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Serotype-Specific Entry of Dengue Virus into Liver Cells: Identification of the 37-Kilodalton/67-Kilodalton High-Affinity Laminin Receptor as a Dengue Virus Serotype 1 Receptor

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Dengue virus, the causative agent of dengue fever, dengue shock syndrome, and dengue hemorrhagic fever, infects susceptible cells by initially binding to a receptor(s) located on the host cell surface. Evidence to date suggests that receptor usage may be cell and serotype specific, and this study sought to identify dengue virus serotype 1 binding proteins on the surface of liver cells, a known target organ. By using a virus overlay protein binding assay (VOPBA), in both nondenaturing and denaturing gel systems, a putative dengue virus serotype 1 binding protein of approximately 37 kDa expressed on the surface of liver (HepG2) cells was identified. Mass spectrometry analysis identified a candidate protein, the 37/67-kDa high-affinity laminin receptor. Entry of the dengue virus serotype 1 was significantly inhibited in a dose-dependent manner by both antibodies directed against the 37/67-kDa high-affinity laminin receptor and soluble laminin. No inhibition of virus entry was seen with dengue virus serotypes 2, 3, or 4, demonstrating that the 37/67-kDa high-affinity laminin receptor is a serotype-specific receptor for dengue virus entry into liver cells.

Dengue virus is a mosquito-borne single-stranded RNA virus that belongs to the genus *Flavivirus*, a genus that includes many important human-pathogenic viruses such as yellow fever virus, tick-borne virus, and several encephalitis viruses (48). Dengue virus comprises four antigenically related serotypes, i.e., serotypes 1, 2, 3, and 4, and infection by any one of the four serotypes can cause either a relatively benign fever (dengue fever) or a more serious disease such as dengue hemorrhagic fever (DHF) or dengue shock syndrome (33). The disease has emerged worldwide throughout tropical and subtropical regions, which are the habitat of the principal transmission vector, *Aedes* mosquitoes (45), and dengue has emerged as a critical globally endemic disease. The primary vectors of dengue virus transmission include *Aedes aegypti*, *A. albopictus*, and *A. polynesiensis* (38).

To infect target cells, dengue virus utilizes its envelope glycoprotein, which contains the component responsible for target cell binding and fusion (27) and for interacting with the receptor(s) on the target cell. Following the penetration step, internalization can occur by either endocytosis or direct fusion (9). In primary dengue virus infection, dengue virus can enter the target cells after the envelope protein attaches to an ill-characterized receptor(s) on target cell surface. In secondary infections, the virus may enter cells through the primary receptor(s), although it may also form immune complexes with preexisting nonneutralizing antibodies and interact with an alternative receptor, such as the immunoglobulin G (IgG) receptor ($Fc_{\gamma}R$), in a process termed antibody-dependent enhancement of infection (24). By this process, the antibody-virus complexes may increase the ability of the virus to bind to and internalize into cells, leading to maximum productive infection. Host molecules involved in virus entry share some properties, such as an ability to bind with high affinity to the virus and a location on the target cell surface (32). Collectively, the results support the notion that dengue virus utilizes multiple cell surface molecules for binding to and infection of target cells, although some receptors may be common to all cells and may be shared among several virus serotypes (4). Table 1 shows putative receptor molecules on various cell types including cells of both human and nonhuman origin.

Instead of monocytes and macrophages, which have long been thought to be the primary target cells, immature monocyte-derived dendritic cells, have recently been identified as the preferential primary target for dengue virus infections (37, 49). Once the dengue virus in infected mosquitoes is transmitted to the patient, the virus utilizes the specific antigen capture receptor lectin, ICAM3-grabbing nonintegrin (DC-SIGN), as a binding site to facilitate entry into the dendritic cells (34). Other tissues from which the virus has been isolated include the liver, which seems to be one of the major target organs of dengue virus infection capable of supporting dengue viral replication, and several lines of evidence have shown that dengue virus directly invades and replicates in the liver (16). Hepatomegaly and liver dysfunction have a higher incidence in DHF patients than in dengue fever patients, suggesting that liver dysfunction may be related to DHF severity (46). Furthermore, from investigations of liver histopathology in fatal cases of dengue virus infection, the dengue viral antigen could be detected in hepatocytes (21). In addition Kupffer cells had mostly been destroyed, suggesting that both hepatocytes and Kupffer cells may be target cells supporting dengue viral replication (21). The unusual increase of alanine aminotransferase (ALT)

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TABLE 1. Characterized and partially characterized dengue virus receptor proteins

Cell type	Species	Serotype	Receptor characteristics	Reference(s)
Vero	Kidney (simian)	D1	Protein	31
HepG2	Hepatocyte (human)	D1	Protein	31
Macrophage	Human	D2	Proteins (27, 45, 67, 87 kDa)	33
CHO	Ovary (hamster)	D2	Glycosaminoglycan	10,17
BHK	Kidney (hamster)	D2	Glycosaminoglycan	23
Vero	Kidney (simian)	D2	Glycosaminoglycan	10,17
Monocytes	Human	D2	FcR, protein	11,14
Monocytes	Human	D2	CD14-associated molecule	12
HepG2	Hepatocyte (human)	D2	GRP78	25
K562	Bone marrow (human)	D2	Protein (100kDa)	39
HL60	Myelomonocyte (human)	D2, D3	Proteins (~40, ~70kDa), GAGs[a]	3
Raji	B cell (human)	D2, D3	Proteins (~34, ~45, ~72 kDa)	4
Molt4	T cell (human)	D2, D3	Proteins (~34, ~45, ~72kDa)	4
LK63	B cell (human)	D2, D3	Proteins (~34, ~45, ~72kDa)	4
C6/36	Whole larva (mosquito)	D4	Proteins (40, 45 kDa)	40
Vero	Kidney (simian)	D4	Proteins (44, 74 kDa)	32
Dendritic cells	Monocyte derived (human)	D1-4	ICAM3-grabbing nonintegrin	34

[a] GAG, glycosaminoglycans.

and aspartate aminotransferase (AST) levels in DHF patients is also evidence suggesting an abnormality of hepatocyte cells and liver dysfunction (28, 35).

Despite the liver being reported as a target organ of dengue virus infection, especially in severe and fatal cases, there is little information on the interaction between dengue virus and liver cells, especially with regard to the nature of the moieties facilitating the entry of the virus. Heparan sulfate, the ubiquitous glycosminoglycan which serves as a host cell binding site for numerous pathogenic agents, appears to be involved in dengue virus entry into liver cells, although the contribution of heparan sulfate to internalization of dengue virus appears to vary in a serotype-specific manner (29, 44). More recently, a dengue virus serotype 2 receptor protein expressed on liver cells has been identified, although evidence would suggest that this is not the major receptor protein for this serotype (25). For other dengue virus serotypes, only an ill-characterized proteineous molecule has been described as a binding molecule for dengue virus serotype 1 on HepG2 cells (31). Therefore, this study sought to isolate and characterize a specific binding molecule for dengue virus serotype 1 on a human hepatoma cell line, one of the natural target cells. In addition, the involvement of heparan sulfate in dengue virus serotype 1 entry was demonstrated and the issue of the serotype specificity of dengue virus binding to hepatoma cells was investigated.

MATERIALS AND METHODS

Cells and viruses. The African green monkey cell line, Vero, was cultivated at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, Utah) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Gibco BRL, Gaitherburg, Md.) and 100 U of penicillin-streptomycin (HyClone) per ml. The human hepatoma cell line HepG2 was cultivated as for Vero cells, except that the medium was supplemented with 10% FBS (Gibco BRL) and the cells were incubated under 10% CO_2. Dengue virus serotype 1 strain 16007 (accession no. AF180817) (20), dengue virus serotype 2 strain 16681 (accession no. U87411) (26), dengue virus serotype 3 strain 16562 (accession no. U11673) (2), and dengue virus serotype 4 strain 1036 (courtesy of Siritorn Butrapet) were propagated in Vero cells. The virus was either partially purified by centrifugation and stored frozen at −80°C or purified through sucrose step gradients as described previously (42).

Enzymatic treatment of HepG2 cells. Pretreatment of HepG2 cells with trypsin prior to infection with dengue virus serotype 1 at a multiplicity of infection of 1 was undertaken as previously described (44), except that the medium was sampled at 24 h postinfection as opposed to 3 days postinfection. A total of 1.08×10^6 HepG2 cells were infected per reaction. Control infections were undertaken identically, except that cells were grown to subconfluence directly in 60-mm culture dishes and infected when the number reached 1.08×10^6. The experiment was undertaken independently in triplicate, with a duplicate assay of the viral titer. For pretreatment of HepG2 cells with both trypsin and heparinase III, HepG2 cells were cultured in 48-well plates at 37°C under 10% CO_2 in DMEM supplemented with 10% heat-inactivated FBS and 100 U of penicillin-streptomycin per ml. When the cell number in each well reached 5.0×10^5, the culture medium was discarded and the cells were washed with serum-free DMEM. Then the cells were treated with 0.1 U of heparinase III (Sigma Chemical Co., St. Louis, Mo.) at 37°C for 1 h. After being washed twice with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4), the cells were incubated with 100 μl of 0.25% trypsin (Gibco BRL) for 5 min and then DMEM supplemented with FBS was added to terminate the trypsinization reaction. The cells were then recovered by centrifugation at $1,200 \times g$ for 5 min and infected with dengue virus serotype 1 diluted with BA-1 (1× medium 199/Earle's balanced salts, 0.05 M Tris-HCl [pH 7.6], 1% bovine serum albumin fraction V, 0.075% $NaHCO_3$, 100 U of penicillin-streptomycin per ml) at a MOI of 1 for 90 min at 37°C. After the period of viral absorption, extracellular virus was inactivated by treating the cells with acid glycine (pH 3.0) (23). The cells were washed again with PBS, and fresh culture medium was added. The infected cells were grown further at 37°C under 10% CO_2, and the medium was assayed for the level of infectious virus after 24 h. The amount of infectious virus was subjected to two titer determinations on Vero cells, and the experiment was undertaken independently in triplicate.

Membrane protein preparation. Confluent HepG2 cells were detached by scraping with TBS buffer (50 mM Tris HCl [pH 7.6], 150 mM NaCl). Cell pellets were pelleted by centrifugation at $500 \times g$ for 3 min in an Eppendorf bench top centrifuge and subsequently resuspended in ice-cold modified buffer M (32) (100 mM NaCl, 20 mM Tris-HCl [pH 8], 2 mM $MgCl_2$, 1 mM EDTA, 0.2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride) and lysed by vigorous vortexing. Nuclei and debris were removed by centrifugation at $600 \times g$ for 3 min, and membranous organelles were pelleted from the supernatant by further centrifugation at $6,000 \times g$ for 5 min. Membrane proteins were pelleted by centrifugation at $20,800 \times g$ for 10 min and resuspended in modified buffer M containing 0.3% Triton X-100. The concentration of protein was quantified by the Bradford method (5).

Western blotting and viral overlay protein binding assay (VOPBA). Cell membrane proteins (60 to 100 μg) were subjected to electrophoresis through either a 10% native polyacrylamide gel or an 8% sodium dodecyl sulfate (SDS)–polyacrylamide gel and transferred to nitrocellulose membranes by using the Mini Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories, Richmond, Calif.) in transfer buffer (15.6 mM Tris base, 120 mM glycine). The membrane containing transferred proteins was blocked with 5% skim milk in TBS at room temperature for 1 h. For Western blotting, the membranes were incubated for 2 h at room temperature with a polyclonal antibody against the 37-kDa/67-kDa

high-affinity laminin receptor (sc-21534; Santa Cruz Biotechnology, Santa Cruz, Calif.) or a monoclonal antibody against actin (MAB1501; Chemicon International, Temecula, Calif.) at a dilution of 1:750 and 1:500 in 5% skim milk in TBS buffer, respectively. After incubation for 2 h at room temperature, membranes were incubated with horseradish peroxidase-conjugated rabbit anti-goat IgG (0031402; Pierce, Rockford, Ill.) or anti-mouse IgG (Sigma Chemical Co.) at a dilution of 1:3,000 in 5% skim milk in TBS buffer for 1 h at room temperature. Finally, the signal was developed using the ECL Plus Western blotting analysis kit (Amersham Pharmacia Biotech, Piscataway, N.J.). For viral overlay, the membranes were incubated with 10^7 PFU of dengue serotype 1 in 1% skim milk in TBS for 2 h at room temperature and washed three times with TBS buffer. Subsequently, the membranes were incubated with a panspecific anti-dengue virus monoclonal antibody produced by hybridoma cell line HB-114 (kindly provided by Siritorn Butrapet) at a dilution of 1:100 in 5% skim milk in TBS buffer. The viral binding band was visualized by incubation with a secondary horseradish peroxidase-conjugated rabbit anti-mouse IgG. The signal was developed as described above.

In-gel proteolysis. Selected bands were excised from gels run in parallel to the VOPBA gels and transferred to individual wells of a polypropylene 96-well plate (Nunc, Roskilde, Denmark). They were then subjected to in-gel proteolysis with trypsin. Briefly, each gel band was washed with 100 mM ammonium bicarbonate (Sigma Chemical Co.) in 50% acetonitrile (BDH, Poole, United Kingdom) for 30 min at room temperature to destain the protein. Protein disulfide bonds were then reduced with 10 mM dithiothreitol (Sigma Chemical Co.) in 100 mM ammonium bicarbonate at 56°C for 30 min followed by alkylation with 55 mM iodoacetamide (Sigma Chemical Co.) for 60 min at room temperature in the dark. The gel pieces were then washed to remove excess reagent with two successive washes each of 100 mM ammonium bicarbonate and 100% acetonitrile for 10 min at room temperature. Finally, the gel pieces were dehydrated with 100% acetonitrile for 10 min and dried completely in a Savant Speed-Vac (Thermo Instruments). Each gel piece was allowed to reswell in an approximate volume of 10 μl of trypsin (modified trypsin; Promega, Madison, Wis.) at a concentration of 10 ng/μl. After 30 min, the gel piece was covered with approximately 25 μl of incubation buffer (50 mM ammonium bicarbonate), and the plate was sealed and incubated at 37°C overnight. Following incubation, the liquid from each well was transferred to a clean 500-μl Eppendorf tube. The gel pieces were then extracted with 50 μl of 60% acetonitrile in 1% formic acid with sonication for 10 min. This solution was transferred and pooled with the first extract and finally concentrated to near dryness in a Speed-Vac. Each sample was then solubilized in 10 μl of 2% methanol in 1% formic acid. The sample was vortexed to aid solubility and centrifuged 15,000 rpm in a bench top centrifuge to settle the contents.

Liquid chromatography-mass spectrometry (MS). A volume of 5 μl of each sample was injected onto a nanoflow liquid chromatography system (nano-LC) coupled directly to a tandem mass spectrometer. The LC system (Ultimate LC-Packings, Amsterdam, Netherlands) was configured with a 300-μm by 5-mm C_{18} reversed-phase trapping cartridge, and the sample was loaded in 0.1% formic acid in high-performance liquid chromatography grade water (BDH) at a flow rate of 25 μl per min. The peptide pool was then washed on this cartridge for 5 min before the flow was switched to 130 nl per min and the peptides were eluted onto a reversed-phase column with a diameter of 75 μm and packed with 10 cm of C_{18} resin. A gradient was developed from 0.1% formic acid in 5% acetonitrile to 0.1% formic acid in 60% acetonitrile in 60 min at a flow rate of 130 nl per min. The elute was fed directly into a Qq-TOF hydbrid tandem mass spectrometer (QSTAR-Pulsar I; ABI-Sciex, Toronto, Canada), and the peptides were ionized by applying a voltage of 2.2 kV distally to the reversed-phase column using platinum wire held in a T-piece connected to the liquid flow. All doubly and triply charged ions greater than 8 counts were isolated and subjected to collisionally induced dissociation with helium as the collision gas. The daughter MS/MS spectra that were produced for each sample were searched against the MSDB nonredundant protein database (Imperial College, London, United Kingdom) using the MASCOT search engine (Matrix Science, London, United Kingdom). No constraint was set for species or for the molecular weight of the intact protein, but allowances for carbamidomethylation of cysteine residues and possible oxidation of methionine residues were made.

Infection inhibition assays. HepG2 cells were grown on six-well plates until the cells number reached 10^6; they were then incubated with a polyclonal antibody against the 37/67-kDa high-affinity laminin receptor or soluble laminin (basement membrane laminin, Engelbreth-Holm-Swarm murine sarcoma; L2020; Sigma Chemical Co.) or polyclonal antibodies against the N terminal of GRP78 (sc1050; Santa Cruz Biotechnology) at various concentration as indicated at 37°C for 1 h. After incubation, the cells were washed twice with PBS and then infected with dengue virus at a multiplicity of infection of 1 at 37°C for 90 min. After viral adsorption, the extracellular viruses were removed by washing with PBS followed by acid glycine treatment (23). The infected cells were washed again with PBS, and fresh growth medium was added. The infected cells were then incubated for a further 24 h, at which point growth medium was taken and assayed for the level of infectious virus by a standard plaque assay. Each sample was subjected to two titer determinations, and the experiment was undertaken three times independently.

Viral assays. Levels of extracellular viruses were determined by a standard plaque assay as described previously (41). Levels of intracellular viruses were determined by release from infected cells by one freeze-thaw cycle followed by 5 min of sonication at 4°C and assay by a standard plaque assay.

Immunofluorescence. A total of 10^5 HepG2 cells were grown on glass slides for 1 day. After being fixed with 1% formaldehyde for 15 min, the cells were incubated with 5 μg of polyclonal antibody against the 37/67-kDa high-affinity laminin receptor or soluble laminin (basement membrane laminin) or polyclonal antibody against the N terminal of GRP78 (sc1050) at 4°C for 1 h. After incubation, the cells were washed twice with PBS and then dengue virus serotype 1 was added at an MOI of 10 at 4°C for 60 min. The excess unbound viruses were removed by being washed twice with PBS, and the cells were incubated for 90 min at 4°C with a 1:10 dilution of panspecific anti-dengue virus monoclonal antibody produced by hybridoma cell line HB-114 (a kind gift of Siritorn Butrapet). The slides were washed twice with PBS and incubated for 30 min at 4°C with a 1:10 dilution of a goat anti-mouse IgG antibody labeled with fluorescein isothiocyanate (FITC) (KPL, Gaithersburg, Md.). After being washed twice with PBS, the slides were air dried and viewed under a fluorescence microscope.

RNA extraction, slot blot analysis, and Northern blot hybridization. Total RNA was extracted from either normal, infected, or mock-infected HepG2 cells grown on 10-cm tissue culture dishes by using TRI reagent (Molecular Research Center, Inc., Cincinnati, Ohio) as specified by the manufacturer. The final RNA pellet was briefly air dried and finally dissolved in diethylpyrocarbonate-treated distilled water. RNA purity and quantity were determined by measuring the absorbance at 260 and 280 nm.

For slot blot analysis, 10 μg of total RNA was applied to the slot blot vacuum manifold apparatus by using a positively charged nylon membrane (GeneScreen Plus; NEN Life Science Products, Inc. Boston, Mass.). Each well was rinsed with 100 μl of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and 10 μg of total RNA was added. Each well was rinsed again with 20× SSC. After the membrane was dried, the RNA was fixed by UV cross-linking. For Northern blot analysis, 20 μg of total RNA was mixed with an equal volume of formaldehyde gel-loading buffer (62.5 μl of formamide, 12.5 μl of 10× morpholinepropanesulfonic acid [MOPS], 20 μl of formaldehyde, 5 μl of ethidium bromide [10 mg/ml]), and 1/10 volume of loading dye (50% glycerol, 1 mM EDTA [pH 8.0], 0.4% bromophenol blue). The samples were incubated at 65°C for 5 min and quickly chilled on ice. After sample loading, electrophoresis was conducted at 100 volts for 60 min in 1× MOPS buffer. After the gel was destained with sterile water, the pattern of RNA was visualized under UV light and photographed. The RNA on the gel was then transferred to GeneScreen Plus, a positively charged nylon membrane, by an upward capillary transfer technique with 10× SSC buffer overnight. After the nylon membrane was rinsed with 2× SSC, RNA was then fixed to the membrane by UV cross-linking and the membrane was baked at 80°C. The membrane was prehybridized in prewarmed prehybridization solution (0.5 M sodium phosphate buffer [pH 6.8], 7% SDS, 1 mM EDTA, 15% formamide) containing 100 μg of denatured salmon sperm per ml at 65°C. After a 2-h prehybridization, prewarmed hybridization buffer containing denatured digoxigenin or ^{32}P-labeled probe was added in place of the prehybridization buffer and the membrane was hybridized overnight. The membrane was washed twice in 2× SSC–0.1% SDS for 15 min at room temperature and then twice in 1× SSC–0.1% SDS for 15 min at 65°C. After being washed, the membrane was placed in plastic wrap and exposed to X-ray film. Prior to reprobing, the membrane was incubated with shaking in boiling 0.1% SDS until the solution had cooled and then soaked in 2× SSC prior to prehybridization. For the slot blot analysis, the membrane was prehybridized at 42°C in hybridization solution (50% deionized formamide, 5× SSC, 0.1% *N*-lauroylsarcosine, 0.02% SDS) containing 100 μg of denatured salmon sperm per ml and then prewarmed hybridization solution containing the denatured digoxigenin-labeled probes was added. After overnight hybrization, the membrane was washed twice with 2× SSC–0.1% SDS for 15 min at room temperature and then twice with 1× SSC–0.1% SDS for 15 min at 68°C. Further detection was then performed as specified by the manufacturer. Briefly, the membrane was equilibrated in washing buffer (0.15 M NaCl, 0.1 M Tris-HCl [pH 7.5], 0.3% Tween 20) and then blocked for 60 min in washing buffer containing 2% skim milk as the blocking solution. The membrane was further incubated for 30 min with a 1:5000 dilution of anti-digoxigenin-AP Fab fragments (Roche Diagnostics GmbH, Mannheim, Germany) in blocking solution and given two

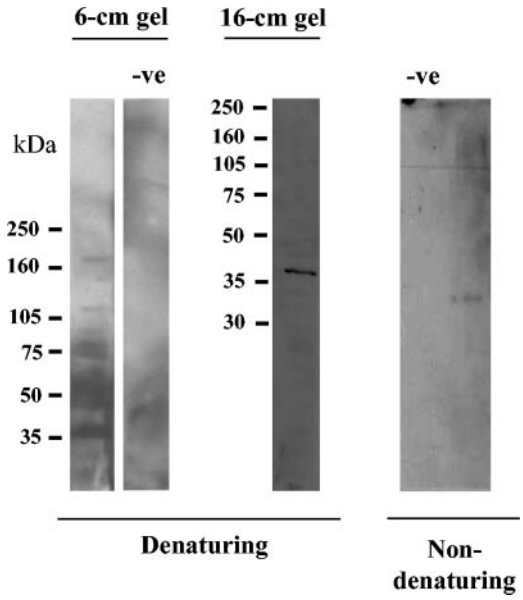

FIG. 1. VOPBA analysis of purified dengue virus serotype 1 on HepG2 cell membrane proteins. HepG2 cell membrane proteins were extracted and separated on denaturing and nondenaturing gels. The membrane proteins were transferred to nitrocellulose membranes and incubated with dengue virus serotype 1. Virus binding protein bands were detected via a panspecific monoclonal antibody directed against the dengue virus E protein. Control reactions were identical, except that no virus was included in the hybridization mix (−ve lanes).

washes for 15 min with washing buffer. It was then preincubated in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$) for 5 min and incubated with 1:100 CDP-star (Roche Diagnostics GmbH) in detection buffer for 5 min. The excess buffer was finally discarded, and the filter was placed on plastic wrap and exposed to X-ray film.

Probe preparation. Probes for both actin and the laminin receptor were prepared from oligo(dT)-primed cDNAs prepared from total RNA from HepG2 cells by PCR using specific primer pairs. The probes were labeled with either digoxyienin (Boehringer GmbH, Mannheim, Germany) or [α-^{32}P]dCTP (Amersham Biosciences, Piscataway, N.J.). The sequence of the primers used are 5′-GAAGATGACCCAGATCATGT-3′ and 5′-ATCTCTTGCTCGAAGTC CAG-3′ for actin and 5′-CACTAACCAGATCCAGGC-3′ and 5′-CCAGCATC CACCACATCA-3′ for the laminin receptor. Probe sizes were 330 bp (actin) and 213 bp (laminin receptor).

Statistical analysis. All data were analyzed using the GraphPad Prism program (GraphPad Software Inc., San Diego, Calif.).

RESULTS

Isolation and characterization of a dengue virus serotype 1 binding protein. To characterize dengue virus serotype 1 binding proteins expressed on the surface of HepG2 cells, 80 to 100 μg of HepG2 cell membrane protein was separated on a 10% native (nondenaturing) polyacrylamide gel and 8% denaturing (SDS) polyacrylamide gels (both 6- and 16-cm-long gels) and transferred to nitrocellulose membranes. The membranes were incubated with purified dengue virus serotype 1 and, to visualize the position of binding, subsequently with a panspecific antibody against dengue virus and a secondary anti-mouse IgG conjugated with horseradish peroxidase. As shown in Fig. 1, a single virus binding band was observed in the VOPBA result from the native (nondenaturing) gel while on the short (6-cm) SDS-polyacrylamide gel, several bands ranging between 35 and 180 kDa were observed (Fig. 1). However, when the denaturing VOPBA was repeated on a second, longer (16 cm) gel, a single prominent band of approximately 37 kDa was observed (Fig. 1), suggesting that some of the bands on the smaller gel result from the virus binding to two or more proteins with similar molecular masses as a complex. Increased separation on the gel possibly results in the inability of the binding complex to form. The virus binding protein bands from both native and denaturing (16-cm) gels were excised from identical parallel gels and subjected to in-gel proteolysis with trypsin. The resultant peptides were analyzed by a Qq-TOF hydbrid tandem mass spectrometer, and the daughter MS/MS spectra that were produced for each sample were searched against the MSDB nonredundant protein database using the MASCOT search engine. Among several matching proteins for each sample (Table 2), two candidates, the 37/67-kDa high-affinity laminin receptor and β-actin, showed significant matches to the spectra obtained in both gel electrophoresis systems. Both of these proteins have approximately similar molecular masses and pIs, explaining their comigration in both gel systems. Given that the 37/67-kDa high-affinity laminin receptor has been characterized as a receptor protein for other arthropod-borne enveloped RNA viruses (30, 47), the role of this protein in dengue virus serotype 1 entry into HepG2 cells was evaluated.

Inhibition of infection. HepG2 cells were preincubated with either various amounts of a polyclonal antibody directed against the 37/67-kDa high-affinity laminin receptor or no antibody (Fig. 2). In addition, HepG2 cells were incubated with

TABLE 2. Results of MS/MS spectra matches from the MSDB nonredundant protein database

Match[a]	Accession no.	Protein description	Mol mass (Da)	Score
1	ATPB_RAT	ATP synthase β chain, mitochondrial precurs	56,318	438
2	ATHUB	Human β-actin	41,710	318
3	S33438	Chinese hamster laminin receptor 34/67 kDa	32,881	184
4	ATHUB	Human β-actin	41,710	512
5	AAH00484	Ubiquinol-cytochrome *c* reductase core protein	48,413	409
6	Q96RS2	Laminin receptor-like protein LAMRL5	32,975	279
7	Q9BWD4	Interleukin enhancer binding factor 2	43,035	267
8	AAH07052	Similar to nuclear ribonuclear protein C	33,578	250

[a] Matches 1 to 3 are from the spectra obtained from the nondenaturing gel sample, while matches 4 to 8 are from the spectra obtained from the denaturing gel sample.

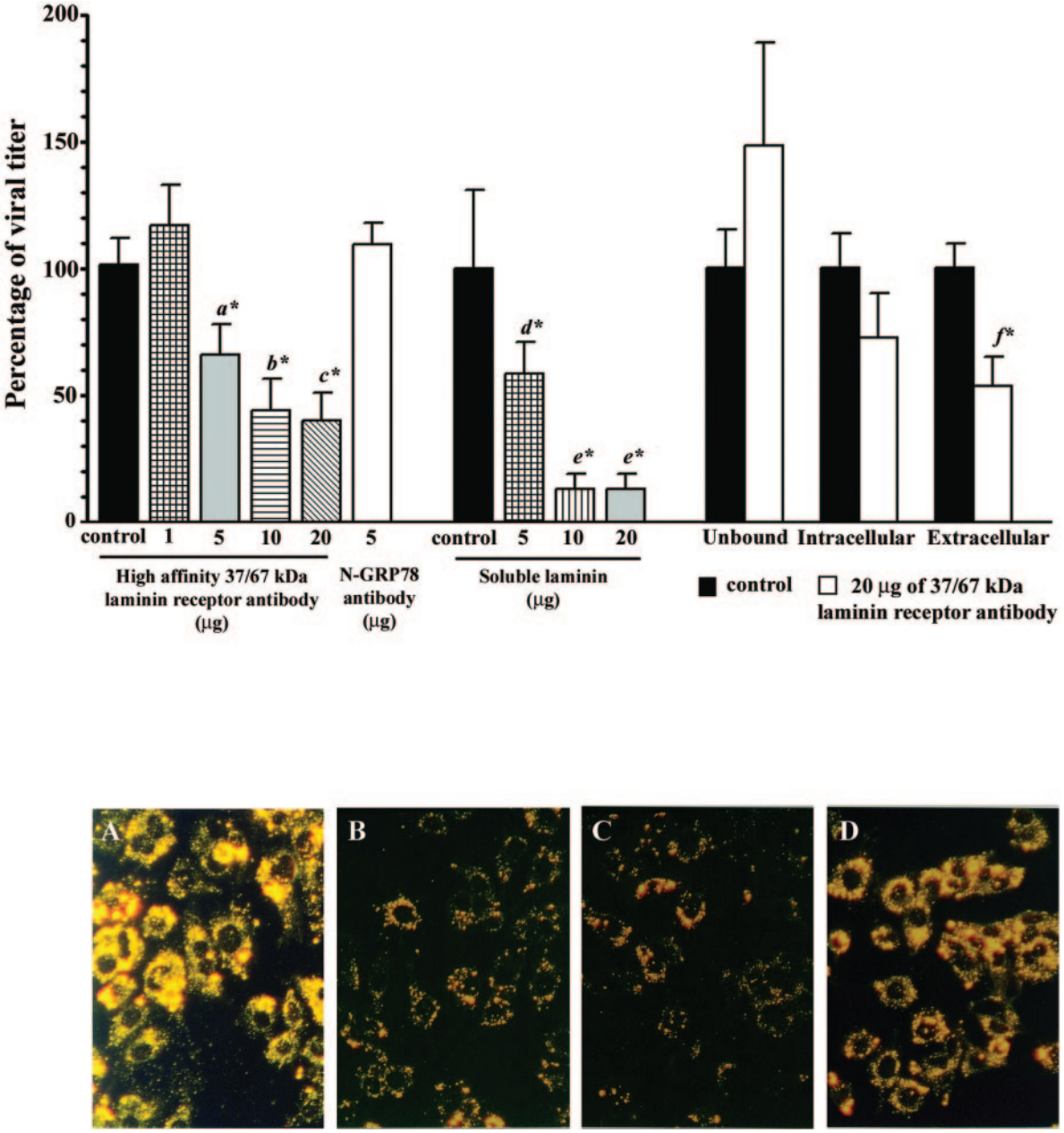

FIG. 2. Inhibition of dengue virus serotype 1 binding and infection of HepG2 cells. (Top) HepG2 cells were preincubated with either no antibody (control) or 1, 5, 10, or 20 μg of an anti-37/67-kDa laminin receptor antibody, 5 μg of an anti-N-terminal GRP78 antibody, or 0 (control) 5, 10, or 20 μg of soluble laminin prior to infection with dengue virus serotype 1 at an MOI of 1. Levels of viral production were assayed by a plaque assay after 1.5 viral replication cycles. Unbound (postinfection) and intracellular and extracellular (after 1.5 replication cycles) virus levels were also independently assayed at the highest level of antibody used (20 μg) in parallel with control (no-antibody) infections. Results are normalized against the level of virus produced with no antibody preincubation. Each experimental point is the sum of triplicate experiments with two assay of titer. Error bars represent standard errors of the mean. Asterisks represent a significant difference from the control (one-sample *t* test); a, $P = 0.0400$; b, $P = 0.0065$; c, $P = 0.0122$; d, $P = 0.045$; e, $P < 0.0001$; f, $P = 0.010$. (Bottom) HepG2 cells were pregrown on glass slides and preincubated either without antibody (A) or with antibodies directed against either the 37/67-kDa high-affinity laminin receptor (B) or the N terminus of GRP78 (D) or with soluble laminin (C) prior to infection with dengue virus serotype 1 at a MOI of 10. The binding of the dengue virus to HepG2 cells was visualized by subsequent and successive incubation with a panspecific monoclonal antibody directed against the dengue virus E protein and an FITC-conjugated anti-mouse monoclonal antibody. The slides were viewed under a fluorescence microscope. Magnification, ×200.

5 μg of a polyclonal antibody directed against the N terminal of GRP 78 (BiP), a protein previously shown to function as a dengue virus serotype 2 receptor expressed on the surface of HepG2 cells (25). The infected cells were grown for 24 h, at which point the growth medium was assayed for the levels of infectious virus by a standard plaque assay. The time of 24 h postinfection was selected since it is insufficient time for viral progeny produced from the initial infection to reinfect the cells

and produce a second round of viral progeny, and so virus levels in the medium reflect only events from the first round of infection (44). The experiment was undertaken independently in triplicate, with a duplicate assay of titers. The results (Fig. 2) show a significant dose-dependent inhibition of infection of HepG2 cells with dengue virus serotype 1 in the presence of antibodies against the 37/67-kDa high-affinity laminin receptor. At 20 μg of antibody, approximately 50% inhibition was observed.

To confirm the role of the 37/67-kDa high-affinity laminin receptor in the internalization process of dengue virus serotype 1, the experiment was repeated at the highest level of antibody inhibition assayed (20 μg of antibody per reaction), but this time the levels of free virus in the medium immediately prior to virus inactivation with acid glycine, as well as the levels of virus in the cell at 24 h, were also assayed. The results (Fig. 2) show a higher level of free (unbound to the cell surface) virus in the medium after incubation of the cells in the presence of antibodies directed against the 37/67-kDa high-affinity laminin receptor as opposed to control cells. Intracellular virus levels also show a difference between control (untreated) and treated (preincubated with anti-37/67-kDa high-affinity laminin receptor antibodies) cells, although the difference of only 25% may reflect a degree of reinfection of the cell by newly produced viruses from the first round of infection. The reduction of extracellular virus levels to 50% of control levels at 24 h was consistent with the earlier observation.

Given that antibodies against the 37/67-kDa high-affinity laminin receptor inhibited dengue virus internalization, we also sought to determine whether the ligand for this receptor was able to block dengue virus serotype 1 internalization. HepG2 cells were therefore incubated in the presence of increasing amounts of soluble laminin prior to infection with dengue virus serotype 1. The results (Fig. 2) show a concentration-dependent inhibition of infection in response to preincubation with soluble laminin compared to the situation for control (nonpretreated) cells. Approximately 90% inhibition was seen with 10 μg of laminin per reaction, and this appeared to be a saturated level since no further increase in inhibition was noted with a higher concentration of laminin.

To verify that incubation of HepG2 cells with either the anti-37/67-kDa antibody or laminin results in a specific reduction of virus binding, the binding of dengue virus to HepG2 cells was directly visualized by immunofluorescence. HepG2 cells were either incubated directly with dengue virus serotype 1 at an MOI of 10 or preincubated with either soluble laminin, a polyclonal antibody against the 37/67-kDa high-affinity laminin receptor, or a polyclonal antibody against the N terminal of GRP78 prior to incubation with dengue virus serotype 1 at the same MOI. Dengue virus binding was visualized by successive incubations with a panspecific anti-dengue virus monoclonal antibody and an FITC-labeled goat anti-mouse IgG. The results (Fig. 2) show a clear reduction of binding compared to control levels with both soluble laminin and the anti-37/67-kDa high-affinity laminin receptor antibody. Levels of binding of the dengue virus after preincubation with an antibody against GRP78, a previously identified dengue virus serotype 2 receptor element (25), were similar to control levels (Fig. 2).

Enzymatic treatment of HepG2 cells prior to dengue virus infection. To assess the relative contributions of protein and nonprotein moieties on the surface of HepG2 cells to the binding and internalization of dengue virus serotype 1, the ability of dengue virus serotype 1 to infect HepG2 cells that lacked extracellular proteins was assessed. HepG2 cells were treated with 0.25% trypsin for 5 min prior to infection with dengue virus serotype 1 in parallel with nontreated cells. After infection, the cells were incubated for 24 h, sufficient time to produce virus but not sufficient time for the virus to reinfect the cells and produce a further round of viral progeny (44), at which point the growth medium was sampled to determine the level of virus produced. Experiment was undertaken in triplicate with a duplicate assay of virus titer. The results (Fig. 3) shows that treatment with trypsin caused an approximately 50% reduction in the amount of virus produced. To further assess the contribution of extracellular elements to dengue virus serotype 1 internalization, cells were treated with a combination of heparinase III and trypsin to remove both extracellular proteins and heparan sulfate. Again, the levels of viral progeny were assessed at 24 h postinfection. The combined treatment of heparinase III and trypsin resulted in an approximately 90% reduction in virus titer (Fig. 3).

Given that pretreatment of HepG2 cells both with trypsin and with an antibody directed against the 37/67-kDa high-affinity laminin receptor resulted in an approximately 50% inhibition of dengue virus serotype 1 internalization, it is possible that the 37/67-kDa high-affinity laminin receptor is the primary protein element regulating the internalization of this

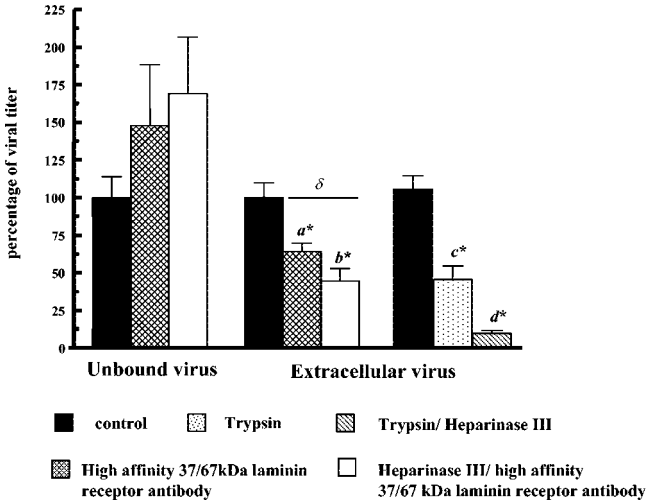

FIG. 3. Effects of enzyme and antibody-enzyme pretreatment on infection of HepG2 cells by dengue virus serotype 1. HepG2 cells were either untreated (controls) or treated with trypsin or a combination of trypsin and heparinase III or preincubated with an antibody against the 37/67-kDa high-affinity laminin receptor or treated with heparinase III and subsequently preincubated with an antibody against the 37/67-kDa high-affinity laminin receptor prior to infection with dengue virus serotype 1 at a MOI of 1. Levels of virus in the medium were assayed either immediately after the period of viral inoculation (unbound virus) or after 1.5 virus replication cycles (extracellular virus). Each experimental point is the sum of triplicate experiments with duplicate assay of titer. Error bars represent standard errors of the mean. Asterisks indicate a significant difference from the control (one-sample *t* test); a, $P = 0.0030$; b, $P = 0.0024$; c, $P = 0.0006$, d, $P < 0.0001$. δ indicates a significant difference between experimental groups ($P < 0.025$, one-sample *t* test).

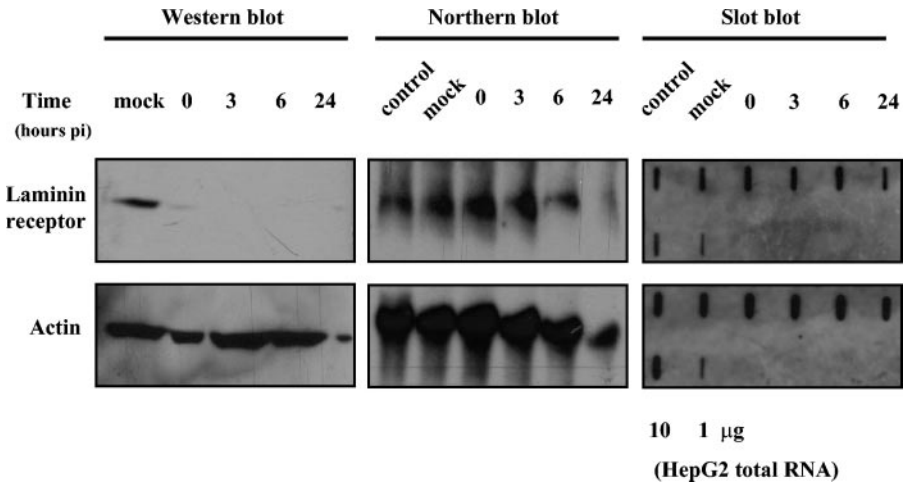

FIG. 4. Expression of the 37/67-kDa high-affinity laminin receptor on dengue virus serotype 1 infection. Cell membrane proteins or RNA were prepared separately from normal HepG2 cells (control), mock-infected HepG2 cells (mock), or dengue virus serotype 1-infected cells (MOI = 1) at the time points indicated. Membrane proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a solid matrix support before being probed sequentially with antibodies directed against the 37/67-kDa high-affinity laminin receptor and actin (Western blots). RNA samples were either slot blotted directly onto a solid matrix (slot blots) or separated by electrophoresis through formaldehyde-agarose gels before being transferred to a solid matrix (Northern blots). Slot blots included a control dilution series of total HepG2 RNA and were probed sequentially with digoxigenin-labeled probes against the 37/67-kDa high-affinity laminin receptor and actin. Northern blots were probed sequentially with α-^{32}P labeled probes against the 37/67-kDa high-affinity laminin receptor and actin.

virus. Prior results showed that treatment with both trypsin and heparinase III resulted in an approximately 90% inhibition of infection. To investigate the combination of the anti-37/67-kDa high-affinity laminin receptor antibodies as well as treatment with heparinase III, HepG2 cells were either incubated directly with 20 μg of antibody directed against the 37/67-kDa high-affinity laminin receptor or preincubated with heparinase III prior to incubation with the antibody. Control cells were untreated with heparinase III and were not preincubated with the antibody. Medium was assayed for viral titer either immediately after incubation with the dengue virus serotype 1 or after 24 h. The results (Fig. 3) show that a higher proportion of the virus remains unbound to the cells in the presence of the antibody, as noted above (Fig. 2), and that the amount of unbound virus increased still further after a combination of antibody plus pretreatment with heparinase III. After 24 h, the level of virus produced from the cells is significantly lower than the control level for both the antibody-treated and the combined antibody-treated plus heparinase III-treated cells. More significantly, the combined antibody plus heparinase III treatment showed a significant reduction ($P < 0.025$; one-sample t test) compared to incubation with the antibody alone.

Expression of the 37/67-kDa high-affinity laminin receptor on dengue virus serotype 1 infection. To investigate the level of expression of the 37/67-kDa high-affinity laminin receptor in response to infection with dengue serotype 1, HepG2 cells were infected with dengue virus serotype 1 at a MOI of 1. At 0, 3, 6, and 24 h postinfection, the infected cells were harvested and cell membrane proteins were extracted. The cell membrane proteins were separated on an SDS–10% polyacrylamide gel and transferred to a nitrocellulose membrane. The level of expression of the 37/67-kDa high-affinity laminin receptor was assessed by Western blot analysis by probing the filter with an anti-37/67-kDa laminin receptor antibody. The same nitrocellulose membrane was subsequently probed with an anti-actin antibody as a control. The results (Fig. 4) showed that the antibody detected a single protein species and demonstrate an immediate reduction in the level of expression of the 37/67-kDa high-affinity laminin receptor in response to infection with dengue virus serotype 1. The level of the 37/67-kDa high-affinity laminin receptor protein in the membrane fraction at time zero (after incubation of the virus and cells for 90 min to allow infection to occur) is undetectable, suggesting that the virus-receptor complex is completely internalized during the infection process. The level of expression of the 37/67-kDa high-affinity laminin receptor remained undetectable for the duration of the experiment, while the level of expression of actin remained constant.

To determine if the level of the 37/67-kDa high-affinity laminin receptor in the membrane fraction was controlled at the level of the mRNA or the protein, both a Northern analysis and a slot blot analysis were undertaken. Total RNA was extracted from control (uninfected), control (mock-infected), and dengue virus serotype 1-infected HepG2 cells at 0, 3, 6, and 24 h postinfection, as above. RNA was either separated by gel electrophoresis before being transferred to a solid matrix (Northern analysis) or bound directly to the solid matrix (slot blot) and hybridized to either digoxigenin-labeled (slot blot) or α-^{32}P-labeled (Northern analysis) probes for the 37/67-kDa high-affinity laminin receptor or actin. The results (Fig. 4) of both the Northern analysis and the slot blot analysis showed no variation of the 37/67-kDa high-affinity laminin receptor with respect to the levels of actin. In the Northern analysis, the signal for the 37/67-kDa high-affinity laminin receptor, as determined by densitometry, was approximately one-third of the actin signal in all lanes (mean arbitrary absorbance of the 37/67-kDa high-affinity laminin receptor as a fraction of actin, 32.78% ± 2.9% [standard deviation]). The slot blot analysis

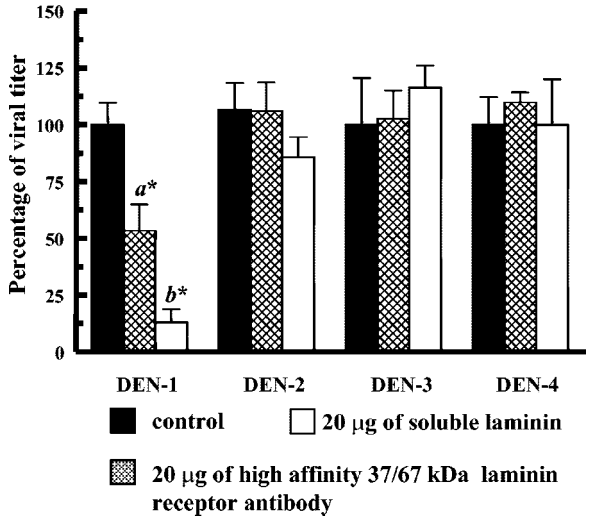

FIG. 5. Serotype specificity of the 37/67-kDa high-affinity laminin receptor. HepG2 cells were incubated with either no antibody or 20 μg of anti-37/67-kDa high-affinity laminin receptor antibody or 20 μg of soluble laminin prior to infection with all four serotypes of dengue virus individually. After an incubation of 1.5 viral replication cycles, the level of virus produced was assayed by plaque assay. Results are normalized against the level of individual serotypes of dengue virus produced with no antibody or ligand preincubation. Each experimental point is the sum of triplicate experiments with duplicate assays of titer. Error bars represent standard errors of the mean. Asterisks represent a significant difference from control (one-sample *t* test); a, $P = 0.0103$; b, $P < 0.0001$

was undertaken using the same samples as above; in addition, a dilution series of RNA from uninfected cells was undertaken to determine sensitivity of the assay. No variation in mRNA levels was seen (Fig. 4), suggesting that the steady-state pool of RNA encoding the 37/67-kDa high-affinity laminin receptor is not altered in response to dengue virus infection, at least over the course of the experiment undertaken.

Serotype specificity of the 37/67-kDa high-affinity laminin receptor. To investigate the dengue virus serotype specificity of the 37/67-kDa high-affinity laminin receptor, the inhibition experiment was repeated with either 20 μg of anti-37/67-kDa high-affinity laminin receptor antibody or 20 μg of soluble laminin for all four dengue virus serotypes. Growth medium was sampled at 24 h postinfection, and the experiment was repeated independently in triplicate, with duplicate assays of virus titer. The results (Fig. 5) show that the inhibitory effect of both the anti-37/67-kDa high-affinity laminin receptor antibody and soluble laminin is specific for dengue virus serotype 1.

DISCUSSION

The initial interaction between a cell and a virus is a critical determinant of viral tropism and thus of pathogenicity; therefore, considerable interest lies in determining the nature of the proteins used by viruses to enter cells. While infections with dengue virus present a significant worldwide problem, which represents a hazard to over two billion people (18), little progress has been made in identifying the extracellular proteins utilized by dengue virus to gain entry into cells. Indeed, to date only three receptor elements have been identified: DC-SIGN used by dengue virus to gain entry into monocyte-derived dendritic cells (34), the $Fc_{\gamma}R$ receptor used in cases of secondary infections to gain entry into monocytes (24), and, more recently, GRP78 used by dengue virus serotype 2 to gain entry into liver (HepG2) cells (25). In addition to these identified receptor elements, a host of poorly characterized proteins and molecules have been implicated in the initial dengue virus-host cell interaction (Table 1).

Using a combination of VOPBA and MS analysis, we have identified a fourth dengue virus receptor element, which is the second one used by the dengue virus to gain entry into liver cells. Perhaps most significantly, we have shown that the interaction between dengue virus and liver cells is serotype specific and that the protein identified in this study, the 37/67-kDa high-affinity laminin receptor protein, is used by dengue virus serotype 1 solely to gain entry into the liver cells studied. Perhaps as significantly, we see no evidence that GRP78, a protein identified as a dengue virus serotype 2 element (25), although probably not the major element, is utilized by dengue virus serotype 1. This precise serotype specificity is somewhat surprising, given the degree of homology between the dengue virus serotypes. However, although the envelope protein (E protein), which plays a role in receptor recognition, is highly conserved among four serotypes, domain III (amino acids 296 to 394), the direct host cell binding domain (13), shows high variation in amino acid sequences. Such variation might lead to the serotype specificity in host cell receptor recognition noted here.

The 37/67-kDa high-affinity laminin receptor is a nonintegrin cell surface receptor that mediates high-affinity interactions between cells and laminin (1) and whose expression has been observed in normal human liver cells as well as being up-regulated in hepatocyte carcinoma cells (36). The 37-kDa molecule is thought to be a precursor protein generating a mature 67-kDa laminin receptor, with the maturation process involving dimerization and acylation of the precursor (7). The 37/67-kDa high-affinity laminin receptor has been reported to serve as a binding protein facilitating the entry of several pathogenic agents including the prion protein (15) as well as some arthropod-borne enveloped RNA viruses (30, 47). In particular, Sindbis virus uses the 37/67-kDa high-affinity laminin receptor as a major receptor to enter baby hamster kidney cells (47), and the 32-kDa C6/36 cells membrane protein identified as the mosquito laminin receptor was found to be a putative receptor for Venezuelan equine encephalitis virus (30).

Dengue virus serotype 1 binding proteins expressed on the surface of the liver cell line HuH-7 have been previously investigated by Hilgard and Stockert (19), and two membrane proteins of approximately 33 and 37 kDa were found to be able to bind to dengue virus serotype 1 by a ligand-blot analysis. In their work, the source of dengue virus serotype 1 was the Western Pacific Nauru strain propagated in C6/36 cells (a mosquito-derived cell line), whereas the present study used dengue virus serotype 1 strain 16007 propagated in Vero cells, a mammalian (African green monkey kidney) cell line. Although different viral strains, viral sources, and liver cell lines were used, it is highly pertinent that a dengue virus serotype 1 binding protein band of approximately 37 kDa was observed in both studies; this suggests that the 37/67-kDa high-affinity

laminin receptor may indeed represent a true liver cell-expressed dengue virus serotype 1 receptor.

The in vitro virus binding ability of the 37/67-kDa high-affinity laminin receptor was verified by infection inhibition assays as well as directly by immunofluorescence. We demonstrated that an anti-37/67-kDa high-affinity laminin receptor antibody as well as soluble laminin inhibited the infection of liver (HepG2) cells in a dose-dependent manner. We further showed that the inhibitory effect of soluble laminin appeared to be more effective than that of the anti-37/67-kDa laminin receptor antibody. The effective inhibition ability of soluble laminin might result from the distinctive properties of laminin, which is a large glycoprotein containing multiple protein binding domains and capable of binding proteins including heparin, collagen, and neurite outgrowth (PHD) fragment (43). Thus, when HepG2 cells were incubated with laminin molecules, laminin would bind not only to the 37/67-kDa high-affinity laminin receptor but also possibly to several other substrates such as heparan sulfate, which is well known to be involved in mediating viral entry (10, 17–19, 23, 29). In addition, the large size of laminin (900 kDa) may more effectively block the 37/67-kDa high-affinity laminin receptor than the polyclonal antibody used in this study.

In response to dengue virus serotype 1 infection, we noted an immediate and significant down-regulation in the level of expression of the 37/67-kDa high-affinity laminin receptor in the membrane fraction as assessed by Western blotting. The loss of a viral receptor from a newly infected cells surface is possibly caused by coendocytosis of the virus-receptor complex after virus-receptor attachment. However, the down-regulation of a virus receptor on infection is a strategy employed by several enveloped viruses to protect the infected cells from superinfection and to allow the virus to efficiently propagate and spread to infect other uninfected cells (6). In addition, there is evidence that the down-regulation of a virus receptor may occur as a viral strategy to prevent interference between the virus and the receptor during virus maturation and secretion. Duck hepatitis B virus uses this strategy (6), and the down-regulation of the virus receptor correlates with the accumulation of newly synthesized viral protein (6). In this case, the virus receptor was found to interact with viral proteins in the biosynthetic pathway, which led to its retention in a pre-Golgi compartment and its subsequent degradation, thus preventing possible receptor interference with the export of duck hepatitis B virus, which uses the same secretory pathway as the receptor (6). In our experiments, we saw no changes in the steady-state RNA pool for the 37/67-kDa high-affinity laminin receptor mRNA, suggesting that a similar regulation at the level of the protein may occur in dengue virus-infected cells.

It has been suggested that heparan sulfate may act either directly as a virus receptor or as a low-affinity virus binding site for virus accumulation prior to transfer to a high-affinity virus receptor (10), and we have recently shown that heparan sulfate is involved in the internalization of all four dengue virus serotypes into HepG2 cells (44), although to a variable degree. For dengue virus serotype 1, a reduction to almost 30% of control levels was observed following heparinase III treatment (44). In this study we noted a significant reduction in the levels of antibody inhibition following pretreatment of the HepG2 cells with heparinase III. Studies of the interaction of the prion protein with the 37/67-kDa high-affinity laminin receptor have also implicated heparan sulfate in the interaction, and it has been proposed that the prion protein binds to the 37/67-kDa high-affinity laminin receptor through both direct and heparan sulfate-dependent interactions (21); as such, our results showing an additive effect between the 37/67-kDa high-affinity laminin receptor and heparan sulfate on dengue virus serotype internalization would be consistent with a similar mechanism occurring in the internalization of dengue virus serotype 1. This is possibly a fairly common mechanism, given that both the 37/67-kDa high-affinity laminin receptor and heparan sulfate have also been implicated in the internalization of Sindbis virus (8).

While we have demonstrated that the 37/67-kDa high-affinity laminin receptor is a receptor protein for dengue virus serotype 1, we have also excluded this receptor protein from playing a role in the internalization of the three remaining serotypes into liver cells. Therefore, many questions remain to be addressed with regard to the nature of the interaction between dengue virus and liver cells.

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