The interaction between claudin-1 and dengue viral prM/M protein for its entry

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ABSTRACT

Dengue disease is becoming a huge public health concern around the world as more than one-third of the world’s population living in areas at risk of infection. In an effort to assess host factors interacting with dengue virus, we identified claudin-1, a major tight junction component, as an essential cell surface protein for dengue virus entry. When claudin-1 was knocked down in Huh 7.5 cells via shRNA, the amount of dengue virus entering host cells was reduced. Consequently, the progeny virus productions were decreased and dengue virus-induced CPE was prevented. Furthermore, restoring the expression of claudin-1 in the knockdown cells facilitated dengue virus entry. The interaction between claudin-1 and dengue viral prM protein was further demonstrated using the pull-down assay. Deletion of the extracellular loop 1 (ECL1) of claudin-1 abolished such interaction, so did point mutations C54A, C64A and I32M on ECL1. These results suggest that the interaction between viral protein prM and host protein claudin-1 was essential for dengue entry. Since host and viral factors involved in virus entry are promising therapeutic targets, determining the essential role of claudin-1 could lead to the discovery of entry inhibitors with attractive therapeutic potential against dengue disease.

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Introduction

Dengue virus (DENV), a mosquito-transmitted single strand RNA virus including four serotypes (DENV-1,-2, -3 and -4), belongs to the genus Flavivirus in the family Flaviiviridae. DENV causes a broad spectrum of clinical manifestations, ranging from mild febrile illness to life threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (WHO, 2012). Each year, 2.5 billion people are under risk of DENV infection, with 50 million infections and 500,000 deaths (WHO, 2012). The formation of intracellular prM/E heterodimers occurs rapidly after translation and is important for the assembly and secretion of immature virus particles. The ‘pr’ retention prevents structural proteins, the capsid (C), envelope (E) and membrane (M) proteins. M is derived from the precursor M protein (prM) via cleavage (Perera and Kuhn, 2008). An internal host derived lipid bilayer encloses an RNA–protein core consisting of genome RNA and C proteins (Kuhn et al., 2002; Perera and Kuhn, 2008). DENV virions attach to the host cell surface receptors/co-receptors and enter the cell via receptor-mediated endocytosis (Lindenbach and Rice, 2003; Mercado-Curiel et al., 2008; Sisney et al., 2009; van der Schaar et al., 2008). Fusion between the viral and cellular membranes requires reassociation of the E protein on the viral surface to form a number of fusogenic trimers via an intermediate structure that consists of E dimers surrounding patches of exposed membrane (Yu et al., 2009; Zhang et al., 2004). Subsequently, the acidic environment of the endosomal vesicles triggers conformational changes in E protein, resulting in fusion of the viral and cellular membranes (Heinz and Allison, 2003). The nucleocapsid is then released into the cytoplasm, and the genomic RNA is translated into a single polyprotein precursor in the order of C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5, which is processed to three structural and seven non-structural (NS) proteins. Virus assembly is initiated by forming immature particles in endoplasmic reticulum (Mackenzie and Westaway, 2001; Yu et al., 2008). The formation of intracellular prM/E heterodimers occurs rapidly after translation and is important for the assembly and secretion of immature virus particles. The ‘pr’ retention prevents...
membrane insertion, suggesting that ‘pr’ is present on the virion in the trans-Golgi network to protect the progeny virus from fusion within the host cell (Yu et al., 2009). During maturation, ‘pr’ peptide is cleaved from prM, and resulting M protein remains in the mature particle as a transmembrane protein beneath the E protein shell (Yu et al., 2009, 2008; Zhang et al., 2003).

DENV entry is a complicated process requiring specific interactions between multiple cell surface proteins and viral proteins (prM/M and E). Cell surface proteins serving as receptors/co-receptors are crucial determinants of tissue tropism during DENV infection. Several cell surface proteins have been identified as receptors/co-receptors in different target cells, however, DENV receptors still remains largely undefined, mainly due to the complexity of different target cells and different virus serotypes (Bielegeldt-Ohmann et al., 2001; Diamond et al., 2000). Macrophages, monocytes, and dendritic cells have been proposed as primary target cells during DENV infection. The binding of E protein to dendritic cell specific ICAM-3 grabbing non-integrin (DC-SIGN) triggers the internalization of DENV into the cells (Lozach et al., 2005; Navarro-Sanchez et al., 2003; Tassaneenrithep et al., 2003). Meanwhile, the mannose receptor (MR) expressed on macrophages has been shown to mediate entry by all four DENV serotypes via binding to the E protein (Miller et al., 2008). Other cell surface proteins involved in DENV entry include APO B100 (Guevara et al., 2010), the chemokine receptors CXCR3 and CXCL10 (Ip and Liao, 2010), and stress proteins related to the heat shock family such as GRP78/Bip (Jindadamrongwech and Smith, 2004) and heat-shock protein 70 and 90 (HSP70/90) (Reyes-Del Valle et al., 2005; Reyes-del Valle and del Angel, 2004). In insect C6/36 cells, prohibitin (Kuadkitkan et al., 2010) and the 45-kD heat-shock related glycoprotein (Salas-Benito et al., 2007) have been shown involved in DENV entry. Nevertheless, the exact cell surface proteins serving as receptors/co-receptors for DENV entry is still not well defined. In the present study, we examined and characterized the essential role of claudin-1 during DENV entry.

**Results**

**Identification of claudin-1 involvement during DENV viral lifecycle:**

Virus entry requires the involvement of many host cell surface factors, including tetraspanin CD 81 (Bartosch et al., 2003; McKeating et al., 2004; Pileri et al., 1998), tight junction protein claudin-1 (Evan et al., 2007; Liu et al., 2009) and occludin (Liu et al., 2009; Ploss et al., 2009), and human scavenger receptor class B type 1 (SR-BI) (Bartosch et al., 2003; Scarselli et al., 2002). To examine the role of claudin-1 during dengue viral lifecycle, we first established a stable cell lines with the knockdown of claudin-1 using shRNA technique. Stable Huh 7.5 cell lines transfected with non-targeting shRNA (NT-shRNA) served as a control cell line, designated as RK1 cells. Cell line with knockdown of claudin-1 was designated as RK4 cells, and further verified by western blot (Fig. 1A) and immunofluorescence staining (Fig. 1B). The immunofluorescent staining of RK1 cells showed clearly observable claudin-1 expression along the cell surface (Fig. 1B, arrow pointed). Claudin-1 expression was significantly reduced in claudin-1 knockdown cells (designated as RK4 cells), as claudin-1 expression in most RK4 cells was hardly observed on the cell surface, or only faint and broken line of claudin-1 expression observed on the surface of a few cells (Fig. 1B). There were some background staining in the cytoplasm in RK4 cells, which were also observed in the RK1 cells, but was not significantly different. This was further confirmed in our western blot analysis showing that there was strong claudin-1 expression in the RK1 control cells, and with less than 10% claudin-1 expression in the RK4 cells (Fig. 1A).

To investigate whether claudin-1 knockdown could affect DENV-induced CPE post infection, the dynamics of cell viability in claudin-1 knockdown RK4 cells was examined in comparison with that in the RK1 control cells after inoculated with DENV-2 at MOI of 1 using the previously established cell viability assay (Fig. 2A) (Che et al., 2009). Knockdown of claudin-1 strongly prevented DENV-2 induced CPE as we observed considerable increase of cell viability in claudin-1 knockdown RK4 cells comparing with that in RK1 control cells. For instance, at 96 h.p.i., cell viability in control cells, including both Huh 7.5 and RK1 cells, were decreased to 18% and 15%, respectively. In claudin-1 knockdown RK4 cells, cell viabilities were kept at high level, with about 66% cells viability at 96 h.p.i. (Fig. 2A). DENV-induced CPE was further examined under the phase contrast microscopic. In control RK1 cells, CPE was observed as early as 96 h.p.i., and became more pronounced at 120 h.p.i., respectively (Fig. 2D-b and -c). In claudin-1 knockdown RK4 cells, CPE was delayed and not observable at 96 h.p.i., and only moderate CPE was observable at 120 h.p.i. (Fig. 2D-f). This observation further confirmed that DENV-2 induced CPE was prevented or delayed in claudin-1 knockdown RK4 cells. Furthermore, cell growth in RK4 cells was not affected due to the knockdown of claudin-1, since the cell growth rate in RK4 cells and the RK1 cells were identical (data not shown). The above results indicated that claudin-1 might play an essential role during DENV infection.

Furthermore, we also examined whether knockdown of claudin-1 could also protect CPEs induced by other DENV serotypes, including DENV-1, –3 and –4. Claudin-1 knockdown RK4 cells were infected with different DENV serotypes (DENV-1, DENV-3, DENV-4), and DENV induced CPE was evaluated at different h.p.i. Interestingly, DENV-1 infection could not induce observable CPE in both RK1 and claudin-1 knockdown RK4 cells (results not shown). Both DENV-3 and DENV-4 induced CPE was inhibited in claudin-1 knockdown RK4

![Fig. 1.](image) The knockdown of claudin-1 expression in RK4 stable cell lines. Knockdown of claudin-1 in Huh 7.5 cells was carried out using claudin-1 specific shRNA, designated as RK4 cells. Cells transfected with non-targeting shRNA (NT-shRNA) served as the control, designated as RK1 cells. (A) Western blot showing the reduction of claudin-1 expression in RK4 cells in comparison with that in the RK1 control cells and the original Huh 7.5 cells. GAPDH expression was also examined serving as loading control. (B) The expression of claudin-1 was disrupted in claudin-1 knockdown RK4 cells in comparison with that in the RK1 cells. Arrow in RK1 cells pointed to the claudin-1 expression around cells, whereas arrow in the claudin-1 knockdown RK4 cells showed that claudin-1 was depleted and there were only very little claudin-1 expression around the cell surface.
cells with up to 60% and 90% cell viabilities at 96 h.p.i., respectively (Fig. 2B and C). This was in agreement with the result when claudin-1 knockdown RK4 cells were infected by DENV-2, suggesting a potential of claudin-1 to serve as a common factor for different DENV serotypes. Collectively, these results demonstrated that loss of claudin-1 suppressed and delayed the development of DENV-induced CPE.

The possible role of claudin-1 during DENV replication

Since knockdown of claudin-1 prevent DENV-2 induced CPE, we examined the dynamics of virus replication in claudin-1 knockdown RK4 cells in comparison with that in the RK1 control cells. Total RNAs were extracted from infected cells and culture medium at indicated h.p.i., respectively, followed by analyses of viral genomic RNA using qRT-PCR. Progeny virus production, expressing as PFU-equivalent RNA copies, in both cell and supernatant samples were examined in both RK1 and RK4 cells at 24 h intervals. Interestingly, we observed a log reduction of progeny virus production (RNA copies) in both cells and supernatant samples of the claudin-1 knockdown RK4 cells starting as early as 24 h.p.i., and remained at one log lower up till 96 h.p.i. (Fig. 3A and B). Although the progeny virus titer was lower in the RK4 knockdown cells, the replication dynamics was similar between the RK1 control and RK4 cells at different h.p.i. This result was also confirmed when we determined the production of infectious progeny viral particles in claudin-1 knockdown RK4 cells using plaque assays. Similarly, we observed a consistent one to two logs reduction of virus titer in both cells and culture medium samples from the RK4 cells, respectively, in comparison with that in the RK1 control cells (Fig. 3C and D). Similarly, the one-log difference in virus titer was observed as early as 24 h.p.i., and the virus replication dynamics kept in the similar fashion in both RK1 and RK4 cells. Collectively, the above results suggested that the effect of claudin-1 knockdown on DENV lifecycle might occurred before 24 h.p.i.

Similar phenomena were observed in the DENV-3 and -4 replication dynamics in RK4 knockdown cells (results not shown).

The effect of knockdown of claudin-1 using siRNA depletion

Due to the possible side effects associated with stable selection of cells expressing shRNA, we further analyzed the effect on DENV viral lifecycle in claudin-1 depleted cells using transient siRNA-mediated knockdown method. To deplete claudin-1 transiently, Huh 7.5 cells were transfected with 100nM human claudin-1 specific siRNA, and NT-siRNA transfection was utilized as control. The inhibition of claudin-1 expression was confirmed using western blot analysis (Fig. 4A). At 48 h post transfection, transfected cells were infected with DENV-2 at MOI of 1, and viral genomic RNA was determined using qRT-PCR at 24 h interval post infection. Consistent with the result in the shRNA knockdown stable cell lines, claudin-1 depleted cells showed an overall one-log reduction in viral RNA copies, but with similar virus replication dynamics when compared to that in the control cells (Fig. 4B). Collectively, both shRNA and siRNA mediated depletion of claudin-1 had showed similar effect on DENV replication dynamics as early as 24 h.p.i., further indicating that claudin-1 might be required for DENV early viral lifecycle.

Claudin-1 was involved in DENV entry

Our results showed that knockdown of claudin-1, using both shRNA or siRNA techniques, could prevent DENV-2 induced CPE and reduce DENV-2 progeny virus production. We next sought to investigate in which viral lifecycle stage claudin-1 was involved. During HCV infection, claudin-1 was utilized as a co-receptor for virus internalization (Evans et al., 2007), and our investigation showed that DENV-2 viral genomic RNA or progeny virus production were one log lower as early as 24 h.p.i. Thus, we first examined whether DENV entry was affected in claudin-1 depleted cells post DENV-2 infection. Viral genomic RNA (vRNA) from infected cells was evaluated at
different time post infection by focusing on the initial 12 h.p.i. (Fig. 4C). In comparison to control NT-siRNA cells, depletion of claudin-1 resulted in a significant decrease of DENV virus entry into the cells in the early stage of infection. At 2 h.p.i., there were only 50% virus entered into the knockdown cells when comparing with that in the control cells, and was consistent at 50% lower up to 8 h.p.i. The difference was even more pronounced at 10 h.p.i. and 12 h.p.i., as we observed over 60 and 80% reduction of viral genomic RNA in the knockdown cells, respectively. These results suggested that virus entry was hampered in claudin-1-depleted cells, suggesting that claudin-1 is required for efficient DENV entry.

To further confirm the involvement of claudin-1 during virus entry, we examined whether the entry of DENV-2 into the claudin-1 knockdown RK4 cells could be rescued by restoring claudin-1 expression in the cells. Claudin-1 knockdown RK4 cells were transiently transfected with plasmid DNA encoding wild-type human claudin-1. An empty vector was also used as negative control. In addition, RK1 control cells were also pre-treated with 1 µM compound 6, a known DENV entry inhibitor (Wang et al., 2009), as a positive control in inhibiting DENV entry. We first confirmed that claudin-1 was expressed in the transfected cells using western blot (Fig. 5A). We then confirmed that DENV-2 genomic RNA and progeny virus production in claudin-1 knockdown cells were reduced in the initial 12 h.p.i., similar to pattern observed in the claudin-1 depleted cells using siRNA knockdown (Fig. 5B).

The claudin-1 knockdown RK4 cells, transfected with human claudin-1 plasmid DNA or empty vector, were inoculated with DENV-2 (MOI of 1) at 48 h post transfection. Viral genomic RNA was then quantified at indicated time points using qRT-PCR. Notably, RK4 cells with restored claudin-1 expression showed increased virus entry into the cells at a level comparable to that in the control RK1 cells (Fig. 5B). On the other hand, the reduction of DENV virus entry in claudin-1 knockdown RK4 cells was comparable to that in RK1 cells treated with entry inhibitor, indicating DENV entry was impaired in claudin-1 knockdown RK4 cells. Similar phenomena were observed in the DENV-3 and -4 replication dynamics in RK4 knockdown cells (results not shown). Taken together, these data strongly suggested that claudin-1 might be essential for efficient DENV entry.

Interaction of claudin-1 with DENV viral protein

It was proposed that via interacting with viral protein, claudin-1 serves as a receptor for recruiting virions to cell surface during HCV entry (Cukierman et al., 2009; Evans et al., 2007; Meertens et al., 2008; Zheng et al., 2007). Since we showed that claudin-1 was also required for efficient DENV entry, we further analyzed whether claudin-1 interacted with DENV viral protein. During DENV infected cells, a larger precursor, PrM of approximately 19 kDa, is synthesized. DENV prM is further processed and cleaved into ‘pr’ and M proteins. DENV M is a small, approximately 10 kDa protein found in the mature virus particle. To determine the possible interaction of claudin-1 with DENV viral protein, we examined the direct binding between claudin-1 and viral glycoproteins prM. We used purified recombinant HIS-tagged prM protein as the bait to fish the protein extracts prepared from whole cell lysate of Huh 7.5 cells. We identified claudin-1 as one of the host proteins that interacted with prM protein (Fig. 6A). This interaction was further verified when purified GST-tagged claudin-1 efficiently interacted with HIS-tagged prM protein in the pull-down assay (Fig. 6B). Strikingly, a robust binding of claudin-1to ‘pr’, M and prM were also observed on our pull-down assay, however, viral E protein does interact with claudin-1 (Fig. 6C). This result suggested a direct interaction between claudin-1 and prM, including pr, M and prM proteins.

ECL1 is essential for the interaction between claudin-1 and prM

Previous studies in HCV entry showed that either ECL1 or ECL2 is essential for the interaction of claudin-1 with viral protein. To further verify the interaction between prM and claudin-1 and to determine the essential domains in claudin-1 for the interaction, we constructed deletion mutants with either ECL1 or ECL2 deleted (Fig. 7A). These mutant proteins were expressed, purified and protein-protein interaction was analyzed using pull-down assays (Fig. 7B). When ECL1 was
deleted, the interaction between prM and claudin-1ΔECL1 was not observed as there was no detectable claudin-1ΔECL1 in the pull-down samples. The deletion of ECL2 did not affect the interaction as claudin-1ΔECL2 mutation could be pulled down by HIS-tagged prM protein, similar to that in the full length claudin-1. This result indicated that ECL1 was essential for the binding of claudin-1 with prM.

Determining the essential amino acids on the ECL1

Early studies showed that amino acids in highly conserved ECL1 motif, W30-GLW51-C54-C64, plays an important role in maintaining its 3D-structure and are important for HCV entry (Cukierman et al., 2009). Another amino acid, isoleucine at position 32 (I32), has been proposed to be involved in ion regulation, and early studies showed exchange of I32 to M (I32M) impaired HCV entry (Evans et al., 2007). Cysteine at position 54 and 64 are known to form disulfide bond in ECL1, which plays an important role in stabilizing ECL1 structure, suggesting the specific structure of ECL1 is required for robust binding. To determine the essential amino acids on the ECL1, a panel of seven point mutations on ECL1 was constructed by alanine substitution of the aforementioned 6 highly conserved amino acids on ECL1 motif, plus the exchange of isoleucine with methionine at position 32. Although ECL2 was not required for prM binding and not engaged in tight junction barrier regulation, we still selected 8 polar amino acids on ECL2 for alanine substitution, including T137, Y140, Q146, Y149, T153, N156, Y159, and Q163. Each point mutation was constructed by site-directed mutagenesis, and the respective recombinant proteins were expressed and purified as described in method section. The interaction of these claudin-1 mutants with prM protein was examined using the pull-down assay. Our results showed that only C54A, C64A and I32M mutations on ECL1 impaired the claudin-1-prM interaction (Fig. 8A), whereas none of the other mutations on ECL1 and ECL2 showed any effect on claudin-1-prM interaction (Fig. 8B). Altogether, above data revealed an essential interface on ECL1 of claudin-1, which might be required for efficient virus-host interaction.

Discussion

In this study, we showed that claudin-1, the major structural component of tight junction, was involved in DENV entry by directly interacting with viral prM protein. Tight junction is an intercellular junctional structure, which functions as a physical barrier with
extremely small openings, allowing the passage of nano-sized or even smaller molecules and restricting the penetration of macromaterials and microbes (Lorenza González-Mariscal and Bautista). Disruption of tight junction contributes to pathogenesis. Extensive disruptions in tight junctions integrity and altered expression and distribution of claudins have been observed in infections of enteropathogenic Escherichia Coli (EPEC) (Guttmann et al., 2006; Yuhan et al., 1997), H. pylori infection (Fedwick et al., 2005), S. flexneri (Sakaguchi et al., 2002), rotavirus (Dickman et al., 2000; Nava et al., 2004; Obert et al., 2000), influenza virus (Armstrong et al., 2012), and HIV-1 virus (Andras and Toborek, 2011). On the other hand, pathogens, especially viruses, have evolved strategies to utilize tight junction to gain access into cells. Clostridium perfringens enterotoxin binds directly to the second extracellular loop on claudin-3, -4 to gain access into cells (Fujita et al., 2000; Sonoda et al., 1999; Takahashi et al., 2005; Van Itallie et al., 2008). claudin-1, -6, -9 and occludin (Benedicto et al. 2009; Liu et al., 2009; Ploss et al., 2009) have been reported as co-receptors for HCV entry (Evans et al., 2007; Meertens et al., 2008; Zheng et al., 2007). Our data showed that depletion of claudin-1 expression hampered DENV-2 entry, reduced progeny virus production and prevent DENV-2 induced CPE. These results strongly suggested that claudin-1 was utilized by DENV-2 to facilitate its entry, which is in agreement with the observation during the entry of other viruses, including HCV (Gao et al., 2010; Harris et al., 2010; Liu et al., 2009; Meertens et al., 2008).

Claudin-1 has been shown to serve as a co-receptor for HCV entry, and deletion of ECL1 or mutation of in highly conserved amino acid W30GLW31-C34-C64 on ECL1 motif hampered the HCV entry (Cukierman et al., 2009). The present study also identified ECL1 as the essential domain for its interaction with DENV prM protein. However, we only identified C34 and C64 as the essential amino acid, but not W30, G, L, and W31. We also identified I32 as the essential amino acid for binding activity. Prior studies suggested that ECL1 regulates the paracellular permeability and ion selectivity, while ECL2 is required for interactions between different claudin isoforms which is the linkage between adjacent cells (Van Itallie and Anderson, 2006). The two cysteines may form an intracellular disulfide bond in ECL1 (Li et al., 2013), a proposed key determinant in pore formation. The disulfide bonds between the two cysteines are critical in maintaining proper folding and stability (Doig and Williams, 1991; Taniyama et al., 1991). Notably, these two cysteines are conserved among all claudin isoforms and are critical in regulating tightness function (Krause et al., 2008;
Wen et al., 2004). Studies using claudin-153-80 peptide, which contains C54 and C64, showed disrupted barrier function both in vitro and in vivo (Msrny et al., 2008). In addition, introduction of I32M into ECL1 also disrupted protein binding. This is in agreement with Evans et al., when introduction of M32I into claudin-7 could render T293 cells partially permissive to HCVpp (Evans et al., 2007), whereas the wt claudin-7 expression on T293 failed to render it permissive. It is known that residue I32 may be involved in regulating the cation passage in tight junction. Since ECL2 was not involved in the interaction between prM and claudin-1, and it was not surprised that deletion of ECL2 or point mutation on ECL2 has no effect on the claudin-1 binding activity.

It is interesting that up to 30% dengue virus secreted from mammalian or insect cells are immature virus particles, with intact prM protein on the virus particle surface (Murray et al., 1993; Rodenhuis-Zybert et al., 2010; Wang et al., 1999; Zybert et al., 2008). In the immature virus, prM and E form heterodimers, protruding from virus surface as 60 trimeric spikes. The pr peptide caps the fusion loop of E protein, prevent the low-pH driven conformational change and pre-membrane fusion before virus release (Heinz and Allison, 2003; Lindenbach and Rice, 2003). The ‘pr’ peptide caps the fusion loop of E protein and prevent conformational change of E protein which is required for subsequent membrane fusion (Yu et al., 2008; Zybert et al., 2008). This is evidenced by inefficient infectivity and high percentage of immature progeny virus in both mammalian and insect cells. Recently, it has been reported that during entry step, the pr-capped fusion loop of E protein could be cleaved by furin, allowing conformational change and fusion of E protein, which renders immature virus infectious (da Silva Voorham et al., 2012; Rodenhuis-Zybert et al., 2011). In the present study, our data provide supporting evidence that immature particles can also interact with claudin-1 protein on the cell surface, and might be capable to entry into the target cells via this interaction. This result is also in agreement with the conclusion of Gao et al. that prM is required for an efficient entry (Gao et al., 2010). Further investigations will be needed to explore how immature virus particles interact with cell surface proteins to entry the target cells, and to understand whether claudin-1 is able to trigger the endocytosis or only plays a role in mediating virus attachment.

Viral entry is initiated by virus attachment, followed by rolling over the cell surface until endocytosis occurs. Apically resided surface proteins to entry the target cells, and to understand whether claudin-1 is able to trigger the endocytosis or only plays a role in mediating virus attachment.

The stable cell lines expression of different shRNAs were established using a lentiviral as described previously (Waninger et al., 2004). Briefly, the target sequences for claudin-1 and the scrambled shRNA were introduced into the U6 promoter/hairpin shRNA expression cassettes using the U6 promoter in psiSilencer (Ambion). The cassettes were inserted into the pHIV-7-Puro vector, respectively. VSV-G-pseudotyped lentivirus was packaged using the lentivirus support kit (Invitrogen). Huh 7.5 cells were transduced by standard methods and subjected to selection with puromycin (0.6 μg/ml) for 10 days.

**Plaque assay**

Virus titer was determined using a plaque assay as described previously (Che et al., 2009). Briefly, BSR cells were seeded in 24-well plate and were inoculated with 10-fold serially-diluted virus. After inoculation, cells were incubated in 37 °C for 4 h for adsorption. The inoculum was removed and cells were washed with PBS twice. An overlay of 1% low-melting-point agarose
**Table 1**

List of primers used for construction of claudin-1 deletion and point mutants.

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<th>Primer ID</th>
<th>Sequence</th>
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RNA purification and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from supernatant and cell pellet from DENV infected cultures using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol. The One Step SYBR Ex Taq RT-PCR Kit (Takara, Clontech Laboratories, Inc., Mountain View, CA), designed for single step real-time RT-PCR using SYBR Green I detection, was utilized following the manufacturer’s protocol. In brief, the 25 µl PCR mixture included 12.5 µl 2× buffer (including dNTP mixture, Mg^2+ and SYBR green I), 0.5 µl (2.5 unit) Ex Taq, 0.5 µl RTase mix, 0.5 µl 10 µM (final concentration 0.2 µM) forward primer (CTTAGGGTTCGCGACGAC), 0.5 µl 10 µM (final concentration 0.2 µM) reverse primer (CAGGGGAACTGGTTTACG), 0.5 µl ROX reference dye II, 2.5 µl RNA sample, and 7.5 µl H2O (Takara Bio Inc). The ten-fold serial dilutions of DENV-2 total RNA (10^3–10^7 copies/ml), prepared from DENV-2 stocks with known viral titer, were used as a reference for standard curve calculation. The mixtures were incubated in a 96-well optical plate (Applied Biosystems), and qRT-PCR was carried out using StepOne Plus Real-Time PCR system (Applied Biosystems, Foster City, CA), following manufacturer’s protocol. RT-PCR reaction performed as followings: reverse transcription was carried out by incubating mixture at 42 °C for 30 min and 95 °C for 5 min, followed by 40 PCR cycles: 95 °C for 10 s, 60 °C for 30 s, and completed by incubation at 95 °C for 15 s. The reaction was then incubated at 60 °C for 1 min and 95 °C for 15 s for melt curve. After the reaction, amplification curve and melting curve were verified, the standard curve established. The Ct value for each sample was determined by default threshold settings of the software.
Cell viability assay

Cell viability was determined using CellTiter-Glo (CTG) Luminescent cell viability assay (Promega, Madison, WI) as described previously (Che et al., 2009). Briefly, cells were seeded at 5000 cells/well in 96-well black plates with clear bottom using DMEM supplemented with 1% FBS, 1% P/S. After incubated overnight, cells were inoculated with DENV at designated MOI as noted in the text. The plates were then incubated at 37 °C with 5% CO2 for 120 h to allow the development of CPE. Cell viability was measured at 120 h.p.i. using CTG reagent (Promega, Madison, WI) by adding equal volume (100 µl/well) of CTG reagent into each well. After 5 min incubation, relative luminescent signals were measured using Synergy 2 plate reader (BioTek Instruments Inc., Winooski, VT). Luminescent signal was normalized to that of control cells, and the percentages of cell viability were determined accordingly.

Protein sample preparation

Cell monolayers, with or without DENV infection, were washed twice with PBS and lysed on ice using lysis buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 1% Nonidet P40 (NP40), 0.02% NaN3, 1% Triton X-100) (Invitrogen, Carlsbad, CA) supplemented with 1 mM PMSF and 1 µl protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO). The cell lysate was further incubated for 30 min on ice and vortex at 10 min interval. After centrifugation at 14,000g for 20 min at 4 °C, cell debris was discarded and supernatant was collected, aliquoted and stored in −80 °C. Protein concentration was determined using Bradford assay (Sigma-Aldrich, St. Louis, MO) prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Western blot analysis

Protein samples were boiled in 1× loading buffer for 5 min prior to load into the SDS-PAGE gel. Proteins were separated in 10% SDS-PAGE at 150 V for 40 min. After transferred onto a PVDF membrane using Mini Trans-Blot cell (Bio-Rad, Hercules, CA), the membranes were blocked using 5% non-fat milk for 1 h at room temperature. Immunoblot analysis was performed using primary antibodies specific against each protein. Mouse monoclonal antibodies (mAbs) specifically against claudin-1 (D-4) was purchased from Santa Cruz biotechnology (Santa Cruz, CA). GAPDH, GST tag and HIS tag mAbs were purchased from GenScript (Piscataway, NJ). Goat anti-mouse IgG-HRP and rabbit anti-goat IgG-HRP were purchased from KPL Inc. (Gaithersburg, Maryland) and Southern Biotechnology Associates (Birmingham, AL), respectively. The ECL reagent (Millipore, Billerica, MA) was used for luminescence detection.

DNA transfection and rescue assay

Plasmids DNA were purified using PureLink quick plasmid midi-prep kit (Invitrogen, Carlsbad, CA), and concentration was quantified using Synergy 2 plate reader (BioTek Instruments Inc., Winooski, VT). For transfection, cells were seeded in 24-well plates at 80% confluence and transfected with 2 µg DNA/well using lipofectamine 2000 (Invitrogen, Carlsbad, CA), as described in manufacturer’s manual. After incubation at 37 °C for 6 h, transfecting medium was replaced with DMEM supplemented with 5% FBS. After incubated at 37 °C for another 48 h, cells were inoculated with DENV-2 at MOI of 1, or treated with compound 6 at concentration of 1 µM prior to inoculation. Cells were harvested and total RNA was extracted using TRizol reagent at time points noted in the text, followed by qRT-PCR analysis for viral genomic RNA level as described above.

Indirect immunofluorescent assay (IFA)

Huh 7.5 cells with or without shRNA knockdown were seeded on chamber slides (Millipore, Billerica, MA). At desired time, cells were washed in PBS twice and fixed in 3.7% formaldehyde (Sigma-Aldrich, St. Louis, MO) at room temperature for 20 min. After being washed three times, cells were permeabilized in 0.2% Triton X-100 in PBS for 20 min. After blocking in 1% BSA in PBS for 1 h, cells were incubated with 200 µl anti-claudin-1 mAb (1:100) for 3 h. After three brief washes in 0.2% BSA in PBS, cells were incubated in goat anti-mouse IgG conjugated polyclonal antibody (1:500) for 1 h. Cells were then washed 5 times and mounted with cover slip using mounting medium (Vector Laboratories, Burlingame, CA), and examined using a Nikon fluorescence inverted microscope (Nikon, Tokyo, Japan).

Expression and purification of GST- and HIS-tagged recombinant proteins

The E. coli strain Rosetta (DE3) LysS (Novagen, Madison, WI) was transformed with pTrEx-4 or pGEX-3T4 expression plasmids by heat-shock method. Cells were cultured at 37 °C in LB medium supplemented with 100 µg/ml Amp until OD600 reached 0.6. To induce protein expression, 1 mM isopropanyl-D-thiogalactopyranoside (IPTG) was added into culture medium, followed by 4 h of incubation at 30 °C. Cells were harvested by centrifuge at 6000g for 30 min at 4 °C, washed in PBS once and stored in −80 °C until purification. To purify HIS-tagged proteins, cells were re-suspended in ice-cold HIS-lysis buffer for HIS-tagged proteins (20 mM phosphate buffer pH 7.4, 10 mM imidazole, 0.5% Triton X-100, 250 mM NaCl). Cells were disrupted on ice using a sonic dismembrator (Fisher Scientific Company, Pittsburgh, PA). Cell debris was removed by spin at 1000g for 20 min at 4 °C. The inclusion bodies were collected by centrifugation at 8000g for 40 min at 4 °C and resuspended in 8 M urea buffer (8 M urea, 40 mM phosphate buffer, 10 mM imidazole, pH 7.4) with gentle rocking at 4 °C for 2 h. Suspension was centrifuged again at 8000g for 1 h at 4 °C, and supernatant with resolubilized proteins was collected and incubated with pre-equilibrated nickel chelate beads (GE Healthcare, Piscataway, NJ) for 2–3 h at 4 °C.

To purify GST-tagged proteins, supernatant was collected using GST-lysis buffer for GST-tagged proteins (1% Sarkosyl, 4.3 mM Na2HPO4, 1.47 mM KH2PO4, 0.137 M NaCl, 2.7 mM KCl, pH 7.3). After removal of the cell debris, supernatants were diluted 100× using GST-wash buffer for GST-tagged proteins (4.3 mM Na2HPO4, 1.47 mM KH2PO4, 0.137 M NaCl, 2.7 mM KCl, 0.5% Triton X-100, pH 7.3), followed by incubation with pre-equilibrated Glutathione Sepharose 4B beads (GE Healthcare, Piscataway, NJ) for 4 h at 4 °C.

To further purify the HIS- and GST-tagged proteins, HIS and GST affinity beads were used (Bio-Rad, Hercules, CA). After incubate the affinity beads with the respective protein samples, the beads were loaded onto an empty column and proteins were purified following the manufacturer’s instruction. Briefly, beads were washed 5–10 times with 2 column volume (CV) of HIS-wash buffer for HIS-tagged proteins (20mM phosphate buffer pH 7.4, 250 mM NaCl, 50 mM imidazole), or GST-wash buffer for GST-tagged protein as described previously. Semi-purified proteins were eluted and collected in fifteen 1 ml fractions, using HIS elution buffer (20 mM phosphate buffer, 350 mM imidazole pH 7.4) for HIS-tagged proteins or GST elution buffer (10 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0) for GST-tagged proteins. Fractions containing target proteins were analyzed using SDS-PAGE. Target proteins were then pooled from identified fractions and dialyzed against 20 mM phosphate buffer, 300 mM NaCl, 20% glycerol, at 4 °C overnight, using dialysis tubing with cut-off value
of 10 kDa. Dialysis product was then aliquoted and stored at –80ºC.

**Pull-down assay**

Total protein concentration of each purified recombinant protein was determined by Bradford assay (Sigma-Aldrich, St. Louis, MO). For in vitro pull-down assays, 12 μg of purified GST-prM was incubated with 1 mg purified HIS-tagged claudin-1 or its deletion mutations or point mutations in GST binding buffer (4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄, 0.137 M NaCl, 2.7 mM KCl, pH 7.3) at 4 ºC with gentle rocking for overnight. A 50 µl pre-equilibrated Glutathione Sepharose 4B beads was added to the mixture and incubated for 4 h at 4 ºC with gentle rocking. Subsequently, the binding mixture was loaded into micro-spin columns (Thermo-Fisher, Rockford, USA). Unbound proteins were removed by 6 washes of 500 µl GST binding buffer. The protein–protein complexes bound to Glutathione Sepharose 4B beads were eluted in 50 µl 1 × GST elution buffer (10 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0) by spin at 14,000g for 2 min. To analyze the pull-down result, 30 µl of each elution was subjected to SDS-PAGE analysis followed by immunoblotting with anti-claudin-1 monoclonal antibody (Santa Cruz, CA).

**Statistical analysis**

All data were analyzed using the GraphPad Prism program (GraphPad Software, San Diego, CA) and Microsoft Excel. Two-tailed Student’s t tests were used to calculate p values.

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**References**


