

Identification of Two Surface Proteins from C6/36 Cells That Bind Dengue Type 4 Virus

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Received 10 March 1997/Accepted 18 June 1997

Dengue viruses infect cells by attaching to a surface receptor, probably through the envelope (E) glycoprotein, located on the surface of the viral membrane. However, the identity of the dengue virus receptor in the mosquito and in mammalian host cells remains unknown. To identify and characterize the molecules responsible for binding dengue virus, overlay protein blot and binding assays were performed with labeled virus. Two glycoproteins of 40 and 45 kDa located on the surface of C6/36 cells bound dengue type 4 virus. Virus binding by total and membrane proteins obtained from trypsin-treated cells was inhibited, while neuraminidase treatment did not inhibit binding. Periodate treatment of cell proteins did not reduce virus binding, but it modified the molecular weight of the polypeptide detected by overlay assays. Preincubation of C6/36 cells with electroeluted 40- and 45-kDa proteins or with specific antibodies raised against these proteins inhibited virus binding. These results strongly suggest that the 40- and 45-kDa surface proteins are putative receptors or part of a receptor complex for dengue virus.

Dengue viruses, mosquito-borne members of the *Flaviviridae* family, are the causative agents of dengue fever and its associated complications dengue hemorrhagic fever and dengue shock syndrome (6, 24). These potentially lethal conditions may be caused by any of the four virus serotypes (types 1 to 4) identified so far (4). Virus attachment to a cell surface receptor is the first step in viral infection, initiating events that lead to release of the viral nucleic acid into the cytoplasm. Understanding of how viral proteins and host cell receptors mediate this initial interaction is required for an understanding of late events, such as viral replication and pathogenesis.

The viral 11-kb RNA genome encodes three structural proteins (capsid, precursor of membrane protein, and envelope) and seven nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) (10, 11, 27, 41).

Dengue virus attachment to the host cell surface is mediated by the viral attachment protein (VAP), which seems to be the glycoprotein E present on the viral membrane. This is supported by the correlation found between the degree of E protein binding and the susceptibility of various cell lines to viral infection (1, 30). The presence of a glycine-rich internal element (amino acids 98 to 111) that is conserved among flavivirus E proteins and involved in low-pH-catalyzed membrane fusion and the evidence of pH-dependent conformational changes (9, 14, 15, 18–20, 33) further suggest that E protein corresponds to the VAP. In addition, a recombinant envelope protein inhibits infection of Vero cells by dengue virus, and the binding motif of this recombinant protein has been identified as being between amino acids 281 and 423 (5).

Dengue virus can infect its host cells via two mechanisms: through the binding of virus complexes to the Fc receptor or through the direct interaction of viral proteins with a specific host cell receptor (8, 23). The first mechanism has been studied extensively because the increase in viral load observed in den-

gue hemorrhagic fever and dengue shock syndrome has been associated with an increase in virus-antibody complexes that bind to Fc- γ receptor-positive cells via the Fc portion of immunoglobulin G (IgG) (16, 23, 25, 28). However, the second mechanism, which produces the primary infection in mosquito or human cells, has been poorly studied, and the cellular receptors have not been yet identified.

Peripheral blood human monocytes and C6/36 cells (an *Aedes albopictus* cell line derived from the larval stage) have proven to be useful models to study early events in dengue type 2 virus infection, such as virus attachment and penetration. Electron microscopy analysis with the cells has shown that attachment is a temperature-independent process which occurs at both 4 and 37°C, while viral penetration proceeds only at 37°C and can occur by membrane fusion in C6/36 cells or by receptor-mediated endocytosis in monocytes (2, 17). Trypsin treatment of adherent human monocytes greatly reduces the ability to support dengue virus replication (8).

The capability of dengue virus to infect human and mosquito cells suggests the presence of a dengue virus receptor. Thus, the identification of the nature, number, and distribution of host cell receptors for dengue virus is important to understand tissue tropism, pathogenesis, and viral replication in the host. Vertical transmission of dengue virus in the genus *Aedes* has been demonstrated, indicating that the virus, and probably its receptor, are present in several mosquito tissues during different stages of the life cycle (12, 22, 34, 35, 37). The C6/36 cell line, which can be infected by dengue virus, was used to partially characterize a putative dengue virus receptor.

In this paper we report that dengue type 4 virus specifically bound to two glycoproteins of 40 and 45 kDa located on the surface of C6/36 cells. Binding was inhibited in total and membrane proteins obtained from trypsin-treated cells, while neuraminidase treatment did not alter virus binding, and neither did sodium periodate, although it modified the molecular weight of the polypeptide. Polyclonal antibodies raised against the 40- and 45-kDa proteins identified the proteins on the surface of C6/36 cells in immunofluorescence assays and blocked the binding of radiolabeled virus to intact C6/36 cells. These data indicate that the 40- and 45-kDa proteins bind

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dengue virus and may in fact be the virus receptor or an important component of the receptor complex.

MATERIALS AND METHODS

Cells and virus. Monolayers of C6/36 cells (from *A. albopictus*), kindly donated by Goro Kuno (Centers for Disease Control, San Juan, P.R.), were grown at 34°C in Joklik modified minimal essential medium (MEM) supplemented with non-essential amino acids, vitamins, 10% fetal calf serum (HyClone), penicillin, and streptomycin. Monolayers of CV-1 cells were grown in Dulbecco's MEM supplemented with 10% fetal calf serum, penicillin, and streptomycin.

Dengue type 4 virus strain H-241, donated by Goro Kuno, was propagated in lactant mice and in C6/36 cells as described previously (13).

Preparation of labeled dengue virus. Radiolabeled dengue virus was obtained from infected C6/36 cells. Briefly, 5×10^7 cells were infected at a multiplicity of infection of 0.1 PFU/cell. At 6 h postinfection the medium was replaced with methionine-free medium (Sigma) containing 1 mCi of [³⁵S]methionine (DuPont), vitamins, gentamicin, and 5% fetal calf serum, and the cells were incubated at 34°C. After 7 days, the supernatant was clarified and centrifuged at $84 \times g$ for 10 min. Viruses were pelleted at $100,000 \times g$ for 2 h at 4°C and stored at -20°C in GTNE buffer (50 mM Tris-HCl, 200 mM glycine, 100 mM NaCl, 1 mM EDTA) supplemented with 50% fetal calf serum. The virus titer was determined by plaque assay in CV-1 cells, as described previously (13). As a control, labeled proteins from uninfected cells were prepared by the same protocol described above.

Binding assay. Direct binding assays were carried out to characterize the attachment of dengue virus to C6/36 cells. For these studies, 12-well plates with 5×10^6 C6/36 cells per well were placed at 4°C for 2 h before incubation with different amounts of radiolabeled dengue virus. Virus-cell interaction was carried out at 4°C to avoid viral penetration. At different times, the medium was removed and the cells were washed twice with fresh medium and lysed with IP buffer (10 mM phosphate-buffered saline [PBS] [pH 7.2], 0.15 M NaCl, 1% Triton X-100, and 0.1% sodium dodecyl sulfate [SDS]) (1). The radioactivity present in both the medium and the cells was measured in a scintillation counter. Assays were conducted in triplicate. Bound counts per minute due to specific binding were obtained after subtracting the bound counts per minute due to nonspecific binding (obtained after cell incubation with labeled proteins from uninfected cells) from the bound counts per minute obtained after incubation with labeled dengue virus. For calculations of nonspecific binding for the saturation experiment, cells were preincubated with 280 µg of unlabeled dengue type 4 virus prior to the addition of different concentrations of labeled dengue virus. Binding assays were performed twice. Each point was determined in duplicate, and values from independent experiments varied by 2 to 5% from the average.

For trypsin treatment, 5×10^6 C6/36 cells were incubated with a 0.075% solution of pancreatic bovine trypsin for 30 min at 34°C (8), followed by incubation for 45 min with fresh medium supplemented with 10% fetal calf serum to inactivate trypsin. Finally, cell monolayers were washed twice with fresh medium, and binding assays were performed as described above.

For sodium periodate treatment, 5×10^6 C6/36 cells were treated with different concentrations of sodium periodate (Sigma) in PBS (pH 5.6) at 4°C for 15 min. The cells were washed twice with PBS and incubated for 15 min with fresh medium supplemented with 10% fetal calf serum. Binding assays were performed as described above.

Preparation of total cell proteins. C6/36 cells were pelleted at $84 \times g$ for 10 min and washed three times with PBS (pH 7.5). The pellet was resuspended in RSB-NP40 (1.5 mM MgCl₂, 10 mM Tris-HCl, 10 mM NaCl, and 1% Nonidet P-40) in the presence of protease inhibitors (2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM benzimidazole, 5 µg of aprotinin per ml, 5 µg of pepstatin per ml, 5 µg of leupeptin per ml, and 5 µg of chymostatin per ml). Nuclei and debris were removed by centrifugation at $9,000 \times g$ for 10 min at 4°C. The amount of protein was quantified by the Bradford method (3).

Trypsin treatment was performed as described above, but incubation was increased to 60 min. After incubation, cells were washed three times with cold PBS, and cell extract was prepared as described above.

For sodium periodate treatment, cells were incubated with 10 mM sodium periodate in PBS (pH 5.6) for 15 min at 4°C. After treatment, cells were washed and cell extract was prepared as described above.

Cell membrane protein preparation. To obtain cell membrane proteins, a phase partitioning with Triton X-114 was performed. C6/36 cells were washed three times with Tris-buffered saline (TBS) (10 mM Tris-HCl [pH 7.5], 150 mM NaCl). Cells were lysed in 4 cell pellet volumes of 1% Triton X-114 in TBS in the presence of the protease inhibitor cocktail at 4°C and centrifuged at $4,000 \times g$ for 30 min. The supernatant was incubated overnight at -20°C and for 10 min at 37°C and centrifuged at $60 \times g$ for 15 min. The pellet was resuspended in the same volume of TBS in the presence of protease inhibitors and incubated at 4°C for 30 min. To ensure good recovery of membrane proteins, the procedure was repeated starting with the incubation at 37°C. Acetone precipitation was carried out to eliminate the detergent, and the amount of protein was quantified by using Bradford's reaction (3).

VOPBA. To identify cell polypeptides involved in virus binding, a virus overlay protein-binding assay (VOPBA) was carried out. VOPBA was performed as described by Jin et al. (21), Ludwig et al. (26), and Crane et al. (7) with some modifications. Briefly, 200 µg of total or membrane proteins from C6/36 cells was

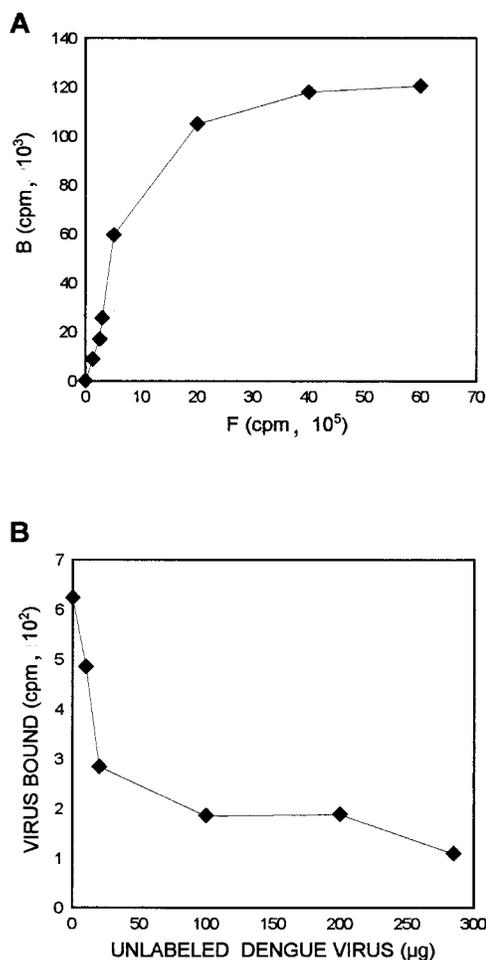


FIG. 1. Binding of ³⁵S-labeled dengue type 4 virus (specific activity, 4×10^4 cpm/µg) to the surface of C6/36 cells. (A) Saturation experiment with different amounts of labeled dengue type 4 virus in the presence of a constant number of cells (5×10^5). Bound virus (B) and free virus (F) were quantitated. (B) Competition of unlabeled dengue virus preincubated with C6/36 cells for 2 h before the addition of labeled dengue virus.

subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes by using a semidry blotting apparatus in 48 mM Tris-39 mM glycine-20% (vol/vol) methanol (39). After overnight renaturation of transferred proteins with 4% bovine serum albumin (BSA) in PBS at 4°C, the membranes were blocked for 1 h at room temperature with 5% low-fat milk in PBS and washed three times with PBS. Membranes were incubated overnight with 5×10^5 cpm of radiolabeled dengue type 4 virus in MEM supplemented with 10% fetal calf serum at room temperature with gentle rocking. Afterwards, membranes were washed four times for 15 min with 2% BSA in PBS and once with 0.1% Nonidet P-40 in PBS at room temperature. Finally, membranes were dried and autoradiographed. To determine the specificity of the virus-cell protein interaction, VOPBAs with a high salt concentration were performed. Briefly, before incubation with the virus, the membranes were washed once for 5 min with PBS-1% skim milk and once in high-salt washing solution (PBS, 1% skim milk, 220 mM NaCl). The incubation with virus was performed under the same conditions described above but in the presence of high-salt washing solution. Finally, membranes were washed three times with high-salt solution prior to exposure to X-ray film (26).

Protein purification. Cell membrane proteins were subjected to SDS-10% PAGE. The 40- and 45-kDa bands were cut from the gel and electroeluted overnight at 25 V in an electroelution apparatus (Blue Tank [ISCO]). The integrity of both proteins was monitored by SDS-PAGE.

DSP cross-linking assay. Cross-linking assays were performed with DSP [di-thiobis(succinimidylpropionate)] (Pierce), 100 µg of dengue type 4 virus and 100 µg of labeled membrane proteins or 100 µg of labeled 40- and 45-kDa proteins obtained after electroelution, according to the manufacturer's protocol. After the cross-linking reaction, samples were treated with 5% β-mercaptoethanol to cleave the DSP reagent.

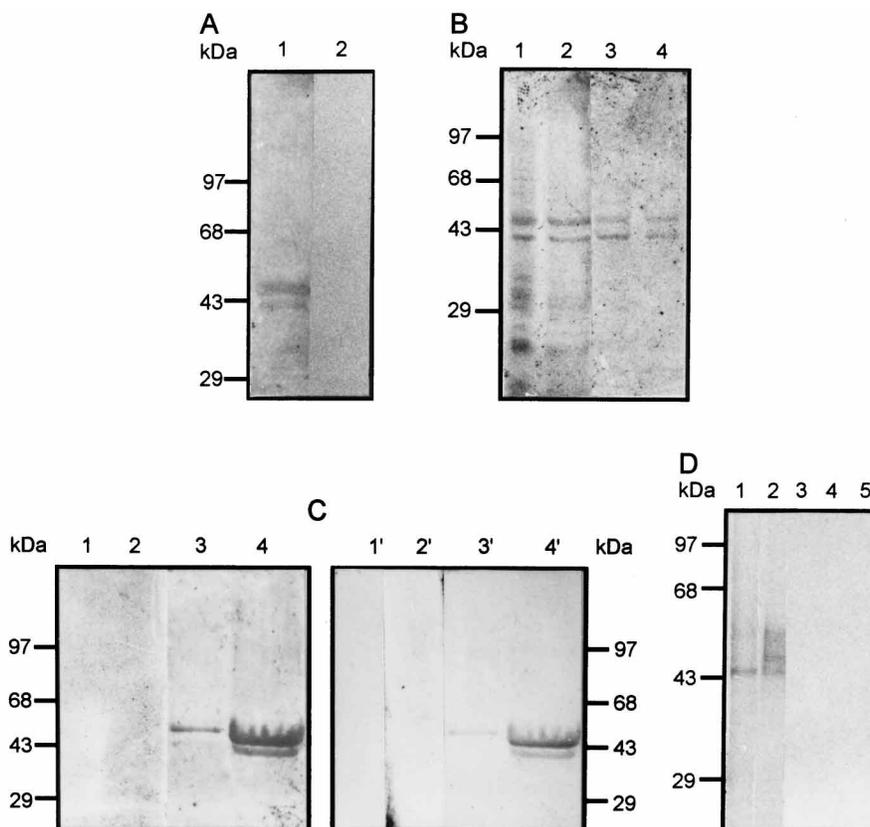


FIG. 2. VOPBA with [35 S]methionine-labeled dengue virus. (A) Total C6/36 cell proteins were subjected to SDS-10% PAGE, transferred to a nitrocellulose membrane, and incubated with 5×10^5 cpm of labeled dengue virus (lane 1) or 5×10^5 cpm of labeled proteins from uninfected cells (lane 2). (B) C6/36 cell membrane proteins were run in the presence (lanes 1 and 3) or in the absence (lanes 2 and 4) of β -mercaptoethanol and incubated with 5×10^5 cpm of labeled dengue type 4 virus under isotonic (lanes 1 and 2) or hypertonic (lanes 3 and 4) conditions. (C) C6/36 cell membrane proteins (lanes 1, 1', 3, and 3') and electroeluted 40- and 45-kDa proteins (lanes 2, 2', 4, and 4') were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with labeled proteins from uninfected cells (lanes 1, 1', 2, and 2') or with labeled dengue virus (lanes 3, 3', 4, and 4'). The membrane was exposed to X-ray film (lanes 1, 2, 3, and 4) and then incubated with the monoclonal antibody 1H10 against dengue type 4 virus E protein (lanes 1', 2', 3', and 4'). (D) DSP cross-linking assay. Labeled membrane proteins from C6/36 cells (lanes 1 and 4) and labeled 40- and 45-kDa proteins (lanes 2 and 5) were cross-linked in the presence (lanes 1 to 3) or absence (lanes 4 and 5) of unlabeled dengue virus. Cross-linked proteins obtained after centrifugation were treated with 5% β -mercaptoethanol, subjected to SDS-10% PAGE, and exposed to X-ray film.

Incubation of cell proteins with lectins. Cell membrane proteins and electroeluted 40- and 45-kDa proteins were subjected to SDS-PAGE and transferred as described above. Membranes were blocked overnight at 4°C in TBS containing 3% (wt/vol) BSA and washed three times in 0.5% (wt/vol) Tween 20 in TBS. Biotinylated agglutinin I (*Ricinus communis*) (Vector) was diluted at 20 μ g/ml in TBS and incubated for 2 h at room temperature. Streptavidin coupled to alkaline phosphatase was diluted 1:5,000 and incubated for 1 h at room temperature in TBS. Color was developed with BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium) and NBT (nitroblue tetrazolium chloride), and the reaction was stopped after 15 min with water.

Polyclonal antibody production. BALB/c mice were immunized six times subcutaneously with 80 μ g of the 40- and 45-kDa proteins, obtained by electroelution, emulsified in Freund's complete adjuvant for the primary immunization and in Freund's incomplete adjuvant for the other five immunizations, at 15-day intervals. Mouse 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.

Indirect immunofluorescence assay. C6/36 cells (5×10^4) were plated in 16-well plates (Lab-Tek), and indirect immunofluorescence assays were performed as described by Meerovitch et al. (31). The assays were done in the presence or absence of Tween 20.

Western blot assay. C6/36 cell proteins were subjected to SDS-PAGE and transferred as described above. Membranes were blocked at room temperature for 1 h 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 protein serum and a monoclonal antibody against dengue type 4 virus E protein (1H10) were diluted 1:1,000 and 1:250 in PBS, respectively, and incubated overnight at 4°C. The second antibody, anti-mouse IgG conjugated to alkaline phosphatase, was diluted 1:4,000 in PBS and incubated at room temperature for 1 h. Color was developed with BCIP and NBT, and the reaction was stopped after 1 h with water.

RESULTS

Binding of dengue type 4 virus to C6/36 cells. To determine the importance of receptor-ligand interactions for the attachment of dengue virus to C6/36 cells, a series of virus-binding experiments was performed. Initially, a constant number of cells was incubated with different amounts of 35 S-labeled dengue virus (Fig. 1A). Bound virus and free virus were quantified in triplicate, and counts were averaged. The data show that under isotonic conditions, C6/36 cells bound 12 to 13% of the total input counts per minute. The counts of cell-bound virus increased proportionally with viral input to a plateau, indicating saturation of cellular binding sites. Moreover, the data show that the binding of dengue virus to C6/36 cells was dose dependent and saturable.

To determine the specificity of cell-virus binding, competition experiments using unlabeled dengue virus were performed. Different amounts of unlabeled dengue virus were preincubated with C6/36 cells, followed by the addition of labeled virus. After incubation with 280 μ g of unlabeled dengue virus, a 90% reduction of specific binding was observed (Fig. 1B), suggesting that the interaction between dengue virus and C6/36 cells is specific.

Identification of dengue virus-binding proteins on cells. To determine the molecules on C6/36 cells which bind to dengue

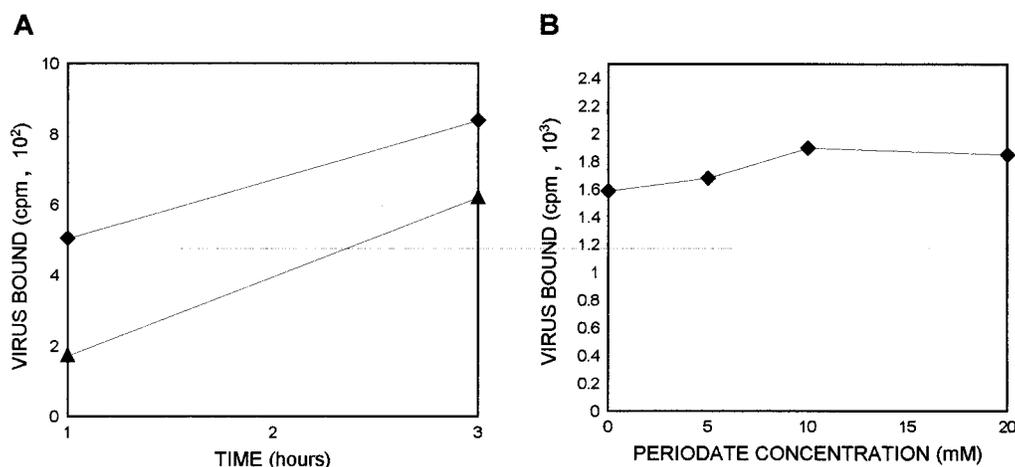


FIG. 3. Binding of ^{35}S -labeled dengue type 4 virus to the surface of C6/36 cells. (A) C6/36 cells nontreated (◆) and treated with trypsin for 30 min (▲) were incubated for up to 3 h with labeled dengue virus. (B) C6/36 cells treated with different concentrations of sodium periodate were incubated with 5×10^3 cpm of labeled dengue virus.

virus, [^{35}S]methionine-labeled viruses were incubated with proteins subjected to SDS-PAGE and transferred to nitrocellulose membranes. Under isotonic conditions, labeled dengue virus bound to two molecules of approximately 40 and 45 kDa present in cell lysates from C6/36 cells (Fig. 2A, lane 1) and to other proteins with lower molecular masses. When labeled proteins from uninfected cells were incubated with transferred proteins from C6/36 cells, no reaction was observed (Fig. 2A, lane 2). Similar bands of 40 and 45 kDa were detected when the binding assay was performed with membrane proteins (Fig. 2B). The presence (Fig. 2B, lanes 1 and 3) or absence (Fig. 2B, lanes 2 and 4) of β -mercaptoethanol did not alter the binding pattern. More stringent conditions with 220 mM NaCl (Fig. 2B, lanes 3 and 4) reduced the nonspecific binding observed with lower-molecular-weight proteins. These results suggest that the 40- and 45-kDa membrane proteins, which do not appear to be disulfide-linked subunits, bind specifically to dengue virus.

To corroborate that the E protein from dengue virus binds to the 40- and 45-kDa molecules, a VOPBA-Western assay was performed. Membrane proteins (Fig. 2C, lanes 1, 1', 3, and 3') and electroeluted 40- and 45-kDa proteins (Fig. 2C, lanes 2, 2', 4, and 4') were incubated with labeled proteins from uninfected cells (Fig. 2C, lanes 1, 1', 2, and 2') or with labeled dengue virus (Fig. 2C, lanes 3, 3', 4, and 4'). The bands of 40 and 45 kDa were detected only after incubation with labeled dengue virus (Fig. 2C, lanes 3 and 4), as is observed in Fig. 2A and B. When a monoclonal antibody against dengue type 4 virus E protein (1H10) was used, the 40- and 45-kDa proteins were revealed (Fig. 2C, lanes 3' and 4'), suggesting that E protein binds the two molecules. No bands were detected in the presence of labeled proteins from uninfected cells (Fig. 2C, lanes 1, 1', 2, and 2').

To demonstrate the specificity of the binding, a cross-linking assay was performed. ^{35}S -labeled membrane proteins and labeled 40- and 45-kDa proteins purified by electroelution were incubated and cross-linked to unlabeled dengue virus. Under these conditions, a 45-kDa protein present in the membrane fraction cross-linked to unlabeled dengue virus (Fig. 2D, lane 1), and 45- and 50-kDa proteins from the electroeluted fraction were observed cross-linked to unlabeled virus (Fig. 2D, lane 2). No bands were detected in the absence of unlabeled

dengue virus (Fig. 2D, lanes 4 and 5) or in the absence of labeled cell proteins (Fig. 2D, lane 3).

Characterization of dengue virus-binding proteins. To initially characterize these molecules, binding assays were performed with preincubation of C6/36 cells with trypsin and sodium periodate. Trypsin digestion for 30 min reduced the binding by 50% after 1 h of incubation with labeled viruses (Fig. 3A), indicating a peptidic nature of the binding molecules. However, after 3 h of incubation, binding was reduced by only 20%, suggesting an active replacement of the binding proteins on the cell membrane.

To analyze the possible role of carbohydrates in dengue virus binding, C6/36 cells were treated with sodium periodate. However, no inhibition of binding was observed (Fig. 3B), suggesting that carbohydrates are not essential for virus binding.

The analysis of the binding molecules was continued with overlay assays, using total proteins obtained after cell treatment with trypsin, neuraminidase, and periodate. No bands were detected when cells were treated with trypsin (Fig. 4A, lane 3), but a doublet of 40- and 45-kDa proteins was observed after neuraminidase treatment (Fig. 4A, lane 2). When radio-labeled virus was incubated with cell proteins obtained after sodium periodate treatment, a single band of 38 kDa was revealed (Fig. 4A, lane 1), suggesting that the 40- and 45-kDa proteins are glycoproteins, although the carbohydrates are not essential for virus binding. To support the hypothesis that the 40- and 45-kDa proteins are glycoproteins, membrane cell proteins and electroeluted 40- and 45-kDa molecules were incubated with agglutinin I (Fig. 4B). Agglutinin I reacted with the 40- and 45-kDa proteins present in the electroeluted fraction and in membranes (Fig. 4B, lanes 1 and 2, respectively), suggesting the presence of β -galactose in these proteins.

Specificity of virus binding. The specificity of the virus interaction with the 40- and 45-kDa proteins was demonstrated by competition assays with unlabeled dengue virus and poliovirus. The overlay mixture was incubated with 280 or 560 μg of unlabeled dengue virus or with 280 μg of poliovirus (Fig. 5, lanes 2, 3, and 4, respectively) before incubation with labeled dengue virus. Labeled virus binding was competed out when dengue virus but not when poliovirus was used as a competitor,

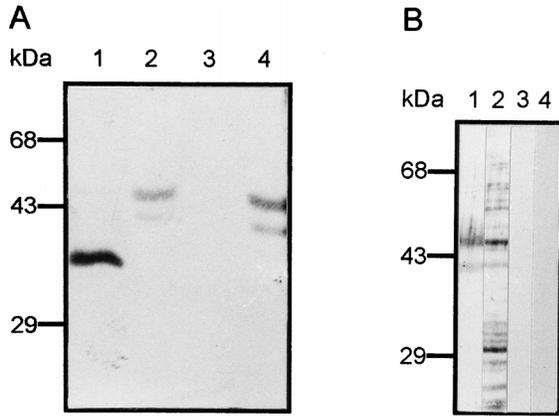


FIG. 4. Characterization of the binding molecule by VOPBA with [³⁵S]methionine-labeled dengue virus. (A) Total proteins obtained from C6/36 cells untreated (lane 4) or treated with sodium periodate (lane 1), neuraminidase (lane 2), or trypsin (lane 3) were subjected to SDS-10% PAGE, transferred to a nitrocellulose membrane, and incubated with 2 × 10⁶ cpm of labeled dengue virus. (B) Electroeluted 40- and 45-kDa proteins (lanes 1 and 3) and cell membrane proteins (lanes 2 and 4) were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and incubated in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of biotinylated agglutinin I and streptavidin coupled to alkaline phosphatase.

indicating that the interaction between dengue virus and the 40- and 45-kDa proteins is specific.

To further analyze the specificity of the interaction between the 40- and 45-kDa proteins and dengue virus, a binding assay with preincubation of labeled dengue virus with the electroeluted 40- and 45-kDa proteins was performed. Preincubation of dengue virus with both proteins prevented by 90% the binding of labeled dengue virus to the C6/36 cells (Fig. 6).

Antibodies against the 40- and 45-kDa proteins block dengue virus binding. Polyclonal antibodies against the 40- and 45-kDa proteins obtained after immunization of mice with the electroeluted proteins were able to detect mainly a 45-kDa protein and to a lesser extent 40-, 32-, 28-, and 20-kDa proteins in Western blot assays (Fig. 7A, lane 1), while no bands were observed after incubation with preimmune serum (Fig. 7A, lane 2). To determine if the antibodies recognized the same proteins that bound dengue type 4 virus, a binding assay was

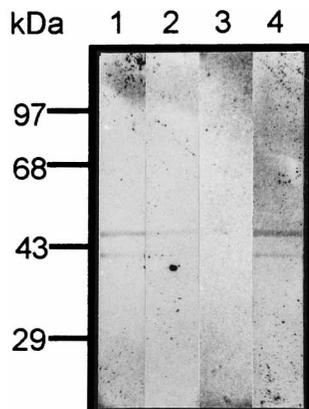


FIG. 5. Total proteins from C6/36 cells subjected to SDS-10% PAGE were preincubated in the absence of virus (lane 1) or in the presence of 280 or 560 µg of unlabeled dengue virus (lanes 2 and 3, respectively) or 280 µg of poliovirus (lane 3) prior to incubation with 3.5 × 10⁵ cpm of labeled dengue type 4 virus.

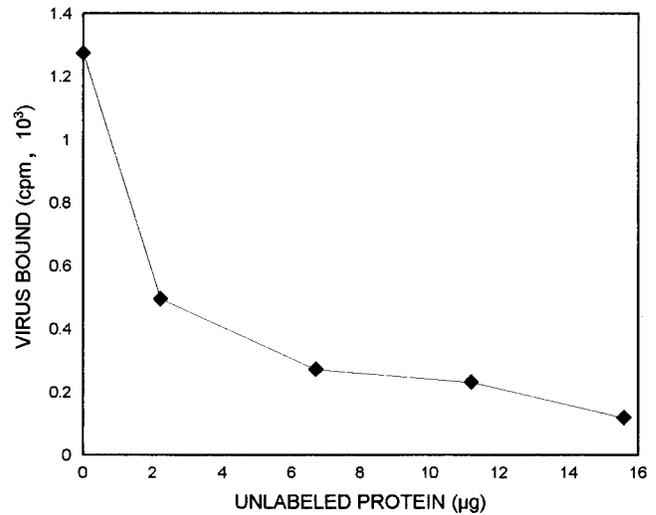


FIG. 6. Competition experiment with unlabeled 40- and 45-kDa proteins. Labeled dengue type 4 virus was preincubated with different amounts of electroeluted 40- and 45-kDa proteins prior to the incubation with C6/36 cells. After virus-cell interactions, the counts per minute for bound and free virus were determined.

performed. IgG against the 40- and 45-kDa proteins blocked by 85% the binding of labeled virus to C6/36 cells, while pre-immune IgG did not alter virus binding (Fig. 7B). A virus binding inhibition similar to that observed with the polyclonal antibodies was also detected after preincubation with an excess of 110 µg of unlabeled dengue virus (Fig. 7B).

Localization of the 40- and 45-kDa proteins on C6/36 cells. The localization of the 40- and 45-kDa proteins on C6/36 cells was analyzed by immunofluorescence. Polyclonal antibodies against both proteins were able to react with the surface of nonpermeabilized C6/36 cells (Fig. 8A) and, to a lesser extent, with the cytoplasm of permeabilized C6/36 cells (Fig. 8B),

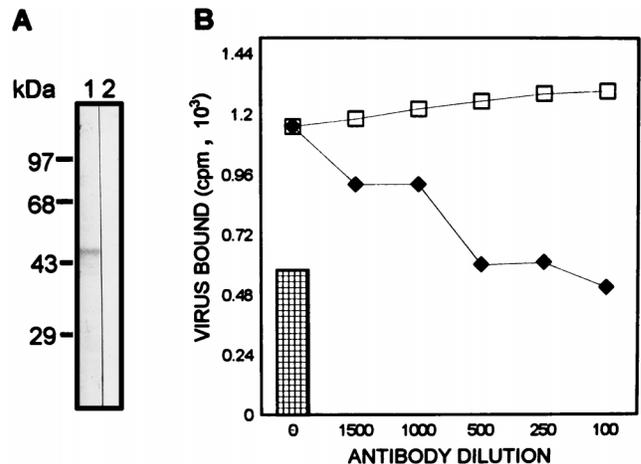


FIG. 7. Binding inhibition induced by polyclonal antibodies against the 40- and 45-kDa proteins. (A) Western blot assay of total proteins from C6/36 cells with the 40- and 45-kDa protein immune (lane 1) and preimmune (lane 2) serum. (B) Binding inhibition induced by the presence of antibodies to 40- and 45-kDa proteins. C6/36 cells were preincubated in the presence of different dilutions of immune (◆) or preimmune (□) IgG against the 40- and 45-kDa proteins at 4°C for 2 h prior to incubation with labeled dengue virus. Bound and free counts per minute were determined. The bar shows the binding inhibition observed after preincubation with 100 µg of unlabeled dengue type 4 virus.

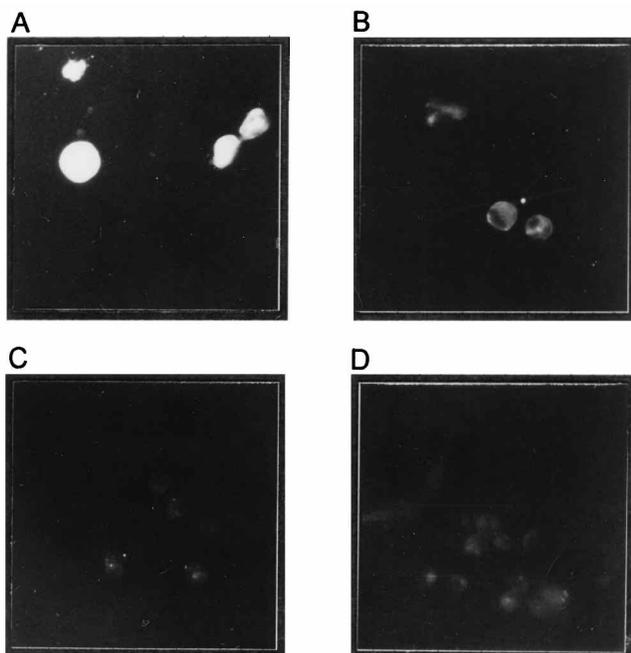


FIG. 8. Immunofluorescence assay with C6/36 cells. Nonpermeabilized (A and C) and permeabilized (B and D) C6/36 cells were incubated with immune (A and B) or preimmune (C and D) serum against the 40- and 45-kDa proteins. As a second antibody, goat anti-mouse IgG coupled to fluorescein was used.

while preimmune serum was negative with Tween 20-treated or nontreated cells (Fig. 8D and C, respectively). This result indicates that the 40- and 45-kDa proteins are located on the surface of C6/36 cells and thus could function as a receptor for dengue virus.

DISCUSSION

The basis of the cell and tissue tropisms of viruses is often related to the ability of the VAP to bind a specific viral receptor. Viral binding is followed by membrane fusion and leads to productive infection in suitable hosts. The envelope protein (E) is the major structural protein exposed on the surface of dengue viruses and has been identified as the VAP (1, 5). An internal domain of the E protein, located between amino acids 281 and 423, contains the binding motif, although its counterpart, the cellular receptor, remains unknown (5). The identification of the receptor is required to understand tissue tropism, pathogenesis, and viral replication in the hosts. To partially characterize the putative dengue virus receptor, we used a mosquito cell line, C6/36, derived from the larval stage of *A. albopictus*, which is susceptible to dengue virus infection.

Our results show that dengue type 4 virus specifically binds to C6/36 cells in a dose-dependent and saturable manner, which is characteristic of specific receptor-ligand interactions, suggesting the presence of a specific receptor for dengue virus on the surface of C6/36 cells. This has also been described for dengue type 1 virus on Vero and HepG2 cell lines (30). Trypsin treatment of the cells inhibited virus binding, while sodium periodate and neuraminidase treatment did not. Protease susceptibility and neuraminidase resistance have also been reported when dengue virus was used in binding assays with Vero, HepG2, and K562 cells and monocytes (8, 29, 30).

These results were supported by overlay assays, where we could demonstrate the binding of labeled dengue type 4 virus

to two molecules of 40 and 45 kDa present in the membrane fraction of C6/36 cells. The susceptibility of the binding molecules to protease treatment was confirmed in these assays, where trypsin treatment prevented the recognition of the 40- and 45-kDa doublet by labeled dengue virus. When C6/36 cells were treated with sodium periodate, dengue virus binding was not altered, but the molecular weights of the polypeptides bound to the labeled virus were modified. This result suggests that the carbohydrate moieties of the binding molecules are not essential for viral attachment. The fact that agglutinin I reacted with both proteins supports the hypothesis that the 40- and 45-kDa proteins are glycoproteins. The presence of a 38-kDa protein, instead of the 40- and 45-kDa proteins, in the overlay assay could be explained if both proteins correspond to glycosylated forms of the 38-kDa protein. Further analysis of the 38-kDa protein and its relation to the 40- and 45-kDa proteins is currently being performed in our laboratory.

Virus-cell interaction was resistant to neuraminidase treatment in binding (data not shown) and overlay assays, suggesting either that sialic acid is not essential for virus binding or that the 40- and 45-kDa proteins do not contain sialic acid. In support of the absence of sialic acid in the 40- and 45-kDa proteins, the lack of a sialyl transferase activity in a cell line from *A. albopictus* has been reported (36, 38, 40). Although this analysis was performed with a different cell line, both lines were obtained from the same insect, and it has been suggested that all insect cell proteins lack sialic acid (40). In addition, resistance of other viral receptors to neuraminidase treatment has also been reported for the minor group receptor of human rhinovirus, where neuraminidase treatment increases rhinovirus binding, suggesting that sialic acid is not required for virus binding (32).

Dengue virus cross-linked with a 45-kDa membrane protein, while 45- and 50-kDa proteins were cross-linked when labeled electroeluted 40- and 45-kDa proteins were used. The 40-kDa protein detected when an overlay assay was performed with membrane proteins was not detected by the cross-linking assay. This could be explained in two different ways. First, even though in the overlay assays proteins of 40 and 45 kDa were able to bind dengue virus, the native foldings of the two proteins are different, with the conformation of the 45-kDa protein being more appropriate for viral attachment. Second, although both proteins are present in a membrane fraction, it is possible that only the 45-kDa protein is exposed on the surface of C6/36 cells. The 50-kDa protein, which was observed when electroeluted 40- and 45-kDa proteins were used in the cross-linking assay, could correspond to a contaminant of the electroeluted 40- and 45-kDa proteins, and its presence in the cross-linked proteins has two explanations. First, this may be a cytoplasmic protein that binds to dengue virus, since it was not detected in our membrane fraction. Second, since DSP cross-links interacting molecules, it is possible that the 50-kDa protein binds to the 45-kDa protein and not to the virus. In support of the second idea, the 50-kDa protein present in total cytoplasmic extracts was not detected in overlay assays (Fig. 2A), suggesting that this protein does not bind directly to dengue virus.

The 40- and 45-kDa proteins do not appear to be disulfide-linked subunits, since in the presence or absence of β -mercaptoethanol both protein bands showed the same molecular weight in VOPBA. Moreover, dengue virus binding does not require a folding dependent on disulfide bridges.

The immunofluorescence assays indicate that 40- and/or 45-kDa proteins are localized on the surface of C6/36 cells. This result correlates with the presence of both proteins in a membrane fraction obtained by Triton X-114 extraction and with

their susceptibility to trypsin and sodium periodate treatments, which were performed with intact cells and not with cell extracts. Monoclonal antibodies raised against each of the proteins are being currently prepared in our laboratory to determine their differential localizations in the cell.

The presence of the 40- and 45-kDa proteins on the surface of the C6/36 cells and the fact that both electroeluted proteins and antibodies against them inhibit dengue virus binding strongly support the idea that the 40- and 45-kDa proteins are putative receptors or part of a receptor complex for dengue virus.

We are aiming our efforts at the complete characterization of the DNA and amino acid sequences of both proteins, to confirm that one or both molecules are indeed the dengue virus receptor. If the 40- and 45-kDa proteins are receptors for dengue virus, it would be of interest to determine their distributions in some susceptible and resistant mosquito tissues (37).

ACKNOWLEDGMENTS

We thank Fernando Medina for the cell cultures and Salvador Charvarría for technical assistance. We also thank Goro Kuno for the C6/36 cell line, David Vaughn for the 1H10 monoclonal antibody against dengue type 4 virus E protein, and Lorena Gutiérrez and Martha Espinosa-Cantellano for critical comments on the manuscript.

This work was supported by a grant from Consejo Nacional de Ciencia y Tecnología. Juan Salas has a scholarship from Consejo Nacional de Ciencia y Tecnología.

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