Arch Virol (2010) 155:1453–1461
DOI 10.1007/s00705-010-0728-7

ORIGINAL ARTICLE

Dengue-2-virus-interacting polypeptides involved in mosquito cell infection

Mandar S. Paingankar · Mangesh D. Gokhale · Dileep N. Deobagkar

Received: 23 April 2009 / Accepted: 12 June 2010 / Published online: 23 June 2010

Abstract For the design of effective antiviral strategies, understanding the fundamental steps of the virus life cycle, including virus–host interactions, is essential. We performed a virus overlay protein binding assay followed by proteomics for identification of proteins from membrane fractions of *A7* (*Aedes aegypti*) cells, C6/36 (*Aedes albopictus*) cells and the midgut brush border membrane fraction of *Ae. aegypti* mosquito that bind to dengue-2 virus. Actin, ATP synthase β subunit, HSc 70, orisis, prohibitin, tubulin β chain, and vav-1 were identified as dengue-2-virus-binding proteins. Our results suggest that dengue-2 virus exploits an array of housekeeping proteins for its entry in mosquito cells.

Introduction

Dengue virus, a causative agent of dengue fever and dengue hemorrhagic fever (DHF), is a mosquito-borne single-stranded RNA virus. *Aedes aegypti* and *Aedes albopictus* are the main vectors of dengue transmission in tropical and subtropical countries. It is known that the transmission efficiency of the vector mosquito is influenced by the virus load, the virulence of the dengue virus and the immunity and susceptibility of the mosquito host. Of these factors, susceptibility of vector mosquito to dengue infection is a critical parameter and is determined by interaction between midgut cell membrane receptors and the virus envelope glycoprotein in receptor-mediated endocytosis [8, 11, 26]. It is known that populations of *Ae. aegypti* differ greatly in their susceptibility to dengue virus [2, 13], and this variability is determined by several genetic factors. Differences in vector competence may, at least in part, be due to the presence of specific midgut epithelial receptors, and their identification and characterization would be a significant step in understanding virus tropism, virulence and differences in vector competence.

Virus overlay protein binding assay (VOPBA) has been used to describe putative dengue virus receptors in mosquito and human cell lines [3, 11, 16–18, 27–29, 34] (see also Supplementary information, Tables S1 and S2). Chee and AbuBakar [5] have suggested that tubulin or tubulin-like protein from *Ae. albopictus* cells (C6/36) may serve as a putative dengue-2 virus receptor. Although, the interaction of dengue virus with mosquito cells has been analyzed, the receptors involved in this process remain to be characterized [3, 16–18, 28, 29]. For example, Salas-Benito et al. [28, 29] employed affinity chromatography and identified two polypeptides, a 45-kDa glycoprotein and a 74-kDa protein, as putative dengue virus receptors. Mercado-Curiel et al. [17, 18] have demonstrated the importance of 67- and 80-kDa proteins in dengue virus infection using VOPBA and specific antibodies. They have also demonstrated that the four serotypes of dengue virus recognize the same putative receptors in *Ae. aegypti* midgut

Electronic supplementary material The online version of this article (doi:10.1007/s00705-010-0728-7) contains supplementary material, which is available to authorized users.

M. S. Paingankar · D. N. Deobagkar (✉)
Molecular Biology Research Laboratory,
Department of Zoology, Centre for Advance Studies,
University of Pune, Pune 411007, India
e-mail: dndeo@unipune.ernet.in

M. D. Gokhale
Department of Medical Entomology and Zoology,
National Institute of Virology, 20-A,
Dr. Ambedkar Road, Pune 411001, India

D. N. Deobagkar
Goa University, Taleigaon Plateau, Goa 403206, India

and *Ae. albopictus* cells. Cao-Lormeau [3] demonstrated four proteins (77, 58, 54 and 37 kDa) from salivary gland extract of *Ae. aegypti* and five proteins (67, 56, 54, 50 and 48 kDa) from salivary gland extracts of *Ae. polynesiensis* as dengue-virus-binding proteins using VOPBA. However, the identity of these cell-surface dengue-2-virus-binding proteins has not been conclusively determined to date.

To understand the interaction between dengue virus with its mosquito host, we investigated the dengue-2-virus-binding proteins in *Aedes* cells using a direct protein–protein interaction approach. Using a proteomics approach, based on VOPBA and a combination of matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/TOF MS) and immunoblotting, we have identified proteins that interact with dengue-2 virus. Here, we describe the identification of polypeptides that appears to serve as important mediators of dengue-2 virus attachment from cell lines of *Ae. aegypti* (A7) and *Ae. albipictus* (C6/36) and midgut brush border membrane fraction (BBMF) of *Ae. aegypti* midgut. The implication of these findings in understanding the biology of dengue-2 virus infection is discussed.

Materials and methods

Virus stock preparation

Dengue-2, Trinidad (TR1751) virus stock was prepared in mice. In order to prepare virus stock, dengue-2 virus was initially reconstituted in 0.5 ml distilled water, and dilutions were prepared in 1.25% bovine serum albumin phosphate saline (BAPS), pH 7.4. One- to two-day-old mice were inoculated intracerebrally with about 0.02 ml of dengue-2 virus suspension (5×10^7 PFU/ml). Mice were monitored for sickness. After about 8–10 days, when mice showed dengue-2 sickness, they were sacrificed, and their brains were homogenized using 1.25% BAPS (20% w/v). The homogenate was centrifuged at $16{,}000 \times g$ for 60 min. The supernatant was distributed in glass vials, lyophilized and stored at −20°C. One vial was used to determine the virus titer (5×10^7 PFU/ml).

Mosquitoes, Aedes cells and virus infection

Adults and larvae of *Ae. aegypti* were obtained from the insectaries of the National Institute of Virology, Pune. *Ae. aegypti* (A7) and *Ae. albopictus* (C6/36) cell lines were continuously maintained in the laboratory on serum-free insect cell culture medium (Hiclone, Australia). Mosquitoes were infected using an artificial membrane feeder. Briefly, the blood meal consisted of equal parts of virus suspension (5×10^5 PFU/ml) and chicken blood. The mixture of blood and virus was incubated at 37°C for 15 min and then placed in membrane feeders maintained at a constant temperature of 37°C. Mosquitoes (3–4 days old) were starved of sucrose and deprived of water for 24 h before blood feeding. They were allowed to feed for 45–60 min. Fully engorged females were selected and kept in the insectary. C6/36 cells were infected with 0.2 ml dengue-2 virus inoculum with an input of 1,000 PFU per plate and incubated at 28°C for 4 days.

Membrane fraction isolation

BBMF from guts of *Ae. aegypti* females and fourth-instar larvae were isolated following a procedure modified from Wolfersberger et al. [38] and Houk et al. [10]. Adult females and larvae were chilled on ice, midguts were extruded by dissection under a binocular microscope, peritrophic membranes and malpighian tubules were removed, and midguts were rinsed in ice-cold buffer A (0.3 M mannitol/5 mM EGTA, 20 mM Tris/HCl, 2 mM PMSF, pH 7.4). Midguts were then placed in 1.5-ml cryogenic vials and stored at −80°C until required. Midguts were homogenized in buffer B (0.3 M mannitol, 5 mM EGTA, 20 mM Tris/HCl, 2 mM PMSF, Triton X-100, pH 7.4). The samples were allowed to stand on ice for 20 min and then centrifuged at $2{,}000 \times g$ for 15 min at 4°C. The supernatant was collected and kept on ice. The pellets were re-extracted in buffer B. The supernatant from both extractions was centrifuged at $23{,}200 \times g$ for 60 min at 4°C. The resulting pellet was then resuspended in buffer A (1 µl/2 midguts). A7 and C6/36 cells were centrifuged and washed twice with PBS and used for membrane fraction isolation.

Two-dimensional gel electrophoresis

Membrane proteins (300 µg) from A7, C6/36 and *Aedes aegypti* midgut were solubilized in 125 µl of IPG strip rehydration buffer (8 M urea, 2% CHAPS, 10 mM DTT, 0.2% Bio-Lyte) at room temperature. A ReadyStrip IPG, pH range 3–10, 7 cm (BioRad Laboratories, USA), was rehydrated overnight at room temperature using rehydration buffer under passive conditions. Subsequently, IEF was carried out as recommended by the manufacturer (4,000 V for 2 h with a gradient until a total of 10,000 volt-hours was reached). The strips were removed from the focusing tray and incubated for 10 min in 1 ml equilibration buffer I (50 mM Tris–HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol, 1% DTT) and 10 min in equilibration buffer II (6 M urea, 0.375 M Tris–HCl, pH 8.8, 2% SDS, 20% glycerol, 2.5% w/v iodoacetamide). Strips were washed with distilled water and placed on the top of the second-dimension gel (12.5% SDS-PAGE). The second dimension

was carried out at a constant current of 35 mA (Mini Protean 3, BioRad Laboratories, USA). The gels were stained with CBB or used for 2D VOPBA as described below.

Virus overlay protein binding assay

VOPBA was performed to identify cell polypeptides involved in virus binding. Membrane proteins (50 μg) were electrophoresed in a SDS-polyacrylamide gel (SDS-PAGE) and transferred to nitrocellulose membranes (Hybond C) using a semidry blotting apparatus (BioRad Laboratories, USA) in 48 mmol Tris, 39 mmol glycine, and 20% (vol/vol) methanol. The membranes were blocked with 2% BSA (Sigma) in PBST (phosphate-buffered saline pH 7.4, 0.5% Tween 20) at 4°C and washed three times for 30 min with PBST. Membranes were then incubated with purified dengue-2 virus in PBS at 37°C for 60 min and washed three times for 30 min with PBST. They were then incubated with an antibody against dengue-2 virus diluted 1:100 in PBS for 60 min. After washing the membranes three times with PBST for 30 min, they were incubated for 60 min at room temperature with a goat anti-rabbit IgG conjugated to peroxidase (diluted 1:3,000 in PBS). Finally, membranes were washed three times for 30 min with PBST. Color was developed with H_2O_2 and DABT. A similar process was followed to identify dengue-2-virus-binding proteins in two-dimensional VOPBA.

Protein identification using MALDI-TOF/TOF MS

Spots from one-dimensional and two-dimensional SDS-PAGE gels corresponding to virus-binding activity in VOPBA were excised and subjected to alkylation, followed by in-gel digestion with trypsin. The resultant peptide masses were analyzed by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF/TOF) on Ultraflex TOF/TOF (Bruker Daltonics, Germany). MALDI TOF/TOF analysis was carried out at the Indian Institute of Science, Bangalore. The mass spectrum produced from each sample was searched against the protein databases (NCBInr, MSDB, and Swissprot) using the MASCOT search engine, and proteins were identified. Sequence coverage greater than 20% was obtained from each protein spot.

Semiquantitative measurement of selected transcripts of *Ae. aegypti* dengue-2-virus-binding proteins

Changes in gene expression of selected genes were monitored by RT-PCR. In adult females of *Ae. aegypti* and C6/36 cells, samples from 48 h p.i. were used as the early time point. Samples from dengue-2 positive adult females (7 days) and C6/36 cells (4 days) were considered as the late time point. *Ae. aegypti* female brain squash preparations were found positive for dengue-2 antigen after 7 days. Similarly, IFA showed the presence of dengue-2 virus antigen in C6/36 cells after 4 days. We expected that dengue-2-positive mosquitoes and C6/36 cells would provide a clear-cut expression profile of these dengue-2-virus-binding proteins. Therefore, these time points were considered late infection time points. Changes in gene expression of selected genes were assessed on RNA pools from dengue-2-virus-infected mosquito carcasses (48 h and 7 days p.i.) and C6/36 cells (48 h and 4 days p.i.). Total RNA of the cells and mosquitoes was isolated using a Micro-to-Midi Total RNA Purification Kit (Invitrogen, USA). An equal amount of RNA from each sample was converted to cDNA using a cDNA synthesis kit (Invitrogen, USA). RT-PCR was performed to measure the transcript levels of actin, tubulin, HSP70 and prohibitin-1. The $MgCl_2$ concentration and exponential range of amplification were determined as described by Marone et al. [14]. Primer sequences for actin, tubulin, HSP70 and prohibitin-1 were designed using the software Primer 3 (http://www-genome.wi.mit.edu) (Table 1).

One microlitre of cDNA was used for RT-PCR using specific primers and 2 U of Gotaq Flexi (Promega, USA) in the buffer provided by the manufacturer. The genes were amplified using gene-specific primers for actin, tubulin, HSc 70, prohibitin and S7 ribosomal protein as described below. Reactions were carried out in a gradient PCR machine (Eppendorf, Germany). A first cycle of 10 min at 95°C was followed by 1 min at 95°C, 1 min at the Tm of respective gene primer set (Table 1) and 2 min at 72°C for 35 cycles and final extension at 72°C for 10 min. The conditions were chosen so that none of the RNAs analyzed reached a plateau at the end of the amplification protocol, i.e., they were in the exponential phase of amplification. The two sets of primers used in each reaction did not compete with each other. A negative control containing

Table 1 Primer sequences used in semi-quantitative PCR

Gene name	Primer	Sequence (5′–3′)	Tm (°C)
S7	PF	GGCGATCATCATCTACGT	58.6
	PR	GTAGCTGCTGCAAACTTCGG	60.6
Actin	PF	CTTCCCATCCATTGTTGGTC	60.4
	PR	CACCGATCCAGACGGAGTAT	62.4
Tubulin	PF	CGGAAACCAAATTGGAGCTA	58.4
	PR	TAACGTTGTTGGGGATCCAT	58.4
HSc70	PF	TACAGTTGCGTGGGTGTGTT	60.4
	PR	TCCTGCACTTCTTCGGACTT	60.3
Prohibitin	PF	GGCTGCTCAGTTCTTTAATCG	53.9
	PR	GATGGTCGATGGCAGATTG	53.5

RNA instead of cDNA was always used in order to rule out contamination with genomic DNA. Images of ethidium-bromide-stained agarose gels of the RT-PCR products were acquired with an AlphaImager 3400 (AlphaInnotech), and quantification of the band intensity was performed using AlphaEase Fc software. Band intensity was expressed as relative absorbance units. The S7 gene was used as an internal control. The mean and standard deviation of all gene expression values were calculated after normalization to the value of S7. Since we have used the semi-quantitative RT-PCR protocol designed by Marone et al. [14], we expect that our results will be comparable to quantitative PCR.

Monoclonal antibodies against actin, tubulin and prohibitin

Monoclonal antibodies against *Drosophila melanogaster* actin (Abcam, USA), β tubulin (Sigma, USA), HSc70 (Sigma, USA) and prohibitin (Abcam, USA) were used to verify the results obtained by MALDI TOF/TOF.

Results

Identification and characterization of dengue-2-virus-binding proteins from Aedes cells

To investigate the molecules that comprise the dengue-2 virus receptor complex, we isolated the membrane fraction from of A7 cells and C6/36 cells. When immobilized membrane fractions were incubated with native dengue-2 virus, six polypeptides were recognized as dengue-2-virus-binding proteins for C6/36 cells (84, 78, 70, 55, 32 and 26 kDa) and an additional 48 kDa protein was detected for A7 cells (Fig. 1a). Similar polypeptides were found in VOPBA of two-dimensional SDS PAGE (Supplementary information, Fig. S1). To identify the dengue-2-virus-binding proteins, protein bands corresponding to dengue-2 virus binding activity from one-dimensional as well as two-dimensional SDS-PAGE gels were excised and analyzed by mass spectrometry (Table 2). In the case of A7 cells, proteins binding to dengue-2 virus were identified as conserved hypothetical protein, vav-1, heat shock cognate 70, ATP synthase β subunit, tubulin β chain, prohibitin and orisis. Similar polypeptides were identified in C6/36 cells except for the tubulin β chain (Table 2). To further corroborate this identification, proteins from the A7 and C6/36 cell membrane fraction were probed with an anti-prohibitin, anti-tubulin or anti-HSc70 monoclonal antibody in a western blot (Supplementary information, Fig. S3). The monoclonal anti-prohibitin antibody interacted with a 32-kDa protein, anti-tubulin antibody recognized a 48-kDa protein and anti-HSc70 antibody recognized a 70-kDa protein in membrane fractions of A7 and C6/36 cells. These results further confirmed the MALDI-TOF/TOF identification.

The effect of dengue-2 virus infection on expression levels of dengue-2-virus-binding proteins was accessed using semi-quantitative RT-PCR. Expression changes of

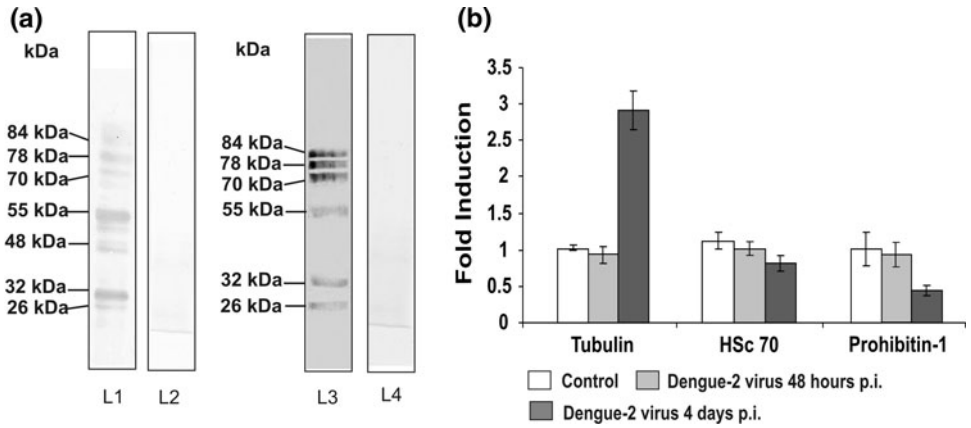

Fig. 1 VOPBA with dengue-2 virus and semi-quantitative expression of dengue-2-virus-binding proteins. **a** VOPBA with dengue-2 virus. Membrane-fraction proteins from A7 cells (*lanes L1 and L2*) and C6/36 cells (*lanes L3 and L4*) were subjected to SDS-12.5% PAGE and transferred to Hybond-C membranes. *Lanes L1 and L3* were incubated with dengue-2 virus with 5×10^5 plaque-forming units of dengue-2 virus, and *lanes L2 and L4* without dengue-2 virus at 37°C. The putative dengue-2-virus-binding proteins became visible after incubation with a rabbit polyclonal antibody to dengue-2 virus and a secondary antibody (peroxidase-conjugated goat anti-rabbit IgG). Reactions were developed using H_2O_2 and DABT. The molecular weights of dengue binding proteins are shown at the left side. **b** Semiquantitative expression of selected dengue-2-virus-binding protein genes from C6/36 cells 4 days after infection. Total RNA was extracted from a pool of control or dengue-2-virus-infected cells, reversed transcribed and amplified by PCR. Results are represented as fold induction in infected cells 48 h p.i. (*grey*), and 4 days p.i. (*black*) relative to control C6/36 cells (*white*). Ribosomal protein S7 mRNA was used to normalize the data. *Bars* indicate standard deviation from three independent experiments

Table 2 Molecular identification of dengue-2-virus-binding proteins from *Ae. aegypti* (*A7*) and *Aedes albopictus* (C6/36) cells

No.	Accession no.	Protein description	Mol. mass (kDa)		Mass values matched
			From Fig. 1	From database	
1	**EAT33460**	Conserved hypothetical protein	84	87.58	12
2	**EAT35349**	Vav-1 protein	78	76.1	14
3	**ABF18332**	Heat shock cognate 70[a]	70	72.24	23
4	**ABF18266**	F0F1-type ATP synthase β subunit	55	53.94	16
5	**EAT45945**	Probable tubulin β chain[a, b]	48	50.17	7
6	**ABF18314**	Prohibitin[a]	32	29.88	12
7	**EAT45780**	Putative orisis	26	22.86	7

[a] The monoclonal anti-prohibitin antibody interacted with a 32-kDa protein, anti-tubulin antibody recognized a 48-kDa protein, and anti-HSc70 antibody recognized a 70-kDa protein in membrane fractions of A7 and C6/36 cells

[b] Detected only in the case of A7 cells

selected genes were monitored at early and late infection time points.

Semiquantitative RT-PCR of selected dengue-2-virus-binding protein mRNA

To determine whether infection with dengue-2 virus induces changes in the steady-state level of transcription of tubulin, prohibitin and HSc 70, semi-quantitative RT-PCR was performed. Expression analysis was carried out using total RNA isolated from uninfected C6/36 cells and dengue-2-virus-infected C6/36 cells, which were harvested at 48 and 96 h p.i., respectively. The expression of tubulin, HSc 70 and prohibitin genes was slightly reduced at 48 h p.i. (Fig. 1b). Tubulin β chain mRNA levels were increased by threefold in dengue-2-virus-infected cells compared to control cells. No consistent difference was found in mRNA levels of HSc 70 in infected and control cells. The prohibitin-1 mRNA level decreased by twofold in infected cells compared to control cells (Fig. 1b).

Identification and characterization of dengue-2-virus-binding proteins from *Ae. aegypti* adult females and fourth instar larvae gut brush border membrane

It has been shown previously that the four serotypes of dengue-2 virus recognize the same putative receptors in *Ae. aegypti* midgut and *Ae. albopictus* cells [17, 18]. Therefore, we investigated whether these dengue-2-virus-binding polypeptides of *Aedes* cells are present in the midgut of mosquitoes. VOPBA analysis showed four proteins of molecular mass of 78, 70, 45 and 32 kDa that interacted with dengue-2 virus in *Ae. aegypti* adult female and fourth-instar larvae gut BBMF (Fig. 2a, Supplementary information, Fig. S2). These proteins were identified as vav-1, heat shock cognate 70, actin and prohibitin (Table 3). Similarly 32-, 45- and 70-kDa proteins were recognized by monoclonal antibodies to prohibitin, actin and HSc70, respectively, on immunoblots. These results were consistent with MALDI TOF/TOF identification. Expressions levels of actin, HSc 70 and prohibitin genes were analyzed in dengue-2-virus-infected mosquitoes by semiquantitative RT-PCR (Fig. 2b). The expression of actin, HSc 70 and prohibitin genes was slightly reduced at 48 h p.i. (Fig. 2b). The relative level of actin mRNA increased by 2.5-fold in response to dengue-2 virus infection. The level of HSc 70 mRNA was equivalent to that of the control, whereas prohibitin-1 transcript levels were reduced by twofold (Fig. 2b).

Discussion

The interaction of the viral attachment protein and its cellular receptors is known to contribute to host range, tissue tropism and viral pathogenesis. The virus–receptor interaction is a multi-step process in itself, where multiple receptors may be used sequentially or in a cell-type-specific manner and, additionally, co-receptors may also be involved. Some proteins have been reported as candidate receptors for the entry of dengue virus into mosquito cells [3, 16–18, 28, 29]. However, their identity has not been conclusively determined. Tubulin or tubulin-like protein from C6/36 cells is the only well-characterized mosquito protein that interacts with dengue-2 virus [5]. The results of earlier studies suggest that while proteins may be denatured as part of VOPBA, the technique is still capable of selecting physiologically relevant binding molecules, possibly as a result of partial renaturation of proteins during the overlay process. While affinity chromatography has been used by other workers to isolate putative dengue receptors proteins [16, 28], these studies have used significantly higher ligand concentrations, and this may lead to isolation of proteins through non-specific binding. In

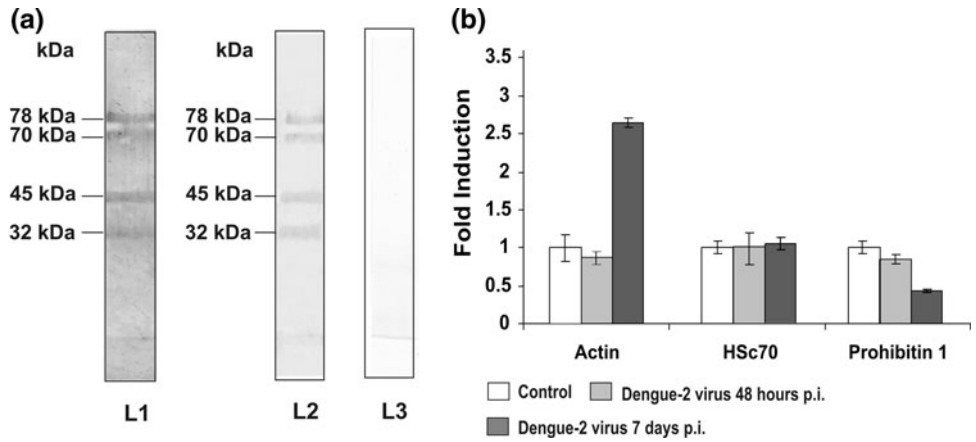

Fig. 2 VOPBA with dengue-2 virus and semi-quantitative expression of dengue-2 virus binding proteins in *Ae. aegypti*. **a** Brush border membrane proteins from *Ae. aegypti* females (*lane L1 and L3*) and fourth-instar larvae (*lane L2*) were subjected to SDS-2.5% PAGE and transferred to Hybond-C membranes. *Lane L1* and *L2* were incubated with dengue-2 virus with 5×10^5 plaque-forming units of dengue-2 virus, and *lane L3* without dengue-2 virus at 37°C. The putative dengue-2-virus-binding proteins became visible after incubation with a rabbit polyclonal antibody to dengue-2 virus and a secondary antibody (peroxidase-conjugated goat anti-rabbit IgG). Reactions were developed using H_2O_2 and DABT. The molecular weights of the dengue-virus-binding proteins are shown on *left side*. **b** Semi-quantitative expression of selected dengue-2-virus-binding protein genes from *Ae. aegypti* adult mosquitoes 7 days after infection. Total RNA was extracted from a pool of control or dengue-2-virus-infected mosquitoes, reversed transcribed and amplified by PCR. Results are represented as fold induction in infected mosquitoes 48 h p.i. (*grey*), 7 days p.i. (*black*) relative to control mosquitoes (*white*). Ribosomal protein S7 mRNA was used to normalize the data. *Bars* indicate standard deviation from three independent experiments

Table 3 Molecular identification of dengue-2virus-binding proteins from *Ae. aegypti* adult females and forth-instar larval gut

No.	Accession no.	Protein description	Mol. mass (kDa)		Mass values matched
			From Fig. 2	From database	
1	**EAT35349**	Vav-1 protein	78	76.1	14
2	**ABF18332**	Heat shock cognate 70[a]	70	72.24	23
3	**EAT42510**	Actin[a]	45	41.61	16
4	**ABF18314**	Prohibitin[a]	32	29.88	12

[a] The monoclonal anti-prohibitin antibody interacted with a 32-kDa protein, anti-actin antibody recognized a 45-kDa protein, and anti-HSc70 antibody recognized a 70-kDa protein in brush border membrane fractions of *Ae. aegypti* midgut

contrast, our method seems to be the most reliable one for identifying putative dengue-2 virus receptors present in *Aedes* cells, as it uses combination of VOPBA, MALDI-TOF/TOF MS and immunoblotting.

A total of eight different proteins were found by this approach to bind to dengue-2 virus. These proteins include tubulin β chain, already described as a dengue-2 virus receptor in C6/36 cells, and seven additional proteins not identified previously as candidate dengue-2-virus-binding proteins (Tables 2, 3). Our results are consistent with those of Mercado-Curiel et al. [18] and Salas-Benito et al. [28], who detected ~70- and 80-kDa dengue-2-virus-binding proteins in the membrane fraction of *Ae. aegypti* midgut, A7 cells and C6/36 cell. Our analysis suggests that the 70-kDa protein could be HSc 70 and the 80-kDa protein could be vav-1.

We observed that transcription of actin, tubulin, HSc 70 and prohibitin genes decreased slightly at 48 h p.i. in the presence of dengue-2 virus (Figs. 1b, 2b). This suggested the existence of a virus host shutoff effect, which has also been shown with other viruses [35, 37]. We further suggest that the increase in the levels of actin and tubulin transcripts at 96 h p.i. in the case of C6/36 cells and 7 days in the case of *Ae. aegypti* adult females (Figs. 1, 2) could be attributed to mechanisms activated by the infected cells to maintain homeostasis and reorganization of cytoskeleton structure during virus infection. A similar suggestion was made by Talavera et al. [33]. Tubulin heterodimers, the major building blocks of cell microtubules, are known to be involved in the assembly and transport of virus particles [9, 12, 22, 23, 31, 32, 39]. It is possible that the initial binding of dengue-2 virus to midgut cells is mediated through a non-protein–protein interaction (i.e., mediated through heparan sulfate, laminin or lectin) and that the subsequent cellular changes allowing contact of dengue E with tubulin facilitate internalization of the virus. It has

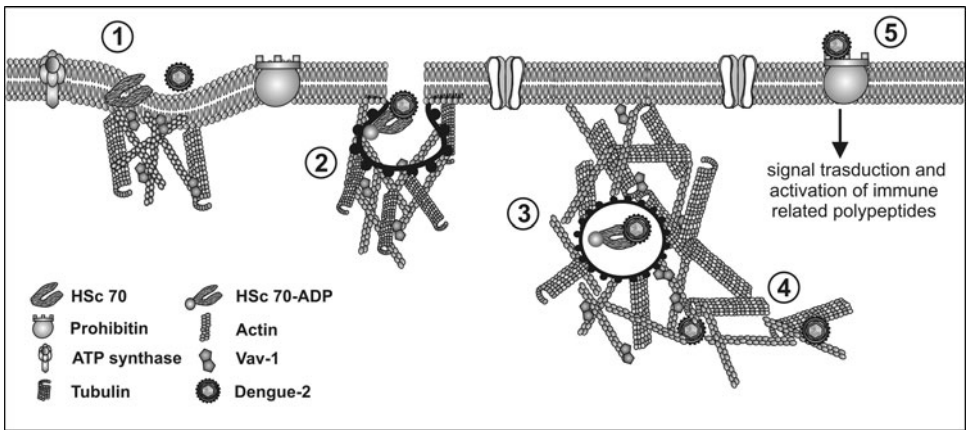

Fig. 3 Proposed model for dengue-2 virus entry and transport. (1) Dengue-2 virus enters the cells via receptor-mediated endocytosis using primary receptors. HSc 70 present in the membrane functions as an attachment molecule and probably concentrates the dengue-2 virus particles at cell surface to allow efficient interaction with primary receptors. (2) In the early endosome, pH change and chaperone activity of HSc 70 play an important role in the transition of the dengue-2 virus envelope protein from a dimer to trimer. (3) Actin-binding proteins and actin polymerization are involved in moving endocytic vesicles. (4) After successful rounds of replication, dengue-2 virus exploits the actin–tubulin network for long-distance vectorial transport. (5) Prohibitin functions as an attachment molecule, probably activating signal transduction to reduce dengue-2 virus infection. Prohibitin may be a non-receptor, innate immunity modulator in mosquitoes

been documented that tubulins of midgut cells can act as a scaffold for dengue-2 virus assembly through binding with the E protein [5], comparable to the model proposed for the transport and assembly of West Nile virus [6] and also Kunjin virus [7, 23]. These results support the hypothesis that actin, actin-associated proteins and tubulin might play a crucial role in the entry and transport of dengue-2 virus.

Heat shock proteins are involved in various steps of the dengue virus life cycle [4, 24, 25, 28]. In the VOPBA assay, we detected HSc 70 as a dengue-2-virus-interacting protein. Furthermore, semi-quantitative studies showed no significant modulation of HSc 70 in response to dengue-2 virus infection (Figs. 1b, 2b). Therefore, we propose that HSc 70 might be involved in concentrating the virus particles on membrane and is not involved in viral replication. Additionally, Modis et al. [21] recently reported that HSP 70 functions as chaperone in the transition of E protein from dimer to trimer. In VOPBA, we found that ATP synthase interacted with dengue-2 virus. ATP-synthase-mediated ribosylation of HSc70 is necessary for the chaperone activity of HSc 70 [1]. Interaction of HSc70s with dengue-2 virus envelope protein might be modulated by the presence and hydrolysis of ATP. Therefore, one could postulate a possible role of the ATP synthase β subunit in infection to provide the ATP in the chaperone process of HSc70.

VOPBA studies showed that prohibitin interacted with dengue-2 virus, while semi-quantitative studies showed suppression of prohibitin in response to dengue-2 virus infection (Figs. 1, 2). Prohibitin is a highly conserved protein in eukaryotes and is assumed to have a multifaceted role in cell physiology [7, 15, 19, 20, 36]. Prohibitin activates the complement pathway [19] and shares several structural features/functional domains with immune-related molecules (see Supplementary information, Table S3). We suggest that dengue-2 virus targets prohibitin-1 in mosquito gut and suppresses early inflammatory responses. A similar strategy is also employed by *Salmonella typhi* in intestinal epithelial caco-2 cells [30]. In immunofluorescence microscopy of C6/36 cells, prohibitin was found to be mainly localized in cell membranes and the nucleus (Supplementary information, S4). These results indicate that prohibitin functions as an attachment molecule, probably activating signal transduction to reduce dengue-2 virus infection. We hypothesize that prohibitin might be a non-receptor, innate immunity modulator in mosquitoes.

Based on above discussion, a model is proposed for dengue-2 virus entry and transport in mosquito cells (Fig. 3). We suggest that dengue-2 virus enters the cells via receptor-mediated endocytosis using primary receptors. HSc 70 present in the membrane perhaps functions as an attachment molecule and probably concentrates the dengue-2 virus particles on the cell surface to allow efficient interaction between primary receptors and dengue-2 virus. In the early endosome, pH change and chaperonic activity of HSc 70 play important role in transition of the envelope protein from a dimer to a trimer. After successful rounds of replication, dengue-2 virus exploits actin, vav-1 and tubulin for vectorial transport. Ion channels might be used by dengue-2 virus to facilitate entry or exit from cells. The exact role of orisis is not clearly understood and requires further investigation. VOPBA studies suggest that, in vivo, similar molecules could be recognized by dengue-2 virus. The VOPBA results needs to be verified further by

analyzing the interaction of these host proteins with virus or individually expressed prM and E in solution.

Acknowledgments The authors acknowledge valuable suggestions given by Deepti Deobagkar. We thank A.C. Mishra, National Institute of Virology, Pune, for the facilities and encouragement and Dipankar Chaterjee, Indian Institute of Science, Bangalore, for the MALDI TOF/TOF facility. This research was supported by UGC-CAS and ICMR grants of DND. MSP is a CSIR Senior Research Fellow.

References

1. Arispe N, De Maio A (2000) ATP and ADP modulate a cation channel formed by Hsc70 in acid phospholipid membranes. J Biol Chem 275:30839–30843
2. Black WC, Bennett KE, Escalante-Gorrochotegui N, Barillas-Mury CV, Fernandez-Salas I, LourdesMunoz M, Farfan-Ale JA, Olson KE, Beaty BJ (2002) Flavivirus susceptibility in *Aedes aegypti*. Arch Med Res 33:379–388
3. Cao-Lormeau VM (2009) Dengue viruses binding proteins from *Aedes aegypti* and *Aedes polynesiensis* salivary glands. Virol J 6:35
4. Chavez-Salinas S, Ceballos-Olvera I, Reyes-del Valle J, Medina F, del Angel RM (2008) Heat shock effect upon dengue virus replication into U937 cells. Virus Res 138:111–118
5. Chee H, AbuBakar S (2004) Identification of a 48 kDa tubulin or tubulin-like C6/36 mosquito cells protein that binds dengue virus 2 using mass spectrometry. Biochem Biophys Res Commun 320:11–17
6. Chu JJH, Ng ML (2002) Trafficking mechanism of West Nile (Sarafend) virus structural proteins. J Med Virol 67:127–136
7. Fusaro G, Dasgupta P, Rastogi S, Joshi B, Chellappan S (2003) Prohibitin induces the transcriptional activity of p53 and is exported from the nucleus upon apoptotic signaling. J Biol Chem 278:47853–47861
8. Heinz FX, Allison SL (2003) Flavivirus structure and membrane fusion. Adv Virus Res 59:63–97
9. Hong SS, Ng ML (1987) Involvement of microtubules in Kunjin virus replication. Arch Virol 97:115–121
10. Houk EJ, Arcus YM, Hardy JL (1986) Isolation and characterization of brush border fragments from mesenterons. Arch Insect Biochem Physiol 3:135–146
11. Hung SL, Lee PL, Chen HW, Chen LK, Kao CL, King CC (1999) Analysis of the steps involved in Dengue virus entry into host cells. Virology 257:156–167
12. Liu WJ, Qi YM, Zhao KN, Liu YH, Liu XS, Frazer IH (2001) Association of bovine papillomavirus type 1 with microtubules. Virology 282:237–244
13. Lourenco-de-Oliveira R, Vazeille M, de Filippis AM, Failloux AB (2004) *Ae. aegypti* in Brazil: genetically differentiated populations with high susceptibility to dengue and yellow fever viruses. Trans R Soc Trop Med Hyg 98:43–54
14. Marone M, Mozzetti S, De Ritis D, Pierelli L, Scambia G (2001) Semiquantitative RT-PCR analysis to assess the expression levels of multiple transcripts from the same sample. Biol Proced Online 3:19–25
15. McClung JK, Jupe ER, Liu XT, Dell'Orco RT (1995) Prohibitin: potential role in senescence, development and tumor suppression. Exp Gerontol 30:99–124
16. Mendoza YM, Salas-Benito JS, Lanz-Mendoza H, Hernandez-Martinez S, del Angel RM (2002) A putative receptor for dengue virus in mosquito tissues: localization of a 45-kDa glycoprotein. Am J Trop Med Hyg 67:76–84
17. Mercado-Curiel RF, WC Black IV, Lourdes Muñoz M (2008) A dengue receptor as possible genetic marker of vector competence in *Aedes aegypti*. BMC Microbiol 8:118
18. Mercado-Curiel RF, Esquinca-Avilés HA, Tovar R, Díaz-Badillo A, Camacho-Nuez M, de Muñoz ML (2006) The four serotypes of dengue recognize the same putative receptors in *Aedes aegypti* midgut and *Ae. albopictus* cells. BMC Microbiol 6:85
19. Mishra S, Moulik S, Murphy LJ (2007) Prohibitin binds to C3 and enhances complement activation. Mol Immunol 44:1897–1902
20. Mishra S, Murphy LC, Murphy LJ (2006) The Prohibitins: emerging roles in diverse functions. J Cell Mol Med 10:353–363
21. Modis Y, Ogata S, Clements D, Harrison SC (2003) A ligand binding pocket in Dengue virus envelop glycoprotein. Proc Natl Acad Sci USA 100:6986–6991
22. Moyer SA, Baker SC, Lessard JL (1986) Tubulin: a factor necessary for the synthesis of both Sendai virus and vesicular stomatitis virus RNAs. Proc Natl Acad Sci USA 83:5405–5409
23. Ng ML, Hong SS (1989) Flavivirus infection: essential ultrastructural changes and association of Kunjin virus NS3 protein with microtubules. Arch Virol 106:103–120
24. Padwad YS, Mishra KP, Jain M, Chanda S, Karan D, Ganju L (2009) RNA interference mediated silencing of Hsp60 gene in human monocytic myeloma cell line U937 revealed decreased dengue virus multiplication. Immunobiology 214:422–429
25. Ren J, Ding T, Zhang W, Song J, Ma W (2007) Does Japanese encephalitis virus share the same cellular receptor with other mosquito-borne flaviviruses on the C6/36 mosquito cells? Virol J 4:83
26. Rey FA (2003) Dengue virus envelope glycoprotein structure: new insight into its interactions during viral entry. Proc Natl Acad Sci USA 100:6899–6901
27. Reyes Del Valle J, Chávez-Salinas S, Medina F, del Angel RM (2005) Heat shock protein 90 and heat shock protein 70 are components of dengue virus receptor complex in human cells. J Virol 79:4557–4567
28. Salas-Benito J, Valle JR-D, Salas-Benito M, Ceballos-Olvera I, Mosso C, Del Angel RM (2007) Evidence that the 45-kD glycoprotein, part of a putative dengue virus receptor complex in the mosquito cell line C6/36, is a heat-shock-related protein. Am J Trop Med Hyg 77:283–290
29. Salas-Benito JS, del Angel RM (1997) Identification of two surface proteins from C6/36 cells that bind dengue type 4 virus. J Virol 71:7246–7252
30. Sharma A, Qadri A (2004) Vi polysaccharise of Salmonella typhi target the prohibitin family of molecules in intestinal epithelial cells and suppress early inflammatory responses. Proc Natl Acad Sci USA 101:17492–17497
31. Smith GA, Enquist LW (2002) Break ins and break outs: viral interactions with the cytoskeleton of mammalian cells. Annu Rev Cell Dev Biol 18:135–161
32. Suomalainen M, Nakano MY, Boucke K, Keller S, Stidwill RP, Greber UF (1999) Microtubule-dependent minus and plus end-directed motilities are competing processes for nuclear targeting of adenovirus. J Cell Biol 144:657–672
33. Talavera D, Castillo AM, Dominguez MC, Gutierrez AE, Meza I (2004) IL8 release, tight junction and cytoskeleton dynamic reorganization conducive to permeability increase are induced by dengue virus infection of microvascular endothelial monolayers. J Gen Virol 85:1801–1813
34. Upanan S, Kuadkitkan A, Smith DR (2008) Identification of dengue virus binding proteins using affinity chromatography. J Virol Methods 151:325–328

35. Villas-Bôas CSA, Conceição TM, Ramírez J, Santoro ABM, Da Poian AT, Montero-Lomelí M (2009) Dengue virus-induced regulation of the host cell translational machinery. Braz J Med Biol Res 42(11):1020–1026
36. Wang S, Zhang B, Faller DV (1999) Rb and prohibitin target distinct regions of E2F1 for repression and respond to different upstream signals. Mol Cell Biol 19:7447–7460
37. Waterboer T, Rahaus M, Wolff MH (2002) Varicellazoster virus (VZV) mediates a delayed host shutoff independent of open reading frame (ORF) 17 expression. Virus Genes 24:49–56
38. Wolfersberger M, Liithy P, Maurer A, Parenti P, Sacchi VF, Giordana B, Hanozel GM (1987) Preparation and partial characterization of amino acid transporting brush border membrane vesicles from the larval midgut of the cabbage butterfly (*Pieris brassicae*). Comp Biochem Physiol 86:301–308
39. Xu A, Bellamy AR, Taylor JA (2000) Immobilization of the early secretory pathway by a virus glycoprotein that binds to microtubules. EMBO J 19:6465–6474