Filamentous particle formation by human parainfluenza virus type 2

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Some paramyxoviruses form long filamentous virus particles; however, the determinants of filament formation and the role of such particles in virus transmission and pathogenicity are not clearly defined. By using conventional immunofluorescence microscopy, we found that human parainfluenza virus type 2 (HPIV2) forms filamentous particles ranging from 5 to 15 µm in length in virus-infected, polarized epithelial cells. The formation of filamentous particles was found to be virus type-specific and was not observed when the same cell types were infected with parainfluenza virus type 3 or Sendai virus, suggesting that different paramyxovirus genera exhibit distinct morphological properties. HPIV2 filamentous particle formation was found to be inhibited by cytochalasin D (CD) or jasplakinolide treatment in a dose-dependent manner. In the presence of 4 µg/ml CD or 1 µM jasplakinolide, the formation of filamentous particles was completely abolished, although similar haemagglutination and p.f.u. titres of virus were found to be released into the culture medium at 24 h post-infection. These observations indicate that host cell components, including the actin microfilament network, are important determinants of the morphology of parainfluenza viruses. The predominance of filamentous particles in polarized epithelial cells may reflect specific pathogenic roles of these particles in infection of human epithelial tissues.

Introduction

The Paramyxoviridae are enveloped, negative-stranded RNA viruses. This family includes the human parainfluenza viruses (PIVs), important human pathogens that cause serious upper and lower respiratory tract infections, especially in infants and small children. Together with respiratory syncytial virus (RSV), PIVs are the most frequent pathogens isolated in children with lower respiratory tract infections. There are three genera in the subfamily Paramyxovirinae, which were classified according to the size and shape of the nucleocapsids, the organization of the genome, the biological activities of the proteins and the sequence relationship of the encoded proteins (Lamb & Kolakofsky, 1996). Human parainfluenza virus type 2 (HPIV2) and simian virus 5 (SV5) are classified in the genus Rubulavirus, while human parainfluenza virus type 3 (HPIV3) and Sendai virus (SeV) belong to another genus, Respirovirus. No differences in virion morphology between these genera have been reported previously. Negatively stained influenza virions are usually pleomorphic enveloped particles about 150 to 300 nm in diameter (Choppin & Compans, 1975; Vainionpaa & Hyypia, 1994). Filamentous virions have been described in several enveloped viruses that infect the respiratory tract, such as influenza viruses (Ada et al., 1958; Roberts & Compans, 1998; Smirnov et al., 1991) and RSV (Armstrong et al., 1962; Berthiaume et al., 1974; Roberts et al., 1995). Thin sections of cells infected with some PIVs including HPIV2 and SV5 have also shown the presence of budding or released filamentous particles (Bäch & Howe, 1973; Compans et al., 1966; Howe et al., 1967), but the determinants of filamentous versus spherical virus morphology for this virus family have not been investigated. Several reports have provided evidence that the morphology of influenza viruses may be modulated by alteration of specific viral structural gene products (Enami & Enami, 1996; Jin et al., 1997; Hughey et al., 1995; Roberts et al., 1998; Smirnov et al., 1991).

The cellular cytoskeleton has been reported to play an important role in transcription, maturation, morphogenesis and budding of a number of enveloped viruses, including Newcastle disease virus (NDV), RSV, SeV and measles virus in the family Paramyxoviridae (Cudmore et al., 1997). Paramyxovirus virion formation and budding are dependent on actin assembly, which may provide a means for structural diversification of influenza virus particles

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viruses were among the first viruses to be reported to contain actin as a component within the virion (Orvell, 1978; Sundqvist & Ehrnst, 1976; Tyrrell & Norrby, 1978; Wang et al., 1976); however, its function in virus structure and replication is not understood. Actin occurs in two forms, a globular, monomeric form that represents the soluble pool of actin and a filamentous form that constitutes the actin microfilaments of the cytoskeletal framework. Actin microfilaments have been reported to be involved in the movement of viral envelope proteins to the cell surface, in communication between envelope proteins and the nucleocapsids in the plasma membrane and in virus budding (Bohn et al., 1986; Stallcup et al., 1983; Tyrrell & Ehrnst, 1979). It was also suggested that the M protein of paramyxoviruses is essential for the incorporation of actin within the virion (Giuffre et al., 1982). In measles virus infection, actin-like microfilaments were reported to project into developing particles at the cell membrane and actin was also found to be present in released measles virions (Bohn et al., 1986). Therefore, it was of interest to investigate further whether the cellular cytoskeleton has any effect on PIV morphology and virus release.

In this study, surface immunofluorescence was used to analyse PIV filament formation. We compared the formation of filamentous virus particles by different PIVs in polarized and non-polarized epithelial cells. Furthermore, we studied the effects of cytochalasin D (CD), an actin-disrupting agent, and jasplakinolide, a potent inducer of actin polymerization, on virus morphology and virus release.

Methods

**Cells, viruses and antibodies.** Monkey kidney Vero C1008, Vero 76 and Madin–Darby canine kidney (MDCK) cells were grown in Dulbecco’s minimal essential medium (DMEM) with 10% foetal bovine serum (FBS) (HyClone Laboratories). HPIV2 stock virus was grown in LLC-MK2 cells, HPIV3 was grown on Vero 76 cells and SeV was grown on MDCK cells in the presence of 1 μg/ml TPCK-treated trypsin (Sigma, type XIII). Titres of the viruses were determined by plaque assay in Vero 76 cells for HPIV2 and HPIV3 and in MDCK cells in the presence of 1 μg/ml trypsin for SeV. Guinea pig anti-HPIV2 and anti-HPIV3 antisera were obtained from the Division of Research and Resources, National Institutes of Health (Bethesda, MD, USA). Rabbit anti-SeV antisera was kindly provided by Laurent Roux (Université de Genève, Geneva, Switzerland).

**Haemagglutination (HA) assay.** HA titres of the released virus particles were determined by incubating equal volumes of serial twofold dilutions of culture medium in PBS-def (PBS deficient in Mg$^{2+}$ and Ca$^{2+}$) with guinea pig red blood cells (final concentration 0.25%) for 1 h at 4 °C.

**Plaque assay.** Serial tenfold dilutions of the culture media collected at designated time-points were added to monolayers of Vero 76 cells. After 1 h incubation at 37 °C, the inoculum was removed and overlaid with 2% white agar mixed at a 1:1 ratio with 2 × DMEM containing 4% FBS. Four days after overlaying, 0.05% neutral red stain was added onto the agar. Plaques were counted after 6 h.

**Virus infection.** Cells were grown to 80% confluence on 12 mm glass coverslips for immunofluorescence studies or 60 mm plastic dishes for virus yield studies and were inoculated with 1 p.f.u. of HPIV2 or HPIV3 stocks per cell. After adsorption for 1 h at 37 °C, the inoculum was removed and the cells were incubated in DMEM supplemented with 2% FBS. A stock solution of CD (5 mg/ml in DMSO, Sigma) was diluted 100-fold and jasplakinolide (500 mM in DMSO, Molecular Probes) was diluted 10-fold in medium just prior to use, added to the virus culture medium after the 1 h incubation period and maintained throughout the infection period. Control cells were incubated with the respective amount of DMSO.

**35S-radiolabelling of HPIV2 and SDS–PAGE.** Vero C1008 cells were grown to 80% confluence on 100 mm tissue culture dishes and were inoculated with 1 p.f.u. of HPIV2 stock per cell. After adsorption for 1 h at 37 °C, the inoculum was removed and the cells were incubated in DMEM supplemented with 2% FBS. CD (5 μg/ml) was added to the culture medium after the 1 h incubation period and maintained throughout the infection period. At 12 h post-infection (p.i.), cells were labelled continuously with 50 μCi [35S]methionine/[35S]cysteine per ml of a mixture of 75% methionine-deficient medium and 25% complete medium in the presence of CD. At 24 h labelling, the supernatant was collected and centrifuged at 2500 r.p.m. in a desktop centrifuge to spin down cell debris. Virus was then pelleted at 120000 g for 1 h and resuspended at 4 °C overnight in a small volume of PBS. Viruses were further purified through a 30–60% sucrose cushion at 240000 g for 1 h. Purified virions were collected from the interface of the sucrose cushion, diluted in PBS and pelleted at 120000 g for 1 h. Pelleted virions were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 1% Triton X-100, 1% SDS, 1 mM EDTA) and the viral protein profile was analysed by SDS–PAGE under reducing conditions followed by autoradiography.

**Surface immunofluorescence.** Vero 76, Vero C1008 or MDCK cells grown on 12 mm glass coverslips were infected with HPIV2, HPIV3 or SeV at an m.o.i. of 1 p.f.u. per cell. At 24 or 48 h p.i., cells were washed three times with ice-cold PBS. Guinea pig anti-HPIV2 antisera at a dilution of 1:500, guinea pig anti-HPIV3 antisera at a dilution of 1:50 or rabbit anti-SeV antisera at a dilution of 1:50 was added onto cell monolayers and cells were then incubated at 4 °C for 30 min. Cells were washed three times with ice-cold PBS and then FITC-conjugated goat anti-guinea pig IgG antibody or rhodamine-conjugated goat anti-rabbit IgG antibody (Southern Biotechnology Associates), at a dilution of 1:100, was added and the monolayers were incubated at 4 °C for 30 min. Cells were then washed and fixed with 2% paraformaldehyde in PBS. Cell surface fluorescence was examined by fluorescence microscopy with a Nikon Optiphot microscope.

**Electron microscopy.** HPIV2- or HPIV3-infected Vero C1008 cells were examined by thin-section electron microscopy at 48 h p.i. Cells were fixed with buffered 1% glutaraldehyde for 30 min, post-fixed for 1 h with 1% osmium tetroxide, dehydrated with a graded ethanol series and embedded for electron microscopy. Thin sections were prepared on a Reichert ultramicrotome, mounted on 300-mesh copper grids, stained with uranyl acetate and lead citrate and examined with a Philips CM10 electron microscope (EM). For negative staining, virions in culture medium were allowed to adhere to carbon/formvar grids and stained with 1% ammonium phosphotungstic acid, pH 7.4. Specimens were viewed with a Philips CM10 EM.

Results

**Filamentous virus particle formation by HPIV2**

To determine whether the production of filamentous HPIV2 virions could be observed by light microscopy, indirect
Filamentous particles in human PIV

Fig. 1. (a) Visualization of filamentous HPIV2 particle formation in Vero C1008 cells by surface immunofluorescence. Cell surface immunofluorescence staining was performed in Vero C1008 cells at 24 h p.i. Cells were incubated at 4 °C for 30 min with guinea pig polyclonal antibody against HPIV2 at 1:500 dilution and then FITC-conjugated anti-guinea pig secondary antibody at 1:100 for another 30 min. Cells were then fixed in 2% paraformaldehyde in PBS and viewed with a Nikon Optiphot microscope. (b) Thin section of HPIV2-infected, polarized Vero C1008 cells showing virus filaments projecting from the cell surface at 48 h p.i. (c) Non-polarized Vero 76 cells infected by HPIV2 showing short, elongated fluorescent particles.

Fig. 2. Polarized Vero C1008 cells infected with HPIV3 or SeV. (a) Vero C1008 cells infected with HPIV3. Cell surface immunofluorescence staining was done at 24 h p.i. Cells were incubated at 4 °C for 30 min with guinea pig polyclonal antibody against HPIV3 at 1:50 dilution and then FITC-conjugated anti-guinea pig secondary antibody at 1:100 for another 30 min. (b) Thin section showing spherical virus particles associated with surfaces of HPIV3-infected Vero C1008 cells at 48 h p.i. (c) Vero C1008 cells infected with SeV. Cell surface immunofluorescence staining was done at 48 h p.i. Cells were incubated at 4 °C for 30 min with rabbit polyclonal antibody against SeV at 1:50 dilution and then FITC-conjugated goat anti-rabbit secondary antibody at 1:100 for another 30 min. Cells were then fixed in 2% paraformaldehyde in PBS and viewed with a Nikon Optiphot microscope.

cell surface immunofluorescence was performed on infected Vero C1008 cells at 24 h p.i. As shown in Fig. 1(a), long filamentous HPIV2 particles ranging from 5 to 15 µm in length were observed by immunofluorescence on the surfaces of the infected epithelial cells. Similar filamentous HPIV2 virions were also seen on the surfaces of HPIV2-infected MDCK cells (data not shown).

To confirm that the filamentous particles observed by immunofluorescence were HPIV2 virions, we also examined thin sections of infected Vero C1008 cells by electron
microscopy and observed the presence of filamentous virus particles. A long HPIV2 virion cut in tangential section at the surface of an infected cell is shown in Fig. 1(a) and contains electron-dense, herringbone-like nucleocapsids surrounded by an envelope with glycoprotein spikes. This result indicates that the filamentous structures observed by immunofluorescence correspond to filamentous virions.

The formation of long filamentous HPIV2 virions was found to be host cell dependent. As can be seen in Fig. 1(a), long filamentous HPIV2 particles were observed predominantly in polarized epithelial cells, and the lengths of particles were up to 15 μm. In contrast, only short, elongated virus particles were seen in Vero 76 cells (Fig. 1(c)). This result indicates that the polarized cells preferentially support the production of long, filamentous HPIV2 virions.

Absence of filament formation in HPIV3 and SeV virions

In order to determine whether filament formation is a general property of other paramyxoviruses, Vero 76, Vero C1008 and MDCK cells were infected with HPIV3 or SeV and were examined by indirect surface immunofluorescence. Fig. 2(a) shows that HPIV3-infected Vero C1008 cells exhibited only punctate surface fluorescence, and a similar fluorescence pattern was also seen in HPIV3-infected MDCK and Vero 76 cells. When EM sections of HPIV3-infected cells were examined, spherical viral particles 0.2 to 0.5 μm in diameter were observed (Fig. 2(b)). Fig. 2(c) shows that only punctate surface fluorescence could be seen in SeV-infected Vero C1008 cells as well. No budding of filamentous particles was observed in SeV-infected Vero 76 or MDCK cells (not shown). Therefore, the formation of filamentous virions is a property of at least two PIVs in the genus Rubulavirus, but was not observed in two members of the genus Respirovirus.

Disruption of the actin microfilament array abolishes HPIV2 filamentous particle formation

To determine whether actin filaments are required for HPIV2 filamentous particle formation, we examined the effects of CD, an actin microfilament-disrupting agent. We found that CD had a dose-dependent inhibitory effect on filament formation. By increasing the CD concentration in the culture medium to 0.5, 1, 2, 4 or 8 μg/ml, a progressive decrease in formation of filamentous particles was observed on the cell surfaces. At CD concentrations of 4 μg/ml or above, no filamentous particles were observed on the cell surface. The pattern of surface immunofluorescence of HPIV2-infected cells changed from numerous filaments to patch-like aggregates of antigen in the presence of CD (Fig. 3(b)). Although this pattern of fluorescence staining differs from the punctate pattern observed with respirovirus-infected cells, no filamentous particles were observed. These results indicate that disruption of the actin microfilament array alters virus antigen distribution in the cells and that the integrity of the actin microfilament network is an important determinant of the formation of filamentous HPIV2 particles.

Jasplakinolide is a macrocyclic peptide that is a potent inducer of actin polymerization in vitro, whereas CD is a natural compound that binds to actin and alters its polymerization. To determine whether jasplakinolide also affects HPIV2 filamentous particle formation by altering the actin microfilaments in a different way from CD, jasplakinolide was added to HPIV2-infected Vero C1008 cell cultures at 100 nM, 1 μM or 10 μM. At 24 h p.i., surface immunofluorescence staining was performed. Decreased numbers of filamentous particles and increasing levels of punctate fluorescence were found in the presence of the compound (Fig. 3(c)). The inhibition of filamentous particle formation by jasplakinolide was also observed to be dose dependent. This result again supports the
Table 1. CD does not reduce HPIV2 specific infectivity

<table>
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<th>Treatment</th>
<th>24 h p.i.</th>
<th>48 h p.i.</th>
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<tr>
<td></td>
<td>p.f.u./ml</td>
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<tr>
<td>- CD</td>
<td>6.5 × 10^5</td>
<td>32</td>
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<tr>
<td>+ CD</td>
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CD and jasplakinolide affect virus morphology but not virus release

To investigate whether the inhibition of HPIV2 filament formation by CD or jasplakinolide reflected an inhibition of virion assembly or budding at the cell surface, released virus HA and infectivity titres were determined. HPIV2-infected culture media were collected at 24 and 48 h.p.i. in the presence or absence of CD treatment. The HA titres of the released viruses were not affected by the CD treatment at 24 h.p.i., but were reduced at 48 h.p.i. (Table 1). Virus infectivity titres in the culture in the presence of CD were only about twofold lower at 24 h.p.i. compared with the control culture infected in the absence of CD. HPIV2 virus production in the untreated culture increased further by about fivefold from 24 to 48 h.p.i., whereas there was no corresponding increase in virus yield in the CD-treated culture. The absence of a further increase in titre after 24 h may result from an effect of CD on the viability of the cells.

As filamentous and spherical particles may differ in their specific infectivity, we also compared the p.f.u. and HA titres of released particles in the presence or absence of CD. Our results (Table 1) show that an increase in infectious units from 24 to 48 h.p.i. corresponded to an increase in HA titre in the absence of the CD treatment. Similarly, a decrease in p.f.u. from 24 to 48 h.p.i. also corresponded to a decline in HA titre in the presence of CD treatment. Thus, the ratio of p.f.u. to HA did not differ substantially in the presence or absence of CD treatment, either at 24 or 48 h.p.i. Since the HA titre is a measure of the total number of virus particles released into the medium, these results indicate that the infectivities of filamentous and spherical particles are similar. The effect of jasplakinolide on HPIV2 virus release was also tested on the HA and p.f.u. titres in the culture supernatant at 24 h.p.i. and the results were similar to those observed with CD (data not shown). Taken together, these results suggest that the morphologically different forms of virions produced in the presence or absence of CD or jasplakinolide are similar in infectivity and that the actin-disrupting agents do not prevent assembly and release of spherical particles.

To determine whether there were any differences in viral protein profiles in the presence of CD treatment, HPIV2 virus produced in the presence or absence of CD was radiolabelled with [35S]methionine/[35S]cysteine, purified and analysed by SDS–PAGE. By comparing control HPIV2 protein profiles with those of HPIV2 produced in the presence of CD, the major viral proteins present in both preparations were found to be similar in profile and relative amounts (data not shown). This result indicates that the change in virus morphology observed as a result of altering the actin microfilament network does not involve changes in parainfluenza virion protein incorporation or virus infectivity.

Discussion

We have observed that HPIV2 forms long filamentous particles ranging from 5 to 15 µm in polarized Vero C1008 or MDCK cells. Previous studies have shown that SV5, another member of the genus Rubulavirus, also forms large numbers of filamentous particles (Compans et al., 1966). In contrast, with two members of the genus Respirovirus, HPIV3 and SeV, we did not observe the formation of filamentous particles in polarized epithelial cells. The morphological differences between HPIV2, HPIV3 and SeV could be readily distinguished by immunofluorescence light microscopy. Previous studies have also shown that two members of the genus Pneumovirus, pneumonia virus of mice and RSV, both form filamentous particles readily (Compans et al., 1967; Miyata et al., 1995; Roberts et al., 1995). Thus, the present results indicate that viruses in different paramyxovirus genera differ in their morphological properties.

Although influenza virus morphology is known to be determined largely genetically (Choppin, 1963; Kilbourne, 1963; Mitnau et al., 1996; Roberts et al., 1998; Smirnov et al., 1991), recent studies (Roberts & Compans, 1998) have provided evidence that host cell factors are also important determinants of the formation of filamentous particles. It has been reported that filamentous influenza particles exhibit a higher specific infectivity and a higher RNA content than spherical virions (Ada et al., 1958; Smirnov et al., 1991). One or
more structural gene products of influenza virus has been linked to the formation of virus filaments (Choppin, 1963; Kilbourne, 1963; Mitnaul et al., 1996; Smirnov et al., 1991; Nishimura et al., 1990; Roberts et al., 1998). Our observation that HPIV2 forms filamentous particles preferentially in polarized epithelial cells is similar to recent results with influenza viruses (Roberts & Compan, 1998), indicating that the host cell is a determinant of filamentous virus production by both of these viruses. It is interesting that the primary site of PIV infection is the mucosal surface of the upper respiratory tract. Thus, it is likely that the polarized phenotype of cells in the respiratory tract is compatible with virus filament formation. Furthermore, we found that CD, an actin microfilament-depolymerizing agent, or the macrocyclic peptide jasplakinolide, a potent inducer of actin polymerization, inhibited HPIV2 filamentous particle formation but had little effect on the HA or p.f.u. titres of released virus particles. These results indicate that the host cell type and the integrity of the cytoskeleton play important roles in PIV morphology.

Filamentous virus morphology may give some advantage to a virus in infecting cells or evading immune responses of the host. Many respiratory tract virus pathogens are filamentous, such as influenza virus and RSV. By their elongated particle size, filamentous particles may be able to infect neighbouring cells prior to release and therefore resist upward mucociliary removal of fluids from the respiratory tract (Roberts & Compan, 1998). A recent report demonstrated that intra-cellular vaccinia virions can utilize host actin microfilaments to project themselves out of the cytoplasm and into neighbouring cells (Cudmore et al., 1995). Filamentous paramyxovirus particles similarly may be able to mediate infection of neighbouring cells. In contrast, spherical particles may be more readily incorporated into aerosols and may play a more important role in person-to-person transmission, while filamentous particles may be important in cell-to-cell transmission, as suggested for influenza virus (Roberts & Compan, 1998).

The finding of actin in many enveloped viruses (Damsky et al., 1977; Naito & Matsumoto, 1976; Wang et al., 1976) triggered studies of the role of the cellular cytoskeleton in virus assembly. Although the cytoskeleton is thought to be involved at some stage in the morphogenesis of enveloped viruses, its exact role has not been defined. It has been reported that treatment of infected cells with CD has little direct effect upon the assembly and release of vesicular stomatitis virus (Gentry & Bussereau, 1980) or influenza virus (Griffin & Compan, 1979; Griffin et al., 1983; Roberts & Compan, 1998). However, treatment of influenza virus-infected cells with CD was found to abolish filamentous particle formation (Roberts & Compan, 1998). Also, release of measles virus particles was inhibited in CD-treated infected cells (Stallcup et al., 1983). CD also caused a blockage in the final release of enveloped vaccinia virus from the cell surface (Payne & Kristensen, 1982) and inhibited the release of murine leukaemia virus (Mousa et al., 1978) and New World hantaviruses (Ravkov et al., 1998). Other studies have reported that CD can stimulate release of some viruses, such as NDV and rotavirus (Bass et al., 1995; Bedows et al., 1983; Morrison & McGinnes, 1985). The role of actin as a determinant of virus assembly may also depend on the virus and cell type. There was no obvious structural rearrangement of F-actin in influenza virus-infected cells (Roberts & Compan, 1998), which contrasts with results in human immunodeficiency virus (HIV)- and vaccinia virus-infected cells. In HIV-infected epithelial cells, F-actin was redistributed into pseudopods and HIV was preferentially released from the pseudopod (Pearce-Pratt et al., 1994). Actin filaments in vaccinia virus-infected cells formed actin tails and projections and the virus particles were propelled to the neighbouring cells on their tips (Cudmore et al., 1995). We did not observe disrupted actin microfilament arrays in HPIV2-infected cells, nor did we observe co-localization of actin with the filamentous virus particles on cell surfaces. This may indicate why there was no marked inhibition of virus release by CD treatment.

Jasplakinolide is a membrane-permeable, F-actin-stabilizing drug. It competes with phalloidin for binding and enhances actin polymerization by inhibiting the depolymerization of actin filaments, leading to a change in actin filament dynamics (Bubb et al., 1994; Senderowicz et al., 1995; Lee et al., 1998). Cells that are treated with jasplakinolide form F-actin aggregates because of their inability to depolymerize the stabilized actin filaments at a normal rate. It was also reported that rearrangement of the actin cytoskeleton of plant cells resulted from treatment with jasplakinolide (Sawitzky et al., 1999). Therefore, treatment with jasplakinolide will alter the normal cellular actin cytoskeleton functions. However, no effects of jasplakinolide on virus morphology or virus assembly have been reported. Here, we found that jasplakinolide can also abolish the filamentous morphology of HPIV2. The patchy fluorescence pattern of viral antigens in the CD- or jasplakinolide-treated, HPIV2-infected cells may also result from disrupted or aggregated cytoskeletal structures. The normal cytoskeleton plays an important role in establishment and maintenance of epithelial cell polarity, and polarized cells preferentially support filamentous virus particle formation. We therefore suggest that CD and jasplakinolide abolish filamentous particle formation by altering the polarized phenotype of the epithelial host cells and the normal function of the cellular cytoskeleton.

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References


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