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Secretion of noninfectious dengue virus-like particles and identification of amino acids in the stem region involved in intracellular retention of envelope protein

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Abstract

DNA plasmids that express flavivirus premembrane/membrane (prM/M) and envelope (E) proteins in the form of virus-like particles (VLPs) have an excellent potential as DNA vaccine candidates against virus infection. The plasmid-expressed VLPs are also useful as safe, noninfectious antigens in serodiagnostic assays. We have constructed plasmids containing the prM/M and E gene regions for DENV-1, -3, and -4 that express and secrete VLPs when electroporated into Chinese hamster ovary cells. Constructs containing the full-length DENV-1 E protein gene did not secrete VLPs into tissue culture fluid effectively. However, a 16-fold increase in ELISA titers of DENV-1 VLPs was achieved after replacing the carboxy-terminal 20% region of DENV-1 E protein gene with the corresponding sequence of Japanese encephalitis virus (JEV). DENV-3 plasmids containing either the full-length DENV-3 E protein gene or the 20% JEV sequence replacement secreted VLPs to similarly high levels. Whereas DENV-4 VLPs were secreted to high levels by plasmids containing the full-length DENV-4 E protein gene but not by the chimeric plasmid containing 20% JEV E replacement. Domain substitutions by replacing prM/M protein stem-anchor region with the corresponding prM/M stem-anchor region of JEV or DENV-2 in the chimeric DENV-4 construct failed to promote the secretion of DENV-4 VLPs. Using the DENV-2 chimeric plasmid with carboxy-terminal 10% of JEV E gene, the sequence responsible for intracellular localization of E protein was mapped onto the E-H1 α-helix domain of DENV-2 E protein. Substitution of three amino acids from the DENV-2 sequence to the corresponding amino acids in the JEV sequence (I398L, M401A, and M412L) in the E-H1 was sufficient to promote extracellular secretion and resulted in detectable titers of DENV-2 VLP secretion.
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Keywords: Dengue virus; prM-E-expressing plasmids; Virus-like particles; Stem-anchor regions

Introduction

The genome of viruses in the genus *Flavivirus* of the family *Flaviviridae* consists of ~11 kb single-stranded, positive-sense RNA genome which encodes capsid (C), premembrane/membrane (prM/M), and envelope (E) structural proteins, and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). Signal sequences located at the carboxy-terminal regions of C as well as prM/M target prM/M and E structural proteins, respectively, for translocation, transport, and secretion through the endoplasmic reticulum (ER) and Golgi apparatus. The prM/M and E proteins are anchored to the ER membrane by their carboxy-terminal domains and associate into heterodimers via interactions between both the ectodomains and the carboxy-terminal stem-anchor regions of each protein (Allison et al., 1999). The amino-terminus (or non-M region) of prM/M prevents premature irreversible low-pH-induced conformational changes in E protein during transit through acidic exocytotic vesicles (Heinz et al., 1994) to ensure the release of functionally active, fusion-competent virus. Structural proteins are subsequently transported through the cell

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secretory pathway, and prM is cleaved to M protein by cellular furin or a furin-like protease just prior to the release of virus particles from the cell (Stadler et al., 1997).

The prM/M stem region, represented by amino acids M-23 to M-37 of dengue virus type 2 (DENV-2), consists of a weakly amphipathic α-helix (M-H) which is partially buried at its carboxy-terminus in the outer lipid leaflet of virion particles, forming a ~20° angle with the lipid membrane surface (Zhang et al., 2003). The two α-helical transmembrane domains (M-T1 and M-T2) of the prM/M anchor region (amino acids M-40 to M-70) partially traverse the lipid bilayer and form an antiparallel coiled-coil structure. The E anchor region (amino acids E-452 to 491) is similar to that of prM/M and consists of two transmembrane regions (E-T1 and E-T2). The E stem region (amino acids E-395 to 450) consists of two consecutive mostly amphipathic α-helices (E-H1 and E-H2) that are separated by the conserved sequence (CS) element, a stretch of amino acids highly conserved among flaviviruses (Stiasny et al., 1996). Similar to M-H, both E-H1 and E-H2 are also partially buried in the outer lipid bilayer (Zhang et al., 2003).

Potential functions for the E protein stem and anchor elements of tick-borne encephalitis virus (TBEV), a member of the *Flavivirus*, were determined based on truncation of each domain and assaying for secretion of prM and E proteins in VLP form (Allison et al., 1999). The E-T2 transmembrane domain, the signal sequence for NS1, is dispensable for the incorporation of E protein into VLPs, whereas E-T1 acts as a membrane anchor and is required for the incorporation of E proteins into VLPs. Stabilization of prM-E heterodimers is conferred by the E-H2 helix, possibly in conjunction with E-T1, although studies of yellow fever virus (YFV) support the speculation that the transmembrane domains of flaviviruses affect the formation of virus but not by prM-E heterodimerization (Op De Beeck et al., 2003). The E-H1 α-helix is involved in irreversible trimerization of soluble E proteins upon exposure to low pH environments (Allison et al., 1999).

During natural flavivirus infections, noninfectious, empty virus-like particles (VLPs) are produced in addition to infectious, mature virions (Russell et al., 1980). These VLPs have structural and physiochemical properties similar to mature virions. Thus, plasmid constructs capable of expressing prM/M- and E-containing VLPs have been the subject of intensive study for their potential as DNA vaccine candidates and as expression plasmids for producing non-infectious serodiagnostic antigens and biosynthetic subunit vaccine candidates. Previously, we reported that pCDJE2-7 and pCBWN plasmid-transformed COS-1 cells express and secrete JEV and WNV VLPs, respectively, into the tissue culture media (Chang et al., 2000; Davis et al., 2001; Hunt et al., 2001). Plasmid DNA containing a eukaryotic transcriptional unit consisting of the human cytomegalovirus immediate early gene promoter, Kozak consensus ribosomal binding sequence, JEV signal sequence located at the carboxy-terminus of C protein, and full-length prM/M and E gene regions of JEV or WNV is sufficient for production of VLPs. More recently, we successfully constructed similar expression vectors for the expression of DENV-2 and St. Louis encephalitis virus (SLEV) prM/M and E proteins (Chang et al., 2003; Purdy et al., 2004). Signal peptidase cleavage at the C-prM junction of the flavivirus polypeptide precursor is dependent on prior processing of the upstream C protein sequence by the viral NS2B/NS3 serine protease to expose the cryptic cleavage site for efficient processing (Stocks and Lobigs, 1998). To eliminate the requirement of NS2B/NS3 for efficient processing, we have incorporated the optimized JEV signal sequence (Chang et al., 2001) into WNV, DENV-2, and SLEV constructs. However, secretion of either DENV-2 or SLEV VLPs from tissue culture cells could be achieved only by constructing chimeric plasmids having an additional replacement of the carboxy-terminal 20% of E protein with the corresponding region derived from JEV. The E protein stem-anchor region of DENV-2 and SLEV may therefore contain a peptide sequence which prevents efficient transport of E protein through the cell secretory network; and replacement with the JEV stem-anchor could alleviate such retention. The region involved is suspected to be localized between amino acids E-397 and E-436 in the DENV-2 E protein, the region corresponding to the E-H1 and E-H2 stem α-helices, since the DENV-2 construct having replacement of the carboxy-terminal 10% of E protein with the corresponding E-T1 and E-T2 domains of JEV alone did not improve VLP secretion (Chang et al., 2003).

Based on these previous findings with DENV-2, we attempted to construct DENV-1, DENV-3, and DENV-4 chimeric plasmids for the expression of viral structural proteins in tissue culture and the secretion of VLPs. To further investigate the E protein region affecting retention and secretion, we mutated 13 amino acids in the E-H1 and E-H2 stem region of the pCB9D2-1J-4-3, a non-secreting plasmid constructed in our previous study (Chang et al., 2003) which consists of the 90% amino-terminal and 10% carboxy-terminal E protein regions of DENV-2 and JEV, respectively, and assayed for the ability of individual or combined mutations that may improve VLP secretion. We describe herein the expression of DENV-1, -3, and -4 prM/M- and E-containing VLPs, and further investigate the important roles of amino acids in α-helix E-H1 of the stem region of DENV-2 E protein that affect intracellular retention and secretion of VLPs.

Results

Transient expression of chimeric DENV VLPs

We previously reported that VLPs, which consist of DENV prM, M, and E proteins and which do not contain any C protein, were secreted from transformed COS-1 cells only after replacement of the carboxy-terminal 20% of

DENV-2 E protein with the corresponding sequence from JEV (Chang et al., 2003). However, VLPs were not secreted by the plasmids with the full-length DENV-2 E protein or with replacement of only the carboxy-terminal 10%. Experiments to construct similar expression plasmids for DENV-1, -3, and -4 were therefore designed based on this carboxy-terminal 20% replacement of the DENV E protein stem-anchor region with the corresponding JEV region (Fig. 1). All constructs expressed E protein in plasmid-transformed CHO cells, as detected by IFA using flavivirus group-reactive MAb (4G2) specific for E protein. However, we observed a variation in staining intensity and morphological pattern depending upon plasmid constructs (Table 1). As expected, expression plasmids pCB8D1-2J and pCB8D3-2J both efficiently secreted DENV-1 and -3 VLPs from transformed CHO cells, respectively, with Ag-ELISA endpoint titers of 1:32 (Table 1). Conversely, the pCB8D4-2J plasmid-transformed cells only secrete DENV-4 VLPs with Ag-ELISA endpoint titers $\leq$1:4.

Interactions between the stem-anchor regions of prM/M and E proteins contribute to overall virus particle stability; thus, suboptimal interactions between the DENV-4 prM/M and JEV E protein stem-anchor regions may have prevented the efficient secretion of chimeric DENV-4 VLPs by pCB8D4-2J. In order to mimic the interactions between these regions previously shown to be associated with efficient secretion of JEV and DENV-2 VLPs, the prM/M stem-anchor region of pCB8D4-2J, containing the three α-helical domains M-H, M-T1, and M-T2 (Fig. 1) of DENV-4 was replaced with that of either DENV-2 or JEV (designated as pCBD2prM8D4-2J or pCBJprM8D4-2J, respectively). Contrary to our expectation, these replacements actually reduced secretion to undetectable levels. Additionally, cells transformed with either the DENV-2 or JEV prM/M protein stem-anchor replacement construct appeared to accumulate E protein in perinuclear regions of transformed cells (data not shown). Conversion of the remaining three amino acids outside of three helical domains of pCBD2prM8D4-2J to the authentic M protein sequence of DENV-2 (designated as pCBD2M8D4-2J) still did not enhance VLP secretion. These results indicated that interactions other than those between the prM/M-E stem-anchor regions were important in the secretion of chimeric DENV-4 VLPs.

Since JEV and WNV VLPs can be expressed efficiently in plasmids containing full-length JEV and WNV prM/M and E proteins, respectively (Chang et al., 2000; Davis et al., 2001), the expression plasmid containing the full-length prM/M and E protein genes of DENV-4 (Sri Lanka) was constructed. In addition, the plasmid containing the full-length prM and E genes of DENV-4 (Honduras) was constructed to determine if strain-specificity sequence played a role in secretion of VLPs. CHO cells transformed with both full-length prM and E of DENV-4 constructs secreted VLPs at Ag-ELISA endpoint titers of 1:32

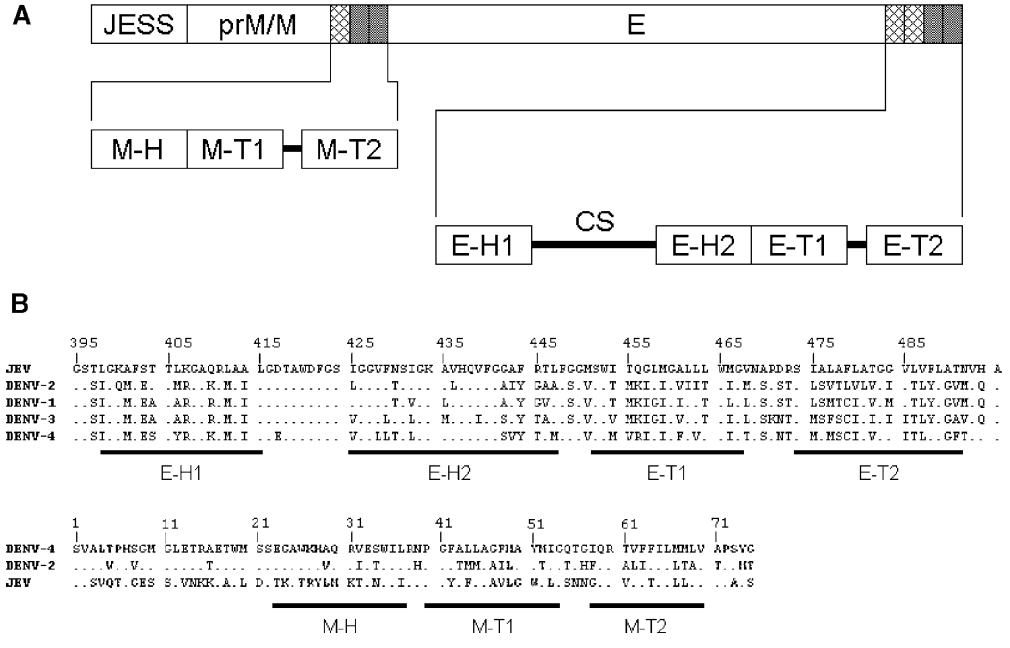

Fig. 1. Comparison of flavivirus premembrane/membrane (prM/M) and envelope (E) protein stem-anchor regions. (A) Flavivirus expression cassettes contain an optimized Japanese encephalitis virus signal sequence (JESS) and viral prM/M and E gene regions for efficient expression of virus structural proteins. The stem α-helical (H; cross-hatched boxes) and transmembrane coiled-coil (T; shaded boxes) domains for each structural protein are indicated. The carboxy-terminal 10% and 20% E protein regions contain amino acids E-442 to E-495 and E-397 to E-495, respectively. (B) Alignment of flavivirus E and prM/M primary amino acid sequences with Japanese encephalitis virus (JEV) or dengue virus type 4 (DENV-4) sequences, respectively. Residues identical to the JEV or DENV-4 sequence are indicated by dots; the single amino acid code is given for variant residues. The stem α-helical and transmembrane coiled-coil domains of prM/M and E proteins, determined by secondary structure prediction from the primary amino acid sequence, are indicated by solid bars.

Table 1
VLP secretion titers for DENV plasmid-transformed CHO cells

Plasmid	Virus (strain)	Chimeric region	IFA pattern	Ag-ELISA titer
pCB8D1-2J	DENV-1 (Panama 1994)	20% JEV-E	Diffuse	32
pCBD1	DENV-1 (Panama 1994)	–	Diffuse	2
pCB8D2-2J-2-9-1	DENV-2 (16681)	20% JEV-E	Diffuse	64
pCB9D2-1J-4-3	DENV-2 (16681)	10% JEV-E	Globular	0
pCBD2-14-6	DEBN-2 (16681)	–	Globular	0
pCB8D3-2J	DENV-3 (Panama 1994)	20% JEV-E	Diffuse	32
pCBD3	DENV-3 (Panama 1994)	–	Diffuse	32
pCB8D4-2J	DENV-4 (Sri Lanka 1994)	20% JEV-E	Diffuse	4
pCBD4	DENV-4 (Sri Lanka 1994)	–	Diffuse	32
pCB8D4-2J-H	DENV-4 (Honduras 1994)	20% JEV-E	Diffuse	4
pCBD4-H	DENV-4 (Honduras 1994)	–	Diffuse	32
pCBD2prM8D4-2J	DENV-4 (Sri Lanka 1994)	20% JEV-E DENV-2 M-H, T1, T2	Globular	0
pCBD2M8D4-2J	DENV-4 (Sri Lanka 1994)	20% JEV-E DENV-2 M	Globular	0
pCBJprM8D4-2J	DENV-4 (Sri Lanka 1994)	20% JEV-E JEV M-H, T1, T2	Globular	0

(Table 1). This unexpected result prompted us to construct expression plasmids containing the full-length DENV-1 and -3 prM and E protein genes (designated as pCBD1 and pCBD3, respectively) in order to understand the affect of the E protein stem-anchor region on VLP extracellular release for each DENV serotype. As observed previously for DENV-2 plasmids containing the full-length prM and E genes, the VLP secretion was significantly reduced by the pCBD1, with Ag-ELISA titers 16-fold lower than those for pCB8D1-2J. Surprisingly, however, pCBD3 secreted VLPs at Ag-ELISA titers similar to those for pCB8D3-2J. These results suggest that replacement of the carboxy-terminal 20% of DENV E protein with that of JEV provides for proper assembly and secretion of VLPs for DENV-1 and -2, yet does not produce any additional benefit to promote DENV-3 VLP secretion, and actually adversely affects the secretion of DENV-4 VLPs.

Characterization of DENV VLPs

The protein compositions of the VLP preparations expressed by constructs pCB8D1-2J, pCB8D2-2J-2-9-1, pCB8D3-2J, and pCBD4 were analyzed by Western blot (Fig. 2) and compared to the corresponding gradient-purified virions. As expected, the appropriate HIAF-detected protein bands corresponding to the M_r for DENV prM and E proteins (~20 and 55 kDa, respectively) in VLP and purified virus preparations for each DENV serotype (Fig. 2A). Bands corresponding to C protein were detected in purified virus preparations for DENV-1 and -3 using the appropriate HIAF while C protein was not detected for either DENV-2 or -4. This may be due to a paucity of antibodies with adequate avidity to bind C protein in the HIAF preparations for DENV-2 and -4. This explanation was supported by the follow-up experiment that the C protein in the same preparation of purified DENV-2 virions was detected by C-specific MAb 1A2A-1 (Fig. 2B). The C protein was not detected in any VLP preparation since the expression plasmids do not contain C protein-encoding sequence. In order to determine the identity of proteins detected by HIAF, DENV-2 VLP and purified virus particle preparations were analyzed with antibodies specific for DENV-2 structural proteins (Fig. 2B). Bands with correct sizes were detected in the DENV-2 VLPs when blotted proteins on the membrane were reacted with a flavivirus E-specific MAb (4G2), prM-specific MAb (2H2), and both prM- and M-reactive antipeptide (M aa 1–34) serum. As expected, C-specific MAb (1A2A-1) detected an appropriately-sized band in purified virion but not in the VLP preparation. Non-specific binding was not detected with any antibody preparation when reacted with normal cell culture antigen prepared identically to VLPs. Additionally, MAb (4G2) detected a band corresponding to the M_r of E protein in VLP preparations for all four DENV serotype constructs (data not shown). These results indicated that DENV prM, M, and E structural proteins were secreted into the tissue culture fluid of plasmid-transformed CHO cells, most likely in the form of VLPs.

Determination of minimal amino acid substitutions required for secretion of chimeric DENV-2 VLPs

We previously reported that neither pCBD2-14-6 nor pCB9D2-1J-4-3 constructs, containing the full-length DENV-2 E or chimeric 90% DENV-2 E-10% JEV E protein gene, respectively, secreted VLPs into tissue culture medium whereas secretion was observed for the 80% DENV-2 E-20% JEV E construct, pCB8D2-2J-2-9-1 (Chang et al., 2003). The carboxy-terminal 20% of flavivirus E protein corresponds to the E-H1, CS, E-H2, E-T1, and E-T2 elements, whereas the carboxy-terminal 10% region corresponds to the carboxy-terminus of E-H2 in addition to the E-T1 and E-T2 domains (Fig. 1). We speculated that the sequence located between amino acids E-397 and E-436 of DENV-2 E protein, corresponding to the E-H1 and E-H2 α-helical domains of the stem region, was responsible for the accumulation of DENV-2 E proteins in the perinuclear region of transformed cells and thus

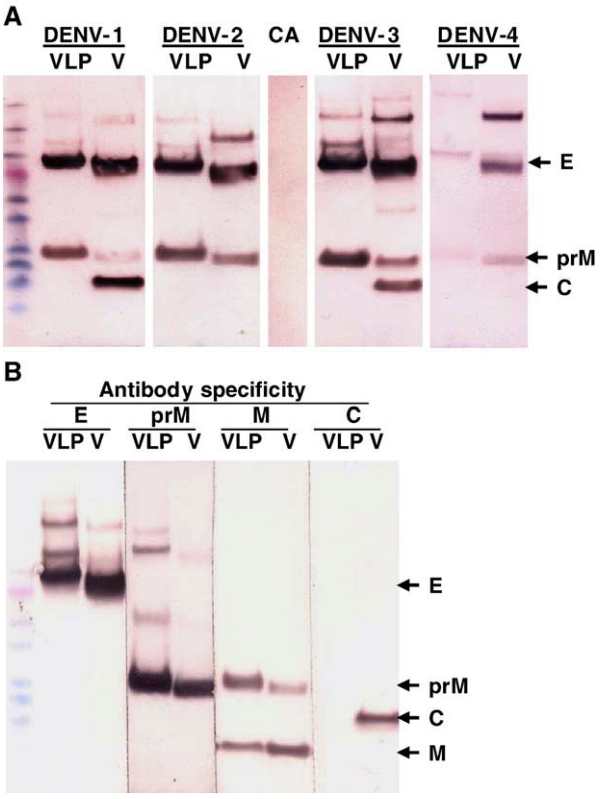

Fig. 2. Characterization of dengue virus (DENV) extracellular virus-like particles (VLPs) and virions. The first lane of each panel contains the SeeBlue Plus2 pre-stained protein standard. Bands corresponding to envelope (E), premembrane (prM), capsid (C), and cleaved membrane (M) structural proteins are indicated with arrows to the right. (A) Reactivity of pelleted VLPs and purified virion particles (V) for each DENV serotype with mouse hyperimmune ascitic fluid for DENV-1 (Hawaii), DENV-2 (New Guinea C), DENV-3 (H-87), and DENV-4 (H-241). Mouse hyperimmune ascitic fluid for DENV-1 (Hawaii) with pelleted normal cell culture antigen from tissue culture fluid (CA) is representative of the lack of reaction for all antibody preparations. (B) Reactivity of DENV-2 VLP and virions with flavivirus group reactive, E-specific MAb (4G2), prM specific MAb (2H2), antipeptide (M protein aa 1–34) serum reacted with DENV-2 prM/M protein, and C specific MAb (1A2A-1).

precluded secretion of VLPs. In order to identify individual amino acids, or combinations thereof, in the stem region that affect this retention of DENV-2 E protein, we substituted by site-directed mutagenesis the 13 amino acids of DENV-2 origin in the pCB9D2-1J-4-3 individually or in pairs with the corresponding JEV sequence (Fig. 3). Most amino acid substitutions did not result in an increase of VLP secretion to detectable levels (data not shown). However, three amino acid substitutions, I398L, M401A, and M412L, in the E-H1 α-helix increased secretion of VLPs to approximately 0.5%, 17%, and 6%, respectively, of titers for that of plasmid pCB8D2-2J-2-9-1 (Fig. 4). Constructs containing the double (I398L-M401A) or triple (I398L-M401A-M412L) substitutions further enhanced VLP secretion compared with those containing a single substitution. The Ag-ELISA titers for double and triple substitutions rose to 25% and 50% of pCBD8D2-2J-2-9-1, respectively. Secondary structure predictions based upon the primary amino acid sequences of DENV-2 and JEV indicated that these three amino acids were located in the hydrophobic face of the amphipathic E-H1 α-helix (Fig. 5).

Localization of DENV structural proteins in CHO cells

Indirect immunofluorescence assay (IFA) analysis of tissue culture cells transformed with DENV expression plasmids revealed two types of fluorescent staining patterns; diffuse low intensity staining with speckling throughout the entire cell or globular high intensity staining localized to perinuclear regions in the cell cytoplasm. The globular pattern was observed in cells transformed with pCBD2-14-6 and pCB9D2-1J-4-3 (Chang et al., 2003, Figs. 6A and C, respectively), as well as with the Q400K and L425I mutants (Figs. 6B and D, respectively), that did not secrete VLPs to detectable Ag-ELISA titers. The diffuse speckled staining pattern was seen in cells transformed with secretion-competent pCB8D2-2J-2-9-1, I398L, M401A, M412L, and triple mutation plasmids. Fig. 6 shows representative cells displaying this fluorescent staining pattern for pCB8D2-2J-2-9-1 and the M401A mutant (Figs. 6E and F, respectively). These results indicated that reduced VLP secretion was most likely due to accumulation of a significant amount of prM/M and/or E proteins in globular clusters within the cell, whereas a more diffuse distribution of proteins that was observed in the cytoplasm of cells that secreted VLPs probably reflected lack of such an accumulation or conversely more secretion.

Discussion

In order to provide a simple method for producing VLPs for all four DENV serotypes, expression plasmids pCB8D1-2J, pCB8D3-2J, and pCBD4 were constructed for the extracellular secretion of DENV-1, -3, and -4 VLPs, respectively, using CHO cells in a method similar to that described previously for DENV-2 (Chang et al., 2003). Indirect immunofluorescence assays using a conformation-dependent MAb (4G2) detected intracellular expression of properly-folded E protein in the cells transformed with expression plasmids for four DENV serotypes. Western blot examination of pelleted VLPs with DENV HIAF revealed protein bands corresponding in size to native virus prM and E proteins for all respective viruses. A panel of antibodies specific to each of the three structural proteins detected the presence of the prM, M (processed prM), and E protein in DENV-2 VLP preparations. These results, in addition to the epitope mapping obtained from previously published results in DENV-2 VLP expression study (Chang et al., 2003) and electron microscope visualization of extracellular recombinant particulate antigen of JEV (Hunt 2001), supported the conclusion that DENV prM and E proteins were expressed in proper conformation in CHO cells, that prM was cleaved to M protein to some extent, and that these structural

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                          395         405         415         425         435         445
                          |           |           |           |           |           |
pCB9D2-1J-4-3             GSSIGQMFET  TMRGAKRMAI  LGDTAWDFGS  LGGVFTSIGK  ALHQVFGGAF  RTLF
S397T                     ..T.......  ..........  ..........  ..........  ..........  ....
I398L                     ...L......  ..........  ..........  ..........  ..........  ....
S397T-I398L               ..TL......  ..........  ..........  ..........  ..........  ....
Q400K                     .....K....  ..........  ..........  ..........  ..........  ....
M401A                     ......A...  ..........  ..........  ..........  ..........  ....
Q400K-M401A               .....KA...  ..........  ..........  ..........  ..........  ....
E403S                     ........S.  ..........  ..........  ..........  ..........  ....
M406L                     ..........  .L........  ..........  ..........  ..........  ....
M406L-R407K               ..........  .LK.......  ..........  ..........  ..........  ....
K410Q                     ..........  .....Q....  ..........  ..........  ..........  ....
M412L                     ..........  .......L..  ..........  ..........  ..........  ....
I414A                     ..........  .........A  ..........  ..........  ..........  ....
L425I                     ..........  ..........  ..........  I.........  ..........  ....
T430N                     ..........  ..........  ..........  .....N....  ..........  ....
L436V                     ..........  ..........  ..........  ..........  .V........  ....
I398L-M401A               ...L..A...  ..........  ..........  ..........  ..........  ....
I398L-M401A-M412L         ...L..A...  .......L..  ..........  ..........  ..........  ....
pCB8D2-2J-2-9-1           ..TL.KA.S.  .LK..Q.L.A  ..........  I....N....  .V........  ....
                                      E-H1                    E-H2
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Fig. 3. Schematic presentation of DENV-2 E protein stem region mutants. Mutations in the E-H1 and E-H2 domains (solid bars; approximately aa 398–415 and 425–447, respectively) of the non-secreting pCB9D2-1J-4-3 construct were made to convert the DENV-2 sequence to the corresponding JEV sequence in order to identify the amino acid(s) affecting secretion of VLPs. Mutant constructs were named based on the amino acid substitution and position. Residues identical to the pCB9D2-1J-4-3 sequence are indicated by dots and the single amino acid code is given for variant residues within each mutant construct. The efficiently-secreting pCB8D2-2J-2-9-1 construct contains the 20% C-terminal JEV E protein replacement.

proteins presumably self-assembled into particle form resembling virion particles.

The VLPs were not efficiently secreted from cells transformed with DENV-4 chimeric plasmid containing replacement of the carboxy-terminal 20% of JEV E protein. The interactions between the prM/M and E protein stem-anchor regions were not involved in improving the efficiency of VLP secretion. Replacement of DENV-4 prM/M stem-anchor region of DENV-2 or JEV in an attempt to re-establish potentially important homologous prM/M-E stem-anchor interactions failed to improve DENV-4 VLP secretion. These results suggest that interactions in addition to those between the prM/M and E stem-anchor regions are important for efficient secretion of VLPs.

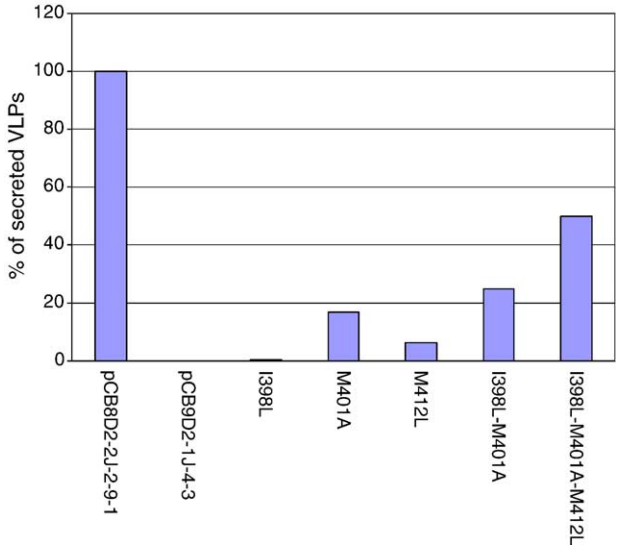

Fig. 4. Effect of E-H1 mutagenesis on secretion of chimeric DENV-2 VLPs. Mutations in the E-H1 domain of pCB9D2-1J-4-3 that resulted in detectable levels of antigen secretion were compared to the secretion of pCB8D2-2J-2-9-1. Relative VLP secretion of each construct, taking that of pCB8D2-2J-2-9-1 as 100%, was calculated for Ag-ELISA endpoint titers from three independent experiments. The Ag-ELISA end-point titers were 64, 0, 4, 16, and 32 for pCB8D2-2J-2-9-1, pCB9D2-1J-4-3, M412L, I398L-M410A, and I398L-M401A-M412L constructs, respectively, in three separate experiments. The Ag-ELISA end-point titers were 0, 0, and 1 for I398L, and were 16, 8, and 8 for M401A constructs.

Previous results demonstrated that replacement of the carboxy-terminal 10% of DENV-2 E protein, containing the carboxy-terminus of E-H2 in addition to the E-T1 and E-T2 domains, with that of JEV sequence in the pCB9D2-1J-4-3 plasmid was unable to secrete detectable levels of VLPs (Chang et al., 2003). Substitution of three DENV-2 amino acids in this plasmid with the corresponding JEV sequence in α-helix E-H1 resulted in the secretion of DENV-2 VLPs. These results strongly implicate that the E-H1 domain, particularly amino acids 398, 401, and 412, is involved in intracellular retention/secretion of E protein, presumably at the endoplasmic reticulum membrane of transformed cells. The mechanism for this retention is unclear. However, epitope mapping using a panel of MAbs did not detect any significant differences in the epitopes expressed by the intracellular E protein (Chang et al., 2003), ruling out the possibility that retention is caused by misfolding of the ectodomain of E. Without concurrent expression of viral nucleocapsid to provide similar interactions during virion assembly, the DENV-2 residues at I398, M401, and M412, under this expression condition, may influence the proper intra- or intermolecular interactions necessary for the assembly and transport of prM- and E-containing VLPs through the cell secretory machinery and hence for efficient VLP secretion.

I398, M401, and M412 residues are distributed along the hydrophobic face of the E-H1 α-helix that is partially buried in the outer leaflet of the lipid membrane (Zhang et al., 2003). An increase in the hydrophobicity of the residues at

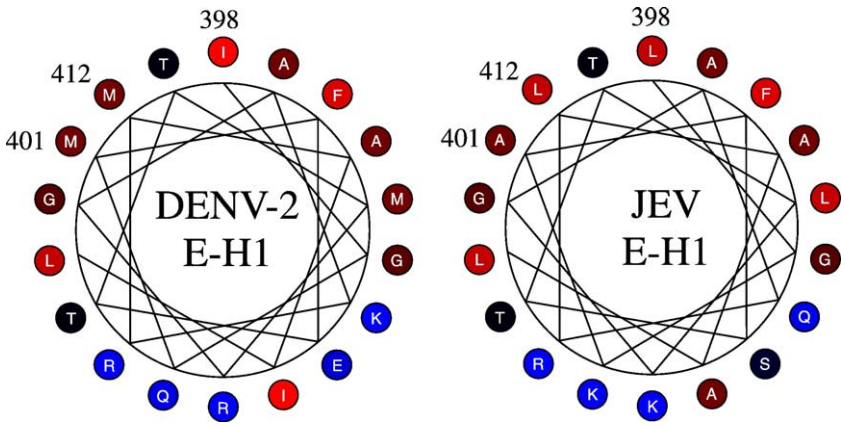

Fig. 5. Comparison of DENV-2 and JEV E-H1 secondary structure arrangements. Helical wheel diagrams representing the E-H1 domains (approximately aa 398-415) for DENV-2 and JEV were constructed using the primary amino acid sequences of pCB9D2-1J-4-3 and pCB8D2-2J-2-9-1, respectively. The strong amphipathic nature of these α-helices is apparent in the segregation of strongly or moderately hydrophobic (red and brown, respectively) and polar or charged (black and blue, respectively) amino acids to distinctly separate faces of the helix. Mutations I398L, M401A, and M412L are localized to a similar region of the hydrophobic face of the helix and are approximately aligned with each other along the hydrophobic face.

positions 401 and 412 (M→A and M→L, respectively) is correlated with a significant increase in DENV-2 VLP secretion, possibly as a result of proper burial of these hydrophobic residues within the lipid membrane. Additionally, shortening of the R-group side-chain length in substitution M401A, and to a lesser extent in M412L, may have increased the flexibility of the E-H1 α-helix with regard to the pitch of the helix with the surface of the membrane, thus allowing a wider range of flexibility of amino acids on the hydrophilic face to interact with ectodomain of E protein. In either case, alterations in the orientation of the hydrophobic face would consequently affect the orientation of the entire helix, including the hydrophilic face of the α-helix. Polar residues on this face may require precise orientation to interact with the underside of the E protein ectodomain domains in E or even prM/M proteins; the two E-H1 domains in E-E homodimers are each located near the E-dimer "hole" region where prM/M is proposed to be located (Zhang et al., 2003). Several polar and charged residues on this α-helix are potentially available for intra- or intermolecular interactions. Additionally, the E-H1 polar face includes a single hydrophobic residue at position 415 that may influence protein interactions by necessitating sequestration from solvent exposure through burial in a hydrophobic pocket. Optimal interactions formed within these regions through modification of the E-H1 domain might in turn influence the curving and bending of the lipid membrane, thereby resulting in the assembly and release of VLPs (Garoff et al., 1998). Similar interactions between the unmodified E-H1 and other domains in the prM and E, resulting in the curving and bending of the lipid membrane, could be provided by the interactions between concurrently expressed nucleocapsid and prM-E during virion assembly in natural viral infection.

As observed previously for DENV-2 and SLEV expression plasmids (Chang et al., 2003; Purdy et al., 2004), VLPs were only secreted to high Ag-ELISA titers after replacement of the carboxy-terminal 20% of the DENV-1 E protein with the corresponding region of JEV. DENV-3 VLPs were secreted equally well with or without the replacement, whereas this replacement actually reduced the secretion of DENV-4 VLPs 8-fold as compared to the full-length DENV-4 E protein construct. The precise interactions within this stem-anchor region of E protein that affect intermolecular prM-E and E-E interactions and efficient secretion of VLPs in this expression system may vary between flaviviruses and remain to be conclusively defined. It was apparent that amino acids 398, 401, and 412 played a significant role in the secretion of DENV-2 VLPs; however, constructs containing all three JEV substitutions only secreted VLPs to 50% the levels of the construct with the complete E-H1 JEV sequence. These three residues are highly conserved between the DENV group, yet substitution of these residues with the replacement of the entire carboxy-terminal 20% of E protein affected secretion to varying degrees for each of the four serotypes. These three residues influenced VLP secretion in DENV-2, and presumably DENV-1 constructs but not in DENV-3 or -4 constructs.

Elucidation of the structural features determining assembly, maturation, secretion, and membrane fusion of flaviviruses is applicable to antiflaviviral drug development. Identification of a DENV-2 E protein hydrophobic pocket, proposed to be a hinge point for conformational changes during membrane fusion, suggested that insertion of small compounds into this pocket might inhibit the fusion transition process and thus inhibit viral infection (Modis et al., 2003). Modis et al. also suggested that peptides derived from the stem sequences could interact with elements in E protein clusters formed during transition to fusion stage, thereby inhibiting the completion of membrane fusion (Modis et al., 2004). We identified here three amino acids in stem helix E-H1 that are involved in VLP secretion; thus,

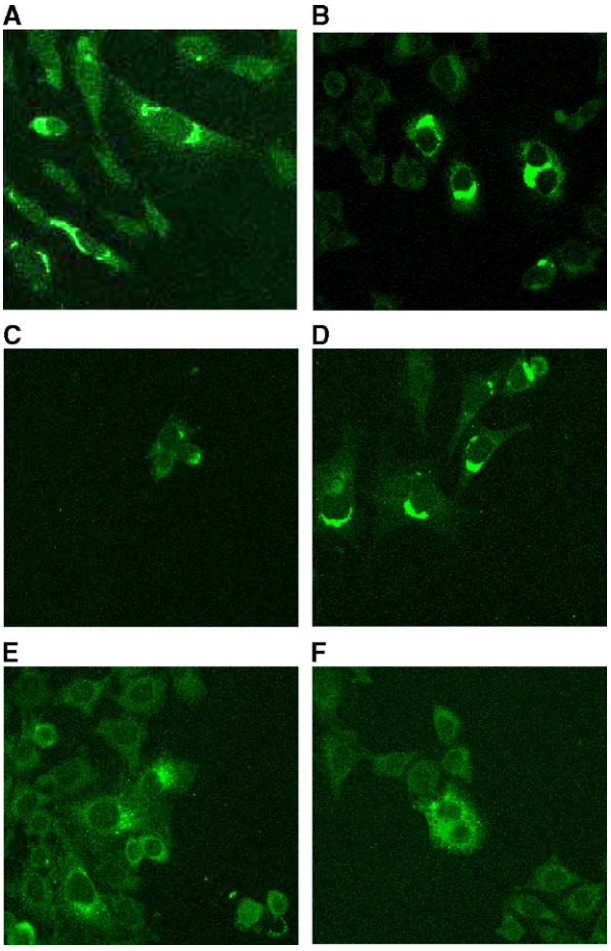

Fig. 6. Comparison of immunofluorescence staining morphologies for secreting and non-secreting plasmid-transformed CHO cells. Rabbit sera containing antibodies against DENV-2 prM, M, and E proteins was used to detect the expression and localization of viral structural proteins in CHO cells transformed with expression plasmids. This rabbit serum was specific for DENV proteins and did not react with normal CHO cell control (data not showed). High-intensity globular expression patterns localized to perinuclear regions in the cytoplasm were representative of retention of structural proteins observed for pCBD2-14-6 (A), pCB9D2-1J-4-3 (C), and for the majority of amino acid substitutions in the E-H1 and E-H2 stem region, represented here by the Q400K (B) and L425I (D) mutant constructs. A low-intensity diffuse staining pattern with specks of concentrated structural proteins was observed throughout the cytoplasm of cells transformed with pCB8D2-2J-2-9-1 (E) and other secretion-competent mutants, represented here by the M401A (F) mutant construct.

the E-H1 peptide might represent a potential target for antiviral drug development.

The transformation of tissue culture cells with plasmid DNA is advantageous for antigen production since these cells secrete viral prM and E proteins in VLPs having proper conformation and presentation of neutralizing-antibody epitopes similar to those of virion particles (Chang et al., 2000, 2003; Davis et al., 2001; Hunt et al., 2001; Konishi and Fujii, 2002; Kroeger and McMinn, 2002; Schalich et al., 1996). These VLP antigens are excellent alternatives to virus-infected suckling mouse brain or tissue culture-derived antigens for use in serodiagnostic assays. In detecting antiflaviviral antibodies in human serum, ELISAs employing WNV, JEV, and SLEV VLP antigens have comparable sensitivities and specificities to those using suckling mouse brain-derived antigens (Davis et al., 2001; Hunt et al., 2001; Purdy et al., 2004). We have obtained similar results in detecting IgM antibody of DENV patient serum panels with the VLP antigens described here (D.A. Holmes, D.E. Purdy, A.J. Noga, and G.J. Chang, submitted for publication).

Expression plasmids encoding prM and E proteins of flaviviruses have excellent potential as DNA vaccine candidates. Plasmids for JEV, WNV, and DENV-2 that secrete VLPs efficiently in tissue culture also elicit neutralizing antibodies and provide protection against virus challenge in mice (Chang et al., 2000, 2003; Davis et al., 2001). Efficient secretion of VLPs in tissue culture might be indicative of efficient VLP production in DNA-vaccinated hosts. Thus, it is considered an important prerequisite for the development of an adequate immune response with neutralizing antibodies to protect vaccinated hosts against virus challenge (Aberle et al., 1999; Chang et al., 2003). In addition to the DENV-2 plasmid, pCB8D2-2J-2-9-1, constructed in a previous study, our current results indicate that expression plasmids pCB8D1-2J, pCB8D3-2J, and pCBD4 express DENV-1, -3, and -4 VLPs efficiently, respectively, thus these four DENV serotypes plasmids could be excellent DNA vaccine candidates for eliciting neutralizing antibodies. We are currently evaluating the immunological responses of these vaccine candidates singly or in combination in mice.

Materials and methods

Cell culture and virus strains

Chinese hamster ovary (CHO-K1) cells (CCL-61; ATCC, Manassas, VA) were grown in Dulbecco's modified Eagle medium (D-MEM)/F12 (Gibco Laboratories, Grand Island, NY) and C6/36 mosquito cells were grown in D-MEM (Gibco Laboratories). All media were supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Inc., Logan, UT), 1 mM sodium pyruvate, 1 mM sodium glutamate, 0.1 mM MEM nonessential amino acids, penicillin (100 U/ml), and 100 μg/ml streptomycin. CHO cells were incubated at 37 °C with 5% CO_2 and *Aedes albopictus*-derived C6/36 cells were maintained at 28 °C without CO_2.

The DENV-1, DENV-3, and DENV-4 strains listed in Table 1 were propagated in C6/36 mosquito cell culture and used for RNA extraction, reverse transcriptase-PCR (RT-PCR), cDNA cloning, and protein analysis. For use in the Western blot, virus was purified by precipitation with 7% PEG-8000 (Fisher Scientific, Fair Lawn, NJ), followed by ultracentrifugation on 30% glycerol–45% potassium tartrate gradients (Obijeski et al., 1976).

Construction of expression plasmids and mutagenesis

Expression plasmids were constructed for each of three DENV serotypes (DENV-1, -3, and -4) by inserting a gene cassette containing the respective DENV serotype full-length prM/M and chimeric E protein gene, consisting of the amino-terminal 80% region of DENV E protein gene and the carboxy-terminal 20% region of JEV E protein gene, into pCBJESS-*Afe*I vector. This pCBJESS-*Afe*I vector was modified from the pCBJESS plasmid (Davis et al., 2001) by introducing a unique *Afe*I cloning site at the carboxy-terminus of the signal peptide sequence for the convenience of cloning as well as for improvement of the cleavage potential of the original JEV-encoded signal peptide sequence (Chang et al., 2000; Davis et al., 2001). The predicted signal peptide probability was significantly improved by altering the cleavage region of the JEV signal peptide sequence at -4 (C) and -2 (G) positions to G and S, respectively, in the pCBJESS-*AfeI* plasmid (Chang et al., 2001).

Briefly, the QIAamp Viral RNA kit (QIAGEN Inc., Valencia, CA) was used according to the manufacturer's recommended procedure to extract genomic RNA from 140 μl of virus-infected C6/36 cell-culture supernatant. Extracted RNA was used as template for production of cDNA encoding the DENV prM/M and amino-terminal 80% E protein gene by QIAGEN OneStep RT-PCR kit (QIAGEN Inc.). Primer sequences containing the appropriate restriction enzyme sites for cloning (Table 2) were designed based on the virus sequences (G.J. Chang, unpublished data). The pCDJE2-7 plasmid (Chang et al., 2000) was used as the PCR template to amplify the JEV carboxy-terminal 20% E protein cDNA sequence using primers J-*Stu*I (5′GGAATT***AGGCCT***GGAAGCACGCTG GGCAAGG) and cSP6 (5′GCGAGCTCTAGCATTTAGG-TGACACTATAG). The PCR products for the DENV prM/M-80% E and the JEV 20% E protein-encoding sequences were digested with *Stu*I and ligated together. Ligation products were then PCR amplified with primers T7 and cSP6. These amplicons were sequentially digested with *Afe*I and *Not*I and then inserted into the *Afe*I and *Not*I sites of the pCBJESS-*Afe*I plasmid to create pCB8D1-2J, pCB8D3-2J, and pCB8D4-2J for use in transient expression of chimeric VLPs of DENV-1, -3, and -4, respectively. Plasmid constructs were designated according to the percent of E protein-encoding sequence derived from the DENV or JEV sequence. For example, the pCB8D1-2J expression plasmid contains the 100% DENV-1 prM and a recombinant E protein-encoding region consisting of the amino-terminal 80% E sequence of DENV-1, designated 8D1, and the carboxy-terminal 20% E sequence of JEV, designated as -2J.

Table 2
Oligonucleotides used for cloning of 100% and 80% DENV prM-E gene regions

Construct and primer	Oligonucleotide sequence[a]
pCB8D1-2J	
D1E-*Afe*I	5′ CTTGCT***AGCGCT***TTCCATCTGACCACCCGAGGGGGAGA
cD1E-*Stu*I	5′ TTGAAA***AGGCCT***TGCCCAGCGTGCTTCCTTTCTTGAACCAGCTTAGT
pCBD1	
D1-*Afe*I-405	5′ CTGGCG***AGCGCT***TTCCATTTGACCACACGAGGGG
cD1-*Not*I-2387	5′ CGAGTC***GCGGCCGC***CGCTTGAACCATGACTCCTAGGTACAGTGTG
pCB8D3-2J	
D3E-*Afe*I	5′ CTTGCT***AGCGCT***TTCCACTTGACTTCACGAGATGGAGA
cD3E-*Stu*I	5′ TTGAAA***AGGCCT***TGCCCAGCGTGCTTCCCTTCTTGTACCAGTT
pCBD3	
D3-*Afe*I-405	5′ CTTGCT***AGCGCT***TTCCACTTGACTTCACGAGATGGAGAG
cD3-*Not*I-2387	5′ CATGTC***GCGGCCGC***AGCTTGCACCACGGCTCCCAG
pCB8D4-2J	
D4E-*Afe*I	5′ CTTGCT***AGCGCT***TTCACTTGTCAACAAGAGATGGC
cD4E-*Stu*I	5′ TTGAAA***AGGCCT***TGCCCAGCGTACTCCCTTTCCTGAACCAATGGAGTGT
pCBD4	
D4-438	5′ GCTTTTTCCTTGTCAACGAGAGAT
cD4-*Not*I-2402	5′ CATGTC***GCGGCCGC***TTATGCATGAACTGTGAAACC
pCBD2prM8D4-2J, pCBJprM8D4-2J, or pCBD2M8D4-2J	
D4-*Kpn*I	5′ TAACTT***GGTACC***GCCGCCGCCATGGGCAAG
cD4-*Ear*I	5′ CTCCTT***CTCTTCC***TGATGACATCCATGTTT
D2-*Ear*I	5′ GATGTC***CTCTTCA***TCAGAAGGGGCCTGGAA
cD2-*Bsa*I	5′ ATTCCT***GGTCTCA***CGCAACGCATTGTCATT
J-*Ear*I	5′ GCTGGA***CTCTTCT***TCAACGAAAGCCACACG
cJ-*Bsa*I	5′ ATTCCC***GGTCTCA***CGCAACGCATACTGTAAGCCGGAGCGACCAAC
D4-*Bsa*I	5′ AATGCG***GGTCTCA***TGCGTAGGAGTGGGGAA
cD4-1151	5′ TCCGGACCTAGGTTCACAATCAAGTGTTAA

[a] Restriction endonuclease sites are indicated by bold, italicized, and underlined font.

The expression plasmids pCBD1, pCBD3, and pCBD4 contain the full-length prM/M and E protein-encoding regions of DENV-1, -3, and -4, respectively. These plasmids were amplified by RT-PCR using the appropriate primers (Table 2) in a similar manner as described above and were cloned directly into *Afe*I–*Not*I-digested pCBJESS-*Afe*I vector.

For replacement of the carboxy-terminal stem-anchor region of DENV-4 prM with the corresponding region of JEV or DENV-2, primers (Table 2) were used to PCR amplify the appropriate regions of pCB8D4-2J, pCDJE2-7, and pCB8D2-2J-2-9-1. These PCR amplicons were digested with *Ear*I and/or *Bsa*I, ligated at a molar ratio of 1:1:1, and cloned into the pCBJESS-*Afe*I vector as described above to create pCBD2prM8D4-2J and pCBJprM8D4-2J which contained the stem-anchor region of prM of DENV-2 and JEV, respectively. Additionally, three additional amino acids of DENV-4 origin in the pCBD2prM8D4-2J were converted to the corresponding DENV-2 sequence (T5V, S8V, and A16T) to generate the pCBD2M8D4-2J, using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), as per the manufacturer's recommended procedure. The primers used for construction of pCBD2M8D4-2J are available upon request.

Thirteen amino acids in the E-H1 and E-H2 amphipathic α-helices region of E in pCB9D2-1J-4-3 differ from the homologous region in pCB8D-2J-2-9-1 (Figs. 1 and 3, Chang et al., 2003). Thus, these amino acids were converted individually or in pairs from the DENV-2 sequence to the corresponding JEV sequence, respectively, using the QuikChange site-directed mutagenesis kit. Primers used in these reactions are also available upon request.

Automated DNA sequencing was performed on CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA) according to the manufacturer's recommended procedure. The chimeric plasmids that had the correct sequence were identified and used for expression studies.

Electroporation of tissue culture cells with plasmid DNA

For transformation, tissue culture cells were grown to 90–100% confluence in 150-cm^2 culture flasks, trypsinized, and resuspended in ice-cold phosphate-buffered saline (PBS) to a final density of 1.5×10^7 cells/ml. For each reaction, 0.5 ml of this cell suspension was electroporated with 30 μg of plasmid DNA in a 0.4 cm electrode gap cuvette using a Bio-Rad Gene Pulser II (Bio-Rad Laboratories, Hercules, CA) set at 250 V and 975 μF. Cells from each electroporation reaction were seeded onto a single 75-cm^2 culture flask containing 20 ml of growth medium. Each plasmid for expression study was tested in triplicate. Tissue-culture medium was harvested 48 h following electroporation and clarified by centrifugation at 10,000 rpm for 10 min at 4 °C. The Ag-ELISA was performed using flavivirus E-specific, group-reactive monoclonal antibodies (MAb) 4G2 (Gentry et al., 1982) and horseradish peroxidase (HRP)-conjugated 6B6C-1 (Roehrig et al., 1983; Jackson Immunological Laboratories, Inc., West Grove, PA.) to capture and detect secreted VLPs, respectively, as previously described (Hunt et al., 2001). Clarified culture medium from each electroporation reaction was tested by Ag-ELISA in duplicate.

Western blot analysis

The DENV VLPs were concentrated and partially purified from clarified tissue culture medium by ultracentrifugation at 19,000 rpm for 8–16 h in a Beckman Coulter Type 19 rotor (Beckman Coulter) at 4 °C. The pellet was resuspended in TN buffer (50 mM Tris, 100 mM NaCl, pH 7.5) to 1/50th the original volume, aliquoted into 1-ml samples, and stored at −70 °C. DENV VLPs and gradient purified virions were electrophoresed under non-denaturing conditions on a NuPAGE 4–12% Bis-Tris gradient gel in an XCell *SureLock* Mini-Cell electrophoresis apparatus (Invitrogen Corp., Carlsbad, CA), followed by electroblotting onto nitrocellulose membranes using an XCell II Blot Module (Invitrogen Corp.). DENV proteins were detected by Western blot using mouse hyperimmune ascitic fluids (HIAF) against DENV-1 (Hawaii), DENV-2 (New Guinea C), DENV-3 (H-87), and DENV-4 (H-241). All DENV-2 structural proteins were individually identified using flavivirus group-reactive MAb (4G2) specific for E protein, MAb (2H2) specific for DENV-2 prM protein, antipeptide mouse serum against DENV-2 M protein peptide (M protein aa 1–34), and MAb (1A2A-1) specific for C protein.

Indirect immunofluorescence antibody assay

Transiently transformed CHO cells were seeded at 100 μl/well onto 96-well plates immediately following electroporation and incubated 24 h. Adherent cells were washed once with PBS and fixed with 3:1 acetone/PBS (−20 °C) for 10 min. Fixed cells were washed twice with PBS, air dried completely ~15–30 min, and then stored at −20 °C in a frost-free freezer until examination. MAb 4G2, diluted 1:200 in PBS, was added at 100 μl/well and incubated at 37 °C in a humidified chamber for 30 min. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories) was diluted 1:100 in PBS-Evans Blue (0.005% as counter-stain, Sigma-Aldrich, St. Louis, MO), applied at 100 μl/well, and incubated at 37 °C for 30 min. Wells were washed twice with PBS, and 3 drops of mounting media from the Prolong Antifade Kit (Molecular Probes, Eugene, OR) was added. Fluorescent cells were examined using Zeiss KS 300 Imaging System (Carl Zeiss, Inc., Thornwood, NY), and E

protein localization pattern and fluorescent intensity were recorded.

Alternatively, for preparation of microscopic slides used in protein localization analysis, cell suspensions from one 75-cm^2 culture flask of electroporated cell culture containing 20 ml growth medium were diluted 1:4 in growth medium, 1 ml was added to each well of a Lab-Tek II Chamber slide (Nalge Nunc International, Naperville, IL), and slides were incubated 48 h until cells reached ~50–75% confluence. The tissue culture fluid was then removed, cells were washed once with PBS and fixed in 3:1 acetone/PBS as above. Slides were air dried completely ~15–30 min then stored at −20 °C in a frost-free freezer until examination. Rabbit serum against DENV-2 prM/M and E proteins was obtained from rabbits vaccinated with pCB8D2-2J-2-9-1 (G. J. Chang, unpublished data). Three hundred microliters of diluted rabbit serum (1:200 in StartingBlock (PBS) Blocking Buffer; Pierce, Rockford, IL) was used to stain each chamber well at RT for 1 h. Slides were washed three times with 1 ml PBS. FITC-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories) was diluted 1:100 in StartingBlock (PBS) Blocking Buffer and 400 μl was added per well and incubated at RT for 1 h in the dark to prevent loss of signal. Wells were washed 3 times with 1 ml PBS, the excess was drained, the well walls were removed, and slides were briefly blotted dry. Cover glass was affixed to slides with minimal volumes (~1 drop) of mounting media from the Prolong Antifade Kit (Molecular Probes).

Analysis of primary amino acid sequences

Helical wheel diagrams were constructed using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Secondary structure prediction using PSI-BLAST PSSM matrices was achieved with the YASPIN program using the DSSP-trained neural network (http://ibivu.cs.vu.nl/programs/yaspinwww/).

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References

Aberle, J.H., Aberle, S.W., Allison, S.L., Stiasny, K., Ecker, M., Mandl, C.W., Berger, R., Heinz, F.X., 1999. A DNA immunization model study with constructs expressing the tick-borne encephalitis virus envelope protein E in different physical forms. J. Immunol. 163, 6756–6761.

Allison, S.L., Stiasny, K., Stadler, K., Mandl, C.W., Heinz, F.X., 1999. Mapping of functional elements in the stem-anchor region of tick-borne encephalitis virus envelope protein E. J. Virol. 73, 5605–5612.

Chang, G.J., Hunt, A.R., Davis, B.S., 2000. A single intramuscular injection of recombinant plasmid DNA induces protective immunity and prevents Japanese encephalitis in mice. J. Virol. 74, 4244–4252.

Chang, G.J., Davis, B.S., Hunt, A.R., Holmes, D.A., Kuno, G., 2001. Flavivirus DNA vaccines: current status and potential. Ann. N. Y. Acad. Sci. 951, 272–285.

Chang, G.J., Hunt, A.R., Holmes, D.A., Springfield, T., Chiueh, T.S., Roehrig, J.T., Gubler, D.J., 2003. Enhancing biosynthesis and secretion of premembrane and envelope proteins by the chimeric plasmid of dengue virus type 2 and Japanese encephalitis virus. Virology 306, 170–180.

Davis, B.S., Chang, G.J., Cropp, B., Roehrig, J.T., Martin, D.A., Mitchell, C.J., Bowen, R., Bunning, M.L., 2001. West Nile virus recombinant DNA vaccine protects mouse and horse from virus challenge and expresses in vitro a noninfectious recombinant antigen that can be used in enzyme-linked immunosorbent assays. J. Virol. 75, 4040–4047.

Garoff, H., Hewson, R., Opstelten, D.J., 1998. Virus maturation by budding. Microbiol. Mol. Biol. Rev. 62, 1171–1190.

Gentry, M.K., Henchal, E.A., McCown, J.M., Brandt, W.E., Dalrymple, J.M., 1982. Identification of distinct antigenic determinants on dengue-2 virus using monoclonal antibodies. Am. J. Trop. Med. Hyg. 31, 548–555.

Heinz, F.X., Stiasny, K., Puschner-Auer, G., Holzmann, H., Allison, S.L., Mandl, C.W., Kunz, C., 1994. Structural changes and functional control of the tick-borne encephalitis virus glycoprotein E by the heterodimeric association with protein prM. Virology 198, 109–117.

Hunt, A.R., Cropp, C.B., Chang, G.J., 2001. A recombinant particulate antigen of Japanese encephalitis virus produced in stably-transformed cells is an effective noninfectious antigen and subunit immunogen. J. Virol. Methods 97, 133–149.

Konishi, E., Fujii, A., 2002. Dengue type 2 virus subviral extracellular particles produced by a stably transfected mammalian cell line and their evaluation for a subunit vaccine. Vaccine 20, 1058–1067.

Kroeger, M.A., McMinn, P.C., 2002. Murray Valley encephalitis virus recombinant subviral particles protect mice from lethal challenge with virulent wild-type virus. Arch. Virol. 147, 1155–1172.

Modis, Y., Ogata, S., Clements, D., Harrison, S.C., 2003. A ligand-binding pocket in the dengue virus envelope glycoprotein. Proc. Natl. Acad. Sci. U.S.A. 100, 6986–6991.

Modis, Y., Ogata, S., Clements, D., Harrison, S.C., 2004. Structure of the dengue virus envelope protein after membrane fusion. Nature 427, 313–319.

Obijeski, J.F., Bishop, D.H., Palmer, E.L., Murphy, F.A., 1976. Segmented genome and nucleocapsid of La Crosse virus. J. Virol. 20, 664–675.

Op De Beeck, A., Molenkamp, R., Caron, M., Ben Younes, A., Bredenbeek, P., Dubuisson, J., 2003. Role of the transmembrane domains of prM and E proteins in the formation of yellow fever virus envelope. J. Virol. 77, 813–820.

Purdy, D.E., Noga, A.J., Chang, G.J., 2004. A noninfectious recombinant antigen for ELISA detection of serum antibodies to St. Louis encephalitis virus. J. Clin. Microbiol. 42, 4709–4717.

Roehrig, J.T., Mathews, J.H., Trent, D.W., 1983. Identification of epitopes on the E glycoprotein of Saint Louis encephalitis virus using monoclonal antibodies. Virology 128, 118–126.

Russell, P.K., Brandt, W.E., Dalrymple, J.M., 1980. Chemical and antigenic structure of flaviviruses. In: Schlesinger, R.W. (Ed.), The Togaviruses. Academic Press, New York, pp. 503–529.

Schalich, J., Allison, S.L., Stiasny, K., Mandl, C.W., Kunz, C., Heinz, F.X., 1996. Recombinant subviral particles from tick-borne encephalitis virus are fusogenic and provide a model system for studying flavivirus envelope glycoprotein functions. J. Virol. 70, 4549–4557.

Stadler, K., Allison, S.L., Schalich, J., Heinz, F.X., 1997. Proteolytic activation of tick-borne encephalitis virus by furin. J. Virol. 71, 8475–8481.

Stiasny, K., Allison, S.L., Marchler-Bauer, A., Kunz, C., Heinz, F.X., 1996. Structural requirements for low-pH-induced rearrangements in the envelope glycoprotein of tick-borne encephalitis virus. J. Virol. 70, 8142–8147.

Stocks, C.E., Lobigs, M., 1998. Signal peptidase cleavage at the flavivirus C-prM junction: dependence on the viral NS2B-3 protease for efficient processing requires determinants in C, the signal peptide, and prM. J. Virol. 72, 2141–2149.

Zhang, W., Chipman, P.R., Corver, J., Johnson, P.R., Zhang, Y., Mukhopadhyay, S., Baker, T.S., Strauss, J.H., Rossmann, M.G., Kuhn, R.J., 2003. Visualization of membrane protein domains by cryo-electron microscopy of dengue virus. Nat. Struct. Biol. 10, 907–912.