รายงานวิจัยฉบับสมบูรณ์

โครงการ

“ ไวรัสเดงกี่ใช้โปรตีน GRP78 ของมนุษย์ เพื่อช่วยในการพับตัวและเพิ่มจำนวนในเซลล์ ”

โดย

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และคณะ

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สนับสนุนโดย

สำนักงานคณะกรรมการอุดมศึกษาและสำนักงานกองทุนสนับสนุนการวิจัย

Abstract

Dengue virus infection is an important mosquito-borne disease and a public health problem worldwide. A better understanding of interactions between human cellular host and dengue virus proteins will provide insight into dengue virus replication and cellular pathogenesis. The glycosylated envelope protein of dengue virus, DENV E, is processed in the endoplasmic reticulum of host cells and therefore reliant on host processing functions. The complement of host ER functions involved and nature of the interactions with DENV E has not been thoroughly investigated. By employing a yeast two-hybrid assay, we found that domain III of DENV E interacts with human immunoglobulin heavy chain binding protein (BiP) or GRP78. The relevance of this interaction was demonstrated by co-immunoprecipitation and co-localization of BiP/GRP78 and DENV E in dengue virus-infected cells. Using the same approach, association of DENV E with two other chaperones, calnexin and calreticulin was also observed. Knocking-down expression of BiP/GRP78, calnexin, or calreticulin by siRNA significantly decreased the production of infectious dengue virions. These results indicate that the interaction of these three chaperones with DENV E plays an important role in virion production, likely facilitating proper folding and assembly of dengue proteins.

บทคัดย่อ

การติดเชื้อไวรัสเดงกี่เป็นปัญหาที่สำคัญทางสาธารณสุข ความรู้พื้นฐานเกี่ยวกับปฏิสัมพันธ์ระหว่างโปรตีนของไวรัสเดงกี่และโปรตีนของเซลล์เจ้าบ้านมีความสำคัญและจำเป็นในการสร้างเข้าใจเกี่ยวกับพยาธิสภาพของโรคไข้เลือดออก จนถึงปัจจุบันความรู้ ความเข้าใจเกี่ยวกับปฏิสัมพันธ์ระหว่างโปรตีนเปลือกหุ้มของไวรัสเดงกี่กับโปรตีนของมนุษย์ใน endoplasmic reticulum ยังไม่ได้ถูกศึกษาในรายละเอียด ดังนั้นผู้วิจัยได้ใช้วิธี yeast two hybrid ในการหาโปรตีนของมนุษย์ที่มีปฏิสัมพันธ์กับโปรตีนเปลือกหุ้มของไวรัสเดงกี่พบว่าโปรตีนของมนุษย์ใน endoplasmic reticulum ที่ชื่อว่า BiP หรือ GRP78 จับกับโปรตีนเปลือกหุ้มของไวรัสเดงกี่ ปฏิสัมพันธ์นี้ได้ถูกยืนยันในเซลล์สัตว์เลี้ยงลูกด้วยนมด้วยวิธี co-immunoprecipitation และ co-localization นอกจากนี้ผู้วิจัยยังพบว่าโปรตีนเปลือกหุ้มของไวรัสเดงกี่จับกับโปรตีนของเซลล์เจ้าบ้านใน endoplasmic reticulum ที่ชื่อว่า calnexin และ calreticulin ที่สำคัญอย่างยิ่งก็คือการทำให้เซลล์เจ้าบ้านไม่สามารถสร้าง BiP หรือ GRP78, calnexin และ calreticulin ได้โดยวิธี RNA interference ทำให้การสร้างไวรัสเดงกี่ในเซลล์ลดลงบ่งชี้ถึงความสำคัญของโปรตีนใน endoplasmic reticulum ของมนุษย์ในการพับตัวและเพิ่มจำนวนของไวรัสเดงกี่ในเซลล์

Executive summary

Dengue virus infection is an important mosquito-borne disease and a public health problem worldwide. A better understanding of interactions between human cellular host and dengue virus proteins will provide insight into dengue virus replication and cellular pathogenesis. The glycosylated envelope protein of dengue virus, DENV E, is processed in the endoplasmic reticulum of host cells and therefore reliant on host processing functions. The complement of host ER functions involved and nature of the interactions with DENV E has not been thoroughly investigated. By employing a yeast two-hybrid assay, we found that domain III of DENV E interacts with human immunoglobulin heavy chain binding protein (BiP) or GRP78. The relevance of this interaction was demonstrated by co-immunoprecipitation and co-localization of BiP/GRP78 and DENV E in dengue virus-infected cells. Using the same approach, association of DENV E with two other chaperones, calnexin and calreticulin was also observed. Knocking-down expression of BiP/GRP78, calnexin, or calreticulin by siRNA significantly decreased the production of infectious dengue virions. These results indicate that the interaction of these three chaperones with DENV E plays an important role in virion production, likely facilitating proper folding and assembly of dengue proteins.

Introduction

Dengue virus (DENV) infection is one of the most important mosquito-borne viral diseases, which is endemic in many countries. Clinical severity ranges from febrile dengue fever (DF) to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). DENV belongs to the *Flaviviridae* family and contains a single positive-stranded RNA genome, encoding a single precursor polypeptide. Host and viral proteases cleave this polypeptide into three structural proteins (capsid, membrane, and envelope) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) [1].

Replication of flaviviruses occurs in association with the endoplasmic reticulum where virons assemble and bud into the lumen of the ER. Virus particles transit through the Golgi where they undergo maturation prior to being released by exocytosis [1]. DENV envelope protein (DENV E) is the major component of the virion surface contains two functionally important glycosylation sites, Asn-67 and Asn-153. DENV E lacking Asn-67 was able to infect cells but the production of new infectious particles was abolished. In addition, DENV E lacking Asn-153 showed reduced infectivity [2]. Although DENV E biosynthesis and assembly is thought to occur in the ER, limited information is available on the involvement of host ER chaperones in the folding and assembly of DENV E. We therefore performed a yeast two-hybrid assay to identify host proteins that interact with DENV E. One of the proteins identified in this screen was human immunoglobulin heavy chain binding protein (BiP) or GRP78, a member of the heat shock protein 70 family found in the ER lumen, the interaction between DENV E and BiP in dengue virus-

infected Vero cells was confirmed by co-immunoprecipitation and co-localization studies. Two additional ER-resident chaperones, calnexin and calreticulin, were similarly examined and both exhibited co-immunoprecipitation and co-localization with DENV E. Significantly, knocking down the expression of BiP, calnexin, or calreticulin by siRNA decreased the yield of infectious dengue virions, indicating that the role of these ER-resident chaperones in the folding and assembly of DENV E is essential for dengue virion production.

Materials and Methods

Yeast two-hybrid screening. Two-hybrid screening was performed by the interaction mating method as described by Finley and Brent [3]. Domain III of DENV E, nucleotides 1819 to 2118 encoding amino acids 295 to 394, was amplified from pBluescript II KS (S1SP6-4497), a plasmid which contains the 5' portion of DENV serotype 2 strain 16681 [4] by a pair of nucleotide primers 5′ AAG CCG GAA TTC AAA GGA ATG TCA TAC 3′ for the forward direction and 5′ GCC CGC GGA TCC CTA TTT CTT AAA CCA G 3′ for the reverse direction. Domain III is an immunoglobulin-like domain and does not contain a trans-membrane domain [5]. The PCR reaction was carried out in a GeneAmp PCR System 9700 (Applied Biosystems) consisting of 35 cycles of denaturation at 94ºC for 45 seconds, annealing at 55ºC for 45 seconds, and extension at 72ºC for 2 minutes. Subsequently, the amplified DNA was cloned as an in frame fusion with the LexA DNA binding domain in the yeast expression vector pEG202, which contains a *HIS3* selectable marker. The resulting bait plasmid, pEG-E,

was transformed into *S. cerevisiae* strain RFY 206 (MATa *his3A200 leu2-3 lys2A201 ura3-52 trp1A::hisG*) containing a Lexop-*lac*Z reporter plasmid, pSH18-34, under *URA3* selection. A galactose inducible HeLa cell cDNA prey library was constructed in plasmid pJZ 4-5 containing a *TRP1* selectable marker and transformed into strain RFY 231 (MATα *his3 leu2::3Lexop-LEU2 ura3 trp1 LYS2*) [6]. The bait strain was mated with the library strains and plated on galactose drop-out medium lacking histidine, tryptophan, uracil and leucine (gal/raf –u, -h, -w, -l) to select for diploids. The production of a DENV E binding protein by a prey plasmid was expected to activate the 3Lexop-*LEU*2 reporter. Putative positive clones were patching to four indicator plates: (glu/–u, -h, -w, -l), (gal/raf –u, -h, -w, -l), (glu/X-Gal–u, -h, -w), and (gal/raf/ X-Gal–u, -h, -w). Prey plasmids were rescued from clones exhibiting a galactose-inducible Leu^+ *lac*Z^+ phenotype by transformation into a Trp^- *Escherichia coli* strain KC8 [3]. To verify the interaction, recovered prey plasmids were introduced into yeast strain RFY 231 along with the *lac*Z plasmid and bait plasmid and again tested on the indicator plates.

Co-immunoprecipitation. Sub-confluent monolayers of 1 x 10^7 Vero cells in a 100-mm dish were infected with DENV serotype 2 strain 16681 at an MOI of 1 for 48 hours. Cells were washed twice with 5 ml of PBS and detached by incubating with 2.5 mM EDTA in PBS for 15 minutes. Detached cells were collected by centrifugation. The cell pellets were lysed with a buffer containing 150 mM NaCl, 20 mM Tris-HCl pH 7.4, 5 mM EDTA, 0.5% deoxycholate, 0.1% SDS, 1% Triton X-100 and protease inhibitors (complete, EDTA-free, Roche) and the

lysate was incubated on ice for 20 minutes. Cell debris was removed by centrifugation at 13,000 x g for 10 minutes at 4°C. Five μg of goat anti-BiP antibody, goat anti-calnexin antibody, rabbit anti-calreticulin antibody or an isotype-matched control antibody (Santa Cruz Biotechnology) were added to lysates and incubated 4 hours in the presence of Protein G Sepharose beads (Amersham Pharmacia Biosciences). Subsequently, the Protein G Sepharose beads were collected by centrifugation at 13,000 x g for 5 minutes and washed twice with washing buffer. Lastly, the bound proteins were eluted by boiling in SDS-PAGE loading buffer, separated by SDS-PAGE, and transferred to nitrocellulose membranes. After blocking with 5% skim milk, the membranes were incubated with mouse anti-DENV E monoclonal antibody (3H5) [7; 8] followed by probing with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse antibody (Dako). The protein bands were detected using ECL reagent (Amersham Pharmacia Biosciences).

Co-localization. Vero cells were grown on cover slips and infected with DENV serotype 2 strain 16681 at an MOI of 1 for 48 hours. Thereafter, the cells were washed, fixed with 4% paraformaldehyde in PBS for 20 minutes, and permeabilized with 0.2% Triton X-100 in PBS for 10 minutes at room temperature. After washing three times with 0.1% Triton X-100 in PBS, the cover slips were incubated for an hour with (i) mouse anti-DENV E antibody (3H5) and goat anti-BiP antibody (Santa Cruz Biotechnology) (ii) mouse anti-DENV E antibody (3H5) and rabbit anti-calnexin antibody (Santa Cruz Biotechnology) (iii) mouse anti-DENV E antibody (3H5) and goat anti-calreticulin antibody

(Santa Cruz Biotechnology). After washing, the cover slips (i and iii) were incubated with both Alexa 488-conjugated donkey anti-goat antibody and Alexa 594-conjugated donkey anti-mouse antibody (Molecular Probes) as secondary antibodies at room temperature for an hour. Cover slip (ii) was incubated with both Alexa 488-conjugated donkey anti-mouse antibody and Cy3-conjugated donkey anti-rabbit antibody (Molecular Probes) as secondary antibodies. Florescent images were captured by a confocal microscope (model LSM 510 Meta, Carl Zeiss).

Knock-down experiments by siRNA and infection assays. The BiP siRNA (5' GCGGAACCTTCGATGTGTCTCTTCT 3'), calnexin siRNA (5' ATAGAATGTGGTGGTGCCTATGTGA 3'), calreticulin siRNA (5' CCCGCTGGATCGAATCCAAACACAA 3') were purchased from Invitrogen, USA and used to knock-down BiP, calnexin and calreticulin by transfection into Vero cells using Lipofectamine™ 2000 reagent (Invitrogen, USA). Transfection with irrelevant siRNA (Invitrogen, USA, Cat. No. 12935-300) was performed as a negative control. After 6 hours of transfection, cells were fed with 10% FBS in MEM medium for 30 hours. Samples were taken for mRNA and protein analysis using real-time PCR (Lightcycler RNA amplification kit, Roche) and Western blot analysis, respectively. Then, siRNA-transfected cells were infected with DENV serotype 2 strain 16681 at an MOI of 1 for 3 hours. The infected siRNA-transfected cells were washed with PBS and fed with 2% FBS in MEM medium for 24 hours. The culture supernatants were collected to measure the amount of DENV production by a focus forming unit (FFU) assay as previously described [9].

Results and Discussion

DENV E interacts with BiP in a yeast two-hybrid system

To identify human proteins that interacted with DENV E, we screened over 10^7 clones from a HeLa cDNA library using DENV E as bait. Forty five putative positive clones were obtained. Sequence analysis of the inserts showed that three of the cDNA inserts were identical and encoded amino acids 467 to 655 of BiP. BiP, also known as glucose regulated protein (GRP78), is an isoform 5 of the heat shock protein 70, which functions as a molecular chaperone involving in folding and assembly of several cellular and viral membrane proteins [10; 11; 12; 13; 14]. The specificity of the interaction between DENV E and BiP is shown in Fig. 1A wherein cells containing the DENV E bait plasmid and BiP prey plasmid exhibited galactose-dependent leucine prototrophy and *lacZ* expression. As controls, cells containing the DENV C bait plasmid and BiP prey plasmid or cells containing the DENV NS5 bait plasmid and BiP prey plasmid did not exhibit galactose-dependent leucine prototrophy and *lacZ* expression.

BiP, in complex with other ER chaperones, facilitates the proper folding of proteins in the secretory system. It has two distinct functional regions. The amino-terminal region of BiP possesses an ATPase activity [15; 16] whereas its carboxyl-terminal region contains an 18 kDa substrate-binding domain and a 10 kDa oligomerization domain [17; 18]. The region of BiP that interacted with DENV E in yeast two-hybrid screening was further mapped by yeast two hybrid system and located between amino acids 467-530 in the substrate-binding domain of the carboxyl-terminal region of BiP (Fig. 1B).

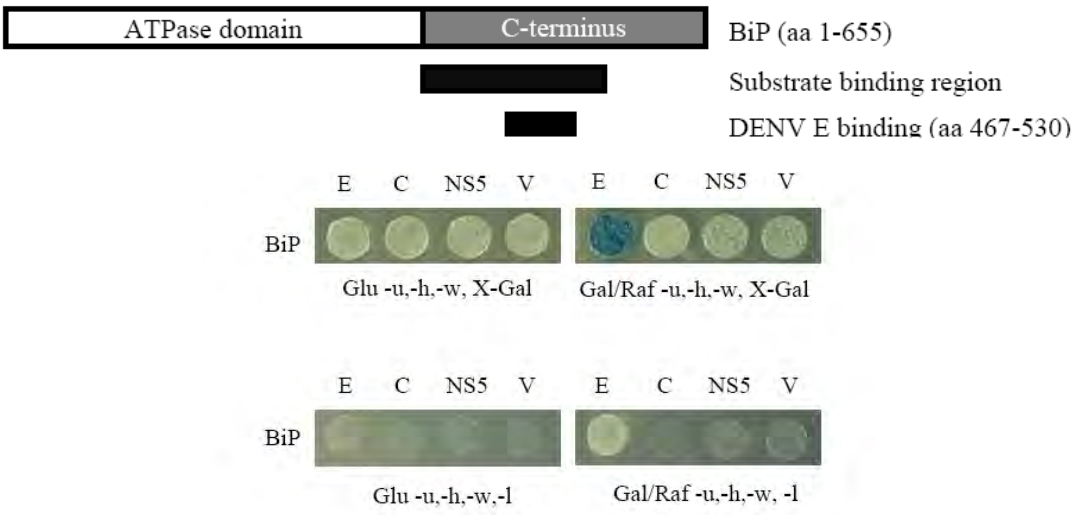


Fig. 1. DENV E-human BiP interaction in a yeast two-hybrid system

(A) Yeast strain RFY231 was co-transformed with a bait plasmid, a BiP prey plasmid and *lacZ* reporter plasmid. The bait plasmids used were pEG-E, expressing the LexA-DENV E fusion protein (E), pEG-C expressing the LexA-DENV C fusion protein (C), pEG-NS5 expressing the LexA-DENV NS5 fusion protein (NS5), and the empty bait plasmid pEG202, expressing the LexA fusion protein (V). A specific interaction was indicated by galactose-dependent β-galactosidase expression, as evidenced by blue colonies on the galactose containing X-Gal plate and white colonies on the glucose containing X-Gal plate, and by galactose-dependent growth on the leucine deficient plate. (B) The region of BiP that interacted with DENV E was located between the amino acid positions 467-530 in the substrate-binding domain of the carboxyl-terminal region of BiP.

DENV E associates with BiP, calnexin, and calreticulin in dengue virus-infected cells

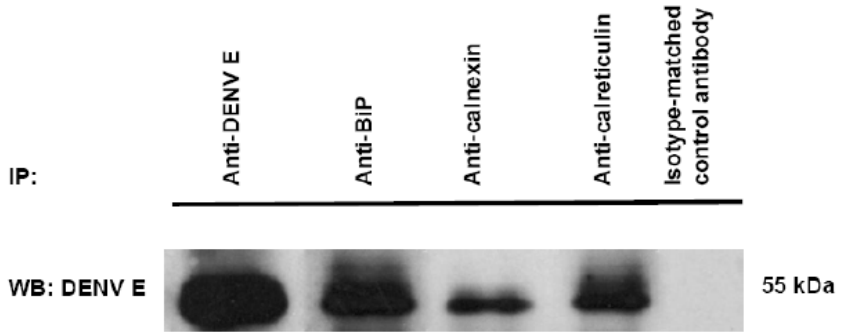
In order to confirm DENV E-BiP interaction in mammalian cells, lysates from dengue virus-infected Vero cells were tested for co-immunoprecipitation of the proteins. As shown in Fig. 2A, immunoprecipitation with anti-BiP antibody pulled down DENV E protein suggesting the association of DENV E and BiP in dengue virus-infected cells. Furthermore, co-localization of DENV E and BiP in the ER was evident when DENV E and BiP fluorescence images were superimposed (Fig. 2B). In hepatitis B virus (HBV), BiP interacts with the large surface protein (L) and plays a role in HBV morphogenesis by regulating proper folding of the L protein and assembly of the envelope protein [19]. The envelope protein of HIV type 1 has also been shown to interact with BiP [11]. Thus the interaction of DENV E and BiP may contribute to DENV morphogenesis by regulating the correct folding of DENV E.

Based on the immunofluoresence intensity of the images in Fig. 2, BiP appeared to be strongly induced by DENV infection. This is consistent with previous studies showing that DENV infection induces the unfolded protein response [20; 21] and that BiP is induced by other flaviviruses [22; 23].

Interestingly, cell surface associated BiP was previously identified as a putative dengue virus receptor in hepatic HepG2 cells [24] and our two-hybrid screen used domain III of DENV E which is thought to bind host cell receptors [5]. Therefore, we tested the ability of anti-BiP antibodies to block DENV infection. However, in our study using Vero cells both polyclonal antibody directed against BiP and

monoclonal antibody directed against the carboxyl-terminal region of BiP, the binding site between DENV E and BiP, failed to inhibit DENV infection (data not shown), suggesting that an alternate or additional receptors are present on Vero cells.

BiP is one of multiple chaperone systems important for ER quality control [25]. Calnexin and calreticulin are lectin-like chaperones that interact with glycosylated proteins and are important for viral glycoprotein processing and maturation [26]. Since DENV E is a glycoprotein, we examined its interaction with calnexin and calreticulin. As shown in Fig. 2A, immunoprecipitation with either anti-calnexin antibody or anti-calreticulin antibody pulled down DENV E protein. Immunoprecipitation of dengue virus-infected cell lysates with an isotype-matched control antibody did not bring down DENV E, demonstrating the specificity of these interactions. As further evidence of interaction, dengue virus-infected Vero cells were examined for co-localizations of the proteins by immunofluorescence and confocal microscopy. Co-localization of DENV E and calnexin or calreticulin in the ER and peri-nuclear regions was clearly evident (Fig. 2B). The intensity of staining with anti-calnexin and anti-calreticulin was strongly enhanced following dengue virus infection (Fig. 2B) suggesting that, like BiP, expression of these two chaperones is induced.



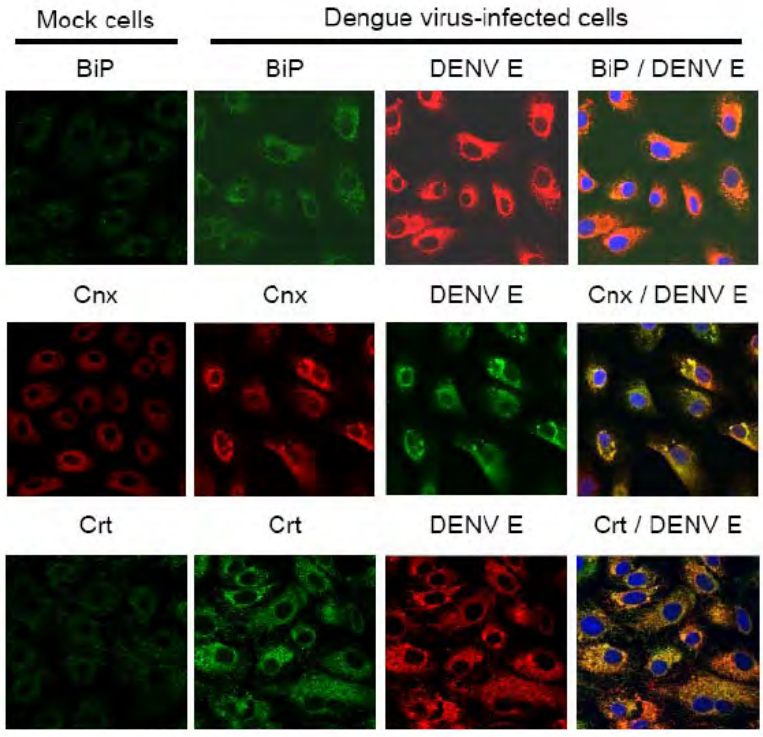


Fig.2. Interaction of DENV E with human BiP, calnexin and calreticulin in dengue virus-infected cells

(A) Lysates of dengue virus-infected Vero cells were immunoprecipitated with mouse anti-DENV E antibody (lane 1), goat anti-BiP antibody (lane 2), goat anti-calnexin antibody (lane 3), goat anti-calreticulin antibody (lane 4) and goat isotype-matched control

antibody (lane 5). Immune complexes were detected by Western blot analysis using anti-DENV E monoclonal antibody. (B) Dengue virus-infected Vero cells at 48 hours after infection were subjected to double immunofluorescence staining for DENV E and chaperones (BiP, calnexin [Cnx], and calreticulin [Crt]) and observed for their colocalization under a laser-scanning confocal microscope. Mock-infected cells served as controls.

Knocking-down the expression of BiP, calnexin and calreticulin by siRNA decreases dengue virus production

Defects in the mechanisms controlling proper protein folding and assembly mediated by ER chaperones affects morphogenesis and production of virions [2; 27; 28; 29]. We asked whether the interaction of BiP, calnexin or calreticulin with DENV E influence dengue virion production. Transfection of Vero cells with siRNA against BiP, calnexin or calreticulin was performed and levels of the corresponding mRNA and proteins were measured by real time PCR and Western blot analysis. The mRNA expression of BiP, calnexin and calreticulin was 15.57%, 27.69% and 7.4%, respectively, relative to irrelevant siRNA-transfected control cells (Fig. 3A). A corresponding decrease in protein expression of BiP, calnexin and calreticulin proteins was observed by Western blot analysis (Fig. 3B).

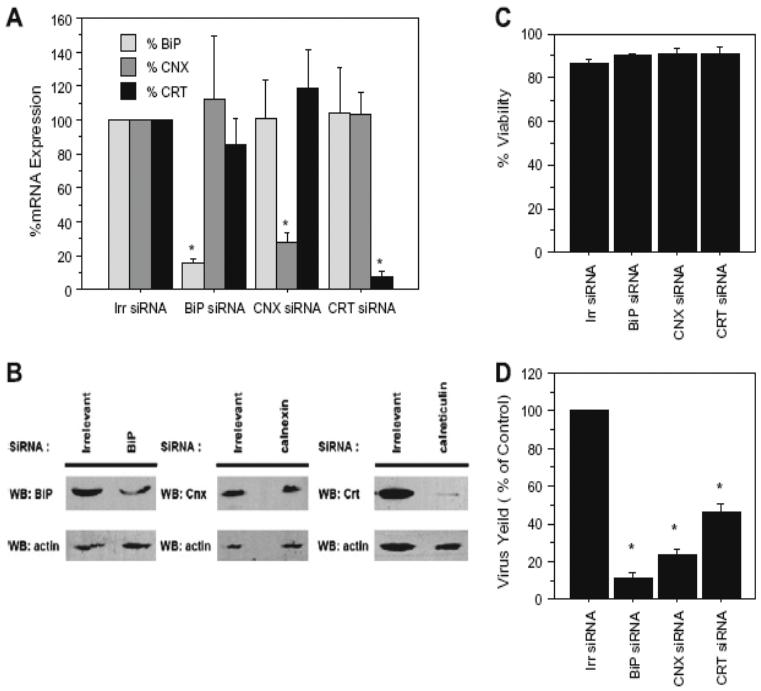


Fig.3. Knocking-down the expression of ER chaperones by siRNA decreases the yield of infectious dengue virus production

Vero cells were transfected with siRNA against BiP, calnexin, calreticulin, or an irrelevant siRNA (negative control) and harvested at 36 hours post transfection. The mRNA and proteins were measured by (A) real time PCR and (B) Western blot analysis using polyclonal antibodies specific to BiP, calnexin, and calreticulin, respectively. The decreased expression of BiP, calnexin and calreticulin was observed in the corresponding siRNA-transfected Vero cells relative to irrelevant siRNA-transfected control cells (* $p \leq 0.05$). The viability of transfected cells with indicated siRNA was measured by trypan blue stanining (C). Transfected cell lysates were then infected with dengue virus at an MOI of 1. At 24 hours post infection, the supernatants were collected to measure the amount of dengue virion production by focus forming unit (FFU) assay. The virus yield is expressed as a percentage of the yield obtained from cells transfected

with irrelevant siRNA (D). The siRNAs to BiP, calnexin and calreticulin decreased the yield of viral progeny, relative to irrelevant siRNA-transfected control cells (* $p \leq 0.05$). Results are derived from three independent experiments.

The transfected cells were subsequently infected with DENV, and 24 hours post-infection virions present in the supernatants were titrated by a focus forming unit assay (FFU). The reduced expression of BiP, calnexin or calreticulin decreased the yield of viral progeny a minimum of 50% (calreticulin) and as much as 90% (BiP) clearly demonstrating the involvement of BiP, calnexin and calreticulin in the production of DENV infectious particles (Fig. 3D). This is in agreement with the observation that BiP, calnexin and calreticulin are essential for replication and infectivity of many human pathogenic viruses [28; 29; 30]. Chaperone proteins are only a part of the host machinery necessary for virus replication and assembly. Recently, silencing of ER-associated degradation (ERAD) components including DERL2, NSFL1C, UBE3A, UFDL1, SEC61G and SEC61A1 was shown to reduce dengue virus infection in the Hela cells [31].

In summary, this work is the first to demonstrate direct interactions between DENV E and the ER-resident chaperones including BiP, calnexin and calreticulin, and the involvement of these chaperones in the production of DENV infectious particles. Future studies will be directed toward elucidating the detailed molecular mechanism by which ER chaperones play a role in dengue virion assembly and cytoplasmic egress.

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Outputs

1. Publications

1.1 International

1.1.1 **Limjindaporn T**, Wongwiwat W, Noisakran S, Srisawat C, Netsawang J, Puttikhunt C, Kasinrerk W, Avirutnan P, Thiemmeca S, Sriburi R, Sittisombut N, Malasit P, and Yenchitsomanus PT. (2009). Interaction of dengue virus envelope protein with endoplasmic reticulum-resident chaperones facilitates dengue virus production. Biochem Biophys Res Commun. 379(2), 196-200.

1.1.2 Noisakran S, Sengsai S, Thongboonkerd V, Kanlaya R, Sinchaikul S, Chen ST, Puttikhunt C, Kasinrerk W, **Limjindaporn T**, Wongwiwat W, Malasit P, and Yenchitsomanus PT. (2008) Identification of human hnRNP C1/C2 as a dengue virus NS1-interacting protein. Biochem Biophys Res Commun. 372(1), 67-72.

1.1.3 **Limjindaporn T**, Netsawang J, Noisakran S, Thiemmeca S, Wongwiwat W, Sudsaward S, Avirutnan P, Puttikhunt C, Kasinrerk W, Sriburi R, Sittisombut N, Yenchitsomanus PT, and Malasit P. (2007). Sensitization to Fas-mediated apoptosis by dengue virus capsid protein. Biochem Biophys Res Commun. 362(2), 334-9.

1.2 National

1.2.1 **Limjindaporn T**, Netsawang J, and Laosutthipong. (2007). Cloning and expression of the anti-apoptotic Bcl-2 gene in human kidney cells. Siriraj Med J, 59 (Suppl 2), S178-180

1.2.2 **Limjindaporn T**, Mairiang D, Khunchai S, and Wongwiwat W. (2007). A novel yeast two hybrid vector, pEG-NRT, for protein protein interaction studies. Siriraj Med J, 59 (Suppl 2), S175-177

1.2.3 **Limjindaporn T,** and Wongwiwat W. (2008). Update on DNA cloning. Reviews. Siriraj Med J, 59 (Suppl 2), S190-191

1.2.4 **Limjindaporn T**, Sawaitbud S, Netsawang J, Mairiang D, Khunchai S, and Wongwiwat W. (2008). Cloning of JNK activation and Fas-binding domain of human apoptotic Daxx gene by homologous recombination in yeast. Thai Journal of Genetics. 1(1), 63-68

1.2.5 **Limjindaporn T**, and Netsawang J. (2008). Cloning and expression of human apoptotic Daxx gene in human kidney cells. Thai Journal of Genetics. 1(1), 57-62

2. Presentations

2.1 International

2.1.1 Wonwiwat W, Noisakran S, Sudsaward S, Netsawang J, Aviruthnan P, Theimmeca S, Puttikhunt C, Kasinrerk W, Sriburi R, Sittisombut N, Yenchitsomanus P, Malasit P, and **Limjindaporn T**. (2008). Association of dengue virus envelope protein and molecular chaperones. Poster presentation. The 6th International Workshop on the Molecular Biology of Stress Responses, Chulabhorn Research Institute, Bangkok, Thailand

2.2 National

2.2.1 **Limjindaporn T**, Wongwiwat W, Noisakran S, Srisawat C, Netsawang J, Puttikhunt C, Kasinrerk W, Avirutnan P, Thiemmeca S, Sriburi R, Sittisombut N, Malasit P, and Yenchitsomanus PT. Oral presentation. การประชุมนักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส สกว. ครั้งที่ 8 ; วันที่ 16-18 ตุลาคม 2551; โรงแรมฮอลิเดย์อินน์ รีสอร์ท รีเจนท์ บีช ชะอำ จังหวัดเพชรบุรี

Appendix

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Interaction of dengue virus envelope protein with endoplasmic reticulum-resident chaperones facilitates dengue virus production

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ABSTRACT

Dengue virus infection is an important mosquito-borne disease and a public health problem worldwide. A better understanding of interactions between human cellular host and dengue virus proteins will provide insight into dengue virus replication and cellular pathogenesis. The glycosylated envelope protein of dengue virus, DENV E, is processed in the endoplasmic reticulum of host cells and therefore reliant on host processing functions. The complement of host ER functions involved and nature of the interactions with DENV E has not been thoroughly investigated. By employing a yeast two-hybrid assay, we found that domain III of DENV E interacts with human immunoglobulin heavy chain binding protein (BiP). The relevance of this interaction was demonstrated by co-immunoprecipitation and co-localization of BiP and DENV E in dengue virus-infected cells. Using the same approach, association of DENV E with two other chaperones, calnexin and calreticulin was also observed. Knocking-down expression of BiP, calnexin, or calreticulin by siRNA significantly decreased the production of infectious dengue virions. These results indicate that the interaction of these three chaperones with DENV E plays an important role in virion production, likely facilitating proper folding and assembly of dengue proteins.

Introduction

Dengue virus (DENV) infection is one of the most important mosquito-borne viral diseases, which is endemic in many countries. Clinical severity ranges from febrile dengue fever (DF) to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). DENV belongs to the *Flaviviridae* family and contains a single positive-stranded RNA genome, encoding a single precursor polypeptide. Host and viral proteases cleave this polypeptide into three structural proteins (capsid, membrane and envelope) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) [1].

Replication of flaviviruses occurs in association with the endoplasmic reticulum where virons assemble and bud into the lumen of the ER. Virus particles transit through the Golgi where they undergo maturation prior to being released by exocytosis [1]. DENV envelope protein (DENV E) is the major component of the virion surface contains two functionally important glycosylation sites, Asn-67 and Asn-153. DENV E lacking Asn-67 was able to infect cells but the production of new infectious particles was abolished. In addition, DENV E lacking Asn-153 showed reduced infectivity [2]. Although DENV E biosynthesis and assembly is thought to occur in the ER, limited information is available on the involvement of host ER chaperones in the folding and assembly of DENV E. We therefore performed a yeast two-hybrid assay to identify host proteins that interact with DENV E. One of the proteins identified in this screen was human immunoglobulin heavy chain binding protein (BiP), a member of the heat shock protein 70 family found in the ER lumen, the interaction between DENV E and BiP in

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dengue virus-infected Vero cells was confirmed by co-immunoprecipitation and co-localization studies. Two additional ER-resident chaperones, calnexin and calreticulin, were similarly examined and both exhibited co-immunoprecipitation and co-localization with DENV E. Significantly, knocking down the expression of BiP, calnexin, or calreticulin by siRNA decreased the yield of infectious dengue virions, indicating that the role of these ER-resident chaperones in the folding and assembly of DENV E is essential for dengue virion production.

Materials and methods

Yeast two-hybrid screening. Two-hybrid screening was performed by the interaction mating method as described by Finley and Brent [3]. Domain III of DENV E, nucleotides 1819 to 2118 encoding amino acids 295 to 394, was amplified from pBluescript II KS (S1SP6-4497), a plasmid which contains the 5′ portion of DENV serotype two strain 16681 [4] by a pair of nucleotide primers 5′ AAG CCG GAA TTC AAA GGA ATG TCA TAC 3′ for the forward direction and 5′ GCC CGC GGA TCC CTA TTT CTT AAA CCA G 3′ for the reverse direction. Domain III is an immunoglobulin-like domain and does not contain a transmembrane domain [5]. The PCR reaction was carried out in a GeneAmp PCR System 9700 (Applied Biosystems) consisting of 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s and extension at 72 °C for 2 min. Subsequently, the amplified DNA was cloned as an in frame fusion with the LexA DNA binding domain in the yeast expression vector pEG202, which contains a *HIS3* selectable marker. The resulting bait plasmid, pEG-E, was transformed into *S. cerevisiae* strain RFY 206 (MATa *his3A200 leu2-3 lys2A201 ura3-52 trp1A::hisG*) containing a Lexop-*lacZ* reporter plasmid, pSH18-34, under *URA3* selection. A galactose-inducible HeLa cell cDNA prey library was constructed in plasmid pJZ 4-5 containing a *TRP1* selectable marker and transformed into strain RFY 231 (MATα *his3 leu2::3-Lexop-LEU2 ura3 trp1 LYS2*) [6]. The bait strain was mated with the library strains and plated on galactose drop-out medium lacking histidine, tryptophan, uracil and leucine (gal/raf-u, -h, -w, -l) to select for diploids. The production of a DENV E binding protein by a prey plasmid was expected to activate the 3Lexop-*LEU2* reporter. Putative positive clones were patching to four indicator plates: (glu/-u, -h, -w, -l), (gal/raf-u, -h, -w, -l), (glu/X-Gal-u, -h, -w) and (gal/raf/X-Gal-u, -h, -w). Prey plasmids were rescued from clones exhibiting a galactose-inducible Leu^+ $lacZ^+$ phenotype by transformation into a Trp^- *Escherichia coli* strain KC8 [3]. To verify the interaction, recovered prey plasmids were introduced into yeast strain RFY 231 along with the *lacZ* plasmid and bait plasmid and again tested on the indicator plates.

Co-immunoprecipitation. Sub-confluent monolayers of 1×10^7 Vero cells in a 100-mm dish were infected with DENV serotype two strain 16681 at an MOI of 1 for 48 h. Cells were washed twice with 5 ml of PBS and detached by incubating with 2.5 mM EDTA in PBS for 15 min. Detached cells were collected by centrifugation. The cell pellets were lysed with a buffer containing 150 mM NaCl, 20 mM Tris–HCl pH 7.4, 5 mM EDTA, 0.5% deoxycholate, 0.1% SDS, 1% Triton X-100 and protease inhibitors (complete, EDTA-free, Roche) and the lysate was incubated on ice for 20 min. Cell debris was removed by centrifugation at 13,000*g* for 10 min at 4 °C. Five microgram of goat anti-BiP antibody, goat anti-calnexin antibody, goat anti-calreticulin antibody or an isotype-matched control antibody (Santa Cruz Biotechnology) were added to lysates and incubated 4 h in the presence of Protein G Sepharose beads (Amersham Pharmacia Biosciences). Subsequently, the Protein G Sepharose beads were collected by centrifugation at 13,000*g* for 5 min and washed twice with washing buffer. Lastly, the bound proteins were eluted by boiling in SDS–PAGE loading buffer, separated by SDS–PAGE, and transferred to nitrocellulose membranes. After blocking with 5% skim milk, the membranes were incubated with mouse anti-DENV E monoclonal antibody (3H5) [7,8] followed by probing with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse antibody (Dako). The protein bands were detected using ECL reagent (Amersham Pharmacia Biosciences).

Co-localization. Vero cells were grown on cover slips and infected with DENV serotype two strain 16681 at an MOI of 1 for 48 h. Thereafter, the cells were washed, fixed with 4% paraformaldehyde in PBS for 20 min, and permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. After washing three times with 0.1% Triton X-100 in PBS, the cover slips were incubated for an hour with (i) mouse anti-DENV E antibody (3H5) and goat anti-BiP antibody (Santa Cruz Biotechnology) (ii) mouse anti-DENV E antibody (3H5) and rabbit anti-calnexin antibody (Santa Cruz Biotechnology) (iii) mouse anti-DENV E antibody (3H5) and goat anti-calreticulin antibody (Santa Cruz Biotechnology). After washing, the cover slips (i and iii) were incubated with both Alexa 488-conjugated donkey anti-goat antibody and Alexa 594-conjugated donkey anti-mouse antibody (Molecular Probes) as secondary antibodies at room temperature for an hour. Cover slip (ii) was incubated with both Alexa 488-conjugated donkey anti-mouse antibody and Cy3-conjugated donkey anti-rabbit antibody (Molecular Probes) as secondary antibodies. Florescent images were captured by a confocal microscope (model LSM 510 Meta, Carl Zeiss).

Knock-down experiments by siRNA and infection assays. The BiP siRNA (5′ GCGGAACCTTCGATGTGTCTCTTCT 3′), calnexin siRNA (5′ ATA GAATGTGGTGGTGCCTATGTGA 3′), calreticulin siRNA (5′ CCCGCTGG ATCGAATCCAAACACAA 3′) were purchased from Invitrogen, USA and used to knock-down BiP, calnexin and calreticulin by transfection into Vero cells using Lipofectamine™ 2000 reagent (Invitrogen, USA). Transfection with irrelevant siRNA (Invitrogen, USA, Cat. No. 12935-300) was performed as a negative control. After 6 h of transfection, cells were fed with 10% FBS in MEM medium for 30 h. Samples were taken for mRNA and protein analysis using real-time PCR (Lightcycler RNA amplification kit, Roche) and Western blot analysis, respectively. Then, siRNA-transfected cells were infected with DENV serotype two strain 16681 at an MOI of 1 for 3 h. The infected siRNA-transfected cells were washed with PBS and fed with 2% FBS in MEM medium for 24 h. The culture supernatants were collected to measure the amount of DENV production by a focus forming unit (FFU) assay as previously described [9].

Results and discussion

DENV E interacts with BiP in a yeast two-hybrid system

To identify human proteins that interacted with DENV E, we screened over 10^7 clones from a HeLa cDNA library using DENV E as bait. Forty five putative positive clones were obtained. Sequence analysis of the inserts showed that three of the cDNA inserts were identical and encoded amino acids 467–655 of BiP. BiP, also known as glucose regulated protein (GRP78), is an isoform five of the heat shock protein 70, which functions as a molecular chaperone involving in folding and assembly of several cellular and viral membrane proteins [10–14]. The specificity of the interaction between DENV E and BiP is shown in Fig. 1A wherein cells containing the DENV E bait plasmid and BiP prey plasmid exhibited galactose-dependent leucine prototrophy and *lacZ* expression. As controls, cells containing the DENV C bait plasmid and BiP prey plasmid or cells containing the DENV NS5 bait plasmid and BiP prey plasmid did not exhibit galactose-dependent leucine prototrophy and *lacZ* expression.

BiP, in complex with other ER chaperones, facilitates the proper folding of proteins in the secretory system. It has two distinct functional regions. The amino-terminal region of BiP possesses an

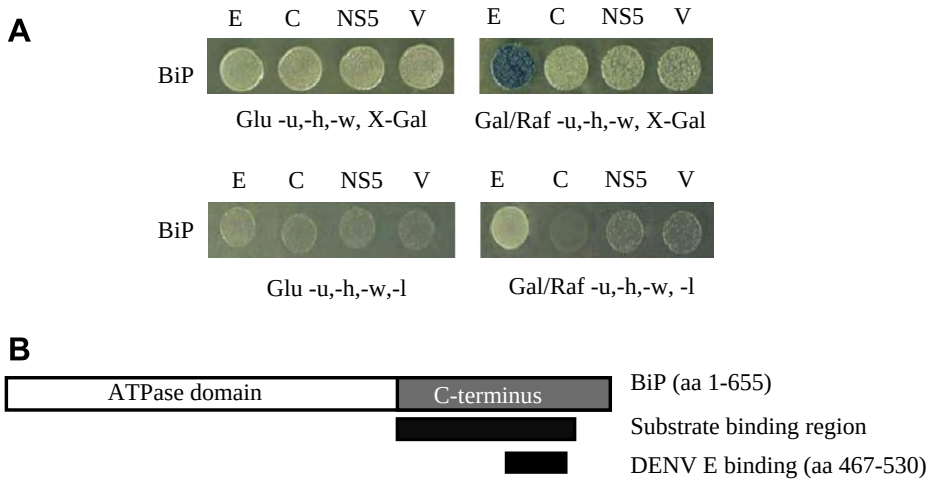

Fig. 1. DENV E-human BiP interaction in a yeast two-hybrid system. (A) Yeast strain RFY231 was co-transformed with a bait plasmid, a BiP prey plasmid and *lacZ* reporter plasmid. The bait plasmids used were pEG-E, expressing the LexA-DENV E fusion protein (E), pEG-C expressing the LexA-DENV C fusion protein (C), pEG-NS5 expressing the LexA-DENV NS5 fusion protein (NS5), and the empty bait plasmid pEG202, expressing the LexA fusion protein (V). A specific interaction was indicated by galactose-dependent β-galactosidase expression, as evidenced by blue colonies on the galactose containing X-Gal plate and white colonies on the glucose containing X-Gal plate, and by galactose-dependent growth on the leucine deficient plate. (B) The region of BiP that interacted with DENV E was located between the amino acid positions 467-530 in the substrate-binding domain of the carboxyl-terminal region of BiP. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

ATPase activity [15,16] whereas its carboxyl-terminal region contains an 18 kDa substrate-binding domain and a 10 kDa oligomerization domain [17,18]. The region of BiP that interacted with DENV E in yeast two-hybrid screening was further mapped by yeast two-hybrid system and located between amino acids 467-530 in the substrate-binding domain of the carboxyl-terminal region of BiP (Fig. 1B).

DENV E associates with BiP, calnexin and calreticulin in dengue virus-infected cells

In order to confirm DENV E-BiP interaction in mammalian cells, lysates from dengue virus-infected Vero cells were tested for co-immunoprecipitation of the proteins. As shown in Fig. 2A, immunoprecipitation with anti-BiP antibody pulled down DENV E protein suggesting the association of DENV E and BiP in dengue virus-infected cells. Furthermore, co-localization of DENV E and BiP in the ER was evident when DENV E and BiP fluorescence images were superimposed (Fig. 2B). In hepatitis B virus (HBV), BiP interacts with the large surface protein (L) and plays a role in HBV morphogenesis by regulating proper folding of the L protein and assembly of the envelope protein [19]. The envelope protein of HIV type 1 has also been shown to interact with BiP [11]. Thus the interaction of DENV E and BiP may contribute to DENV morphogenesis by regulating the correct folding of DENV E.

Based on the immunofluorescence intensity of the images in Fig. 2, BiP appeared to be strongly induced by DENV infection. This is consistent with previous studies showing that DENV infection

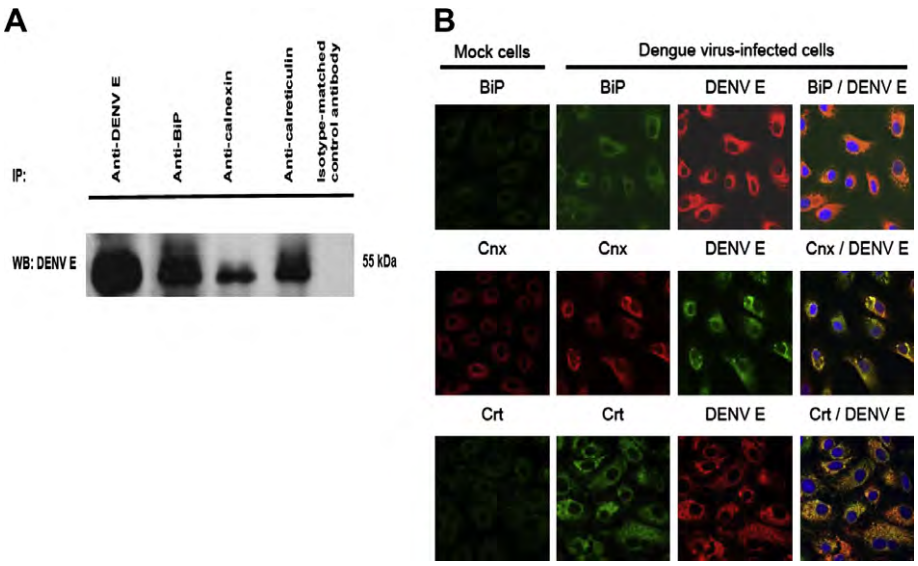

Fig. 2. Interaction of DENV E with human BiP, calnexin and calreticulin in dengue virus-infected cells. (A) Lysates of dengue virus-infected Vero cells were immunoprecipitated with mouse anti-DENV E antibody (lane 1), goat anti-BiP antibody (lane 2), goat anti-calnexin antibody (lane 3), goat anti-calreticulin antibody (lane 4) and goat isotype-matched control antibody (lane 5). Immune complexes were detected by Western blot analysis using anti-DENV E monoclonal antibody. (B) Dengue virus-infected Vero cells at 48 h after infection were subjected to double immunofluorescence staining for DENV E and chaperones (BiP, calnexin [Cnx] and calreticulin [Crt]) and observed for their co-localization under a laser-scanning confocal microscope. Mock-infected cells served as controls.

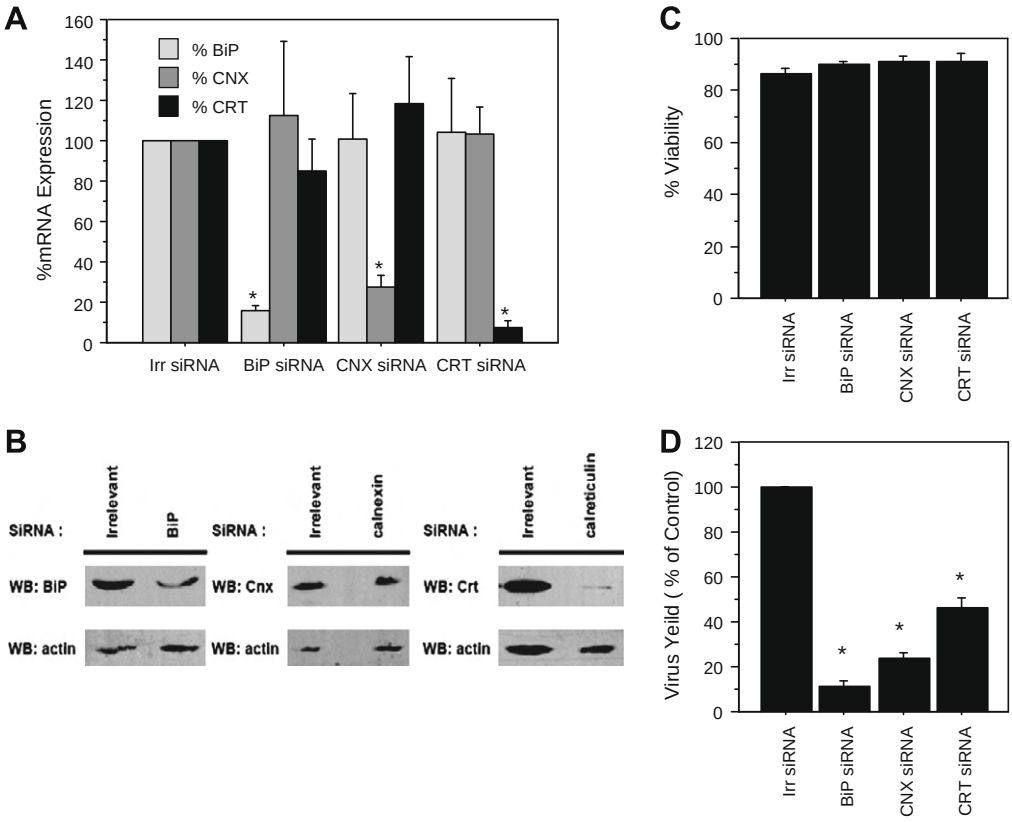

Fig. 3. Knocking-down the expression of ER chaperones by siRNA decreases the yield of infectious dengue virus production. Vero cells were transfected with siRNA against BiP, calnexin, calreticulin or an irrelevant siRNA (negative control) and harvested at 36 h post-transfection. The mRNA and proteins were measured by (A) real-time PCR and (B) Western blot analysis using polyclonal antibodies specific to BiP, calnexin and calreticulin, respectively. The decreased expression of BiP, calnexin and calreticulin was observed in the corresponding siRNA-transfected Vero cells relative to irrelevant siRNA-transfected control cells ($^{*}p \leqslant 0.05$). The viability of transfected cells with indicated siRNA was measured by trypan blue stanining (C). Transfected cell lysates were then infected with dengue virus at an MOI of 1. At 24 h post-infection, the supernatants were collected to measure the amount of dengue virion production by focus forming unit (FFU) assay. The virus yield is expressed as a percentage of the yield obtained from cells transfected with irrelevant siRNA (D). The siRNAs to BiP, calnexin and calreticulin decreased the yield of viral progeny, relative to irrelevant siRNA-transfected control cells ($^{*}p \leqslant 0.05$). Results are derived from three independent experiments.

induces the unfolded protein response [20,21] and that BiP is induced by other flaviviruses [22,23].

Interestingly, cell surface associated BiP was previously identified as a putative dengue virus receptor in hepatic HepG2 cells [24] and our two-hybrid screen used domain III of DENV E which is thought to bind host cell receptors [5]. Therefore, we tested the ability of anti-BiP antibodies to block DENV infection. However, in our study using Vero cells both polyclonal antibody directed against BiP and monoclonal antibody directed against the carboxyl-terminal region of BiP, the binding site between DENV E and BiP, failed to inhibit DENV infection (data not shown), suggesting that an alternate or additional receptors are present on Vero cells.

BiP is one of multiple chaperone systems important for ER quality control [25]. Calnexin and calreticulin are lectin-like chaperones that interact with glycosylated proteins and are important for viral glycoprotein processing and maturation [26]. Since DENV E is a glycoprotein, we examined its interaction with calnexin and calreticulin. As shown in Fig. 2A, immunoprecipitation with either anti-calnexin antibody or anti-calreticulin antibody pulled down DENV E protein. Immunoprecipitation of dengue virus-infected cell lysates with an isotype-matched control antibody did not bring down DENV E, demonstrating the specificity of these interactions. As further evidence of interaction, dengue virus-infected Vero cells were examined for co-localizations of the proteins by immunofluorescence and confocal microscopy. Co-localization of DENV E and calnexin or calreticulin in the ER and peri-nuclear regions was clearly evident (Fig. 2B). The intensity of staining with anti-calnexin and anti-calreticulin was strongly enhanced following dengue virus infection (Fig. 2B) suggesting that, like BiP, expression of these two chaperones is induced.

Knocking-down the expression of BiP, calnexin and calreticulin by siRNA decreases dengue virus production

Defects in the mechanisms controlling proper protein folding and assembly mediated by ER chaperones affects morphogenesis and production of virions [2,27–29]. We asked whether the interaction of BiP, calnexin or calreticulin with DENV E influence dengue virion production. Transfection of Vero cells with siRNA against BiP, calnexin or calreticulin was performed and levels of the corresponding mRNA and proteins were measured by real-time PCR and Western blot analysis. The mRNA expression of BiP, calnexin and calreticulin was 15.57%, 27.69% and 7.4%, respectively, relative to irrelevant siRNA-transfected control cells (Fig. 3A). A corresponding decrease in protein expression of BiP, calnexin and calreticulin proteins was observed by Western blot analysis (Fig. 3B). The transfected cells were subsequently infected with DENV, and 24 h post-infection virions present in the supernatants were titrated by a focus forming unit assay (FFU). The reduced expression of BiP, calnexin or calreticulin decreased the yield of viral progeny a minimum of 50% (calreticulin) and as much as 90% (BiP) clearly demonstrating the involvement of BiP, calnexin and calreticulin in the production of DENV infectious particles (Fig. 3D). This is in agreement with the observation that BiP,

calnexin and calreticulin are essential for replication and infectivity of many human pathogenic viruses [28–30]. Chaperone proteins are only a part of the host machinery necessary for virus replication and assembly. Recently, silencing of ER-associated degradation (ERAD) components including DERL2, NSFL1C, UBE3A, UFDL1, SEC61G and SEC61A1 was shown to reduce dengue virus infection in the Hela cells [31].

In summary, this work is the first to demonstrate direct interactions between DENV E and the ER-resident chaperones including BiP, calnexin and calreticulin, and the involvement of these chaperones in the production of DENV infectious particles. Future studies will be directed toward elucidating the detailed molecular mechanism by which ER chaperones play a role in dengue virion assembly and cytoplasmic egress.

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Sensitization to Fas-mediated apoptosis by dengue virus capsid protein

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Abstract

Dengue fever (DF) and dengue hemorrhagic fever (DHF) are important public health problems in tropical regions. Abnormal hemostasis and plasma leakage are the main patho-physiological changes in DHF. However, hepatomegaly, hepatocellular necrosis and fulminant hepatic failure are occasionally observed in patients with DHF. Dengue virus-infected liver cells undergo apoptosis but the underlying molecular mechanism remains unclear. Using a yeast two-hybrid screen, we found that dengue virus capsid protein (DENV C) physically interacts with the human death domain-associated protein Daxx, a Fas-associated protein. The interaction between DENV C and Daxx in dengue virus-infected liver cells was also demonstrated by co-immunoprecipitation and double immunofluorescence staining. The two proteins were predominantly co-localized in the cellular nuclei. Fas-mediated apoptotic activity in liver cells constitutively expressing DENV C was induced by anti-Fas antibody, indicating that the interaction of DENV C and Daxx involves in apoptosis of dengue virus-infected liver cells.

Keywords: Dengue virus capsid protein; Human Daxx; Protein interaction; Apoptosis

Dengue virus is a mosquito-borne member of the family *Flaviviridae*. Infection with dengue virus produces variable clinical illness ranging from non-specific viral infection to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DHF/DSS) [1]. DHF patients generally present with continuous fever lasting 2–7 days, with hemorrhagic tendencies, plasma leakage, thrombocytopenia and hemoconcentration. Liver injury with an elevation of aminotransferases and reactive hepatitis are common in adult patients with dengue virus infection [2,3]. Councilman bodies in liver biopsies of DHF/DSS cases correspond to hepatocytes in apoptosis and this may be a key element in the pathophysiology of hepatic failure associated with DHF/DSS [4].

Apoptosis of liver HepG2 cells during dengue virus infection has been observed [4–8]. Because the capsid protein of other members of the family *Flaviviridae* have multiple functions including either induction or inhibition of apoptosis [9–12], we suspected that dengue virus capsid protein (DENV C) might be involved in apoptosis. DENV C is a

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small, highly positively charged, 12-kDa protein that is required for the maturation of viral particles and assembly of the nucleocapsid core [13]. A proportion of DENV C localizes to the nucleus [14–16] and interacts with heterogeneous nuclear ribonucleoprotein K (hnRNP K) [17]. By using a yeast two-hybrid screen, we showed in this study that DENV C interacts with human death domain-associated protein Daxx, a modulator of Fas-mediated apoptosis [18]. The interaction between DENV C and Daxx was examined by immnuoprecipitation, and co-localization studies in dengue virus-infected HepG2 cells. Given the pro-apoptotic role of Daxx, the effect of DENV C expression on viability of HepG2 cells was examined. HepG2 cells expressing DENV C became sensitive to Fas-mediated apoptosis, suggesting the interaction with Daxx may be physiologically significant and relevant to pathogenesis of dengue virus infection.

Materials and methods

Yeast two-hybrid screening. Two-hybrid screening was performed as described by Finley and Brent [19]. DENV C was amplified from pBluescript II KS (S1SP6-4497), a plasmid which contains the 5′ portion of dengue virus serotype 2 strain 16681 [20] , with a pair of primers, 5′-GCG CTG AAT TC ATG AAT GAC CAA CGG AAA-3′ and 5′-GAT ACG GGA TCC CTA TCT GCG TCT CCT AT-3′. The PCR was carried out in a GeneAmp PCR System 9700 (Applied Biosystems), starting with an initial denaturation step of 94 °C for 5 min and followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s, extension at 72 °C for 2 min, and one cycle of final extension at 72 °C for 10 min. Subsequently, the amplified DNA was cloned as an in-frame fusion with the LexA DNA binding domain in the yeast expression vector pEG202, which contains a *HIS3* selectable marker. The resulting bait plasmid, pEG-C, was transformed into *S. cerevisiae* strain RFY 206 (MATa *his3Δ200 leu2-3 lys2Δ201 ura3-52 trp1Δ::hisG*) containing a Lexop-*lacZ* reporter plasmid, pSH18-34, under *URA3* selection. A galactose inducible HeLa cell cDNA prey library in plasmid pJG4-5 containing a *TRP1* selectable marker was transformed into strain RFY 231 (MATα *his3 leu2::3Lexop-LEU2 ura3 trp1 LYS2*) [21]. The bait strain was mated with the library strains and plated on galactose drop-out medium lacking histidine, tryptophan, uracil, and leucine (gal/raf -u, -h, -w, -l). The production of a DENV C binding protein by a prey plasmid was expected to activate the 3Lexop-*LEU2* reporter. Putative positive clones were patched to four indicator plates: (glu/-u, -h, -w, -l), (gal/raf -u, -h, -w, -l), (glu/X-Gal -u, -h, -w), and (gal/raf/X-Gal -u, -h, -w). Prey plasmids were rescued from clones exhibiting a galactose-inducible Leu^+ $lacZ^+$ phenotype by transforming into a Trp^- *Escherichia coli* strain KC8 [19]. To verify the interaction, recovered prey plasmids were introduced into yeast strain RFY 231 along with the *lacZ* plasmid and bait plasmid and again tested on the indicator plates.

Co-immunoprecipitation. HepG2 cells were grown in DMEM (Gibco-BRL) supplemented with 10% heat inactivated fetal bovine serum (Hyclone), 1 mM sodium pyruvate (Sigma), 1 mM non-essential amino acids (Gibco-BRL), and 1.2% penicillin G-streptomycin at 37 °C in 5% CO_2. Up to 3×10^6 HepG2 cells were infected with dengue virus strain 16681 at a MOI of 1 for 32 h. The cell pellets were lysed with RIPA buffer. Five microgram of purified mouse anti-DENV C monoclonal antibody produced in the laboratory or 5 μg of rabbit anti-Daxx polyclonal antibody (Santa Cruz Biotechnology) or 5 μg of isotype-matched control antibodies were added to lysates. The mixture was incubated at 4 °C overnight and then protein G Sepharose beads (Amersham-Pharmacia Biosciences) were added. The bound proteins were eluted, subjected to SDS–PAGE and performed immunoblot analysis. The membranes were incubated with mouse anti-DENV C antibody or rabbit anti-Daxx antibody and followed by probing either with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse Ig antibody or HRP-conjugated swine anti-rabbit Ig antibody, respectively. The protein bands were detected by using ECL reagents (Amersham-Pharmacia Biosciences).

Co-localization. HepG2 cells were grown on cover slips as described above. Subconfluent monolayer of these cells were infected with dengue virus strain 16681 at a MOI 1 for 32 h, washed, fixed with 4% formaldehyde in PBS for 20 min, and permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. After washing three times with 0.1% Triton X-100 in PBS, cover slips were incubated both with mouse anti-DENV C antibody and rabbit anti-Daxx antibody for an hour. After washing, the cover slips were incubated both with Alexa 488-conjugated rabbit anti-mouse Ig antibody (Molecular Probes) and Cy3-conjugated donkey anti-rabbit Ig antibody (Jackson Immunoresearch laboratories) as secondary antibodies at room temperature for an hour. Fluorescent images were captured with a confocal microscope (model LSM 510, Carl Zeiss).

Generation of HepG2 cells constitutively expressing DENV C. The DENV C gene was amplified from pBluescript II KS (S1SP6-4497) [20] using primers CapBamHI: 5′-GTAGGATCCATGAATGACCAACGG AAAAAG-3′ and CapXhoI: 5′-GCACTCGAG CTATCTGCGTCTCCT AT-3′. The PCR was carried out as described above for construction of the two-hybrid bait plasmid. The PCR product was cloned into the plasmid pcDNA3.1/His C (Invitrogen). The integrity of positive clones was verified by DNA sequencing and an evaluation of DENV C expression. HepG2 cells were transfected with either plasmid pcDNA3.1 his c/DENV C or empty vector pcDNA3.1/His C using Lipofectamine (Invitrogen). Twenty-four hours post transfection, stable cell colonies were selected in DMEM containing 1 mg/ml G418 (Calbiochem) for 1 month. The isolated G418-resistant colonies were maintained in DMEM containing 0.5 mg/ml G418 and examined for the expression of DENV C. To compare the level of DENV C expression in dengue virus-infected HepG2 cells and G418-resistant clones, Western blot analysis was performed using totally 75 μg of total protein and probed with β-actin (Santa Cruz Biotechnology) and DENV C antibodies.

Apoptosis assays. Up to 1×10^6 of stable HepG2 transformants were treated with 0.5 μg/ml anti-Fas mAb (Sigma) and 1 μg/ml cycloheximide for 24 h in culture medium containing 2% FBS. Two clones of HepG2 cells constitutively expressing DENV C; HepG2C1 and HepG2C6, and a clone of HepG2 cells containing the empty vector; HepG2His, were examined for the presence of apoptotic cells by harvesting both detached and adherent cells, staining for cell surface phosphatidyl serine, assaying for caspase-3 activation, and performing the DNA fragmentation assay.

For annexin V/propidum iodide staining, samples were suspended in annexin V buffer and incubated on ice with FITC-conjugated annexin V (BD Biosciences) for 15 min. Immediately prior to analysis, propidum iodide was added and samples were analyzed by flow cytometry (Becton–Dickinson).

In the assessment of caspase-3 activation, the cells were lysed with RIPA buffer and subjected to SDS–PAGE analysis as previously described. The blots were incubated with goat anti-caspase-3 antibody (Santa Cruz Biotechnology) followed by a HRP-conjugated rabbit anti-goat Ig antibody. Protein bands were detected with ECL reagents.

The DNA fragmentation assay was performed using the SDS-high salt extraction method [22]. Cell pellets ($\sim 10^6$ cells) were suspended in 80 μl of PBS and gently mixed with 300 μl of buffer containing 10 mM Tris–HCl (pH 7.6), 10 mM EDTA, and 0.6% SDS. The lysates were mixed with 100 μl of 5 M NaCl and incubated overnight at 4 °C. Supernatants were treated sequentially with RNase A (1 mg/ml) and proteinase K (0.2 mg/ml) for 30 min at 37 °C. Precipitated DNA was resuspended in TE buffer, electrophoresed in 1.5% agarose prepared in Tris–borate–EDTA buffer, and stained with ethidium bromide.

Results and discussion

DENV C interacts with Daxx in a yeast two hybrid system

To identify human proteins that interact with DENV C, 10^7 clones from a HeLa cDNA library were screened using DENV C as bait. Sixty putative positive clones were

obtained. Restriction endonuclease analysis indicated that 23 unique genes were represented (data not shown). Sequence analysis of the inserts showed that one of the cDNA inserts encoded an amino acid stretch (529–740) of Daxx. We demonstrated the specificity of the two-hybrid interaction between DENV C and Daxx by showing galactose-dependent activation of the reporters in cells containing the Daxx prey along with the DENV C bait but not a domain III of dengue virus envelope protein DENV E bait (Fig. 1A). After sequencing the prey plasmid containing Daxx obtained from our yeast two-hybrid screening, the region of Daxx that interacts with DENV C was the 211 carboxyl-terminal residues, a region which binds to multiple cellular proteins including Fas and Pml [18,23–25] (Fig. 1B). Interestingly, the nucleocapsid protein of hantavirus, which, like dengue virus, causes a hemorrhagic fever, has also shown to interact with the 240 carboxyl-terminal residues of Daxx [26].

Daxx is a 740 amino acid protein that contains two amino-terminal amphipathic helices (PAH1, PAH2), a coiled-coiled domain (CC), an acidic domain (D/E), and a carboxyl-terminal serine/proline/threonine rich domain (S/P/T). It is localized both in the cytoplasm and in the nucleus, although the majority is present in the nucleus.

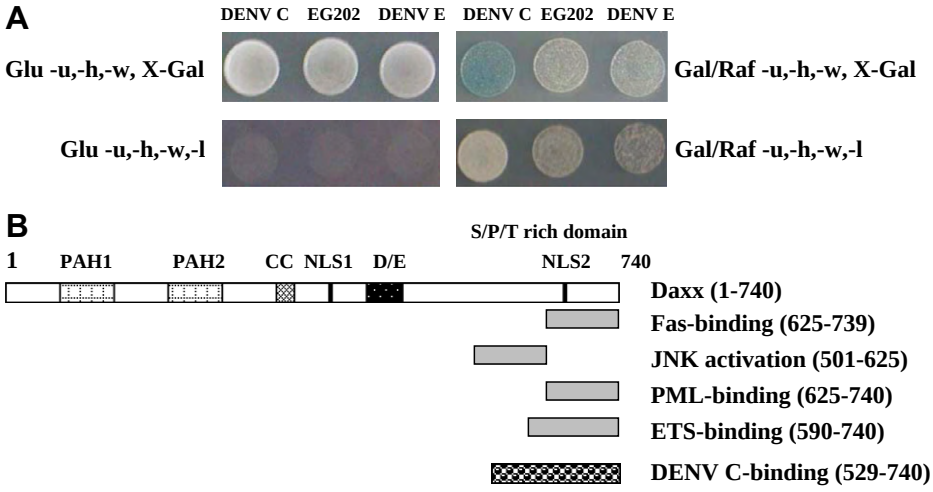

Fig. 1. DENV C interacts with Daxx in a yeast two-hybrid system. (A) Yeast strain RFY231 was co-transformed with a bait plasmid, a Daxx prey plasmid and *lacZ* reporter plasmid. The bait plasmids were pEG-C, expressing the LexA-DENV C fusion protein, the empty bait plasmid pEG202, or an unrelated bait plasmid pEG-E. A specific interaction was indicated by galactose-dependent β-galactosidase expression, as evidenced by blue colonies on the galactose containing X-Gal plate, and by galactose-dependent growth on the leucine deficient plate. (B) The region of Daxx that interacts with DENV C was the 211 carboxyl-terminal residues, a region which binds to Fas and Pml. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

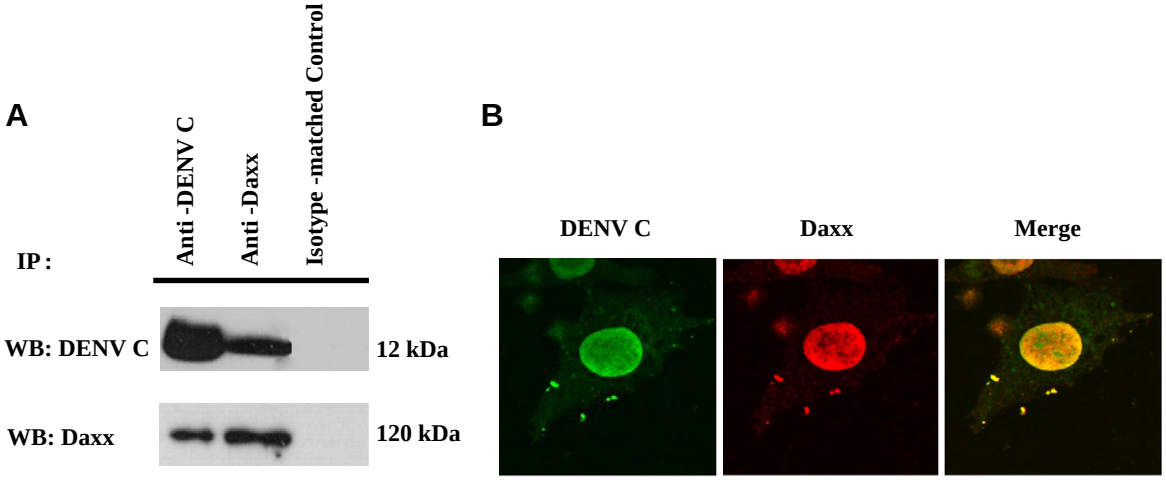

Fig. 2. Interaction between DENV C and Daxx in dengue virus-infected HepG2 cells. (A) The cell lysates from dengue virus-infected HepG2 cells were immunoprecipitated either with purified mouse anti-DENV C antibody or rabbit anti-Daxx antibody or isotype-matched control antibodies. The complexes were detected either with mouse anti-DENV C antibody or rabbit anti-Daxx antibody. (B) Dengue virus-infected HepG2 cells were fixed and immunostained with mouse anti-DENV C antibody (green in A) and rabbit anti-Daxx antibody (red in B). The merged image (yellow in C) demonstrated co-localization between DENV C and Daxx. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In the cytoplasm, Daxx interacts with Fas at the plasma membrane [18]. Stimulation of Fas causes translocation of Daxx from the nucleus to the cytoplasm, where it interacts with ASK1 and promotes JNK activation [27]. In the nucleus, Daxx shuttles between two different sub-nuclear structures, nucleoplasm and the promyelocytic leukemia nuclear bodies (PML-NB) [23,24]. In the nucleoplasm, Daxx acts as a transcriptional repressor [28,29]. Daxx asso-

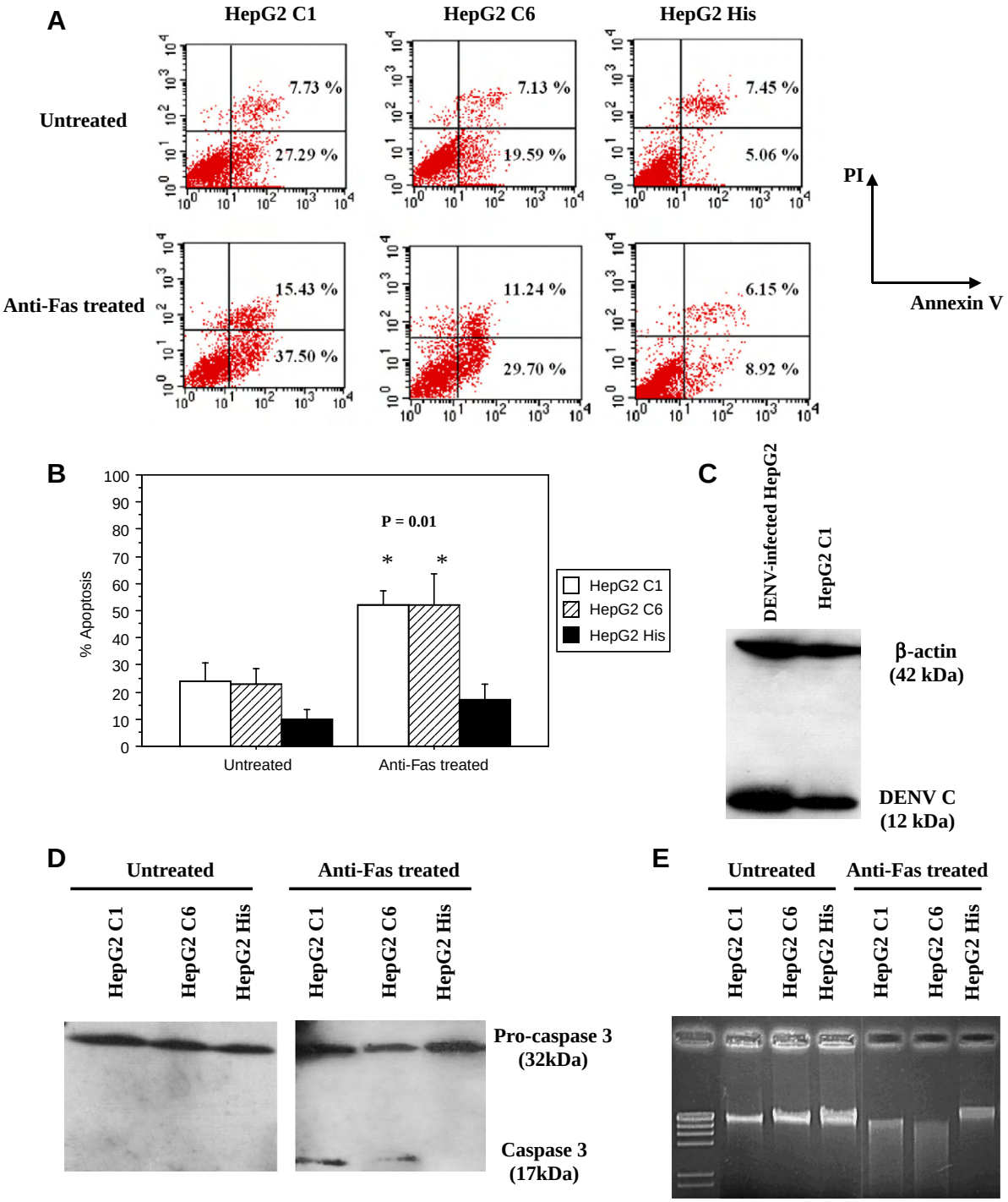

Fig. 3. Sensitization of Fas-mediated apoptosis by DENV C. (A) The untreated and anti-Fas antibody treated of HepG2C1, HepG2C6, and HepG2His were incubated with FITC-conjugated annexin V. Immediately prior to analysis, propidum iodide was added and samples were analyzed by flow cytometry. (B) The percentage of cells undergoing apoptosis as measured by the percent of annexin V positive from three independent experiments was shown. (C) The expression levels of DENV C in dengue virus-infected HepG2 and HepG2C1 were compared. (D) In the assessment of caspase-3 activation, untreated and anti-Fas antibody treated HepG2C1, HepG2C6, and HepG2His were incubated with goat anti-caspase-3 antibody. Three independent experiments were conducted and gave the similar results. (E) The DNA fragmentation assay using the pellets from untreated and anti-Fas antibody treated of HepG2C1, HepG2C6, and HepG2His. Similar results were obtained from three independent experiments.

ciates with proteins that are critical for transcriptional repression, such as histone deacetylase II [30]. The ability of Daxx to repress transcription is inhibited by its localization to the PML-NB [28].

DENV C associates with Daxx in dengue virus-infected HepG2 cells

Clinical studies have implicated liver in the pathogenesis of dengue virus infection [2,3]. In addition, a transformed liver cell line, HepG2, is permissive to dengue virus and infection of HepG2 cells with dengue virus results in the induction of apoptosis [4–8]. In order to confirm DENV C-Daxx interaction in liver cells, the lysates from dengue virus-infected HepG2 cells were tested for coimmunoprecipitation of the two proteins. As shown in Fig. 2A, anti-DENV C antibody pulled down Daxx protein and, conversely, anti-Daxx antibody co-precipitated DENV C in immunoprecipitation. Immunoprecipitation with isotype-matched control antibodies did not bring down Daxx or DENV C. These results suggest that DENV C and Daxx interact during dengue virus infection of HepG2 cells.

As further evidence of interaction, dengue virus-infected cells were examined for co-localization of the proteins by an immunofluorescence confocal microscopy. Daxx was primarily localized in the nucleus of dengue virus-infected HepG2 cells (Fig. 2B). A similar nuclear distribution of DENV C was detected in these cells. Co-localization of DENV C and Daxx was clearly evident when the DENV C and Daxx images were superimposed (Fig. 2B). These data support the physical interaction of DENV C and Daxx and further suggest that association occurs in the nucleus of dengue virus-infected HepG2 cells.

DENV C expression sensitizes HepG2 cells to Fas-mediated apoptosis

Dengue virus infection induces apoptosis in HepG2 cells [4–8] and Daxx has been implicated in a myriad of interactions controlling apoptosis and cell death [31,32]. It is possible that DENV C, through an interaction with Daxx, may influence apoptosis. To test this possibility, HepG2 cells constitutively expressing DENV C were generated and examined for apoptosis. Two DENV C expressing cell lines were constructed; HepG2C1 and HepG2C6. The DEN C expression in HepG2C1 is comparable to dengue virus-infected HepG2 cells (Fig 3C). While both expressed DENV C, expression was about twofold higher in HepG2C1 than HepG2C6 and no DENV C expression was detected in the negative control strain HepG2His.

As an indicator of apoptosis, cells were stained with FITC-conjugated annexin V and propidium iodide and analyzed by flow cytometry [33]. As seen in Fig. 3A and B, cell lines HepG2C1 and HepG2C6 had a 2- to 3-fold increase in the intensity of annexin V staining relative to the control cells, HepG2His, but comparable numbers of propidium iodide-stained cells. This result suggests that DENV C- expressing cells are in an early stage apoptosis before any evidence of caspase 3 activation or DNA laddering is apparent (Fig. 3D and E).

Since Daxx participates in Fas-mediated apoptosis, the effect of Fas activation in DENV C-expressing cells was examined. Treatment with anti-Fas antibody had no effect on HepG2His control cells as measured by annexin V staining, caspase activation or DNA laddering (Fig. 3). This is in agreement with prior work demonstrating that HepG2 cells do not respond to Fas activation [7]. However, both HepG2C1 and HepG2C6 showed a significant increase in staining with annexin V and propidium iodine upon treatment with anti-Fas antibody (Fig. 3A and B). Apoptosis in response to this treatment was also evident in the development of activation of caspase 3 and DNA ladder in these cells (Fig. 3D and E).

The forgoing indicates that DENV C affects the HepG2 cells and sensitizes them to Fas-mediated apoptosis. Presumably this is due to the interaction of DENV C with Daxx, which may disrupt one or more of the many interactions of Daxx with other proteins controlling cell death [31,32]. DENV C may not act alone in inducing apoptosis. The apoptotic process in HepG2 cells can be induced by the dengue virus ectodomain of the small membrane protein ApoptoM [5]. Apoptosis pathways other than the Fas-mediated pathway are also involved in these cells, including activation of NF-κB and TNF-related apoptosis-inducing ligand [4,7]. However, the apoptotic process appears to be independent of p53 [8].

In summary, this work is the first to demonstrate an interaction between DENV C and human Daxx and the pro-apoptotic function of DENV C in liver cells. Future studies will be directed toward elucidating the detailed molecular mechanisms by which DENV C contributes to Fas-mediated apoptosis.

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