Role of the actin cytoskeleton during influenza virus internalization into polarized epithelial cells

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Summary
The in vivo site of influenza virus infection is a polarized epithelium, and it is well established that the virus preferentially enters from the apical surface of polarized epithelial cells; however, many of the molecular events involved during the endocytosis of the virus into polarized epithelia remain unclear. Here we examined the role of actin microfilaments and the myosin VI motor protein during influenza entry into a panel of polarized and non-polarized cells. By treatment of cells with cytochalasin D and jasplakinolide, we show that influenza virus entry into all the polarized epithelial cells tested requires actin dynamics, with a specific role for the actin cytoskeleton in the process of virus internalization from the plasma membrane. In contrast, influenza could still efficiently enter and infect all non-polarized cells tested after disruption or stabilization of the actin cytoskeleton. To examine the role of the actin motor protein, myosin VI, we expressed a dominant-negative construct in both polarized and non-polarized cells. Influenza virus infectivity in myosin VI tail mutant-transfected cells was significantly decreased in polarized epithelial cells, but not in non-polarized cells. As a whole, our data suggest indispensable roles of a dynamic actin cytoskeleton for influenza virus entry into polarized epithelial cells, a feature not shared with non-polarized cells.

Introduction
Influenza virus is a common pathogen of the upper respiratory tract of humans and a wide variety of animal species. It is the member of Orthomyxoviridae and includes three basic types: influenza A, B and C viruses (Cox and Kawaoka, 1998; Lamb and Krug, 2001). During virus infection, influenza virus first binds to cell surface sialic acid receptors followed by receptor-mediated endocytosis (Matlin et al., 1981; Yoshimura et al., 1982; Yoshimura and Ohnishi, 1984; Marsh and Helenius, 1989; Skehel and Wiley, 2000), with the virus apparently being able to use either clathrin-mediated endocytosis, or a clathrin and caveolin-independent route of entry (Sieczkarski and Whittaker, 2002; Rust et al., 2004). The acidic environment of the late endosome then triggers viral fusion and uncoating (Matlin et al., 1981; Yoshimura et al., 1982; Yoshimura and Ohnishi, 1984; Skehel et al., 1995; Bui et al., 1996; Sieczkarski and Whittaker, 2003), and the uncoated viral ribonucleoproteins are released into the cytosol before being transported into the nucleus to initiate virus replication (Martin and Helenius, 1991).

Intracellular trafficking of influenza virus has been explored as a model of host–pathogen interaction, culminating in the recent characterization of the uptake of individual influenza virions (Lakadamyali et al., 2003). Notably however, such molecular studies have been carried out using influenza virus infection in non-polarized cell lines and such cells are not the in vivo target of viral infection, which is the polarized respiratory epithelium (Matrosovich et al., 2004). Since there are significant differences between polarized and non-polarized cells with regard to receptor distribution, cytoskeletal structure and the mechanism of endocytosis, it is possible that our current knowledge of influenza virus intracellular trafficking does not completely apply to in vivo viral infection. An understanding of influenza virus entry into polarized cells will not only extend our understanding of in vivo viral entry mechanism, but also help exploit possible new targets for antiviral therapy.

For epithelial cells, the actin cytoskeleton is important for establishment and maintenance of cell polarity. During cell development, cell-cell and cell-extracellular matrix contacts stimulate the reorganization of actin network (Yeaman et al., 1999). Actin forms cables both in apical microvilli and at the basolateral side of the cell, and immediately beneath the microvilli is the actin meshwork of the terminal web (Hirokawa et al., 1982). Although the importance of the cytoskeleton in cell motility, mitosis and vesicles transport has been well recognized, the role of actin in endocytosis is not unequivocal due to the discrepancy of the studies in different cell lines (Fujimoto et al., 2000). So far, the most accepted view is that actin is important for apical endocytosis in polarized cells, but is...
dispensable for endocytosis at the basolateral surface of polarized cells and in non-polarized cells (Gottlieb et al., 1993; Jackman et al., 1994; Maples et al., 1997). A study of influenza virus entry involving tracking individual, DiD-labelled virus particles in living CHO cells showed that actin filaments and microtubules were involved in stepwise manner during viral endocytosis (Lakadamyali et al., 2003). However, this study did not connect actin- and microtubule-dependent trafficking with viral infectivity and was carried out in non-polarized cells. It is quite possible that in non-polarized cells influenza virus trafficking between membrane compartments can be facilitated by the existence of intact cytoskeleton structure, but is not absolutely required for viral infection – in contrast to the situation in polarized cell types.

There are several possible explanations for the requirement of actin filaments during apical endocytosis in polarized epithelial cells. These include promoting membrane deformation, facilitating endocytic vesicle transportation, as well as severing of invaginated vesicles (da Costa et al., 2003). Recently, the actin motor protein myosin VI has been analysed extensively, and its role in endocytosis has been partially revealed (Hasson, 2003). Like other proteins in the myosin family, myosin VI consists of four structural domains, which include an N-terminal conserved motor domain, a single IQ motif for interacting with the calcium-binding protein calmodulin, and a coiled-coil domain for dimerization, as well as C-terminal globular tail domain involved in cargo binding (Hasson and Mooseker, 1994). However, unlike other members in the myosin family, myosin VI moves backwards – towards the minus ends of actin filaments (Wells et al., 1999). Based on the fact that actin filaments tend to be oriented with their plus ends at the cell periphery and minus ends pointing inwards, it has been hypothesized that trafficking of endocytic vesicles could be facilitated by myosin VI (Aschenbrenner et al., 2003).

In this article, we carried out a systematic analysis of the mechanism of influenza virus entry into a panel of polarized epithelial cells, as well as non-polarized cells. Using both cell biological and biochemical techniques, we find that influenza virus entry into polarized cells is absolutely actin-dependent, with virus entry arrested at the level of virus internalization at the plasma membrane. In contrast, actin played a dispensable role in non-polarized cells. We also show that the actin motor protein, myosin VI, plays an important role in influenza virus infection of polarized cells.

**Results**

*Influenza virus infection is actin-dependent in polarized cells, but not in non-polarized cells*

To study the entry mechanism of influenza virus into polarized cells and to compare it with that in non-polarized cells, we chose a panel of several polarized epithelial cells, as well as non-polarized cells, for viral infection. The polarity of the polarized epithelial cells was confirmed by the detection of transepithelial electrical resistance > 300 Ω cm², by the expression of the tight junction protein ZO-1, and by the failure of cells to be infected by VSV when added from the upper (apical) chamber (data not shown). Among the cell lines we tested, MDCKII, LLC-PK1::μ1B, Calu-3, and Caco-2 were all considered well-polarized cells based on our criteria. However, other cell lines including NCI-H292, A549, H441, H1299, which have all been reported as models for polarized cells, showed discontinuous tight junction protein ZO-1 staining when grown on filters, and were infectable by VSV from the upper chamber, and so were not used further in this study. The non-polarized cells BHK, CHO, HeLa and Mv1 Lu cells were also used for viral infection, and typically gave a resistance of approximately 20–25 Ω cm² when grown on filters.

We first examined the effects of the actin-disrupting drug cytochalasin D. We carried out a dose–response experiment in all the polarized cell types used, using TRITC-phalloidin to visualize the integrity of the actin cytoskeleton and ZO-1 labelling to visualize tight junctions. A typical example for actin-disruption is shown in Fig. 1, which depicts MDCKII cells treated with 20 μM cytochalasin D. Under these conditions, there is a distinct loss of actin cables, as well as a loss of integrity of tight junctions. This concentration of drug was sufficient to induce essentially the same effects in LLC-PK1::μ1B, Calu-3 and Caco-2 cells (not shown), as well as a distinct loss of actin cables in BHK, CHO, HeLa and Mv1 Lu cells (not shown).

To determine the effects of cytochalasin D on influenza virus infection, cells were treated with drug for 30 min before infection. Influenza viruses (1–5 pfu cell⁻¹) were incubated with actin-disrupted cells, or control cells that were not drug-treated, for 30 min at 37°C followed by 4 h infection in the presence of 20 μM monensin to block any further viral entry once cytochalasin D was withdrawn. Actin-disruption in all the well-polarized cells tested (MDCKII, LLC-PK1::μ1B, Calu-3 and Caco-2) resulted in a significant decrease in viral infectivity as measured by single-hit infection assays of nuclear NP by immunofluorescence microscopy (Fig. 2A). In all cases, there were minimal numbers of cells where the viral NP was localized to the nucleus in actin-disrupted polarized epithelial cells. In the presence of cytochalasin D, viruses appeared to be arrested in a punctate distribution in the cell periphery. These data are quantified in Fig. 2B. In contrast to the situation with polarized cells, non-polarized cells (BHK, CHO, HeLa and Mv1 Lu) could still be fully infected after actin filaments were disrupted with cytochalasin D (Fig. 2C). In some cases
Fig. 1. Effects of cytochalasin on the actin cytoskeleton. MDCKII cells were pretreated with 20 μM cytochalasin D for 30 min at 37°C (+CD), or were untreated. The actin cytoskeleton was visualized with TRITC-phalloidin (red) and tight junctions labelled with anti-ZO-1 antibody (green). Samples were processed by deconvolution microscopy, and representative merged slices shown.

Fig. 2. The actin cytoskeleton is crucial for influenza virus infection in polarized cells, but dispensable for influenza virus infection in non-polarized cells.

A. Polarized MDCKII, LLC-PK1::m1B, Calu-3 and Caco-2 cells were pretreated with 20 μM cytochalasin D for 30 min at 37°C and then infected with influenza virus at 1–5 moi of infectious unit per cell. The cells were analysed by indirect immunofluorescence microscopy at 4 h post infection using mouse anti-NP, and rat or rabbit anti-ZO-1 antibodies. Secondary antibodies included Alexa 488-labelled (green) anti-mouse IgG and Alexa 568-labelled (red) anti-rat or anti-rabbit IgG.

B. For quantification, > 100 cells were scored in three independent experiments. Error bars represent the standard deviation of the mean.

C. Non-polarized BHK, CHO, Mv1 Lu and HeLa cells were pretreated with 20 μM cytochalasin D for 30 min at 37°C and then infected with influenza virus at 1–5 moi of infectious unit per cell. The cells were analysed by indirect immunofluorescence microscopy at 4 h post infection using mouse anti-NP antibody and Alexa 488-labelled anti-mouse IgG.

D. For quantification, > 100 cells were scored in three independent experiments. Error bars represent the standard deviation of the mean.
there was a small increase in the level of infection in the presence of cytochalasin D; the reason for this is unknown. These data are quantified in Fig. 2D. Overall, these results indicate that actin microfilaments play different roles in influenza virus infection in polarized epithelial cells and non-polarized cells. In contrast to their dispensable role in viral infection of non-polarized cells, intact actin filaments are obligatory for influenza virus infection in polarized epithelial cells.

As the inhibitory role of cytochalasin D treatment in influenza virus infection was universal in all well-polarized cells tested, we decided to use MDCKII cells as a representative polarized cell type in our following studies with this agent because of their uniform morphology and the relative simplicity of maintenance. As our results demonstrated an effect of cytochalasin D on infection of polarized cells, we wished to determine how the effects of the drug were manifested. We first examined virus binding. MDCKII cells were treated with 20 μM cytochalasin D for 30 min, and were exposed to influenza virus at 4°C for 60 min. Bound virus was then visualized by immunofluorescence microscopy, and quantified (Fig. 3A and B). As expected, there was no significant difference in the binding levels of the virus when the actin cytoskeleton was disrupted with cytochalasin D (data not shown). Thus, inhibition of actin polymerization by two distinct mechanisms showed similar effects of entry of influenza virus into polarized versus non-polarized cells. As cytochalasin D showed more specific inhibition, this was used for all subsequent inhibitor studies.

Influenza virus entry is inhibited by cytochalasin D during the early steps of endocytosis in polarized epithelial cells

To examine how influenza virus infection in actin-disrupted polarized cells was inhibited, we used confocal microscopy to examine the location of the viruses during endocytosis. We first used confocal microscopy to examine incoming viruses in the Z-dimension of MDCKII cells. Cells were infected with a high multiplicity of infection (moi) of virus (approximately 100 pfu cell⁻¹) in the presence or absence of cytochalasin D. Confocal sections were assembled into a Z-stack to show the relative location of viruses with respect to the apical and basal surfaces of the cell. In control MDCKII cells, influenza viruses were transported to the nucleus after 3 h of viral infection and viral replication was initiated, as indicated by the concentrated viral NP signal in the centre of the cell – a location that correlated with the position of the nucleus (Fig. 4a and c). However, when polarized epithelial cells were treated with cytochalasin D, influenza viruses failed to be transported to the nucleus for replication and instead viruses seemed to be trapped near the apical plasma membrane, which was revealed by the viral NP signal around the cell surface (Fig. 4b and d). The results indicated that intact actin cytoskeleton structure was crucial for influenza virus entry in polarized epithelial cells; when
Actin microfilaments were disrupted by cytochalasin D, influenza virus entry was arrested at the early stage of endocytosis.

Influenza viruses cannot internalize into polarized cells in which actin filaments have been disrupted

Due to the limited resolution of confocal microscopy, we could not determine whether the influenza virus trapped near the apical surface after cytochalasin D treatment was on the cell surface, or had been internalized into endocytic vesicles. To resolve this issue, we took a more biochemical approach, in combination with deconvolution microscopy. We labelled influenza viruses with biotin using a modification of a method developed to study SV40 internalization (Pelkmans et al., 2002). After biotin labelling, influenza viruses were still fully infectious based on haemagglutination and single-hit infection assays (not shown). One of the advantages of using biotinylated influenza in the study of virus entry is that the internalized viruses can be easily distinguished from the cell surface-bound viruses. After incubating biotinylated influenza with cells at 4°C for 1 h, cells are shifted to 37°C for different period of time to allow virus internalization. Then cells are transferred back to 4°C and are either treated with membrane-impermeable reducing agent (TCEP) to cleave the disulphide bonds in the conjugated biotin molecules or incubated with unconjugated streptavidin (2 mg ml\(^{-1}\)) to block biotin from surface-bound viruses. The internalized viruses could then be visualized by microscopy by using fluorescently conjugated streptavidin following fixation and permeabilization. From our preliminary studies, we found that preincubating cells with streptavidin was more efficient than TCEP treatment with regard to blocking biotin signal from uninternalized viruses (not shown). Therefore, the method of preincubating cells with streptavidin before fluorescence microscopy was used in all further studies.

We examined entry of biotinylated influenza virus in both MDCKII cells (polarized) and Mv1 Lu cells (non-polarized), which had been treated with cytochalasin D, or were untreated (Fig. 5). Biotinylated virus (approximately 100 pfu cell\(^{-1}\)) was bound to the cells at 4°C, and in all cases could be readily detected in the absence of streptavidin (not shown). After shifting to 37°C before streptavidin treatment, virus was detectable in internal vesicles of Mv1 Lu cells in the presence or absence of cytochalasin D (Fig. 5A and C), with increasing signal based on the time of temperature shift. However, for MDCKII cells, only limited numbers of internalized viruses were observed in the presence of cytochalasin D (Fig. 5B and D), whereas virus was efficiently internalized in control cells. The results confirmed our previous confocal microscopy data and showed that the infection block at actin-disrupted MDCKII cells was at the level of virus internalization.

The actin-stabilizing agent jasplakinolide also specifically blocks influenza virus entry into polarized cells versus non-polarized cells

Jasplakinolide is a cyclic peptide that binds to and stabilizes filamentous actin in vitro and enhances actin polymerization in vivo; it therefore has a very different mechanism of action to cytochalasin D. To determine the effects of jasplakinolide on influenza virus infection, cells were treated with drug for 30 min before infection. Influenza viruses (1–5 pfu cell\(^{-1}\)) were incubated with drug-treated cells, or control cells that were not drug-treated, for 30 min at 37°C followed by 4 h infection in the presence of 20 μM monensin to block any further viral entry once jasplakinolide was withdrawn. Actin stabilization in all the
well-polarized cells (MDCKII, Calu-3 and Caco-2) resulted in a marked decrease in viral infectivity as measured by single-hit infection assays of nuclear NP by immunofluorescence microscopy (Fig. 6A). In all cases, there were minimal numbers of cells where the viral NP was localized to the nucleus and viruses tended to be arrested in a punctate distribution in the cell periphery. In contrast to the situation with polarized cells, non-polarized cells (CHO, HeLa and Mv1 Lu) could still be fully infected after actin filaments were stabilized with jasplakinolide (not shown). We also examined the effects of jasplakinolide treatment on influenza virus internalization into polarized MDCKII cells. Using a quantitative assay of biotinylated virus, we observed that influenza virus internalization was eliminated when actin dynamics were arrested with jasplakinolide (Fig. 6B). We observed little or no effect of jasplakinolide in non-polarized Mv1 Lu cells, and jasplakinolide treatment did not affect viral binding in any cell type tested (not shown). As with data for cytochalasin D treatment, these results indicate that actin microfilaments play different roles in influenza virus infection in polarized epithelial cells and non-polarized cells. However, experiments using jasplakinolide show that not only are intact actin filaments obligatory for influenza virus infection in polarized epithelial cells, but actin dynamics need to be operational.

The actin motor protein myosin VI is involved in influenza virus infection of polarized cells, but not of non-polarized cells

To address more specific requirements for actin-dependent endocytosis in polarized versus non-polarized cells, we next examined the functional role of the actin
motor protein myosin VI. Using a dominant negative mutant (myosin VI tail), it has been shown previously that myosin VI can facilitate the trafficking of endocytic vesicles along actin filaments in the cell periphery (Aschenbrenner et al., 2003). We first explored the role of myosin VI in influenza virus entry in polarized cells, to determine if actin played a role beyond the internalization step. MDCKII cells were transfected with myosin VI full length (wild type) or a myosin VI tail mutant containing only the cargo-binding domain (myosin VI tail) before influenza virus infection. Viral infectivity was measured by single-hit infection assay monitoring the presence of nuclear NP after 3 h of infection (Fig. 7). Whereas expression of GFP-tagged myosin VI wild type had only limited effect on influenza virus infection, viral infectivity was measured by single-hit infection assay monitoring the presence of nuclear NP after 3 h of infection (Fig. 7). Whereas expression of GFP-tagged myosin VI wild type had only limited effect on influenza virus infection, expression of the GFP-tagged myosin VI tail mutant showed a drop in infectivity.

Fig. 6. Jasplakinolide prevents infection and internalization of influenza virus into polarized cells. A. Polarized MDCKII, Caco-2 and Calu-3 and cells were pretreated with 1 μM jasplakinolide for 30 min at 37°C and then infected with influenza virus at 5 moi of infectious unit per cell. The cells were analysed by indirect immunofluorescence microscopy at 4 h post infection using mouse anti-NP, and rat or rabbit anti-ZO-1 antibodies. Secondary antibodies included Alexa 488-labelled (green) anti-mouse IgG. B. Polarized MDCKII cells were pretreated with 1 μM jasplakinolide (+ jasp) or were untreated (− jasp). Biotin-labelled influenza viruses were bound to cells for 1 h at 4°C and were induced to internalize virus by warming to 37°C for 0–20 min. To visualize only the viruses that have been internalized, cells were incubated with unconjugated streptavidin (2 mg ml$^{-1}$) for 1 h at 4°C to block biotin from surface-bound viruses. Cells were then fixed, permeabilized and processed for fluorescence microscopy using Alexa 488-conjugated streptavidin. Samples were processed by deconvolution microscopy, and for each time point at least six different fields were used for quantification of fluorescence intensity. A non-linear fitting curve was generated using single 3-parameter exponential rise to maximum method.

Fig. 7. Myosin VI is involved in influenza virus infection of polarized cells, but not of non-polarized cells. A. MDCKII cells were transfected with GFP-myosin VI full length or tail mutant plasmids and allowed to become polarized for 12 h before infection. Cells were fixed and expressing cells identified based on GFP-expression. Cells were labelled with monoclonal anti-influenza NP antibodies to detect infection. Cell nuclei were counterstained with Hoechst 33258. A merge of the GFP (green) and anti-NP (red) signal is shown. B. Viral infectivity in both polarized MDCKII and non-polarized Mv 1 Lu cells transfected with myosin VI full length was normalized to 100% and the viral infectivity in cells transfected with myosin VI tail mutant was expressed as a percentage. More than 100 cells were scored in three independent experiments. Error bars represent the standard deviation of the mean. Data were analysed using SAS software (version 9.1) by analysis of variance (ANOVA), with least squares means adjusted for multiple comparisons by the Bonferroni method. With $a = 0.01$, the decrease in the infectivity of myo-tail plasmid transfected MDCKII cells was extremely significant ($***P = 0.0008$) compared with that in myo-wt plasmid transfected cells. In non-polarized Mv 1Lu cells, the reduction in myo-tail transfected cells was not significant ($P = 0.3620$).
Role of actin during influenza entry into polarized cells

Quantification of infectivity was scored as the percentage of GFP-expressing cells that were also infected with influenza virus, which showed that expression of the GFP-tagged myosin VI tail mutant reduced infectivity by approximately 50% in MDCKII cells, compared with cells expressing GFP-tagged myosin VI wild type (Fig. 7B). Statistical analysis of the infectivity levels of myosin VI tail plasmid-transfected MDCKII cells showed an extremely significant decrease ($P = 0.0008$), compared with that in myosin VI wild-type plasmid-transfected cells. As a comparison, we also transfected GFP-tagged myosin VI wild type and the GFP-tagged myosin VI tail mutant into non-polarized Mv1Lu cells. In this case the reduction of infection in myosin VI tail-transfected cells was not significant ($P = 0.3620$); see Fig. 7B. Overall, these results suggest that myosin VI was important for influenza virus infection of polarized epithelial cells, but was dispensable in non-polarized cells.

**Discussion**

In this study, we examined the role of the actin cytoskeleton in influenza virus entry in polarized versus non-polarized cells. We found that in polarized MDCK II cells, influenza virus failed to internalize into cells and form endocytic vesicles in cells where intact actin filaments had been disrupted. Of the possible scenarios for the role of actin during endocytosis, our studies are most consistent with a role of actin microfilaments during fission of endocytic vesicles, i.e. in our experiments, influenza viruses were trapped in nascent endocytic vesicles at the plasma membrane and could not enter cells. However, our results do not rule out additional possible roles played by actin, which might include facilitation of clathrin-coated vesicle delivery through apical actin meshwork beneath the microvilli, or further endocytic vesicle movement near the nuclei.

Studies in various cell types have shown that actin plays a variable, but not obligatory role in endocytosis (Fujimoto et al., 2000); in most cases the cell lines studied were non-polarized. In polarized epithelial cells, it is generally considered that actin plays a more fundamental role in endocytosis (Apodaca, 2001). The findings reported here concur with these previous reports; inhibition of actin polymerization played a critical role in influenza virus entry into polarized cells (MDCK II, LLC PK1:11B, Caco-2 and Calu-3), but not into non-polarized cells (Mv1Lu, BHK, HeLa and CHO). In the case of influenza virus, previous reports have shown that infection of polarized MDCK cells is inhibited by cytochalasin D treatment (Gottlieb et al., 1993), whereas the related compound cytochalasin B does not affect entry into non-polarized chick embryo fibroblasts (Patterson et al., 1979); however, the molecular mechanism of this infection block was not addressed in previous studies. In non-polarized CHO cells, cytochalasin D was more recently shown to reduce intracellular movement of internalized viruses (Lakadamyali et al., 2003), although how this movement correlates with virus infectivity is presently unknown.

In addition to actin, the motor protein myosin VI has been shown to be involved in endocytosis (Hasson, 2003; Buss et al., 2004). Different cell types express different forms of myosin VI, as a result of alternative splicing. (Hasson, 2003; Buss et al., 2004) and it is thought that different isoforms of myosin VI might have varying roles in different tissues or even in the same cells during development (Buss et al., 2004). Irrespective of the form of myosin VI present in a particular cell type, the overexpression of the globular tail of myosin VI tail results in a significant delay in transferrin uptake in epithelial cells (Aschenbrenner et al., 2003). Importantly, myosin VI tail expression results only in a kinetic, and not an absolute, block in transferrin endocytosis (Aschenbrenner et al., 2003). In the case of influenza virus entry, we show that expression of the myosin VI tail only gave a statistically significant reduction of virus entry in polarized cells such as MDCKII. At present we can show a role for myosin VI during influenza virus infection in polarized epithelial cells, however, we were unable to carry out a more detailed analysis of the role of myosin VI during influenza entry due to the limitations imposed by transfection of polarized MDCK cells. Future work will address this using different means of plasmid delivery or alternative myosin VI-specific reagents.

Recent live cell imaging has also shown a role for the cytoskeleton in virus entry via the process of ‘surfing’. In this process, the retroviruses murine leukaemia virus and avian leucosis virus, as well as VSV, were shown to associate with the dense microvilli of polarized epithelia and move on the cell surface in an actin and myosin-dependent manner prior to internalization (Lehmann et al., 2005). Although influenza virus was not analysed in these studies, it is noteworthy that large numbers of influenza virus particles have been documented to be adsorbed onto the cilia of tracheal organ culture cells (Dourmashkin and Tyrrell, 1970) and may travel along the actin bundles in the microvilli down to the bottom of microvilli, from where endocytosis occurs (Matlin et al., 1981). It is therefore likely that in polarized epithelial cells influenza will display actin-dependent movement or ‘surfing’ on the cell surface, in addition to the actin-dependent internalization described here. It remains to be determined how essential such alternative actin-based machineries are for influenza virus infection. In the case of ‘surfing’, movement is controlled by myosin II, rather that the myosin VI motor described here.

Current information on the role of actin during endocytosis has focused on the connection between actin and
clathrin-mediated endocytosis (Yarar et al., 2005). It is known that influenza can enter cells via clathrin-dependent or clathrin-independent pathways (Sieczkarski and Whittaker, 2002; Rust et al., 2004), although to date these studies have only been carried out in non-polarized cells. Therefore, at present it is unclear if the requirement for actin in polarized cells is specific to any particular route of virus internalization (e.g. clathrin-mediated endocytosis).

In polarized epithelial cells, apical endocytosis preferentially occurs at the base of the microvilli (Apodaca, 2001). Immediately below these plasma membrane domains is the actin network of the terminal web, which nascent endocytic vesicles must traverse to reach the early endosome. For influenza virus infection of a variety of polarized epithelial cells (but not in non-polarized cells) both the stability of the actin network and the motor protein myosin VI are important for delivery of incoming virus particles to the endosomal system, where viral fusion occurs. Future work will address the relative use of actin and myosin VI in different routes of virus internalization, both for influenza and other viruses where the in vivo site of infection is a polarized epithelium.

**Experimental procedures**

**Cells, viruses and infections**

MDCK type II cells (Nichols et al., 1986) were kindly provided by Dr Colin Parrish, Cornell University. Mv1 Lu, BHK, HeLa and CHO cells (American Type Culture Collection), as well as MDCK II cells, were maintained in Dulbecco’s modified MEM (Cellgro) containing 10% fetal bovine serum, 100 μM penicillin and 10 μg ml⁻¹ streptomycin. Caco-2 (human colon epithelial cells) and Calu-3 cells (human lung epithelial cells) were both obtained from the American Type Culture Collection and were maintained in Dulbecco’s modified MEM (Cellgro) containing 20% fetal bovine serum, 100 μM penicillin and 10 μg ml⁻¹ streptomycin. LLC-PK1::μ1B cells (porcine kidney epithelial cells) (Folsch et al., 2001) were provided by Dr Ira Mellman (Yale University) and tested for transepithelial electrical resistance (TEER) at 30–21 days after seeding and cells with TEER higher than 300 Ω cm² were used for viral infection. When VSV failed to give infection from the apical surface, virus (VSV) when applied from the upper (apical) chamber (Gottlieb et al., 1993). Cells were only considered to be fully polarized when VSV failed to give infection from the apical surface.

Influenza virus A/WSN/33 (H1N1) was propagated in MDCK cells. Infections were performed essentially as described previously (Sieczkarski and Whittaker, 2002). Briefly, viral stocks were diluted in RPMI 1640 medium containing 0.2% BSA and buffered to pH 6.8 with HEPES. Viruses were absorbed for 30 min at 37°C. Cells were then maintained in growth medium containing 2% serum for 4 h at 37°C before analysis. For cytochalasin D treatment, 20 μM cytochalasin D was used to pretreat cells for 30 min followed by the addition of virus inoculum for 30 min at 37°C. For jasplakinolide treatment, 1 μM jasplakinolide was used to pretreat cells for 30 min followed by the addition of virus inoculum for 30 min at 37°C. Cells were then incubated with growth medium containing 2% serum and 20 μM monensin to block viral infection after withdrawing cytochalasin D or jasplakinolide. Monensin was obtained from Calbiochem and a stock solution made in ethanol. Cytochalasin D and jasplakinolide were obtained from Calbiochem and stock solutions made in DMSO.

Plasmid constructs of GFP-myosin VI and GFP-myosin VI-tail were kindly provided by Dr Tama Hasson (U.C. San Diego). Transfections were performed using the Lipofectamine 2000 (Invitrogen) according to manufacturer’s protocols. Transfections were typically allowed to proceed for 12 h before infection.

**Fluorescence microscopy**

Fluorescence microscopy was performed as described previously (Sieczkarski and Whittaker, 2002). Influenza virus nucleoprotein (NP) was detected using the monoclonal antibody H16, L10–4R5 (ATCC). Monoclonal and polyclonal antibodies against human and mouse ZO-1 were obtained from Chemicon International and Zymed Laboratories respectively. Actin filaments were stained with TRITC-phalloidin (Sigma Chemical). Secondary antibodies used were Alexa 488-labelled (green) or Alexa 568-labelled (red) goat anti-mouse IgG (Molecular Probes). Biotin-labelled virus was visualized with Alexa 488-labelled streptavidin. Cells were mounted in Mowiol and viewed on a Nikon Eclipse E600 fluorescence microscope, using a 40× Plan Apo objective (NA 0.95) or a 20× Plan Apo objective (NA 0.75), and images captured with a SPOT RT camera and SPOT software (version 3.5.4) before transfer into Adobe Photoshop (version 7.0). For quantification, >100 cells were scored for each sample.

For deconvolution, a 60× Plan Apo objective (NA 1.4) was used for image acquisition and 0.25-μm z-slices collected using QED image software (QED Imaging, Pittsburgh, PA, USA). AutoDeblur Gold (AutoQuant Imaging, Watervliet, NY, USA) was used for deconvolution, and images transferred into Adobe Photoshop version 7.0 (Adobe Systems, San Jose, CA, USA).

Confocal microscopy was performed using an Olympus Fluoview confocal confocal station. Samples were fixed and incubated with antibodies. Alexa 488 was excited with the 488 nm line of an Argon laser and Alexa 568 was excited with the 568 nm line of a Krypton laser. Cells were viewed with a 60× Plan Apo objective (NA 1.4) and images were captured with Fluoview software (Olympus) before being transferred into Adobe Photoshop.

**Biotinylation of influenza virus**

Influenza virus A/WSN/33 (H1N1) was grown in 11 d-old embryonated eggs and allantonic fluid was collected to prepare concentrated virus stock. To label virus with biotin, 1 ml of concentrated influenza virus (2 mg ml⁻¹) was mixed with 10 μl of freshly prepared 10 mM Sulfo-NHS-SS-biotin (Pierce) for 2 h at 4°C. Viruses were then washed with PBS followed by ultracentrifugation at 50 000 g for 60 min to remove any unincorporated biotin. Labelled virus was tested by both haemagglutination and infec-
tion assays to evaluate virus integrity and infectivity before use in binding and internalization assays.

Influenza virus internalization using biotinylated virions

Biotinylated influenza viruses were incubated with cells in binding medium for 1 h at 4°C to allow virus binding. Cells were then washed with viral binding medium before temperature was shifted to 37°C for indicated time periods to induce viral internalization. To discriminate internalized viruses from surface-bound viruses, cells were incubated with unconjugated streptavidin (2 mg ml⁻¹) at 4°C for 1 h to quench surface-bound biotin-labelled viruses. Cells were then fixed and processed for immunofluorescence with Alexa 488-labelled streptavidin to visualize internalized influenza virus. Cells were mounted in Mowiol and viewed on a Nikon Eclipse E600 fluorescence microscope using a 60 × Plan Apo objective (NA 1.4). For analysis of virus internalization we performed deconvolution microscopy. Images were captured with a Sensiscam EM camera (Cooke) driven by IPLab software (Scanalytics). For each sample, z-stage images were collected with a step size of 0.25 μm and AutoDeblur Gold 9.1 software (AutoQuant Imaging) was used to producing maximum projection images. For each time point, at least six different fields were used for further quantification. The maximum projection images were quantified for fluorescence intensity by IPLab (Scanalytics) and the data were transferred to Sigma Plot 9.0 (Systat Software).

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