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Evidence that the 45-kD Glycoprotein, Part of a Putative Dengue Virus Receptor Complex in the Mosquito Cell Line C6/36, Is a Heat-Shock–Related Protein

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Abstract. Dengue virus (DENV) is transmitted to humans by mosquitoes of the genus *Aedes*. Although several molecules have been described as part of DENV receptor complex in mosquito cells, none of them have been identified. Our group characterized two glycoproteins (40 and 45 kD) as part of the DENV receptor complex in C6/36 cells. Because identification of the mosquito cell receptor has been unsuccessful and some cell receptors described for DENV in mammalian cells are heat-shock proteins (HSPs), the role of HSPs in DENV binding and infection in C6/36 cells was evaluated. Our results indicate that gp45 and a 74-kD molecule (p74), which interact with DENV envelope protein, are immunologically related to HSP90. Although p74 is induced by heat shock, gp45 apparently is not. However, these proteins are relocated to the cell surface after heat-shock treatment, causing an increase in virus binding without any effect on virus yield.

INTRODUCTION

Dengue virus (DENV), a member of the family *Flaviviridae*, is the causative agent of dengue fever and dengue hemorrhagic fever.[1] It is present in tropical and subtropical areas[2] and is transmitted by mosquitoes of the genus *Aedes*.[3,4] Because DENV can be cultured *in vitro* in human, primate, and mosquito cells, it is likely that some host proteins mediating binding, entry, translation, and/or replication of this virus may be conserved in different hosts. With regard to all arthropodborne virus infections, the study of cellular proteins required for attachment and entry in mosquito cells is essential for understanding virus tropism and virulence and may suggest a theoretical basis for prophylaxis.

In a previous report[5] with C6/36 cells, a cell line derived from *Aedes albopictus* larvae,[6] we showed that virus particles of DENV type 4 (DENV4) bind to two glycoproteins of 40 kD (gp40) and 45 kD (gp45) present on the cell surface. Additionally, polyclonal antibodies directed against gp45 were able to block virus binding and a cytopathic effect. Antibodies to gp40/45 detected a 45-kD protein present in different development mosquito stages and in permissive tissues of *Ae. aegypti* mosquitoes,[7] which suggested that gp45 is present in susceptible tissues in mosquitoes and is part of a receptor complex for DENV in mosquito cells. Unfortunately, although we have been isolated this protein by affinity chromatography using the envelope (E) protein as a receptor, the identification of gp45 has been unsuccessful.

Several heat-shock proteins (HSPs) have been implicated as receptors for viruses such as coxsackie and rotavirus with their host cells.[8–10] Specifically for DENV, HSP90 (84 kD) and HSP70 (72 kD) have been described as part of DENV receptor complex in human monocytes-macrophages and in promonocytic (U937) and neuroblastoma (SH-SY-5Y) human cell lines.[11] The glucose-regulated protein GRP 78 (BiP) was identified as part of DENV receptor complex in the human hepatoma cell line HepG2.[12] However, the effect of stress conditions such as heat shock on virus entry is unknown.

In most organisms, the cellular stress response is characterized by an increase in synthesis and/or relocation of HSPs. Under physiologic conditions, HSPs are expressed at low levels.[13] However, a wide variety of stressful stimuli including environmental, pathologic, or physiologic factors induce a marked increase in HSP synthesis[14] known as the stress response. Because some putative receptors for DENV in mammalian cells are HSPs, which are highly conserved molecules in mammalian and mosquito cells, the role of heat shock in DENV infection in C6/36 cells was evaluated.

MATERIALS AND METHODS

Virus and cells. Dengue 2 virus, which was kindly provided by the Instituto Nacional de Diagnóstico y Referencia Epidemiológicos, was propagated in newborn BALB/c mice as previously described.[15] C6/36 cells (from *Ae. albopictus*)[6] adapted to grow at 35°C[16] were cultured as previously described.[5] Baby hamster kidney (BHK)–21 cells were growth at 37°C in Petri dishes with minimal essential medium (MEM) supplemented with non-essential amino acids, 10% fetal calf serum, penicillin, and streptomycin.

Affinity chromatography and immunoblots. Total protein extracts from C6/36 cells were obtained as previously described[11] and passed through a NiNTA resin (Qiagen, Valencia, CA) coupled to a recombinant DENV E protein following the procedure previously reported.[17] Briefly, 2 mg of total protein from C6/36 cells were passed over an affinity column that consisted of full length DENV-4 recombinant E protein immobilized by its amino-terminal His-tag to an NiNTA resin (Qiagen). After extensive washing with interaction buffer (50 mM NaH_2PO_4, 200 mM NaCl, 1% Triton X-100, pH 8) the DENV receptor molecules were eluted with high ionic strength buffer (50 mM NaH_2PO_4, 500 mM NaCl, pH 8). The eluted molecules were concentrated in a Centricon 10 centrifuge microconcentrator (Amicon, Beverly, MA) and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomasie blue. The 45-kD band was excised and digested with trypsin. The peptides

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were subjected to by matrix-assisted laser desorption/ionization time of flight (MALDI-ToF) analysis at the Protein Core Laboratory Facility (Columbia University, New York, NY). Edman N-terminal sequencing of the protein was also performed.

The elution fraction was analyzed by Western blot assays using a monoclonal antibody to HSP90 diluted 1:20,000 (SPA-830; Stressgen Biotechnologies, Victoria, British Columbia, Canada), a polyclonal antibody to gp45 diluted 1:10,000,[5] and a monoclonal antibody to HSP70 diluted 1:5,000 (MA3-007; Affinity Bioreagents, Golden, CO). An anti-mouse horseradish peroxidase (HRP)–conjugated antibody (Amersham, Piscataway, NJ) diluted 1:30,000 were used as a secondary antibody. Briefly, 20 μL or 60 μg of either elution fractions or total protein extracts from C6/36 cells were subjected to a SDS-PAGE on a 10% gel (Mini PROTEAN System; Bio-Rad Laboratories, Hercules, CA) and transferred to a nitrocellulose membrane by using a semidry blotting apparatus (Bio-Rad Laboratories) with 48 mM Tris, 39 mM glycine, 20% (v/v) methanol. Transfer efficiency was monitored by staining with 0.1% Ponceau red in 7% trichloracetic acid. The membrane was blocked with low-fat milk for 1 hour at 37°C with gentle shaking and then incubated for 1 hour at 37°C with primary antibody diluted in phosphate-buffered saline (PBS), 0.5% Tween 20. The membrane was washed three times with PBS, 0.5% Tween 20 and incubated for 1 hour at 37°C with the secondary antibody diluted in PBS, 0.5% Tween 20. After exhaustive washing with PBS, 0.5% Tween 20, the reaction was developed using a chemiluminescence kit (Supersignal West Femto Maximum Sensitivity Substrate; Pierce, Rockford, IL) according to the manufacturer's protocol and X-OMAT film (Eastman Kodak, Rochester, NY).

Heat-shock treatment and detection of gp45. Monolayers of C6/36 cells were incubated at 35°C or heat-shocked at 41°C for 1 or 3 hours. The culture medium was then removed, cells were resuspended in PBS, transferred to a 1.5-mL microcentrifuge vials, and centrifuged in a microcentrifuge (5417R; Eppendorf, Hamburg, Germany) at 61.6 × *g* for 6 minutes at room temperature. Cells were then fixed with PBS, 4% paraformaldehyde for 1 hour at room temperature and then blocked with 100% fetal bovine serum overnight at 4°C. The cells were then washed once with washing buffer (PBS, 2% fetal bovine serum) at room temperature, centrifuged as described above, and incubated for 1 hour at room temperature with a mouse polyclonal antibody to gp45 diluted 1:50 in washing buffer. The cells were then washed three times and incubated with an anti-mouse FITC-conjugated antibody (Zymed, South San Francisco, CA) diluted 1:50 in washing buffer as secondary antibody. Finally, the cells were washed three times, resuspended in 0.5 mL of PBS, 1% paraformaldehyde, 2% fetal bovine serum, and analyzed by flow cytometry in a FACScalibur apparatus (Becton Dickinson, Franklin Lakes, NJ).

Immunoprecipitation assay. C6/36 cells incubated at 35°C or heat-shocked at 41°C were labeled with 100 μg of biotin 3 sulfo-N-hydroxisuccimide (Sigma, St. Louis, MO) per 2×10^6 cells for 1 hour at room temperature. After washing with PBS, the cells were homogenized and the total protein extract was obtained as described previously.[11] Thirty micrograms of each protein extract was incubated with 5 μL of polyclonal antibody to gp45 and 15 μL of agarose-coupled G protein (Sigma) overnight at 4°C with gentle shaking. After four washing steps (10 minutes per wash) with TNT buffer (20 mM Tris-HCl, 300 mM NaCl, 0.1% Triton X-100, pH 7.2), the proteins bound were eluted with 200 mM glycine (Sigma), pH 2, for 2 minutes at room temperature, subjected to SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was blocked overnight at 4°C with 4% fetal calf serum in PBS with gentle shaking. Finally, the membrane was incubated with strepatavidin-HRP (Amersham) diluted 1:500,000 in PBS, 1% Tween 20 for 1 hour at room temperature, washed three times with PBS, 1% Tween 20, and developed with chemiluminescence (Supersignal West Femto Maximum Sensitivity Substrate-Pierce).

Virus overlay protein binding assay (VOPBA). C6/36 cells were cultured in 60 cm-diameter culture dishes (Corning, Corning, NY) overnight. The monolayers were washed with culture medium and subjected to heat shock at 41°C for 1 or 3 hours. Cells were then incubated for 1 hour at 35°C. One of the dishes was incubated at 35°C and used as a control. Total protein extracts from cells were obtained and used in VOPBA as previously described.[11]

Virus binding assay. Binding of DENV to C6/36 cells was assayed by flow cytometry following the procedure reported by Triantafilou and Triantafilou[18] with some modifications. Briefly, C6/36 cells were placed in two 24-well plates (3×10^5 cells/well) and incubated at 35°C in culture medium. One of the plates was then incubated at 41°C for 1 hour and the other was incubated at 35°C. After heat shock, the cells treated were incubated for 1 hour at 35°C. Culture medium was removed, PBS, 4% paraformaldehyde was added, and the plates were incubated at room temperature for 30 minutes. The monolayers were washed twice with binding buffer (PBS, 0.5% fetal bovine serum) and incubated with DENV-2 diluted in binding buffer at a multiplicity of infection (MOI) of 0.1 for 2 hours at room temperature with gentle shaking. The monolayers were washed twice with binding buffer, re-fixed as described above, washed twice with washing buffer, and blocked with fetal bovine serum overnight at 4°C. The cells were washed once with washing buffer and incubated with monoclonal antibody to DENV-2 (Chemicon, Temecula, CA) diluted 1:100 in washing buffer for 1 hour at room temperature. Subsequent to three washes, the monolayers were incubated with an anti-mouse FITC-conjugated antibody (Zymed) diluted 1:200 in washing buffer for 1 hour at room temperature with gentle shaking. Finally the cells were washed three times with washing buffer and then detached using PBS, 1% paraformaldehyde, 2% fetal bovine serum buffer and transferred to 1.5-mL microcentrifuge vials. Samples were analyzed by flow cytometry in a FACScalibur cytometer (Becton Dickinson).

Dengue virus infection assay. C6/36 cells were cultured in 25-cm^2 culture flasks (Corning). One flask was incubated at 35°C and then infected, and the other was subjected to heat shock for 1 hour at 41°C and then infected with DENV-2 at an MOI of 0.1 for 40 minutes at 35°C with gentle shaking. Virus was then removed and the cells were washed once with culture medium. Forty-eight hours later, the supernatants were recovered and virus titer was determined by plaque assay.

Virus titration. BHK-21 cells were seeded in a 24-well plates (2×10^5 cells/well) with 0.5 mL of culture medium/well. C6/36 infected cell supernatants were serially diluted and 50 μL of the inoculum was incubated on BHK-21 cells for 4

hours at 37°C. Afterwards, 0.5 mL of the overlay medium was added (2× MEM supplemented with non-essential amino acids, 3% carboxymethyl cellulose, 10% fetal calf serum, penicillin, and streptomycin). The cultures were incubated for 6 days at 37°C and then counted for plaque formation after fixation with 10% formalin and staining with 0.5% naphthol-blue-black (Sigma) in 165 mM sodium acetate, 6% acetic acid solution.

Confocal microscopy. Cells grown on coverslips were left untreated or heat-shock treated for 1 hour. The cells were then washed with PBS, fixed with 4% of paraformaldehyde for 1 hour, and washed and incubated with the blocking solution (PBS, 10% fetal bovine serum) for 1 hour at room temperature. After this treatment, cells were washed and incubated overnight at 4°C with the mouse anti-gp45 antibody diluted 1:50. Cells were washed and incubated with FITC-conjugated anti-mouse IgG 1:80 (Zymed) for 1 hour at room temperature. After a washing step, nuclei were stained with propidium iodide solution (Sigma). Slides were mounted with Vectashield® (Vector Laboratories, Burlingame, CA) and analyzed using a Leica (Wetzlar, Germany) TCS SP2 confocal microscope.

RESULTS

Sharing of an epitope by gp45 and HSP90. In previous studies, using an affinity chromatography approach with recombinant DEN E protein as a receptor and staining with Coomasie blue, we detected two proteins of 40 kD and 45 kD (gp40 and gp45) in the C6/36 mosquito cell line.[17] These two proteins were also detected by VOPBA using labeled virus particles. To identify the 45-kD protein, the protein band in the gel was excised and the trypsin-digested peptide mass map was analyzed by mass spectrometry. The mass values of four peptides were similar to the GTP binding site of translation elongation factor 1α (EF1α), a G protein of the arthopod *Lithophane hemina*. The amino terminal sequence of the 45-kD protein was obtained and a Basic Local Alignment Search Tool (BLAST) analysis was performed with the obtained amino acid sequence [XEK(Q/T)LNADQ(D/G/A)V(G/L)K(E/A)(Y/G)RL]. This analysis showed that the amino acids residues EKQLNAD are present and highly conserved in the amino terminal of several G proteins from nematodes. These data suggest that the 45-kD protein may be a G protein. However, additional studies are required to identify this protein.

To further characterize the proteins in the elution fraction from the affinity chromatography column, these fractions were subjected to SDS-PAGE and analyzed by Western blot assay using monoclonal antibodies against HSP90 and HSP70, the two proteins identified as part of DENV receptor complex in monocytes.[11] Antibody to gp45 was used as a control. As was previously reported,[17] gp45 was detected in the elution fraction (Figure 1A, lane 1), which corroborated that virus particles and recombinant E protein attached to the column are able to interact with the same group of molecules. A commercial monoclonal antibody to HSP90 detected two proteins in the elution fraction, gp45 and an additional protein of 74-kD (p74) (Figure 1A, lane 2). This finding suggested that gp45 and p74 may share an epitope with human HSP90. The epitope for this antibody was mapped between amino acids 604 and 697 in human HSP90.[19] Conversely, the monoclonal antibody to HSP70 did not detect any band in the elution fraction (Figure 1A, lane 3), although it recognized a band of approximately 70 kD in total protein extracts from C6/36 cells and BHK-21 cells (Figure 1B).

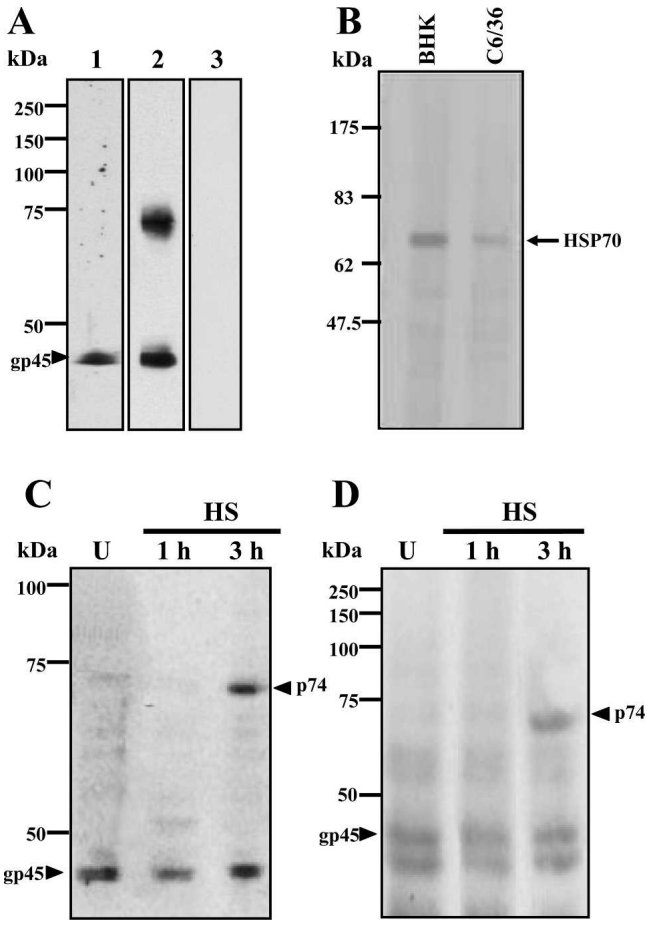

FIGURE 1. Affinity chromatography using dengue virus envelope (E) recombinant protein and Western blot assay with untreated and heat-shock–treated cells. **A**, Total proteins from C6/36 cells were passed through an affinity column with recombinant E protein coupled to NiNTA-agarose and 50 μL of the elution fractions (lanes 1–3) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel and transferred to a nitrocellulose membrane. Membranes were incubated with polyclonal antibody to gp45 (lane 1), monoclonal antibody to heat-shock protein 90 (HSP90) (lane 2), and monoclonal antibody to HSP70 (lane 3) and with a secondary antibody (peroxidase-conjugated goat anti-mouse IgG). **B**, Total protein extracts from baby hamster kidney 21 (BHK) cells and C6/36 cells (C6/36) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with monoclonal antibody to HSP70 and with a secondary antibody (peroxidase-conjugated goat anti-mouse IgG). The reaction was developed by chemiluminiscence. The migration of HSP70 is indicated by an **arrow**. C6/36 cell extracts from untreated (lane U) cells and heat-shock (HS)–treated cells for 1 and 3 hours (lanes 1 h and 3 h) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were incubated with polyclonal antibody to gp45 (**C**) or monoclonal antibody to HSP90 (**D**) and with a secondary antibody (peroxidase-conjugated goat anti-mouse IgG). Reactions were developed by chemiluminiscence. **Arrowheads** indicate the gp45 and 74-kD proteins. Molecular weight markers are indicated on the left.

Lack of overexpression of gp45 under heat-shock conditions. In several organisms, stress conditions induce a marked increase in the synthesis of several HSPs such as HSP70,[14]

while others such as HSP90 are not overexpressed. To determine if gp45 and p74 are HSPs, the relative amounts of gp45 and p74 were measured under a stressful condition (heat shock). Total extracts of C6/36 cells grown under standard conditions or at 41°C for 1 or 3 hours were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and incubated with the antibodies to gp45 and HSP90. Polyclonal antibody to gp45 detected this protein in untreated cells and in cells heat-shocked for 1 and 3 hours (Figure 1C). However, p74 was detected mainly after 3 hours of heat-shock treatment, which suggested that p74 is a heat-shock–inducible protein, but gp45 is not. Similar results were observed when antibody to HSP90 was used. The gp45 protein was present in the extract obtained from untreated cells, as well as from heat-shock–treated cells, and p74 was detected mainly after 3 hours of heat-shock treatment (Figure 1D). These results indicate that DENV E protein interacts with at least two proteins: p74, which shares an epitope with HSP90 and is induced after heat-shock treatment and gp45, a constitutive protein that also shares the same epitope.

To corroborate that gp45 and heat-shock–induced p74 are able to interact with the virus particle, a VOPBA was performed. Dengue 2 virus bound to gp45, as well as to 60-, 74-, and 80-kD proteins in untreated and heat-shock–treated cells (Figure 2, lanes 1–3). However, the amount of virus bound to the 74-kD protein was markedly increased in the extract from cells heat-shocked for 3 hours (Figure 2, lane 3). Proteins with similar molecular weights have also been detected in the four DENV serotypes, C6/36 cell extracts, and mosquito tissue extracts by the same approach.[20]

Increase in gp45 on the cell surface after heat-shock treatment. The HSPs are mostly intracytoplasmic proteins; however, under some conditions these proteins have been detected on the cell surface. Because we previously reported gp45 to be a membrane protein,[5] we analyzed whether its presence on the cell membrane could be enhanced under heat-shock conditions using two different approaches. Initially, we used a flow cytometry assay on non-permeabilized cells. Antibody to gp45 detected a shift in the FACS histogram, and the shift observed increased after the cells were heat-shocked for 1 hour. This finding suggested that this protein was relocated to the cells surface during heat shock (Figure 3A). Because gp45 and p74 share a common epitope with HSP90, we cannot conclude that the enhancement observed is caused by either protein.

To clarify this finding, we detected increased amounts of both proteins on the cell surface after heat-shock treatment by immunoprecipitation of biotinylated proteins. Untreated and heat-shock–treated cells were surface-labeled with biotin and the cell extracts were immunoprecipitated with the antibody to gp45. The immunoprecipitated proteins were separated by SDS-PAGE and incubated with peroxidase-conjugated streptavidin. The presence of both molecules on the cell surface of untreated cells was inferred by their labeling with biotin (Figure 3B, lane 1). Moreover, a higher amount of both proteins was observed on the cell surface of heat-shock–treated cells (Figure 3B, lane 2), which supports the conclusion that p45 and p74 are present on the cell surface under normal conditions and that their presence on the cell surface is enhanced by heat-shock treatment.

To further support our conclusion, the location of gp45 and p74 was analyzed by confocal microscopy using non-permeabilized cells and antibody to gp45. In untreated cells, a punctuate arrangement of the proteins on the cell surface was observed (Figure 3C), and a patched distribution was detected on heat-shock–treated cells (Figure 3D). Thus, the distribution and arrangement of the proteins on the cell surface is modified after heat-shock treatment. Our data suggest that under normal conditions gp45 and p74 are present on the cell surface, and after heat-shock treatment their amounts on the cell surface increase and they acquire a patch distribution.

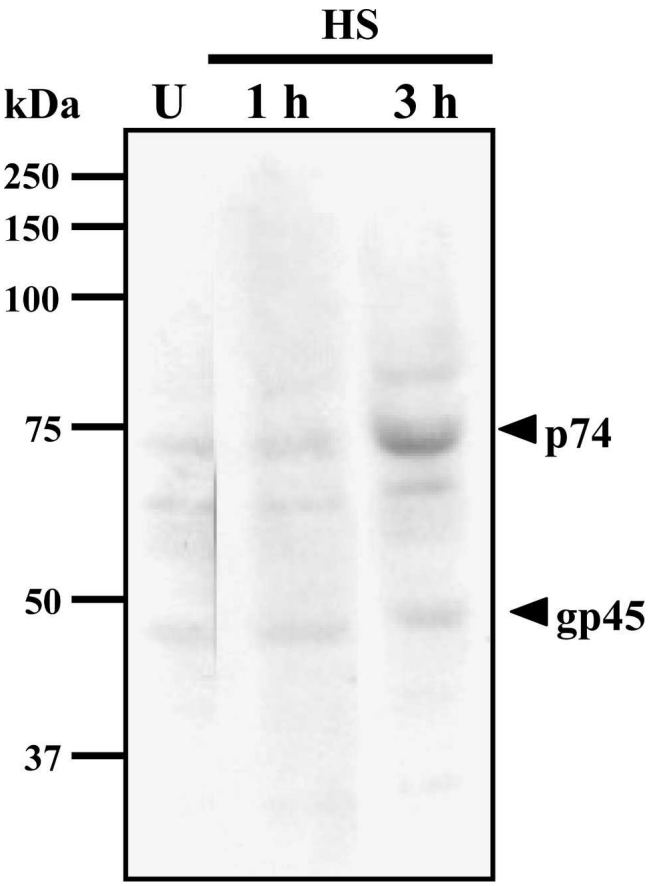

FIGURE 2. Virus overlay protein binding assay of untreated and heat shock (HS)–treated (for 1 and 3 hours) C6/36 cells. The C6/36 cell extracts from untreated (lane U) and HS-treated cells (lanes 1h and 3h) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was incubated with 2×10^4 plaque-forming units of dengue 2, a mouse polyclonal antibody to dengue envelope protein, and a secondary antibody (peroxidase-conjugated goat anti-mouse IgG). Reactions were developed by chemiluminiscence. **Arrowheads** indicate the gp45 and 74-kD proteins. Molecular weight markers are indicated on the left.

Effect of heat shock on DENV binding to the cell surface. Our previous studies indicated that gp45 is involved in DENV infection because antibodies against this protein inhibited DENV binding and infection in a dose-dependent manner.[5,7] The gp45 and p74 proteins may be required for DENV binding and/or entry. We took advantage of their higher amounts on the cell surface in heat-shock–treated cells and analyzed their role in both steps. Because a 1-hour heat-shock treatment increases the amount of gp45 and p74 on the cell surface, we analyzed DENV binding in untreated cells and in cells heat-shocked for 1 hour. The amount of virus

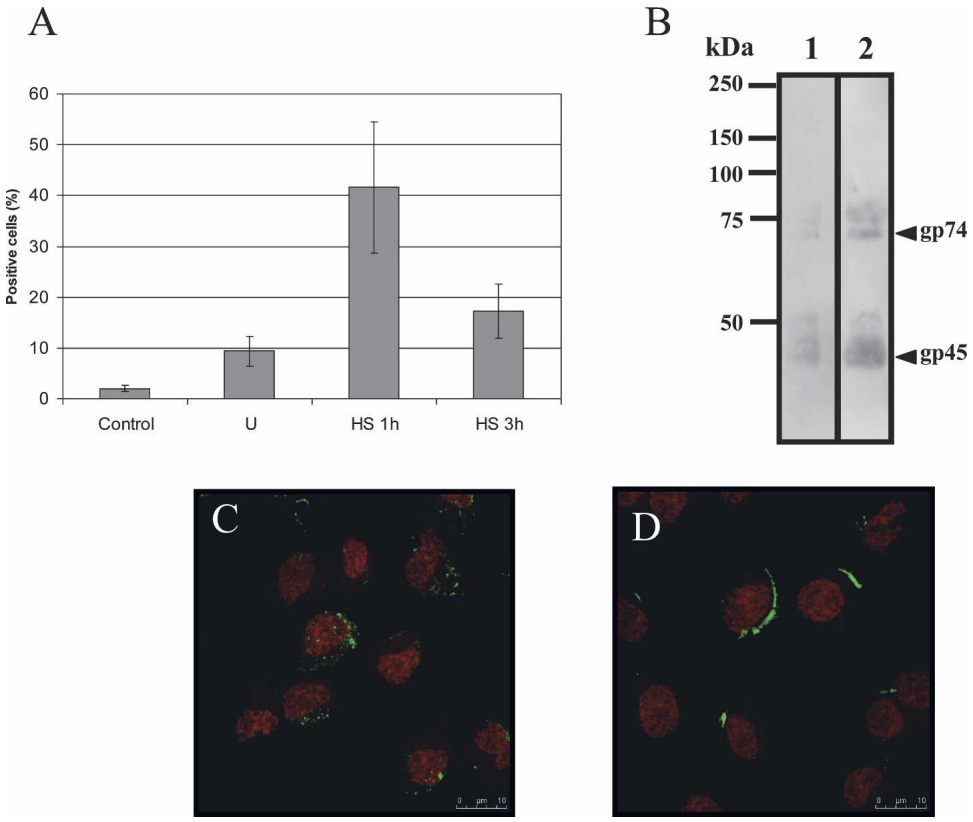

FIGURE 3. Enhancement of the amount of gp45 on the surface of C6/36 cells by heat shock (HS). **A**, C6/36 cells were incubated at 41°C (heat shock) for 1 and 3 hours or at 35°C (U), and the amount of gp45 was determined on the surface of non-permeabilized cells using a polyclonal antibody to gp45, a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG as secondary antibody, and flow cytometry. Percentage of gp45 on the surface of untreated and heat-shock–treated cells is shown. Each experimental point is mean ± SD of three separate experiments performed in triplicate. **B**, Surface proteins of C6/36 cells untreated (lane 1) or heat-shock–treated at 41°C (lane 2) were labeled with biotin and immunoprecipitated with a polyclonal antibody to gp45 and agarose-coupled G protein. After elution, the samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was incubated with horseradish peroxidase–conjugated streptavidin and the reaction was developed by chemiluminescence. **Arrowheads** indicate the gp45 and p74 proteins. Molecular weight markers migration is indicated on the left. Analysis of untreated (**C**) and heat-shock–treated (**D**) non-permeabilized C6/36 cells by confocal microscopy using a polyclonal antibody to gp45 and an FITC-conjugated goat anti-mouse antibody. Nuclei were stained with propidium iodine. This figure appears in color at www.ajtmh.org.

bound under these conditions was determined in paraformaldehyde-fixed non-permeabilized cells by flow cytometry.[8] The results showed that DENV 2 bound to the cell surface more efficiently after heat-shock treatment than under normal conditions (Figure 4), which suggested that a higher virus binding efficiency correlated with the increase of gp45 and p74 on the cell surface.

Effect of heat shock on amount of DENV. To analyze the effect of heat shock in virus infection, the amount of virus in untreated and heat-shock–treated cells was determined by plaque assay. Although heat shock treatment induced an increase in virus binding to the cell surface, the amount of virus in untreated and heat-shock–treated cells was not altered (Figure 5).

DISCUSSION

Dengue virus is transmitted to humans by the bite of an infected mosquito. A productive infection in mosquitoes requires DENV to replicate efficiently in mosquito cells, which makes our study of cellular receptors required for virus entry in these cells relevant. This virus can be grown in human, primate, and mosquito cells; thus, the role of some cellular proteins that mediate binding and/or entry may be conserved in different hosts. Although several studies have attempted to identify DENV receptor complex in mosquito cells, its identity has not been completely determined. Antibodies against 70-kD and 95-kD proteins in C6/36 cells blocked infection with West Nile virus, Japanese encephalitis virus, and DENV,[21] antibodies against an 80-kD protein in C6/36 cells inhibited DENV infection,[22] and antibodies against 67-kD and 80-kD proteins in *Ae. aegypti* mosquito midguts and C6/36 cells purified by affinity chromatography inhibited virus infection.[20] Antibodies against human 37/67–kD high-affinity laminin receptor and laminin inhibited virus infection, which suggests that a laminin-binding protein may be involved in DENV infection in C6/36 cells.[23]

Our group has characterized two glycoproteins of 40 and 45 kD (gp40 and gp45) as part of DENV receptor complex in C6/36 cells.[5] We showed that antibodies directed against gp45 can inhibit DENV binding to C6/36 cells and that semipurified protein gp45 could compete with C6/36 cells for virus binding. Moreover, antibodies against gp45 inhibit DENV infection. We showed that gp45 in mosquito tissues correlated

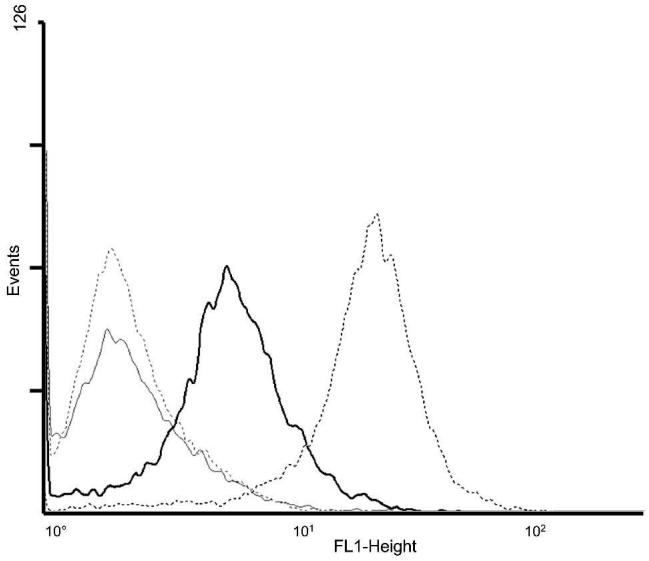

FIGURE 4. Binding of dengue virus in untreated and heat-shock (HS)–treated (for 1 hour) treated C6/36 cells. The C6/36 cells were incubated at 41°C (heat shock) (dotted line) or at 35°C (solid line) without dengue virus (gray line) or with dengue virus (black line). Cells were fixed and the amount of envelope (E) protein–positive cells was determined using an antibody to E protein and a fluorescein isothiocyanate–conjugated goat anti-mouse IgG as secondary antibody and flow cytometry on non-permeabilized cells.

with susceptibility to DENV infection[7], which supports the idea that gp45 plays an important role in DENV infection. Our group and other investigators have purified putative receptors from mosquito cells or tissues; however, the role of these receptors has not been identified.

We have used amino terminal sequencing and mass spectrometry of tryptic peptides by MALDI-ToF analysis to investigate purified proteins. Eight of 16 amino terminal amino acids have homology with those of several G proteins. Additionally, four peptides obtained by MALDI-ToF analysis correspond to the GTP binding domain from a G protein. The G proteins have important functions as receptors, as well as signals transducers, and these functions are compatible with a virus-binding and/or receptor protein.[24] Although these findings suggest that the gp45 is a G protein, further analysis, including mass spectroscopy, must be conducted to identify this protein.

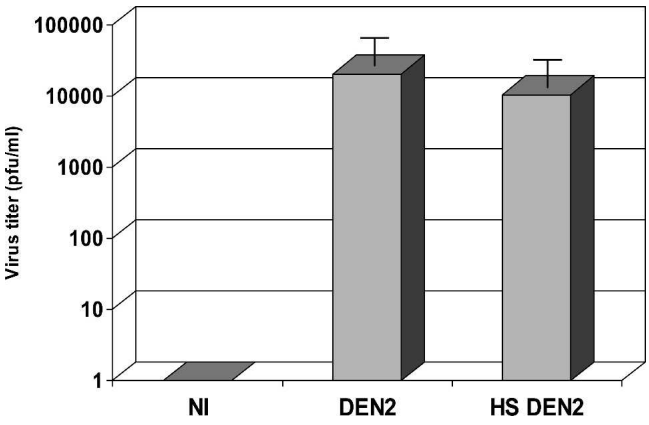

FIGURE 5. Dengue virus titers in heat-shock–treated and untreated C6/36 cells. The C6/36 cells were incubated at 41°C with DEN 2 virus (HS DEN2) or at 35°C (DEN2) before the incubation with DEN virus at a multiplicity of infection of 0.1 for 40 minutes at 35°C. Forty-eight hours post-infection, supernatants were recovered and viral yield was determined by plaque assay. Each column is the mean of results obtained from three separate experiments performed in triplicate. Error bars show the standard deviation. pfu = plaque-forming units; NI = not infected.

Because some HSPs such as HSP90, HSP70, and GRP 78 have been implicated in the first interaction between DENV and host cells,[11,12] we analyzed the role of HSPs in mosquito cells, as well as a possible relationship between gp45 and HSP. Proteins from C6/36 cells with affinity to DENV E protein were isolated by affinity chromatography and analyzed by Western blot with human antibodies to HSP90 and HSP70.[17] Monoclonal antibody to HSP90 detected gp45 and a 74-kD protein from the elution fraction. The epitope recognized by the antibody has been mapped between amino acids 604 and 697 in the C-terminal domain of human HSP90. This domain is important in HSP90 because it is involved in dimerization and binds to tetratricopeptide repeat co-chaperones.[25] The amino acid sequence recognized by the antibody was used to find a similar sequence in a mosquito protein database. Although we found some open reading frames and proteins whose sequences could be similar to the one detected by the antibodies, additional experiments are required to identify them.

The fact that p74 is recognized by a monoclonal antibody against HSP90 and is induced and relocated to the cell surface after heat shock is consistent with the idea that p74 could be an HSP. Conversely, gp45, which was also recognized by the monoclonal antibody against HSP90, was not induced by heat shock. However, it was relocated to the cell surface after heat-shock treatment, which suggests that gp45 could also be a heat-shock–related protein. Heat shock and other stress conditions can induce production and relocation of different HSPs.[26–28] Several inducible and non-inducible HSPs in mosquito cells have similar molecular weights to the proteins described in this work.[29]

The C6/36 cells used in this study were adapted to grow at 35°C.[16] This temperature is 7°C warmer than conventional growth temperatures (28°C). Although these cells may be in a permanent heat-shocked state, this is not the case because they were gradually adapted and can still respond to stress conditions. Over-expression of stress proteins such as p74 was observed when the cells were heat-shocked.

We have demonstrated that antibodies against gp45 could inhibit DENV binding and infection. In this study, we showed that gp45 and p74, which bind to E protein, relocate to the cell surface and this relocation correlates with an increase in virus binding after heat-shock treatment. These results indicate that gp45 and p74 function as attachment molecules, probably concentrating DENV particles at the cell surface to allow efficient interaction with an as yet unidentified entry factor. However, we were unable to detect an increase in the amount of virus after heat-shock treatment. Because the amount of virus is the result of serial events that include adequate virus attachment, entry, translation, replication, and morphogenesis, enhanced viral attachment after heat-shock treatment may not be necessary for an increase in the amount of virus. Moreover, we cannot rule out the possibility that HSPs induced after heat-shock treatment might function as regulators

in translation, replication, or morphogenesis of dengue virus. Because susceptibility to other virus infections such as Chinkunkunya virus was observed under heat shock in mosquitoes,[30] it would be interesting to analyze the effect of heat shock in susceptibility to DENV.

The role of HSPs in different steps of the virus replicative cycle in host cells suggests that stress conditions may regulate viral synthesis.[31–38] The ability of p74 to interact with the E recombinant protein from DENV and its relocation to the cell surface by heat shock suggests that this protein may play a role in DENV binding. This proposal is currently being analyzed. Thus, HSPs may be involved in virus infection, pathogenesis, immune response, and tropism.

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