Unbound VLPs were removed by extensive washing with PBS. Prewarmed 37°C media was added to the cells and time points were taken at the indicated times. Cells were stained with 5B6 and FITC-conjugated donkey anti-mouse IgG in PBS, 2% FBS, 0.1% NaN_3. The logarithmic fluorescent intensity was analyzed on a FACSCalibur flow cytometer (Becton–Dickinson).

ELISA

During the flow cytometric analysis experiment described above, the supernatants from each time course were

reserved to determine the off rate and quantify the amount of VLPs that dissociate from the cell surface prior to internalization. The reactivity of 5B6 against the BPV-1 VLP preparation was determined as previously described (Wideroff et al., 1995). Two hundred microliters of the supernatant (400 μ_l total volume) was bound to the plate (Immunolon 2HB, Dynex Technologies). The 5B6 ascites were used at a dilution of 5×10^4. The detection of VLPs at this dilution of antibody was determined to be sensitive down to 5 ng of antigen.

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