Generation and Preliminary Characterization of DENV-4/2 EDIII Chimeric Virus-like Particles

การสร้างและศึกษาคุณสมบัติเบื้องต้นของอนุภาคคล้ายไวรัสลูกผสมระหว่างเชื้อไวรัสเด็งกี่ชนิดที่ 4 และ 2

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ABSTRACT

Dengue virus (DENV) infection has dramatically increased and becoming a severe public health problem. However, a licensed vaccine does not provide efficient protection against dengue. Virus-like particles (VLPs) have shown considerable promise for use as vaccine candidate. In this study, we generated a DENV-4/2 EDIII chimeric VLPs from C6/36 cells transfected with a chimeric plasmid containing the prM and E genes of DENV-2, domain III of DENV-4 E protein and stem anchor of Japanese encephalitis virus. Selection with blasticidin and limiting dilution yielded stably transfected C6/36 cells in which extracellular and intracellular E protein could be detected by using an anti-E monoclonal antibody. Electrophoretic separation of partially purified particulate materials revealed three major protein bands corresponding in size to the viral structural proteins E, prM and M. Electron microscopic study showed two size classes of spherical particles with peak frequencies at 34-39 nm and 41-48 nm.

บทคัดย่อ

การติดเชื้อไวรัสเด็งกี่มีแนวโน้มที่จะพบการติดเชื้อเพิ่มขึ้นและอาจกลายเป็นปัญหาทางด้านสาธารณสุขที่สำคัญและรุนแรง ในปัจจุบัน วัคซีนที่ได้รับอนุญาตให้ใช้ในการป้องกันการติดเชื้อไวรัสยังไม่มีประสิทธิผลสูง อนุภาคคล้ายไวรัสถือเป็นแนวทางที่น่าสนใจสำหรับการนำไปพัฒนาเป็นวัคซีนตัวเลือก ดังนั้นในการศึกษานี้จึงได้ทำการสร้างอนุภาคคล้ายไวรัสลูกผสม ระหว่างเชื้อไวรัสเด็งกี่ชนิดที่ 4 และ ชนิดที่ 2 จากเซลล์ยุงที่ได้รับพลาสมิดที่มีส่วนประกอบของยีน prM และ E ของเชื้อไวรัสเด็งกี่ชนิดที่ 2, ส่วนย่อย domain III ของเชื้อไวรัสเด็งกี่ ชนิดที่ 4 และส่วนของ stem anchor ของเชื้อไวรัส Japanese encephalitis การตรวจหาโปรตีน E ภายในเซลล์และนอกเซลล์ โดยใช้แอนติบอดีที่จำเพาะต่อโปรตีน E สามารถตรวจพบได้จากเซลล์ที่มีความคงตัวหลังจากได้รับพลาสมิดและถูกคัดเลือกด้วยยา blasticidin ผลการวิเคราะห์การแยกโปรตีนกึ่งบริสุทธิ์ที่ได้จากการทดลอง พบแถบของโปรตีน 3 แถบที่มีขนาดสอดคล้องกับโปรตีนโครงสร้างของเชื้อไวรัสเด็งกี่ ได้แก่ โปรตีน E, prM และ M จากการศึกษาด้วยกล้องจุลทรรศน์อิเล็กตรอนพบอนุภาครูปร่างกลม สามารถแบ่งอนุภาคตามขนาดได้เป็น 2 กลุ่มคือ กลุ่มอนุภาคขนาดประมาณ 34-39 นาโนเมตร และ 41-48 นาโนเมตร

Keywords: Dengue virus, Virus like particles (VLPs), Domain III

คำสำคัญ: เชื้อไวรัสเด็งกี่ อนุภาคคล้ายไวรัส ส่วนย่อย domain III ของโปรตีน E

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Introduction

Dengue virus (DENV) causes one of the most important mosquito-borne viral diseases in human. DENV belongs to the genus Flavivirus in the family *Flaviviridae*, There are four antigenically distinct serotypes of dengue virus (DENV-1 to -4), all of which can cause undifferentiated fever, dengue fever, dengue hemorrhagic fever or dengue shock syndrome. DENV is an enveloped virus with a single-stranded, positive-sense RNA genome. The viral genomic RNA encodes three structural proteins [capsid (C), premembrane/membrane (prM/M) and envelope (E)] and seven non-structural proteins (NS1, 2A, 2B, 3, 4A, 4B and 5). In the endoplasmic reticulum of infected cells, viral structural proteins assemble into immature particles, about 60 nm in diameter, which contain the two envelope glycoproteins, prM and E on the surface. When the immature particles are transported to the trans-Golgi network, the pr portion is cleaved from prM and immature particles become mature particles. The mature viral particles contain M and E proteins on the outer envelope (Lindenbach, 2001; Kuhn et al., 2002). The envelope glycoprotein, E, is involved in receptor binding, membrane fusion, and represents the target for virus neutralizing antibodies. There are three outer domains in the dengue E protein: EDI, II and III. The EDIII domain is responsible for host receptor binding as well as the induction of virus neutralizing antibodies; EDIII is considered to be an antigenic target of particular relevance for dengue vaccine development (Wang et al., 2006; Crill et al., 2009).

Currently, several dengue vaccine candidates have been developed, including live-attenuated vaccines, inactivated whole virus particles, subunit vaccines, DNA vaccines and virus-like particles (VLPs). A number of live-attenuated dengue vaccine candidates are now being tested in several clinical trials (Daniel et al., 2009). However, concerns have been raised on the possibility that these attenuated virus may revert to virulent virus, which could lead to a risk of disease. VLPs represent an alternative approach in the development of vaccine against dengue, and have been shown to be of considerable promise in the prevention of many viral diseases, such as carcinomas of the cervix and liver (Mao et al., 2006; Zhang et al., 2011). VLPs exhibit similar structure to infectious virions, they may be able to stimulate the host immune system in the same manner as native virus particles. As VLPs lack virus-specific genetic materials required for infection. VLPs should be safer than conventionally attenuated vaccine candidates (Noad, Roy, 2003).

Recently, a number of strategies has been used in the generation of dengue VLPs. In an approach, the genes encoding DENV prM and E proteins are placed together for the construction of VLPs. A recombinant vector carrying a cDNA fragment encoding the prM and E proteins of DENV-2 was integrated into the genome of *Pichia pastoris*, and a high level constitutive expression of recombinant DENV proteins was achieved (Liu et al., 2010). In another approach, chimeric VLPs are generated by incorporating dengue sequences into pre-existing heterologous VLPs aiming for an efficient stimulation of the host immune responses against the foreign protein epitopes (Shang et al., 2012). Chimeric particles in which DENV-2 EDIII had been inserted into the c/e1 loop of HBcAg were shown to be highly immunogenic eliciting high titer antibodies (Arora et al., 2012). This is an important

finding as EDIII has a very low intrinsic potential for inducing cross-reactive antibodies implicated in the pathogenesis of dengue hemorrhagic fever (Simmons et al., 1998, 2001). Ideally, the generation of dengue VLPs for use as a vaccine candidate should result in a product that can induce neutralizing antibody against all four dengue serotypes. The production of VLPs ought to achieve high level of VLPs. In a previous study, DENV-2 VLPs were generated from stably (prM+E)-expressing C6/36 cells (Suphatrakul et al., 2015). This DENV-2 VLPs preparation was constructed by introducing modifications at the pr-M cleavage junction and the fusion loop of protein E as well as the replacement the E stem–anchor region with that of Japanese encephalitis virus. Studies in mice showed that the DENV-2 VLPs preparation could induce high titer of neutralizing antibody in mice when administered with adjuvants. Whether similar constructs for other dengue serotypes will be as successful has not yet been delineated.

In this study, DENV-4/2 EDIII chimeric VLPs were generated in C6/36 cells by employing the DENV-2 VLPs construct from the previous study (Suphatrakul et al., 2015) as the backbone. The domain III of DENV-2 E protein in the DENV-2 VLPs construct was substituted with the homologous region of DENV-4 with an aim for the induction of high level of antibody response to DENV-4 EDIII domain during the immunization with chimeric VLPs. Following the construction of expression plasmid and derivation of stably expressing clones, the VLPs were partially purified and their morphology was studied by electron microscopy.

Materials and Methods

prM+E expression plasmid

A recombinant plasmid employed for the expression of the DENV-4/2 EDIII chimeric VLPs (Fig. 1) was based on pIE1-SP-prME-blasticidin (Charoensri et al., 2014). This plasmid contained an insect ribosomal binding site, the defensin A signal sequence, the prM and the EDI-II domains of the E gene of DENV-2, and the E stem-anchor region of Japanese encephalitis virus. The prM and E genes of DENV-2 were modified by introducing the pr (E203A) and E (L387H) mutations in order to enhance maturation and release of the particles, respectively (Charoensri et al., 2014). Codon usage of the coding regions was optimized to that of *A. albopictus* (Nakamura et al., 2000). This plasmid also encoded a blasticidin resistant gene for the selection of transfected cells.

Antibody

A murine IgG1 monoclonal antibody specific for the dengue virus and Japanese encephalitis virus E protein, clone 1D10 (Puttikhunt, Kasinrerk and Malasit, unpublished results), was used in the detection of intracellular and extracellular E protein in transfected cells and the culture media. An alkaline phosphatase-conjugated goat anti-mouse IgG antibody and a Cy3-conjugated goat anti-mouse IgG antibody (Jackson Immunoresearch, West Grove, PA, USA) were used as the secondary antibody in the dot blot immunoassay and indirect immunofluorescence assay, respectively.

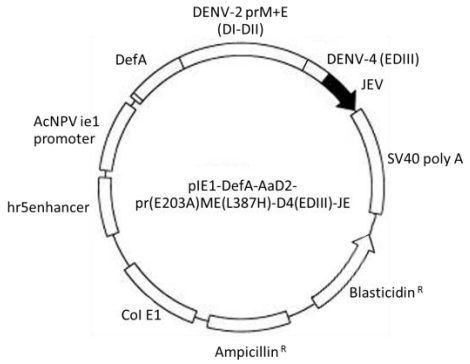

Fig. 1 Schematic diagram of the expression vector employed in this study. The chimeric plasmid contains defensin A signal sequence, prME of DENV-2 with the E domain III from DENV-4, the stem-anchor region of Japanese encephalitis virus E protein and the poly A-addition sequence of simian virus 40. The antibiotics resistance genes are ampicillin- and blasticidin-resistant genes for the selection of plasmid-expressing *E.coli* and insect cells, respectively.

Cells and transfection

C6/36, an *Aedes albopictus* cell line (Igarashi, 1978), was grown at 29 ˚C in Leibowitz's L-15 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 0.29% tryptose phosphate broth, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin solution. For transfection, C6/36 cells were seeded into a 24-well plate and the monolayer transfected with 1 µg of plasmid using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Twenty-four hours after transfection, the intracellular expression of E protein was examined by using the monoclonal antibody 1D10 in an indirect immunofluorescence assay. Extracellular E protein was detected by dot blot immunoassay.

Production and purification of chimeric VLPs

After transfection with plasmid, transfected C6/36 cells were selected initially with 50 ug/ml of blasticidin (Invitrogen, Carlsbad, CA, USA) in L-15 media supplemented with 10% FBS and subsequently by two rounds of limiting dilution in 96-well plates. Several clones with high proportion of intracellular E protein expression (80-90% of 1D10-positive cells in indirect immunofluorescence assay) as well as readily detectable level of extracellular E protein by dot blot immunoassay were selected for expansion. In the purification, the culture media of several selected clones were harvested, pooled, concentrated by filtration using a membrane with the molecular weight cut off of 300-500 kDa and subjected to ultracentrifugation using a 20%-55% (w/w) step sucrose gradient (32,000 rpm for 2 hours in the SW41 rotor). Materials collected from the 20% and 55 % (w/w) sucrose interface were then separated by rate zonal centrifugation using a 5–25% (w/w) sucrose gradient (32,000 rpm for 3 hours in the SW41 rotor).

Several fractions obtained by an upward displacement method were dotted onto a nitrocellulose membrane for the detection of E protein by dot blot immunoassay. Two fractions with high E protein signal were pooled, washed with 10 mM Tris pH (7.2), 150 mM NaCl and 2mM EDTA, and concentrated using a centrifugal filter unit with the molecular weight cut off of 100 kDa (Millipore, Billerica, MA, USA).

Indirect immunofluorescence assay

Transfected C6/36 monolayer on a glass cover slip was fixed with 3.7% formaldehyde in phosphate-buffered saline, permeabilized with 2% triton X-100 in phosphate-buffered saline, and then incubated for 1 hour at 37°C with an anti-E domain I/II monoclonal antibody (clone 1D10). Following a washing step using phosphate-buffered saline, intracellular E proteins were visualized by reacting with a Cy3-conjugated goat anti-mouse IgG antibody at 37°C for 1 hour. Nuclei were counterstained with 4, 6-diamino-2-phenylindole hydrochloride (DAPI, Sigma-Aldrich, St. Louis, USA).

Dot blot immunoassay

From 1-4 µL of the sucrose fractions from the rate zonal centrifugation were dotted manually onto nitrocellulose membrane. Non-specific protein binding was blocked with 5% (w/v) skim milk in phosphate-buffered saline. The presence of dengue virus E protein was detected with using the antibody 1D10, followed by alkaline phosphatase-conjugated goat anti-mouse IgG antibody. The signal was visualized by adding a mixture of nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate.

SDS-PAGE and determination of protein concentration

The rate zonal sucrose fractions were lysed with 0.1% sodium docecylsulfate and then separated in a 5-15% sodium docecylsulfate-polyacrylamide gel electrophoresis. For the visualization of protein, the gel was stained with silver nitrate, and the size of viral structural proteins was determined by comparing to a prestained protein ladder marker (Fermentas, Hanover, MD, USA). Total protein concentration in the rate zonal centrifugation fraction was determined by using a fluorometric kit (Qubit, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Quantitation of prM cleavage

The level of the prM cleavage level was measured from a signal of prM and M in the SDS-PAGE gel. After staining with silver nitrate, the SDS-PAGE gel was scanned with scanner (CanoScan 9000F, Melville, NY, USA). The density of the prM and M bands were subtracted with the background signals derived from equal areas of the gel image by using the GeneTools image analysis software (Syngene, Cambridge, UK) and then adjusted by dividing with the amino acid content (166 residues for prM and 75 residues for M). The extent of the prM cleavage was calculated by using the following formula: prM cleavage (%) = [M adjusted signal/ (prM adjusted signal + M adjusted signal)] x 100 (Junjhon et al., 2008).

Transmission electron microscopy

For the visualization of chimeric VLPs, 1.6 µg of the selected rate zonal centrifugation fraction were placed on a formvar/carbon-coated copper grid (Electron Microscopy Sciences, Hatfield, PA, USA), fixed with 1% glutaraldehyde, and stained twice with 1% uranyl acetate. The grids were then examined with a transmission electron microscope (JEM-2200FS; JEOL, Tokyo, Japan) at a magnification of 50,000×. In the determination of size, the diameter of a particle was determined from the distances measured in two perpendicular directions (Junjhon et al., 2008).

Results and Discussion

Expression of DENV-4/2 EDIII chimeric E protein in transfected C6/36 cells

When near-confluent monolayer of C6/36 cells was transfected with a chimeric plasmid (Fig. 1), intracellular expression of dengue E protein was observed in about 2.5% of the transfected C6/36 cells at 24 hours post transfection by using the monoclonal antibody 1D10 in an indirect immunofluorescence assay (data not shown). Following selection with blasticidin and two rounds of limiting dilution cloning, several clones with relatively high proportion (80-90%) of 1D10-positive cells were identified (Fig. 2). The intensity of the fluorescence signal observed in these selected clones was comparable to that of DENV-2, strain 16681, infected C6/36 cells. This result indicated that the chimeric DENV-4 EDIII domain within the DENV-2 prME-JE background did not interfere with the expression of this chimeric E protein in C6/36 cells. It is likely that the overall conformation of the chimeric E protein was not adversely affected as 1D10, which recognizes a conformational epitope in the EDI/II domain (Puttikhunt, Kasinrerk and Malasit, unpublished results), could react well with this protein. However, additional testing with other monoclonal antibodies will need to be performed to verify this interpretation. This result suggests that the chimeric E protein can be expressed in an insect cell line without deleterious changes in the protein conformation.

Detection of extracellular E protein in the culture of chimeric (prM+E)- expressing C6/36 cells

The presence of the DENV-4/2 chimeric E protein in the particulate form in the culture media of transfected C6/36 cells was examined by subjecting concentrated media that had been pooled from several selected clones to centrifugation using 20%-55% sucrose step gradient followed by a rate zonal centrifugation.

Dot blot immunoassay with 1D10 revealed that almost all rate zonal centrifugation fractions contained E protein, but there were six fractions (#14-19) with comparatively higher levels than the others (Fig. 3).

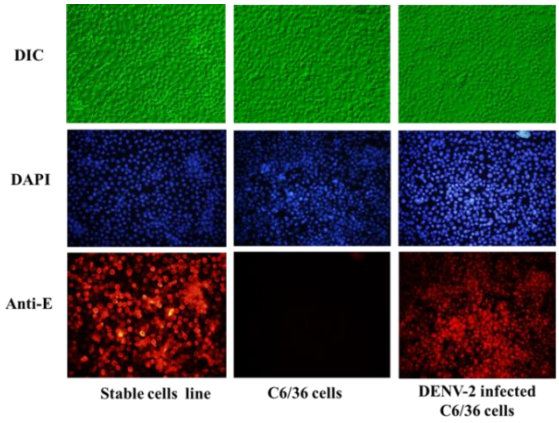

Fig. 2 Immunofluorescence staining of C6/36. The monolayer of C6/36 clone were fixed, permeabilized and reacted with monoclonal antibody 1D10 (anti-E panel). Expression of E protein was visualized with Cy3-conjugated goat anti-mouse IgG antibody. Nuclei were stained with DAPI (DAPI panel). DIC, light microscopy employing differential interference contrast (DIC panel). C6/36 cell were infected with DENV-2 strain 16681 and C6/36 cells as positive and negative control respectively.

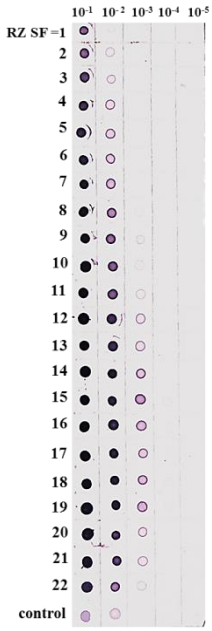

Fig. 3 Detection of extracellular E protein. The four fractions (2-5) from sucrose step gradient were separated by rate zonal centrifugation using a 5–25% (w/w) sucrose gradient. Fractions 1-22 were employed at several dilutions for the detection of extracellular E protein by anti-E (1D10) antibody and alkaline phosphatase-conjugated goat anti-mouse IgG antibody.

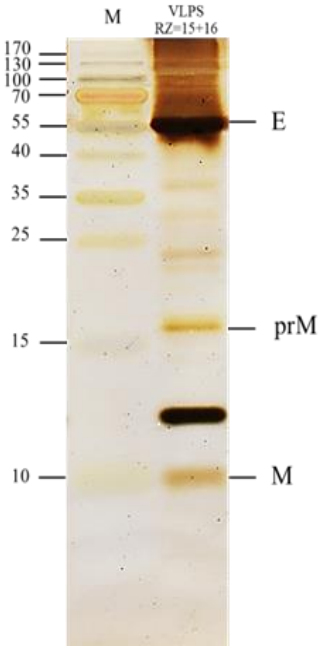

Fig. 4 SDS-PAGE analysis and silver staining. The rate zonal separation (fractions 15+16) were disrupted with 0.1% SDS, separated in a 5- 15% sodium docecylsulfate poly-acrylamide gel electrophoresis and stained with silver nitrate (lane 2). The viral structural proteins were indicated based on size. Sizes (in kD) of the protein marker bands (lane M) are indicated.

This result suggests that, in the presence of co-expressing prM, the DENV-4/2 EDIII chimeric E protein can assemble into particles that are released from transfected cells.

Characterization of VLPs

Several fractions derived from rate zonal centrifugation in sucrose density gradient were lysed using sodium dodecylsulfate and subjected to electrophoretic separation in sodium dodecylsulfate-polyacrylamide gel. Staining of the gel with silver nitrate revealed three major bands corresponding in size to the three viral proteins: E (54 kDa), prM (19 kDa) and M (8 kDa) (Fig. 4).

The simultaneous presence of these viral structural proteins in the same selected fractions form rate zonal centrifugation further indicated assembly of the chimeric E proteins into particulate form. The extent of prM cleavage among these particles as determined from the proportion of adjusted prM and M intensity signal was 71.8%, which was slightly

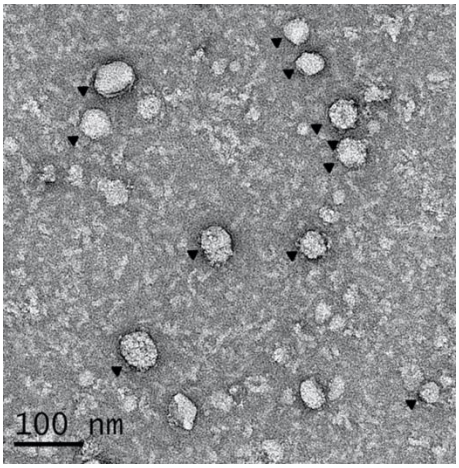

Fig. 5 Transmission electron microscopic visualization of chimeric VLPs particles. The rate zonal sucrose fractions 15+16 were fixed with 1% glutaraldehyde and stained with 1% uranyl acetate. Bar represents 100 nm. Black arrows indicate particles.

lower than the level of 83.2% observed in the parental DENV-2 VLPs (Suphatrakul et al., 2015). This result suggests that the substitution of DENV-4 EDIII domain in DENV-2 VLPs does not strongly affect the efficiency of prM cleavage. The result is consistent with a close similarity between DENV-2 and DENV-4 structural proteins as compared with other serotypes (Volk et al., 2007).

Analysis of the rate zonal sucrose fractions 15+16 by negative staining and transmission electron microscopy revealed spherical particles with wide variations (range 22-68 nm) in the diameter (Fig. 5). When the frequency distribution of 461 particles was plotted according to size, the distribution pattern was compatible with either a single size class of 34-48 nm particles, or two size subclasses of medium (34-39 nm) and large (41-48 nm) particles (Fig. 6).

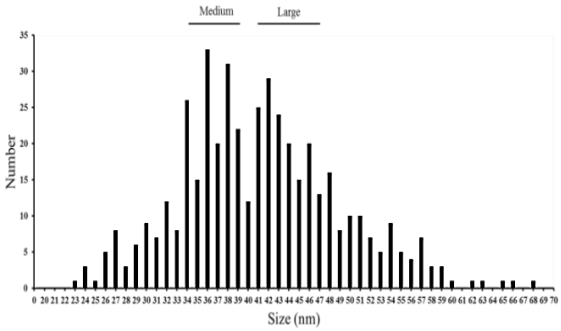

Fig. 6 The frequency distribution of particles. The particles (n= 461) were separated into 1 nm increment of the diameter. Bars indicate the peak frequency of the two possible size subclasses.

Large spherical particles detected in this preparation were similar in size to those previously described in the parental DENV-2 VLPs (Suphatrakul et al., 2015), DENV-4 VLPs (Zhang et al., 2011) and native viral particles of DENV-2 strain 16681Nde(+) (Junjhon et al., 2008). Whether the medium size particles represent a new class of VLPs, or derived from large VLPs by drying during the processing of the specimen for electron microscopy will need to be investigated further.

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