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ORIGINAL ARTICLE

Surface proteins of C6/36 cells involved in dengue virus 4 binding and entry

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Abstract Dengue virus (DENV) is the causative agent of the most important mosquito-borne viral disease, which is endemic to over 100 countries in tropical and subtropical areas of the world. It is transmitted to humans by *Aedes* mosquitoes. The first step in the viral infection of host cells is virion attachment to the plasma membrane, which is mediated by specific surface molecules. There are several molecules that participate in DENV infection of mosquitoes, but only a few have been identified. In this work, we co-purified 4 proteins from C6/36 cells using a recombinant DENV 4 E protein and identified them as 70 kDa Heat Shock and 70 kDa Heat Shock cognate proteins (HSP70/HSc70), Binding immunoglobulin protein (BiP), Thioredoxin/protein disulphide isomerase (PDI), and 44 kDa Endoplasmic reticulum resident protein (ERp44) via matrix-assisted laser desorption/ionisation time of flight (Maldi-ToF) analysis. Using immunofluorescence and flow cytometry assays, we observed re-localisation of HSP70/HSc70 and, to a lesser extent, BiP to the plasma membrane under stress conditions, such as during DENV infection. By performing binding and infection assays independently, we found that all 4 proteins participate in both processes, but to differing extents: HSP70/HSc70 is the most critical component, while ERp44 is less important. Viral infection was not inhibited when the cells were incubated with antibodies against all of the surface proteins after virus binding, which suggests that DENV entry to C6/36 cells is mediated by these proteins at the same step and not sequentially.

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Introduction

Dengue is the most important mosquito-borne viral disease in humans. It has become a major international public health problem because its incidence has grown dramatically due to several factors, such as the expanding geographical distribution of the virus and its mosquito vector, the co-circulation of multiple virus serotypes in the same geographic area, urbanisation associated with poor living conditions and water supplies, a lack of vector control, and climate change [1].

Dengue is endemic in over 100 countries located in tropical and subtropical areas of the world, and approximately 2.5 billion people live in regions where Dengue virus (DENV) can be transmitted [1, 2].

The clinical manifestations of DENV infection vary from an asymptomatic or flu-like illness to serious diseases, such as dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) [2].

There are four closely related DENV serotypes (DENV 1-DENV 4), which belong to the *Flaviviridae* family in the genus *Flavivirus* [1]. These serotypes consist of single-stranded enveloped RNA viruses that encode 3 structural (C, prM, and E) and 7 non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins [3].

The first step in viral infection is interaction between virus attachment proteins (VAP) and cellular receptors. This process contributes to defining the host range, tissue tropism, and viral pathogenesis. The most recent findings suggest that a viral particle can use multiple cellular molecules during entry. For example, virions can bind to molecules on the surface of host cells that allow rapid attachment of the viral particle to the cell surface, and other receptors can then interact with the virions to permit viral entry. These secondary interactions are termed post-binding, post-attachment, entry, fusion or internalisation processes [4], and these molecules are used sequentially [5].

DENV can infect monocytes and macrophages in an indirect manner through a process referred to as antibody-dependent enhancement (ADE) wherein which pre-existing anti-viral antibodies present at sub-neutralising concentrations cause DENV internalisation through Fc receptors (FcR) [6–8]. This mechanism has also been observed in primary human dendritic cells [9], and human platelets [10].

Several molecules in mammalian cells are involved in the binding and entry of DENV. Although heparin sulphate was initially described as a DENV 2 or DENV 4 receptor in Vero cells [11] and endothelial cells [12], respectively, most DENV receptors are proteins [13, 14]. The DENV receptors identified to date include the following proteins: DC-SIGN in dendritic cells for all four DENV serotypes [15, 16]; a 37/67 kDa high-affinity laminin receptor for DENV 1 in HepG2 cells [17]; DENV 1, 2, and 3 in porcine kidney cells [18]; the mannose receptor for all DENV serotypes in macrophages [19]; GRP78 (BiP) for DENV 2 in HepG2 cells [20, 21]; heat shock proteins 70 and 90 for DENV 2 in monocytes, macrophages, U937, and human neuroblastoma cells [22, 23]; and β3 integrin for DENV 2 in HMEC-1 cells [24].

DENV is transmitted to humans by mosquitoes of the *Aedes* genus, mainly *Ae. aegytpi* and *Ae. albopictus* [1]. After a blood meal from a patient suffering from viraemia, viral replication first takes place in the midgut in the haematophagous female mosquito. Then, the virus spreads to other organs and tissues, such as the tracheal system, body fat, salivary glands, nervous system, oesophagus, haemocytes, the ommatidia of compound eyes, and malpighian tubules [25, 26]. The identification of molecules involved in DENV binding of and entry into mosquito cells has been the subject of several studies. Previous experiments performed in our laboratory discovered that DENV 4 interacts with two glycoproteins with molecular weights of 40 and 45 kDa that are present on the surface of C6/36 (*Ae. albopictus*) cells [27]. Antibodies raised against these molecules inhibited DENV 4 attachment to the surface of C6/36 cells [27] and reduced the cytopathic effect (CPE) observed in DENV 4-infected C6/36 cells in a dose-dependent manner [28], which supported the idea that these glycoproteins are DENV receptors. Moreover, both DENV 4 and antibodies against the 45 kDa glycoprotein were able to recognise a 45 kDa protein in extracts obtained from different developmental stages of *Ae. aegypti* mosquitoes and in different susceptible mosquito tissues (the salivary gland, ovaries and midgut), but not in the non-dengue vector *Anopheles albimanus,* suggesting that this protein participates in DENV infection and vertical transmission [28]. Further experiments performed under heat shock conditions suggested that the 45 kDa protein might be related to the heat shock protein family. Additionally, a 74 kDa protein that is up regulated under heat shock conditions also binds to DENV 2 [29].

In other studies performed with C6/36 cells, it was demonstrated that DENV 2 interacts with two surface proteins with molecular weights of 67 and 80 kDa [30]. These proteins are localised in the *Ae. aegypti* midgut and are apparently able to interact with all DENV serotypes. Moreover, antibodies against both proteins inhibited DENV 2 binding and infection, especially antibodies against the 67 kDa protein [31]. Because the presence of the 67 kDa protein in the midgut correlates with DENV infection susceptibility, this molecule may be a vector competence marker [32].

In virus overlay protein binding assays (VOPBA) using the four DENV serotypes and protein extracts from salivary glands of *Ae. aegypti* and *Ae. polynesiensis,* a total of 4 or 5 proteins was detected, respectively, with molecular weights ranging between 37 to 77 kDa [33].

More recent studies have identified some molecules in mosquito cells that appear to be involved in the first step of DENV infection. These molecules include heparin sulphate for DENV 1 and DENV 2, a 50 kDa protein identified as a 37/67 kDa human high-affinity laminin-related receptor for DENV 3 and 4 [34], Vav-1, HSc70, ATP synthase alpha subunit, prohibitin, osiris, actin and the tubulin beta chain [5, 35]. All of these results strongly suggest that either DENV requires more than one molecule for binding of and entry into mosquito cells, most likely in a sequential manner, or that several proteins can be used as receptors for the virus.

Materials and methods

Cells and viruses

Monolayers of C6/36 cells from *Ae. albopictus* [36] adapted to grow at 35 °C [37] were maintained in Jocklik modified minimal essential medium (MEM) supplemented with nonessential amino acids, vitamins (Gibco), 0.034 % sodium bicarbonate (Sigma), 10 % foetal calf serum

(Gibco), penicillin (100 U/mL, Sigma), and streptomycin (100 μg/mL, Sigma). The cells were grown at 35 °C without CO_2 (Instrumental Lab Line incubator).

Monolayers of BHK-21 cells were cultured in MEM supplemented with 10 % foetal calf serum, nonessential amino acids, 0.034 % sodium bicarbonate (Sigma), penicillin (100 U/mL, Sigma), and streptomycin (100 μg/mL, Sigma). The cells were grown at 37 °C under 5 % CO_2 (Lab Line incubator). The dengue type 4 virus strain H-241 (Centers of Disease Control, USA) was propagated in newborn mice and C6/36 cells [38] and titrated in BHK-21, as reported previously [39].

Antibodies

The monoclonal antibodies used in this study were mouse monoclonal antibody against 70 Heat shock protein (HSP70) (mouse monoclonal antibody SPA-820, (Stressgen), 90 Heat shock protein (HSP90) (mouse monoclonal antibody SPA-830, Stressgen), Thioredoxin/protein disulphide isomerase (PDI) (rabbit monoclonal antibody C81H6- 3501, Cell Signaling), Binding immunoglobulin protein (BiP) (rabbit monoclonal antibody C50B12- 3177, Cell Signaling) and rabbit monoclonal antibody Glyceraldehyde 3-phosphate dehidrogenase (GAPDH) (rabbit monoclonal antibody AM4300, Ambion). A rabbit polyclonal antibody against the endoplasmic reticulum (ER) resident protein (ERp44) (rabbit polyclonal antibody 2886S, Cell Signaling) and anti-actin antibody (rabbit polyclonal antibody H-196, Santa Cruz) were also used.

Preparation of total cell proteins

Monolayers of C6/36 cells were incubated at 35 °C or 41 °C for 2 h. After 1 h of recovery at 35 °C, the cells were pelleted at 1,000 rpm for 10 min at room temperature (Eppendorf 5804R Centrifuge). The pellet was then incubated for 10 min on ice and resuspended in RSB-Igepal (1.5 mM $MgCl_2$, 10 mM Tris-HCl, 10 mM NaCl, and 1 % Igepal CA-630, Sigma) in the presence of a protease inhibitor cocktail (Complete, Roche). Nuclei and debris were removed by centrifugation at 10,000 rpm for 10 min at 4 °C (Eppendorf 5415R Microfuge), and the protein content was quantified via the Bradford method [40].

Bacterial transformation and expression of the DENV 4 recombinant E protein

The recombinant plasmid pJR-Er, into which the DENV 4 envelope protein was cloned, was used to transform *Escherichia coli* (strain DH5α), and the recombinant protein was obtained using the protocol previously reported by Reyes del Valle and del Angel [41], with some modifications. Briefly, 10 mL of Luria Broth containing ampicillin (Sigma) was inoculated with 100 μL of a saturated overnight culture and grown at 37 °C until an optical density (OD) of 0.6 was reached. Then, isopropyl-thiogalactoside (IPTG) (Invitrogen) was added to a final concentration of 0.6 mM to induce protein expression for 3 h. Non-transformed bacteria were also included as a negative control. The bacteria were pelleted by centrifugation at 12,000×*g* for 15 min at 4 °C and resuspended in 1.5 mL of lysis buffer (20 mM Tris, 750 mM NaCl, 1 % Triton X-100, 5 mM 2-mercaptoethanol, pH 8.0). Next, the bacteria were lysed by incubation with 1 mg/mL lysozyme in 10 mM Tris (pH 8.0) for 30 min at 4 °C and sonicated five times for 30 s each. To recover the soluble fraction, the mixture was centrifuged at 12,000×*g* for 25 min at 4 °C. For Western blot assays, proteins obtained from the supernatant were separated via 10 % denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and the presence of the DENV 4 recombinant E protein was detected with a specific antibody (see below). To obtain the large yields of recombinant E protein needed to perform affinity chromatography, the initial expression conditions were modified. Bacteria transformed with the recombinant plasmid pJR-Er were used to inoculate 250 mL of medium, and bacterial growth was permitted for 12 h at 37 °C. When the stationary growth phase was reached, two volumes (500 mL each) of fresh medium containing 0.6 mM IPTG was added to induce expression for 5 h at 30 °C, and the recombinant E protein was obtained under non-denaturing conditions as described below.

Purification of recombinant DENV 4 E protein

To purify recombinant E protein, the protocol reported previously by Reyes del Valle and del Angel [41] was followed, with some modifications. Briefly, immobilised metal affinity chromatography was performed under native conditions. Initially, 500 μL of nickel-nitrilotriacetate agarose (Ni-NTA) (Qiagen) was equilibrated with an equal volume of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) for 5 min at room temperature. Afterwards, the resin was mixed by gentle shaking overnight at 4 °C with 15 mL of clarified lysate from bacteria that had been induced. Then, the resin was added to a column and washed 4 times with 5 mL of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 10 mM 2-mercaptoethanol, 0.5 % Tween-20, pH 8.0) for 10 min at 4 °C with constant shaking. The recombinant E protein was eluted with 1 mL of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole). Three elution fractions were collected and separated via 10 % SDS-PAGE. The presence of recombinant E protein was verified by a Western blot assay as described in the following section.

Western blot assay

The recombinant E protein and proteins from C6/36 cells were analysed in Western blot assays using the following antibodies: mouse polyclonal anti-DENV (diluted 1:10,000, MS X Dengue Complex MAB8705, Chemicon International); anti-Histidine (diluted 1:10,000, His probe H-3, Santa Cruz biotechnology); polyclonal antibodies against Gp45 (diluted 1:10,000), [27]; anti-ERp44 (diluted 1:3,000); monoclonal anti-HSP70 and anti-HSP90 (diluted 1:7,000); anti-PDI and anti-BiP (diluted 1:2,000); anti-GAPDH (diluted 1:6,000) and anti-Actin (diluted 1:5,000). An anti-mouse horseradish peroxidase (HRP)–conjugated antibody (goat anti-mouse IgG-HRP, Zymed; diluted 1:20,000) and an anti-rabbit HRP-conjugated antibody (goat anti-rabbit IgG-HRP, Invitrogen; diluted 1:15,000) were used as secondary antibodies. Briefly, extracts of recombinant E protein or total protein extracts from C6/36 cells were subjected to SDS-PAGE in a 10 % gel (Mini PROTEAN System, Bio-Rad Laboratories) and transferred to a nitrocellulose membrane using a semidry blotting apparatus (Trans-Blot SD Cell, Bio-Rad Laboratories) with transfer buffer (48 mM Tris, 39 mM glycine, and 20 % (v/v) methanol). The transfer efficiency was monitored by staining with 0.1 % Ponceau red in 7 % trichloracetic acid. The membrane was blocked with 5 % casein (Casec, Nestle nutrition) in phosphate-buffered saline (PBS) for 2 h at room temperature with gentle shaking and then incubated overnight at 4 °C with one of the primary antibodies diluted in PBS-0.5 % Tween 20. The membrane was washed 4 times with PBS-0.5 % Tween 20 and incubated for 2 h at room temperature with the appropriate secondary antibody diluted in PBS-0.5 % Tween 20. After 4 washes with PBS-0.5 % Tween 20, the reaction was developed using a chemiluminescence kit according to the manufacturer's protocol (Super Signal West Pico Chemiluminiscent Substrate, Pierce) and Medical X-ray (General Purpose blue, Kodak). Densitometry analysis was performed with Gel Quant Express 3.1 software (2005) DNR Ltd. by comparing the sample with the loading control anti-Actin or anti-GAPDH respectively.

Purification of C6/36 cell proteins that interact with the recombinant DENV 4 E protein and mass spectrometry analysis

Purification of surface proteins that interact with the recombinant DENV 4 E protein was performed as previously described by Reyes del Valle and del Angel [41]. Briefly, Ni-NTA agarose (Qiagen) was saturated with recombinant E protein by incubating 25 mL of a lysate made from induced bacteria with 500 µL of pre-equilibrated Ni-NTA resin for 1 h at 4 °C. The unbound fraction was discarded, and the resin was washed 5 times with 20 volumes of washing buffer for 10 min at 4 °C with constant mixing on a rocking platform (Platform Vari Mix). Afterwards, the resin was packed into a column and equilibrated with 10 volumes of isotonic buffer (20 mM Tris, 125 mM NaCl, pH 8.0). Subsequently, 7 mg of C6/36 total protein extract was diluted in 5 mL of isotonic buffer with 20 mM imidazole and passed through the column for 30 min at room temperature (0.5 mL/min). Nonspecific binding was avoided by washing the column with 5 volumes of isotonic buffer and 5 volumes of hypertonic buffer (20 mM Tris, 200 mM NaCl, 10 mM imidazole, pH 8.0). Proteins bound to the recombinant E protein were eluted in 2 fractions of 200 µL each with 500 mM and 1 M NaCl. The eluted molecules were analysed via 10 % SDS-PAGE and stained with Coomassie blue. Bands of 40, 45 and 74 kDa were cut out of the gel, and the peptides were analysed using matrix-assisted laser desorption/ionisation time of flight (MALDI-ToF) mass spectrometry at the Protein Core Lab Facility at Columbia University, New York, N.Y.

Detection of C6/36 cell surface proteins under stress conditions by flow cytometry

C6/36 cells were seeded into 12-well plates (6×10^5 cells/well) and incubated at 35 °C with culture medium for 24 h. Two plates were incubated with DENV 4 at a multiplicity of infection (MOI) of 0.1 for 2 h at 37 °C, and two other plates were incubated without virus at the same temperature. The culture medium was removed, and the monolayers were washed with PBS. Then, 4 % paraformaldehyde in PBS was added, and the plates were incubated at room temperature for 30 min. The monolayers were washed twice with binding buffer (PBS, 0.5 % foetal bovine serum) and blocked with foetal bovine serum overnight at 4 °C. The cells were then washed once again with binding buffer and incubated with or without monoclonal antibodies against HSP70, HSP90, BiP, PDI (diluted 1:100), and ERp44 (diluted 1:50) or secondary antibody alone diluted in binding buffer for 2 h at room temperature. After 3 washes, the monolayers were incubated with a goat anti-rabbit FITC-conjugated antibody (diluted 1:200; Invitrogen) in binding buffer for 2 h at room temperature with gentle shaking, protected from light. Next, the cells were washed 3 times with washing buffer, detached using scrapers (Corning) and resuspended in PBS plus 2 % foetal bovine serum. Finally 10,000 events were counted in a flow cytometer (FACScalibur, Becton Dickinson). Analysis of the results was performed using WinMDI 2.8 software. The experiment was performed twice in triplicate.

DIL-labelled DENV 4

C6/36 cells (8×10^6) were seeded into a 75 mm^2 flask (Corning), infected with DENV 4 at an MOI of 1, diluted in 5 mL of medium, and incubated for 1 h at 37 °C with constant mixing on a rocking platform. Then, 10 mL of culture medium was added, and the cells were incubated for 4 days at 35 °C. The cells were lysed through 3 cycles of freezing and thawing, and the supernatant was collected and centrifuged at 5,000 rpm for 30 min at 4 °C. Then, the lysate was aliquoted into 1 mL Eppendorf tubes, and 40 µL of 1,1′,-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DIL) (25 µg/µL, Invitrogen; diluted 1:500) was added, followed by incubation for 1 h at 37 °C with constant mixing on a rocking platform protected from light. Then, the lysate was centrifuged at 13,000 rpm for 10 min at 4 °C, and the supernatant was collected and stored at −70 °C (Sankyo Ultrafrezer).

Determination of virus binding by flow cytometry

Viral binding assays were performed according to the protocol employed by Triantafilou and Triantafilou [42], with some modifications. Briefly, C6/36 cells were transferred to 12-well plates (6×10^5 cells/well) and incubated at 35 °C with culture medium. The culture medium was removed, and the monolayers were washed 3 times with PBS. Then, 4 % paraformaldehyde in PBS was added, and the plates were incubated at room temperature for 30 min. The monolayers were washed 3 times with binding buffer (0.5 % foetal bovine serum in PBS) and incubated independently with mAbs against HSP70, HSP90, BiP, PDI, and ERp44, or anti-mouse IgG (diluted 1:100) as a control in binding buffer for 2 h at 4 °C with constant mixing. A cell monolayer incubated only with binding buffer was also included as a control. Then, we added 100 µL of DIL-labelled DENV 4 and incubated the cells for an additional 2 h at 4 °C with constant mixing on a rocking platform, protected from light. The monolayers were washed 3 times with binding buffer and re-fixed as described above. Finally, the cells were processed as described previously. The experiment was performed 5 times in triplicate.

Evaluation of virus binding by immunofluorescence

C6/36 cells were grown in an immunofluorescence chamber (0.4×10^5 cells/well, Lab-Tek) and incubated at 35 °C with culture medium for 48 h. The culture medium was removed, and the wells were washed 3 times with PBS and incubated with mAbs against HSP70, HSP90, BiP, PDI, ERp44, and anti-mouse IgG (diluted 1:100) as a control for 2 h at 4 °C with constant mixing on a rocking platform. Some cells were maintained in PBS alone as a staining control. The monolayers were washed 3 times with PBS and incubated with DENV 4 diluted in PBS at an MOI of 0.1 for 2 h at 4 °C with gentle shaking, followed by washing 3 times and fixation at room temperature for 30 min with 4 % paraformaldehyde in PBS. Then, the cells were blocked with 1 % bovine serum albumin in PBS for 30 min, washed 3 times with PBS and incubated overnight at 4 °C with a mouse polyclonal anti-DENV antibody (diluted 1:200, MS X Dengue Complex, Chemicon international). After 4 washes with PBS, the monolayers were incubated with a goat anti-mouse FITC-conjugated antibody (diluted 1:500, Invitrogen) in PBS for 2 h at room temperature in the dark. The cells were washed 4 times with PBS, and the nuclei were stained with 2 µL of vectashield-4', 6-diamidino-2-phenylindole (Vector Laboratories). Finally, the cells were observed under an immunofluorescence microscope (Eclipse 80i, Nikon). The relative fluorescence was measured using the NSI Advanced Research 3.0 program (Nikon).

Evaluation of viral infection by flow cytometry

C6/36 cells were transferred to 12-well plates (6×10^5 cells/well) and processed as described previously. After incubation with the various monoclonal antibodies, the monolayers were washed 3 times with binding buffer and incubated with DENV 4 diluted in the same buffer at an MOI of 0.1 for 1 h at 37 °C with gentle shaking. Then, the monolayers were washed 5 times with binding buffer and incubated at 35 °C for 48 h. The supernatant was subsequently removed and used to perform a Platelia™ assay (see below). To detect DENV, the cells were washed 3 times with binding buffer and fixed with 4 % paraformaldehyde in PBS at room temperature for 30 min. The monolayers were then washed 3 times with binding buffer and blocked with foetal bovine serum overnight at 4 °C and subsequently washed 3 times again and incubated with a mouse anti-DENV polyclonal antibody (diluted 1:200, MS X Dengue Complex, Chemicon international) in binding buffer for 2 h at room temperature. After 3 washes, the monolayers were incubated with an anti-mouse FITC-conjugated antibody (diluted 1:500) in binding buffer for 2 h at room temperature, protected from light. Finally, the cells were washed 5 times with wash buffer and processed for flow cytometry as described previously. The experiment was performed 5 times in triplicate.

Determination of viral infection by immunofluorescence

C6/36 cells were grown in an immunofluorescence chamber (0.4×10^5 cells/well, Lab-Tek) as described

above. After incubation with monoclonal antibodies, the monolayers were washed 3 times with PBS and incubated with DENV 4 diluted in PBS at an MOI of 0.1 for 1 h at 37 °C with gentle shaking. The monolayers were then washed 5 times with PBS, incubated at 35 °C for 24 h and processed for immunofluorescence as described previously.

Post-attachment virus inhibition assay

C6/36 cells were transferred to 12-well plates (6×10^5 cells/well) and processed as described previously. Then, the monolayers were washed 3 times with binding buffer and incubated with DENV 4 diluted in binding buffer at an MOI of 0.1 for 2 h at 4 °C with constant shaking. After 3 washes with binding buffer, the cells were incubated independently with mAbs against HSP70, HSP90, BiP, and PDI or a non-related anti-mouse IgG (diluted 1:100) as a control in binding buffer for 2 h at 4 °C with gentle shaking. The monolayers were subsequently washed 5 times with binding buffer and incubated at 37 °C for 1 h with gentle shaking. The monolayers were washed as described previously and incubated at 35 °C for 48 h. To detect DENV 4, the cells were processed for flow cytometry as described previously. The experiment was performed 5 times in triplicate.

Evaluation of viral infection using the Platelia assay

The supernatant obtained from the flow cytometry assay performed to detect viral infection was used to determine the DENV NS1 protein content using the Platelia™ Dengue NS1 Ag assay (BIORAD). This assay is a one-step sandwich format microplate enzyme immunoassay that allows qualitative or semi-quantitative detection of the dengue NS1 antigen in human serum or plasma [43]. The assay was performed according to the manufacturer's instructions, and the results are expressed as the OD. The OD was read at 450/620 nm on an automatic ELISA plate reader (Stat fax).

Statistical analysis

The one way Holm-Sidak test was used as a post-hoc test to determine statistical significance of each Ab treatment in comparison to the control (experiments performed with the unrelated antibody). It was selected because is more powerful than other tests and it is able to detect differences that others can not [44]. The data are expressed as the mean ± SD of independent experiments. P values of 0.05 or less were considered to be statistically significant. Statistical analysis was performed using SIGMA STAT 3.11 (Systat software, Inc.)

Results

Purification of proteins from C6/36 cells that interact with the DENV 4 recombinant envelope protein

Previous experiments have demonstrated that conducting affinity chromatography using the DENV 4 [21] or the recombinant DENV E protein [41] as a ligand allows purification of proteins implicated in viral binding of and entry into C6/36 cells. Therefore, affinity chromatography was performed, and the DENV recombinant E protein was used as bait (Fig. 1A). Extracts from non-transformed bacteria were used as a control (Fig. 1B). To enrich the cell protein fraction that bound to the recombinant E protein, elution with 250 mM imidazole was performed (Fig. 1C). The identities of the DENV E and C6/36 Gp45 (previously reported by our group [27, 28]) proteins in the elution fraction were confirmed in Western blot assays using an anti-DENV (MS X Dengue Complex MAB8705) and mouse anti-Gp45 polyclonal antibodies (data not shown). Additionally, several proteins with sizes ranging from 25 to 75 kDa were also observed in the purified elution fraction. Some of these proteins were most likely of bacterial origin. In accordance with previous reports describing mosquito proteins that interact with DENV, we selected the 40, 45, and 74 kDa proteins for further analysis. Proteins with molecular weights below 40 kDa were also detected; however, these proteins were not examined further because they have been previously analysed [35]. The 40, 45 and 74 kDa bands were excised from the gel and analysed via Maldi-ToF mass spectrometry (Table 1). Proteins from the heat shock protein family (HSP70/HSc70 and BiP), ER chaperones (ERp44 and PDI), and cytoskeleton (actin, alpha- and beta-tubulin) proteins were identified. Given that cytoskeletal proteins are not present on the cell surface, they were not analysed in further experiments.

Detection of proteins on the surface of C6/36 cells during cell infection

Commercial antibodies against the molecules identified in the Maldi-ToF analysis were selected to perform further experiments. Because these antibodies were primarily raised against mammalian proteins, we decided to verify their pattern recognition and specificity in the C6/36 protein extracts via Western blot assays. As shown in Fig. 2, all of the antibodies except anti-ERp44 detected a single band of the expected molecular weight. A possible explanation for this one discrepancy is that the antibody used to detect ERp44 was polyclonal, and one of the bands observed might be the result of a cross-reaction with a related protein. Only one isoform of ERp44 has been reported in *Ae. aegypti*; in mammals, there are two

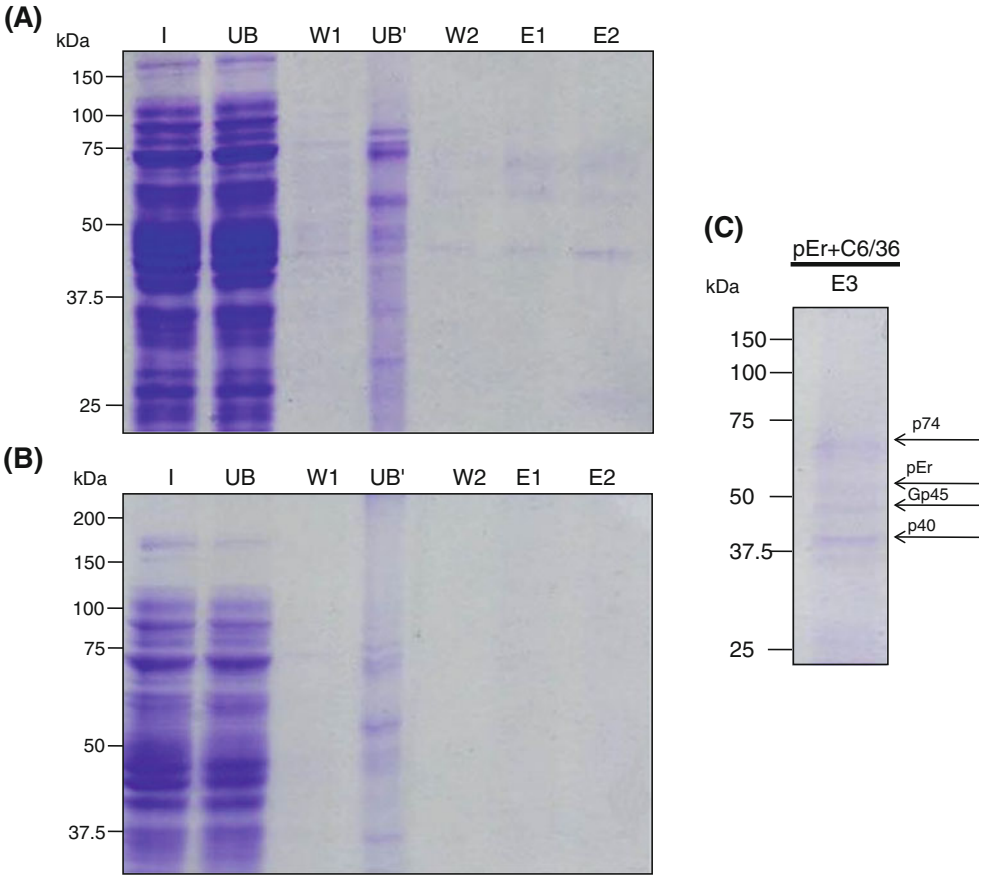

Fig. 1 Purification of C6/36 cell proteins by affinity chromatography with the DENV 4 recombinant E protein. Total protein extracts from C6/36 cells was added to either DENV 4 recombinant E protein (pEr, **A**) or total protein extracts from non-transformed bacteria (NTB, **B**), coupled with Ni-NTA agarose affinity chromatography, as described previously (see material and methods), followed by analysis via 10 % SDS-PAGE. I, total bacterial protein extracts; UB and UB', the unbound fractions of total bacterial or C6/36 protein extracts, respectively. W1 and W2, final washes; E1 and E2, elution fractions with 0.5 and 1 M sodium chloride, respectively. The elution fraction containing 250 mM imidazole (E3) was analysed via 10 % SDS-PAGE and is shown in **C**. The molecular markers (BioRad) are indicated on the left side of each gel

Table 1 Maldi-ToF/mass spectrometry analysis

Band	Protein	Match
74 kDa	Heat shock 70 Ca	17
	Heat shock 70 Aa	16
	Heat shock cognate 70	12
	BiP (78-kDa glucose-regulated protein)	7
45 kDa	Alpha-tubulin	2
	Beta-tubulin	1
40 kDa	ERp44 (endoplasmic reticulum–resident protein)	11
	PDI (thioredoxin/protein disulfide isomerase)	3
	Actin	11

Proteins from C6/36 cells purified by affinity chromatography with DENV E recombinant protein were excised from a polyacrylamide gel and analysed at the Core Protein Facility of Columbia University, N.Y., USA. The number of peptides that matched each protein is indicated in the right column

isoforms with different molecular sizes. However, additional studies will be necessary to clarify whether one of these possibilities is correct.

Although these proteins are known to be located on the cell surface in other cell lines, their location in C6/36 cells is unknown. Thus, we performed flow cytometry using non-permeabilised cells and specific antibodies against each protein under both normal and DENV 4 infection conditions, as described in the "Materials and methods". An anti-HSP90 antibody was included because previous studies have detected a cross-reaction of this antibody with the 45 and 74 kDa proteins detected as part of the DENV receptor complex in C6/36 cells [29]. The epitope recognised by the anti-HSP90 antibody is present in *Ae. aegypti* HSP70, but not in HSc70 (data not shown).

Under normal conditions, all of the examined proteins were detected on the cell surface. BiP was the most highly expressed, while HSP70/HSc70 showed the lowest expression. However, under infection with DENV 4, the cell surface expression of HSP70/HSc70 and PDI increased, while that of ERp44 and BiP did not change (Fig. 3). To determine if the change of expression on the cell surface was due to a overexpression of the proteins studied, a semi-quantitative Western blot assay was performed with total protein extracts from C6/36 cells recovered in both conditions (Fig. 4). None significant modification in the protein amounts was observed (Fig. 4B) suggesting that the increase of HSP70/HSc70 and PDI on the cell surface was likely a re-location more than an overexpression event.

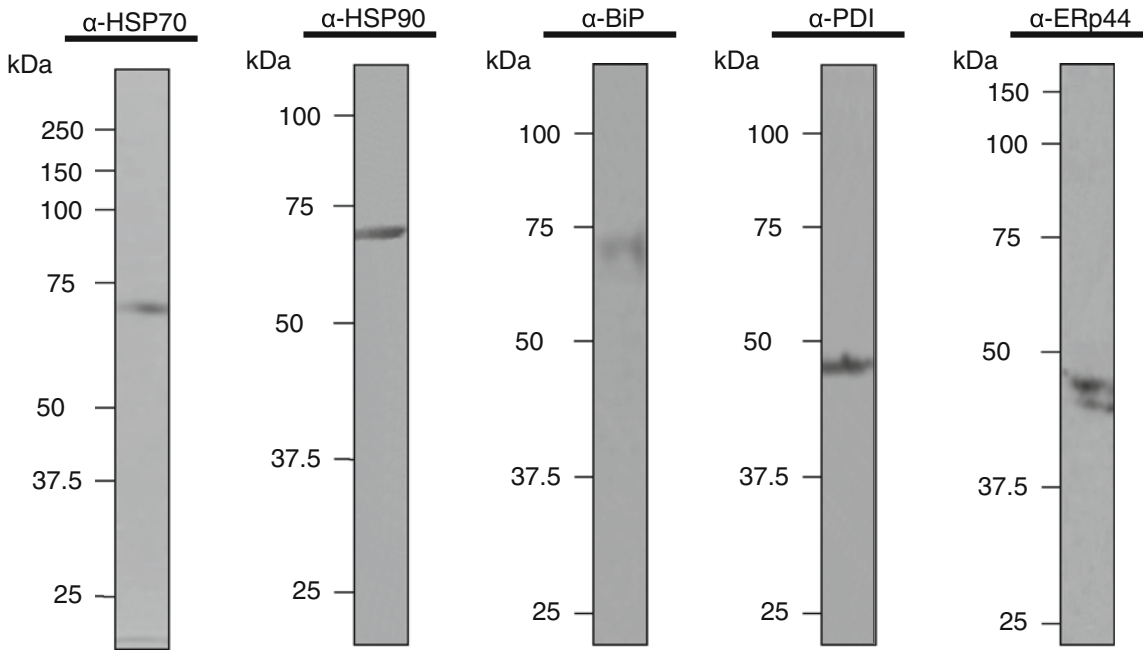

Fig. 2 Western blot assays. Fifty micrograms of total protein extract from C6/36 cells were separated via 10 % SDS-PAGE, transferred to a nitrocellulose membrane and incubated with different antibodies (see "Materials and methods"), as indicated in each lane. The molecular markers (BioRad) are indicated at the left side of each lane

Evaluation of virus binding

To evaluate the role of these proteins in DENV 4 binding, immunofluorescence (Fig. 5A) and flow cytometry (Fig. 6A) assays were performed. The virus was incubated with C6/36 cells at 4 °C to inhibit entry, and the various virus-protein interactions were blocked by pre-incubating the cells with specific antibodies. An unrelated antibody was used as a negative control. To detect DENV 4 on the cell surface by immunofluorescence, we used an anti-DENV complex antibody. In this experiment, all of the antibodies used had an inhibitory effect on virus binding, but anti-HSP70 and anti-HSP90 had the strongest effects (57.3 % and 58.8 %, respectively compared with the unrelated antibody) (Fig. 5B). In addition, a flow cytometry assay using virus labelled with DIL was performed. Again, the anti-HSP70 and anti–HSP90 antibodies were able to induce a statistically significant reduction in viral binding to the cell surface (41 % and 29.5 %, respectively compared with the unrelated antibody, Fig. 6B), indicating that these proteins might be directly involved in the interaction between DENV 4 and the C6/36 cell surface. Incubation with antibodies against PDI and BiP did not cause a statistically significant reduction in virus binding, and incubation with anti-ERp44 antibodies also did not have any inhibitory effect, suggesting that the participation of these proteins in the cell surface-DENV 4 interaction is minimal. Taken together, these results suggest that all of the proteins analysed, except ERp44, are involved in DENV 4 binding to the C6/36 surface, but HSP70 and HSc70 appear to be the most critical.

Evaluation of viral infection

To evaluate the effects of these proteins on DENV 4 infection rates, immunofluorescence (Fig. 7A) and flow cytometry (Fig. 8A) assays were performed. C6/36 cells were pre-incubated at 4 °C with antibodies against the proteins, and DENV 4 was then added followed by incubation at 37 °C to allow entry. An unrelated antibody was used as a negative control. To detect DENV 4, an anti-DENV complex antibody was used in cells that had previously been permeabilised with Triton X-100. Interestingly, pre-incubation with all five antibodies induced a statistically significant reduction in DENV infection. The strongest inhibition occurred in the presence of anti-HSP70 and anti-HSP90 antibodies: infection was inhibited by these antibodies by 54.6 % and 53.9 %, respectively, in the immunofluorescence assays (Fig. 7B) and 48.2 % and 53.3 %, respectively, in the flow cytometry assays (Fig. 8B) compared with the unrelated antibody (IgG). Taken together, these results suggest that the viral binding and entry process might be mediated by different molecules, some of which are involved in binding (HSP70/HSc70), while others are required for a post-attachment step (BiP, PDI and ERp44). An additional similar inhibitory assay was performed, but DENV 4 infection was evaluated 48 h later via detection of the viral NS1 protein

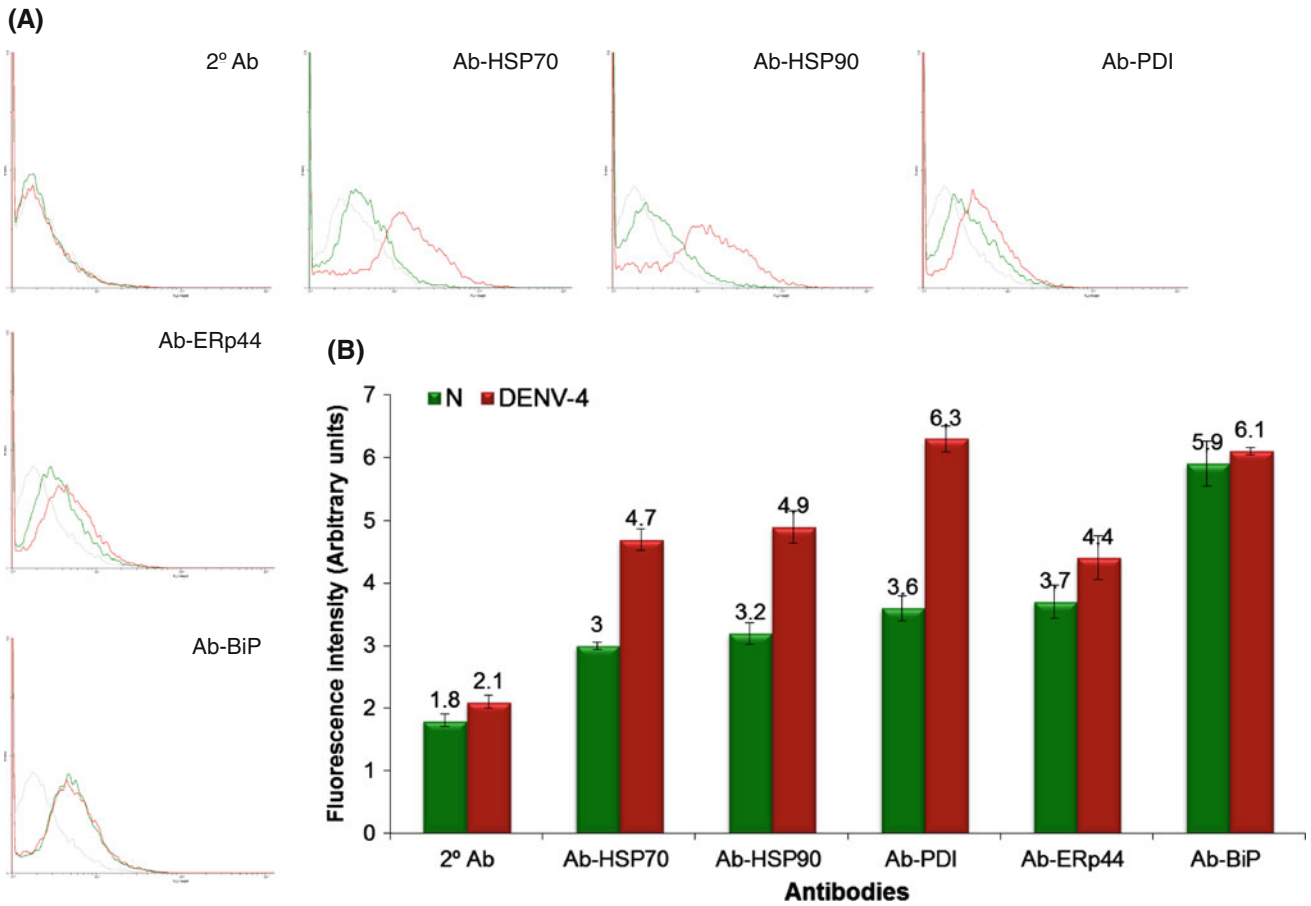

Fig. 3 Surface proteins in C6/36 cells detected by flow cytometry. Non-permeabilised C6/36 cells were incubated separately with antibodies against HSP70, HSP90, BiP, PDI, and ERp44. A secondary FITC-coupled antibody was included as a control (2° Ab). The mean of the data obtained in experiments performed in triplicate ± SD is shown. **A,** Representative histograms for each experiment; green line, uninfected C6/36 cells; red line, C6/36 cells infected with DENV 4; and grey line, C6/36 cells incubated with secondary antibody alone. **B**, Graphic representation of flow cytometry assays; uninfected C6/36 cells (gren bars) and C6/36 cells under viral infection (red bars)

in the supernatant using the Platelia™ Dengue NS1 AG assay. We also examined a further treatment in which the C6/36 cells were pre-incubated with all of the antibodies at the same time (Fig. 9A). This assay has been used to evaluate DENV infection previously, and the results obtained with this method are equivalent to a virus plaque assay [43]. As shown in Fig. 9B, a significant reduction (81.4 %) in the production of NS1 protein was observed when the cells were incubated with all of the antibodies compared to both of the controls (cells not incubated without any antibodies and cells incubated with a non-related antibody). Again, the results suggested that the viral binding and entry process might be mediated by different molecules at different stages.

Post-attachment inhibition assay

To differentiate the role of C6/36 surface proteins in DENV 4 binding and/or entry, cells were incubated with blocking antibodies following viral binding. In these experiments, the virus was added to the cells at 4 °C, after which the antibodies was added, and the cells were then incubated at 37 °C for 1 h to allow entry. At 48 h post-infection, detection of DENV 4 was performed by flow cytometry in Triton X-100 permeabilised cells. Although there is a slight reduction in the viral infection between 12.9 and 24.2 % in the presence of the antibodies tested (see Fig. 10), when the results were analysed by the one way Holm-Sidak test, none were able to induce a statistically significant reduction in DENV 4 infection, demonstrating that all of the proteins analysed appear to play a role in viral binding and entry.

Discussion

The first step in viral infection of host cells is the virus-receptor interaction on the cell surface. Although several proteins in mosquito cells have been reported to interact with DENV, only a few of these proteins have been

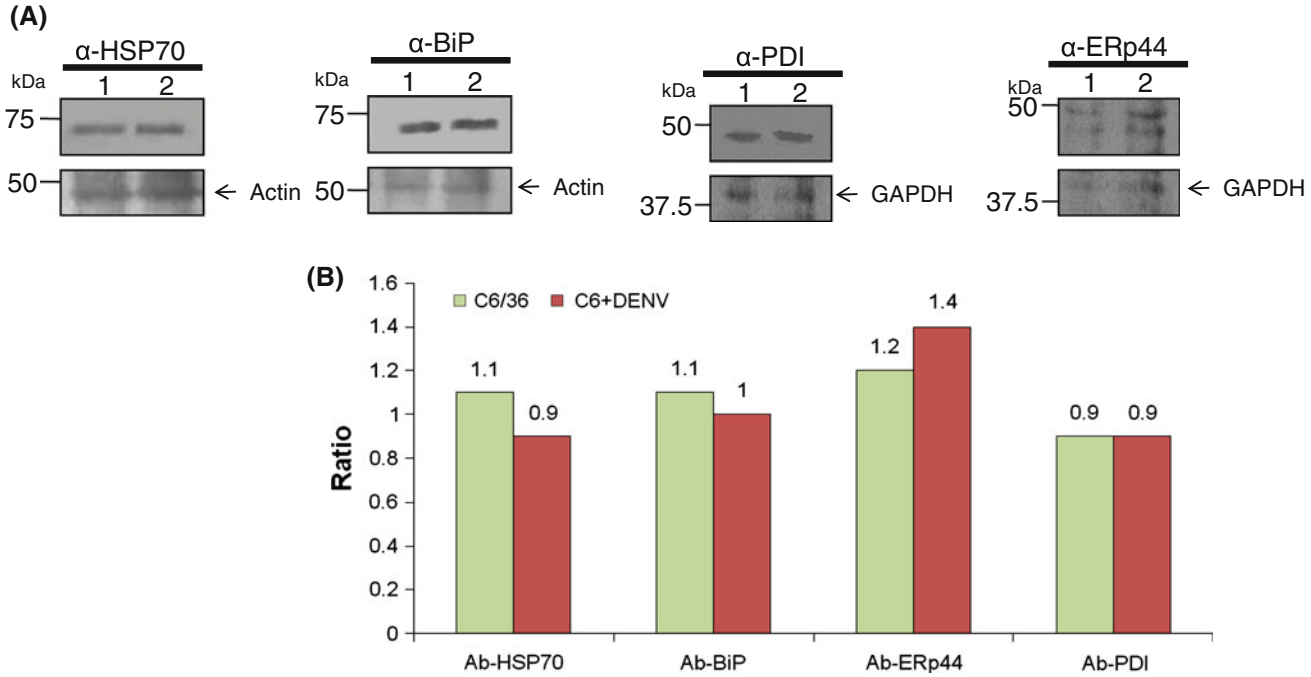

Fig. 4 Expression of proteins evaluated by Western blot assays. **A** Fifty micrograms of total protein extract from C6/36 cells in normal conditions (lane 1) or infected for two hours with DENV (lane 2) were separated via 10 % SDS-PAGE, transferred to a nitrocellulose membrane and incubated with different antibodies (see "Materials and methods"), as indicated in each lane. The molecular markers (BioRad) are indicated at the left side of each panel. **B** Densitometry analysis performed with Gel Quant Express 3.1 software (2005) DNR Ltd. comparing each sample with the respective loading control anti-Actin or anti-GAPDH respectively

identified [27, 29–33]. One of these proteins is a 37/67-kDa human high-affinity laminin receptor-related protein; however, this protein participates in the infection of only some DENV serotypes [34]. Proteins such as Vav-1, HSc70, ATP synthase beta subunit, prohibitin and osiris have been found in membrane fractions from the *Ae. aegypti* midgut, C6/36 cells and A7 cells, but their participation in DENV binding/entry is still unclear [5]. Finally, the best-characterised molecule involved in DENV infection of C6/36 and CCL-125 cells (*Ae. aegypti)* is prohibitin [35]. Because several proteins in mosquito cells are able to interact with the DENV E protein, it is possible that the virus uses more than one molecule in the binding and entry processes.

To identify molecules that may participate in viral binding and internalisation, affinity chromatography was performed using protein extracts from C6/36 cells and a DENV 4 recombinant E protein, as described previously [41]. Given that the 40, 45 and 74 kDa proteins have been previously detected in association with DENV infection and were also observed in our experiments with the E protein [27, 29], these molecules were further characterised. Because prohibitin (38 kDa) has been previously studied [35], we excluded it from this study. Mass spectrometry analysis revealed 9 cellular proteins that interacted with the virus. Of these proteins, three are cytoskeleton proteins (actin, alpha-tubulin, and beta-tubulin [5, 45]) and are related to the clathrin-mediated endocytosis mechanism of DENV entry into C6/36 cells [46]; three belong to the heat shock family (HSP70/HSc70 and BiP (GRP78)); and two belong to the PDI family (PDI and ERp44).

Although the heat shock proteins have been described as a intracellular resident molecules, several studies have now revealed that they could be associated with the plasma membrane of several cells lines despite that the mechanism is still unknown [47]. For example, The HSP70 protein has been located on the cell surface of U937 [23] and Huh7 [48] cells by flow cytometry and by biotinylation of surface proteins in U937 and neurobalstoma cells [22]. By flow cytometry analysis, the HSc70 protein has been identified in the surface of MA 104, Caco-2, Hep2 and BHK cells and confirmed by immunofluorescence on MA 104 cells [49], and by VOPBA in membrane fractions of C6/36 and A7 mosquito cells [5]. HSP70 protein systems have been strongly implicated in several phases of the viral life cycle such as entry, virion disassembly, replication and morphogenesis [47]. In previous experiments, we observed an increase in the expression of a 74 kDa protein that bound to DENV 2 in C6/36 cells under heat shock conditions, and this finding correlated with an increase in viral binding to the cell surface [29]. In this work, the 74 kDa protein was purified by affinity chromatography and identified as HSP70/HSc70. Using specific antibodies against HSP70,

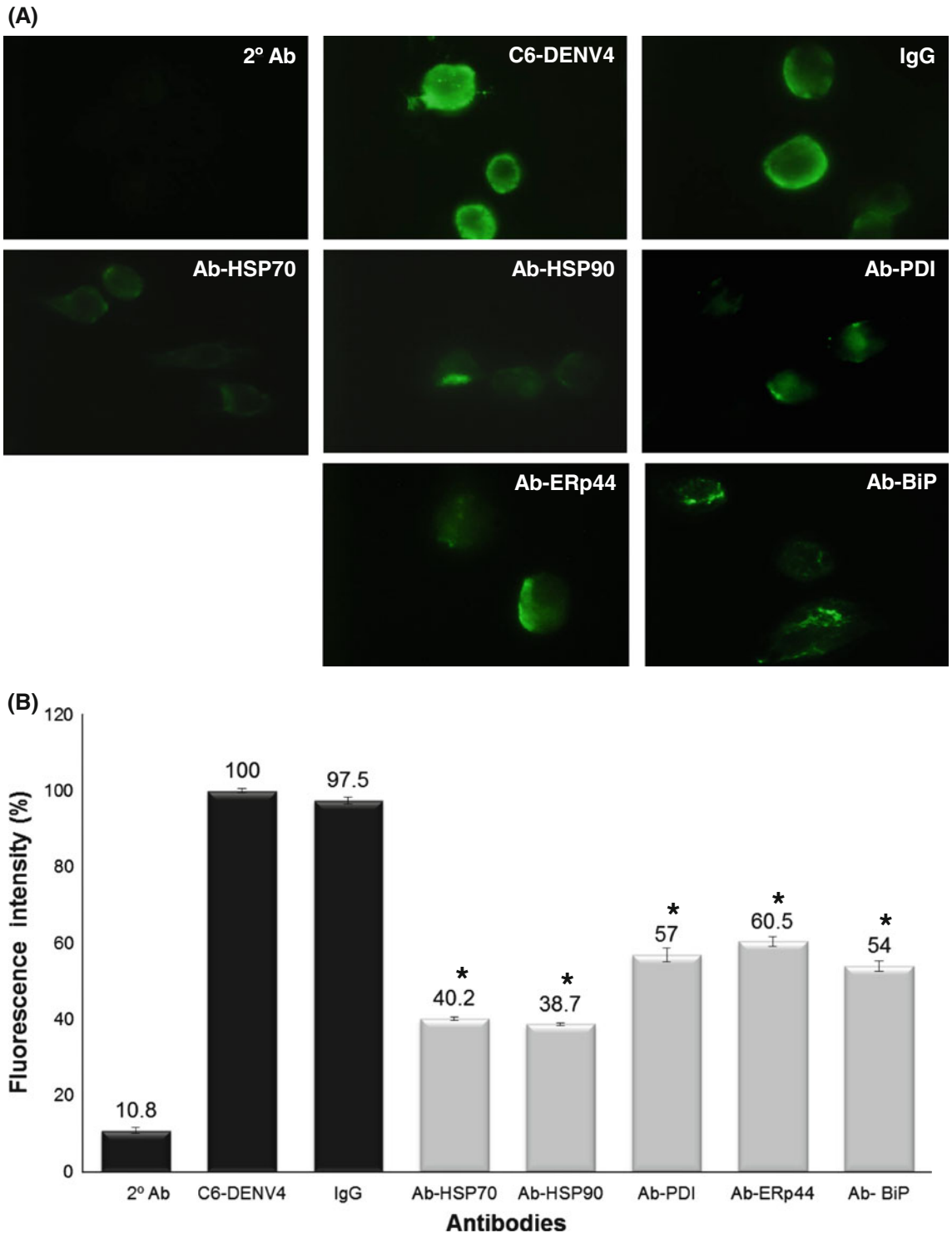

Fig. 5 Viral binding determined by immunofluorescence assay. **A**, C6/36 cells were incubated at 4 °C with specific antibodies against heat shock, PDI, and ERp44 proteins, or a non-related antibody (IgG), or without antibodies (2° Ab and C6-DENV4). Then, DENV 4 was added, followed by incubation at 4 °C and detection with an anti-DENV complex antibody in non-permeabilised cells. C6/36 cells without virus were included as a control (2° Ab). The nuclei were stained with DAPI (see supplemental material 1), and the cells were visualised using an immunofluorescence microscope. **B**, Immunofluorescence levels were measured with a Nikon microscope in 30 cells selected randomly and are expressed as a percentage compared to the positive control. Data are expressed as the mean ± SD. * indicates a statistically significant difference ($p < 0.001$)

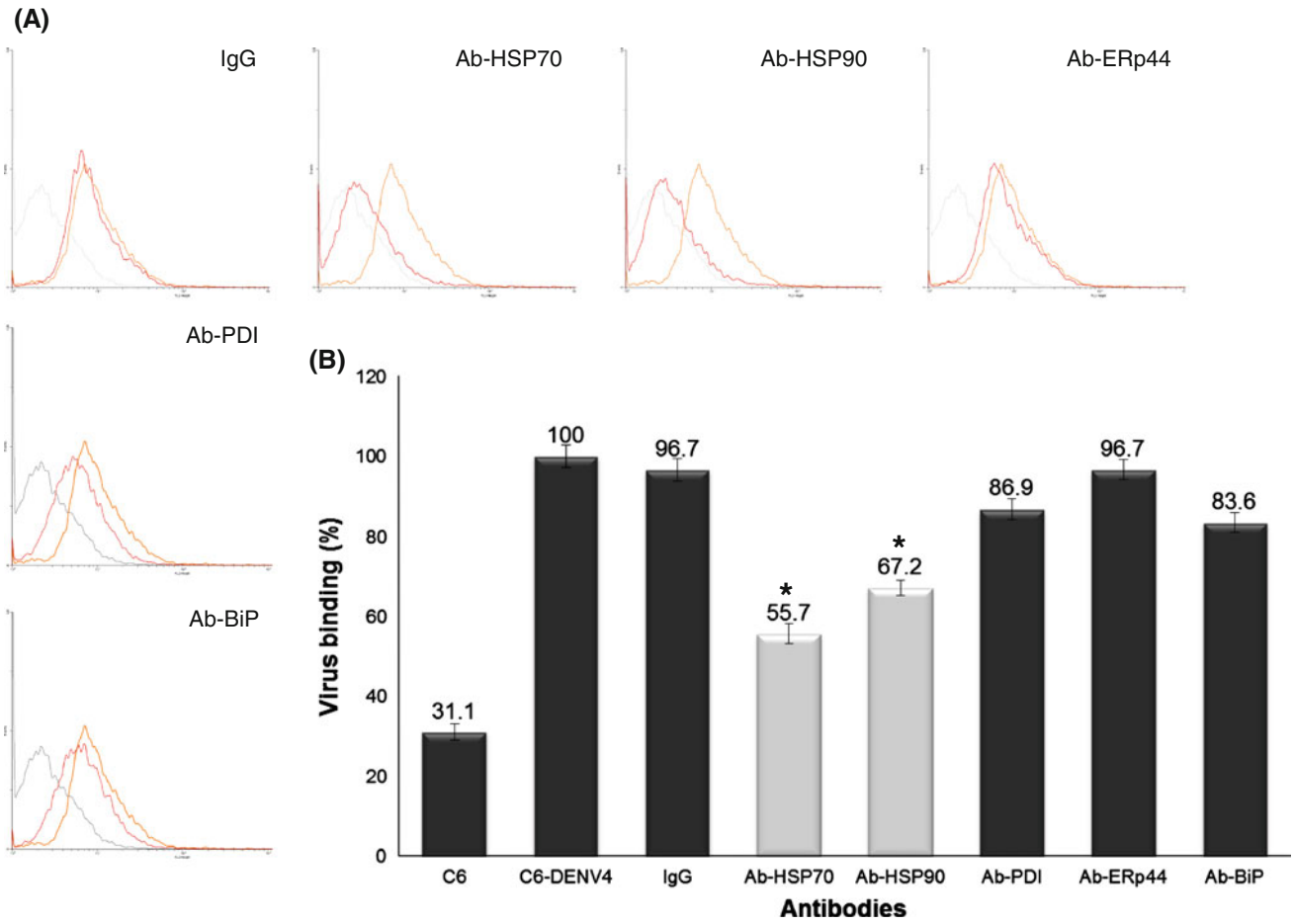

Fig. 6 Viral binding as determined by flow cytometry. C6/36 cells were incubated at 4 °C with specific antibodies against HSPs, PDI, and ERp44, or a non-related antibody (IgG), or without antibodies (C6-DENV4). Then, DIL-labelled DENV 4 was added, after which the cells were incubated at 4 °C, and DENV 4 binding was detected by flow cytometry in non-permeabilised cells. **A**, Representative histograms of each experiment; orange line, C6/36 cells incubated with DENV; red line, C6/36 cells pre-treated with specific (HSP70, HSP90, ERp44, PDI or BiP) or non-related (IgG) antibodies; grey line, C6/36 cells without virus. **B**, Graphic representation of the mean values from of experiments performed in triplicate. The SD is indicated on each bar. * indicates a statistically significant difference ($p < 0.001$)

we detected a change in its presence on the cell surface during DENV 4 infection without an apparent increase in the expression of the protein. Several reports have described the re-location of HSP70 to the lipid rafts during viral infection as a mechanism to recruit molecules involved in virus binding and entry. For example, there is an increase in the association of HSP70 with lipid rafts during *Japanese Encephalitis virus* (JEV) to Huh-7 cells [48] and a similar phenomenon has been observed in U937 cells when interact with a recombinant E protein from DENV 4 [22]. However the participation of lipid rafts in DENV infection to C6/36 cells wait to be elucidated. Even in the human monocytic cell line THP-1, the infection with DENV triggered an increased level of HSP70 expression [50], in C6/36 cells infected with the same virus the expression levels of HSc70 is not modified [5], according with our results. Taken together, these results suggest that the overexpression of HSP70 on the surface of C6/36 cells observed after DENV infection seems to be more a re-location process than an increase in the protein synthesis and probably facilitates the viral infection as has been suggested for DENV infection to endothelial cells where a gradually increase in the expression levels of β3 integrin has been observed after infection [24].

Antibodies against HSP70 and HSP90 had the strongest inhibitory effects on virus binding and infection, achieving inhibition of approximately 50 %. These results strongly indicate that HSP70 is part of the receptor complex for DENV 4 in C6/36 cells. HSP70 has been implicated in both JEV [4, 46] and DENV infections, particularly in DENV 2 infection of monocytes, macrophages [23], U937, human neuroblastoma [22], C6/36 cells, A7 cells and the *Ae. aegypti* midgut [5]. Recently, increased expression of HSP70 in the midgut of *Aedes sp*. during a blood meal has been

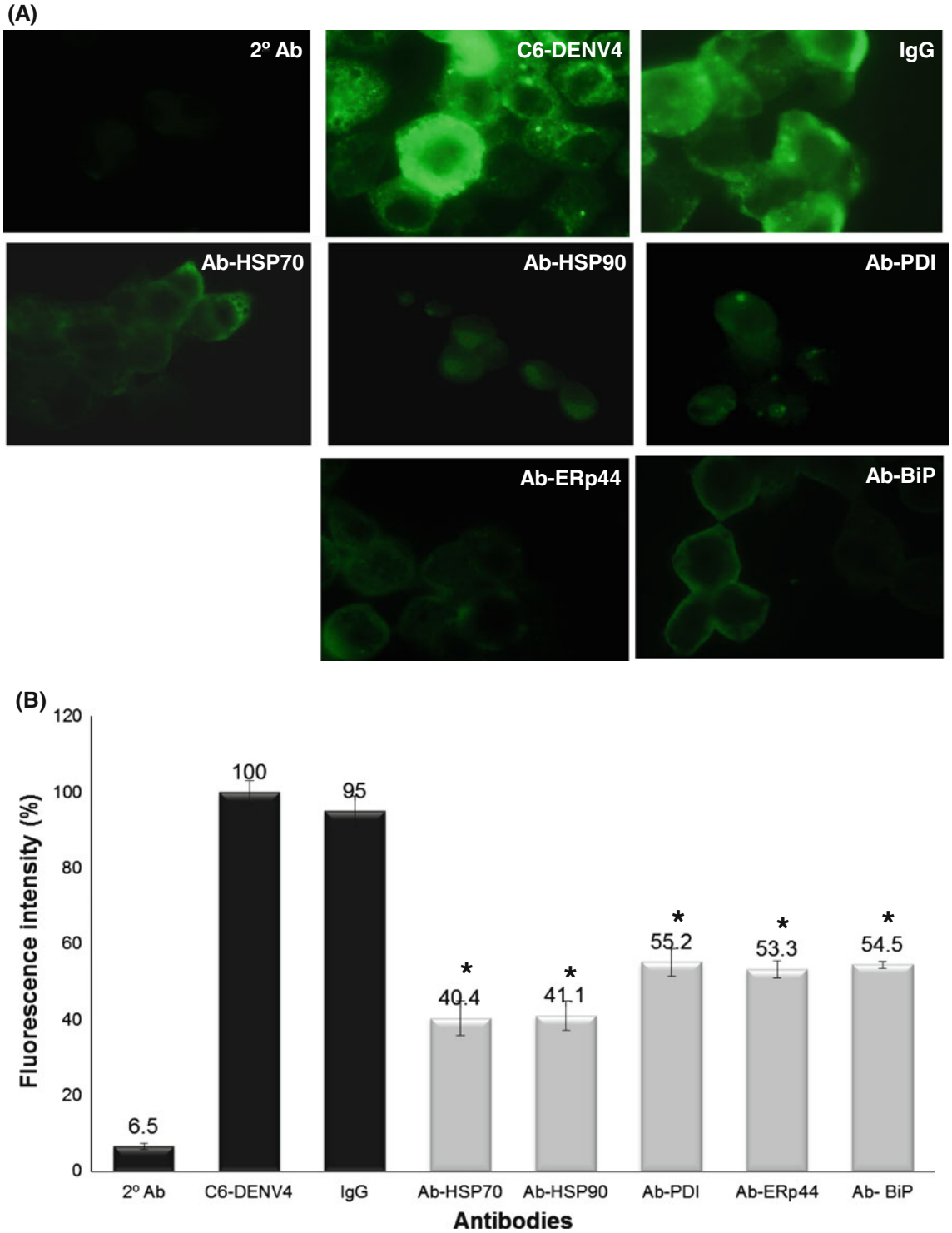

Fig. 7 Viral infection as determined by immunofluorescence. **A**, C6/36 cells were incubated at 4 °C with specific antibodies (indicated in each photograph), a non-related antibody (IgG) or without antibodies (2^{o} Ab and C6-DENV4). Then, DENV 4 was added and detected 24 h post-infection with an anti-DENV complex antibody. Non-infected cells were included as a control (2° Ab). The nuclei were stained with DAPI (see supplemental material 2), and the cells were visualised with an immunofluorescence microscope. **B**, Immunofluorescence levels were measured with a Nikon microscope in 30 cells selected randomly and are expressed as a percentage compared to the positive control. The SD is indicated. * indicates a statistically significant difference ($p < 0.001$)

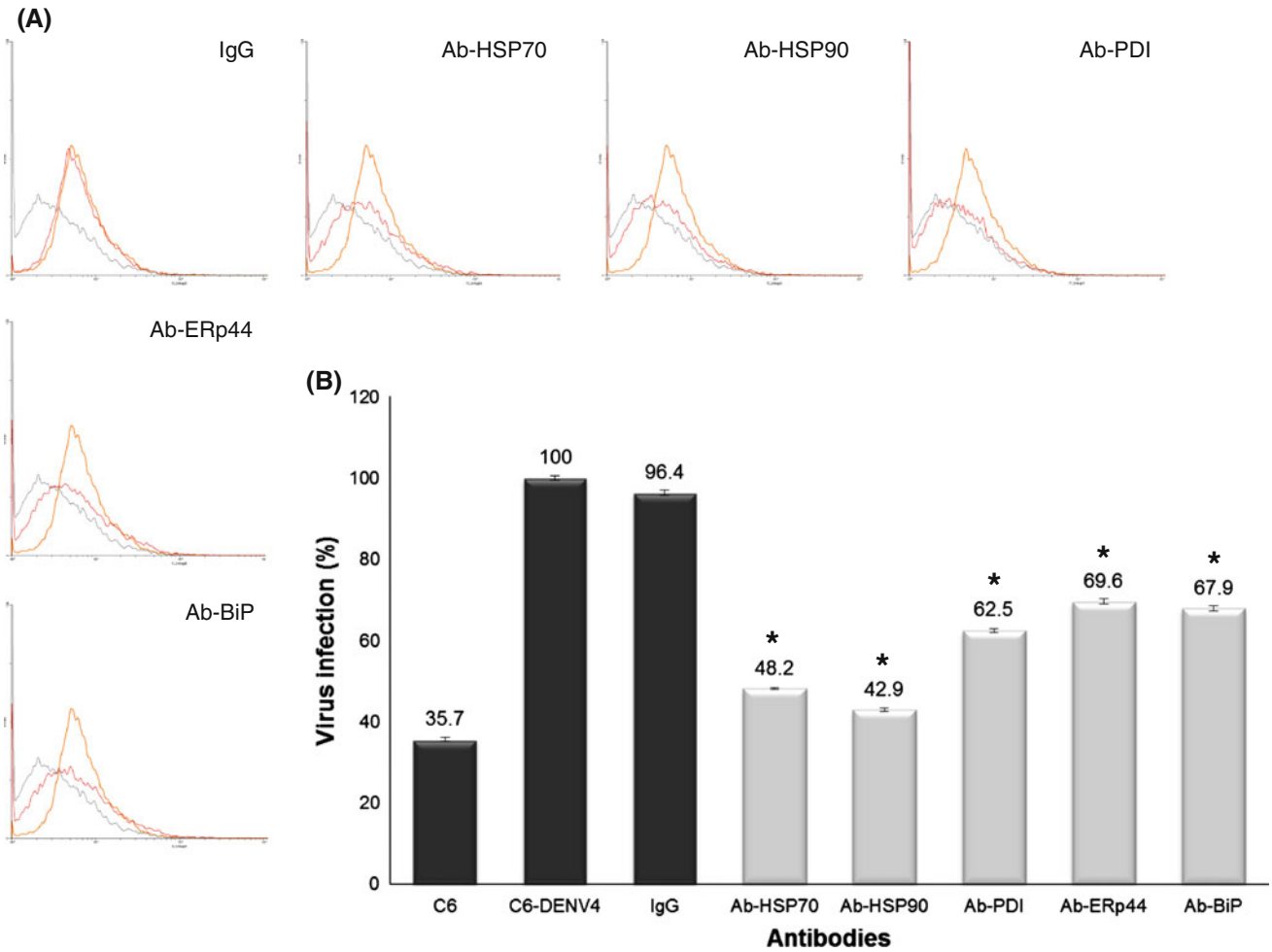

Fig. 8 Viral infection as determined by flow cytometry. C6/36 cells were incubated at 4 °C with specific antibodies, a non-related antibody (IgG) or without antibodies (C6-DENV4). Then, DENV 4 was added and detected 48 h post-infection by flow cytometry in permeabilised cells. **A**, Representative histograms for each experiment; orange line, C6/36 cells incubated with DENV; red line, C6/36 cells pre-treated with specific (HSP70, HSP90, ERp44, PDI, and BiP) or non-related (IgG) antibodies; grey line, C6/36 cells without virus. **B**, Graphic representation of the mean values from experiments performed in triplicate. The SD is indicated on each bar. * indicates a statistically significant difference ($p < 0.001$)

reported. Given that the midgut is the first target of DENV during infection, this interaction might be relevant to the natural course of viral infection in *Aedes* mosquitoes [51]. Additionally, previous studies with Huh7 cells have associated the activation of PI3 K/Akt pathway with HSP70 in the early stage of JEV infection [48]. The phosphatidilylinositol-3 kinase (PI3 K) has been implicated in the interferon beta (INF-beta) gene expression NF-kappaB-dependent activation in cells infected with DENV 2 during the initiation of host innate immune response [52] and in the inhibition of caspases 9 and 6-dependent apoptosis during the early steps of viral infection in the mice neuroblastoma cell line N18 infected by DENV 2 [53]. It is activated in early stages of viral infection by JEV to the mouse neural stem cell line C17.2 [54] and it participates in the cellular signal transduction that leads to overexpression of metalloproteinase-9 (MMP-9), an enzyme that is involved in the disruption of brain blood barrier and neuroinflammatory response, in rat brain astrocytes infected with JEV [55]. Using a tandem affinity purification assay, the interaction of West Nile virus (WNV) E protein with PI3 K from C6/36 cells has been evidenced [45]. Although this interaction was not observed with DENV proteins, an increase in the expression of PI3 K was observed in C6/36 infected with DENV2 and the treatment with the PI3 K inhibitor LY294 diminished the viral infection in the same cell line [45]. Moreover, the genes involved in PIK3 K signaling were up-regulated in refractory *Aedes aegypti* mosquitoes infected with DENV2 [56] suggesting that de PI3 K/Akt pathway plays an important role during DENV infection in mosquitoes. Since the apoptosis has been implicated in mosquito response to infection by several arboviruses [56], the PI3 K/Akt is involved in the inhibition of the apoptotic

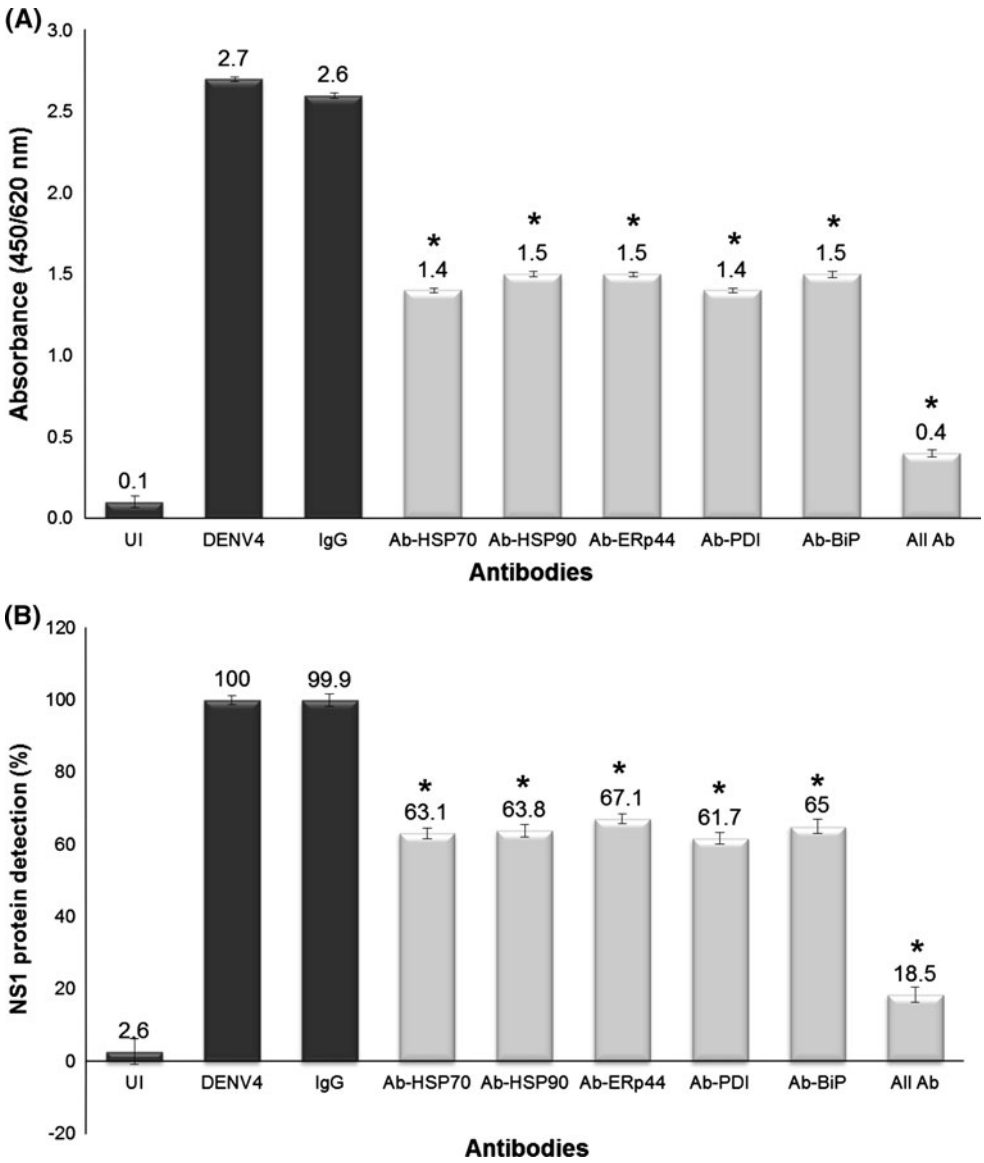

Fig. 9 Viral infection as determined by the Platelia™ Dengue NS1 AG assay. C6/36 cells were incubated at 4 °C with specific antibodies either individually or in combination (*grey bars*), with a non-related antibody (IgG), without antibodies (DENV4) or without virus (UI) (*black bars*). Then, DENV 4 was added, and NS1 was detected 48 h post-infection in the supernatant using with the Platelia™ Dengue NS1 AG kit. **A** Graph of absorbance values. **B** Graph indicating the Platelia results as percentages. Average percentages from experiments performed in triplicate ± SD are shown. * indicates a statistically significant difference ($p < 0.001$)

process during the early stages of DENV infection, probably triggered during virus binding [53]; and apparently the E protein of DENV does not interact directly with PI3 K, we might speculate that the activation of the PI3 K/Akt pathway could be archived though the interaction of DENV with HSP70 in order to delay the apoptotic process in C6/36 allowing viral replication, however more studies will be necessary to clarify this point.

We performed the same experiments for HSP70 using an anti-HSP90 antibody because previous Western blot assays showed a relationship between Gp45, p74, and this protein [29] with similar results. Moreover, a bioinformatics analysis revealed that the epitope recognised by the anti-HSP90 antibody used in our experiments is present in *Ae. aegypti* HSP70, which supports the existence of a cross-reaction between HSP70 and 90. The protein detected in C6/36 cells is likely HSP70. A highly homologous protein, HSc70, has been shown to be involved in infections with viruses such as *Rotavirus* [49] and JEV [4]. We identified these proteins in our Maldi-ToF analysis. Because the anti-HSP70 antibody used in our assays cannot differentiate between HSP70 and HSc70 and the two proteins share high homology (95 %), we cannot confirm which molecule is participating in DENV binding and entry. HSc70 plays an important role in the transition of the envelope protein from a dimer to a trimer during membrane fusion [57]. Because HSc70 shares a high homology with HSP70, its participation in the binding and entry processes is likely similar.

BiP (GRP78) is primarily an ER resident chaperone member of the HSP70 family that recognises hydrophobic residues in the unfolded regions of several proteins participating in the unfolded protein response [58, 59], translocation of newly synthesized polypeptides across the ER

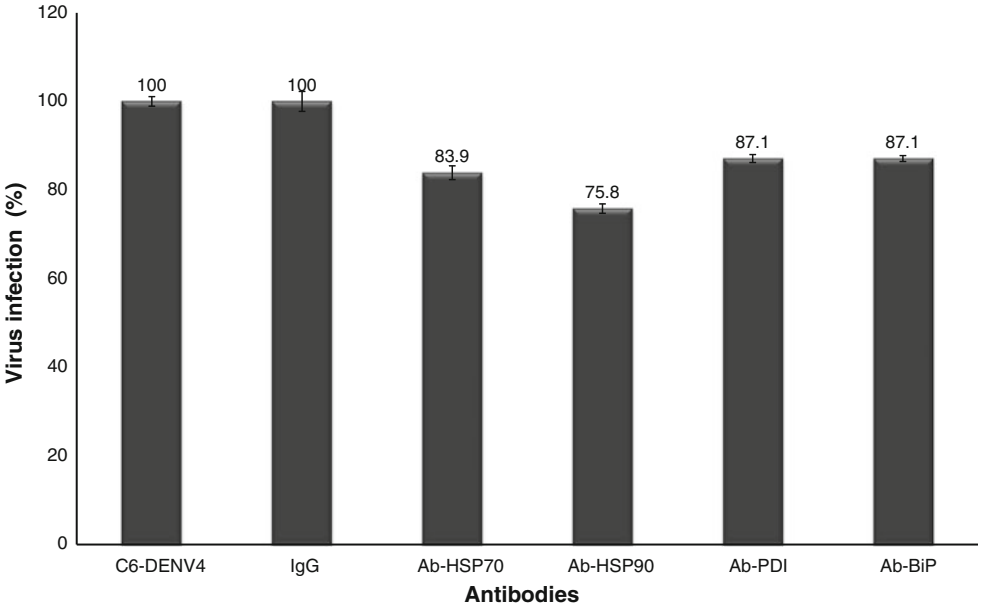

Fig. 10 Post-attachment inhibition as determined by flow cytometry. C6/36 cells were incubated at 4 °C with DENV 4 for 2 h. Then, the cells were incubated with specific antibodies, a non-related antibody (IgG) or without antibodies (C6-DENV4). DENV 4 internalisation was allowed to proceed at 37 °C for 1 h and was detected 48 h post-infection by flow cytometry in permeabilised cells. The mean values from experiments performed in triplicate ± SD are shown

membrane, targeted of misfolded proteins for degradation, regulation of calcium homeostasis and ER stress sensor [60]. It has been implicated in *Coxsackie virus* infection of GMK cells [61], DENV 2 infection of HepG2 cells [20, 21], and Borna disease virus (BDV) infection in human oligodendroglioma OL cells [62]. Previous reports have shown that domain III of DENV E interacts with BiP, and the knockdown of BiP by siRNA significantly decreases the production of infectious dengue virions, suggesting its participation in the folding and assembly of dengue proteins [63]. Similar functions have been suggested for JEV [64]. Although GRP78 has been typically described as a ER resident chaperone, recent studies have demonstrated its presence in cytoplasm, mitochondria, and nucleus [65]. It possesses 4 potential transmembrane domains and is expressed on the cell surface, mainly [60, 66, 67] but not only, during ER stress [60]. It has been found on the surface of several cancer cell lines [48, 65, 68, 69] but also in B cells [70], in mouse hippocampus neurons [62], murine brain endothelial cells (bEND.3), murine macrophage cells (RAW) [71], spermatocytes, and spermatids [72]. In our experiments, we found that BiP is expressed on the surface of C6/36 cells too, and its expression increased slightly under viral infection condition. Specific antibodies against BiP were able to block viral binding (13.1 % reduction) and infection (28.5 % reduction), suggesting a possible role of this protein in a post-attachment step.

PDI is a resident protein of the ER that belongs to a large protein family characterised by the presence of a thioredoxin domain that catalyses reduction and oxidation reactions. This protein plays an important role as a chaperone in protein folding via its redox activity (thiol/disulphide interchange) on cysteine residues [73]. PDI is also expressed on the cell surface and associates with lipid rafts, where it can cause redox modifications [73–75]. PDI was clearly detected on the surface of C6/36 cells in the present study and, according to other reports, has also been observed on lymphocytes [74], platelets [76, 77], and MA104 and Caco-2 cells [73]. Furthermore, its expression on the cell surface increases during viral infection without an apparent change in the total amount of the protein, suggesting again a re-localisation mechanism. PDI has been shown to be involved in the entry of other viruses, such as *Polyomavirus* [78], *Newcastle Disease virus* (NDV) [79], *Rotavirus* [73] and *Human Immunodeficiency virus* (HIV) [74, 80, 81]. This enzyme participates in important conformational changes in viral proteins during entry [73, 74], and our experiments revealed that PDI plays an important role in DENV binding and entry, similar to BiP, though it has weaker effect on virus binding (9.8 %) and viral infection (33.9 %).

Another member of the PDI family detected in our Maldi-ToF analysis was ERp44. This protein is composed of an amino-terminal thioredoxin (Trx) domain and a carboxyterminal ER retrieval signal. ERp44 participates in the folding and transport of proteins in the secretory pathway and is induced during ER stress [82]. Relocation of this protein to the surface of platelets following their activation has been reported recently [77]. ERp44 is present on the surface of C6/36 cells under normal conditions, and only small amount localises to the plasma membrane during viral infection. However, ERp44 does not participate in DENV binding, and antibodies against ERp44 only inhibited viral infection up to 26.8 %. Because ERp44 is a resident protein of the ER, its role in DENV 4 entry is not as relevant as that of the other proteins studied here.

Interestingly, when C6/36 cells were pre-incubated with all of the antibodies at the same time prior to viral infection, a significant reduction in DENV 4 infection was observed, as measured by the production of NS1 viral protein. These results suggest that DENV 4 uses several surface proteins to infect C6/36 cells and that these proteins might participate at different levels. For instance, HSP70/HSc70 is involved in viral binding, while BiP and PDI play a role in viral entry. To confirm these roles, a post-attachment experiment was performed in which the virus was incubated with C6/36 cells at 4 °C before the specific antibodies was added. Then, internalisation was allowed to proceed at 37 °C for 1 h, and the infection was evaluated by flow cytometry 48 h later. Interestingly, none of the antibodies used in the previous experiments had an inhibitory effect on viral infection. These results suggest that the virus, after interacting with the proteins, is internalised when the temperature is optimal, and the proteins do not participate in a two-step process. However, we observed that anti-BiP and anti-PDI antibodies were able to inhibit viral infection, but not viral binding, which suggests that the proteins participate in a post-attachment step. Because both molecules are ER resident proteins, a possible explanation for this finding is that their participation in DENV entry is secondary. The inhibition of infection observed using these antibodies was only 26.8-33.9 %, compared with the 48.2-53.3 % inhibition obtained with the anti-HSP70 and anti-HSP90 antibodies. These results suggest that among the proteins studied here, HSP70/HSc70 is the most important element in the receptor complex for DENV 4, which is in agreement with previous reports [5, 22, 23]. The absence of significant inhibition of viral binding when using the anti-BiP and anti-PDI antibodies was likely due to the high affinity of the virus for HSP70/HSc70. However the anti-HSP70 antibodies only inhibited the infection by 48.2 %, suggesting that this protein likely functions together with other proteins in the DENV binding and entry processes, such as prohibitin [35], a laminin-like receptor, and heparin sulphate [34]. However, the exact contribution and participation of these molecules in DENV infection of mosquito cells needs to be further investigated using silencing technology with specific siRNAs.

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