**Echovirus 11 infection induces dramatic changes in the actin cytoskeleton of polarized Caco-2 cells**

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Binding of echovirus 11 strain 207 (EV11-207) to Caco-2 monolayers results in rapid transfer of the virus to tight junctions prior to uptake. Using a confocal microscopy based-method, this study quantified the spatiotemporal distribution of actin during the time course of infection by EV11-207 in Caco-2 polarized cells. It was found that binding of EV11-207 to the apical surface resulted in rapid rearrangement of the actin cytoskeleton, concomitant with transport of the virus particles to tight junctions. By interfering with the actin network dynamics, the virus remained trapped at the cell surface, leading to abortion of infection. In addition, it was observed that at 4 h post-infection, concomitant with the detection of virus replication, actin filament was depolymerized and degraded. Finally, it was shown that the mechanisms leading to loss of actin were independent of viral genome synthesis, indicating a potential role for the viral protein synthesis seen in late infection. These data confirmed a previous study on the requirement for an intact actin cytoskeleton for EV11-207 to infect cells and reinforce the notion of actin cytoskeleton subversion by picornaviruses during infection in polarized epithelial cells.

**INTRODUCTION**

Viruses succeed as intracellular parasites because of their ability to invade cells and appropriate the cellular machinery required during their life cycle. The actin cytoskeleton of the host cell does not escape virus infection unscathed and is often co-opted by the virus at many stages of its life cycle to facilitate the infection process (Arakawa et al., 2007; Cudmore et al., 1997; Taylor et al., 2011). Many investigations during the past decade have revealed the mechanisms evolved by several viruses to exploit the actin cytoskeleton for their cell-surface movements following attachment to their receptor, prior to endocytosis and at different stages of their life cycle (Burckhardt & Greber, 2009; Haidari et al., 2011; Jolly et al., 2007; Miazza et al., 2011; Sun & Whittaker, 2007; Taylor et al., 2011).

The family *Picornaviridae* is composed of non-enveloped viruses including polioviruses and other enteroviruses, hepatitis A virus, foot-and-mouth-disease virus and rhinoviruses. Although these viruses initiate infection at respiratory or intestinal epithelial surfaces (Coyne & Bergelson, 2006), the early stages of infection have been studied intensively in non-polarized systems and much is known of the initial interactions between the viruses and cell-surface receptors (Bella & Rossmann, 1999; Belnap et al., 2000a, b; Wien et al., 1996; Xing et al., 2000). The echoviruses (enteric cytopathic human orphan viruses), a group of human virus isolates that cannot be grown in suckling mice, are grouped with coxsackie B viruses to form a subgenus within the genus *Enterovirus* of the family *Picornaviridae* (Stuart et al., 2002b).

Infection with enteroviruses can cause meningitis, encephalitis, rash, and mild respiratory and enteric infections (Grist et al., 1978; Rhoades et al., 2011). Occasionally, they cause overwhelming disease and death in neonates. Of the 32 types of echovirus, type 11 is the most frequent cause of serious neonatal morbidity and mortality, often presenting as fulminant hepatitis, infection of the central nervous system, or both (Modlin, 1986; Ventura et al., 2001).

How enteroviruses initiate infection at intestinal epithelial surfaces is a question of major importance. These surfaces are lined by polarized epithelial cells that are connected by a variety of specialized cell–cell contacts to form a barrier that separates the inside of the organism from its...
environment. Key to the formation of this barrier are the tight junctions that separate the plasma membrane into two domains, the apical and basolateral membranes with different protein and lipid compositions. Tight junctions are employed as targets by a variety of infectious agents including viruses (Guttman & Finlay, 2009) and are known to be dynamic structures with a baseline protein turnover that varies for the different protein components (Ivanov, 2008; Samarin & Nusrat, 2009; Tsukita et al., 2001). Entry of coxsackievirus B3 (CVB3) into these epithelial cells has been addressed with Caco-2 cell-culture models (Coyne & Bergelson, 2006; Coyne et al., 2007). An interesting connection between actin dynamics and polarized entry was found for CVB3 in differentiated human Caco-2 cells (Coyne & Bergelson, 2006). CVB3 binds to the glycosyl-phosphatidylinositol (GPI)-anchored decay-accelerating factor (DAF or CD55) at the apical membrane. DAF is an important inhibitor of the complement cascade and blocks the C3 convertase on the apical membrane (Burchhardt & Greber, 2009). It has been reported that attachment of CVB3 to DAF induces simultaneous activation of the c-Abl tyrosine kinase and reorganization of the actin cytoskeleton, leading to CVB3 targeting to tight junctions between polarized cells (Coyne & Bergelson, 2006). However, it is not known whether CVB3 uses active actin-dependent transport to the tight junction.

We have studied strain 207 of echovirus 11 (EV11-207), isolated directly on human colon epithelial cells during an outbreak of diarrhoea (Patel et al., 1998; Stuart et al., 1985). We showed that this virus, like several other enteroviruses, binds the GPI-anchored cell-surface protein DAF. This protein was shown to be necessary for virus entry, but purified DAF failed to induce the conformational change in the virus capsid that is thought to be required for EV7 and EV11 uncoating (Powell et al., 1997, and our own observations). Indeed, EV11-207 has an affinity for DAF in the micromolar range and displays high on and off rates when compared with enterovirus receptors capable of causing conformational change to the capsid (Lea et al., 1998; Stuart et al., 2002b). We also demonstrated that, in non-polarized systems, the entry pathway of EV11-207 is dependent on lipid rafts and the actin cytoskeleton. Recently, we showed that, following binding of EV11-207 to the apical surface of human polarized Caco-2 cells, the virus was rapidly transferred to the tight junctions prior to internalization (Sobo et al., 2001). Here, we investigated the requirement of the actin cytoskeleton in the replication cycle of EV11-207 in these polarized epithelial cells. We found that, following attachment of EV11-207 to DAF at the apical surface, the actin cytoskeleton was rapidly rearranged, leading presumably to transfer of the virus to tight junctions prior to internalization. By interfering with the actin network dynamics, the virus remained trapped at the cell surface, leading to abortion of infection. Finally, we observed that at 4 h post-infection (p.i.), concomitant with the probable peak of viral protein synthesis, actin filaments were depolymerized and degraded.

**RESULTS**

**Bound EV11-207 at the apical surface and the actin network**

We initially designed experiments to observe bound EV11-207 at the apical surface of polarized Caco-2 cells and to determine the morphological localization of actin from the apical to the basal poles in EV11-207-bound cells and in mock-infected controls, using indirect immunofluorescence and a confocal microscopy method. EV11-207 at an m.o.i. of 100 was added to medium in contact with the apical surface of monolayers growing on Transwell inserts. After 30 min at 4 °C to allow virus attachment, the cells were washed to remove unbound viruses. Fixed, permeabilized cells were stained with an anti-ZO-1 mAb specific for the tight junction protein ZO-1, anti-EV11-207 polyclonal antibody (pAb) and DAPI. Filamentous actin (F-actin) was stained with Alexa Fluor 647-conjugated phalloidin. Bound EV11-207 showed a diffuse staining pattern at the apical surface. As expected, no virus was detected at the middle and basal poles of the cells (Fig. 1a) or in the control cells (Fig. 1b).

Because cellular F-actin exists in several forms and is found in a number of locations within polarized epithelial cells (Cooper, 1987; DuBose & Haugland, 1993; Gottlieb et al., 1993; Wells et al., 1998), we sought to assess morphologically the locations of F-actin in the apical, middle and basal poles within EV11-207-bound polarized Caco-2 and control cells. We found that F-actin at the basal pole of both EV11-207-bound polarized Caco-2 and control cells was present as organized stress fibres (Fig. 1a, b) (Gill et al., 2008). In the middle pole, F-actin was found in the regions of intercellular contact forming a peripheral ring surrounding the cells, whereas the apical pole content was organized as a peripheral ring at tight junctions, as indicated by the anti-ZO-1 mAb, and in less organized networks in the apical cytoplasm (DuBose & Haugland, 1993; Gottlieb et al., 1993; Wells et al., 1998).

**EV11-207 binding induces actin rearrangement**

In many cases, virus-induced signalling leads to dynamic changes in the actin cytoskeleton (Cudmore et al., 1995; Li et al., 1998; Miazza et al., 2011; Pelkmans, 2005), and we have previously shown that cytochalasin D, an agent that disrupts the actin cytoskeleton, inhibited infection by EV11-207 in non-polarized cells (Stuart et al., 2002a). Therefore, we monitored F-actin dynamics in polarized Caco-2 cells following binding of EV11-207 to the apical membrane. Monolayers growing on Transwell inserts were exposed to EV11-207 (m.o.i. of 100) at 4 °C for 30 min to allow virus attachment. The cells were then washed to remove unbound viruses and warmed at 37 °C for 10 min. Fixed, permeabilized cells were stained with anti-ZO-1 mAb, anti-EV11-207 pAb and DAPI. F-actin was stained with phalloidin. We found that, in contrast to the control (Fig. 1b) where F-actin was organized in dense stress-fibre
networks, the F-actin present at the basal pole of the cells was rearranged in a less dense ring-like network surrounding the cells (Fig. 2). At the middle pole, F-actin, as in the control, was organized as peripheral structures surrounding the cells. Compared with the control (Fig. 1b), the actin at the apical pole presented a notable change in terms of intensity and general organization. At that pole of the cells, the less organized cytoplasmic network disappeared and the ring network present at tight junctions showed a visible increase in intensity (Fig. 2). More importantly, the overall change in the apical F-actin organization was concomitant with the transfer of EV11-207 to tight junctions (Sobo et al., 2011) where a clear co-localization of the virus, F-actin and ZO-1 was observed (Fig. 2). A similar change in actin organization was observed when a low m.o.i. of 1 or 5 was used (see Supplementary Fig. S1, available in JGV Online).

**F-actin depolymerization and degradation**

The remarkable actin rearrangement observed 10 min after binding of EV11-207 to the apical surface led us to continue monitoring the dynamics in late infection. Monolayers were exposed to EV11-207 at 4 °C for 30 min to allow virus attachment and then washed. Fixed, permeabilized cells were stained with anti-EV11-207 pAb (green), anti-ZO-1 mAb (red) and DAPI (blue). Actin filaments were stained with Alexa Fluor 647-conjugated phalloidin (white). Typical patterns are presented for apical, middle and basal cell poles. Bars, 10 μm.

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**Fig. 1. Spatial distribution of actin in EV11-207-bound cells.** Polarized Caco-2 monolayers were exposed to EV11-207 at an m.o.i. of 100 (a) or mock-infected (b) at 4 °C for 30 min to allow virus attachment and then washed. Fixed, permeabilized cells were stained with anti-EV11-207 pAb (green), anti-ZO-1 mAb (red) and DAPI (blue). Actin filaments were stained with Alexa Fluor 647-conjugated phallocidin (white). Typical patterns are presented for apical, middle and basal cell poles. Bars, 10 μm.
localization at the middle pole. At the 4 h time point, when replication was detectable, F-actin staining was dramatically reduced (Fig. 3b) with no apparent organization, suggesting that the protein was degraded or depolymerized (phalloidin selectively labels polymerized F-actin), or both. Moreover, actin staining with a mAb (see Supplementary Fig. S2, available in JGV Online) confirmed the loss of actin. The cells lost their polarized organization, as revealed by the presence of ZO-1 throughout the length of the cells (Fig. 3b) and the mislocalization of occludin, a tight junction protein (see Supplementary Fig. S3, available in JGV Online). Infection at a low m.o.i. of 1 and 5 also led to the loss of F-actin staining (see Supplementary Fig. S4, available in JGV Online).

**Quantitative analysis of F-actin dynamics**

Given the morphological observation that F-actin staining fluctuated during infection by EV11-207, we first quantified the total amounts (fluorescence intensity) at 10 min and at 1, 2, 4 and 6 h p.i. and expressed these as percentages of the total amount in the control. The results showed an increase in F-actin (130 % of the control) after 10 min, suggesting actin polymerization following binding of EV11-207 to the apical surface, and in keeping with the morphological reorganization and targeting of the virus to tight junctions at this time point (Fig. 4a). At 1 and 2 h p.i., actin staining remained similar to that of the control. At 4 and 6 h p.i., the intensity of the staining decreased to approximately 5 % of that of the control, suggesting a dramatic decrease in F-actin levels, presumably resulting from F-actin depolymerization and/or degradation. We further set up a computational strategy using Metamorph/ MetaXpress software (see Methods) to quantitatively determine fluctuation of the actin ring (surrounding the cell) during infection. This strategy yielded a cell–cell contact area zone height of 1 μm (Fig. 4c, d). The actin ring in this zone (cortical actin) was quantified and expressed as percentage of total actin. The results depicted in Fig. 4(b) showed that the actin ring in the control was about 40 % of the total actin. At 10 min p.i., this value increased to 75 %, confirming the remarkable morphological changes that we observed at this time point (Fig. 2). At 1 and 2 h p.i., no significant change could be observed in the ratio of cortical actin to total actin compared with the control.

**Biochemical analysis of G- and F-actin**

Cellular actin exists in several forms within polarized epithelial cells. Monomeric actin forms a globular actin (G-actin) pool distributed diffusely throughout the cell cytoplasm, whilst F-actin is found in a number of locations including tight junctions, the cell periphery, the cytoplasm and focal adhesion sites (Cooper, 1987; DuBose & Haugland, 1993; Gottlieb et al., 1993; Wells et al., 1998). To test whether the F-actin decrease observed with fluorescence quantification resulted from degradation and/or depolymerization leading to an increase of the G-actin pool, we used a well-established protocol (Cramer et al., 2002) based on Triton X-100 (TX-100) extraction. This allowed us to separate the G- and F-actin pools during infection. We analysed the contents of the two pools and the total amount of actin during infection by Western blotting and quantified the results by densitometry. We found that total actin decreased at 4 and 6 h p.i., confirming the fluorescence quantification (Fig. 5a). However, the increase in the levels of phalloidin staining (30 %) within 10 min of infection was not seen in the total actin (Fig. 5a). This suggested that it was not new synthesis of actin but a change in the G- to F-actin ratio, which we confirmed (data not shown) by the use of cycloheximide, a protein-synthesis inhibitor (Abrami et al., 1998).
2006). The G-actin level (TX-100 soluble) remained constant during infection, with just a minor increase at 4 and 6 h p.i. (Fig. 5b). γ-Catenin was used as an equal loading marker (Fig. 5c). The F-actin analysis displayed in Fig. 5(d) revealed a minor increase at 10 min p.i. and a remarkable decrease in the protein at 4 and 6 h p.i., confirming once again the fluorescence quantification. Taken together, these data indicated that, during infection by EV11-207, the F-actin pool was principally degraded, but that a minor part contributed, presumably by depolymerization, to a minor increase in the G-actin pool.

Loss of actin is dependent on viral protein expression

To investigate whether the loss of actin observed during infection by EV11-207 was dependent on RNA replication or protein expression, we made use of cycloheximide, a protein-synthesis inhibitor (Abrami et al., 2006). Cycloheximide (35 μM) (Croons et al., 2008; Dang et al., 2011) was added to infected cells 3 h after virus attachment, the time point at which poliovirus exhibits its peak of RNA replication (Cooper, 1964). In addition, it has been demonstrated that protein synthesis in polyomavirus-infected cells is inhibited by 99% within 4 min of exposure to cycloheximide (Yu & Cheevers, 1976). At 6 h p.i., fixed, permeabilized cells were stained with anti-EV11-207 pAb and anti-ZO-1 mAb, and actin was stained with phalloidin. As expected, the results showed a loss of actin in control infected cells (Fig. 6). Interestingly, cycloheximide treatment appeared to prevent the loss of actin in infected cells, showing that the loss was independent of RNA replication but presumably dependent on accumulation of viral proteins.

Fig. 3. Spatial distribution of actin following internalization of EV11-207 in Caco-2 monolayers. Polarized Caco-2 monolayers were exposed to EV11-207 at 4 °C for 30 min to allow virus attachment. The cells were washed and warmed to 37 °C for 1 h (a) and 4 h (b). Fixed, permeabilized cells were stained with anti-EV11-207 pAb (green), anti-ZO-1 mAb (red) and DAPI (blue). Actin filaments were stained with Alexa Fluor 647-conjugated phalloidin (white). Typical patterns are presented for apical, middle and basal cell poles. Bars, 10 μm.
particles on the cell surface were detected before permeabilization with pAb to EV11-207 capsid and secondary antibody conjugated to a red fluorophore (Fig. 7). Cells were then permeabilized to expose cytoplasmic particles and restained with pAb to EV11-207 capsid and secondary antibody conjugated to a green fluorophore (Fig. 7). Thus, internalized virus particles, which could be stained with the green fluorophore-conjugated antibody only after permeabilization, appeared uniquely green and were typically distant from any red signal. Surface-associated virus particles, which could be stained both before and after permeabilization, appeared either red or yellow (green + red) (Patel et al., 2009). The results showed a diffuse staining at the cell surface in cells treated with or without (control) latrunculin A at 0 min p.i., as revealed by a yellow colour (Fig. 7a, arrows). At 1 h p.i., virus particles were detected in a perinuclear localization in control cells, whereas they formed clusters (arrows) at the cell surface in latrunculin A-treated cells (Fig. 7a, arrows). These data strongly indicated the requirement for F-actin dynamics for EV11-207 uptake in polarized epithelial cells.

To confirm the requirement for actin in the uptake of EV11-207, we performed a time-of-addition experiment in which latrunculin A treatment was applied either 1 h before virus attachment or during infection at different time points. Monolayers treated or not were exposed to EV11-207 (m.o.i. of 1, 5, 10, 50 and 100) at 4 °C for 30 min to allow virus attachment. At 0, 10 and 30 min and 1 and 2 h p.i., latrunculin A treatment was applied. At 6 h p.i., fixed, permeabilized cells were stained with pAb to EV11-207 capsid. Cells that displayed diffuse cytoplasmic staining were considered to be infected (positive). Fig. 7(b) shows the percentage of infected cells compared with the total after latrunculin A treatment at different time points. The results revealed that latrunculin A treatment for 1 h before attachment of the viruses, as well as drug addition at 0 min p.i., totally inhibited infection. Addition at 10 min p.i. (when the virus is in the tight junctions) led to 95% inhibition of infection, as replication could be detected in only 5% of the cells. When latrunculin A was added at 30 min p.i., this led to virus replication in 50% of the cells, whereas treatment at 1 h p.i. had no visible effect on virus replication (replication was observed in 100% of the cells, similar to the control). It is important to note that, at this time point, virus particles were internalized (Figs 3a and 7a). Interestingly, the inhibition of infection by latrunculin A was independent of m.o.i.

F-actin dynamics required for EV11-207 entry

Actin dynamics contribute to virus internalization, in most cases by endocytosis (Pelkmans, 2005). Following the above observations, we dissected the contribution of actin dynamics to the uptake of EV11-207. Monolayers were treated with 2.5 μM latrunculin A, a specific inhibitor of actin filament assembly (Abrami et al., 2010; Cramer et al., 2002; Spector et al., 1983; Yarmola et al., 2000). To examine virus-particle entry, we used a serial staining approach (Patel et al., 2009) to differentiate internalized virus particles from virus on the cell surface (see Methods). Cells treated with latrunculin A for 1 h were exposed to EV11-207 (m.o.i. of 10) for binding. At 0 min and 1 h p.i., cells were fixed with 4% paraformaldehyde, a non-permeabilizing agent. Virus

DISCUSSION

Whilst almost all picornaviruses characterized to date initiate infections at respiratory or intestinal surfaces, it is not clear how these viruses undergo internalization following receptor attachment. Respiratory or intestinal surfaces are lined by polarized epithelial cells that are connected by a variety of specialized cell–cell contacts to

Fig. 4. Quantification of actin during the time course of infection by EV11-207. Polarized Caco-2 monolayers were exposed to EV11-207 or were mock-infected (control) at 4 °C for 30 min to allow virus attachment. The cells were washed and warmed to 37 °C for different time periods. Fixed, permeabilized cells were stained with DAPI and Alexa Fluor 647-conjugated phalloidin to detect actin filaments. (a) The fluorescence intensity of total actin was measured during the time course of infection and expressed as a percentage of the control (mean ± SD for six monolayers). (b) The fluorescence intensity of cortical actin (cell–cell contact) was measured and expressed as a percentage of total actin intensity during the time course of infection as well as in the control (mean ± SD for six monolayers). (c, d) Metamorph analysis of confocal images at 10 min after binding. Cell interior and border (black) masks (c) were built based on an introduced manual contour. In (d), the inner and outer scanned actin staining of the surfaces presented in (c) is shown. A more detailed description of (c) and (d) can be found in Methods.
form a barrier that separates the inside of the organism from its environment. Key to the formation, organization and maintenance of polarity of these epithelial cells are actin dynamics (Grusche et al., 2009; Winder & Ayscough, 2005). The current study demonstrated that, following attachment of EV11-207 to the apical surface of polarized Caco-2 cells, actin polymerization was slightly increased and, more importantly, actin filaments were rearranged. This reorganization was presumably required for the virus transfer to tight junctions (Sobo et al., 2011) and internalization. Viruses have been observed to undergo three types of surface motion: random diffusion, retrograde drifts (also called retrograde flow) and confined motions (Burckhardt & Greber, 2009). Many of them manipulate actin dynamics to accomplish such motions at the cell surface following binding to their receptor. For example, some retroviruses (avian leukosis virus, human immunodeficiency virus type 1 and murine leukemia virus), polyomaviruses [human papillomavirus 16 (HPV-16) and murine polyomavirus-like particles] and poxviruses (vaccinia virus) have been reported to undergo drift motions on filopodia and microvilli in an actin rearrangement-based manner (Cudmore et al., 1995; Ewers et al., 2005; Lehmann et al., 2005; Mercer & Helenius, 2008; Schelhaas et al., 2008; Sherer et al., 2007). Moreover, our data demonstrated that actin was degraded during infection by the time point when viral protein expression was detectable. EV11-207 therefore apparently exerts two different and opposite effects on actin filaments. Firstly, just after binding to the apical surface, EV11-207 induces a slight polymerization and a remarkable rearrangement of F-actin (Figs 2 and 4). Secondly, EV11-207 induced actin depolymerization and degradation (Figs 3, 4 and 5) during late infection.

**EV11-207 induces F-actin polymerization and rearrangement**

Extracellular particles take advantage of directional movements inside cells by coupling to the retrograde flow of F-actin (Burckhardt & Greber, 2009). Our data clearly demonstrated a requirement for F-actin dynamics during EV11-207 internalization in polarized epithelial cells. However, the precise role that actin filaments play in EV11-207 uptake is not yet clear. It may promote not only EV11-207 motion from the apical surface to tight junctions, but also the subsequent internalization. This was clearly shown in our experiments where, by interfering with F-actin dynamics using latrunculin A, virus particles were trapped at the cell surface leading to abortion of infection, whereas drug treatment after virus-particle internalization did not

**Fig. 5.** Biochemical analysis of F- and G-actin in Caco-2 monolayers during the time course of infection. F- and G-actin were separated by TX-100 extraction in live Caco-2 cells. (a) The total amount of actin was determined. (b, d) Equal volumes of TX-100-soluble G-actin (b) and -insoluble F-actin (d) pools were analysed by Western blotting and quantified by densitometry. (c) γ-Catenin was used as an equal loading marker. Results are shown as mean ± SD for three monolayers.
affect infection (Fig. 7b). The present findings confirmed our previous observations, according to which treatment with cytochalasin D, a drug that acts by inducing depolymerization of the actin cytoskeleton, inhibited infection by EV11-207 in non-polarized systems (Stuart et al., 2002a). It has also been established that destruction of the actin cytoskeleton with cytochalasin D blocked endocytosis of membrane-bound and fluid-phase markers from the apical surface of polarized cells (Gottlieb et al., 1993). The first non-enveloped virus shown to use F-actin flow for infection was HPV-16 (Burckhardt & Greber, 2009; Schelhaas et al., 2008), which infects mucosal tissue, preferably in wounded epithelium (Burckhardt & Greber, 2009; Longworth & Laimins, 2004). It binds to heparan sulfate proteoglycans, such as transmembrane syndecans, or GPI-linked proteoglycans and additional receptors (Morgan et al., 2007; Selinka et al., 2002; Shafti-Keramat et al., 2003). Processive movements of HPV-16-like particles were observed on filopodia at a rate similar to the F-actin flow of 1–5 μm min⁻¹; these movements supported infection and were inhibited by actin depolymerization or stabilizing agents (Schelhaas et al., 2008). Similarly, adenoviruses, which are a significant cause of acute respiratory, ocular and gastrointestinal diseases in humans, have been reported to undergo F-actin-dependent uptake (Li et al., 1998). An interesting connection between actin dynamics and uptake of CVB3, a non-enveloped enterovirus that binds to DAF at the apical membrane, has been reported (Coyne & Bergelson, 2006).

Although we do not know yet how EV11-207 is coupled to F-actin dynamics, mechanisms might presumably link to actin dynamics and actin-based uptake signalling pathways and provide the mechanical constraint that drives the transport of EV11-207 from the apical membrane to tight junctions and subsequent internalization. Polymerized actin could provide the mechanical force that drives virus motions at the cell surface (Burckhardt & Greber, 2009) or helps sever vesicles from the plasma membrane (Kelly, 1995; Li et al., 1998). A growing number of studies indicate not only that the actin cytoskeleton is required for cell motility and cell shape (Chant & Stowers, 1995; Li et al., 1998), but also that it plays a significant role in receptor-mediated endocytosis (Li et al., 1998; Moreau et al., 1996; Mulholland et al., 1994).

Fig. 6. Protein-synthesis inhibition does not abolish the loss of actin. Infected Caco-2 monolayers (m.o.i. of 10) were treated or not (control) with cycloheximide at 3 h p.i. At 6 h p.i., fixed, permeabilized cells were stained with anti-EV11-207 pAb (green), anti-ZO-1 mAb (red) and DAPI (blue). Actin filaments were stained with Alexa Fluor 647-conjugated phalloidin (white). Bars, 10 μm.
Fig. 7. Actin filaments are required for EV11-207 internalization in Caco-2 cells. (a) Caco-2 monolayers were treated or not (control) with latrunculin A, an actin filament-destabilizing agent, for 1 h before exposure of the monolayers to EV11-207 (m.o.i. of 10). Initially (0 min) and at 1 h p.i., fixed (and non-permeabilized) cells were submitted to indirect immunofluorescence using anti-EV11-207 pAb to visualize virus (red) at the cell surface (Pα-EV11 Unperm). Cells were then permeabilized and stained with the same anti-EV11-207 pAb to detect intracellular virus particles (green) (Pα-EV11 Perm). Images are three-dimensional projections from the multi-section scannings. xy and xz represent top and side views, respectively. Bars, 10 μm. (b) Caco-2 monolayers were treated or not (control) with latrunculin A for 1 h at the indicated time points before or following exposure of Caco-2 monolayers to EV11-207 (m.o.i. of 1, 5, 10, 50 and 100). At 6 h p.i., fixed, permeabilized cells were stained with anti-EV11-207 pAb and DAPI. Cells that displayed diffuse cytoplasmic staining of EV11-207 were considered to be infected. Typically, an m.o.i. of 1 led to infection of 60% of cells. Infected cells were counted and expressed as a percentage of the latrunculin A-untreated control (mean±sd for five monolayers).
EV11-207 mediates actin depolymerization and degradation

Our current data clearly showed that actin was depolymerized and degraded by 4 h p.i. when viral protein synthesis was observed. The question therefore arises as to what role(s) actin depolymerization and degradation might play in the EV11-207 life cycle. The finding that G-actin was not degraded raises the possibility that EV11-207 might specifically trigger F-actin degradation during infection. However, EV11-207 did not induce F-actin degradation until late in infection when viral protein expression was observed. It is likely that the effects on actin seen reflect the much higher levels of viral gene products, particularly proteases, seen at late times in infection. It has been shown that poliovirus protease 3C, in addition to proteolytically processing the polyprotein encoded by the viral genome, mediates cleavage of different cellular factors, including cytoskeletal elements such as the microtubule-associated protein 4 (Joachims et al., 1995; Uzunbegu & Carrasco, 1989). Furthermore, the time course of degradation observed in the present study appears incompatible with host-cell shut-off resulting from infection by picornavirus and cellular degradative turnover of actin, as biochemical evidence suggests a half-life for actin of 6 days (Glacy, 1983; Rubinstein et al., 1976) or 4 days (Antecol et al., 1986). By analogy with the ways that other viral and intracellular pathogens subvert the actin cytoskeleton (Cudmore et al., 1997; Elton et al., 2001), roles in intracellular trafficking of viral components, virus replication and assembly, and release of virions are possible. Consistent with this is the release of virions observed by 4 h p.i. (data not shown). More subtle exploitations of F-actin degradation have not yet been ruled out. Work is in progress to determine the mechanism(s) leading to the loss of actin, to characterize the general effects of EV11-207 infection on cytoskeletal components and to investigate the specific roles of F-actin degradation in the EV11-207 life cycle in these epithelial cells.

METHODS

Cells. Caco-2, a human colon adenocarcinoma cell line (ATCC), was cultured as monolayers on filters. Cells were seeded in 6.5 mm Transwell-COL inserts (0.4 μm pore size; Costar) at a density of 6 × 10⁴ cells cm⁻² and were re-fed every second day. The cells were maintained in high-glucose Dulbecco’s modified Eagle’s medium containing 10% FCS and 1% penicillin/streptomycin at 37 °C and 5% CO₂. Monolayers were used between days 8 and 12 post-seeding. Transepithelial electrical resistance (RT) was measured across monolayers using an EVOM ohmmeter (World Precision Instruments). Confluent monolayers consistently displaying a high RT (>300 Ω cm⁻²) were used in experiments. HT29 (human colon adenocarcinoma) cells were obtained from the European Collection of Cell Culture and maintained in RPMI 1640 medium as described previously (Stuart et al., 2002a).

Virus. The EV11-207 isolate has been described elsewhere (Stuart et al., 2002a). Briefly, EV11-207 was isolated on HT29 cells from a clinical sample, as described previously (Patel et al., 1985). A stock of virus was produced in HT29 cells. Confluent HT29 monolayers were infected with EV11-207 at an m.o.i. of 10. Infection was allowed to proceed overnight at 37 °C. Cells were scraped into the culture medium, which was then subjected to freezing and thawing three times, followed by centrifugation at 2000 g for 5 min. The supernatant was harvested and titres were determined by plaque assay on HT29 cells.

Reagents and antibodies. Latrunculin A (Invitrogen) was dissolved in DMSO and used at 2 μg ml⁻¹. Cycloheximide was from Sigma. Alexa Fluor 647-conjugated phalloidin and DAPI were from Invitrogen. Primary antibodies were anti-EV11 rabbit pAb (Stuart et al., 2002a), rabbit anti-γ-catenin pAb (Santa Cruz Biotechnology), and mouse anti-ZO-1 and anti-actin mAbs (Zymed). The secondary antibodies for immunofluorescence were FITC-conjugated goat anti-rabbit and TRITC-conjugated goat anti-mouse (Jackson ImmunoResearch). Secondary antibody conjugated to HRP for Western blot analysis was from Dako and comprised goat anti-rabbit and anti-mouse antibodies.

Time course of EV11 infection. Between 8 and 12 days post-seeding, the cells were infected with EV11-207 at an m.o.i. of 100, unless stated otherwise. The infected cells were incubated at 4 °C for 30 min to allow virus attachment and then washed three times with PBS before incubation at 37 °C to initiate virus entry. At different time points, the cells were washed twice with PBS containing 1% newborn calf serum (NCS) and fixed with PBS containing 4% paraformaldehyde for 10 min at room temperature. The cells were then prepared for immunofluorescence as described below.

Immunofluorescence analysis. Fixed cells were permeabilized with 0.05% TX-100 in PBS and incubated at room temperature for 2 min before being washed three times with PBS/NCS. The cells were incubated with the indicated primary antibodies and Alexa Fluor 647-conjugated phalloidin (to stain F-actin) for 45 min at room temperature, washed three times with PBS/NCS, incubated with fluorochrome-conjugated secondary antibodies for 30 min at room temperature, washed three times with PBS/NCS, incubated with DAPI for 5 min, washed three times and mounted on glass slides. Images were acquired in multiple sections (0.5 μm each section) from the apical pole to the basal pole using a confocal laser-scanning microscope (LSM 510 Meta; Zeiss) and analysed by Imaris software. xy and xz represent the top and side views, respectively.

Image acquisition. Confocal images were acquired on a Zeiss LSM 510 Meta microscope. The exposure settings (appropriate values for the size of the microscope pinhole and for the gain of laser) were maintained throughout image acquisition to allow direct comparisons of images. To minimize the bleed-through effect and avoid loss of image data needed for quantification, images were acquired using only sequential scanning and saved in TIFF graphics file format (Zinchuk et al., 2007). For serial optical section, stacks with a z-step of 0.5 μm were collected (Miazzia et al., 2011).

Actin remodelling and quantification. To quantify the observed pattern of F-actin during the time course of infection by EV11-207, we classified the filaments according to their precise position (apical, middle and basal) along the z-axis of cellular stacks. Measurement of cellular staining intensity along this axis was performed using Metamorph/MetaXpress software (Molecular Devices). At different time points, each type of actin filament was expressed as a percentage of the total population. Moreover, we separated thin bundles in the interface between neighbouring cells and the cytoplasmic filaments. A statistical validation of the cytoplasmic versus cell–cell contacts of the actin filaments was also performed with Metamorph/MetaXpress software. The analysis was performed as follows. The stack of images taken for 2 positions from −1 to +1 was dilated by 16 pixels until neighbouring nuclei touched each other to obtain a ‘best-focus’ image that reflected the best structure for basic definition of borders. On the
Preparation of TX-100-soluble and -insoluble cellular pools from live cells. We used a well-established protocol (Cramer et al., 2002; Shurety et al., 1998) for partitioning cellular actin into TX-100-soluble (G-actin) and -insoluble (F-actin) pools from live cells. Briefly, to obtain the G-actin pool, polarized Caco-2 cells grown on Transwell inserts were incubated for 5 min at room temperature in 100 μl PBS containing 1 % TX-100, protease inhibitor cocktail and 1 μg phalloidin ml⁻¹ to prevent filament disassembly. Cells were then washed briefly in PBS. The TX-100-insoluble pool was prepared by solubilizing and harvesting cells from the filters with a cell scraper in an equal volume (100 μl) of PBS containing 1 % TX-100, 2 % SDS and Complete Protease Inhibitor Cocktail (Sigma). Similarly, cellular total actin was prepared by lysing Caco-2 monolayers in 100 μl PBS containing 1 % TX-100, 2 % SDS and Complete Protease Inhibitor Cocktail. Each cellular actin pool was then passed through a fine-gauge syringe needle and an equal volume was loaded onto an SDS-PAGE gel.

Immunoblotting and protein quantification. Proteins were separated by SDS-PAGE (10 % acrylamide), unless stated otherwise, and transferred onto PVDF membranes. Western blot results were revealed using an ECL Western Blotting System (Amersham) and quantified by densitometry.

ACKNOWLEDGEMENTS

D. Nkwe and J. Grove are acknowledged for critical reading of the manuscript. S. Startchik is acknowledged for computer help and Metamorph software use. This work was supported by grants from the Swiss National Science Foundation to T. A. M. and K. S.

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