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Identification of a 48 kDa tubulin or tubulin-like C6/36 mosquito cells protein that binds dengue virus 2 using mass spectrometry

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Abstract

Binding of dengue virus 2 (DENV-2) to C6/36 mosquito cells protein was investigated. A 48 kDa DENV-2-binding C6/36 cells protein (D2BP) was detected in a virus overlay protein-binding assay. The binding occurred only to the C6/36 cells cytosolic protein fraction and it was inhibited by free D2BP. D2BP was shown to bind to DENV-2 E in the far-Western-binding studies and using mass spectrometry (MS) and MS/MS, peptide masses of the D2BP that matched to β-tubulin and α-tubulin chains were identified. These findings suggest that DENV-2 through DENV-2 E binds directly to a 48 kDa tubulin or tubulin-like protein of C6/36 mosquito cells.

Keywords: Dengue virus; Mass spectrometry; Mosquito; Tubulin

Dengue virus (DENV) is an enveloped positive single-stranded RNA virus belonging to the *Flaviviridae* family [1]. The virus is the causative agent for the mosquito-borne disease, dengue fever and the more severe diseases dengue hemorrhagic fever and dengue shock syndrome. The virus has been suggested to establish an infection by binding to cells surface molecule such as heparan sulfate using the envelope protein (E) as the virus attachment protein [2]. DENV E is also suggested to act as the fusion protein allowing the virus to fuse to cells membrane [3,4], resulting in the release of the nucleocapsid into the cytoplasm. In addition to heparan sulfate, a number of other putative DENV receptors have also been described [5–7] but most of them were identified simply by their molecular mass and with no specific protein identification. More recently, DC-SIGN or CD209 molecule, a lectin, was shown to mediate DENV infection of human dendritic cells [8]. This suggests the potential importance of DC-SIGN in the establishment of DENV infection in human cells. Whether equivalent or functionally equivalent molecule is also present in other cells commonly infected with DENV, especially mosquito cells, are not known. While the cells surface proteins could be important for DENV attachment the possibility that there are other cellular proteins that could be important for DENV entry and assembly are yet to be described. In other flavivirus infections, the virus E has been shown to bind to intracellular proteins associated with virus transport and assembly [9], hence highlighting the potential role of E not only in the entry processes but also in the assembly of the virions. In efforts to better understand the interactions between DENV with its mosquito host, in the present study, we investigated the potential DENV-2-binding proteins (D2BP) of C6/36 mosquito cells using direct protein–protein interaction approaches.

Materials and methods

Preparation of cells lysate. Total C6/36 cells proteins were prepared by solubilizing cells in cold homogenization buffer consisting of 10 mM Tris–HCl, pH 7.5, 150 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 6 mM 3- [(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO), 15 mM *n*-octylglucoside, 0.1% SDS, and anti-proteases

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(leupeptin, pepstatin A, and aprotinin) overnight at 4 °C. Intact cells and cell debris were removed by centrifugation at 800*g*. For preparation of cytosolic and membrane protein fractions, cells were freeze–thawed in the homogenization buffer containing anti-proteases and sonicated in ice-cold water three times at output level 4 (Sonifier 250; Branson, USA). Intact cells and cells nuclei were removed by centrifugation at 800*g*. The supernatant was then centrifuged at 40,000*g* and the resulting supernatant was designated as the cells cytosolic protein fraction. The remaining pellet, designated as the crude membrane fraction, was solubilized in homogenization buffer containing anti-proteases and detergents as above for overnight at 4 °C and then centrifuged at 40,000*g*. The supernatant recovered was designated as the membrane fraction. All centrifugations were performed at 4 °C. Protein concentration was determined using the Micro BCA Protein Assay Reagent Kit (Pierce, USA) before glycerol was added to a final concentration of 10%.

VOPBA. Protein fractions (50 μg/well) were separated on 10% SDS–PAGE under reducing (with DTT) or non-reducing (without DTT) conditions and electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked with 5% skim milk in TBS (TBS-S; 50 mM Tris–HCl, pH 7.6, 150 mM NaCl) and then incubated for 2 h with sorbitol gradient-purified DENV-2 New Guinea C prepared as previously described [10]. The membrane was washed vigorously three times in TBS-T (TBS containing 0.05% Tween 20) and incubated with clarified culture supernatant of the 3H5-1 hybridoma (anti-DENV-2 E [11]). After several washings, alkaline phosphatase-conjugated goat anti-mouse IgG (Promega, USA) was added to the membrane at 1:7500 dilution in TBS-S. The membranes were then developed using BCIP/NBT (KPL, USA). All incubations were performed at room temperature with constant agitation.

Gel elution of DENV-2 C6/36 cells binding protein. C6/36 cells cytosolic protein fraction was separated on a preparative 10% SDS–PAGE under reducing condition. Protein band of estimated molecular mass of 49 kDa was excised from the gels and minced into small cubes. The gel cubes were transferred into microcentrifuge tubes and incubated overnight at 4 °C in one part water or PBS (w/v). After the incubation, samples were centrifuged at 40,000*g* at 4 °C and the resulting supernatant was transferred into a new microcentrifuge tube. The eluted protein was concentrated to 1/10 of its initial volume using SpeedVac Concentrator (Savant Instrument, USA) and then treated with SDS-Out Sodium Dodecyl Sulfate Precipitation Kit (Pierce, USA) to remove SDS. Following that protein concentration was determined as mentioned above and the eluted protein fraction was designated as the gel-eluted D2BP.

Inhibition of DENV-2-binding assay. C6/36 cells cytosolic protein fraction was separated on a preparative 12.5% SDS–PAGE under reducing condition. Separated proteins were electrophoretically transferred onto nitrocellulose membrane and blocked with TBS-S. The membrane was then placed onto Mini-Protean II Multi Screen (Bio-Rad, USA). Sorbitol gradient-purified DENV-2 or sorbitol gradient-purified C6/36 cells protein (2 μg each) was either left untreated or pre-incubated with the gel-eluted D2BP (20 μg) for 1 h at room temperature. The samples were then added to the membrane on multi-screen apparatus. After 1 h incubation, the samples were removed and the membrane was washed three times with TBS-T. The presence of bound DENV was detected using anti-DENV-2 E monoclonal antibody and alkaline phosphatase (AP)-conjugated goat anti-mouse IgG as previously described.

Production of polyclonal antibody. The gel-eluted D2BP prepared in PBS (50 μg) was emulsified with an equal volume of complete Freund's adjuvant (Sigma–Aldrich, USA). Six-week-old female Balb/cJ mice were then injected subcutaneously. Booster injections were given 3 times at 2 weeks interval with the same amount of protein emulsified with incomplete Freund's adjuvant (Sigma–Aldrich, USA). Antiserum was obtained at 2 weeks after the last booster injection.

Far-Western-binding study. The potential binding of DENV-2 E to D2BP was investigated using the on membrane protein–protein-binding analysis (far-Western) [12]. In this study, the sorbitol gradient-purified DENV-2 and sorbitol gradient-purified C6/36 cells protein were separated on 12.5% SDS–PAGE under non-reducing conditions and electrophoretically transferred onto nitrocellulose membrane. The membrane was then probed using the gel-eluted D2BP or left untreated and the binding of the D2BP to E was then detected using polyclonal antibodies (1:40 in TBS-S) raised against the gel-eluted D2BP. Anti-DENV-2 E monoclonal antibody was also used for the detection of DENV-2 E blotted onto the membrane and the membrane was developed as described previously. Image of the membrane was analyzed for protein bands and their respective molecular masses were determined using BioNumerics ver. 3.0 (Applied Maths, Belgium).

Two-dimensional gel electrophoresis. The specificity of the polyclonal antibodies and the constituents of the gel-eluted D2BP fractions were examined using two-dimensional gel electrophoresis (2-DE). The gel-eluted D2BP in water was added to rehydration buffer containing 8 M urea, 2% 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS; Pierce, USA), 18 mM dithiothreitol (DTT; Amersham Biosciences, Sweden), 0.5% carrier ampholytes (Bio-Rad, USA), and 0.0007% bromophenol blue. The mixture was incubated at 4 °C for 1 h before subjecting to isoelectric focusing (IEF) on a 7 cm ReadyStrip IPG strip pH 3–10 using a Protean IEF Cell (Bio-Rad, USA). The strips were overlaid with mineral oil (Bio-Rad, USA) and rehydrated at 50 V for 12 h at 20 °C. IEF was performed using the following parameters: 250 V for 250 V/h at slow mode; 500 V for 500 V/h at slow mode; 4000 V for 4000 V/h at slow mode; 4000 V for 12,000 V/h at linear mode, and finally hold at 500 V at rapid mode. The strips were then equilibrated with equilibration solution 1 containing 6 M urea, 0.375 M Tris–HCl, pH 8.8, 2% SDS, 0.065 M DTT, and 20% glycerol followed with another equilibration step in solution 2 containing 6 M urea, 0.375 M Tris–HCl, pH 8.8, 2% SDS, 0.26 M iodoacetamide, 20% glycerol, and finally rinsed with SDS–PAGE running buffer. The strips were placed onto a 10% SDS–PAGE gel and electrophoresed at 120 V for approximately 2 h. The gel was stained with either silver nitrate or electrophoretically transferred onto PVDF membrane (Millipore, USA) and probed with polyclonal antibody raised against the gel-eluted D2BP or pre-immune serum (1:40 in TBS-S) as the negative control.

Mass spectrometry analysis. Further identification of the D2BP was made using a combination of the far-Western-binding studies and mass spectrometry. In this study, DENV-2 E was eluted from preparative acrylamide gels (gel-eluted), electrophoresed, and transferred onto nitrocellulose membrane. Small vertical strips were cut from the membrane and the position of DENV-2 E was identified following immunoblotting of the strips with anti-DENV-2 E monoclonal antibody. The position of DENV-2 E on the remaining blot was then identified after precisely aligning the membrane with the anti-DENV-2 E-developed strips and the nitrocellulose membrane was cut across the membrane saving only the membrane portion aligned with the position of DENV-2 E. The strip containing the DENV-2 E was then cut into equal sized strips (3 mm) and incubated separately with either the gel-eluted D2BP or left untreated in the TBS. Strips containing only bovine serum albumin (BSA) prepared in parallel with the DEN2 E were also incubated with D2BP. After the incubation, the strips were washed rigorously, first with TBS-T, followed by TBS, then with water and finally rinsed in 50 mM NH_4HCO_3. The strips were then digested with mass spectrometry grade trypsin (Promega, USA) and the tryptic peptides were subjected to mass spectrometric analysis using a matrix-assisted laser desorption/ionization-time of flight (MALDI-ToF) mass spectrometry (Amersham Biosciences, Sweden) and liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS) performed at Bioprocessing Technology Institute, Singapore. For MALDI-ToF MS, the peptide mass values obtained from the D2BP-incubated DENV-2 E strips were compared to that incubated with either BSA or untreated controls. All peptide mass values found in common to all samples were removed and the remaining peptide mass values were searched against the SwissProt and NCBInr databases using several available web engines (ProFound; http://prowl.rockefeller.

edu/cgi-bin/ProFound, PeptIdent; http://us.expasy.org/tools/peptident.html, and MS-Fit; http://prospector.ucsf.edu/ucsfhtm14.0/msfit.htm). Searches were done either against databases for all Taxa or limited to Insecta or *Anopheles gambiae*, depending on the search engines used. As for LC-MS/MS, protein was identified by MS/MS ion search from MSDB databases using Mascot MS/MS ion search (http://www.matrixscience.com/search_form_select.html).

Sequence alignment. All sequences used in this study were obtained from the GenBank and aligned using CLUSTAL X version 1.81 [13]. Sequences were displayed using GENEDOC version 2.5 [14].

Results and discussion

A 48 kDa C6/36 cells protein was detected bound to DENV-2 in a VOPBA using total solubilized C6/36 cells protein (Fig. 1A, arrow). The 48 kDa DENV-2-binding protein (D2BP) was not detected when the blot was probed with a similarly prepared sorbitol gradient-purified C6/36 cells protein (Fig. 1A). Bound DENV-2 was detected by VOPBA only in the cells cytosolic protein fraction prepared under both reduced and non-reduced conditions (Fig. 1B). The molecular mass of the D2BP in the reduced cytosolic protein samples, however, was approximately 49 kDa (Fig. 1B, arrowhead), reflecting the unfolded or denatured state of the protein. Under our experimental conditions (cells lysate was prepared in high salt buffer), no DENV-2 binding was detected to the cells membrane protein fraction (Fig. 1C). Binding of the sorbitol gradient-purified DENV-2 to the membrane immobilized D2BP was inhibited when sorbitol gradient-purified DENV-2 was pre-incubated with the gel-eluted D2BP fraction prior to VOPBA (Fig. 2). The 49 kDa protein was also not detected when the blot was probed with the sorbitol gradient-purified C6/36 cells protein either pre-incubated or without pre-incubation with the D2BP fraction (Fig. 2). These results suggested that the binding of

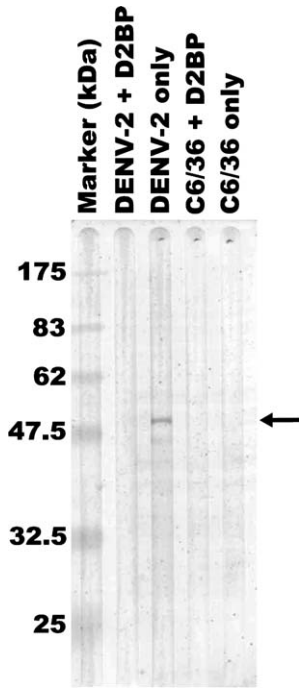

Fig. 2. Inhibition of DENV-2 binding to membrane immobilized C6/36 mosquito cells protein. C6/36 cells cytosolic protein fraction (100 μg) was separated on 12.5% SDS–PAGE under reduced conditions. The protein was electrophoretically transferred onto nitrocellulose membrane and the membrane was placed onto a Mini-Protean II Multi Screen apparatus. The membrane was probed with the gel-eluted D2BP-incubated sorbitol gradient-purified DENV-2 (DENV-2 + D2BP), sorbitol gradient-purified DENV-2 (DENV-2 only), the gel-eluted D2BP-incubated sorbitol gradient-purified C6/36 cells protein (C6/36 + D2BP) or sorbitol gradient-purified C6/36 cells protein (C6/36 only). Anti-DENV-2 E monoclonal antibody was used to detect bound DENV-2. Alkaline phosphatase-conjugated goat anti-mouse IgG was used as secondary antibody and developed with BCIP/NBT. Arrow indicates the approximately 49 kDa D2BP.

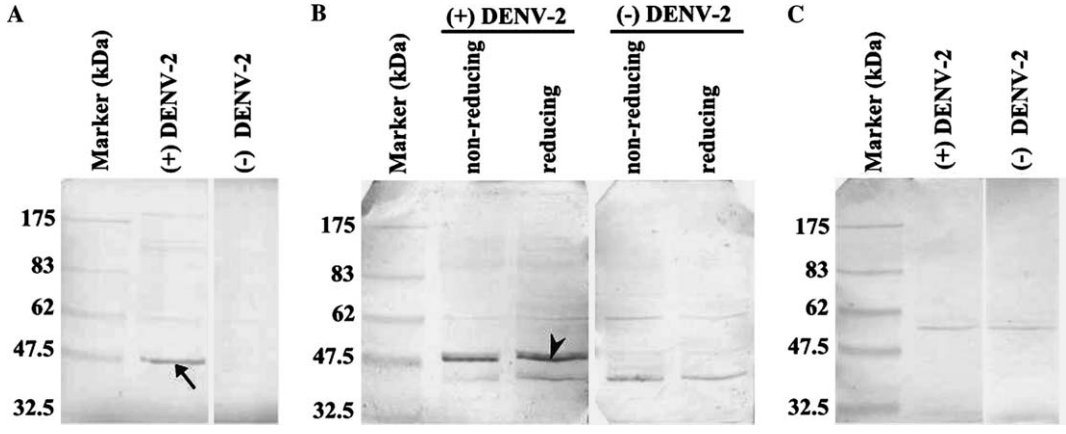

Fig. 1. Virus overlay protein-binding assay (VOPBA). Total solubilized C6/36 cells protein (A), cytosolic (B), and membrane (C) protein fractions at 50 μg/well were electrophoresed and transferred onto nitrocellulose membrane. The membrane was probed with sorbitol gradient-purified DENV-2 [(+) DENV-2] or sorbitol gradient-purified C6/36 cells protein [(−) DENV-2] and developed using anti-DENV-2 E monoclonal antibody. Arrow indicates the approximately 48 kDa DENV-2-binding protein (D2BP) and arrowhead shows the reduced form of the protein.

DENV-2 to the C6/36 cells D2BP was specific and was inhibited by competing D2BP.

The interaction between the 48 kDa C6/36 cells D2BP to DENV-2 E was subsequently examined using the gel-eluted D2BP. Sorbitol gradient-purified DENV-2 or sorbitol gradient-purified C6/36 cells proteins were separated by electrophoresis and transferred onto nitrocellulose membranes. The membrane was then probed using the gel-eluted D2BP fraction. Bound D2BP was detected using mice polyclonal antibodies raised against the gel-eluted D2BP fraction. A protein band with an estimated molecular mass of approximately 55 kDa (54.9 kDa) was uniquely detected in the sorbitol gradient-purified DENV-2 probed with the gel-eluted D2BP (Fig. 3, lane 1, arrow). No corresponding band was detected in control samples (Fig. 3, lanes 2 and 3). The corresponding blot developed using anti-DENV-2 E specific 3H5-1 monoclonal antibody showed the presence of a protein with an estimated molecular mass of approximately 55 kDa (Fig. 3, lane 4, arrowhead) comparable to the estimated size of DENV-2 E. The presence of DENV-2 E was later confirmed using mass spectrometry (peptide mass values 1045.577, 1252.683, 1425.05, 1594.376, and 1940.537; DENV-2 E GenBank Accession No. P14339). The polyclonal sera used in the far-Western studies however, also detected a number of other protein bands. As the sera were raised against the gel-eluted D2BP fraction, it suggested that the gel-eluted D2BP fraction contained several other antigenic C6/36 cells proteins and these proteins were also present in the sorbitol gradient-purified DENV-2 fractions. Results from a high resolution 2-DE performed using the gel-eluted D2BP and immunoblot developed using the polyclonal antibodies confirmed the presence of these other antigenic proteins (Fig. 4B).

Using mass spectrometry, peptide mass fingerprints of proteins bound to the membrane-immobilized DENV-2 E were determined. After subtracting all the common mass values, only 18 peptide mass values were obtained from samples treated with the gel-eluted D2BP (Table 1). A search of the peptide mass databases (NCBInr and Swiss-Prot) using only these peptide mass values showed that at least 13 of the peptide mass values consistently matched with the β-tubulin chain family of proteins from several different species including *Drosophila melanogas-*

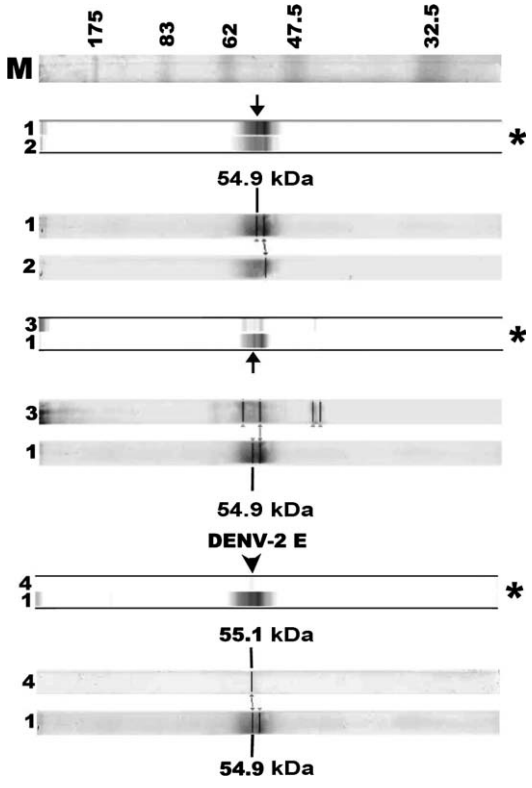

Fig. 3. Far-Western blotting of DENV-2 with the gel-eluted C6/36 cells D2BP as a probe. Schematic of a pairwise comparison of the strips analyzed using BioNumeric ver. 3.0 is shown. DENV-2 was electrophoresed and transferred onto nitrocellulose membrane. The nitrocellulose strips were probed with the gel-eluted D2BP (1) or without it (2). Similarly prepared control C6/36 cells protein strip was also probed with the gel-eluted D2BP (3). Bound D2BP was detected using polyclonal antibody derived from mice inoculated with the gel-eluted D2BP. DENV-2 E (4) was detected using anti-DENV-2 E monoclonal antibody. Arrowhead indicates the position of DENV-2 E at approximately 55 kDa. Arrows indicate D2BP bound to a protein band of approximately 55 kDa. Lanes with asterisk are the schematic images generated by BioNumeric ver. 3.0. M, molecular mass marker in kiloDaltons.

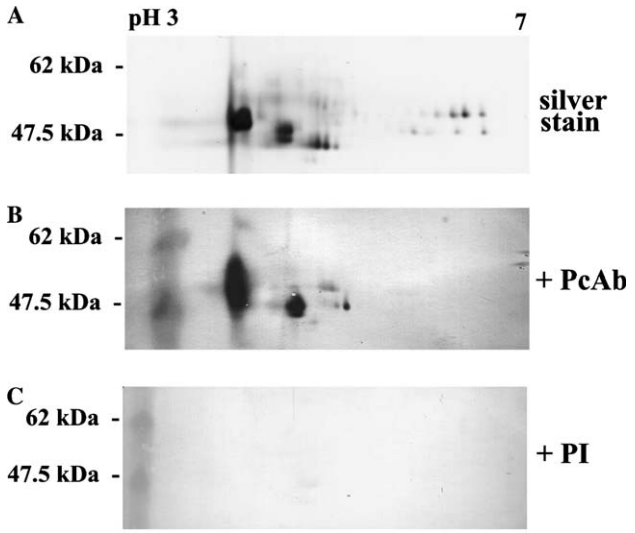

Fig. 4. Two-dimensional gel electrophoresis of gel-eluted C6/36 cells D2BP. The gel-eluted D2BP fraction (15 μg) was subjected to isoelectric focusing on a 7 cm ReadyStrip IPG strip pH 3–10. The focused proteins were then separated on 10% SDS–PAGE and the gels were either stained with silver nitrate (A) or electrophoretically transferred onto PVDF membrane (B,C). The PVDF membranes were probed with polyclonal antibodies (+ PcAb) raised in mice against the gel-eluted D2BP or with the pre-immune serum (+ PI). Alkaline phosphatase-conjugated goat anti-mouse IgG was used as the secondary antibody and the blot was developed with BCIP/NBT.

Table 1
Identification of C6/36 mosquito cells protein bound to DENV-2 E

Peptide mass value	*C. elegans* (NP_509313)	*R. norvegicus* (P04691)	*D. melanogaster* (Q24560)	*A. gambiae* (EAA05547)	*A. gambiae* (XP_309084)	*A. gambiae* (XP_314718)	*D. melanogaster* (P06603)	*Mus musculus* (P05214)	*A. gambiae* (EAA05546)
1025.540	*								
1039.579		*	*	*					
1078.436	*	*	*	*	*	*			
1130.383	*	*	*	*	*	*			
1143.335	*	*	*	*	*	*			
1159.406	*	*	*	*	*	*			
1229.269	*	*	*	*	*	*			
1246.283	*	*	*	*	*				
1341.257	*	*	*	*		*	†	†	†
1368.279		*							
1404.188		*	*			*			
1410.128							†	†	†
1592.880					*				
1601.067	*		*	*	*				
1611.133							†	†	†
1687.072							†	†	†
1718.047							†	†	†
1763.983							†	†	

Results shown are the peptide mass values of D2BP bound to DENV-2 E that matched tubulin of several different organisms and the uncharacterized proteins of *A. gambiae*.

Far-Western blotting of DENV-2 E with the gel-eluted C6/36 cells D2BP as a probe was performed as described in the legend to Fig. 3. * and † indicate the peptide mass values that matched β-tubulin and α-tubulin, respectively. Results from selected relevant organisms are presented. Mass tolerance used for the database search was ±1 Da.

ter, *Caenorhabditis elegans*, *Rattus norvegicus*, and a few uncharacterized proteins from the mosquito *A. gambiae*, the only mosquito genome database presently available (Table 1). Multiple alignments of the amino acid sequence of the uncharacterized *A. gambiae* proteins (GenBank Accession Nos. EAA05547, XP_309084, and XP_314718) with the β-tubulin chain family of proteins from other species (Table 1) revealed 81–94% amino acid sequence similarity (Fig. 5A), suggesting that the uncharacterized *A. gambiae* protein was also a β-tubulin chain. This supported the assertion that D2BP is β-tubulin or tubulin-like protein as β-tubulin chain from different species shared at least 60% amino acid similarity [15]. Using LC-MS/MS, two peptide sequences IREEYPDR and INVYYNEASGGK that matched to β-tubulin from various insect species including *Schistosoma japonicum* (MSDB Accession No. Q7Z114), *D. melanogaster* (MSDB Accession No. Q9V8V3), and *Bombyx mori* (MSDB Accession No. Q8T8B2) were also identified, supporting unequivocal presence of tubulin or tubulin-like C6/36 cells protein bound to DENV-2 E. The remaining at least five peptide mass values, on the other hand, showed consistent matches with the peptide mass values of α-tubulin chain of several different species including a yet to be characterized *A. gambiae* protein (Table 1). This uncharacterized *A. gambiae* proteins shared approximately 94–97% amino acid sequence similarity to α-tubulin protein from other species (Fig. 5B). Hence, added further support to the presence of tubulin or tubulin-like protein bound to DENV-2 E. The presence of both β- and α-tubulin chains in the D2BP fraction is not surprising considering that both the tubulin chains had almost similar molecular mass and p*I*, and therefore, were most likely to be co-eluted from the gel as the D2BP fraction. The β- and α-tubulin chains are also known to assemble as repeating α/β-tubulin heterodimers in their native state [16] and this complex can be precipitated by the rotavirus NSP4 recombinant protein [17].

Results presented in the study provide evidence that DENV-2 interacts directly with the C6/36 mosquito cells D2BP. The binding was specific as it was inhibited by soluble D2BP and using the highly sensitive MS and MS/MS methods, it was identified that D2BP had peptide mass values that matched tubulin or a tubulin-like 48 kDa cells protein. The binding was shown to be mediated by the virus E, as D2BP was identified bound directly to the approximately 55 kDa DENV-2 E by mass spectrometry. This finding represents perhaps the first time that a specific cellular protein bound to DENV-2 E was identified directly from a protein–protein-binding study, as in all other earlier studies only the molecular mass of cells proteins that interacted with DENV was shown [5–7,18] or inhibition of dengue virus infection was observed only following treatments with known antibodies [8].

Tubulin heterodimers, the major building block of cells microtubule, have long been known to be involved

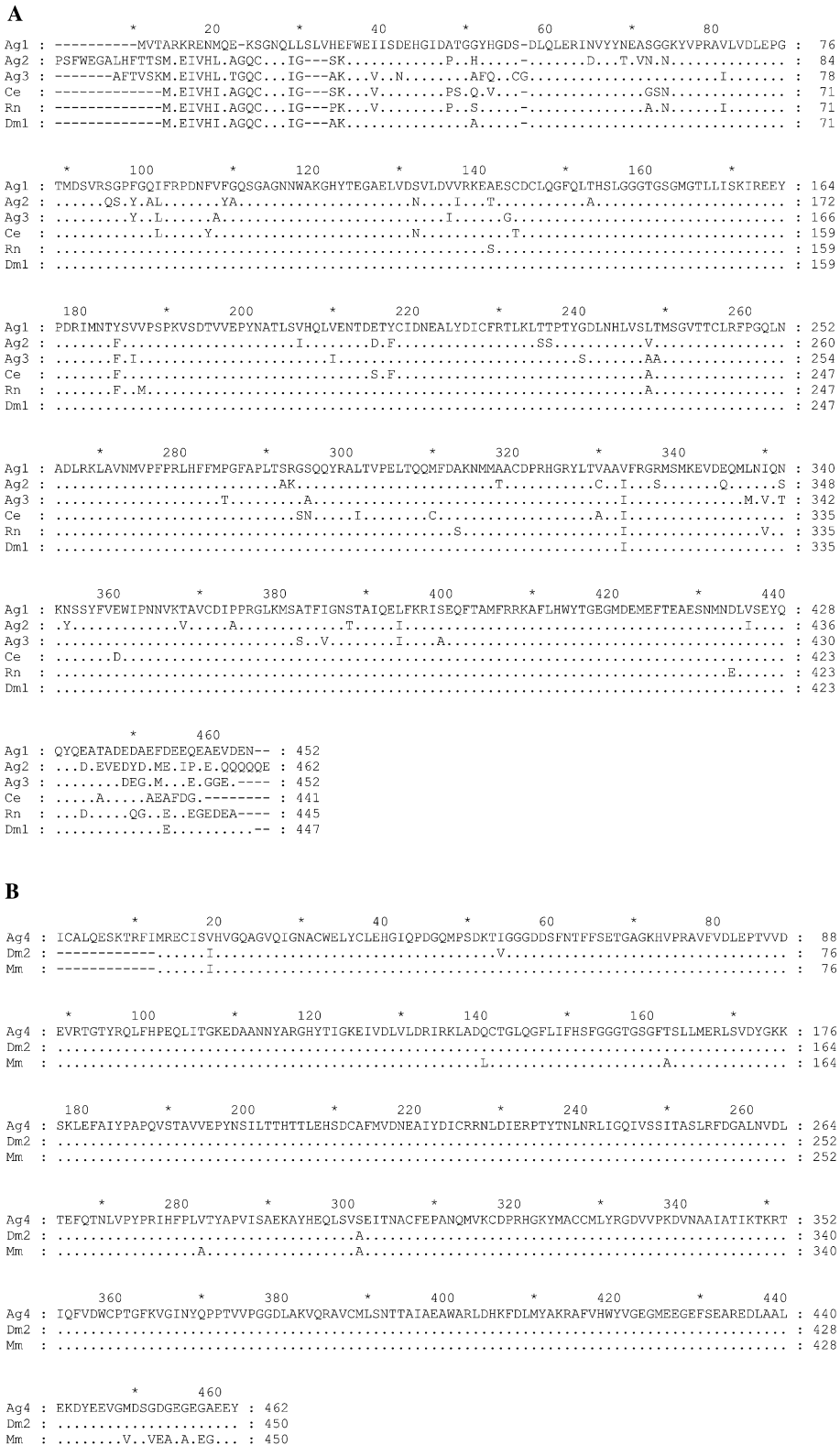

Fig. 5. Alignment of amino acid sequences of *A. gambiae* proteins that matched the 48 kDa C6/36 mosquito cells protein. The amino acid sequences were aligned against selected β- (A) and α-tubulin (B) chains using CLUSTAL X version 1.81 and displayed using GENEDOC version 2.5. Sequence identity is indicated by (●). Ag1 (*A. gambiae*, GenBank Accession No. EAA05547), Ag2 (*A. gambiae*, GenBank Accession No. XP_309084), Ag3 (*A. gambiae*, GenBank Accession No. XP_314718), Ce (*C. elegans*, GenBank Accession No. NP_509313), Rn (*R. norvegicus*, GenBank Accession No. P04691), Dm1 (*D. melanogaster*, GenBank Accession No. Q24560), Ag4 (*A. gambiae*, GenBank Accession No. EAA05546), Dm2 (*D. melanogaster*, GenBank Accession No. P06603), and Mm (*M. musculus*, GenBank Accession No. P05214).

in the assembly and transport of virus particles [19–21]. Some of these interactions are mediated by microtubule-associated protein but direct interaction of viral proteins with tubulin has also been reported [17,22,23]. In DENV-2 infection it is not likely that the binding of E to cells microtubules mimics that of the microtubule-associated proteins (MAPs) or the microtubule-binding domain of kinesin and dynein [24–27] as no DENV-2 E sequence similarity to these proteins was noted. It is not presently known, however, how the interaction between DENV-2 E and tubulin occurs and how the interaction relates to the entry of DENV into cells. It is possible that the initial binding of DENV-2 to C6/36 cells is mediated through a non-protein–protein interaction (i.e., mediated through heparan sulfate or lectin) and that the subsequent cellular changes allowing contact of E with tubulin facilitate internalization of the virus. Tubulin may also be involved in the assembly and transport of the virions to the extracellular environment later after infection. It is envisaged that tubulin of C6/36 cells could act as a scaffold for DENV-2 assembly through binding with E, comparable to the model proposed for the transport and assembly of West Nile virus [9] and also Kunjin virus [19,20]. Hence, the findings presented here highlighted the potential importance of tubulin or tubulin-like 48 kDa protein in DENV-2 infection of mosquito cells.

Acknowledgments

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