Generation and Characterization of DENV-1/2 EDIII Chimeric Virus-like-Particles

การสร้างและศึกษาคุณสมบัติของอนุภาคคล้ายไวรัสลูกผสมระหว่างเชื้อไวรัสเด็งกี่ ชนิดที่ 1 และ ชนิดที่ 2

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ABSTRACT

Dengue virus (DENV) infection causes dengue fever, dengue hemorrhagic fever, and dengue shock syndrome. In this study C6/36 cells were employed in the generation of chimeric DENV-1 (EDIII)/DENV-2 virus-like particles (VLPs) by transfecting with an insect cell expression plasmid encoding the prM and E genes of DENV-2 in which the domain III and the stem-anchor region of E protein had been substituted with corresponding regions of DENV-1 and Japanese encephalitis virus, respectively. Selection with blasticidin resulted in transfected cells with high level of expression of E protein in the cytoplasm. SDS-PAGE analysis of partially purified extracellular E-containing materials revealed three structural protein bands (E, prM and M) and unknown protein band. Electron microscopy analysis demonstrated the presence of spherical particles with size heterogeneity. Cleavage of prM in these particles was reduced from that of the parental DENV-2 VLPs. The results indicated that the DENV-1/2 EDIII chimeric E protein can assemble into spherical particles of various class sizes in stably transfected C6/36 cells.

บทคัดย่อ

การติดเชื้อไวรัสเด็งกี่ ก่อให้เกิดโรคไข้เด็งกี่ ไข้เลือดออก และอาจทำให้เกิดภาวะช็อกได้ ในการศึกษานี้ใช้เซลล์ C6/36 เพื่อสร้างอนุภาคคล้ายไวรัสลูกผสมระหว่างเชื้อไวรัสเด็งกี่ ชนิดที่ 1 และ ชนิดที่ 2 โดย transfect ด้วยพลาสมิดที่สามารถแสดงออกโปรตีนในเซลล์แล้ว และมียีนก่อเหตุการสร้างโปรตีน prM และ E ของเชื้อไวรัสเด็งกี่ ชนิดที่ 2 ซึ่งถูกแทนที่ด้วยการสร้างโปรตีน E ในโดเมน domain III ของเชื้อไวรัสเด็งกี่ ชนิดที่ 1 และส่วนปลายของเชื้อไวรัส Japanese encephalitis ตามลำดับ การคัดเลือกด้วยยา blasticidin ทำให้ได้เซลล์ที่มีการแสดงออกของโปรตีน E ภายในไซโทพลาซึมในระดับสูง โปรตีน E ที่ถูกปล่อยออกมาถูกทำให้บริสุทธิ์ขึ้น โดยการปั่นแยกด้วยความร้อนสูง และคืนมาด้วย SDS-PAGE พบโปรตีนโครงสร้าง 3 แบบ (E, prM และ M) และแยกโปรตีน prM ในการศึกษานี้กลุ่มตัวอย่างอิเล็กทรอนิกส์พบอนุภาคทรงกลมมีขนาดที่หลากหลาย เกิดขึ้นจากโปรตีน prM ในอนุภาคคล้ายไวรัสลูกผสมของเชื้อไวรัสเด็งกี่ ชนิดที่ 2 ที่มีการแยกส่วนขยายน์ domain III จากเชื้อไวรัสเด็งกี่ ชนิดที่ 1 สามารถประกอบด้วยอนุภาคทรงกลมหลายขนาดในเซลล์ C6/36 ที่มีการแสดงออกของโปรตีนอย่างคงที่ได้

Keywords: Dengue virus, Chimeric virus like-particles, Envelope (E) protein

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Introduction

Dengue virus (DENV) is a member of the *Flaviviridae* genus within the family *Flaviviridae*. Dengue viruses are composed of four distinct serotypes (DENV1–4). The dengue virus genome is a single-stranded, positive-sense RNA molecule of approximately 11 kb, which encodes three structural proteins, capsid (C), premembrane/membrane (prM/M), envelope (E) proteins, and seven non-structural proteins (Lindenbach et al., 2007). The E protein is a major envelope protein on the surface flavivirus virions. The flavivirus E protein consists of three distinct external domains - central domain (domain I), dimerization domain (domain II) and immunoglobulin like-domain (domain III) - as well as the stem-anchor region (Lindenbach et al., 2007). The domain II contains a fusion loop and is involved in the homodimerization of E. The domain III appears to be involved in receptor binding and is a target of virus neutralizing antibodies, which may represent a correlate of protection (Crill et al. 2001; Wahala et al., 2009; Pierson et al., 2008). Depending on the extent of prM cleavage, there are three types of extracellular dengue particles (immature, partially mature and mature particles) distinguishable by surface morphology (Junjhon et al., 2010). Dengue virus infection may result in asymptomatic infection and a number of illnesses, including undifferentiated fever, dengue fever, dengue hemorrhagic fever and dengue shock syndrome. These diseases are found throughout tropical and subtropical regions of the world.

The most advanced vaccine candidate developed by Sanofi Pasteur is based on four recombinant viruses containing the two envelope protein genes, prM and E, from each of the four DENV serotypes and the Yellow Fever virus 17D genetic background (Osorio et al., 2011). Recent phases IIb-III clinical trials employing a three-dose immunization schedule revealed low efficacy against diseases caused by DENV-1 and -2, and in children younger than nine years of age (Hadinegoro et al., 2015). There are, however, safety concerns in using this and other live-attenuated dengue vaccine candidates in children.

Virus-like particles (VLPs) are multi-subunit proteins with structures that relate to the native conformation of virus particles. VLPs lack viral genome, making them non-infectious and unable to replicate (Rodriguez-Limas et al., 2013). The VLPs can represent an alternative choice in the development of dengue vaccine. VLPs have come into focus with the recent success of the VLP-based vaccine in the protection of human papilloma virus-associated carcinoma (Mao et al., 2006). Although DENV VLPs have attracted a lot of interest in the past decade (Bisht et al., 2001; Konishi, Fujii, 2002; Purdy, Chang, 2005) they were not strongly immunogenic in experimental animals and it was difficult to produce them in large quantity. Recently, DENV-2 VLPs were generated in stably prM+E gene-transfected insect cells and tested in mice and monkeys (Suphatrakul et al., 2015). In the presence of adjuvant, DENV-2 VLPs prepared with an enhanced prM cleavage strongly induced virus neutralizing antibody in mice and monkeys that had been primed, respectively, with VLPs and homologous, live attenuated virus (Suphatrakul et al., 2015).
In an attempt to prepare additional VLPs for use in the study of immunization against disease caused by DENV-1, chimeric DENV-2 VLPs containing the substituted EDIII domain from DENV-1 were generated. A recombinant expression plasmid encoding the EDIII of DENV-1, the prM and remaining E sequence of DENV-2, and the stem-anchor region of Japanese encephalitis virus E protein and the polyA-addition sequence of simian virus 40. Two antibiotics resistance genes include ampicillin- and blasticidin-resistant genes for the selection of plasmid-expressing E.coli and insect cells, respectively. ColE1 represents the origin of replication.

Fig. 1 Schematic diagram of an expression vector employed in this study. The pIE-1-based plasmid contains an immediate early promoter (ie1) and the hr5 enhancer from Autographa californica nuclear polyhedrosis virus (AcNPV), defensin A signal sequence, prME of DENV-2 with the E domain III from DENV-1, the stem-anchor region of Japanese encephalitis virus E protein and the polyA-addition sequence of simian virus 40. Two antibiotics resistance genes include ampicillin- and blasticidin-resistant genes for the selection of plasmid-expressing E.coli and insect cells, respectively. ColE1 represents the origin of replication.

Materials and methods

Cells and antibody

An Aedes albopictus cell line, C6/36 (Igarashi, 1978), was grown in Leibovitz-15 medium (L15; GIBCO BRL, California, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; GIBCO BRL, California, USA), 0.29% tryptose phosphate broth (Sigma-Aldrich, USA), 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (GIBCO BRL, California, USA) at 29°C. A monoclonal antibody, 1D10, was kindly provided by Drs. Chunya Putthikhunt, Watchara Kasinrerk and Prida Malasit for the detection of flavivirus E protein (Keelapang et al., 2004).

Plasmid

A recombinant plasmid was constructed for the expression of dengue prM+E protein in insect cells. The expression of the chimeric prM+E gene is regulated by the Autographa californica nuclear polyhedrosis virus (AcNPV) ie1 promoter and the hr5 enhancer (Huynh, Zieler, 1999; Charoensri et al.,
The coding region is composed of the defensin A signal sequence, the DENV-2-derived prM and EDI plus EDII sequence, the DENV-1-derived EDIII sequence, and the E stem-anchor region from Japanese encephalitis virus (Fig 1). The plasmid was a derivative of pIE1-SP-prME, into which a prM cleavage-enhancing mutation, pr(E203A), and a fusion loop mutation (L387H) had been introduced (Charoensri et al., 2014). Codon usage of the coding regions was optimized to that of A. albopictus (Nakamura et al., 2000).

**Transfection and selection**

C6/36 cells in 24-well culture plate were transfected with 1 µg/well of chimeric plasmid by using lipofectamine 2000 reagent (Invitrogen, California, USA) according to the manufacturer’s protocol (Invitrogen). Transfected cells were dissociated and plated at low density (1.5–2 cells per well) in 96-wells plate. Clones containing the plasmid were selected with 50 µg/ml of blasticidin (Thermo Scientific, Massachusetts, USA) in the growth media.

**Indirect immunofluorescence assay and dot blot immunoassay**

Intracellular E protein was detected by an indirect immunofluorescence assay (IFA). C6/36 cells were fixed on the glass cover slip with 3.7% formaldehyde in phosphate buffered saline and then permeabilized with 2% triton X-100 in phosphate buffered saline. The cell monolayer was incubated successively with 1D10 monoclonal antibody, Cy3-conjugated goat anti-mouse IgG antibody at the 1:1000 dilution (Jackson Immunoresearch, Philadelphia, USA), and 0.1 µg/ml of 4’,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, USA). Examination of the fluorescence signal was performed with a fluorescent microscope (Olympus Provis AX70, Tokyo, Japan).

Extracellular E proteins were detected by a dot blot immunoassay. The fractions derived ultracentrifugation were dotted manually on a nitrocellulose membrane. Non-specific binding was blocked with 5% skim milk in phosphate buffered saline. The membrane was incubated successively with 1D10 monoclonal antibody and alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) antibody (Thermo Scientific, Massachusetts, USA) at the dilution of 1:5,000 for 1 hour at room temperature. Bound E proteins were then visualized by development with 5-bromo-4-chloro-3-indolyl phosphate (Fermentas, Maryland, USA) and nitroblue tetrazolium chloride (Bio Basic, Ontario, Canada) substrates diluted in 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl and 5 mM MgCl2.

**Purification of VLPs**

C6/36 clones stably expressing chimeric VLPs were expanded in T75 flasks to confluence. The growth media were substituted with L15 supplemented with 1.5% FBS for 6 or 7 days, harvested, and concentrated approximately 30-fold by ultrafiltration with using a membrane with the cut off value of 500 kDa. The concentrated supernatant was subjected to centrifugation in a 20 g% and 55 g% (w/w) sucrose step gradient using the SW41 rotor (Beckman Coulter, Palo Alto, California) at 32,000 rpm for 2 hours. Materials at the sucrose interface were then collected and washed with 10 mM Tris (pH 7.2), 2 mM EDTA and 150 mM NaCl employing a centrifugal filter unit with the cutoff value of 100 kDa (Millipore, Massachusetts, USA). The particulate
materials were then purified by the second rate zonal ultracentri-fugation step employing a 5-25 g% (w/w) sucrose gradient at 32,000 rpm for 3 hours. Fractions (0.5 ml) were removed from the bottom of the tube by using an upward displacement method. Then, the fractions were washed with NTE as described above. Total protein concentration was determined by fluorometry (Qubit, Invitrogen, California, USA).

Electrophoresis and determination of prM cleavage
The rate zonal sucrose fractions 15-16 were pooled and separated by electrophoresis in a 0.1% sodium dodecyl sulfate (SDS), 5-15% polyacrylamide gel in non-reducing condition. Proteins were stained with 0.1% silver nitrate. Protein sizes were determined by comparing to a prestained protein ladder marker (Fermentas, Hanover, USA).

Following silver nitrate staining, the protein bands were scanned by scanner (CanoScan, New York, 900F, USA) and the density of each band was analyses by using GeneTools software (Syngene, Cambridge, UK). The density signals of E, prM and M were deduced with the local background signals derived from equal areas of the gel image and then adjusted by dividing with the amino acid content of each protein (E, 495 residues; prM, 166 residues; M, 75 residues). The percentage of prM cleavage was calculated by using the following formula: prM cleavage (%) = [M adjusted signal/(prM adjusted signal + M adjusted signal)] × 100 (Junjhom et al., 2008). The proportion of VLPs in the preparation was calculated by using the formula: [(E+prM+M signal/total signal) × 100].

Negative staining and electron microscopy
Approximately 600 ng of purified chimeric VLPs preparation were adsorbed onto a glow-discharged, Formvar/carbon-coated copper grid (Electron Microscopy Sciences, Pennsylvania, USA). The grid was washed with 1 ml of deionized water, fixed with 1% glutaraldehyde, and then stained twice with 1 g% uranyl acetate. Air-dried grids were examined using a transmission electron microscope (JEM-2200FS; JEOL, Tokyo, Japan) at 200 kV. In the determination of size, a mean diameter of the particles was determined from the distances measured in two perpendicular directions (Junjhom et al., 2008).

Result and Discussion
Generation of C6/36 cell lines expressing DENV-1 EDIII chimeric VLPs construct
In an attempt to generate a C6/36 cell line stably expressing the EDIII chimeric VLPs, C6/36 cells were transfected with a chimeric plasmid. Detection of the intracellular E protein in the transfected C6/36 cells at about 24 hours after the transfection revealed 17.4% (n=1,251) of cell with cytoplasmic red fluorescent signal. Following blasticidin selection, 77 clones were identified with indirect immunofluorescence and dot blot immunoassay for the expression of intracellular and extracellular E protein, and one clone was employed in subsequent selection at low cell density using 96 wells-plates. A relatively high expression of intracellular E protein in up to 80-95% of the cells was detected in several clones following the third round of selection (Fig. 2). This result indicates that the DENV-1 EDIII chimeric prM+E gene construct can be expressed in C6/36 cells
Intracellular E protein in C6/36 cells was detected by using an anti-E antibody, 1D10, and Cy3-conjugated goat anti-mouse IgG antibody. Nuclei were stained with DAPI. C6/36 cells infected with a DENV-2 strain 16681 and uninfected C6/36 cells were employed as the positive control and negative control, respectively. DIC, differential interference contrast.

Detection of E protein in the particulate fraction following ultracentrifugation

The culture media of several stably expressing clones were pooled, concentrated and subjected to ultracentrifugation employing two sets of sucrose density gradients. Following the first centrifugation using 20 g% and 55 g% step sucrose gradient, the E protein was detected by dot blot immunoassay to be present in the 20 - 55g% sucrose interface as well as in the 20 g% sucrose fraction and the culture media fraction (data not shown). Materials from the 20 g% and 55 g% sucrose interface were subjected to a rate zonal separation using 5-25 g% sucrose gradient, and all fractions were dotted on nitrocellulose membrane for the detection of E protein by dot immunoassay employing the monoclonal antibody 1D10. As shown in Fig. 3, the E protein was detected in all fractions with relatively higher concentration in the fractions 13-19 from the bottom of the tube. The migration of the E protein in this rate zonal separation was similar to that of the subviral particles of DENV-2 (Junjhon et al., 2008), suggesting that the chimeric E protein is present in the form of subviral particle-like materials.
The protein component of the rate zonal fractions 15-16 was examined by electrophoresis in the 5-15% polyacrylamide gel and staining with silver nitrate. Three major bands that were detected with the size of approximately 50, 20 and 10 kDa (Fig. 4) correspond to the expected size of the three viral proteins E (54 kDa), prM (19 kDa) and M (8 kDa) (Chang, 1997). An additional band of about 12 kDa may represent an unknown host/serum protein or an M dimer. The extent of prM cleavage of the prM protein in this preparation as calculated from the adjusted prM and M band density was 41%. This prM cleavage is higher than that of DENV-1 (Keelapang et al., 2013) but less than that of the DENV-2 VLPs employed as the backbone (Suphatrakul et al., 2015).

This result indicates that the substituted EDIII domain derived from DENV-1 down modulated the cleavage of prM. Whether these proteins were able to assemble into particles was next examined by negative staining and transmission electron microscopy.

Electron microscopy study of the fractions 15+16 revealed spherical particles of various sizes (Fig. 5), the majority of which is distributed into two class sizes: small (26-30 nm, 48.8%) and medium (35-38 nm, 13.2%) (Fig. 6). In addition, there were larger particles of 42-60 nm that did not cluster into distinctive peaks. Small particles of 26-30 nm were similar to DENV-2 native subviral particles (Junjhon et al., 2008) and DENV-2-JE chimeric virus-like particles with minimal prM cleavage (Charoensri et al, 2014; Suphatrakul et al., 2015). The medium size particles may represent an artifact resulting from the contraction of the larger particles during negative staining as these particles are devoid of the capsid
protein, or a distinct class of particles. Similar broad distribution of virus-like particles had been described previously with tick born encephalitis virus (Ferlenghi et al., 2001). Taken together, these results indicate the assembly of virus-like particles of various sizes and subsequent release of the particles from transfected C6/36 cells.

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References


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Fig. 6 Frequency distribution of particles according to size (n=441). Particles were separated into classes of 1 nm increments based on the mean diameter and the frequency at each increment plotted against the diameter. Bars indicate the peak frequency of the three size classes.


