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A PUTATIVE RECEPTOR FOR DENGUE VIRUS IN MOSQUITO TISSUES: LOCALIZATION OF A 45-KDA GLYCOPROTEIN

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Abstract. Dengue virus (DENV) infects target cells by attaching to various cell receptors, many of which are still unknown. In C6/36 cells (*Aedes albopictus* cell line), DENV-4 bound to two glycoproteins of 40 and 45 kDa, located on the cell surface. Preincubation of cells with polyclonal antibody against the 45-kDa protein specifically blocked DENV-4 infection of C6/36 cells. The antibody and purified DENV-4 detected the 45-kDa molecule in total extracts from eggs, larvae, and pupae as well as from the midgut, ovary, and salivary glands from adult-stage *Aedes aegypti* mosquitoes, whereas in malphigian tubules it was absent. This suggests that the distribution of the 45-kDa protein correlates with tissue tropism of DENV infection in mosquitoes. The 45-kDa molecule was not detected in *Anopheles albimanus* mosquito. The relevance of our findings is discussed from the pathogenetic and vector competence viewpoints.

INTRODUCTION

Dengue virus (DENV), a mosquito-borne member of the *Flaviviridae* family, causes a serious febrile illness in humans known as dengue fever and its associated complications: dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS).[1,2] Dengue fever affects more than 100 million people worldwide, and there are still no vaccines or antiviral agents available.[3]

Mosquitoes from *Aedes* genus (*Aedes aegypti* and *Aedes albopictus*) play an important role in DENV transmission. The adult female obtains DENV from an infected human during blood feeding, the virus replicates in the salivary glands, and after some days the infected mosquitoes transmit the virus to another human during the next blood feeding. The virus ingested by the female has its first replicative cycle in the midgut, and then the virus reaches the hemocele and hemolymph, thus having access to different insect tissues.[4] However, not all mosquito tissues are infected. Ultrastructural studies have shown viral particles in nervous system, salivary glands, foregut, and midgut as well as in the fat body, epidermal cells, the interior body wall lining cells, ovary, and hemocytes but not in the muscle, diverticula, hindgut, and malpighian tubules, suggesting the existence of a rather selective tissue tropism in insects as it occurs in human.[5,6]

On the other hand, several studies have shown the existence of transovarial transmission in *Aedes* females infected with DENV, allowing virus propagation through the insect progeny. This mechanism has been implicated in the virus maintenance during interepidemic periods without human or other vertebrate host participation.[4,7–10]

Because the first step in DENV infection is the interaction between virus and host cells through a cellular receptor, followed by an endocytic or direct cell membrane penetration process, the ability of DENV to infect mosquitoes at different developmental stages might depend on the presence of a viral receptor in mosquito tissues.[11,12]

The envelope (E) protein exposed on the surface of the viral membrane contains structural and functional elements that participate in the virus–host cell receptor interaction and is hence known as the viral attachment protein.[13–18] Infection of Vero cells by DENV-2 is inhibited specifically by the recombinant E protein antibodies, and the binding domain of E protein has been identified between amino acids 281 and 423.[19] In addition, studies with lectins suggest that carbohydrates such as α-mannose residues present on the E protein also contribute to the binding and penetration of the virus into BHK and C6/36 cells.[20]

Three types of molecules have also been postulated as DENV receptors on different host cells: a highly sulfated type of heparan (HS) present on Vero and BHK cells[20,21]; two glycoproteins of 40- and 45-kDa identified on C6/36 cells (*Aedes albopictus* larvae cell line)[22,23]; and two proteins ranging from 40 to 45 kDa and 70 to 75 kDa, respectively, localized in the myelomonocytic cell line HL60 and in the non-Epstein-Barr virus transformed B cells.[24] It is, therefore, possible that DENV uses different kinds of molecules for binding and entry into different cell lines.

The two glycoproteins of 40 and 45 kDa present on the surface of C6/36 cells bound DENV-4 specifically. DENV-4 binding to C6/36 can be blocked by antibodies raised against the 40- and 45-kDa molecules or by the partially purified 40- and 45-kDa proteins, suggesting their participation in the virus–receptor complex.[23] Both the 40- and the 45-kDa proteins are immunologically related because antibodies induced against one of them react with the other. Sodium periodate treatment of C6/36 cell proteins causes a reduction in the molecular weight of the proteins detected by DENV-4; instead of the 40- and 45-kDa proteins, we detected a 38-kDa protein. This effect suggests that the 40- and 45-kDa proteins from C6/36 are glycoproteins and that the oxidation of vicinal hydroxyl groups on sugars induced by the sodium periodate treatment might reduce the size of the molecule but not its ability to bind DENV. In addition, in C6/36 cells, the carbohydrate moiety may not be relevant to the interaction with DENV because the 38-kDa protein bound efficiently with DENV even after periodate treatment.[23]

Although the 40- and 45-kDa glycoproteins were described in a mosquito cell line, it may not necessarily indicate their presence in mosquito tissues. To determine the presence of the 40- and 45-kDa glycoproteins in mosquito tissues, we analyzed the different stages of the life cycle of mosquitoes from *Aedes* and *Anopheles* genera using polyclonal antibodies against these glycoproteins. In addition, using a virus overlay protein binding assay (VOPBA), the molecules involved in

DENV-4 binding were also studied at the different stages in the mosquito life cycle.

We report that DENV infection of C6/36 cells is specifically blocked by the presence of antibodies directed against the two glycoproteins of 40 and 45 kDa. These antibodies were able to detect a protein of 45 kDa in total protein extracts from eggs, larvae, pupae, and the adult. In the adult, the 45-kDa protein was present in significant quantity in midgut, ovary, and salivary glands from *Aedes*. Furthermore, DENV-4 also bound to a protein with similar electrophoretic migration to the one detected by the antibodies.

These results suggest that the 45-kDa glycoprotein initially characterized on the surface of C6/36 cell line is also present in native mosquito tissues. The presence of the 45-kDa protein, which binds DENV, correlates with tissue tropism of DENV infection of mosquitoes, suggesting that the molecule could function as a receptor complex for the virus in mosquito tissues.

MATERIALS AND METHODS

Cells and virus. Monolayers of C6/36 cells (from *Aedes albopictus*) were grown at 34°C in Jockling modified minimal essential medium (MEM) (Gibco) supplemented with nonessential aminoacids, vitamins, 10% of fetal calf serum (Hyclone), penicillin, and streptomycin.

DENV-4 strain H-241 was propagated in suckling mice and in C6/36 cells as described previously.[25]

Mosquitoes. Eggs, larvae, pupae, and mosquitoes of *An. albimanus* (white-striped pupal phenotypes) and *Ae. aegypti* were obtained from the insectaries of the Instituto Nacional de Salud Pública and the Instituto Nacional de Diagnóstico y Referencia Epidemiológica, respectively. Three-to 4-day-old adult female and male mosquitoes were used in this study. *An. albimanus* mosquitoes and their different stages were maintained as previously described,[26] whereas *Ae. aegypti* was essentially maintained as described by Munstermann.[27]

Infection inhibition assay. 6×10^4 C6/36 cells seeded in a 96-well plate were incubated at 34°C overnight. Culture media were removed by aspiration, and cells were incubated for 3 hours at 4°C with 100 μL of MEM containing different amounts (0.5–10 μg) of 40- and 45-kDa affinity purified antibodies or goat antimouse IgG (Gibco-BRL). The cells were then incubated with DENV-4 at a multiplicity of infection (MOI) of 0.1 for 1.5 hours at 4°C, and cells were washed once to remove unattached virus. The infection was allowed for 5 days at 34°C and the cytopathic effect over C6/36 was monitored by phase contrast microscopy. Photographs were taken with TriX-Pan film (Kodak) in a Nikon microscope. The experiment was performed twice in duplicate.

Preparation of DENV. DENV-4 was purified in infected C6/36 cells. Briefly, 5×10^7 cells were infected at a MOI of 0.1 plaque forming units/cell. After 4 to 6 days, the supernatant was harvested and clarified by centrifugation at 11,000 *g* for 10 min. Viruses were pelleted at 17,700 *g* for 30 min after incubation with 10% (wt/vol) polyethylene glycol 8,000 in 1.5 mol of sodium chloride (NaCl) at 4ºC for 24 hours. The pellet was resuspended in 1/10 of the original volume with GNTE buffer (50 mmol Tris HCl, 200 mmol glycine, 100 mmol NaCl, 1 mmol Ethilene diamine tetracetate [EDTA]) and applied into a discontinuous gradient of 60% and 30% (wt/vol) sucrose in GNTE. Sucrose gradients were centrifuged at 136,500 *g* for 2.5 hours at 4ºC. The visible band that contained the viruses was harvested, diluted with GTNE, and pelleted at 47,500 *g* for 2 hours at 4°C. Finally, the viral pellet was resuspended in GTNE containing 1% bovine serum albumin (BSA) (SIGMA), aliquoted, and stored at -20ºC. The virus titer was determined by plaque assay in BHK21 cells, as described previously.[25]

Preparation of total cell proteins. C6/36 cells were pelleted at 84 *g* for 10 minutes and washed three times with phosphate-buffered saline (PBS) pH 7.5. The pellet was resuspended in RSB-NP40 (1.5 mmol magnesium chloride, 10 mmol Tris HCl, 10 mmol NaCl, and 1% NP-40) in the presence of protease inhibitors cocktail (PIC) (2 mmol EDTA, 0.5 mmol phenylmethyl sulfonyl fluoride [PMSF], 2 mmol benzonidine, 5 μg/mL aprotinin, 5 μg/mL pepstatin, 5 μg/mL leupeptin, and 5 μg/mL chymostatin). Cell debris were removed by centrifugation at 9,000 *g* for 10 minutes at 4°C. The amount of protein was quantified following Bradford's method.[28]

Preparation of total proteins from eggs, larvae, pupae, and adult mosquito. *Ae. aegypti* and *An. albimanus* eggs, obtained after oviposition, or 50 larvae or pupae were put in 100 μL or 150 μL of PBS containing PIC, respectively.[29] For adults, 50 to 100 mosquitoes were placed in 3 mL of PBS with proteases inhibitors cocktail. Head, thorax, and abdomen were separated and placed in 200 μL of PIC. [29] Samples were frozen in liquid nitrogen for 1 minute and thawed at room temperature three times. Later, samples were broken with five to 10 strokes in a Dounce homogenizer. Debris were removed by centrifugation at 9,000 *g* for 15 minutes at 4°C, and the protein concentration in the supernatant was quantified following the Bradford method.[28]

Preparation of total proteins from mosquito tissues. Mosquitoes were briefly stunned on ice and placed in a drop of cold PBS. Salivary gland, midgut, ovary, and malpighian tubules were carefully removed and collected in 50 μL of PBS with PIC and frozen in liquid nitrogen for 1 minute and thawed at room temperature three times.[29] Samples were degraded with five to 10 strokes in a Dounce homogenizer. Cell debris were removed by centrifugation at 9,000 *g* for 15 minutes at 4°C. The protein concentration was determined as described previously.[28]

VOPBA. To identify cell polypeptides involved in virus binding, a VOPBA was performed. Briefly, 70 μg of total or membrane proteins from C6/36 cells or total proteins from mosquito tissues were ran in a SDS-polyacrylamide gel (SDS-PAGE) and transferred to nitrocellulose membranes using a semidry blotting apparatus in 48 mmol Tris, 39 mmol glycine, and 20% (vol/vol) methanol.[30] After overnight renaturalization of transferred proteins with 4% BSA (SIGMA) in PBS at 4°C, the membranes were blocked for 1 hour at room temperature with 5% low-fat milk in PBS and washed three times with PBS. Membranes were incubated overnight with 50 μg of purified DENV-4 in PBS, 1% skim milk, and 220 mmol NaCl. Then membranes were washed once for 5 minutes with PBS, 1% skim milk, and 220 mmol NaCl and three times with PBS. Later, membranes were incubated with a monoclonal antibody against DENV-4 E protein 1H10 diluted 1:500 in PBS overnight at 4°C. After washing the membranes three times with PBS, they were incubated for 1 hour at room temperature with a second antibody antimouse IgG conjugated to peroxidase (Zymed), diluted 1:4000 in PBS. Finally, membranes were washed three times with PBS and placed in 1 mL

of solution A and 25 μL of solution B (Boeringher ECL plus). Membranes were exposed to an x-ray film.

Polyclonal antibody production. BALB/c mice were immunized six times subcutaneously with 80 μg of the 40- and 45-kDa protein obtained by electroelution and emulsified in Freund's complete adjuvant for the primary immunization and in Freund's incomplete adjuvant for the other five immunizations at 15-day interval. Mice sera were obtained 6 days after the last immunization, and immunoglobulins were purified in protein G columns (Gibco, BRL), dialyzed against PBS, and lyophilized. Sera were tested by Western blot assays.

Western blot assay. Seventy micrograms of tissue proteins from *Ae. Aegypti* and *An. albimanus* mosquitoes and C6/36 cell proteins were run in SDS-PAGE and transferred to nitrocellulose membrane as described previously. Transference efficiency was determined by Ponceau red staining. Membranes were blocked at room temperature for 1 hour in PBS containing 5% (wt/vol) skim milk and washed three times in 0.5% (wt/vol) Tween 20 in PBS. The anti-40- and 45-kDa serum and the monoclonal antibody against DENV-4 E protein 1H10 diluted 1:1000 and 1:250 in PBS, respectively, were incubated with the membranes overnight at 4°C. The second antibody, antimouse IgG conjugated to alkaline-phosphatase (Zymed), was diluted 1:4000 in PBS and incubated at room temperature for 1 hour. Color was developed with 5-bromo-4-chloro-3-indolylphosphate toluidinium (BCIP) and nitroblue tetrazolium chloride (NBT) (Gibco-BRL), and the reaction was stopped after 1 hour with water.

RESULTS

Virus infection inhibition in C6/36 cell by antibodies against 40- and 45-kDa glycoproteins. To analyze the role of the 40- and 45-kDa proteins during DENV-4 infection, antibodies against these molecules (anti-p40/45) were tested for their abilities to block DENV infection. Monolayers of C6/36 cells were preincubated for 3 hours with different amounts of protein G-purified antibodies against 40- and 45-kDa glycoproteins or a nonrelated IgG and then incubated with DENV-4. The infection was permitted for 5 days at 34°C, and the CPE was analyzed by phase contrast microscopy. The CPE in the infected C6/36 cells was clearly observed in the absence (Figure 1A, infected) or in the presence of each 0.5 μg of anti-p40/45 antibodies or with the nonrelated antibody, and it was completely distinguishable from the noninfected cells (see Figure 1A). However, increasing concentrations of the anti-p40/45 (from 1 to 10 μg) gradually reduced the CPE, whereas the nonrelated antibody at the same concentrations did not

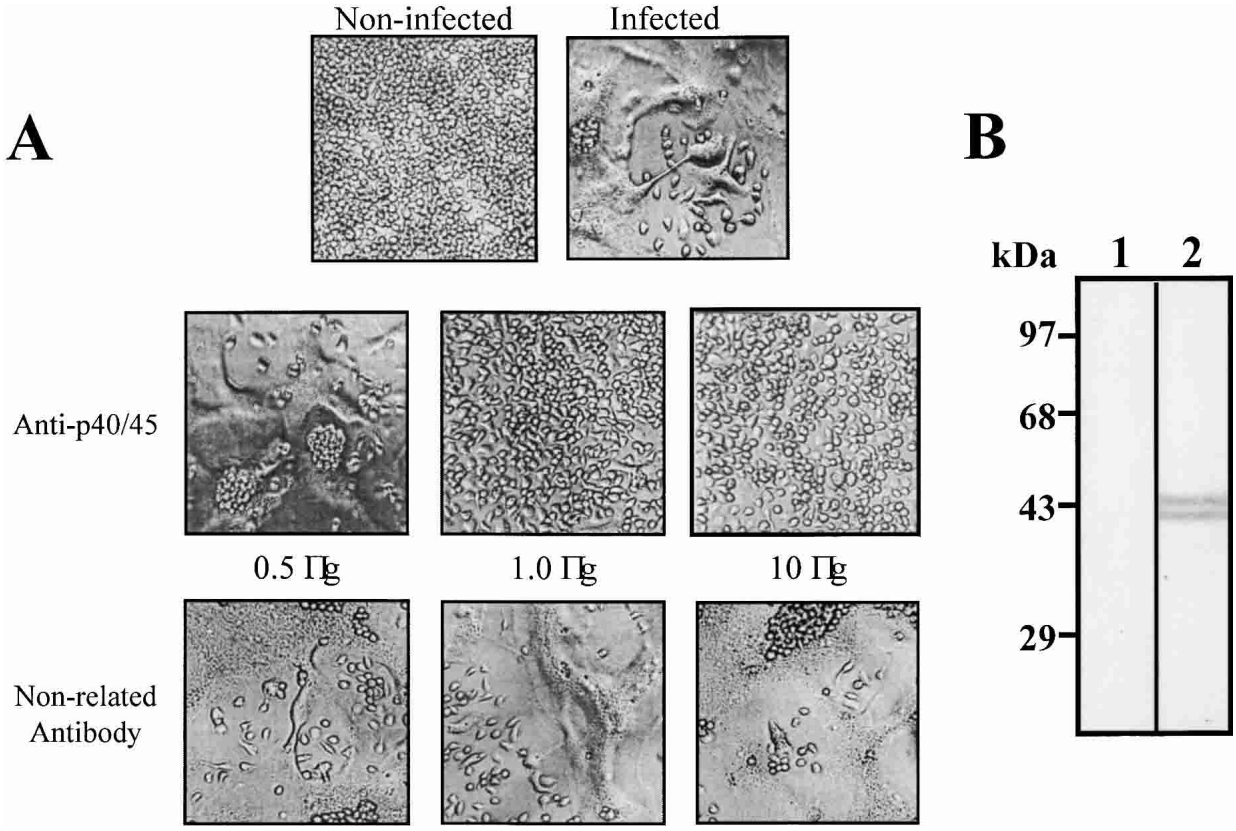

FIGURE 1. Inhibition of dengue virus (DENV) infection by antibodies against the 40- and 45-kDa proteins. **A,** 6×10^4 C6/36 cells per well were placed in a 96-well plaque and incubated at 34°C overnight. Then the culture media were eliminated by aspiration and fresh media in the absence (infected) or presence of 0.5 μg, 1 μg, and 10 μg of anti-p40/45 or a nonrelated antibody were added. Cells were incubated with the antibodies for 3 hours at 4°C before the addition of DENV-4 (except noninfected). The virus–cell interaction was permitted for 90 minutes at 4°C. CPE induced by the infection was analyzed by phase contrast microscopy 5 days after infection. **B,** Purified DENV-4 (lane 1) and total proteins from C6/36 cells (lane 2) were subjected to SDS 10%–PAGE, transferred to nitrocellulose membrane, and incubated with the anti-p40/45 antibody overnight at 4°C and with a second antibody, an antimouse IgG antibody coupled to alkaline phosphatase. Color was developed by NBT and BCIP. Migration of the molecular weight markers in kilodaltons is indicated on the left side of the membrane.

have any effect on virus infection (Figure 1A). This result clearly indicate that anti-p40/45 antibodies were able to block the CPE induced by DENV at 5 days after infection; however, it does not discount that the lower amount of virus that could have infected the cells could induce CPE later during infection. The effect induced by the anti-p40/45 antibody in DENV-4 infection was not related with an nonspecific reaction against DENV, because the anti-p40/45 is not able to react with purified DENV-4 virus in a Western blot assay (Figure 1B, lane 1), whereas it detected efficiently the 40- and 45-kDa proteins present in C6/36 total extract (Figure 1B lane 2). This result suggests that the 40- and 45-kDa glycoproteins are not only involved in virus-cell binding but could also be involved in the internalization process, supporting their role as putative DENV receptors.

DENV-4 interacts with proteins from different stages of the life cycle of *Ae. aegypti.* The inhibition of DENV infection induced by anti-p40/45 antibodies supports the importance of both proteins in the viral infection of C6/36 cells. However, the presence of the 40- and 45-kDa proteins and their role in DENV infection have not been analyzed in the mosquito. Because vertical transmission of DENV in *Ae. aegypti* and *Ae. albopictus* females has been reported to occur,[4,7–10] both the virus and its receptor are likely to be present at the different developmental stages of the mosquito life cycle. Initially, total proteins from C6/36 cells were incubated with purified DENV-4 and with a polyclonal anti-DENV-4 antibodies. The interaction was revealed with a second antibody coupled to alkaline phosphatase. DENV-4 specifically bound to the 40- and 45-kDa proteins as previously described (Figure 2A, lane 2), whereas no band of protein was detected in the absence of the anti-DENV-4 antibody (Figure 2A, lane 1), indicating that our assay revealed specific binding of DENV-4 to the doublet protein molecules. To support the specificity of the assay, proteins present in total extract from C6/36 cells and in total extract from eggs, larva, pupae, head, thorax, and abdomen of *Ae. aegypti* were used in a Western blot assay using only the second antibody antimouse IgG coupled to alkaline phosphatase. The second antibody was unable to react with any protein band in C6/36, eggs, larva, pupae, head, thorax, and abdomen total extracts (data not shown). This result strongly suggests that our assay is specific to detect cell proteins that bind DENV-4.

When proteins obtained from total extracts from eggs, larva, and pupae from *Ae. aegypti* were analyzed by VOPBA using purified DENV-4 as well as total protein extract from C6/36 cells, which was used as a positive control, we could detect that purified DENV-4 bound to the two molecules of 40- and 45-kDa present in the total extract from eggs (see Fig 2B, lane E). These two molecules comigrate with the 40- and 45-kDa proteins, which bound DENV-4 in C6/36 extract (see Figure 2B, lane C6).[23] However, in the larvae and pupae,

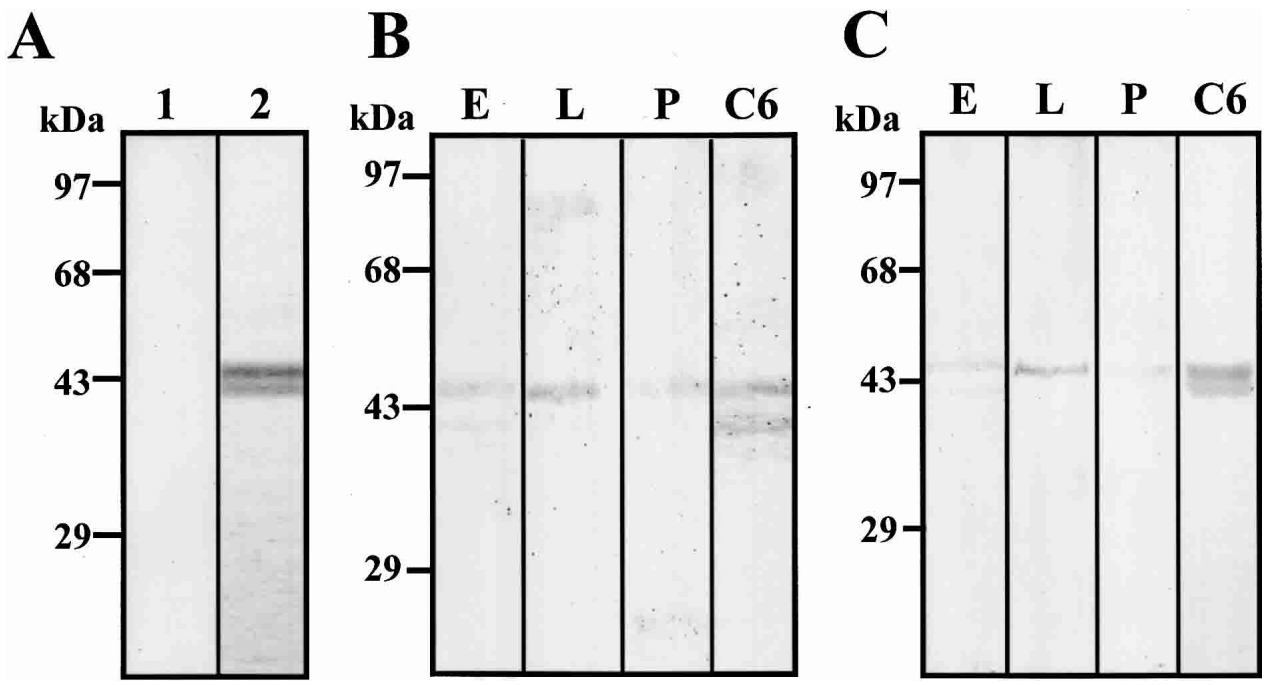

FIGURE 2. Virus overlay protein binding assay (VOPBA) and Western blot assays with protein extracts from different developmental stages of *Aedes aegypti.* **A,** Total proteins from C6/36 cells were subjected to SDS 10%–PAGE, transferred to nitrocellulose membrane, and incubated with 50 μg purified DENV-4 overnight at room temperature. The membranes were washed and incubated in the absence (lane 1) or in the presence of an anti-DENV-4 antibody (lane 2) overnight at 4°C. An antimouse IgG antibody coupled to alkaline phosphatase was used as a second antibody. Color was developed by NBT and BCIP. Migration of the molecular weight markers in kilodaltons is indicated on the left side of the membrane. **B,** Total proteins from C6/36 cells (C6), eggs (E), larvae (L), and pupae (P) of *Ae. aegypti* were subjected to SDS 10%–PAGE, transferred to nitrocellulose membranes, and incubated with 50 μg purified DENV-4 overnight at room temperature. The membranes were incubated with an anti-DENV-4 antibody overnight at 4°C, and an antimouse IgG antibody coupled to peroxidase was used as a second antibody. Finally, membranes were placed in 1 mL of solution A and 25 μl of solution B (Boeringher ECL plus) and were exposed to an x-ray film. **C,** Western blot assay was performed with total proteins from C6/36 cells (C6), eggs (E), larvae (L), and pupae (P) of *Ae. aegypti* subjected to SDS 10%–PAGE and transferred to nitrocellulose membranes. Membranes were incubated with the polyclonal antibodies against the 45-kDa C6/36 glycoproteins overnight at 4°C. An antimouse IgG antibody coupled to alkaline phosphatase was used as a second antibody. Color was developed by NBT and BCIP. Migration of the molecular weight markers in kilodaltons is indicated on the left side of the membrane.

DENV-4 bound only to the 45-kDa molecule (see Figure 2B, lanes L and P, respectively).

To determine a possible relationship between the proteins recognized by the virus and the proteins present on C6/36 cells, the anti-p40/45 antibody was used in a Western blot assay on protein extracts from eggs, larva, and pupae. The anti-p40/45 antibody recognized the 40- and 45-kDa molecules present in the protein extract from eggs and C6/36 (see Figure 2C, lanes E and C6, respectively). Both molecules comigrated with the proteins detected by DENV-4. In larvae and pupae, the anti-p40/45 antibody reacted with a 45-kDa molecule (see Figure 2C, lanes L and P, respectively), which also comigrated with the molecules detected by VOPBA. These results indicate that DENV-4 bound to a 45-kDa molecule present in total extract from different stages in the mosquito life cycle. The molecule detected by VOPBA comigrated with the one detected by the anti-p40/45 antibody from C6/36 cells.

Interaction of DENV-4 with different body segments of adult *Ae. aegypti.* To determine whether the 45-kDa molecule is also present in adult, a VOPBA was performed using the protein extracts from the three main segments of *Ae. aegypti* mosquito. A major protein of 45-kDa was localized by the purified DENV-4 in the head, thorax, and abdomen extracts (Figure 3A, lanes H, T and A). This molecule had electrophoretic mobility similar to the homologous molecule in C6/36 cells (see Figure 3A, lane C6). The 40-kDa protein was detected only in extracts from abdomen (see Figure 3A, lane A). Similar to what was noted previously, the anti-p40/45 antibody also reacted with a 45-kDa molecule in the three body segments (see Figure 3B, lanes H, T, and A) and with the molecule of 40-kDa presents in abdomen (see Figure 3B, lane A). Additionally, the anti-p40/45 antibody also reacted with a band of 80 kDa present in extracts from head and thorax (see Figure 3B, lanes H and T). Consistent with our previous results, DENV-4 bound specifically to a molecule that had similar electrophoretic migration to the one detected by the anti-p40/45 antibody in the C6/36 cell line.

Presence of the 40- and 45-kDa molecules in protein extracts from female and male from *Ae. aegypti.* It is well known that the hematophagous female of *Ae. aegypti* is responsible for DENV transmission to humans. However, it has been reported that the male is also susceptible to DENV infection.[31] Although the participation of male mosquito in DENV transmission to humans has continued to be disregarded, it could have a role in sexual DENV transmission among mosquitoes. To analyze whether there were differences in the concentration of 40- and 45-kDa proteins in tissues from females and males of *Ae. aegypti,* a Western blot assay with the anti-p45 antibody was performed (see Figure 3B and 3C, respectively). The 45-kDa protein was detected in tissues from female and male (see Figure 3B and 3C, lanes H,

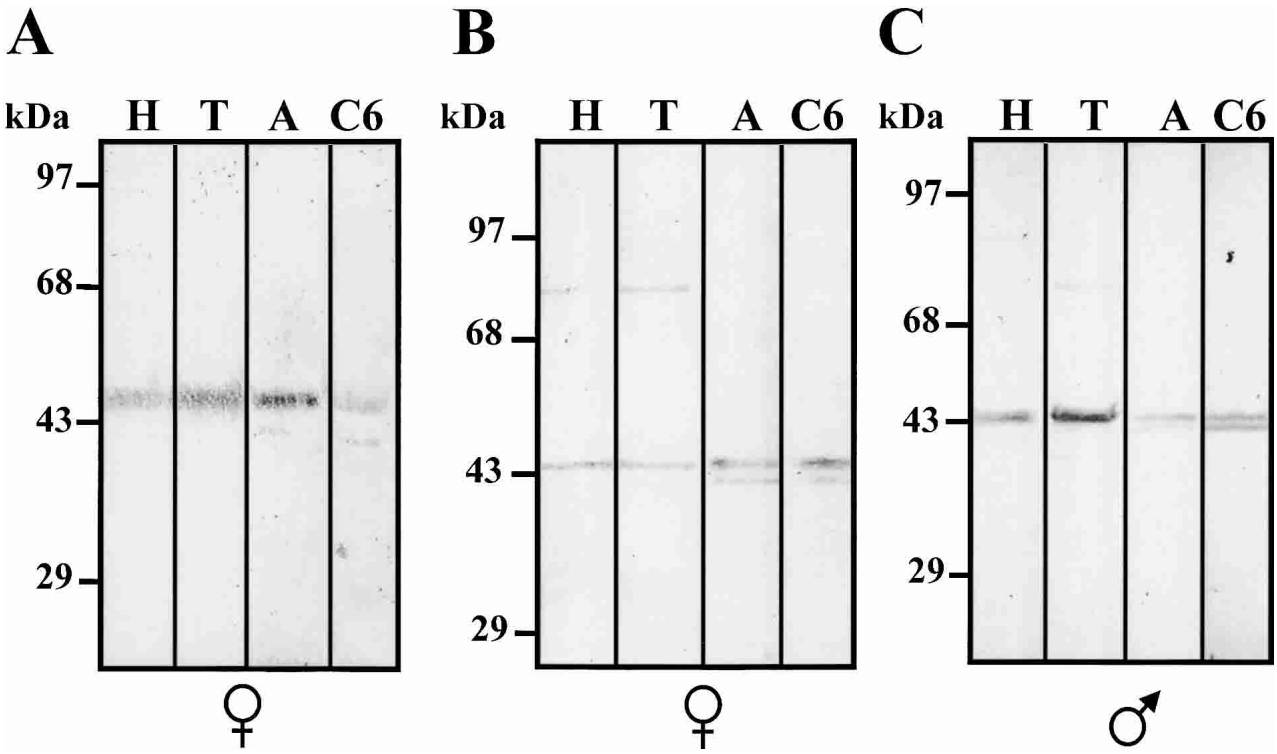

FIGURE 3. Virus overlay protein binding assay and Western blot on protein extracts from different body segments of *Ae. aegypti.* **A,** Total proteins from C6/36 cells (C6) or head (H), thorax (T), and abdomen (A) of *Ae. aegypti* were subjected to SDS 10%–PAGE, transferred to nitrocellulose membranes, and incubated with 50 μg purified DENV-4 overnight at room temperature. Membranes were subsequently incubated with an anti-DENV-4 antibody overnight at 4°C and with an anti-mouse IgG antibody coupled to peroxidase. Finally, membranes were placed in 1 mL of solution A and 25 μl of solution B (Boeringher ECL plus) and were exposed to an x-ray film. Western blot assay performed with total proteins from C6/36 cells (C6), head (H), thorax (T), and abdomen (A) of *Ae. aegypti* females **(B)** and males **(C)**. Total proteins transferred to nitrocellulose membranes were incubated with polyclonal antibodies against the 45-kDa C6/36 glycoproteins overnight at 4°C and with an antimouse IgG antibody coupled to alkaline phosphatase. Color was developed by NBT and BCIP. Migration of the molecular weight markers in kilodaltons is indicated on the left side of each membrane.

T, and A). Although the antibody detected the 80-kDa protein in the thorax and head from females, this protein was detectable only in the thorax extract from male (see Figure 3C, lane T) and was absent in the head extracts (see Figure 3C, lane H). The 40-kDa molecule was present only in the abdomen of female and male mosquitoes (see Figure 3B and 3C, lanes A).

Tissue protein recognition by antibodies against 40- and 45-kDa C6/36 glycoproteins in *Ae. aegypti.* The property of the virus to infect only certain tissues has been associated with the presence of the viral receptor in those tissues, and the tissue tropism of DENV in mosquitoes has been reported previously.[5,6] To determine whether the distribution of 40- and 45-kDa glycoproteins in different mosquito tissues correlates with the organ distribution of DENV-4 infectivity, protein extracts from salivary glands (thorax), midgut (abdomen), ovary (abdomen) and malpighian tubules (abdomen) were obtained and analyzed in a Western blot assay. Two proteins with molecular weight of 40 and 45 kDa were detected in the salivary glands, midgut, and ovary but not in the malpighian tubules (Figure 4A, lanes Sg, M, Ov, and Mt, respectively). An additional band of about 30-35 kDa was observed in salivary gland extracts (Fig. 4A, lane Sg) and also, although to a less extent, in the ovary (see Figure 4A, lane Ov) but not in the other tissues tested. These results strongly suggest a correlation between the organ distribution of virus infectivity and the presence of the 40- and 45-kDa proteins in mosquito tissues.[5,6]

To corroborate the results obtained with antibodies against the 40- and 45-kDa molecules on mosquito tissues, the same protein extracts were used for a VOPBA with purified DENV-4. Virus was able to bind to a 45-kDa molecule and 30- to 35-kDa molecules in salivary glands (thorax), midgut (abdomen), and ovary (abdomen) (see Figure 4B, lanes Sg, M, and Ov, respectively). DENV-4 bound with higher efficiency to the 30- to 35-kDa molecules from midgut (abdomen) and ovary (abdomen) than to that from the salivary gland (thorax) but was unable to bind to any of the molecules in malpighian tubules (abdomen) (see Figure 4B, lane Mt).

Analysis of DENV binding molecules in tissues from both female and male *An. albimanus.* Rosen et al[32] reported that mosquito species vary in their susceptibility to DENV infection; the members of the *Aedes* genus were the most susceptible, whereas *Culex* and *Armigeres* are relative refractory to DENV infection. Specifically, *An. sinensis* and *stephensi* were nonsusceptible to any serotype of DENV virus after parenteral inoculation. In an attempt to determine the presence of proteins similar to the 40- and 45-kDa molecules (from *Aedes)* in *An. albimanus,* a malaria vector, a Western blot assay with the anti-p40/45 antibodies was performed.[32] The anti-p40/45 antibodies were unable to react with any of the proteins present in the extracts from egg, larvae, and pupae of *An. albimanus* (data not shown), whereas the 40- and 45-kDa proteins were clearly detected in C6/36 cell extract. Likewise, when the same extracts from *An. albimanus* were used in VOPBA using the purified DENV-4, the virus was unable to bind to either of the molecules in the eggs, larvae, and pupae (data not shown).

Because we were unable to detect any molecule in eggs, larvae, and pupae stages of *An. albimanus,* we further ana-

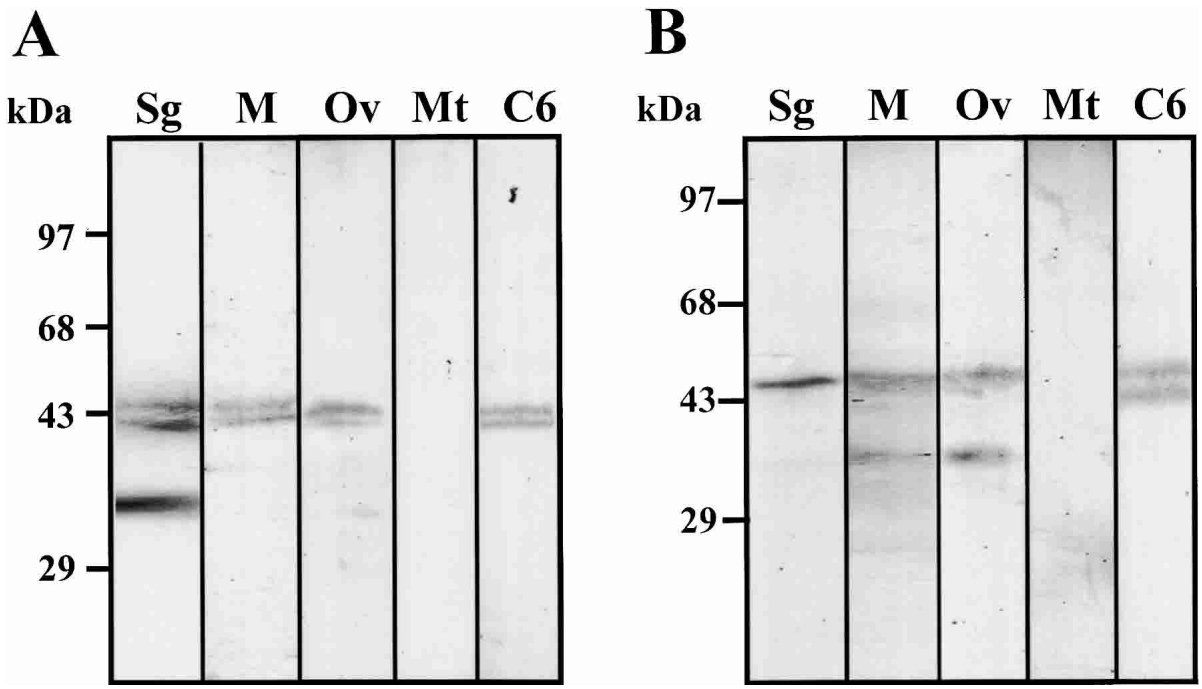

FIGURE 4. Western blot and virus overlay protein binding assays of protein extracts from different tissues of *Ae. aegypti.* Total proteins from C6/36 cells (C6), salivary glands (Sg), midgut (M), ovary (Ov), or malpighian tubules (Mt) of *Ae. aegypti* were subjected to SDS 10%–PAGE and transferred to nitrocellulose membranes. **A,** Membrane was incubated with an antibody against the 45-kDa C6/36 glycoprotein and with an antimouse IgG antibody coupled to alkaline phosphatase. Color was developed by NBT and BCIP. **B,** Membrane was incubated with 50 μg purified DENV-4 overnight at room temperature, with an anti-DENV-4 antibody overnight at 4°C, and with an antimouse IgG antibody coupled to peroxidase as second antibody. Finally, membranes were placed in 1 mL of solution A and 25 μL of solution B (Boeringher ECL plus) and were exposed to an x-ray film. Migration of the molecular weight markers in kilodaltons is indicated on the left side of each membrane.

lyzed proteins from the head, thorax, and abdomen of the adult mosquito using the Western blot assay. Only one protein of 40 kDa was revealed in the thorax and abdomen (Figure 5A, lanes T and A) but not in the head (see Figure 5A, lane H) of females. In males, the 40-kDa molecule was observed only in the thorax; however, an additional band of 80 kDa was also detected in this extract (see Figure 5B, lane T). We could not detect any other molecule in head and abdomen (see Figure 5B, lanes H and A). Our results indicate a significant difference in the protein pattern recognized by the anti-p40/p45-antibody between males and females. The 40-kDa molecule, detected in thorax and abdomen protein extracts from females, was not detected in protein extracts from the same tissues of males. However, DENV-4 was unable to bind to either of the doublet proteins in the female or male tissue extracts (data not shown). Although the 40-kDa molecule from *Anopheles,* detected in the thorax and abdomen of females and in the thorax of males, is immunologicaly related to the 40- and 45-kDa protein from *Aedes* and C6/36, it was not able to bind DENV-4, indicating the specificity of DENV-4 receptors on cells of *Aedes aegypti* mosquito.

DISCUSSION

During the previous years, several groups have been studying DENV infections in mosquitoes from *Aedes* genus; however, little is known about the molecules involved in virus–cell interactions in these insects. In this article, we studied the binding of DENV-4 to proteins present in different developmental stages in the life cycle and in different tissues of mosquitoes in the *Aedes* and *Anopheles* genera.[5,6,32–34]

In a previous study, we reported two glycoproteins with molecular weights of 40 and 45 kDa present on the surface of C6/36 cell (a cell line from larvae of *Ae. albopictus*) that bind DENV-4 specifically. Antibodies against both proteins as well as the partially purified molecules were able to block viral binding to C6/36 cells. However, some relevant questions about the characteristics of the glycoproteins and their roles in DENV infection in the mosquito have not been addressed. The first important question is the ability of the antibodies against the 40- and 45-kDa proteins to block DENV-4 infection in C6/36 cells. Our results clearly indicated that the anti-p40/45 antibodies were able to inhibit the CPE induced by DENV in C6/36 cells in a dose-dependent manner. This result strongly suggests that the 40- and 45-kDa proteins play an important role in DENV-4 binding and internalization, properties that are totally compatible with the function of a putative viral receptor.

C6/36 cells is a cell line obtained from larvae of *Ae.albopictus;* thus, we analyzed the presence of the 40- and 45-kDa proteins in the early developmental stages in the life cycle of *Ae. aegypti:* egg, larvae, and pupae. Antibodies against 40- and 45-kDa glycoproteins detected a 45-kDa molecule in the three stages and the doublet 40- and 45-kDa molecules in eggs. Purified DENV-4 bound to molecules with the same molecular weights similar to the one detected by the antibodies and to the 45-kDa molecule in larvae and pupae as well as to the doublet molecules in egg. The ability of DENV-4 to

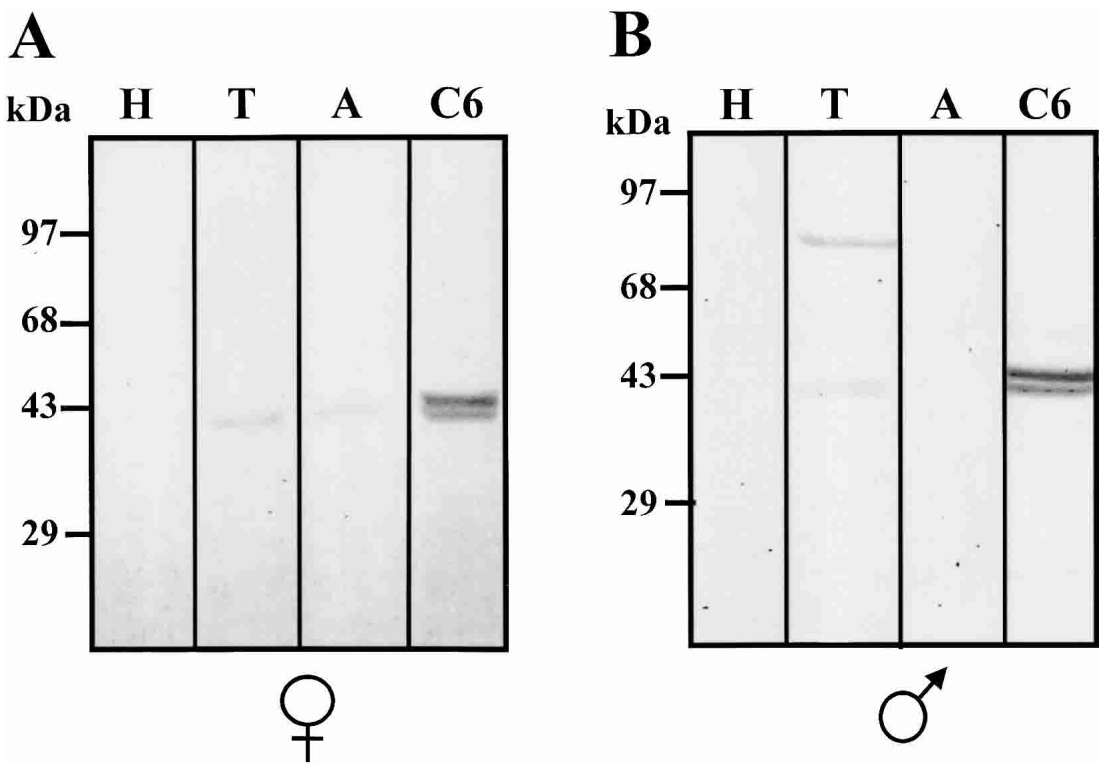

FIGURE 5. Western blot assay of protein extracts from female and male of *An. albimanus.* Total proteins from head (H), thorax (T), abdomen (A), and C6/36 cells (C6) from females **(A)** and males **(B)** were subjected to SDS 10%–PAGE and transferred to nitrocellulose membranes. Membranes were incubated with antibodies against the 40- and 45-kDa C6/36 glycoprotein and with an antimouse IgG antibody coupled to alkaline phosphatase. Color was developed by NBT and BCIP. Migration of the molecular weight markers in kilodaltons is indicated on the left side of each membrane.

bind to the same molecules that could play a role as receptors in C6/36 makes it possible to assume that they could also participate in DENV entry during mosquito ontogenic development. Several lines of evidence support the vertical transmission of DENV from *Aedes* females to the progeny, explaining the maintenance of DENV during interepidemic periods without human or non human primate participation.[7–10] This process of vertical transmission would require the presence of a putative viral receptor during the different developmental stages of infected mosquito. The 45-kDa protein may thus function as the putative receptor for DENV in mosquito tissues at all stages of the mosquito life cycle. The 45-kDa protein, which is present in all the three developmental stages, was consistently detected by the virus and the antibodies but localized as a weak band in pupae extract. Because the same amount of tissue protein was loaded in to each electrophoretic lane, this result could be explained only by a possible reduction in the protein expression during this developmental stage. The detection of the 40-kDa protein observed on C6/36 cells can only be achieved with the egg extracts, which conflicts with the fact that the larvae stage of this mosquito is the source of C6/36 cells. One plausible explanation for this discrepancy could be that C6/36 cells might be one type of larvae cells that express the two forms of the protein.

In our previous work, we demonstrated that periodate treatment of C6/36 protein extract modified the molecular weight of the 40- and 45-kDa glycoproteins to a 38-kDa protein, which was also able to bind DENV-4.[23] Therefore, the 40- and 45-kDa molecules are probably the glycosylated forms of the 38-kDa molecule. The absence of 40-kDa protein in some stages could be associated with differences in the pattern of glycosylation during insect development.

The experiments described previously were also performed on protein extracts from different body regions of adult *Ae. aegypti* of both sexes. The 45-kDa protein was clearly detected in the head, thorax, and abdomen extracts, whereas the 40-kDa was only observed in abdomen. This observation correlates with what we noted with different mosquito tissues, where two bands were detected in extracts from ovary and midgut, both of which are located in the abdominal region, whereas in the salivary glands located in thorax the 45-kDa protein was the only molecule detected. These results probably indicate a differential expression or nature of glycosylation of these proteins in the different organs, and there seems to be a higher preference for the expression of the 45-kDa molecule.

An additional band of approximately 80 kDa was detected by Western blot assays on the head and thorax extracts but was not detected in VOPBA with DENV-4. Muñoz et al.[35] reported that DENV2 bound to a 80-kDa protein present in C6/36 cells. Although we may not know at this stage whether the 80-kDa protein detected by our antibodies in insect tissues is the same molecule reported by Muñoz et al.,[35] our results indicate that the protein is immunologicaly related to the 40- and 45-kDa glycoproteins even though it fails to interact with DENV-4. On the contrary, when we analyzed specific tissues from mosquito, we were not able to detect the 80-kDa protein. It is possible that the protein is present only in tissue that we did not analyze. Further analysis of the role of this molecule in the DENV infection or tropism is necessary. Because a similar observation was made with the salivary glands, where an additional band of approximately 35 kDa was also detected, this protein molecule could be the nonglycosylated form of the 40- and 45-kDa proteins, although further studies will be required to confirm this hypothesis.

Interestingly, the same molecules were detected by DENV and antibodies in male and female mosquitoes, suggesting that both sexes could be susceptible to DENV infection. This result correlates with previous observations that male mosquitoes could indeed be susceptible to DENV infection.[31] Notwithstanding that we did not study the virus–tissue interaction in males in more detail, this result possibly suggests that similar molecules could be involved in DENV infection in both genders.

Ultrastructural studies have shown a viral tissue tropism for DENV in members of the *Aedes* genus; the nervous system, salivary glands, ovary, and midgut are the most susceptible tissues, whereas the malphigian tubules appear relatively nonsusceptible.[5,6] Using different tissue extracts and antibody anti-p40/45, our Western blot results revealed a positive correlation between tissue tropism and the presence of at least the 45-kDa protein. Except for the midgut and ovary, in which both proteins were detected, only the 45-kDa protein was detected in salivary glands, whereas none was localized in malpighian tubules.

Interesting results were obtained when similar experiments were performed with protein extract from *An. albimanus,* the malaria vector, a nonsusceptible DENV insect. None of the proteins was recognized by the antibodies in egg, larvae, and pupae. However, the 40-kDa protein was detected in the thorax and abdomen of adults, suggesting that the expression of this protein in *An. albimanus* is probably stage specific. In males, the anti-p40/45 antibodies revealed two bands in the thorax of 40 and 80 kDa. This pattern was similar to what was observed in *Ae. aegypti* males with one exception: the 45-kDa protein was detected instead of the 40-kDa protein. This result might lend support to the idea expressed previously that the 45-kDa glycoprotein is more relevant in DENV infection than the 40-kDa molecule. The inability of DENV-4 to bind to the 40-kDa molecule present in the thorax and abdomen of females and thorax of males also supports this idea. Ultrastructural studies directed to analyze the precise location of these molecules in the insect tissues in vivo are necessary to understand their role in DENV infection.

Our results showed positive correlations between DENV protein recognition, antibody-protein recognition, and virus-tissue tropism, supporting the idea that the 45-kDa protein is, indeed, one of the molecules involved in DENV infection. The 40- and 45-kDa molecules might be functioning in insect tissues as a receptor complex and are also an important factor in the determination of the competence of DENV insect vector. Further analyses including the 40- and 45-kDa proteins isolation, sequencing and gene identification are currently being performed in our laboratory.

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