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

Pulin Che [a,b], Hengli Tang [c], Qianjun Li [a,b,*]

[a] Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294, United States
[b] University of Alabama at Birmingham, Department of Medicine, Division of Infectious Diseases, BBRB 562, 845 19th Street South, Birmingham, AL 35294, United States
[c] Department of Biological Science, Florida State University, 3063 King Life Sciences, Stadium Drive, Tallahassee, FL 32306, United States

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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.

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 *Flaviviridae*. 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 cases of severe dengue with over 5% case fatality rate (WHO, 2012). In the past two decades, all four serotypes DENV extended their distribution geographically and circulated around tropical and sub-tropical countries, including those in Southeast Asia, the Pacific, Africa, Eastern Mediterranean and the Americas (Guzman et al., 2010). Unfortunately, there are no effective antiviral drugs or licensed vaccine currently available against DENV, and dengue diseases become a huge public concern (Halstead and Deen, 2002).

The DENV ssRNA genome is approximately 11 kb in length (Kinney et al., 1997), embedded in a DENV particle which contains three 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; Stiasny 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

* Corresponding author at: University of Alabama at Birmingham, Department of Medicine, Division of Infectious Diseases, BBRB 562, 845 19th Street South, Birmingham, AL 35294, United States. Fax: +205 934 5600.
E-mail address: liq@uab.edu (Q. Li).

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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 (Bielefeldt-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; Tassaneetrithep 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 (Evans 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 immunofluorescent 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).

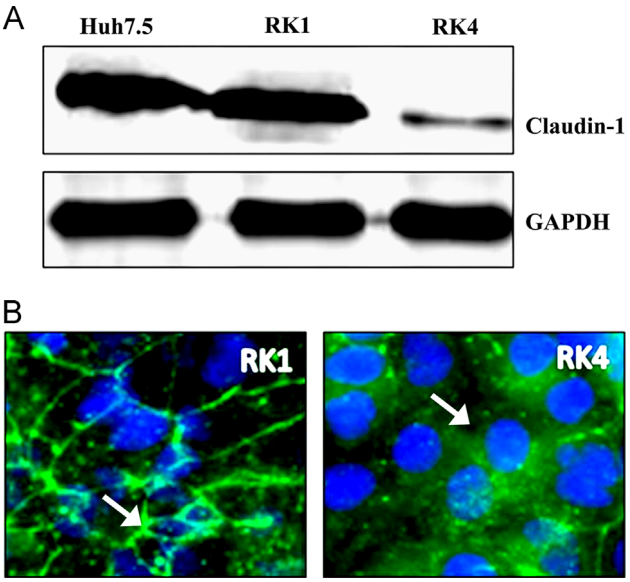

Fig. 1. The knockdown of claudin-1 expression in RK4 stable cell lines. Knockdown of claudin-1 in Huh 7.5 cells was carried out using claudin-1 specific shRNA, designated as RK4 cells. Cells tranfected with non-targeting shRNA (NT-shRNA) served as the control, designated as RK1 cells. (A) Western blot 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 caludin-1 was depleted and there were only very little claudin-1 expression around the cell surface.

Knockdown of claudin-1 prevent DENV induced CPE

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., (Fig. 2D-e) and only moderate CPE was observed 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