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

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ABSTRACT

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 a 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 coimmunoprecipitation 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 sub tropical 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 family *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; Sakoonwatanyoo 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

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Table 1
Proposed mosquito dengue virus receptor proteins.

Cell	Cell type	Serotype; strain	Receptor characteristics	Ref.
C6/36	*Aedes albopictus* cell line	DEN-4; H-241	Two glycoprotein 40 and 45 kDa	Salas-Benito and del Angel (1997)
C6/36	*Aedes albopictus* cell line	DEN-2; NGC	80 and 67 kDa protein	Munoz et al. (1998)
C6/36	*Aedes albopictus* cell line	DEN-2; NGC	Tubulin protein or like-tubulin protein	Chee and AbuBakar (2004)
C6/36	*Aedes albopictus* cell line	DEN-3, 4	Laminin-binding protein	Sakoonwatanyoo et al. (2006)
A. aegypti	Midgut, ovary and salivary gland cell, eggs, larvae and pupae cell extract	DEN-4; H-241	A 45 kDa glycoprotein	Yazi Mendoza et al. (2002)
A. aegypti	Salivary gland	DEN-1, 2, 3 and 4	37, 54, 58 and 77 kDa protein	Cao-Lormeau (2009)
A. polynesiensis	Salivary gland	DEN-1, 4	48, 50, 54, 56, and 77 kDa protein	Cao-Lormeau (2009)

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 and 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., 2006). 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. Attempts 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