

Cross-talk between Rac1 and Cdc42 GTPases regulates formation of filopodia required for dengue virus type-2 entry into HMEC-1 cells

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Infection with dengue virus type-2 (DENV-2) begins with virus adherence to cell surface receptors. In endothelial cells (HMEC-1), a cell model for DENV-2 infection, $\alpha 5\beta 3$ integrin has been identified as a putative receptor for the virus. Previous work had suggested that the actin cytoskeleton of HMEC-1 cells plays an important role in virus entry and infection. In the present work, fixed and living HMEC-1 cells expressing enhanced green fluorescent protein–actin were monitored for actin reorganization after virus inoculation, utilizing fluorescence and time lapse microscopy. Cell infection and production of infective viruses were quantified using an anti-E protein antibody and by measuring the p.f.u. ml⁻¹. Specific drugs that antagonize actin organization and regulate actin–signalling pathways were tested in viral adhesion and infection assays, as were the expression of dominant-negative Rac1 and Cdc42 proteins. Disorganization of actin precluded infection, while microtubule depolymerization had no effect. Activation of Rac1 and Cdc42 signalling, which occurs upon virus binding, induced reorganization of actin to form filopodia in the cellular periphery. Formation of filopodia was a requirement for virus entry and further cell infection. Expression of the dominant-negative proteins Rac1 and Cdc42 confirmed the role of these GTPases in the actin reorganization that is required to form filopodia. In addition, inhibition of the ATPase activity of myosin II greatly decreased infection, suggesting its participation in filopodial stability. We show here, for the first time, that internalization of DENV-2 into endothelial cells requires viral induction of dynamic filopodia regulated by Rac1 and Cdc42 cross-talk and myosin II motor activities.

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INTRODUCTION

Dengue fever (DF) is an important public health problem in tropical and subtropical countries; worldwide, it is the most important mosquito-transmitted viral disease of humans (Guzmán-Tirado *et al.*, 1999). It is caused by the single-stranded RNA virus dengue virus (DENV), which is transmitted to humans by the *Aedes* mosquito. Transmission of the virus could result in either asymptomatic infection, undifferentiated febrile illness, DF or the life-threatening dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS) (Kliks *et al.*, 1989). However, it is not fully understood how the virus can produce mild or more severe manifestations of the disease (Leitmeyer *et al.*, 1999).

DENV is an enveloped RNA virus formed by nine proteins, of which the three named C, E and M are structural

components of the viral particles. The nucleocapsid is surrounded by a lipid bilayer in which M and E, both glycoproteins, are inserted. E-protein is assembled as homodimers on the surface of mature virions and mediates early interactions for the virus entry into target cells through specific receptors (Henchal & Putnak, 1990; Chambers *et al.*, 1990; Seema & Jain, 2005).

In naturally DENV-infected human tissues, high copy numbers of positive-strand viral RNA have been detected in endothelial cells (Jessie *et al.*, 2004). *In vitro* infection of human endothelial cells by DENV has been utilized to study viral pathogenesis, due to the pathological vascular leakage reported to occur in dengue patients (Wei *et al.*, 2003; Talavera *et al.*, 2004; Peyrefitte *et al.*, 2006; Basu & Chaturvedi, 2008).

Infection of human dermal microvascular endothelial cells, HMEC-1, by DENV-2 has been shown to cause disorganization of tight junctions, leading to increased permeability

Five videos showing HMEC-1 cells expressing EGFP–actin following various treatments are available as supplementary material with the online version of this paper.

and disruption of the cell monolayer cytoarchitecture and functions. These alterations were accompanied by notorious actin rearrangements in infected and non-infected cells and release of the inflammatory cytokine interleukin 8 (Talavera *et al.*, 2004).

Recent studies with HMEC-1 cells have identified the membrane protein $\alpha 5 \beta 3$ integrin as a receptor for DENV-2 (Zhang *et al.*, 2007). Integrins are heterodimeric receptors that play an important role in cellular migration, which is an essential function of vascular endothelial cells for wound repair, angiogenesis and the maintenance of vascular integrity (Boudreau & Jones, 1999). Integrins can transduce signals that activate regulatory pathways involved in actin organization (Huvener & Danen, 2009).

Several reports have documented that some enveloped viruses induce the formation of diverse actin structures to facilitate their internalization into host cells (Cudmore *et al.*, 1997; Higley & Way, 1997; Ploubidou & Way, 2001; Chu *et al.*, 2003, 2006; Eash & Atwood, 2005). Viral particles of murine leukemia virus induce actin reorganization to form filopodia to which viruses have been reported to associate before cell infection (Lehmann *et al.*, 2005).

Having demonstrated that DENV-2 infection of HMEC-1 cells induces actin reorganization (Talavera *et al.*, 2004), we investigated the participation of the actin cytoskeleton in the initial stages of DENV-2 infection. We report here that DENV-2 binding to these cells induced the formation of filopodia, a motile function that allows the viral particles to move towards the cell body for internalization. The actin reorganization taking place upon the binding step is regulated by activation of Rac1 and Cdc42 GTPases and it is essential for the formation and function of filopodia and a productive viral infection.

METHODS

Cell lines and culture. The HMEC-1 cell line was generously donated by E. W. Ades and F. J. Candal from the CDC (Atlanta, GA, USA) and T. J. Lawley (Emory University, Atlanta, GA, USA) and cultured as described previously (Talavera *et al.*, 2004). Cells were used at passages 17–25. C6/36 HT cells were utilized for virus propagation and BHK-21 (clone 15) cells for titration of virus infectivity and were grown at 34 and 37 °C, respectively, as previously described (Tesh, 1979).

Transfection. HMEC-1 were plated at 2×10^6 cells per plate and transfected with 20 μ g purified plasmid DNA encoding enhanced green fluorescent protein (EGFP)-tagged β -actin (Clontech) or plasmid DNA encoding dominant-negative versions of Rac1-N17 and Cdc42-N17 (kindly donated by Dr J. Chernoff, Fox Chase Cancer Center, Philadelphia, PA, USA). Electroporation assays were performed as described previously (Hernández *et al.*, 2004). Transfection efficiency of the HMEC-1 cells was determined by flow cytometry, measuring the fluorescence signal of EGFP-actin inside the cells. For the dominant-negative Rac1 or Cdc42, FITC-labelled anti-C-myc was used to detect the C-myc tag in the transfection vectors. Transfection efficiencies for cells transfected with EGFP-actin and the GTPase mutants were 70 and 43 %, respectively.

Virus stock and titration. DENV-2 was obtained from the Institute of Diagnostic and Epidemiological Reference in Mexico City, Mexico. The strain was isolated from a patient who developed DF. Monolayers of C6/36 HT were inoculated with the virus and incubated at 34 °C for up to five passages. After the last passage, cells were lysed by freeze–thaw cycles and stored in aliquots at –70 °C. The virus was titrated by plaque assays using BHK 21 monolayers. The p.f.u. ml⁻¹ was calculated as described by Meiklejohn *et al.* (1952) and Fujita *et al.* (1975). Supernatants of HMEC-1 infected with DENV-2 were also titrated by this method.

Virus inactivation. Frozen virus stocks were thawed at 37 °C. For virus inactivation, AT-2 (Sigma-Aldrich) was added to purified virus preparations to a final concentration of 50 μ M in DMSO. After 1 h at 37 °C, AT-2 was removed by ultracentrifugation and virus particles were used within 2 h (Rossio *et al.*, 1998). AT-2-treated virus showed less than 2 % productive infectivity.

Virus fluorescent labelling. The lipophilic dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine (DiD) (Molecular Probes) was added to the virus suspension at 2 nM concentration and incubated for 20 min at 22 °C. Unbound dye was removed by ultracentrifugation (Lakadamyali *et al.*, 2003). DiD-labelled virus retained the same infectivity as unlabelled particles.

Virus binding assay. Cells were grown on glass coverslips in regular culture medium before virus inoculation. Prior to the binding experiment, cells were washed with 1 \times PBS. Subsequently, DiD-labelled DENV-2 particles were added to the cells and incubated for 30 min at 4 °C. Unbound viral particles were removed by washing the cells with 1 \times PBS. Coverslips were mounted with VectaShield H-1000. DENV-2 DiD particles were visualized with an Olympus fluorescent confocal microscope using a 60 \times Planapo objective.

Cell infection. HMEC-1 cells (3.5×10^5 cells ml⁻¹) seeded on glass coverslips and cultured for 24 h were inoculated with active or inactivated virus at an m.o.i. of 3.0 and kept on ice for 30 min to allow virus adhesion and synchronizing of the infection. After this time, cells were cultured at 37 °C for 90 min to allow virus internalization. Viral inocula were then removed and fresh culture medium was added for an additional 48 h. In some cases, cells were treated with cytochalasin D (CD; 5 μ g ml⁻¹), nocodazole (20 μ M), NSC23766 (100 μ M), Y-27632 (30 μ M) or blebbistatin (100 μ M) for 60 min prior to DENV-2 inoculation. After removal of the drugs and the viral inoculum, cells were cultured for 48 h at 37 °C to continue the infection.

Detection of viral E-protein. Virus-infected HMEC-1 grown on glass coverslips were fixed in 3.7 % formaldehyde and permeabilized with 0.05 % Triton X-100 in PBS. Cells were blocked with PBS/2 % BSA and exposed for 1 h to a monoclonal antibody (mAb) to DENV-2 E-protein (donated by the Pedro Kourí Institute, Havana, Cuba) at 1:10. Cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG at 1:200 for 1 h. Coverslips were mounted with VectaShield H-1000 and analysed with an Olympus epifluorescence microscope.

Flow cytometry analysis. At 48 h post-inoculation (p.i.), DENV-2-infected and control cells were harvested and fixed with 2 % formaldehyde in PBS/FBS for 20 min at room temperature and permeabilized with 0.05 % Triton X-100. DENV-2 E-protein mAb, at 1:50, was added for 60 min at 4 °C. Cells were washed with PBS/FBS and incubated with FITC-conjugated anti-mouse IgG for 30 min at room temperature. Fluorescence intensity of the cells was determined in a FAC-Scalibur flow cytometer and data were analysed using FlowJo software (Tree Star). As a negative control, endothelial cells were only treated with the secondary antibody.

Visualization of F-actin after viral infection. Cells grown on coverslips were fixed with 3.7% paraformaldehyde. F-actin was stained with rhodamine-phalloidin following the manufacturer's instructions (Molecular Probes). After rinsing with $1 \times$ PBS, coverslips were mounted with Vecta-Shield. Images were acquired with a colour digital camera and processed with the Image-Pro Plus-5 software (Media Cybernetics).

Time-lapse fluorescent microscopy. For live cell imaging, 3×10^4 EGFP-actin-transfected endothelial cells were plated on Lab-Tek chamber slides for 24 h at 37°C . DENV-2 DiD particles were added to cells which were kept on ice, under the experimental conditions described for the virus binding assay (4°C). Subsequently, they were transferred to 37°C (time point=0 min) and kept at the same temperature throughout the monitoring of actin reorganization. Cells were continuously monitored with a colour digital video camera and the complete sequences were acquired for imaging.

Assay for activated GTPase proteins. Activation of Rac1 and Cdc42 GTPases was assessed by the CRIB pull-down method (Benard *et al.*, 1999). Briefly, endothelial cells lysates were prepared from DENV-2-inoculated serum-starved cells and transferred to 37°C for 5, 10 and 15 min. Cells were lysed with buffer A (50 mM Tris/HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 10 mM MgCl_2), containing a complete protease-inhibitor cocktail (Roche). Supernatants were incubated with the GST-CRIB domain of PAK1, an effector of Rac1/Cdc42. Pellets and aliquots from the cell lysates were separated by 15% SDS-PAGE electrophoresis. After electro-transfer, membranes were reacted with a polyclonal antibody (pAb) to Rac1 or with a pAb to Cdc42 (both diluted 1:1000) and incubated with peroxidase-labelled goat anti-rabbit IgG (diluted 1:2000). The 21 kDa band revealed by enhanced chemiluminescence corresponded to pulled down activated forms of Rac or Cdc42. As positive controls, HMEC-1 cells were stimulated with insulin (agonist of Rac1) or bradikinin (agonist of Cdc42) for 20 min at 37°C and cells were subjected to the same treatment. Densitometry analyses were performed using image analyser SigmaGel.

Statistical analysis. Data are presented as means \pm SD. In all cases, they represent at least three independent determinations. The significance of the results was calculated by Student's *t* test utilizing the software Sigma Stat. *P* values ≤ 0.05 were considered significant with respect to controls.

RESULTS

Organization of the actin cytoskeleton is required for infection of HMEC-1 cells by DENV-2

It has been reported that some viruses modify the host cell cytoskeleton presumably to facilitate their internalization (Radtko *et al.*, 2006). We used CD and nocodazole to disrupt microfilaments and microtubules of HMEC-1 cells during the first stages of the infection process with DENV-2, to determine the cytoskeleton participation. Cytoskeleton patterns were assessed in cells fixed and stained with rhodamine-phalloidin or with a mAb to β -tubulin. Fig. 1a (i, iii) shows the normal pattern of actin filaments and microtubules. CD-treated cells show microfilaments collapsed in the cytoplasm and actin aggregates in the cell periphery instead of straight filaments and stress fibres (Fig. 1a, ii). Treatment with nocodazole (Fig. 1a, iv) shows the disrupted microtubule network and its retrac-

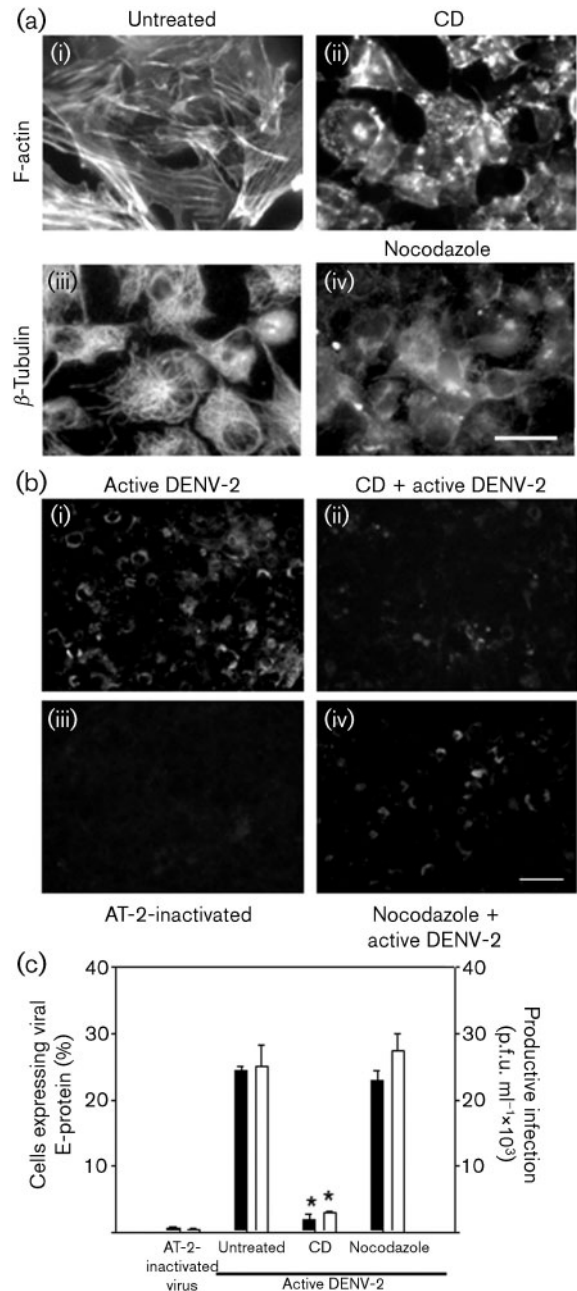


Fig. 1. DENV-2 infection of HMEC-1 cells in the presence of CD or nocodazole. Cells were pretreated with CD or nocodazole. (a) Pattern of actin filaments and microtubules in untreated (i, iii) and treated (ii, iv) cells. Bar, 20 μm . (b) E-protein expression in control and CD- or nocodazole-pretreated cells (i, ii, iv) and in cells inoculated with AT-2-inactivated virus (iii). Bar, 100 μm . (c) Virus entry and productive infection in each condition was analysed by flow cytometry (black bars) and p.f.u. ml^{-1} (white bars), respectively. **P* ≤ 0.05 . Error bars, SD.

tion from the cell periphery. Expression of the viral envelope E-protein in the cytoplasm of cells inoculated with DENV-2 and cultured for an additional 48 h was considered an indicator of virus entry into the cells

(Fig. 1b). The results showed that very few cells pretreated with CD and then inoculated with the virus were positive for E-protein (Fig. 1b, ii) and that cells treated with nocodazole contain E-protein in the cytoplasm at similar levels to untreated cells (Fig. 1b, iv). Cells inoculated with AT-2-inactivated virus (negative control of virus entry) did not show visible E-protein expression (Fig. 1b, iii). Quantification of the fluorescent E-protein signal in three experiments (Fig. 1c) revealed that 26% of virus-inoculated cells were stained with the E-protein mAb, while the percentage of E-protein-positive cells decreased to 3% in the presence of CD. The expression of E-protein was not affected by nocodazole, but it dropped to 0.5–1.0% when cells were inoculated with AT-2-inactivated viruses. Productive viral infection under the above conditions was assessed by titration of infective viral particles released to cell culture media, which showed a direct correlation between the expression of E-protein in infected cells and the outcome of productive infection (Fig. 1c).

These data show that productive infection of HMEC-1 cells with DENV-2 requires active viruses, is sensitive to CD and is not affected by nocodazole, indicating that virus infection requires normal organization and dynamism of the actin cytoskeleton.

The initial contact or binding of DENV-2 to HMEC-1 cells is independent of actin organization

Having shown that actin organization is an important factor for the outcome of DENV-2 infection of HMEC-1 cells, we utilized CD as a tool to dissect actin participation in the initial stages of the viral cycle, i.e., the binding and internalization of the virus. Binding of the viral particles to HMEC-1 cells was analysed utilizing DiD-labelled DENV-2 particles. HMEC-1 cells (control and pretreated with CD) were inoculated with fluorescent active virus and cultured at 4 °C to allow binding but avoid cell internalization. After 30 min, unbound virus was removed and cells were visualized by confocal fluorescence microscopy. Fig. 2(a) shows two representative microscope fields, out of 15 for each condition, in which viral particles are bound to the cells. These can be seen as bright white spots on the surface of control cells and CD-treated cells (Fig. 2a, i, ii). In the corresponding phase-contrast images (Fig. 2a, iii, iv), viral particles and the apical surface of the cells were focused at the same focal plane. Quantification of the particles bound to the cell surface in each condition is shown in Fig. 2(b).

These data show that binding of viral particles was not significantly decreased by CD, indicating that in the first stage of the virus infection of cells, actin organization is not essential.

DENV-2 binding induces the formation of actin-containing filopodia which associate to the viral particles

Although CD had a negative effect in the outcome of cell infection, it did not block virus binding. Thus, it is

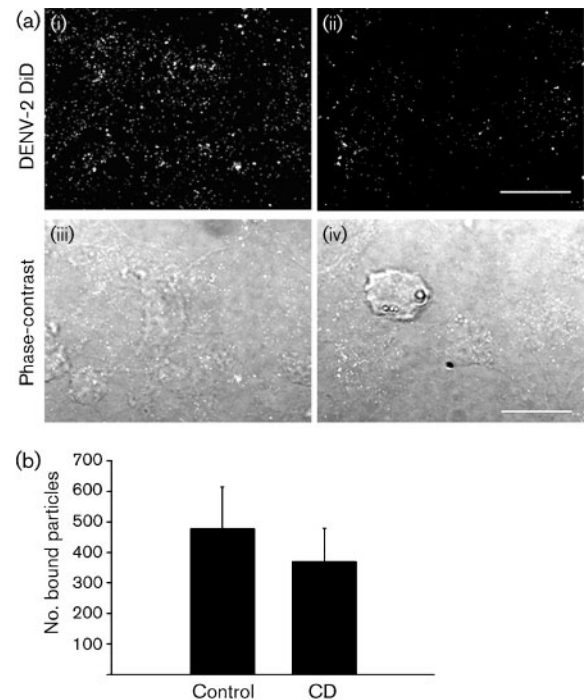


Fig. 2. Binding of DiD-labelled viral particles to HMEC-1 cells. (a) Two representative microscope fields, out of 15 for each condition, showing DENV-2 DiD particles bound to the HMEC-1 cells (white, bright spots) (i, ii) and the corresponding phase-contrast images of the same cells (iii, iv), where viral particles and the apical surface of the cells were focused at the same focal plane. (i) and (iii) show control cells inoculated with DENV-2-DiD; (ii) and (iv) show cells pretreated with CD then inoculated with DENV-2 DiD. Bar, 5 μ m. (b) Quantification of cell-bound particles in each condition in 15 confocal microscope fields. Error bars, sd.

plausible that actin organization is required in subsequent steps of the infection process. Actin organization after binding of DENV-2 particles was followed in live HMEC-1 cells expressing EGFP-actin using time-lapse fluorescence microscopy. Fig. 3(a) shows the normal actin pattern observed in control cells not infected with the virus. In a cell inoculated with DiD-labelled DENV-2, actin-containing filopodia protruding from the cell surface are clearly seen (Fig. 3b). The 12 min frame (left) shows two viral particles (red) associated with filopodia, while other particles are floating free close to the cell. At 17 min, one particle that had already reached the cell body but was out of focus in the previous frame (yellow) is seen and the two particles associated to filopodia have moved closer to the cell body. By 20 min, only one of the particles is still visible and still associated to the filopodium, while the other particles have moved to different focus planes. Trajectories of the two particles throughout the 8 min period are shown in the last frame. Calculation of viral movement indicated a velocity of 0.19 μ m min⁻¹ for the top particle and of 0.06 μ m min⁻¹ for the bottom one. The movements of filopodia and viral particles during the 20 min

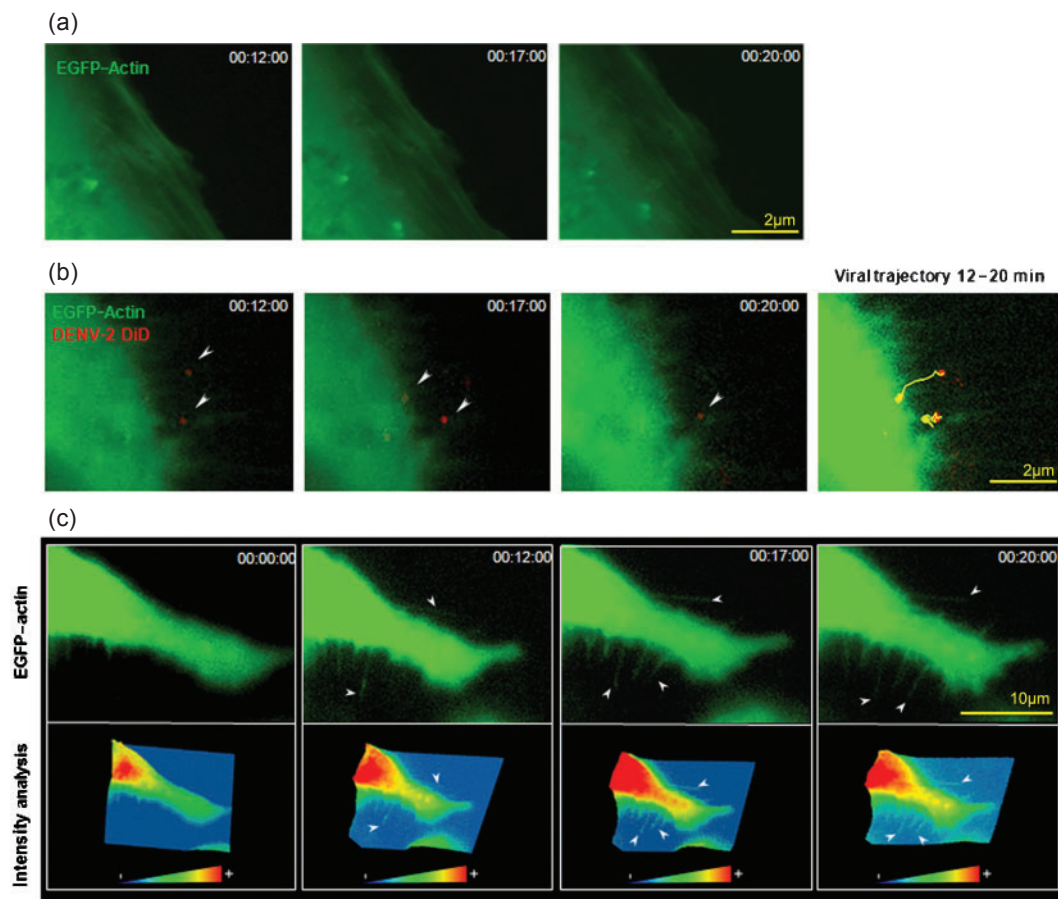


Fig. 3. Time-lapse microscopy of DENV-2 interaction with EGFP-actin-expressing cells. (a, b) Distribution pattern of EGFP-actin in a representative control cell (a) and in a cell inoculated with DENV-2 DiD (b). The frames show the position changes of two viral particles associated with filopodia (arrowheads). The last frame shows the trajectory of the particles. The time is shown in min. (c) The upper frames show actin organization in the cytoplasm, at the cell periphery and in filopodia (arrowheads) of a cell inoculated with DENV-2. The lower frames show the fluorescence intensity analysis of EGFP-actin in the same cell. The colour scale indicates variations of EGFP-actin concentration. The sequences show a representative cell selected from 20 microscope fields analysed in each condition. These sequences are shown in Supplementary Video S1, available in JGV Online.

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The fluorescence intensity analysis (Fig. 3c) provides a quantitative appreciation of actin distribution during the formation of filopodia. The frame at 0 min, the time that cells were transferred to 37 °C, shows a representative cell, where a higher concentration of actin is seen in the centre, a lower concentration is seen in the cell extension and small protrusions are being formed in the periphery. At 12 min, actin has flowed into the cell extension and filopodia have become longer and clearly visible (arrowheads). The different position of the arrowheads in relation to the cell body at 17 and 20 min shows the dynamism of the virus-induced filopodia. The intensity changes of actin concentration in the cell extension can also be appreciated in this figure.

Induction of filopodia is required for virus internalization and cell infection

As described above, interaction of DENV-2 with HMEC-1 cells induced a fast reorganization of the actin cytoskeleton leading to formation of filopodia. The formation of these structures in mammalian cells is regulated by the small GTPases of the Rho family. HMEC-1 cells expressing EGFP-actin were pretreated with NSC23766 (inhibitor of Rac1 GTPase) and Y-27632 (inhibitor of RhoA GTPase) and then inoculated with the virus and monitored by time-lapse fluorescence microscopy (Fig. 4). In control cells that were not inoculated with DENV-2 or treated with drugs, the actin cytoskeleton pattern normally seen in HMEC-1 cells remained the same throughout the recording time (Fig. 4a). When cells were inoculated with the virus in the absence of any of the drugs (Fig. 4d), many filopodia were

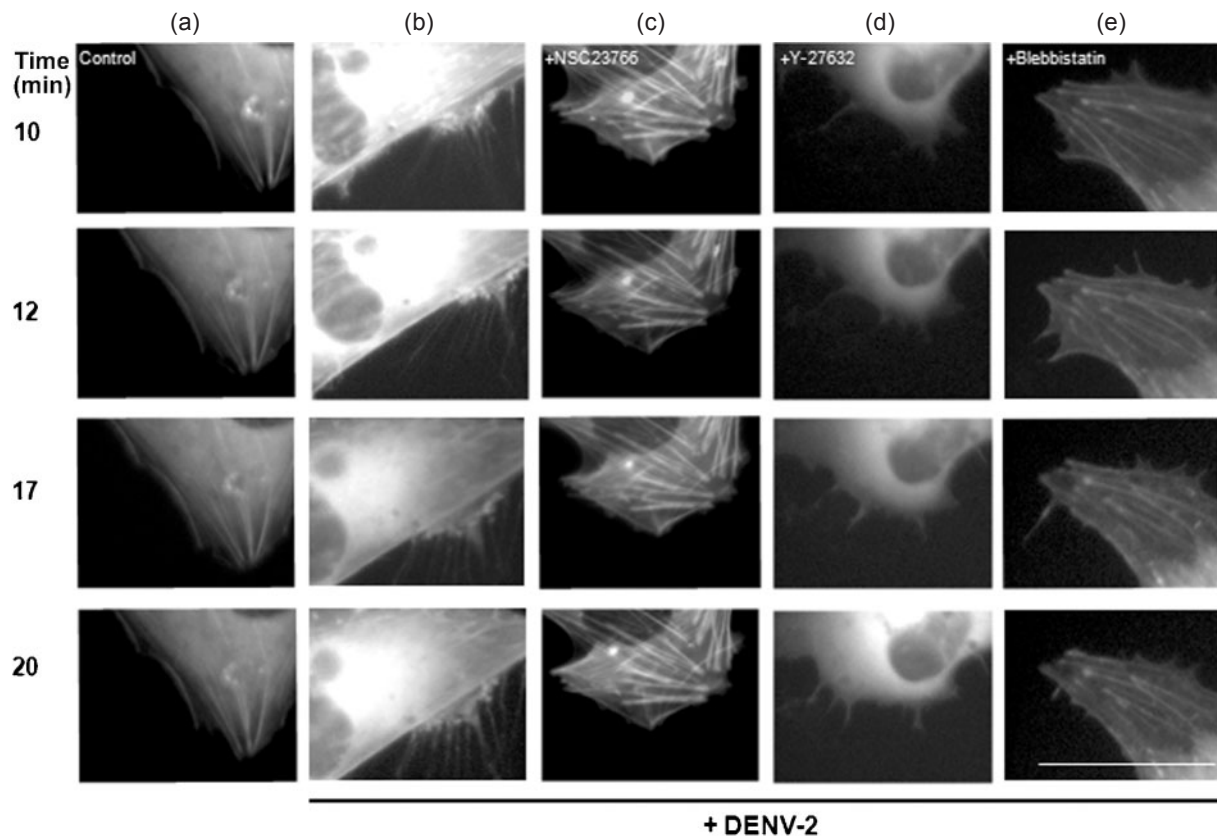


Fig. 4. Effect of inhibitors of actin organization in the induction of filopodia. Cells expressing EGFP-actin were pretreated with NSC23766 (c), Y-27632 (d) or blebbistatin (e) before inoculation with DENV-2. A control cell not inoculated with virus (a) and a drug-untreated cell inoculated with virus (b) are also shown. Bar, 20 μm . The sequences show a representative cell selected from 20 microscope fields analysed in each condition. These sequences are shown in Supplementary Videos S2–S5, available in JGV Online.

formed in approximately 10 min (also shown in Fig. 3). The elongation and change in position of the filopodia that occurred in the cell at 12, 17 and 20 min can be seen. In contrast, cells pretreated with NSC23766 (Fig. 4c) did not form filopodia at any time and instead, prominent stress fibres were organized in the cytoplasm. Pretreatment with Y-27632 (Fig. 4d) had no effect, as filopodia were seen in the periphery of the monitored cell, although stress fibres were not present in the cytoplasm. Additionally, cells were also pretreated with blebbistatin (inhibitor of myosin II ATPase activity), as this molecule has been reported to participate in the function of filopodia during viral infection (Lehmann *et al.*, 2005). Pretreatment with blebbistatin led to the formation of actin fibres, but few apparently unstable filopodia (Fig. 4e). The movement and organization of actin in these cells can be followed in Supplementary Videos S1–S5.

The specific effect of these drugs on actin organization suggested that filopodia induction by DENV-2 involves activation of Rac1 signalling and that myosin II contractile activity is required for their stability. RhoA participation was not necessary at this stage.

The formation or absence of filopodia and their consequences on viral infection were tested in cells pretreated with drugs for 60 min before inoculation with DENV-2. Fig. 5 shows the percentage of cells expressing the viral E-protein in each condition and the p.f.u. ml^{-1} of viral particles recovered from culture media of infected cells. When the cells were pretreated with CD or NSC23766, the percentage of infected cells and the p.f.u. ml^{-1} drastically decreased to values around 2–3%. Blebbistatin decreased infection to 5–7%. No effect was observed when the cells were pretreated with Y-27632 (Fig. 5).

These data suggested that the specific reorganization of actin into filopodia that is triggered by DENV-2 binding to HMEC-1 cells is mainly regulated by activation of Rac1 signalling pathways and corroborated that the absence of filopodia precluded virus entry and a productive infection.

Formation of filopodia also requires the activation of Cdc42 GTPase

The formation of filopodia has been generally assigned to the regulation of the Cdc42 GTPase (Passey *et al.*, 2004;

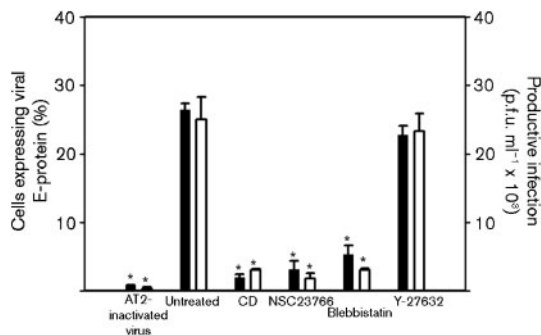


Fig. 5. Effect of NSC23766, blebbistatin and Y-27632 on the outcome of DENV-2 infection. CD-, NSC23766-, blebbistatin- or Y-27632-pretreated HMEC-1 cells were inoculated with DENV-2. Inoculation with AT-2-inactivated virus was used as a negative control. Virus entry and productive infection in each condition were analysed by flow cytometry (black bars) and p.f.u. ml⁻¹ (white bars), respectively. **P* ≤ 0.05.

Mattila & Lappalainen, 2008). As a reliable specific inhibitor for Cdc42 GTPases is not currently available, we used a different strategy to establish the participation of this other member of the Rho family.

First, the levels of activated Rac1 and Cdc42 were determined during the induction of filopodia utilizing pull-down assays of cell lysates obtained from virus-inoculated cells; these were then transferred to 37 °C and cultured for different times. Fig. 6(a) shows representative immunoblots of the assays. Densitometric measurements obtained from three independent experiments (Fig. 6b) revealed that activation of Rac1 could be detected 5 min after the transfer and reached its peak around 10 min after this. For Cdc42, activation at a lower level was also detected around 5 min and peaked at 15 min after transfer. The peaks of activity coincided with the times when filopodia were formed in virus-inoculated cells, as shown in Figs 3 and 4. Insulin and bradikinin, well known agonists of Rac1 and Cdc42, respectively, were used as positive controls for GTPase activity in the assays.

As a second approach, HMEC-1 cells were transiently transfected to express the dominant-negative GTPase mutants Rac1-N17 and Cdc42-N17. Cells expressing EGFP-actin reacted to DENV-2 inoculation by forming filopodia. Exposure to bradikinin or insulin induced formation of filopodia and lamellae, respectively, as expected from the drug-induced activation of signalling pathways. Cells expressing Cdc42-N17 (Fig. 7a, middle row) did not develop filopodia when inoculated with DENV-2 nor when treated with bradikinin, but formed lamellae when stimulated with insulin. Cells expressing Rac1-N17 (Fig. 7a, bottom row) did not form filopodia when inoculated with DENV-2, although filopodia were formed by stimulation with bradikinin; cells did not respond to insulin. These results showed that expression of the dominant mutated versions of Rac1 or Cdc42 GTPases

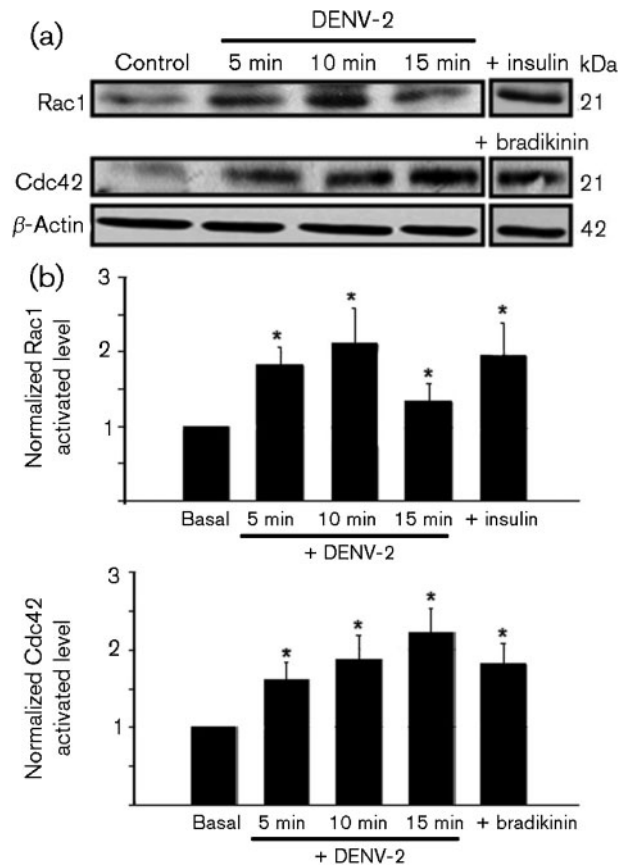


Fig. 6. Activation of Rac1 and Cdc42 GTPases during the formation of filopodia. HMEC-1 cells treated with insulin (10 nM) or bradikinin (3 µg ml⁻¹) and drug-untreated cells were inoculated with DENV-2. (a) Representative Western blot of pulled-down activated Rac1 and Cdc42 GTPases. (b) Densitometry analysis of activated Rac1 and Cdc42 in three independent experiments. **P* ≤ 0.05. Error bars, SD.

in HMEC-1 cells specifically inhibited the formation of filopodia induced by DENV-2.

The percentage of cells expressing viral E-protein and the p.f.u. ml⁻¹ quantified in cells or the culture media of cells expressing dominant-negative mutants of Rac1 or Cdc42 were very low (2%) (Fig. 7b). However, in cells pretreated with bradikinin in the absence of viral inoculation, expression of E-protein, as well as productive infection, were increased above the values determined in cells which were only inoculated with the virus. The approaches above provide evidence for the participation of Cdc42 in the formation of filopodia induced by DENV-2 inoculation and confirmed that Rac1 activation also has a role in this process.

DISCUSSION

The present data from experiments with drugs that alter actin organization and microtubule dynamics show that

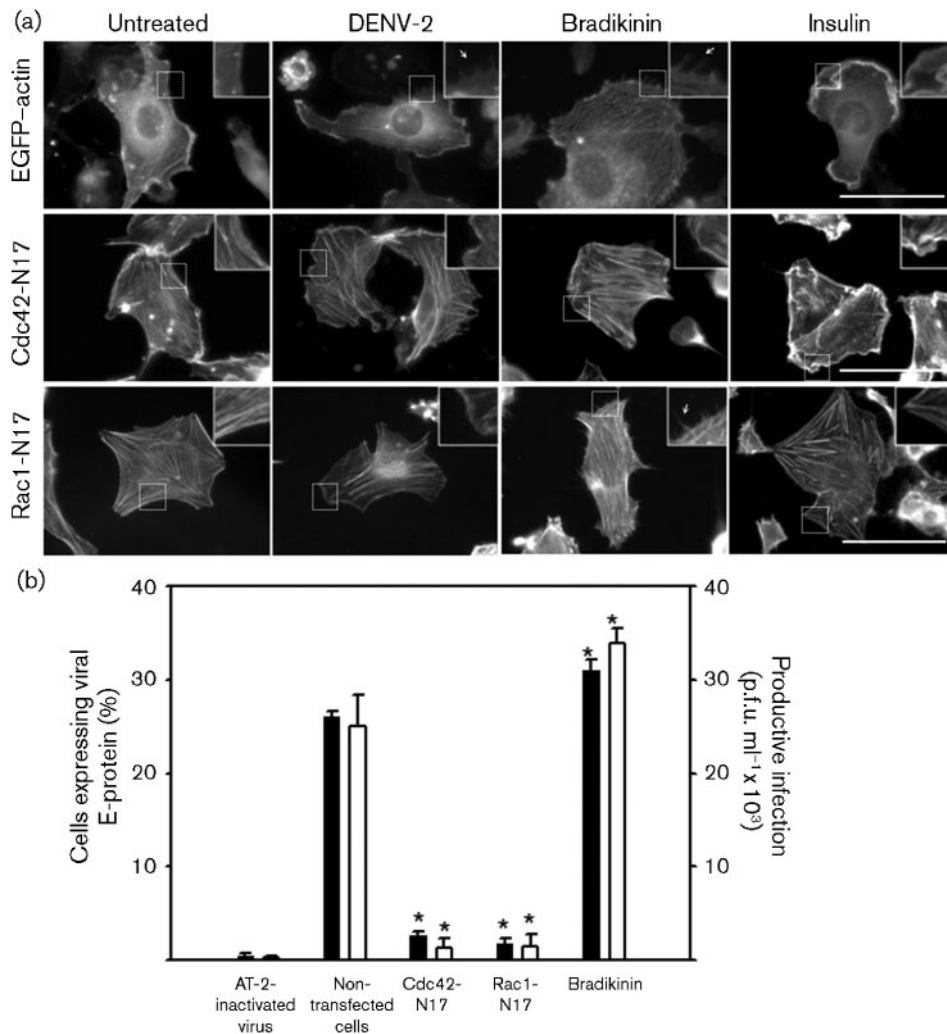


Fig. 7. Effect of the expression of dominant-negative Rac1-N17 and Cdc42-N17 proteins on filopodia induction and infection. HMEC-1 cells expressing EGFP-actin, Rac1-N17 or Cdc42-N17 mutated proteins were inoculated with DENV-2 or stimulated with bradikinin or insulin. (a) Untreated cells were not infected with virus nor treated with drugs. DENV-2, bradikinin or insulin indicate that the cell was inoculated with virus or treated with bradikinin or insulin, respectively. Insets show magnification of the cell periphery (arrows point to filopodia). Bar, 20 μm. The images show a representative cell selected from 20 microscope fields analysed in each condition. (b) Expression of E-protein (black bars; measured by flow cytometry) and productive infection (white bars; p.f.u. ml⁻¹) in cells expressing the mutant forms of Rac1 and Cdc42 or stimulated with bradikinin. Results represent three independent experiments; error bars indicate SD. * $P < 0.05$.

exposure to CD during the initial stages of DENV-2–HMEC-1 cell interaction blocked viral infection, while infection progressed unaltered when microtubules were disorganized. These data support the idea that actin organization plays an important role in the virus infection cycle.

However, when binding of DENV-2 to HMEC-1 cells was tested in the presence of CD, binding to the cell surface was not significantly affected, although bound particles were not internalized. This suggests that blockage of the viral infection by disorganization of the actin cytoskeleton could occur downstream of the binding step. Similar

results have been reported with influenza virus internalization in MDCK epithelial cells using CD (Sun & Whittaker, 2007).

DENV penetrates several types of cells, but a receptor has only recently been identified in HMEC-1 cells (Zhang *et al.*, 2007). It has been characterized as an integrin, a family of protein receptors known to be inducers of actin cytoskeleton rearrangements (Zhang *et al.*, 2007). Therefore, we propose that binding of DENV-2 to an integrin receptor may induce reorganization of the actin cytoskeleton. As the latter is sensitive to CD, this explains why internalization of the virus after binding is inhibited in its presence.

Monitoring actin dynamics and organization in live HMEC-1 cells revealed that filopodia were formed upon contact with inoculated active viruses. Filopodia are rich in lipid rafts where many receptors concentrate and can be utilized for virus adhesion. The role of filopodia could then be to transport receptors and bound viruses to the cell body where viral particles will be internalized by clathrin-mediated endocytosis (Mattila & Lappalainen, 2008). Very recently, it has been reported that DENV-2 entry into human endothelial ECV304 cells occurs via clathrin-mediated endocytosis without participation of filopodia. These and previous data with non-mammalian cells suggest that internalization of DENV-2 is likely to be cell-type-dependent (Peng *et al.*, 2009). Our present data show that filopodia formed after initial binding of DENV-2 are necessary for further binding and transport of viruses to the cell body. When filopodia were not formed, as in cells pretreated with CD, viral entry and productive infection were inhibited. However, in HMEC-1 cells, the process for internalization of viral particles is not yet documented.

It has been reported that herpes simplex and Epstein–Barr viruses activate Rac1 and Cdc42 GTPases to form lamellipodia and filopodia in epithelial cells and fibroblasts (Melamed *et al.*, 1994; Favoreel *et al.*, 2007). Thus, signalling pathways, known to regulate the formation of filopodia, were analysed after DENV-2 binding to HMEC-1 cells. When cells were pretreated with NSC23766, a specific inhibitor of Rac1 activation, and then inoculated with the virus, filopodia were not formed, suggesting the participation of Rac1 in their formation. These cells showed prominent stress fibres in the cytoplasm that could have been formed by activation of RhoA, as reported to occur by shutting off the Rac1 pathway (Mackay & Hall, 1998). In contrast with previous work with herpes virus, where RhoA GTPase has been reported to induce filopodia in infected cells (Clement *et al.*, 2006), our results showed that inhibition of RhoA signalling by Y-27632 had no effect on the formation of filopodia. Quantification of viral entry and productive infection confirmed that filopodia formation is regulated by activation of the Rac1 signalling pathway.

Filopodia are connected to the cortical actin network which, in association with myosin II, controls their contractility at their base. In cells pretreated with blebbistatin, some filopodia were induced by DENV-2, although the filopodia that were formed were unstable. It has been reported that filopodial activity and surfing of murine leukemia virus in host cells depend on actin dynamics and motor activity of myosin II and other non-conventional myosins such as myosin V and myosin X (Lehmann *et al.*, 2005; Favoreel *et al.*, 2007; Ikebe, 2008; Mattila & Lappalainen, 2008). Unfortunately, myosins in HMEC-1 cells have not been characterized. From our results, we can only suggest that motor activity of myosin II is required to regulate the proper assembly and stability of filopodia and that inadequate function would hamper the transport of viral particles. This could partially explain the very low percentage of virus entry and productive infection of HMEC-1 cells in the presence of blebbistatin.

The pull-down assays of virus-inoculated cells indicated that Rac1 activation was initiated immediately before filopodia appeared on the cell surface and peaked before Cdc42 reached maximal activity values. These assays suggested the possibility of Rac1 upstream activation of Cdc42 through cross-talking between the two signalling pathways, as is reported to occur in other cells (Tomasevic *et al.*, 2007). It has also been reported that stimulation of integrin receptors activates a signalling cascade in which PI3K and TIAM (a specific activator of Rac1) participate (Mackay & Hall, 1998); TIAM has been implicated in the cross-talk between Rac1 and Cdc42 (Chhabra & Higgs, 2007). The results obtained after cells were pretreated with NSC23766, which specifically blocks interaction between Rac1 and TIAM, suggest that binding of DENV-2 to an integrin receptor (already identified in HMEC-1 cells) could activate PI3K and trigger signalling, leading to the formation of filopodia. In this scheme, activation of PI3K would occur upstream of the activation of Rac1. Preliminary results have shown that Wortmannin, an inhibitor of PI3K activity, decreased expression of E-protein in DENV-2-infected cells, providing support for the participation of PI3K in this pathway. Rac1 would activate PAK, a kinase known to phosphorylate the specific guanine nucleotide exchange factor of Cdc42. Cdc42, in turn, will activate effectors such as WAS, WASP or ERK, involved in the organization of filopodia (Chhabra & Higgs, 2007).

The present findings provide the first evidence for the operation of actin-regulating signalling pathways in HMEC-1 cells that are activated by DENV-2 enabling the formation of filopodia. Filopodia are essential for the transport of the viruses toward the cell body for their entry into the cells and to obtain a productive infection. Filopodia are induced after virus binding to a cell receptor(s) linked to the signalling pathways of Rac1 and Cdc42 GTPases. These signalling pathways, which normally regulate the organization and function of actin in the cells under the stimulus of DENV-2, are used to facilitate viral infection.

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