Identification and characterization of prohibitin as a receptor protein mediating DENV-2 entry into insect cells

Atichat Kuadkitkan, Nitwara Wikan, Chanida Fongsaran, Duncan R. Smith *

Molecular Pathology Laboratory, Institute of Molecular Biosciences, Mahidol University, Thailand

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Dengue is transmitted primarily by mosquitoes of the Aedes genus. Despite a number of studies, no insect dengue virus receptor protein has been clearly identified and characterized. Using a number of separation methodologies and virus overlay protein binding assays we identified the 35 kDa protein that segregated with susceptibility to dengue serotype 2 (DENV-2) infection in two mosquito species and two mosquito cell lines. Mass spectroscopy identified the protein to be prohibitin, a strongly conserved and ubiquitously expressed protein in eukaryotic cells. Antibody mediated inhibition of infection and siRNA mediated knockdown of prohibitin expression significantly reduced infection levels and subsequent virus production in both Aedes aegypti and Aedes albopictus cell lines. Confocal microscopy showed a significant degree of intracellular colocalization between prohibitin and DENV-2 E protein, and communoprecipitation confirmed that prohibitin interacts with dengue E. Prohibitin is the first characterized insect cell expressed dengue virus receptor protein.

Introduction

Dengue viruses (DENV) are the causative agent of the most common mosquito-borne viral disease in human and are distributed over 100 countries especially in tropical and subtropical regions (Gubler, 1997; Guzman and Kouri, 2002). Approximately 2.5–3 billion people live in areas potentially at risk of DENV transmission and each year there are estimated to be 100 million new infections resulting in around 24,000 deaths, and there is still no vaccine or antiviral agent available (Guzman and Kouri, 2002; Rigau-Perez et al., 1998). The consequences of DENV infection in humans ranges from a self-limiting illness known as dengue fever (DF) to a severe hemorrhagic fever (DHF) which can progress to dengue shock syndrome (DSS) (Halstead, 1988). DENV belong to the genus Flaviviridae, genus Flavivirus. There are four antigenically related viruses referred to as DENV-1, DENV-2, DENV-3 and DENV-4. The virion is characterized as a small (50 nm in diameter) enveloped particle containing a single positive sense polarity strand of RNA of approximately 11 kb in length (Chang, 1997). The mature DENV virion consists of three structural proteins: envelope (E), capsid (C) and membrane-associated protein (M) (Chang, 1997).

The principal vectors of DENV are mosquitoes of the Aedes genus, predominantly Aedes aegypti and Aedes albopictus, which are wildly distributed in both tropical and subtropical regions of the world, particularly in Asia and America. The female mosquito obtains DENV from a viremic animal or human during a blood meal resulting in the dengue viruses infecting and replicating in several mosquito tissues and eventually the salivary gland (Gubler and Rosen, 1976). The virus can be transmitted to eggs by vertical transmission (Diallo et al., 2000; Fontenille et al., 1997; Rosen, 1987).

In order to infect insect host cells, DENV utilizes its envelope (E) protein which contains the components responsible for host cell binding and fusion (Klasse et al., 1998) to interact with host cell receptors, followed by receptor-mediated endocytosis (Acosta et al., 2008; Mosso et al., 2008). Numerous studies in mammalian cells have reported cell surface receptors used by DENV to facilitate cell entry and the data suggests that receptor usage in mammalian cells is both cell type and serotype specific (Cabrera-Hernandez and Smith, 2005). Mammalian receptor proteins are predominantly proteins involved in either mediating cell: cell contacts such as DC-SIGN (Tassaneetrithep et al., 2003) and the 37/67 kDa high affinity laminin receptor (Thepparit and Smith, 2004) or are chaperone proteins such as HSP70/90 (Reyes-del Valle and del Angel, 2004). In contrast, no DENV insect receptor protein has been identified, although several proteins, predominantly characterized only by molecular weight (see Table 1), have been implicated in the virus entry process (Cao-Lormeau, 2009; Chee and AbuBakar, 2004; Munoz et al., 1998; Sakoonwatyoo et al., 2006; Salas-Benito and del Angel, 1997; Yazi Mendoza et al., 2002). Significantly, the majority of these studies have failed to distinguish between dengue virus binding or interacting proteins, of which there may be a number in a cell, and bona fide receptor proteins in that there is no functional analysis of the role of the identified protein in internalizing the DENV. It can be assumed that at least some of the bands identified will represent either non-specific binding proteins or cellular proteins such as chaperones. In an earlier...
study, we implicated a laminin binding protein expressed by *A. albopictus* C6/36 cells as mediating the entry of dengue serotypes 3 and 4 (Sakoonwatanyoo et al., 2006). However, whether this protein represents a receptor actually used in insects, or is a protein that becomes expressed as a consequence of cellular transformation or immortalization is capable of being used by DENV to enter cultured cells remains unclear. As both immortalization and transformation of cells involve both the expression of proteins not normally expressed, as well as the down-regulation of proteins normally expressed, results on virus entry cell culture systems need to be interpreted with caution. In a recent study, we showed that CCL-125 cells (an *A. aegypti* cell line) are DENV permissive (Wikan et al., 2009). This study therefore sought to determine whether there was a DENV-2 binding membrane protein expressed by both C6/36 and CCL-125 cells, as well as by a DENV-2 susceptible mosquito species (*A. aegypti*) but, critically, not by a DENV refractory mosquito species (*Culex quinquefasciatus*). Such a protein that segregated with susceptibility to dengue virus would represent a candidate dengue virus receptor protein and would be further characterized.

### Results

#### Investigation of DENV-2 membrane binding proteins

To investigate the DENV-2 binding proteins expressed on the surface of C6/36, CCL-125 and adult *A. aegypti* mosquito cells, 100 μg of membrane proteins of each cell type were separated on 10% SDS-polyacrylamide gels, in parallel with membrane proteins extracted from adult *C. quinquefasciatus* mosquitoes. The extracted membrane proteins were transferred to PVDF membranes, followed by incubation with DENV-2. The membranes were subsequently incubated with a pan specific anti-dengue virus E protein monoclonal antibody and a secondary anti-mouse IgG conjugated with horseradish peroxidase. As shown in Fig. 1a, a prominent binding band at approximately 35 kDa was present in C6/36, CCL-125 and adult *A. aegypti* mosquito cells, but was absent from the non-dengue susceptible *C. quinquefasciatus* membrane protein preparation. The segregation of the 35 kDa band with susceptibility to dengue infection is an indication that this protein may function as a dengue receptor protein. Several other dengue virus binding bands were clearly observed. None of these bands however segregated with susceptibility to infection, and may represent non-specific binding proteins.

#### Characterization of a DENV-2 binding protein

To characterize the 35 kDa binding protein, two dimensional Virus Overlay Protein Binding Assay (2D-VOPBA) was used. CCL-125 cell membrane proteins were separated through IPG strips and 10% SDS-polyacrylamide gels (Fig. 1b) and DENV-2 binding proteins detected as for 1D VOPBA (above). As shown in Fig. 1c, a virus binding spot was present at approximately 35 kDa, comparable to the result from the 1D VOPBA (Fig. 1a). The protein spot from a parallel gel was excised and sent for protein identification by mass spectrometry (Table 2).

The MS/MS data were searched against the MSDB database using the MASCOT search engine, the result showed that the candidate protein is prohibitin with coverage of some 84%.

To further confirm identity of the 35 kDa protein, ion-exchange column chromatography was used fractionate the membrane proteins before VOPBA analysis. CCL-125 membrane proteins were loaded onto a cation-exchange column and after washing, the proteins were eluted from the column using 2 column volume of a step gradient of cation buffer containing NaCl concentrations of 0.1, 0.5, 0.8 and 1.0 M. The eluted protein fractions were concentrated by TCA precipitation at 4 °C overnight and subsequently pelleted by centrifugation. The pellets were washed with pre-cooled acetone and finally resuspended with lysis buffer. The proteins were loaded onto two parallel 10% SDS-polyacrylamide gels and the separation was carried out at constant 100 V. After electrophoresis the proteins in one gel were transferred to PVDF membrane and VOPBA was performed as described above, while the parallel gel was stained with Coomassie Brilliant Blue (Fig. 1d). The result from the VOPBA analysis (Fig. 1e) showed a prominent dengue virus binding band at 35 kDa in the lanes of crude extract (C), wash fraction (W), 0.8 M NaCl fraction and 1.0 M NaCl fractions. Protein bands from the parallel gel were excised and sent for mass spectroscopy analysis and N-terminal sequencing. N-terminal sequencing of the binding protein from the 0.8 M NaCl fraction gave no sequence, suggesting the protein is N-terminally blocked. The remaining bands were identified as prohibitin with peptide coverages of 59% (from crude fraction; Table 2), and 58% (from wash fraction; Table 2). The third band from the 1.0 M NaCl fraction did not have prohibitin as a major candidate, but had peaks present in the peak list that corresponded to some 22% coverage of prohibitin. The lack of a clear identification of prohibitin in the 1.0 M NaCl fraction might indicate a large number of similarly sized proteins eluting at this molarity. Overall however, the 35 kDa DENV-2 binding band was therefore identified as prohibitin in four separate analyses, with isolation by two independent methodologies.

Prohibitin is composed of 2 proteins, with molecular weights of ~30 kDa (prohibitin 1 or PHB1) and ~37 kDa (prohibitin 2 or PHB2) which share more than 50% amino-acid identity (Mishra et al., 2006). The two proteins function together as hetero-oligomers which is required for protein stability, and loss of one prohibitin protein in the cell leads to loss of the other protein (Artal-Sanz et al., 2003; Berger and Yaffe, 1998; He et al., 2008; Kasashima et al., 2008; Merkwirth et al., 2008), and this is controlled at the level of the protein, and is independent of RNA levels (Kasashima et al., 2008). While the mass spectroscopy results did not allow unambiguous differentiation between prohibitin 1 and prohibitin 2, our results are consistent with dengue binding to prohibitin 2, given the larger molecular weight of this protein.

The role of prohibitin in DENV-2 infection

To investigate the role of prohibitin in dengue virus infection, siRNA mediated-gene silencing was utilized. Attemps were initially undertaken to directly down-regulate prohibitin 2 but, despite extensive attempts, the prohibitin 2 message proved refractory to silencing. The reasons for some mRNAs being refractory to silencing

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<th>Proposed mosquito dengue virus receptor proteins.</th>
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<td>A. aegypti</td>
<td>Midgut, ovary and salivary gland cell, eggs, larvae and pupae cell extract</td>
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<td>A. polynesiensis</td>
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are unclear, but probably relate to the formation of stable secondary structures in the mRNA (Kurreck, 2006). However, given that stability of prohibitin 2 protein requires the presence of prohibitin 1 protein (Artal-Sanz et al., 2003; Berger and Yaffe, 1998; He et al., 2008; Kasashima et al., 2008; Merkwirth et al., 2008), silencing of prohibitin 1 at the mRNA level would functionally silence prohibitin 2 at the protein level.

To down-regulate the expression of PHB1 in C6/36 and CCL-125 cells, each cell type was transfected with 500 pmol of siPHB1_3 in parallel with lipofectamine alone (mock transfection). The cells were harvested for RNA extraction every day for 5 days post-transfection (p.t.). Multiplex PCR was performed to detect the expression level of PHB1 and PHB2 concurrently, and the results were analyzed by agarose gel electrophoresis. As the interdependence of prohibitin is

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**Fig. 1.** VOPBA analysis of DENV-2 binding to insect cell membrane proteins. (a) Membrane proteins from C6/36 cell (lane 1), CCL-125 cell (lane 2), whole adult Aedes aegypti (lane 3) and whole adult Culex quinquefasciatus (lane 4) were subjected to 10% SDS-PAGE and transferred to PVDF membranes. Virus overlay was used to detect the position of DENV-2 binding proteins. A prominent DENV-2 binding band that segregated according to DENV-2 susceptibility was present at approximately 35 kDa (black arrow). (b and c) CCL-125 membrane proteins were extracted and subjected to first dimension isoelectric focusing on 7 cm IPG (pH 3–10) strips and second dimension 10% SDS-PAGE. One of two parallel gels was stained with Coomassie Brilliant Blue R-250 (b), and the second was transferred to a PVDF membrane and the position of dengue virus binding spots determined by virus overlay (c). A virus binding spot was present at approximately 35 kDa (arrows, b, c). The corresponding spot on the parallel Coomassie stained gel was excised and analyzed by mass spectrometry. (d,e) CCL-125 membrane proteins were fractionated by cation-exchange chromatography with elution at the indicated NaCl concentrations. Samples were concentrated and run on two parallel gels (d, e) together with un-fractionated crude extract (lane c) and the eluted wash fraction (lane w). One gel was stained Coomassie Brilliant Blue R-250 (d) while the second was transferred to solid matrix support before detection of dengue virus binding bands (e). The 35 kDa band (e, arrowed) from crude, wash and 1.0 M NaCl were excised from the parallel Coomassie Brilliant Blue R-250 stained gel and subjected to mass spectroscopy analysis.

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found at the protein level but not at the level of the RNA (Artal-Sanz et al., 2003; Berger and Yaffe, 1998; Kasashima et al., 2006). PHB2 mRNA level was used as an internal control in silencing PHB1. Results (Fig. 2) showed a constant signal for PHB1 and PHB2 in mock transfected cells while a significant reduction in the level of PHB1 mRNA was observed in response to transfection with siPHB1_3 by days 3 to 5 p.t. and the greatest reduction was observed on day 4 p.t., which was selected as the optimal time point for subsequent experiments.

CCL-125 cells were transfected with siPHB1_3 as described above, as well as with siGP as a siRNA control as well as mock infected with lipofectamine alone. On day 4 post-transfection, cells were harvested and proteins extracted which were then separated by electrophoresis as well as with siGFP as a siRNA control as well as mock infected with lipofectamine alone. On day 4 post-transfection, cells were harvested and proteins extracted which were then separated by electrophoresis in 10% polyacrylamide gel transferred to solid matrix support following which membranes were probed with anti-human prohibitin 1 and anti-human prohibitin 2 antibodies as well as actin as an internal control. While the anti-human prohibitin 1 antibody was specific for prohibitin 1 in CCL-125 cells, the anti-human prohibitin 2 antibody cross reacted with both prohibitin 1 and 2 and showed a slightly higher affinity for prohibitin 1 (Fig. 2b). However, reduction in the expression of both prohibitin 1 and prohibitin 2 was observed in the siPHB1_3 transfected cells, while no reduction was seen in normal control, mock transfected or siGP transfected cells (Fig. 2b).

CCL-125 and C6/36 cells were mock transfected and transfected with both siPHB1_3 and siGP as above and on day 4 post-transfection were infected with DENV-2. Given that the times for de novo DENV-2 production are different for infected C6/36 and CCL-125 cells, namely 17 h for DENV-2 from C6/36 cells (Sakoonwatanyoo et al., 2006) and 20 h for DENV-2 from CCL-125 cells (Wikan et al., 2009), infected cells were cultured for one and a half virus replication cycle for each cell type (25.5 h post-infection for C6/36 cells and 30 h for CCL-125 cells) which maximizes virus output but ensures that the virus produced comes only from the initial round of infection. Subsequently the cells were harvested for protein extraction and Western blot analysis, while the culture medium was collected for standard plaque assay. The result of western blot assay showed a reduction of PHB1 in CCL-125 cells and a reduction of PHB2 in both C6/36 and CCL-125 (Fig. 3a) cells. Interestingly the antibody used failed to detect prohibitin 1 in C6/36 cells (data not shown), but given that this antibody is an anti-human prohibitin antibody this is perhaps not unsurprising. In the VOPBA analysis, the result showed a decrease in DENV-2 binding in the siPHB1_3 treated samples as compared to the siGP or mock transfected samples (Fig. 3a) confirming that prohibitin is, at least, a DENV-2 binding protein and that it is prohibitin 2, and not prohibitin 1 that binds DENV-2. The levels of reduction of prohibitin expression in both siPHB treated C6/36 (approximately 50%) and CCL-125 cells (approximately 60%) were equal to the reduction seen in the VOPBA analysis (Fig. 3b). Extracellular virus production was significantly decreased by approximately 90% in both CCL-125 and C6/36 cells treated with siPHB1_3 (Figs. 3c, d) compared to both siGP and mock transfected cells.

### Antibody mediated inhibition of DENV-2 entry

To more precisely define the role of prohibitin in mediating DENV-2 internalization, C6/36 and CCL-125 cells were pre-incubated with various amounts of a polyclonal anti-PHB2 antibody (sc-67045, Santa Cruz Biotechnology) or no antibody or a control polyclonal antibody against STAT5 (sc-28685, Santa Cruz Biotechnology) before infection with DENV-2 as described above. The anti-STAT5 antibody was selected as both it and the anti-PHB2 antibody are rabbit polyclonal antibodies. The infected cells were again cultured for one and a half virus replication cycles of each cell type (25.5 h post-infection for C6/36 and 30 h for CCL-125 cells) and the culture medium and the cells were collected separately for assay of viral extracellular and intracellular virus levels, respectively. The experiments were done independently in triplicate, with duplicate plaque assay. Intracellular virus levels showed a significant difference between prohibitin antibody treated cells and controls in both cell types (Figs. 4a, b) with a reduction of approximately 90%, similar to the reduction seen in the silencing experiments. Similar to intracellular virus level, extracellular virus levels show a significant decrease of viral production in antibody pre-treated cells (Figs. 4c, d), although while C6/36 cells showed a reduction of 90% consistent with the silencing experiments, CCL-125 cells showed a slightly smaller reduction of only 75%. The results strongly suggest that PHB2 act as a DENV-2 receptor present on the surface of both cell types. Moreover as both down-regulation of prohibitin through siRNA and blocking of cell surface expressed prohibitin with an antibody produce approximately 90% reductions in both virus output and intracellular virus levels (with the exception of antibody inhibition of CCL-125 cells on virus output) the results would suggest that the major contribution of prohibitin is as a receptor protein as opposed to a chaperone protein.

The infection of DENV-2 in the presence of anti-PHB antibodies as well as the specificity of prohibitin for dengue was confirmed by flow cytometry. C6/36 cells were pre-incubated with antibodies directed against PHB2, integrin αv/β5 or no antibody
before infection with DENV-2 at MOI 10 or with STAT5, PHB2 or no antibody before infection with JEV at MOI 10. After incubation for 15 h for JEV infected cells or 16 h for DENV-2 infected cells, cells were subjected to flow cytometry after staining with primary antibodies directed against dengue E protein or an antibody specific for flavivirus E proteins. Results (Fig. 5) showed that anti-PHB2 antibodies specifically reduced the number of infected cells by approximately 50% while not affecting JEV entry.

To provide further evidence that PHB acts as a receptor protein, we investigated binding of DENV-2 to C6/36 cells in the presence or absence of anti-PHB2 antibodies. C6/36 cells were either pre-treated with a rabbit polyclonal antibody directed against PHB2 or not pre-treated before incubation with DENV-2 or mock infection. All steps were undertaken at 4 °C. DENV-2 was visualized by successive incubations with a pan specific anti-dengue E protein monoclonal antibody followed by a FITC conjugated secondary antibody and observation under a fluorescent microscope. Results showed a considerable reduction of DENV-2 binding when cells were pre-treated with the anti-PHB antibody as compared to non-treated cells (Fig. 6), confirming that DENV-2 binds to PHB at the cell surface and that blocking of PHB2 blocks attachment of the virus.

Intracellular colocalization between prohibitin and DENV-2 E protein

To determine whether there is an intracellular interaction between prohibitin and DENV-2 E protein, C6/36 cells were seeded on glass cover slips and infected with DENV-2 in parallel with mock infected cells. At 24 h post-infection, the cells were fixed with pre-cooled methanol and then permeabilized with 0.3% Triton X-100 in PBS. Subsequently the cells were incubated with an anti-dengue virus E protein monoclonal antibody and a goat polyclonal anti-PHB antibody or anti-dengue virus E protein monoclonal antibody and a rabbit polyclonal anti-PHB2 antibody followed by the appropriate secondary antibody conjugated with fluorescence dyes. The results (Fig. 7) show colocalization between both DENV-2 and PHB1 and DENV-2 and PHB2.

Intracellular interaction between prohibitin and dengue E protein

To demonstrate a direct interaction between prohibitin and DENV-2, coimmunoprecipitation experiments were undertaken. C6/36 cells were either infected with DENV-2 or mock infected and at 3 days post-infection cells were harvested and lysed before incubation with a protein G sepharose slurry to remove non-specific binding proteins, followed by the addition of a rabbit polyclonal anti-PHB2 antibody and subsequently a second round of protein G sepharose slurry, before coimmunoprecipitated proteins were run on a polyacrylamide gel. After electrophoresis, proteins were transferred to solid matrix together with crude lysate fractions, and dengue E protein was detected with a monoclonal antibody. Results (Fig. 7c) showed that dengue E protein was coimmunoprecipitated together with prohibitin.

Discussion

To date only two studies have reported the partial characterization of putative DENV receptor proteins expressed by C6/36 cells (an A. albopictus cell line). In 2004, using VOPBA coupled with mass spectrometric analysis Chee and AbuBakar (2004) identified a 48 kDa tubulin-like protein able to bind DENV-2. However a DENV-2 binding band was found only in the cytosolic protein fraction and not in membrane fraction of C6/36 cells. It is possible therefore that tubulin...
may be involved in the later steps of DENV internalization, but not the initial binding. Using western blot, VOPBA analysis and an inhibition of infection assay, we have previously shown evidence that a laminin binding protein is a candidate DENV-3 and DENV-4 receptor protein (Sakoonwatanyoo et al., 2006).

In this study, again using VOPBA analysis, we found a prominent DENV-2 binding band at approximately 35 kDa that was observed in membrane proteins from C6/36 and CCL-125 cells and from adult *A. aegypti* mosquitoes, but critically, was not present in membrane proteins from adult *C. quinquefasciatus*, a non-susceptible species.

While one report has shown that *C. quinquefasciatus* can be infected with DENV-2 (Vazeille-Falcoz et al., 1999), this was only obtained using intrathoracic injection of extremely high titers of DENV-2 and no infection was observed when *C. quinquefasciatus* mosquitoes were exposed orally to DENV-2 under conditions in which 100% of *A. aegypti* mosquitoes became infected. This would be consistent with other authors who have shown *C. quinquefasciatus* to be a non-susceptible species to dengue infection (Huang et al., 1992). Overall, the evidence suggests that *C. quinquefasciatus* may be infected under conditions of exposure to extremely high virus titers, it is unlikely to play a role as a dengue vector, and our use of this species as a non-susceptible species is not unwarranted.

![Fig. 3. DENV-2 infection of prohibitin silenced insect cells. (a) Membrane proteins from control or prohibitin silenced DENV-2 infected C6/36 or CCL-125 cells were subjected to 10% SDS-PAGE and transferred to PVDF membranes before virus overlay. Filters were subsequently stripped and analyzed for levels of prohibitin 1, prohibitin 2 and actin. A cross reacting PHB1 band is indicated (*). Intensity of prohibitin 2 (gray bars) and VOPBA band (cross-hatched bars) was determined by densitometry and is plotted after normalization against actin (b). Virus production from mock transfected, siGFP transfected and siPHB1_3 transfected C6/36 or CCL-125 cells (c) or CCL-125 cells (d) infected with DENV-2. Virus yield is shown at one and a half DENV-2 replication cycles post-infection and plotted as virus titer (PFU/ml) ± SD. The experiments were undertaken independently in triplicate with duplicate plaque assays. The statistical significance of virus output compared with the mock transfected cells is shown. *Signifies P<0.001 as compared to mock transfected cells.

As such, the 35 kDa protein segregates with susceptibility to DENV-2 infection, suggesting it is a viable candidate for a receptor protein. Interestingly, the band we previously identified as a laminin binding protein (Sakoonwatanyoo et al., 2006) was present in all membrane extracts, and indeed, a number of non-specific binding bands were present in the range 55 to 200 kDa (Fig. 1). While the 35 kDa protein band identified in our study is distinct from proteins that have been implicated previously by ourselves (Sakoonwatanyoo et al., 2006) or by other groups (Chee and AbuBakar, 2004; Munoz et al., 1998; Salas-Benito et al., 2007; Salas-Benito and del Angel, 1997; Yazi Mendoza et al., 2002) and as such represents a novel candidate, evidence for a 35–37 kDa DENV binding protein in *A. aegypti* insect tissues is seen in the VOPBA analysis of at least two other groups (Cao-Lormeau, 2009; Yazi Mendoza et al., 2002).

In our VOPBA analysis, we clearly detected the 40/45 kDa DENV binding protein previously reported as putative DENV-4 receptor (Reyes-del Valle and del Angel, 2004; Salas-Benito and del Angel, 1997) in C6/36 and CCL-125 membrane proteins, however these protein bands were not present in our preparations of membrane proteins from adult *A. aegypti* mosquitoes (Fig. 1). It is possible that as C6/36 and CCL-125 cells are derived from mosquito larvae different receptor proteins are expressed at different stages of maturity.
Mass spectroscopy fingerprint analysis of the 35 kDa candidate receptor protein after isolation by two independent techniques identified the candidate protein as prohibitin. Prohibitin (PHB) is a ubiquitously expressed and highly conserved protein in eukaryotic cells (Morrow and Parton, 2005). Initial investigations of PHB showed that this protein acted as an inhibitor of cell proliferation and hence the name prohibitin (McClung et al., 1989). PHB is composed of 2 proteins with different molecular weights. Prohibitin1 (PHB1) has a mass of ~30 kDa while prohibitin2 (PHB2) has a mass of ~37 kDa (Mishra et al., 2006). PHB1 and PHB2 are highly homologous to each other and share more than 50% identical amino-acid residues (Mishra et al., 2006). These two proteins have a propensity to form oligomers and hetero-oligomerization has been proposed to be required for the stability of the proteins. The loss of PHB1 protein leads to the loss of PHB2, and vice versa (Artal-Sanz et al., 2003; Berger and Yaffe, 1998; He et al., 2008; Kasashima et al., 2008; Merkwirth et al., 2008). However, this interdependence of PHB is found at the level of the protein, and not at the level of the RNA (Kasashima et al., 2006). PHB1 is present in many compartments of the cell, mainly the mitochondria (Ikonen et al., 1995; Merkwirth and Langer, 2009; Nijtmans et al., 2000) but is also present in the cytoplasm and nucleus (Thompson et al., 2001; Wang et al., 2002). In 2004, Sharma and Qadri (Sharma and Qadri, 2004) reported that the Vi capsular polysaccharide of Salmonella typhi can bind to PHB on a human intestinal epithelial cell membrane resulting in S. typhi resistance to phagocytosis and attenuation of IL-8 production via the MAPK pathway. In the same year Koloinin et al. (2004) identified a peptide motif which binds to PHB on the white fat vasculature of mice. Collectively these two studies indicate that PHB can function as a cell surface expressed binding protein.

Previous studies have also elucidated a chaperonin function for prohibitins in mitochondria (Nijtmans et al., 2000, 2002). As such, the reduction of DENV-2 production seen in prohibitin silenced cells could reflect the reduction of chaperone activity of PHB, and indeed, colocalization analysis suggested that there is a significant intracellular interaction between prohibitin and dengue E protein, and this was confirmed by coimmunoprecipitation experiments, providing confirmation of a physical interaction between dengue E protein and prohibitin. Pertinently however, DENV-2 entry was significantly reduced in the presence of anti-prohibitin antibodies, suggesting a direct interaction at the cell surface, which is supported by the reduction of DENV-2 binding to cells pre-treated with anti-prohibitin antibodies. The possibility of multiple interactions between DENV receptor proteins and DENV proteins does however have precedents. We have previously identified the chaperone protein (GRP78) as a DENV-2 receptor protein in liver cells (Jindadamrongwech et al., 2004) and more recently the GRP78 has been implicated as a direct DENV E protein chaperone in various cell types (Wati et al., 2009). Given the multifunctional nature of many cellular proteins, it is perhaps not unsurprising that viruses can interact with particular proteins at multiple stages in its replication cycle.

We have shown here that DENV-2 interacts with Aedes prohibitin but not Culex prohibitin, and moreover that the interaction is specific for DENV-2 and not for a closely related flavivirus, Japanese encephalitis virus despite prohibitin being a highly conserved protein. As noted previously, oligomerized PHB1/PHB2 proteins are believed to form ring structures (Ikonen et al., 1995; Merkwirth and Langer, 2009; Nijtmans et al., 2000) with a diameter of 20 to 25 nm. The regular arrangement of the PHB1/PHB2 heterodimers around the ring provides the possibility of...
multiple interactions with the larger (60 nm) DENV, with a portion of the DENV virion possibly sitting in the space in the ring. As the dengue virion is composed of multiple E subunits (Khun et al., 2002) it is possible that individual E proteins in the virion could interact with individual PHB1/PHB2 heterodimers. In this way species and virus specificity could be modulated by relatively small changes in the highly conserved prohibitin protein. In this way, the small number of non-conserved amino-acid residues between PHB2 from A. aegypti and C. quinquefasciatus may be the primary mediators of virus specificity. The high conservation of PHB enabled the use of anti-human PHB antibodies in the experiments here. While the respective anti-human PHB polyclonal antibodies are specific for human PHB1 and PHB2, we observed cross reactivity with the anti-human PHB2 polyclonal antibody detecting both PHB1 and PHB2 from both A. albopictus and A. aegypti, while the anti-human PHB1 antibody did not detect A. albopictus PHB1. A. aegypti and human prohibitins are approximately 75% homologous (both PHB1 and PHB2), and as noted previously human PHB1 and PHB2 share 50% amino-acid identity (Mishra et al., 2006). Differences in cross reactivity

**Fig. 5.** Antibody mediated inhibition of infection assessed by flow cytometry. C6/36 cells were pre-treated with antibodies against integrin αV/β5 or PHB2 or no antibody (a, c) before infection with DENV-2 at MOI of 10, or treated with antibodies against STAT5 or PHB2 or no antibody (b, d) before infection with JEV at MOI of 10. After 15 (JEV) or 16 (DENV-2) hours percent infection was determined by flow cytometry. Raw data profiles (a, c) and tabular analysis (b, d) from three independent experiments are shown.

**Fig. 6.** Analysis of DENV-2 binding to C6/36 cells. C6/36 cells were either mock infected (Mock) or infected with DENV-2 in the absence (DEN-2) or presence of antibodies directed against PHB2 (DEN-2 + anti-PHB Ab) at 4°C. Samples were subsequently incubated with a monoclonal directed against dengue E protein (HB114) and an appropriate secondary antibody before visualization under a fluorescence microscope.
between the anti-human PHB2 polyclonal antibody and PHB2 from A. aeegyti and A. albopictus may also explain the somewhat unequal inhibition seen between C6/36 and CCL-125 cells when the antibody is used to block virus entry. Until the development of specific antibodies against insect prohibitins, research in this area will still need to use the sub-optimal anti-human monoclonal antibodies.

Fig. 7. Interaction of prohibitin with DENV-2 E protein. (a, b) C6/36 cells were grown on glass cover slips and either mock infected or infected with DENV-2 and examined for the localization of PHB1 (a; red) or PHB2 (b; red) and DENV-2 E protein (green). Fluorescent signals were observed using an Olympus FluoView 1000 confocal microscope. Representative, non-contrast adjusted unmerged and merged images are shown. Merged images have bright-field view added. (c) C6/36 cells were either mock infected or infected with DENV-2 for 3 days after which cells were lysed. Lysates were either analyzed directly or after coimmunoprecipitation using an anti-PHB2 polyclonal antibody for the presence of DENV-2 E protein.
This study has identified and characterized the first insect cell expressed DENV-2 receptor protein, prohibitin. It is clear however that prohibitin interacts with DENV-2 at several points in its life cycle, and our identification of prohibitin as a receptor protein relies primarily upon the significant levels of antibody mediated inhibition of infection seen in the presence of anti-prohibitin antibodies, and this was confirmed by two different methodologies. The gold standard of receptor identification is using the identified receptor protein to make a non-permissive cell line permissive. However, in the absence of a well characterized dengue non-permissive cell line this becomes problematic. In addition, prohibitin acts as a complex and, to obtain permissiveness to dengue in a non-permissive cell line, it would probably be necessary to drive expression of both PHB1 and PHB2 over endogenous prohibitin expression. Further, more detailed studies will be required to dissect out the contributions of prohibitin at different stages of the dengue virus life cycle. At this point in time we have only characterized the interaction with DENV-2, and as such further studies will seek to determine if this protein is used by other dengue serotypes. As a highly conserved and ubiquitous protein, it is also possible that this protein interacts with DENV in infections in mammalian cells, which would point towards conserved pathways in DENV replication in insect and mammalian cells.

Material and methods

Cell culture and virus stocks and titration

Dengue virus serotype 2 (strain 16681) was propagated in C6/36 cells as described elsewhere (Sakoonwatanyoo et al., 2006). Japanese encephalitis virus (Beijing-1 strain, accession No. L48861) was propagated as described previously (Boonsanay and Smith, 2007). C6/36 cells were cultured at 28 °C in minimum essential medium (MEM, Gibco™ Invitrogen, Carlsbad, California, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco™ Invitrogen), and 100 U/ml of penicillin and 100 µg/ml streptomycin (PAA Laboratories, Linz, Austria). CCL-125 cells were cultured at 28 °C in L-15 medium (Leibovitz’s L-15 medium, Gibco™ Invitrogen) supplemented with 20% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. LLC-MK2 cells were cultured at 37 °C/5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM, Gibco™ Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. All work with DENV infected cells was undertaken under BSL2 containment. Plaque assays were undertaken as previously described (Sithisarn et al., 2003).

Mosquitoes

The two species of mosquitoes, A. aegypti (Linnaeus) and C. quinquefasciatus (Say), were laboratory-reared and maintained at 28 °C until adults. The A. aegypti mosquitoes were from a colony originally established by the Department of Medical Sciences, National Institute of Health, Thailand from eggs collected in Bangkok, Thailand in 1989 and laboratory-reared since then. The C. quinquefasciatus mosquitoes were from a colony originally established by the Department of Medical Sciences, National Institute of Health, Thailand from immature stages (larvae/pupae) collected in Bangkok, Thailand in 1978 and laboratory-reared since then.

C6/36 and CCL-125 cell membrane protein preparation

Cell membrane preparations were undertaken as previously described (Theparat and Smith, 2004). Briefly, twenty 100 × 20-mm culture plates of confluent C6/36 or CCL-125 cells were washed twice with Tris-buffered saline (TBS, 50 mM Tris–HCl [pH 7.6], 150 mM NaCl). The cells were then collected by scraping and pelleted by centrifugation at 610 × g for 3 min followed by resuspension in pre-cooled lysis buffer (Buffer SF, 0.5% Triton X-100, 60 mM DTT, 2 mM PMSF). The cells were lysed by vortexing and sonicating at 4 °C. The solution was centrifuged at 610 × g to remove cell debris. The supernatant was transferred into a new tube. The pellets were lysed again until no intact cells remained. The supernatants were pooled and centrifuged at 6000 × g for 5 min to remove membranous organelles and then at 20,800 × g for 10 min to pellet the plasma membrane proteins. The pellet was resuspended in buffer SF and protein concentration was determined by the Bradford assay (Bradford, 1976). The proteins were kept at −80 °C until used.

Mosquito membrane preparation (whole insects)

Pooled adult mosquitoes (1.5–2 g) were ground in liquid nitrogen and then suspended in pre-cooled lysis buffer (Buffer II, 20 mM Tris–HCl [pH 7.5], 300 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2 mM PMSF). Subsequent membrane protein extraction was undertaken as described above.

Western blotting and virus overlay protein binding assay (VOPBA)

Approximately 100 µg of membrane proteins from C6/36 cells, CCL-125 cells, A. aegypti and C. quinquefasciatus mosquitoes were separated by electrophoresis through 10% SDS-polyacrylamide gels and proteins were transferred to solid matrix support (PVDF membranes) using the Mini Trans-blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, Richmond, CA). The membranes containing transferred proteins were blocked with 5% skim milk in TBS buffer for 2 h at room temperature. For Western blotting, the membranes were incubated with a 1:500 dilution of a goat polyclonal anti-prohibitin 1 (PHB1) antibody (sc-18196, Santa Cruz Biotechnology, Santa Cruz, CA) or a 1:500 dilution of a rabbit polyclonal anti-PHB2 antibody (sc-67045, Santa Cruz Biotechnology) or a 1:500 dilution of a goat polyclonal anti-actin antibody (sc-1616, Santa Cruz Biotechnology) at room temperature for 4 h. The membranes were then incubated with a 1:3000 dilution of a horseradish peroxidase-conjugated rabbit anti-goat IgG (31402, Pierce, Rockford, Ill, USA) or a 1:4000 dilution of a horseradish peroxidase-conjugated goat anti-rabbit IgG (31460, Pierce) at room temperature for 1 h. Finally, the signals were developed by using the ECL Plus™ Western blotting analysis kit (Amersham Bioscience, NJ, USA), followed by autoradiography. For VOPBA, the membranes were incubated with 106 PFU of DENV-2 at 4 °C over night. After three washes with TBS buffer, the membrane was further incubated with a 1:100 dilution of a pan specific anti-dengue virus E protein monoclonal antibody, produced by hybridoma cell line HB114 (Henchal et al., 1982), in 5% skim milk in TBS buffer for 2 h at room temperature. Then the membranes were incubated with a 1:8000 dilution of a horseradish peroxidase-conjugated rabbit anti-mouse IgG secondary antibody (Sigma Chemical Co., St. Louise, MO, USA) in 5% skim milk in TBS buffer for 1 h at room temperature. The signal was developed as described above.

Two-dimensional virus overlay protein binding assay

Membrane proteins of CCL-125 cells were prepared for 2 dimensional gel electrophoresis using the 2-D Sample Prep for Membrane Proteins kit (Pierce, Rockford, Ill, USA) according to the manufacturer’s instruction. Then 150 µg of membrane proteins were loaded on IPG strips (Immobiline™ DryStrip pH 3–10 NL, 7 cm, Amersham Bioscience) and the rehydration step was performed at 50 µA for 15 h using the IPGphor IEF system (Amersham Bioscience). Subsequently, IEF was carried out for 30 min at 300 V, 30 min at 1000 V and 2 h at 5000 V with a step-and-hold gradient until it reached to 8000 volt-hours. The strips were equilibrated with 5 ml of equilibration buffer (2% SDS, 50 mM Tris–HCl [pH 8.8], 6 M urea, 30% glycerol, 0.002% bromphenol blue) containing 50 mg of dithiothreitol (DTT) for 15 min at room temperature followed by 5 ml of equilibration buffer containing 125 mg of iodoacetamide for 15 min at room temperature. The equilibrated strips were placed on the top surface of a 10% SDS-polyacrylamide gel.
Molecular weight markers (161–0317, Broad range protein marker, Bio-rad) were applied to small pieces of chromatography paper and inserted next to each strip on the top of the gel, then the strips and marker were sealed with hot sealant (0.5% agarose in 1× SDS-PAGE running Buffer, 0.001% Bromophenol blue). The second dimension was carried out at a constant 100 V. The gels were stained with Coomassie Brilliant Blue R-250 or transferred to PVDF membranes for VOPBA with DENV-2 as described above.

Ion-exchange column chromatography and virus overlay protein binding assay

Membrane proteins from CCL-125 cells (1.5 mg) prepared as described above were applied onto cation-exchange chromatography columns (Macro-prep High S Support, Bio-Rad Laboratories). The columns were washed with two column volumes of NaCl-free cation buffer (20 mM Na2HPO4) and then proteins were eluted with two column volumes of cation buffer containing 0.1, 0.5, 0.8, 1.0 M NaCl respectively. Trichloroacetic acid (TCA; Sigma Chemical Co.) was added to eluted fractions to precipitate proteins and samples were incubated at 4 °C overnight. Solutions were centrifuged at 15,000×g for 15 min at 4 °C. The pellets were washed with pre-cooled acetone and then centrifuged at 15,000×g for 15 min at 4 °C. After the supernatant was removed, the proteins were resolubilized in lysis buffer and divided into 2 aliquots for SDS-PAGE separation. One gel was stained with Coomassie Brilliant Blue R-250 and the other was transferred to solid matrix support and analyzed by VOPBA with DENV-2 as described above.

Peptide mass fingerprint analysis (PMF)

Peptide mass fingerprint analysis was used to further identify DENV-2 binding proteins. Protein bands or spots that corresponded to the VOPBA result were excised from the gel and sent to the Australian Proteome Analysis Facility (APAF, Macquarie University, Australia) for mass spectrometry analysis.

siRNA design and generation

The target site on A. aegypti PHB 1 (GenBank accession number XM_001653742.1) and the green fluorescent protein (GFP; GenBank accession number U50974) were determined using the online tool from Ambion, Austin, TX (http://www.ambion.com/techlib/misc/siRNA_finder.html). The sequences were subjected to siRNA template design to generate DNA oligonucleotide sequences for use for Silencer® siRNA construction Kit (Ambion). Templates for siRNA generation were selected:

siPHB1_3: 5′-AAGACATTGCTCCTACAgATgA-3′;
siGFP: 5′-AAAgATgAcCGgAACTACAAG-3′.

siRNAs were synthesized using the Silencer® siRNA construction Kit (Ambion, Austin, TX, USA) based on in vitro transcription and the concentrations were determined by spectrophotometry.

siRNA transfection

The CCL-125 or C6/36 cells were maintained in antibiotic-free L-15 or antibiotic-free MEM respectively. The cells were transfected with siRNAs using Lipofectamine™RNAiMAX (Invitrogen, Carlsbad, CA, USA) by the reverse transfection method according to the manufacturer’s instruction. Briefly, 500 pmol of siPHB1_3 or siGFP were incubated with 2.5 μL of Lipofectamine™RNAiMAX in 12-well tissue culture plate for 20 min. Then 6×105 cells of CCL-125 or C6/36 were seeded into the wells and samples incubated under standard conditions. At 24 h post-transfection, the culture medium was changed and the cells were further cultured for up to 5 days. Mock transfections (lipofectamine only) were undertaken in parallel.

RNA extraction and RT-PCR analysis

The transfected cells were harvested every day for 5 days and total RNA extracted. The cells in each well were homogenized and incubated with 1 ml of Tri reagent (Molecular Research Center, Cincinnati, OH) at room temperature for 5 min. The mixture was vigorously vortexed in the presence of 200 μl chloroform and allowed to stand at room temperature for 15 min, followed by centrifugation at 12,000×g for 15 min. The aqueous phase solution was transferred to a new tube and RNA precipitated with the addition of 500 μl of isopropanol at −30 °C overnight. The solution was centrifuged at 12,000×g for 15 min and the RNA pellets were washed with 75% ethanol with subsequent centrifugation at 7500×g for 5 min. The pellets were briefly air-dried and finally dissolved in DEPC-treated water. Total RNA concentrations were determined by spectrophotometry. For RT-PCR analysis, first strand cDNA was synthesized using an oligo(dt)17 primer. The cDNA was then amplified in multiplex reactions with 2 specific primers for PHB1 (PHB1_F: 5′-TCACgCTgCTATgTgTTAC-3′; PHB1_R: 5′-CTgTgTGACgACgACCgTTCA-3′), and PHB2 (PHB2_F: 5′-AgATgTgTgTgggACgAAGATC-3′; PHB2_R: 5′-TCTgTgACgATgggCACgTTAC-3′). The expected products were 305 bp for PHB1 and 246 bp for PHB2. The cycle conditions were 94 °C for 5 min, followed by 22 cycles of 94 °C for 20 s, 60 °C for 10 s and 72 °C for 15 s, followed by a final extension of 72 °C for 5 min. The PCR products were analyzed on 2% agarose gels.

Infection assay, Western blot assay and VOPBA on PHB silenced cells

CCL-125 and C6/36 cells were mock transfected or transfected with 500 pmol of siPHB1_3 or siGFP as described above. On day 4 post-transfection, cells were infected with DENV-2 at MOI of 10 for CCL-125 or at MOI of 1 for C6/36 at 28 °C for 2 h. The cells were washed with 1× PBS and treated with acid glycin, pH 3 for 1 min to remove uninternalized virus (Hung et al., 1999). Cells were washed with 1× PBS again and fresh growth medium was added. After one and a half virus replication cycles of each cell type (Sakoonwatanyoo et al., 2006; Wilkan et al., 2009), the culture medium was collected and the viral titer was determined by standard plaque assay (Sathitorn et al., 2003). Each sample was analyzed in duplicate and the experiment was done independently in triplicate. The transfected-infected cells were collected for protein extraction and Western blot assay and VOPBA as described above.

Antibody mediated infection inhibition assay

CCL-125 and C6/36 cells were grown in six-well plates and the cells were then incubated with a rabbit polyclonal antibody against PHB2 at various concentration or a polyclonal antibody against STAT5 (sc-28685, Santa Cruz Biotechnology) or no antibody at 28 °C for 1 h. After incubation, the cells were infected with DENV-2 at MOI of 10 for CCL-125 or at MOI of 1 for C6/36 at 28 °C for 2 h. The cells were washed with 1× PBS and treated with acid glycin, pH 3 for 1 min to remove uninternalized virus (Hung et al., 1999). The cells were washed with 1× PBS and fresh growth medium was added. The culture medium was collected after one and a half virus replication cycle of each cell type (Sakoonwatanyoo et al., 2006; Wilkan et al., 2009) for viral titration and the viral titer was determined by standard plaque assay (Sathitorn et al., 2003). Each sample was analyzed in duplicate and the experiment was done independently in triplicate.
**Infection inhibition assay by flow cytometry**

C6/36 cells were grown on six-well plates under standard conditions for 24 h; the cells were then incubated with 20 μg of an antibody directed against PHB2 (sc-67045, Santa Cruz Biotechnology) or 20 μg of an antibody directed against STAT5 (sc-28685, Santa Cruz Biotechnology) or 20 μg of antibody directed against integrin αV/β5 (sc-13588 Santa Cruz Biotechnology) or no antibody at 28 °C for 1 h. After incubation, the cells were infected with DENV-2 or JEV at MOI of 10 at 28 °C for 2 h. The cells were washed with 1× PBS and treated with acid glycine pH 3 for 1 min to remove the excess virus. The cells were further cultured at standard condition for 15 h for a JEV infected cell or 16 h for a DENV-2 infected cell. Then the cells were harvested by scraping and fixed in 4% paraformaldehyde in 1× PBS for 20 min at room temperature. Cells were subsequently washed twice with 1% BSA in PBS and permeabilized with 0.2% saponin. The cells were incubated with a 1:10 dilution of a pan specific anti-dengue virus E protein monoclonal antibody (HB114) or a 1:10 dilution of a monoclonal anti-flavivirus antibody (HB112, Henchal et al., 1985). After washing, the cells were incubated with a 1:10 dilution of a goat anti-mouse IgG conjugated with fluorescein for 1 h at room temperature. Then the cells were analyzed by flow cytometry (BD FACalibur).

**Analysis of cell surface binding of DENV-2**

C6/36 cells were grown on 12-mm coverslips (Menzel-Glaser, Braunschweig, Germany) at 28 °C for 1 day. Cells were subsequently washed twice with PBS followed by fixation with 4% paraformaldehyde for 15 min. After washing twice with PBS, cells were incubated with or without 15 μg of a rabbit polyclonal antibody directed against PHB2 (sc-67045, Santa Cruz Biotechnology Inc.) at 4 °C for 1 h. After incubation, cells were washed twice with PBS, and then approximately 2 × 10^7 pfu DENV-2 per coverslip was added and samples were incubated at 4 °C for 1 h. The excess unbound viruses were removed by washing twice with PBS followed by fixation with 4% paraformaldehyde for 15 min. After a further two washes with PBS, the cells were incubated for 90 min at 4 °C with a 1:10 dilution of pan specific anti-dengue virus monoclonal antibody produced by hybridoma cell line HB114 (Henchal et al., 1985). The coverslips were washed twice with PBS and subsequently 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, USA) followed by a further two washes with PBS. Signals were observed under an Olympus fluorescence microscope.

**Immunofluorescence**

C6/36 cells were seeded on glass coverslips and cultured under standard condition for 24 h, and then the cells were infected with DENV-2 at MOI of 1. After 24 h post-infection, the cells were washed with PBS and fixed with 100% pre-cooled methanol for 20 min. The cells were subsequently permeabilized with 0.3% Triton X-100 in PBS for 10 min and then washed twice with 0.3% Triton X-100 in PBS. The cells were then incubated with a 1:10 dilution of a pan specific anti-dengue virus E protein monoclonal antibody (HB114) and a 1:100 dilution of a goat polyclonal anti-PHB1 antibody or a 1:100 dilution of a rabbit polyclonal anti-PHB2 antibody (sc-67045, Santa Cruz Biotechnology Inc.) at 4 °C overnight. After primary antibody incubation, the cells were washed four times with 0.03% Triton X-100 in PBS and incubated with secondary antibody for 1 h at room temperature. Subsequently the cells were washed six times with 0.03% Triton X-100 in PBS and then mounted onto glass slides. The cells were observed under a confocal microscope (Olympus FluoView 1000). The secondary antibodies used were a 1:10 dilution of a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (KPL), a 1:100 dilution of a Cy5-conjugated rabbit anti-goat IgG (Invitrogen) and a 1:100 dilution of a Rhodamine Red X-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch).

**Commmunoprecipitation**

C6/36 cells were grown in 10-mm tissue culture plates. The cells were infected with DENV-2 at MOI of 10 at 28 °C for 2 h or mock infected. At day 3 post-infection, the culture medium was discarded and the cells were washed twice with cooled-PBS and then lysis buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM NaVO₄, 1 mM PMSF) was added. The lysed cells were collected by scraping and solutions centrifuged at 14,000 × g for 10 min at 4 °C. The lysates were transferred to new tubes and kept at –80 °C until use. 200 μl of lysates were incubated with 20 μl of a 50% slurry of protein G sepharose beads (Protein G Sepharose™ 4 Fast Flow, Amersham Bioscience, NJ, USA) with gentle rocking at 4 °C for 1 h. The mixture was centrifuged at 6000 × g for 3 min and the supernatant was transferred to a new tube, then 1 μg of a rabbit polyclonal anti-PHB2 antibody (sc-67045, Santa Cruz Biotechnology Inc.) was added. The mixture was incubated with gentle agitation overnight at 4 °C, following which a further 20 μl of 50% slurry of protein G sepharose beads was added and samples incubated at 4 °C for 3 h. After centrifugation, the supernatant was discarded and the pellet was washed twice with 1× lysis buffer and resuspended with 20 μl of 3× SDS sample buffer and heated at 100 °C for 5 min and then centrifuged at 14,000 × g for 2 min. The presence of DENV-2 E protein was detected by Western blotting as described above using a 1:200 dilution of a mouse anti-dengue complex monoclonal antibody (MAB8705, Millipore, MA, USA) followed by a 1:8000 dilution of a horseradish peroxidase-conjugated rabbit anti-mouse IgG secondary antibody (Sigma).

**Statistical analysis**

Viral production data were analyzed using the GraphPad Prism program (GrapPad Software). Statistical analysis of significance was done by a paired sample test using SPSS (SPSS Inc., Chicago, IL) using a value of P = 0.05 for significance.

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