Animal RNA

Functional entry of dengue virus into *Aedes albopictus* mosquito cells is dependent on clathrin-mediated endocytosis

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Entry of dengue virus 2 (DENV-2) into *Aedes albopictus* mosquito C6/36 cells was analysed using biochemical and molecular inhibitors, together with confocal and electron microscopy observations. Treatment with monodansylcadaverine, chlorpromazine, sucrose and ammonium chloride inhibited DENV-2 virus yield and protein expression, whereas nystatin, a blocker of caveolae-mediated endocytosis, did not have any effect. Using confocal microscopy, co-localization of DENV-2 E glycoprotein and the marker protein transferrin was observed at the periphery of the cytoplasm. To support the requirement of clathrin function for DENV-2 entry, overexpression of a dominant-negative mutant of Eps15 in C6/36 cells was shown to impair virus entry. The disruption of actin microfilaments by cytochalasin D significantly also affected DENV-2 replication. In contrast, microtubule disruption by colchicine treatment did not impair DENV-2 infectivity, suggesting that DENV-2 does not require transport from early to late endosomes for successful infection of mosquito cells. Furthermore, using transmission electron microscopy, DENV-2 particles of approximately 44–52 nm were found attached within electron-dense invaginations of the plasma membrane and in coated vesicles that resembled those of clathrin-coated pits and vesicles, respectively. Together, these results demonstrate for the first time that DENV-2 enters insect cells by receptor-mediated, clathrin-dependent endocytosis, requiring traffic through an acidic pH compartment for subsequent uncoating and completion of a productive infection.

INTRODUCTION

Dengue virus (DENV) is an arthropod-borne member of the genus *Flavivirus*, family *Flaviviridae*, and can cause either dengue fever, a relatively benign, self-limited, febrile disease, or the severe and potentially fatal disease dengue haemorrhagic fever/dengue shock syndrome. The virion is an enveloped particle containing a single, positive-stranded RNA and three structural proteins: the capsid protein (C), a small membrane protein (M) and the envelope glycoprotein (E). There are four serotypes of DENV (DENV-1 to -4), which circulate in nature between their vectors, the mosquitoes *Aedes aegypti* and *Aedes albopictus*, and the vertebrate hosts. Currently, it is estimated that the virus is endemic in more than 100 countries, producing about 50 million infections each year (Gubler, 2002). Despite the importance and increasing incidence of DENV as a human pathogen, there is no antiviral chemotherapy and little is known about its cell biology.

The initial event in virus infection involves virion attachment to cell-surface-specific receptors followed by internalization into the cytoplasm. After binding to the host cell, enveloped viruses exploit two main pathways for entry and uncoating: direct fusion between the viral envelope and the plasma membrane, and endocytosis. The most common and well-known mode of endocytosis is the classical clathrin-mediated pathway with penetration in early or late endosomes where the exposure to low pH conditions triggers conformational changes in a viral glycoprotein leading to fusion of the endosomal membrane with the viral envelope. However, recent studies have demonstrated a variety of non clathrin-coated endocytic routes utilized by viruses, including predominantly caveolae-dependent pathways, but also the less frequent processes of macropinocytosis and micropinocytosis (Bishop, 1997; Pelkmans & Helenius, 2003; Sieczkarski & Whittaker, 2002).

The identity of the cellular receptor and the entry process for DENV in vertebrate and invertebrate cells is poorly understood. For mosquito cells, two glycoproteins of 40 and 45 kDa present on the membrane of *A. albopictus*-derived C6/36 cells have been found to be involved in DENV-4 binding (Reyes-del Valle & del Angel, 2004; Salas-Benito & del Angel, 1997), polypeptides of 80 and 67 kDa have been described for DENV-2 binding to the same cells (Muñoz *et al.*, 1998) and a laminin-binding protein has

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been identified as a candidate DENV-3 and DENV-4 receptor protein (Sakoonwatanyoo *et al.*, 2006). For mammalian cells, a wide diversity of proteins and heparan sulfate proteoglycans have been reported as receptor candidate molecules (Bielefeldt-Ohmann *et al.*, 2001; Chen *et al.*, 1997; Germi *et al.*, 2002; Hilgard & Stockert, 2000; Jindadamrongwech & Smith, 2004; Martínez-Barragán & del Angel, 2001; Moreno-Altamirano *et al.*, 2002; Navarro-Sánchez *et al.*, 2003; Reyes-del Valle *et al.*, 2005; Thepparit & Smith, 2004; Wei *et al.*, 2003). The mechanism of penetration is also currently a subject of controversy. Electron microscopic studies have shown that DENV-2 penetrates directly into the cytoplasm of C6/36 (Hase *et al.*, 1989) and BHK cells (Lim & Ng, 1999) by fusion at the plasma membrane. By contrast, experiments of virus inhibition with acidotropic agents, as well as the formation of syncytia by cell-to cell fusion of mosquito cells infected with DENV exposed at low acidic pH, have provided evidence of viral uptake through receptor-mediated endocytosis (Randolph & Stollar, 1990; Summers *et al.*, 1989). More recently, the entry of DENV to HeLa cells was shown to take place by clathrin-dependent endocytosis (Krishnan *et al.*, 2007).

In this report, we analysed the entry pathway of DENV-2 into C6/36 cells by the utilization of biochemical and molecular inhibitors together with microscopic observations. This systematic study confirmed the acid pH sensitivity of DENV internalization and the functional requirement of clathrin-mediated endocytosis in DENV entry into mosquito cells.

METHODS

Cells and virus. The C6/36 HT mosquito cell line from *A. albopictus* adapted to grow at 33 °C was cultured in Leibovitz L-15 medium (Gibco) supplemented with 0.3 % tryptose phosphate broth, 0.02 % glutamine, 1 % minimum essential medium (MEM) with non-essential amino acids and 5 % fetal calf serum. Vero (African green monkey kidney) cells were grown in Eagle's MEM (Gibco) supplemented with 5 % calf serum. For maintenance medium (MM) of L-15 and MEM, the serum concentration was reduced to 1.5 %.

DENV-2 strain NGC was provided by Dr A. S. Mistchenko (Hospital de Niños Dr Ricardo Gutierrez, Buenos Aires, Argentina). Virus stock was prepared in C6/36 cells and titrated by plaque formation on Vero cells.

Antibodies and reagents. The anti-DENV-2 antibody was a mouse monoclonal antibody against E glycoprotein purchased from Abcam. Monoclonal antibody to tubulin, goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) or rhodamine (TRITC), FITC-labelled phalloidin, and FITC-labelled cholera toxin B subunit were purchased from Sigma-Aldrich. TRITC-labelled human transferrin was purchased from Molecular Probes.

Monodansylcadaverine, chlorpromazine, nystatin, ammonium chloride, colchicine, cytochalasin D, sucrose, acridine orange and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich.

Inhibition of DENV-2 infection by inhibitor treatment. The mechanism of DENV-2 entry was investigated using different inhibitors of endocytosis. The range of concentrations and time of treatment appropriate for each inhibitor was first determined by a cell viability assay in order to avoid any toxic effects. C6/36 cells grown in 24-well microplates were incubated in MM with or without the compound, and cell viability was measured after each treatment using the MTT method, as described previously (Talarico *et al.*, 2005).

The treatment conditions (compound concentration and time of incubation) in the DENV-2 inhibition assay were chosen for each drug according to the viability data determined previously (data not shown). For chlorpromazine (10–50 μM), colchicine (10–200 μM), cytochalasin D (5–40 μM) and ammonium chloride (10–50 mM), C6/36 cells grown on cover slips in 24-well microplates were pre-treated with each compound for 2 h at 33 °C, and the cells were then infected with DENV-2 at an m.o.i. of 0.1 in the presence of drug. Alternatively, cells were pre-treated before infection with mono-dansylcadaverine (30 min, 25–200 μM), sucrose (30 min, 200–500 mM) or nystatin (3 h, 12.5–200 μM) and then washed and infected with DENV-2 in the absence of any compound. For all drug treatments, the virus inoculum was removed after 1 h of infection at 33 °C, when approximately 90 % of adsorbed virus had been internalized, as determined by a proteinase K-resistant infectious centre assay (data not shown). The cultures were then washed and further incubated at 33 °C in MM without any compound. At 48 h post-infection (p.i.), supernatants were harvested to determine extracellular virus yields by plaque assay and cells were fixed for immunofluorescence staining.

To confirm that the effect of each drug was restricted to the virus entry process, another set of cultures was infected with DENV-2 at an m.o.i. of 0.1 and incubated for 1 h at 33 °C to allow virus entry. Cells were then washed with PBS and incubated with each compound at 33 °C (the time of treatment depended on the compound being tested, as described above). The supernatants were then removed and the cultures were incubated at 33 °C in MM without compound. At 48 h p.i., cells were fixed for immunofluorescence staining.

To asses the effect of ammonium chloride on the pH of acidic intracellular vesicles, C6/36 cells, treated or not with compound for 2 h at 33 °C, were stained with acridine orange (1 μg ml^{-1} in MM without serum) for 15 min at 33 °C. Cells were washed twice with PBS, mounted on PBS and visualized under a fluorescence microscope (Olympus BX51).

For cytosol acidification, 1 M acetic acid (pH 5.0), diluted 1 : 100 in L-15 medium adjusted to pH 5.0 with HCl, was added to C6/36 monolayers, whilst control cells were incubated in L-15 medium (pH 5.0) only. After 10 or 30 min of treatment, cells were infected with DENV-2 (m.o.i. of 0.1) in L-15 medium (pH 7.0). Cell supernatants were titrated at 48 h after infection as described above.

Indirect immunofluorescence and co-localization assays. Compound-treated and untreated infected cells were fixed with methanol for 10 min at −20 °C, washed with PBS and stained with monoclonal antibody against the E glycoprotein followed by FITC-labelled goat anti-mouse IgG. The percentage of fluorescent cells in each sample was calculated from 20 randomly selected fields.

Transferrin and cholera toxin uptake control assays were performed in the presence or absence of entry inhibitors. C6/36 cells were incubated with TRITC-labelled transferrin (15 μg ml^{-1}) or FITC-labelled cholera toxin (0.3 μg ml^{-1}) for 20 min at 4 °C for binding and then shifted to 33 °C for 40 min for internalization. Cells were washed to remove any uninternalized ligand and then fixed with methanol for transferrin, or with 4 % paraformaldehyde followed by permeabilization with 0.2 % Triton X-100 for cholera toxin.

For co-localization studies, pre-chilled cells were infected with DENV-2 (m.o.i. of 10) in the presence of TRITC-labelled transferrin

and incubated at 4 °C for 60 min. Cells were then shifted to 33 °C for 25 min, washed with PBS and fixed with methanol. Immunofluorescence assays for DENV-2 E glycoprotein staining were performed as described above. Cells were visualized using a confocal laser-scanning microscope (Olympus FluoView) with a 60× objective lens.

Transfection of Eps15 constructs. Plasmid constructs of Eps15 (a conserved protein necessary for clathrin-dependent endocytosis) fused with the green fluorescent protein (GFP) gene (the dominant-negative mutant GFP–EΔ95–295, and GFP–DIIIΔ2 used as a control) were kindly provided by C. Shayo (IBYME, Argentina). C6/36 cells, grown on cover slips until 70 % confluency, were transfected with the constructs using Lipofectamine 2000 (Invitrogen). Briefly, 4 μg of each construct was diluted in 50 μl Opti-MEM and combined with 50 μl Opti-MEM containing 2.5 μl Lipofectamine 2000. After 40 min incubation at room temperature, the DNA–liposome complexes were added to the cells and cultures were incubated for 6 h at 33 °C. The medium was then replaced with MM and cells were incubated until 24 h post-transfection. Cells were then infected with DENV-2 (m.o.i. of 10) and, after 1 h of incubation at 33 °C to allow virus internalization, cultures were fixed with methanol. Staining of the E glycoprotein with a mouse monoclonal antibody and TRITC-labelled goat anti-mouse IgG was performed. Cultures were examined using a confocal laser-scanning microscope as described above.

Electron microscopy. C6/36 cells grown in six-well microplates were infected with DENV-2 (m.o.i. of 60), incubated for 60 min at 4 °C and then shifted to 33 °C for 25 min. Cells were washed with cold PBS and fixed with 1.5 % glutaraldehyde in 0.1 M phosphate buffer for 4 h at 4 °C. Cells were scraped from the plates, incubated overnight at 4 °C with 0.32 M sucrose, and then pelleted and incubated with 1.5 % OsO_4 containing 0.32 M sucrose for 2 h at 4 °C. Thereafter, cells were pelleted and washed with distilled water. Cells were incubated overnight with 2 % uranyl acetate and dehydrated with a series of ethanol gradients followed by propylene oxide, embedded in Epon 812 resin mixture (TAAB) and polymerized at 70 °C for 2 days. Ultrathin sections were restained with 2 % uranyl acetate and observed under an electronic microscope (C10; Zeiss).

RESULTS

DENV-2 uses a clathrin-mediated endocytic pathway to enter mosquito cells

The two best-defined endocytic pathways for viral entry are clathrin- and caveolae-mediated internalization (Pelkmans & Helenius, 2003; Sieczkarski & Whittaker, 2002). Thus, our first experimental approach was to determine whether DENV-2 enters C6/36 cells via a receptor-mediated or clathrin- or caveolae-mediated endocytic pathway by analysing the effects of monodansylcadaverine, chlorpromazine and nystatin on virus infection. Monodansyl-cadaverine is an extensively used pharmacological inhibitor of receptor-mediated endocytosis (Chu & Ng, 2004; Wei et al., 2007). Chlorpromazine is a cationic, amphiphilic molecule that acts by disturbing the assembly of clathrin lattices at cell surfaces and endosomes (Wang et al., 1993). Nystatin is a sterol-binding drug that sequesters cholesterol, a prominent component of lipid rafts involved in caveolae formation (Anderson et al., 1996; Pelkmans et al., 2001). Treatment of cells with concentrations of

monodansylcadaverine of between 100 and 200 μM reduced DENV-2 yield by 65 % (Fig. 1a). A dose-dependent inhibition of virus production was obtained in chlorpromazine-treated cells with a maximum inhibition of 84 % at a concentration of 50 μM (Fig. 1a). By contrast, nystatin treatment lacked any inhibitory effect on virus yield (Fig. 1a).

We also assayed the effect of these three inhibitors on the expression of DENV-2 E glycoprotein using an indirect immunofluorescence assay at 48 h p.i. (Fig. 1b). When cells were treated with monodansylcadaverine (200 μM) or chlorpromazine (50 μM) before virus infection, a significant reduction in DENV-2 infection was again observed, with 60 and 98 % inhibition of viral antigen-positive cells, respectively. However, neither monodansylcadaverine nor chlorpromazine seemed to exert any inhibitory effect on DENV-2 protein expression when added 1 h after infection, indicating that these compounds effectively blocked an early event during the entry process of DENV-2. In accordance with the infectivity data, nystatin had no effect on DENV-2 protein expression (Fig. 1b).

The internalization of FITC-labelled transferrin, a ligand known to enter into the cell by clathrin-mediated endocytosis, was used as a functional control assay to demonstrate that the action of monodansylcadaverine and chlorpromazine was effectively exerted on receptor-mediated endocytosis from clathrin-coated pits. In control cells, a bright dotted fluorescence pattern was observed in the cell cytoplasm, whilst cultures treated with chlorpromazine (Fig. 1c) or monodansylcadaverine (data not shown) exhibited a faint widespread fluorescence only at the cell surface, indicating that transferrin uptake was arrested efficiently. The specific effect of nystatin on caveolae-mediated endocytosis was also tested using FITC-labelled cholera toxin as a marker of internalization through caveolae. Untreated cells showed a clear dotted perinuclear cytoplasmic fluorescence due to accumulation of the toxin in the endoplasmic reticulum and Golgi apparatus, whilst treatment with nystatin resulted in a diffuse green fluorescence pattern indicative of the impairment of toxin incorporation (Fig. 1c).

The susceptibility of C6/36 cells to DENV-2 infection was also analysed under particular biophysical conditions reported to affect clathrin-dependent endocytosis, such as hyperosmolarity and cytosol acidification. Treatment of cells with high concentrations of sucrose creates a hypertonic environment that leads to the blockade of formation of clathrin-coated pits for endocytosis, whereas cytosol acidification prevents invaginated clathrin-coated domains from pinching off (Hansen et al., 1993). When C6/36 cells were pre-treated with 300–500 mM sucrose and then infected with DENV-2, an inhibition of virus yield of approximately 70 % was observed at 48 h p.i. Furthermore, acidification of the cytosol induced by acetic acid also reduced DENV-2 production by 64 and 83 % after 10 and 30 min of treatment, respectively (data not shown).
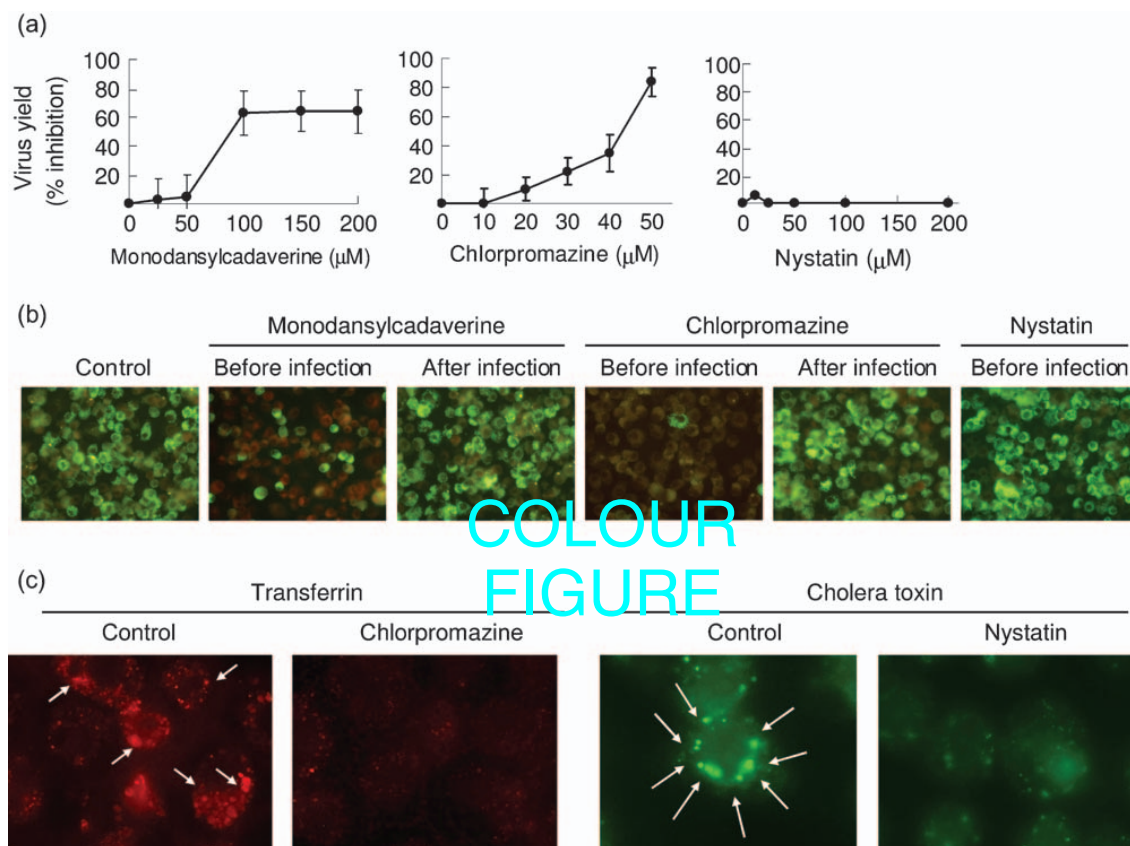
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Fig. 1. Effect of endocytosis inhibitors on infection of C6/36 cells with DENV-2. (a) Cells were treated with various concentrations of monodansylcadaverine, chlorpromazine and nystatin, and then infected with DENV-2. Virus yields were quantified by plaque assay at 48 h p.i. and results are expressed as percentage inhibition with respect to infected cells without drug treatment. Each point shows the mean ± SD of two independent experiments. (b) Cells were treated with 200 μM monodansylcadaverine, 50 μM chlorpromazine or 200 μM nystatin before or after infection with DENV-2, or were untreated (control). At 48 h p.i., immunofluorescence staining was carried out using mouse anti-E glycoprotein antibody. (c) Cells were treated with 50 μM chlorpromazine or 200 μM nystatin and incubated with TRITC-labelled transferrin or FITC-labelled cholera toxin, respectively. Controls without drugs were performed and samples were visualized by fluorescence microscopy. Arrows indicate internalized transferrin and perinuclear accumulation of cholera toxin, respectively.

Overall, the infectivity and immunostaining assays with chemical and biophysical inhibitors were indicative of the involvement of clathrin in DENV-2 internalization. Additional evidence was obtained in co-localization studies of viral E glycoprotein and transferrin by confocal microscopy. C6/36 cells were simultaneously incubated with DENV-2 and TRITC-labelled transferrin for 60 min at 4 °C to ensure binding without endocytosis, and synchronized entry was then initiated by incubating the cells at 33 °C. After 25 min of the shift up to 33 °C, co-localization of DENV-2 E glycoprotein and the marker protein transferrin was observed at the periphery of the cytoplasm (Fig. 2), providing more strong evidence of the key role of clathrin-mediated endocytosis as the entry route for DENV-2.

Finally, a specific molecular inhibitor in the form of a dominant-negative mutant was also used to confirm the role of clathrin-mediated endocytosis in DENV-2 entry into mosquito cells. Eps15, a conserved protein necessary for clathrin-dependent endocytosis, is organized into three domains: the N-terminal domain (DI), which contains three EH domains involved in protein–protein interactions, the central domain (DII) and the C-terminal domain (DIII), which contains the binding site for the plasma membrane adaptor complex AP-2. It has been proposed that Eps15 acts as a bridge between AP-2 and other EH domain-interacting proteins in the process of coated vesicle formation. We made use of EΔ95–295, a dominant-negative form of Eps15 lacking the second and third EH domains, which specifically interferes with clathrin-coated vesicle formation at the plasma membrane (Benmerah et al., 1999). We performed a virus entry assay in cells expressing the GFP-tagged version of the dominant-negative EΔ95-295 (GFP–EΔ95–295); a construct that does not affect clathrin-mediated uptake (GFP–DIIIΔ2) was

Fig. 2. Co-localization of DENV-2 E glycoprotein with transferrin. Cells were incubated with DENV-2 and TRITC-labelled transferrin for 60 min at 4 °C and 25 min at 33 °C. Cells were fixed and processed for immunofluorescence with mouse anti-E glycoprotein antibody and FITC-conjugated anti-mouse antibody. Cells were visualized with a confocal microscope.

used as a control (Benmerah *et al.*, 1998). To this end, C6/36 cells were transiently transfected with each construct and, at 24 h post-transfection, cells were incubated with DENV-2 for 1 h at 33 °C and processed for immunofluorescence staining and confocal microscopy. As expected, GFP–DIIIΔ2-transfected cells exhibited a speckled virus staining within the cytoplasm indicating efficient virus uptake (Fig. 3a, b). In contrast, a slight and disperse red fluorescence at the cell surface, demonstrative of a blockage in virus entry, was observed in cells expressing the dominant-negative GFP–EΔ95–295 (Fig. 3c, d), providing additional strong evidence that DENV-2 internalization into cells occurs via a clathrin-dependent pathway.

Involvement of acid pH in DENV-2 entry

After budding into the cytoplasm, clathrin-coated vesicles lose their clathrin coat and become acidified. Most enveloped viruses that enter the host cell via endocytosis require this low-pH step to trigger fusion of the viral envelope with the endosome membrane and release the nucleocapsid into the cytosol. To obtain evidence for a role of acid pH in DENV-2 entry into mosquito cells, the effect of ammonium chloride on DENV-2 infection was investigated. This drug is a lysosomotropic weak base that raises the pH of intracellular acidic vesicles (Castilla *et al.*, 1994; Mizzen *et al.*, 1985). Treatment of C6/36 cells with ammonium chloride before infection and during the first hour of infection at 33 °C significantly reduced virus yield (Fig. 4a). Accordingly, the expression of E glycoprotein was almost completely blocked under the same drug treatment, with a greater than 90 % inhibition in the number of virus-antigen-positive cells (Fig. 4b). No inhibition in DENV-2 protein staining was detected when ammonium chloride was added after 1 h of virus infection (Fig. 4b), indicating that the compound affects the early step of virus internalization. As a control, a virucidal assay was performed to verify that ammonium chloride did not
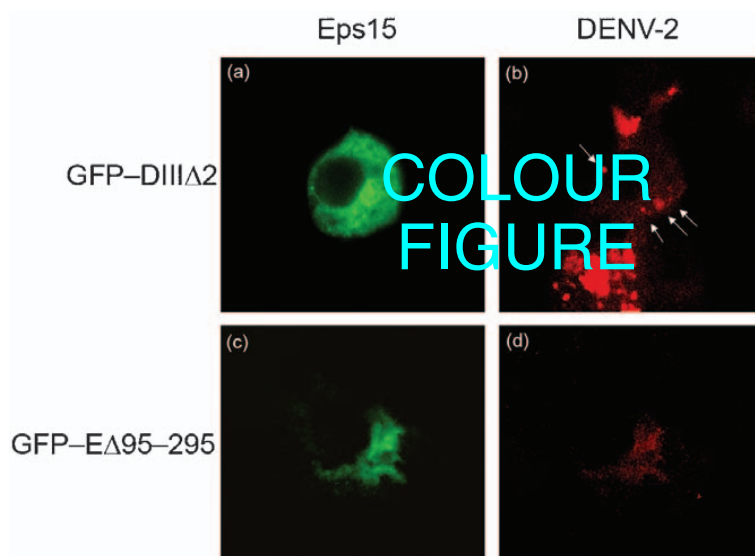

Fig. 3. GFP–EΔ95–295 effectively inhibits DENV-2 internalization. Cells transiently transfected with GFP–DIIIΔ2 (a, b) or GFP–EΔ95–295 (c, d) were infected with DENV-2 at 24 h post-transfection. After 1 h incubation at 33 °C, GFP expression was examined and virus internalization was visualized by an immunofluorescence assay using mouse anti-E glycoprotein antibody and TRITC-conjugated anti-mouse antibody. Cells were visualized with a confocal microscope. Arrows show internalized DENV-2 antigen.
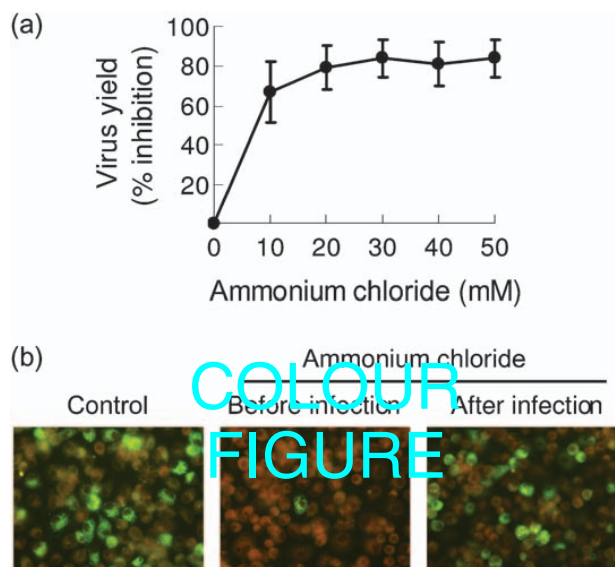
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Fig. 4. Inhibition of endosomal acidification on DENV-2 entry into C6/36 cells. (a) Cells were treated with increasing concentrations of ammonium chloride and infected with DENV-2. Virus yields were determined at 48 h p.i. Results are expressed as percentage inhibition with respect to infected cells without drug treatment. Each point shows the mean ± SD of two independent experiments. (b) Cells were treated with 50 mM ammonium chloride before or after infection with DENV-2, or were untreated (control). At 48 h p.i., immunofluorescence staining was carried out using mouse anti-E glycoprotein antibody.

destroy virus infectivity by direct inactivation of virions (data not shown).

To ensure that the ammonium chloride concentrations used effectively increased the pH of intracellular vesicles, we performed acridine orange staining. Untreated cells showed the typical perinuclear orange fluorescence of the acid compartments, whereas cells treated with ammonium chloride did not exhibit this fluorescence pattern (data not shown). Thus, it can be concluded that DENV-2 entry into mosquito cells is a low-pH-dependent process.

Role of the cytoskeleton network

Involvement of the host cellular cytoskeleton network in the endocytosis pathway of DENV-2 was next investigated by treatment with the cytoskeleton-disrupting drugs cytochalasin D and colchicine, which act by inducing depolymerization of actin filaments and microtubules, respectively (Flanagan & Lin, 1980; Hamel, 1996). The concentration of both drugs able to affect the integrity of the cytoskeleton without affecting cell viability was first determined by MTT assay and fluorescent staining. The organization of microtubules and microfilaments in C6/36 cells was similar to that described for mammalian cell lines. The distribution of actin-containing microfilaments as typical bundles of thin fibres was resolved in control cultures using FITC-labelled

phalloidin; treatment with cytochalasin D (2.5–40 μM) totally destroyed this pattern (Fig. 5a). Microtubules were examined using a monoclonal antibody to tubulin. The staining pattern observed as a network of well-defined tubules radiating from the perinuclear region was transformed after 12.5–200 μM colchicine treatment into an arrangement of condensed amorphous aggregations in the cytoplasm surrounding the nucleus (Fig. 5b).

Virus inhibition assays revealed that the presence of cytochalasin D during virus entry caused a significant reduction in virus titres over the range of concentrations tested, whereas no inhibition of virus production was observed in colchicine-treated cultures (Fig. 5c).

The differential requirement of microtubule and microfilament integrity for DENV-2 entry to mosquito cells was confirmed by analysis of the expression of E glycoprotein in cultures treated with both compounds either before or after virus infection. When cultures where treated with cytochalasin D (40 μM) before DENV-2 infection, the number of cells expressing E glycoprotein was drastically reduced from 72 to 7 %, whereas the addition of this microfilament-disrupting agent after 1 h of infection did not impair the level of DENV-2 antigen-positive cells (Fig. 5d), suggesting that DENV-2 entry into mosquito cells is dependent on microfilament integrity. Expression of the viral protein was not affected by the presence of colchicine, whether added before or after infection (Fig. 5d).

Ultrastructural study of virus entry

To follow the entry route of DENV-2 into C6/36 cells, electron microscopy studies were performed. Cells infected with DENV-2 were first incubated at 4 °C for virus adsorption and then warmed to 33 °C for 25 min to allow virus internalization. Thereafter, infected cells were processed for electron microscopy. The virus particles observed were, as expected, in the size range of 44–52 nm (Damonte et al., 2004) and presented an electron-dense core surrounded by a membrane bilayer (Fig. 6). Although microscopic observation was performed after prolonged adsorption at 4 °C and 25 min of incubation at 33 °C, we could find virions at different stages of the entry pathway, indicating that it is not a process easy to synchronize. Virus particles were found in the extracellular space (Fig. 6a) or attached within electron-dense invaginations of the plasma membrane that resembled those of clathrin-coated pits (Fig. 6b, c). In addition, single virus particles were also observed within coated vesicles with a diameter of around 120 nm (Fig. 6d), which is in the size range described for clathrin-coated vesicles (Bishop, 1997). No direct fusion of virus particles with plasma membrane was observed.

DISCUSSION

There has been increased interest in recent years in understanding the cellular mechanisms that viruses exploit
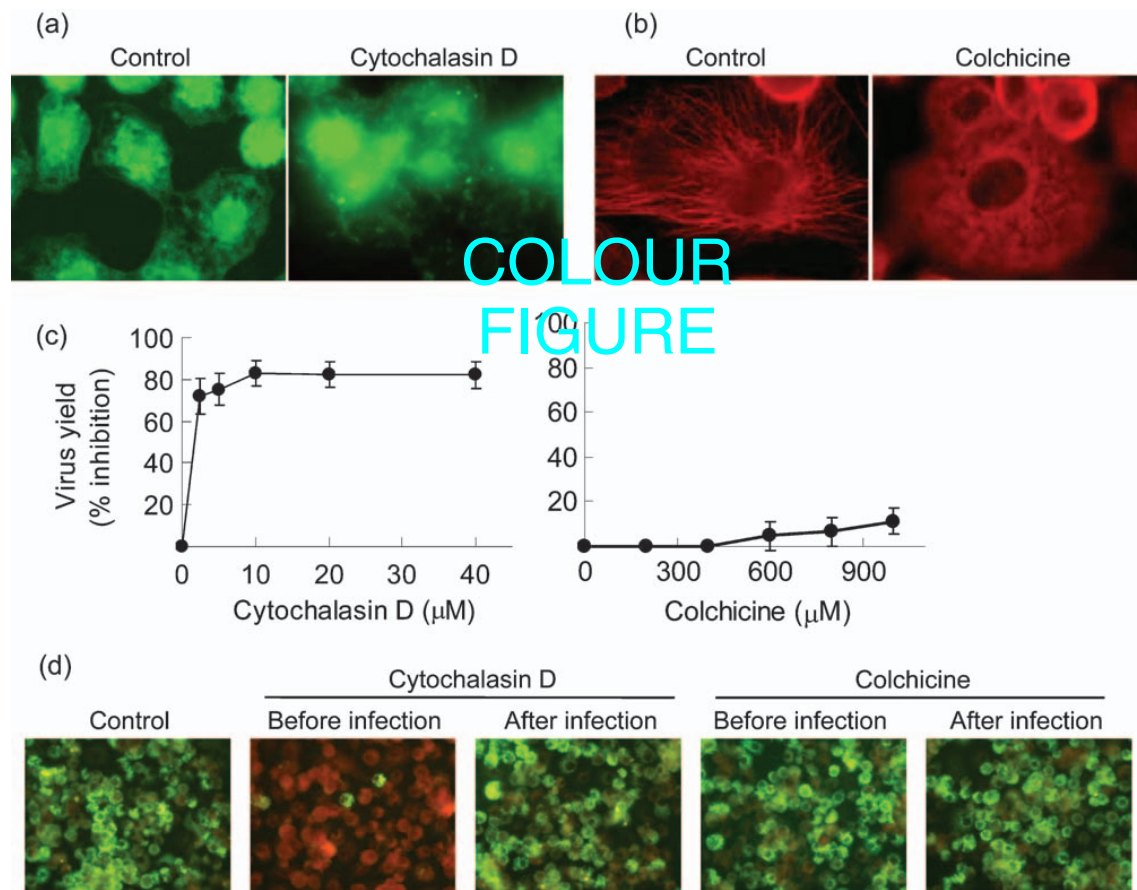
Fig. 5. Effect of cytoskeleton-disrupting drugs on DENV-2 entry into C6/36 cells. (a, b) Cells were untreated (control) or treated with 40 μM cytochalasin D or 200 μM colchicine and then stained with FITC-labelled phalloidin (a) or monoclonal anti-tubulin antibody and TRITC-labelled anti-mouse antibody (b). (c) Cells were treated with increasing concentrations of cytochalasin D or colchicine and infected with DENV-2. Virus yields were determined at 48 h p.i. Results are expressed as percentage inhibition with respect to infected cells without drug treatment. Each point shows the mean ± SD of two independent experiments. (d) Cells were treated with 40 μM cytochalasin D or 200 μM colchicine before or after infection with DENV-2, or were untreated (control). At 48 h p.i., immunofluorescence staining was carried out using mouse anti-E glycoprotein antibody.

to enter the host cell. The best-characterized pathway is receptor-mediated, clathrin-dependent endocytosis, which is used by several viruses to invade the cell through invaginations of the plasma membrane coated with clathrin (Pelkmans & Helenius, 2003; Sieczkarski & Whittaker, 2002). However, although this route of entry has been thoroughly analysed in mammalian cells for viruses of different families, only a few studies have documented the endocytic entry pathway of viruses into mosquito cells. In this field, endocytosis has been reported for nucleopolyhedrovirus baculoviruses in Sf21 cells (Long *et al.*, 2006), the reovirus rice dwarf virus in NC-24 cells (Wei *et al.*, 2007), California encephalitis virus in C6/36 cells (Hacker & Hardy, 1997) and Japanese encephalitis virus and West Nile virus, members of the family *Flaviviridae*, in C6/36 cells (Chu *et al.*, 2006; Mizutani *et al.*, 2003; Nawa, 1998). In particular, the mode of entry of a flavivirus in an insect cell culture is representative of the route of entry in its

insect vector in nature and, consequently, this knowledge will provide information relevant to designing control strategies. In this paper, we used diverse chemical inhibitors, dominant-negative mutant gene overexpression, confocal immunofluorescence and electron microscopy to characterize the entry of DENV-2 in C6/36 cells. To our knowledge, the studies reported here demonstrate for the first time that DENV-2 enters insect cells by receptor-mediated, clathrin-dependent endocytosis, requiring traffic through an acidic pH compartment for the subsequent uncoating and completion of a productive infection.

To demonstrate the route of DENV-2 internalization in mosquito cells, C6/36 cells were initially treated with a set of chemical agents that inhibit endocytosis and clathrin function. DENV-2 infection was significantly inhibited when cells were treated before infection with monodansylcadaverine, chlorpromazine and sucrose, as shown by
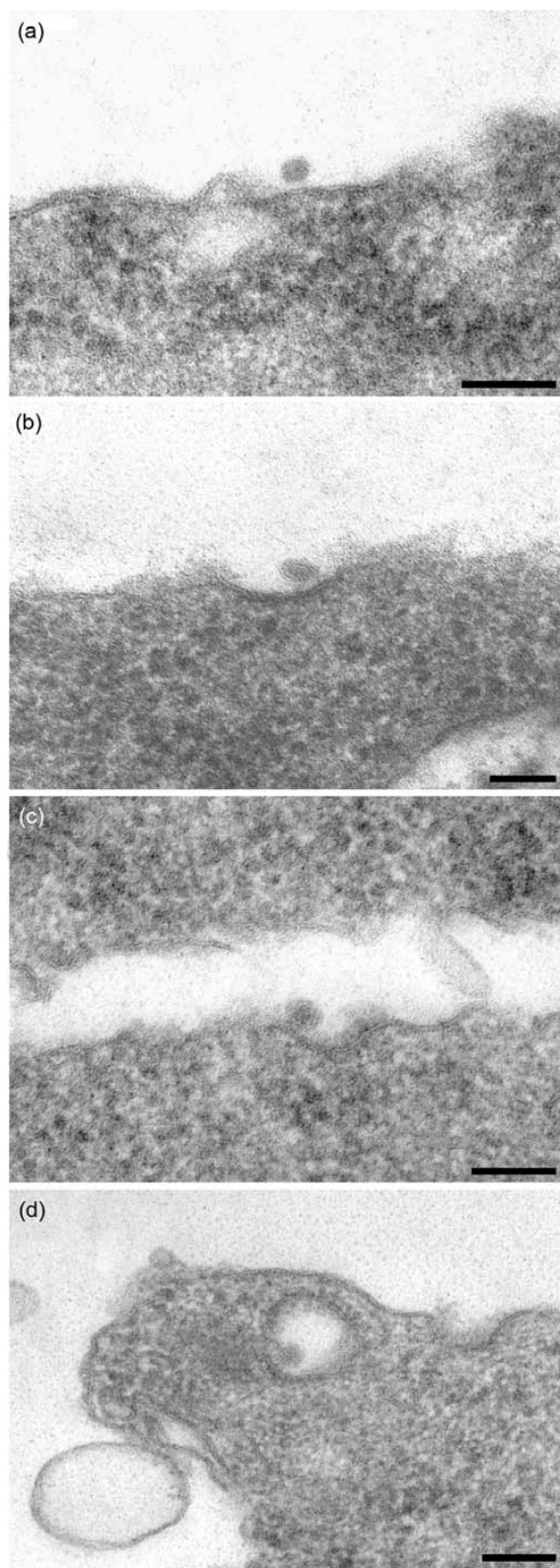
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Fig. 6. Ultrastructural study of DENV-2 entry into mosquito cells. Cells were infected with DENV-2 and after 1 h adsorption at 4 °C, cultures were shifted to 33 °C to allow internalization. After 25 min, samples were fixed and processed for electron microscopy. (a) Virus particle in the extracellular medium. (b, c) Virus particles attached within electron-dense invaginations of the plasma membrane. (d) Virus particle within an endocytic coated vesicle. Bars, 100 nm.

Although these inhibitors may have other non-specific side effects inside the cell, the consistent DENV-2 inhibition obtained using compounds acting through diverse mechanisms indicate that DENV-2 internalization proceeds by clathrin-mediated endocytosis. Moreover, the results of clathrin-coated vesicle involvement obtained with these chemical inhibitors were further supported by other experimental approaches. First, using immunofluorescence assays, the DENV-2 E glycoprotein was found to co-localize with transferrin, a marker ligand of clathrin-dependent internalization. Secondly, we performed electron microscopy experiments and found that DENV-2 was always observed inside clathrin-coated pits and vesicles. Finally, a dominant-negative mutant of Eps15, EΔ95–295, was used as a molecular inhibitor that specifically interferes with clathrin-coated vesicle formation. Expression of EΔ95–295 in C6/36 cells strongly altered the internalization of DENV-2 and this specific interference provided strong and confirmatory evidence of the functional role of the clathrin-mediated pathway of endocytosis for DENV-2 infection of mosquito cells.

As clathrin-coated vesicles deliver their cargo in acidic early endosomes, we also verified that a low-pH-dependent compartment was necessary to accomplish DENV-2 entry. Early treatment with ammonium chloride, which instantly raised the endosomal pH, prevented DENV-2 infection of C6/36 cells, as shown by infectivity and immunofluorescence assays. This is consistent with recent structural studies, which have shown that the envelope glycoprotein E of DENV, as well as that of other flaviviruses, undergoes a conformational transition under acidic conditions from a native dimeric form to a fusogenic trimeric form (Kuhn et al., 2002; Modis et al., 2004; Stiasny & Heinz, 2006). Each monomer contains three structural domains: domain I contains the N terminus and is the central domain that organizes the structure, domain II is an elongated structure providing most of the dimerization contacts and domain III has an Ig-like fold and constitutes the C terminus. Domain III has been proposed to contain the receptor-binding site, whereas a loop structure at the tip of domain II has been described as the fusion peptide. In the native dimeric form of the E glycoprotein, the fusion peptide is buried in the inner surface and becomes exposed as consequence of the irreversible conformational changes initiated by exposure to the endosomal low pH (Modis et al., 2003), here shown to be required for successful entry of infective DENV-2 into C6/36 cells.

virus yield reduction, as well as reduced viral antigen expression. In contrast, nystatin failed to affect DENV-2 replication, indicating that DENV-2 does not use an alternative caveolae-dependent endocytosis for entry.

The host cytoskeleton has been involved in the trafficking of endosomes containing ligand–receptor complexes, and a sequential participation of microfilaments and microtubules has been proposed. Actin filaments appear to facilitate the uptake of ligands internalized via coated pits and the subsequent degradative pathway, whereas microtubules are involved in maintaining the endosomal traffic between peripheral early and juxtanuclear late endosomes (Durrbach *et al.*, 1996). In our experiments, DENV-2 replication was significantly affected by pre-treatment of cells with cytochalasin D concentrations able to prevent actin polymerization without causing cytotoxicity. The requirement for microfilament integrity as a mediator of DENV-2 internalization in mosquito cells is in accordance with recent evidence that the actin cytoskeleton is associated with moving endocytic vesicles from the plasma membrane into the cytosol through the motor action of myosin VI (Buss *et al.*, 2001; Qualmann & Kessels, 2002), and provides additional support for clathrin-dependent DENV-2 entry. In contrast, microtubule disruption by colchicine treatment did not impair DENV-2 infectivity in mosquito cells, suggesting that DENV-2 does not require transport from early to late endosomes for successful infection. This result is indicative of the location at early endosomes of the pH-dependent fusion between the viral envelope and the endosomal membrane leading to virus uncoating and release of the nucleocapsid into the cytosol. Krishnan *et al.* (2007) achieved a similar conclusion for DENV-2 infection of HeLa cells using dominant-negative mutant overexpression of Rab 5 and 7 GTPases, which regulate the transport to early and late endosomes, respectively (Schimmoller *et al.*, 1998).

According to our data, DENV-2 appears to use a similar internalization route in mosquito cells to other studied members of the genus *Flavivirus* in mosquito and mammalian cells, such as West Nile virus (Chu & Ng, 2004; Chu *et al.*, 2006; Gollins & Porterfield, 1985; Mizutani *et al.*, 2003) and Japanese encephalitis virus (Nawa, 1998; Nawa *et al.*, 2003). Furthermore, the entry of members of others genera in the family such as hepatitis C virus (Blanchard *et al.*, 2006; Meertens *et al.*, 2006) and the pestivirus bovine viral diarrhea virus (Krey *et al.*, 2005; Lecot *et al.*, 2005) was also recently shown to occur by clathrin-mediated endocytosis. In contrast, our findings do not agree with the unique previous study reporting that DENV-2 enters C6/36 cells by direct fusion with the plasma membrane (Hase *et al.*, 1989). Although the same serotype and host cell were used in both studies, the divergent results may be due to the fact that the conclusions of Hase *et al.* (1989) were based on ultrastructural electron microscopy studies, whilst we undertook a detailed analysis of DENV entry at the biochemical and molecular level. The information available about the mode of DENV entry into vertebrate cells is scanty and controversial. An early study reported the occurrence of direct fusion at the plasma membrane in BHK cells (Lim & Ng, 1999), whereas a recent study demonstrated clathrin-dependent endocytosis

for DENV-2 in HeLa cells (Krishnan *et al.*, 2007). As the four DENV serotypes can replicate in a wide range of host cells, further investigation is required to elucidate whether there is any dependence of the entry process on the cell type and serotype. In fact, the four serotypes have been shown to exhibit a differential susceptibility to inhibitors of virus binding and penetration: DENV-2 and DENV-3 were the more susceptible serotypes, whereas DENV-1 and DENV-4 showed very weak inhibition (Lin *et al.*, 2002; Talarico & Damonte, 2007; Talarico *et al.*, 2005). In accordance with these results, a recent study reported important structural differences in E domain III of DENV-4 in comparison with those of DENV-2 and DENV-3 (Volk *et al.*, 2007). A detailed understanding of the early interactions between DENV serotypes and the host cell may reveal new targets for anti-flavivirus therapy and the prevention of mosquito-to-human transmission.

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