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Novel binding between pre-membrane protein and claudin-1 is required for efficient dengue virus entry

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ABSTRACT

Flavivirus pre-membrane (prM) protein is important for proper folding and secretion of envelope (E) protein. However, other functions of prM protein in relation to virus life-cycle are poorly characterized. In this study, we aimed to elucidate if dengue virus (DENV) prM protein interacts with host proteins and contributes to viral pathogenesis by screening human liver cDNA yeast two-hybrid library. Our study identified claudin-1 as a novel interacting partner of DENV prM protein. Virus production was significantly attenuated in claudin-1 knock-down cells. We showed that claudin-1 expression is up-regulated at the early phase of infection to facilitate DENV entry and down-regulated at the late stage of infection probably to prevent super-infection. Our study also demonstrated that DENV C protein played an important role in down-regulating claudin-1 expression during DENV infection.

Introduction

Dengue virus (DENV) is a single-stranded RNA virus belonging to *Flaviviridae* family. There are four DENV serotypes that are antigenically different, namely DENV1, DENV2, DENV3, and DENV4. Dengue virus infection causes a broad spectrum of clinical manifestations ranging from asymptomatic fever to potentially fatal hemorrhagic manifestations as in Dengue Hemorrhagic Fever and Dengue Shock Syndrome [1,2].

The DEN virions attach to the host cell receptors/co-receptors and enter the cell by receptor-mediated endocytosis. The acidic environment of the endosomal vesicles trigger conformational changes in the E protein that results in fusion of the viral and cell membranes. Nucleocapsid is then released into the cytoplasm which dissociate into the capsid protein and RNA. The genomic RNA is translated into a single polypeptide which is subsequently processed by virus and host proteases to form three structural and seven non-structural proteins in the order C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 [3–5]. The capsid protein undergoes oligomerization and interacts with viral RNA to form nucleocapsid. The pre-membrane (prM) and envelope (E) proteins synthesized at endoplasmic reticulum form heterodimers and further interact with virus nucleocapsid to generate immature virus particles. The immature virus particles are transported through trans-Golgi network and cleaved by host protease furin to form infectious mature virus particles. Mature virions are subsequently released from the host cell by exocytosis [4,5].

In this study, we aimed to investigate the function of flavivirus prM protein since its interaction with cellular proteins is poorly characterized. We identified claudin-1 as a novel binding partner of DENV prM protein. Functional studies revealed that claudin-1 influenced DENV production. We showed that claudin-1 expression is differentially regulated during DENV infection to facilitate DENV entry and to prevent super-infection. This is the first study reporting the association of DENV prM protein with claudin-1 and sheds light on the functional significance of this interaction.

Materials and methods

Cells and viruses. Huh-7 cells were maintained in Dulbecco's Modified Eagle medium (Sigma) containing 10% fetal calf serum (Invitrogen) and incubated at 37 °C with 5% CO_2. Dengue 2 virus (New Guinea C) propagated in C6/36 cells was used throughout this study. Huh-7 cells were infected with DENV at MOI of 0.1.

Plasmid construction. Full-length DENV prM was cloned into pGBKT7 vector (Clontech) using NdeI and BamHI restriction enzyme sites. The resulting constructs were sequenced to ensure correct reading frames (Supplementary Fig. S1) and were subsequently used as bait in yeast two-hybrid (Y2H) library screening. Full-length DENV prM and claudin-1 were also cloned into pGADT7/pGBKT7 (Clontech) vectors, respectively. All three

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structural and seven non-structural genes were cloned into pcDNA3.1-V5-TOPO vector (Invitrogen) to produce V5-C, V5prM, V5-E, V5-NS1, V5-NS2A, V5-NS2B, V5-NS3, V5-NS4A, V5-NS4B, and V5-NS5, respectively.

Yeast two-hybrid (Y2H) screening. Y2H mating assays were carried out using prM as bait and pre-transformed human liver cDNA yeast library (Clontech) following manufacturer's instructions. The resulting positive colonies were picked to isolate the library plasmids using Yeast plasmid extraction kit (Clontech) and sequenced for BLAST analysis (GenBank).

Co-immunoprecipitaion. Huh-7 cells (5×10^5) were infected with DENV. At 24 h post-infection, cell lysate was pre-mixed with 2 μg of anti-DENV prM-/anti-claudin-conjugated magnetic microbeads and purified using IMACs column (Miltenyi Biotec). Samples obtained from co-immunoprecipitation assays or whole cell lysates were subjected to Western blotting using anti-DENV prM (1:300)/anti-claudin (1:500)/anti-actin (1:2000) antibodies (Ab).

Knock-down of claudin-1. Double-stranded siRNAs targeted against human claudin-1 (sense, 5′-UAACAUUAGGACCUUAGAA-3′) were commercially synthesized and transfected into Huh-7 cells using RNAimax transfection reagent (Invitrogen). The resulting Huh-7 cells were named claudin-1KD cells. The effect of gene silencing was examined by Western blotting using anti-claudin Ab.

Virus growth kinetics. Huh-7, scrambled and claudin-1KD cells were infected with DENV or transfected with RNA *in vitro* transcribed from full length infectious clone of DENV. At the indicated timings, culture supernatants were collected for plaque assay.

Immunofluorescence studies. Huh-7, scrambled and claudin-1KD cells were infected with DENV or transfected with RNA *in vitro* transcribed from full length infectious clone of DENV. DENV-infected/RNA-transfected cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton-X. The anti-prM (1:300) or anti-claudin-1 (1:500) antibodies were used as primary antibodies and secondary goat antibodies conjugated to FITC or Texas red (Chemicon) were used. The nuclei were stained with DAPI (Molecular Probes). The specimens were viewed with a laser scanning confocal inverted microscope (Leica TCS SP2).

Percoll fractionation of cell homogenates. Huh-7, scrambled and claudin-1KD cells were infected with ^{35}S-labeled DENV for 1 h at 4 °C and were subsequently allowed to internalize DENV for 5, 10, 20 and 30 min at 37 °C. The cells were detached by gentle scraping. After centrifugation for 10 min at 1000 rpm, cell pellet was resuspended in homogenization buffer (10 mM Tris–HCl, 0.2 mM sucrose, 1 mM EDTA, protease cocktail inhibitor [pH 7.4]). The suspension was subjected to 20 strokes in a tight-fitting homogenizer (Jensons). The homogenate was centrifuged for 10 min at 1000 rpm to remove the nuclei. For the separation of sub-cellular particles in the post-nuclear supernatant, 20% Percoll was prepared. The post-nuclear supernatant samples were centrifuged at 32,000*g* for 30 min. Density marker beads (Pharmacia Biotech) were used as external standards for the density gradients in the Percoll solution. A total of 26 fractions were collected and processed for liquid scintillation counting of ^{35}S-labeled DENV radioactivity in a Beckman LS6500 liquid scintillation counter.

Western blotting analysis of claudin-1 during DENV infection. Total cellular protein was extracted from mock-infected and DENV-infected cells or from various structural and non-structural genes-transfected Huh-7 cells. Twenty micrograms of cellular protein extract was fractionated on SDS–PAGE gel and then transferred onto PVDF membrane. The membrane was incubated overnight at 4 °C with anti-claudin-1 or anti-actin Ab. After three vigorous washings with PBST, the membrane was further incubated with horseradish peroxidase-conjugated secondary Ab at room temperature and developed using chemiluminescence detection kit (Invitrogen). The blots were scanned and quantitated using the Biorad Quantity one Program.

Results

Identification of claudin-1 as a binding partner of prM protein

Using DENV prM protein as bait, Y2H screening was performed against human liver cDNA library. Three independent clones that encoded claudin-1 protein (sequences were shown in Supplementary Fig. S2) were found to interact with prM protein. To confirm this interaction, full-length claudin-1 was cloned into the yeast vector pGBKT7 to serve as bait taking DENV prM protein as prey. Claudin-1 showed strong interaction with DENV prM protein as revealed by α-galactosidase assay (Fig. 1A). These results indicated that DENV prM protein interact with claudin-1 in the yeast system. To eliminate the possibility of auto-activation, the bait and prey plasmids were co-transformed with empty pGADT7/pGBKT7 vector. No intrinsic or non-specific activation for either the bait or the prey was observed (Table 1). This indicated that Y2H analysis for prM-claudin-1 interaction is specific and reliable.

To confirm prM-claudin-1 association in mammalian system, co-immunoprecipitation was performed. Huh-7 cells were infected with DENV. At 24 h p.i., cell lysates were immunoprecipitated with anti-claudin-1 Ab followed by immunoblotting using anti-DENV prM Ab. As shown in Fig. 1B(i), immuno-reactive bands were observed only with DENV-infected Huh-7 cells (Lane 3). The reciprocal co-immunoprecipitation performed showed consistent results [Fig. 1B(ii)]. The precipitation controls were included to ensure successful precipitation in all groups [Fig. 1B(iii and iv)]. The expression of prM and claudin-1 proteins in whole cell lysates (WCL) was shown in Fig. 1B(v and vi). Co-immunoprecipitation was also performed using mouse/rabbit isotype control Ab to eliminate the possibility of non-specific precipitation (data not shown). This demonstrated for the first time that DENV prM protein interacted specifically with claudin-1.

Claudin-1 influences DENV production

To examine the role of claudin-1 in relation to virus production, claudin-1 knock-down (claudin-1KD) Huh-7 cells were generated. The effect of gene silencing in Huh-7 cells was confirmed by Western blotting and densitometry (Quantity One, Biorad). The gene knock-down decreased the endogenous claudin-1 level by 60% as revealed by densitometry (Fig. 2A). The effect of claudin-1KD on DENV production was examined in Huh-7 cells by infecting scrambled and claudin-1KD cells with DENV. At the indicated time points, culture supernatants were collected for plaque assay. Obvious reduction in virus production was observed with DENV-infected claudin-1KD cells (Fig. 2B) at as early as 1-day post-infection although 2 log units reduction in virus titres were obtained at 3 days post-infection ($P < 0.05$). This indicated that claudin-1 influenced DENV production.

Claudin-1 influences the early events in DENV life-cycle

To test if claudin-1-induced changes in virus titres were due to enhanced virus entry and genome uncoating, these processes were bypassed by transfecting scrambled and claudin-1KD cells with RNA *in vitro* transcribed from full length infectious clone of DENV. At the indicated time points, culture supernatants were collected for plaque assay. There is no significant difference in virus titres obtained from claudin-1KD cells transfected with full-length DENV RNA compared to scrambled cells (Fig. 3A). This showed that claudin-1 influenced the early events in DENV life-cycle.

Huh-7, scrambled and claudin-1KD cells were infected with DENV and cells were assayed for infectious entry of DENV by counting the number of virus prM protein expressing cells.

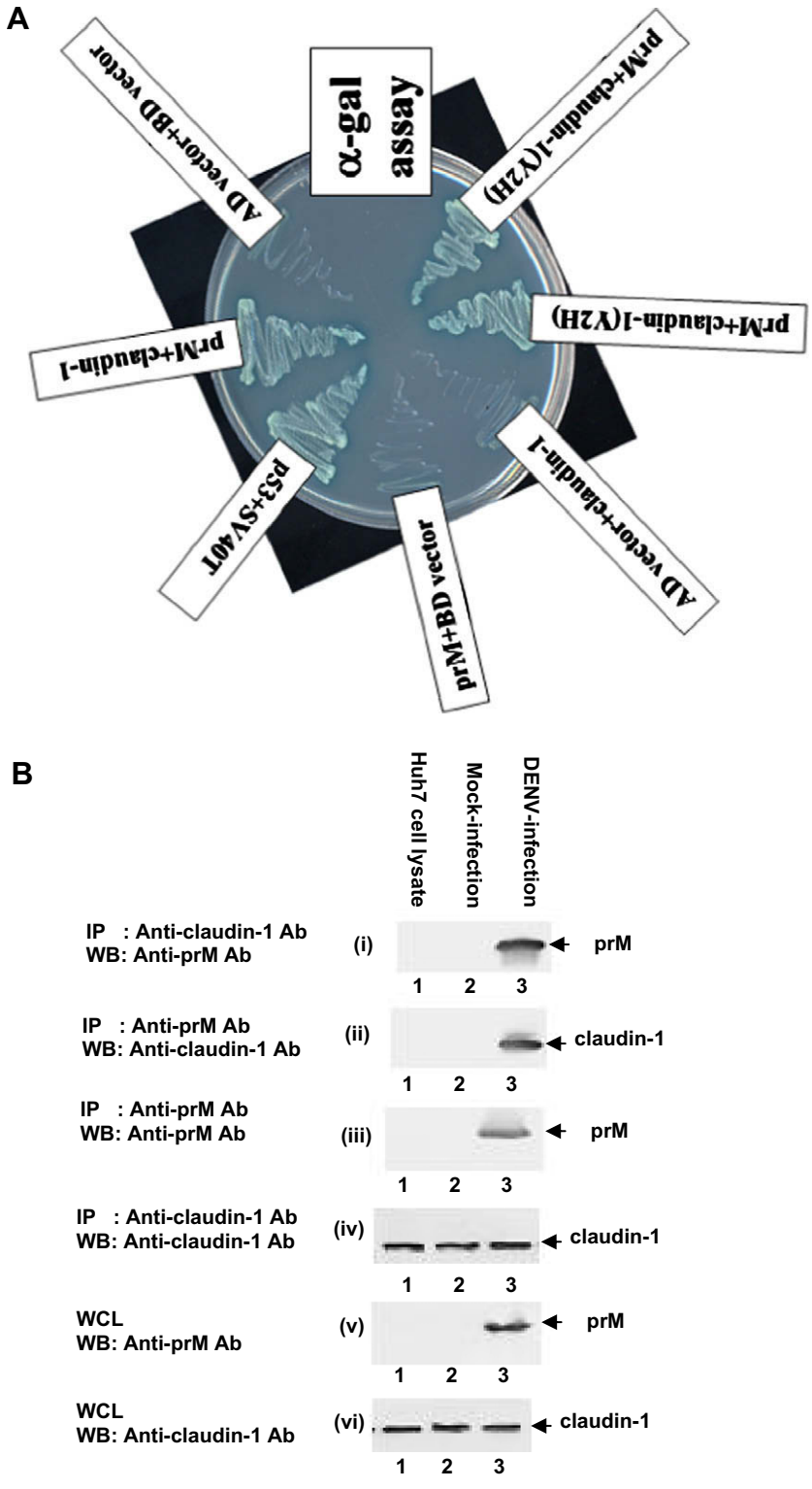

Fig. 1. Interaction between claudin-1 and prM. (A) Interaction between DENV prM protein and claudin-1 in yeast two-hybrid system, assayed for α-galactosidase activity. Full-length claudin-1 fused with BD-domain was co-transformed with prM protein fused with AD-domain into the yeast strain *S. cerevisiae* AH109 (prM + claudin-1). The truncated claudin-1 clones (AD-fusions) isolated from Y2H library plasmids were also co-transformed with prM protein fused with BD-domain [(prM + claudin-1(Y2H)]. Co-transformation was also performed with control vectors (AD vector + BD vector, prM + BD vector, AD vector + claudin-1). The resulting transformation mixtures were plated onto synthetic dropout media lacking tryphtophan, leucine, histidine and adenine and tested for α-galactosidase production. Strong α-galactosidase signals were obtained for the interaction pairs such as prM + claudin-1 and prM + claudin-1 (Y2H). Interaction of p53 with SV40 large T-antigen served as positive control (p53 + SV40T). (B) Co-immunoprecipitation. (i) Huh-7 cells were infected with DENV and immunoprecipitated with anti-claudin-1 Ab. Presence of band in Lane 3 confirms the binding between prM and claudin-1 in Huh-7 cells. (ii) Reciprocal Co-IP performed using anti-prM Ab. (iii–vi) Immunoprecipitation controls and input controls.

Approximately 200 cells were counted at each experiment and repeated for three times. Knock-down of claudin-1 caused a marked reduction of DENV infection ($P < 0.05$) (Fig. 3B). Huh-7, scrambled and claudin-1KD cells transfected with DENV RNA did not show

Table 1
Interaction between DENV prM protein and claudin-1 in yeast two-hybrid system, assayed for *HIS*3 autotrophy.

AD fusion	BD fusion Claudin-1	Lamin-C	BD vector	p53
prM	+++	–	–	
AD vector	–	–	–	
SV40 T		+++		

All the constructs (AD and BD fusions) were assayed for non-specific reporter gene activation by transforming singly or with control pGBKT7-Lamin-C or BD vector and plated onto SD-His plates. Interaction of p53 with SV40 large T-antigen served as positive control. Growth was recorded after 2 days when the positive control showed clear growth. (+++): clear growth (strong interaction); (–): no growth (no interaction). As depicted in the table, non-specific activation for all the fusions was not observed.

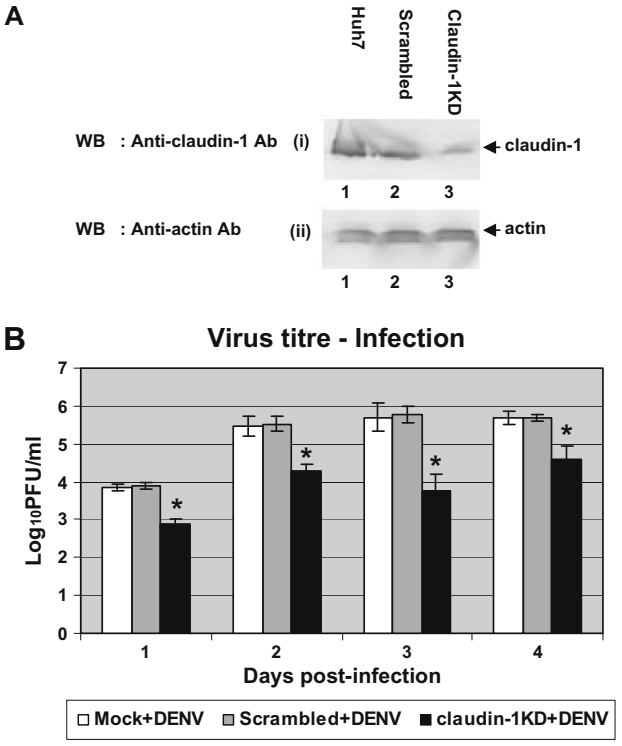

Fig. 2. Claudin-1 influences DENV production. (A) Western blot analysis showing the effect of claudin-1 gene silencing in Huh-7 cells. (B) Huh-7, scrambled and claudin-1KD cells were infected with DENV and supernatants harvested at indicated timings were subjected to plaque assay. Reduced virus titres are observed with claudin-1KD cells compared to scrambled/Huh-7 cells. The results represented the mean activity of three independent experiments ± standard deviation. The *P*-values were calculated using student's *t* test (*$P < 0.05$).

significant changes ($P > 0.05$) in the expression of prM protein (Supplementary Fig. S3). This indicated that claudin-1 influenced the early steps in DENV life-cycle.

We performed cellular fractionation in 20% Percoll gradient (as described in Materials and methods) to examine the distribution of DENV in specific location in the entry process following the gene silencing of claudin-1. Cellular fractionation in 20% Percoll gradient allowed the separation of sub-cellular particles based on buoyant density. Density marker beads facilitated the density measurement of the separated sub-cellular particles in the gradient. The densities of the endosomes, plasma membrane, endoplasmic reticulum and lysosomes were determined to be 1.036, 1.047, 1.06, and 1.079 g/ml, respectively (data not shown). A total of 26 fractions were collected and analyzed for radioactivity counting. The densities of the radioactive peaks were determined relative to the density bead markers based on their positions in the Percoll gradient (Supplementary Fig. S4). A single peak of radioactivity counts was detected at the density corresponded to the plasma membrane fraction when Huh-7 cells incubated with DENV at 37 °C for 5 min were subjected to cellular fractionation [Fig. 3C(i)]. This demonstrated that DENV attached to the plasma membrane of Huh-7 cells. At 10 min upon warming to 37 °C, two peaks of radioactivity densities corresponded to endosome marker fraction (first peak) and lysosome maker fraction (second peak) were determined [Fig. 3C(ii)]. Much higher radioactivity counts observed with first peak suggested that majority of DENV were localized within the early endosomes of Huh-7 cells at 10 min p.i.

Three radioactivity count peaks were obtained at 20 min p.i. The densities of the three peaks corresponded to early endosomes, endoplasmic reticulum, and lysosome markers, respectively [Fig. 3C(iii)]. The radiolabeled DENV was observed to localize predominantly within the lysosome fraction (as suggested by highest radioactivity counts). This demonstrated that DENV traveled along the endosomal–lysosomal pathway. Similar results were obtained from DENV-infected scrambled cells [Fig. 3C(i–iii)]. In contrast, a single peak of radioactivity counts was detected at the density corresponded to the plasma membrane fraction even after 20 min incubation with claudin-1KD cells at 37 °C [Fig. 3C(i–iii)]. This demonstrated that claudin-1 influenced DENV entry from the plasma membrane to endosomes.

DENV infection alters the expression of claudin-1

We went further to investigate if claudin-1 expression is altered during DENV infection by Western blotting the whole cell lysates of DENV-infected Huh-7 cells. The band intensity obtained with anti-claudin-1 Ab was quantified after normalization against those obtained with anti-actin Ab. As shown in Fig. 4A, the expression of claudin-1 was significantly increased at 6 and 12 h p.i. ($P < 0.05$). Interestingly, the claudin-1 level was down-regulated from 24 h p.i. onwards.

In order to elucidate which viral protein is causing this differential expression of claudin-1, we expressed V5-tagged DENV structural and non-structural proteins in Huh-7 cells. Although the expression of all DENV structural proteins (C, prM, and E) inhibited the expression of claudin-1 ($P < 0.05$), C protein expression significantly decreased the expression level of claudin-1 ($P < 0.01$, Fig. 4B).

Discussion

The *Flaviviridae* family consists of several medically important pathogens such as dengue virus (DENV), West Nile virus (WNV), Japanese encephalitis virus (JEV) and yellow fever virus (YFV). These viruses spread globally and pose great threat to human health. Although these viruses account for few hundred million human infections annually, there are no effective therapeutic options currently available. Although there are licensed vaccines for JEV and YFV, these viruses still claim thousands of life across their vast endemic areas [1,2,6]. There are no approved vaccines available for DENV and WNV. Hence, there is a need to look for effective antiviral drugs to control the disease. In order to design an appropriate anti-viral drug, it is essential to understand the virus–host protein interactions.

In this study, DENV prM protein was used to elucidate the biological role of prM protein in the pathogenesis of DENV. Human liver cDNA library was screened using DENV prM protein as bait. We demonstrated for the first time that DENV prM protein interacted

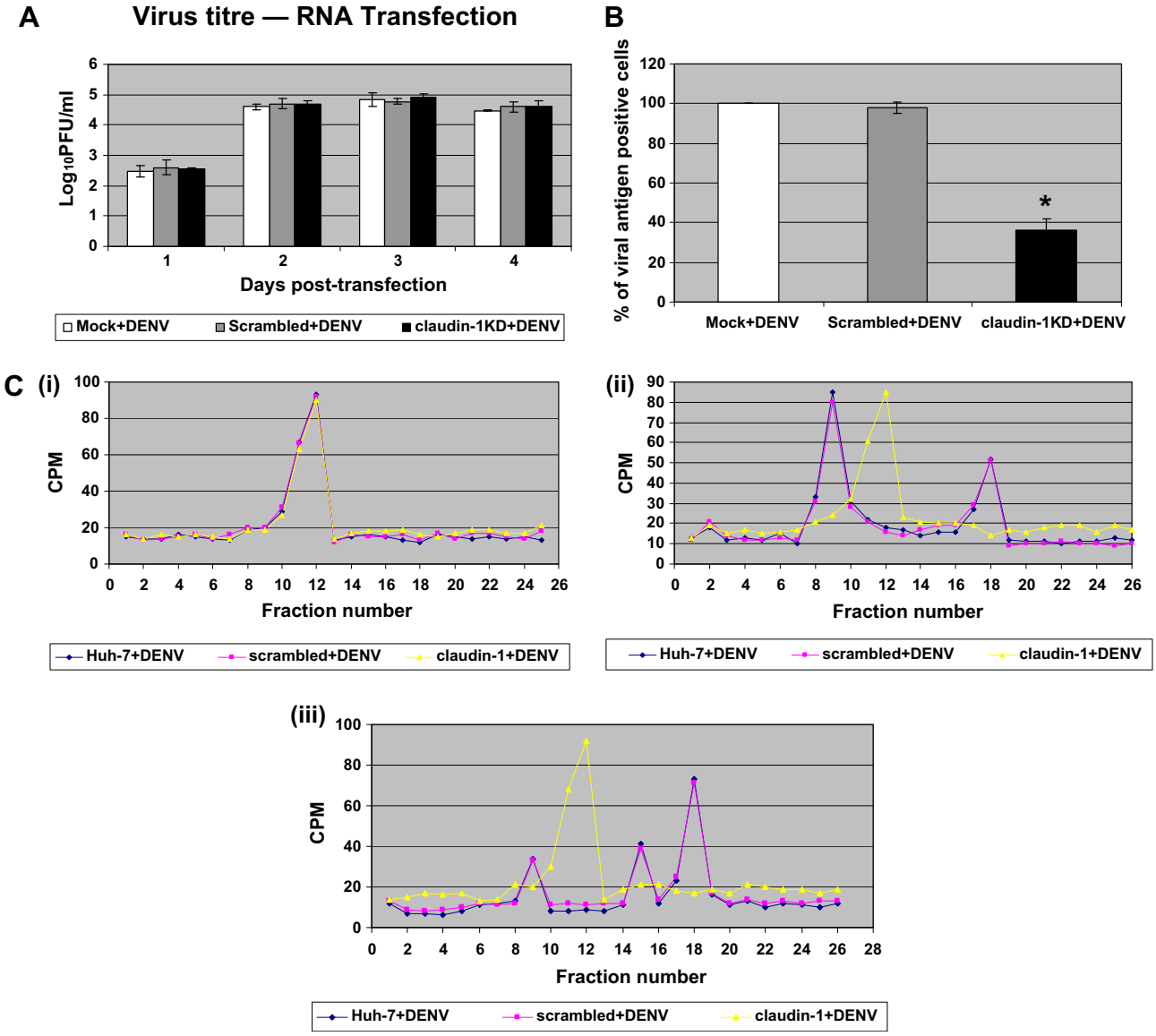

Fig. 3. Claudin-1 influences the early events in DENV life-cycle. (A) Huh-7, scrambled and claudin-1KD cells were transfected with DENV RNA and supernatants harvested at indicated timings were subjected to plaque assay. There is no significant difference ($P > 0.05$) in virus titres obtained from claudin-1KD cells transfected with full-length DENV RNA compared to scrambled cells. (B) Huh-7, scrambled and claudin-1KD cells infected with DENV were analyzed for the number of virus prM protein expressing cells. Knock-down of claudin-1 caused a marked reduction of DENV infection (*$P < 0.05$). (C) (i) Huh-7, scrambled and claudin-1KD cells were allowed to bind ^{35}S-radiolabeled DENV at 37 °C for 5 min and subjected to cellular fractionation. DENV is detected predominantly in association with the plasma membrane fraction of Huh-7, scrambled and claudin-1KD cells. (ii, iii) A procedure similar to that for panel (i) was carried out except that Huh-7, scrambled and claudin-1KD cells were incubated at 37 °C for a period of 10 and 20 min, respectively. Trafficking of the DENV particles along the endocytic pathway is noted in Huh-7 and scrambled cells. In contrast, DENV is detected predominantly in association with the plasma membrane fraction of claudin-1KD cells even after 20 min incubation at 37 °C.

directly with claudin-1. Claudins are a family of tight junction membrane proteins that create a paracellular barrier in epithelial and endothelial cells to protect them from the external environment [7,8]. Differential expression of claudin-1 has been reported in several metabolic disorders such as diabetes [9]. Claudins have also been shown to play an essential role in the life-cycle of several viruses such as Hepatitis C virus (HCV), WNV and Adenovirus [10–16]. By modulating the expression of claudins, these viruses moved to the tight junctions of epithelial and endothelial cells and induced the pathogenesis associated with the virus infection [15,16].

It is generally believed that prM is important for the proper folding and secretion of E protein and that cleavage of prM to M are required to render the virus particle infectious [17–20]. Dissecting the biological significance of prM-claudin-1 interaction illustrated that claudin-1 could function as a receptor/co-receptor for DENV entry. It is not surprising since a high proportion of DENV was shown to contain uncleaved prM and prM-containing DENV particles are infectious [21–23]. Uncleaved prM on the surface of DENV might interact with claudin-1 to facilitate the entry of the virus. Although claudin-1 has been shown to function as an essential co-factor for HCV entry [10,11,13,14], we are the first to demonstrate that the entry of arthropod-borne flaviviruses such as DENV is influenced by claudin-1.

We also showed that the expression of claudin-1 was significantly increased at the early phase of infection and decreased at the late stages of infection (Fig. 4). This differential expression of claudin-1 during DENV infection could be the virus strategy to facilitate efficient virus entry at the early stages and to prevent the danger of super-infection at the late stages of infection. However, this warrants further investigation.

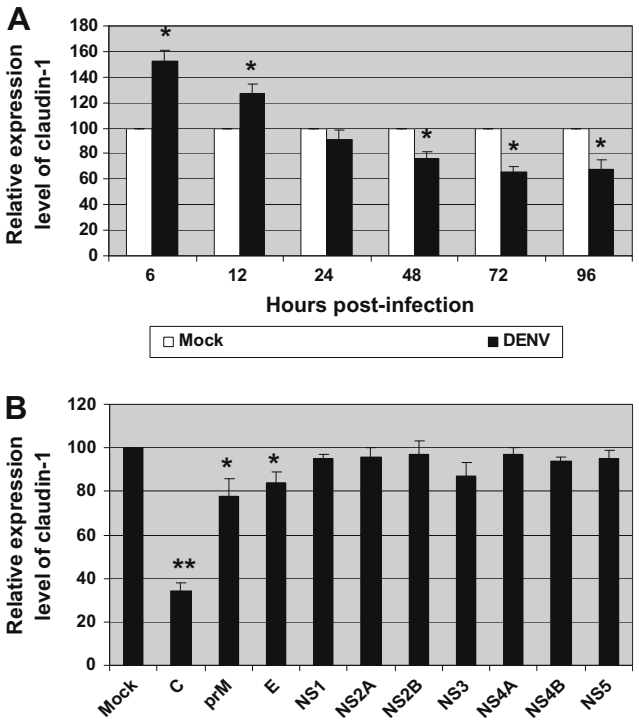

Fig. 4. Expression of claudin-1 during DENV infection: (A) Densitometry analysis of claudin-1 expression during DENV infection. The expression of claudin-1 is up-regulated at 6 and 12 h p.i. and down-regulated from 24 h p.i. onwards. (*$P < 0.05$). (B) Densitometry analysis of claudin-1 expression in various viral protein-transfected Huh-7 cells. Exogenous expression of C protein significantly decreased the expression level of claudin-1. (*$P < 0.05$ and **$P < 0.01$).

In conclusion, our study identified a novel interaction between claudin-1 and prM protein and this association is critical to mediate efficient virus entry. This study broadened our current understanding on the functions of flavivirus prM protein. Moreover, this study also identified a new anti-flaviviral target (claudin-1) and formed the platform towards the discovery of new anti-flaviviral drugs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.172.

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