

A conserved region in the prM protein is a critical determinant in the assembly of flavivirus particles

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Flaviviruses are assembled to bud into the lumen of the endoplasmic reticulum (ER) and are secreted through the vesicle transport pathway, but the details of the molecular mechanism of virion assembly remain largely unknown. In this study, a highly conserved region in the prM protein was identified among flaviviruses. In the subviral particle (SP) system of tick-borne encephalitis virus (TBEV) and Japanese encephalitis virus, secretion of SPs was impaired by a mutation in the conserved region in the prM protein. Viral proteins were sparse in the Golgi complex and accumulated in the ER. Ultrastructural analysis revealed that long filamentous structures, rather than spherical SPs, were observed in the lumen of the ER as a result of the mutation. The production of infectious virions derived from infectious cDNA of TBEV was also reduced by mutations in the conserved region. Molecular modelling analysis suggested that the conserved region is important for the association of prM–envelope protein heterodimers in the formation of a spike of immature virion. These results are the first demonstration that the conserved region in the prM protein is a molecular determinant for the flavivirus assembly process.

INTRODUCTION

Enveloped viruses bud through cellular membranes, such as the plasma membrane, the membrane of the endoplasmic reticulum (ER) and the Golgi complex. Flaviviruses are generally thought to bud into the ER of virus-infected cells (Lindenbach *et al.*, 2007). Individual particles are subsequently transported to the Golgi complex in transport vesicle and released by exocytosis via the *trans*-Golgi network (Mackenzie & Westaway, 2001). However, intermediate budding structures have not been observed and the details of the mechanism of assembly and secretion are still largely unknown.

Flaviviruses, belonging to the family *Flaviviridae*, include many clinically important human pathogens, such as dengue (1–4) serotype viruses, West Nile virus, Japanese encephalitis virus (JEV), yellow fever virus and tick-borne encephalitis virus (TBEV) (Lindenbach *et al.*, 2007). The flavivirus genome consists of a positive-polarity ssRNA of approximately 11 kb, which encodes three structural proteins: the core (C), pre-membrane (prM)/membrane (M) and envelope (E) proteins. It also encodes seven non-structural (NS)

proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5, within a single long ORF (Chambers *et al.*, 1990). The ORF is translated into a large polyprotein, which is co- or post-translationally cleaved by cellular and viral proteases.

Flavivirus virions are 40–50 nm in diameter, spherical in shape and contain a nucleocapsid and an envelope. The envelope has two viral proteins: the major envelope protein E and the small membrane protein prM/M. Cryo-electron microscopy analysis of the immature dengue virus revealed 60 spikes, each comprising three prM/E heterodimers, that are organized icosahedrally on the surface of the particles (Zhang *et al.*, 2003), in contrast to the smooth surface of mature virions reported previously (Kuhn *et al.*, 2002).

The M protein is synthesized as a precursor protein, prM, in the ER and contains one to three *N*-linked glycosylation sites (Chambers *et al.*, 1990). Newly synthesized E and prM proteins associate to form heterodimers that are incorporated into immature virions (Allison *et al.*, 1995b; Wengler & Wengler, 1989). One of the major functions of the prM protein is a chaperone-like activity aiding folding and maturation of E proteins (Konishi & Mason, 1993; Lorenz *et al.*, 2002). During transport of the virions

A supplementary figure and table are available with the online version of this paper.

through the ER and Golgi complex to the cell surface, the prM proteins of the heterodimers protect the E proteins from premature fusion by the low-pH conditions in the transport vesicles (Guirakhoo *et al.*, 1991, 1992; Heinz *et al.*, 1994; Zhang *et al.*, 2003). Prior to release from the cell, the cellular furin protease cleaves the pr portion from prM, resulting in rearrangement of the E proteins into head-to-tail homodimers on the surface of the mature virus particles (Elshuber *et al.*, 2003; Stadler *et al.*, 1997). Other roles of the prM protein are not well understood.

In flaviviruses, it has been demonstrated that subviral particles (SPs) are assembled and secreted from cells expressing the viral prM and E genes (Allison *et al.*, 1995b; Fonseca *et al.*, 1994; Mason *et al.*, 1991; Op De Beeck *et al.*, 2003; Yamshchikov & Compans, 1993). These SPs are similar to the slowly sedimenting haemagglutinin particles that are released from flavivirus-infected cells in addition to infectious virions (Gritsun *et al.*, 1989; Heinz & Kunz, 1977). SPs contain viral E proteins but lack the nucleocapsid protein and viral RNA. Because the characteristics of the envelope proteins in SPs are structurally and functionally similar to those of authentic virions, SPs have been used in research of the viral E proteins (Allison *et al.*, 1995a, 1999, 2001; Lorenz *et al.*, 2003).

Despite recent advances in our understanding of flaviviruses, the functional residues of the prM ectodomain that are important for assembly and secretion of the virions remain almost unknown. In our previous study, a single point mutation in the prM protein was found to reduce the secretion of TBEV particles, suggesting that prM plays a crucial role in the virus budding process (Yoshii *et al.*, 2004). In the current study, a highly conserved region in the prM protein was identified among flaviviruses. Several amino acid substitutions were introduced into this conserved region by site-directed mutagenesis in the SP systems of TBEV and JEV to examine their effects on the

assembly and secretion of SPs. Some of these mutations were then introduced into an infectious cDNA clone of TBEV to confirm the role of the conserved region in the virus replication cycle.

RESULTS

Effect of point mutations in the conserved region of prM proteins on SP secretion

From a multiple-sequence analysis of the flavivirus prM protein, a highly conserved region was identified among flaviviruses (analysis of >4000 strains using Pfam: <http://pfam.sanger.ac.uk/>; Finn *et al.*, 2010). Table 1 shows the sequence of the conserved region among representative flaviviruses. To investigate the importance of this region in virus replication, alanine substitutions were engineered in this region of the pCAG-TBEME plasmid, which expresses the prM and E proteins of TBEV (Table 2). Alanine substitutions were also engineered at cysteines 68 and 70, but no expression of viral proteins was observed in cells transfected with these plasmids (data not shown). This may have been due to a critical conformational change in the prM protein, as both cysteine residues stabilize the prM structure by disulfide bonds (Li *et al.*, 2008).

pCAG-TBEME plasmids containing the different mutations were transfected into 293T cells. After incubation for 24 h, extracellular E proteins were detected by ELISA. As presented in Fig. 1(a), the secretion of E protein was reduced considerably by prM mutations at proline-63, aspartate-65, valine-66, aspartate-67 and phenylalanine-69. An alanine substitution of glutamate-62 did not affect the kinetics of the E protein. Interestingly, the conservative valine-to-alanine substitution at residue 66 reduced the secretion of the E protein. At position 66, a branched-chain amino acid (valine, isoleucine or leucine) is conserved, and a

Table 1. A conserved sequence in flavivirus prM protein

The non-conserved amino acid in the sequence is shown in non-bold.

Virus*	GenBank accession no.	Amino acid sequence									Amino acid range†
TBEV	AB062063	E	P	V	D	V	D	C	F	C	62–70
POWV	NC_003687	E	P	V	D	V	D	C	F	C	62–70
LANV	AF253419	E	P	V	D	V	D	C	F	C	58–66
JEV	AF069076	D	P	E	D	V	D	C	W	C	61–69
WNV	AB185914	D	P	E	D	I	D	C	W	C	61–69
DENV	U87411	E	P	E	D	I	D	C	W	C	60–68
YFV	X03700	E	P	D	D	I	D	C	W	C	57–65
APOIV	NC_003676	E	P	V	D	L	D	C	F	C	57–65
Consensus		E/D	P	–	D	V/I/L	D	C	F/W	C	

*POWV, Powassan virus; LANV, Langat virus; DENV, dengue virus; YFV, yellow fever virus; APOIV, Apoi virus.

†Numbers indicate the amino acid position in the prM protein.

Table 2. The amino acid difference in prM proteins between the parental virus and the plasmids used in this study

The non-conserved amino acid in the sequence is shown in non-bold.

Virus/ mutant	Position*								
	62	63	64	65	66	67	68	69	70
TBEV (pCAG-TBEME and Oshima-IC)									
Wild type	E	P	V	D	V	D	C	F	C
prE62A	A	–	–	–	–	–	–	–	–
prP63A	–	A	–	–	–	–	–	–	–
prD65A	–	–	–	A	–	–	–	–	–
prV66A	–	–	–	–	A	–	–	–	–
prD67A	–	–	–	–	–	A	–	–	–
prF69A	–	–	–	–	–	–	–	A	–
prV66I	–	–	–	–	I	–	–	–	–
prF69W	–	–	–	–	–	–	–	W	–
	Position†								
	61	62	63	64	65	66	67	68	69
JEV (pcJEME)									
Wild type	D	P	E	D	V	D	C	W	C
prP62A	–	A	–	–	–	–	–	–	–
prD64A	–	–	–	A	–	–	–	–	–
prV65A	–	–	–	–	A	–	–	–	–
prD66A	–	–	–	–	–	A	–	–	–
prW68A	–	–	–	–	–	–	–	A	–

*Based on TBEV strain Oshima 5-10 (GenBank accession no. AB062063).

†Based on JEV strain Nakayama (GenBank accession no. EF571853).

valine-to-isoleucine substitution was engineered in pCAG-TBEME. Unlike the alanine substitution, the isoleucine substitution did not affect secretion of the E protein, indicating the importance of the branched-chain amino acid at this residue. At position 69, the aromatic amino acid phenylalanine is conserved in tick-borne and no-known-vector flaviviruses, whilst tryptophan is conserved here in mosquito-borne flaviviruses. A phenylalanine-to-tryptophan substitution was engineered at this residue in pCAG-TBEME and this substitution reduced secretion of the E protein.

To confirm the importance of the conserved region in other flaviviruses, similar alanine substitutions were engineered in the pcJEME plasmid expressing the prM and E proteins of JEV. pcJEME plasmids containing the different mutations were transfected into 293T cells. After incubation for 24 h, intracellular and extracellular E proteins were detected by Western blotting. The substitutions of proline-62, aspartate-64, valine-65, aspartate-66 and tryptophan-68 in the prM protein resulted in reduced secretion of the E protein, as observed in TBEV (Fig. 1b). These data indicated that the conserved region in the prM protein of flaviviruses has an important role in the assembly and secretion of the virus particles.

Effect of mutations in the conserved region on prM–E interaction

Interaction between the prM and E proteins is necessary for assembly of the virus particle. To investigate whether the mutations in the conserved region affected the prM–E association, 293T cells transfected with the pCAG-TBEME plasmids were lysed, and co-immunoprecipitation of the prM and E proteins was carried out. As shown in Fig. 2, prM and E proteins were detected in eluates from co-immunoprecipitation samples from cells transfected with pCAG-TBEME carrying the prP63A, prD65A, prV66A or prF69A mutation and in the wild-type plasmid. These data demonstrated that heterodimerization between prM and E was not disrupted by mutations at positions 63, 65, 66 or 69. Following alanine substitution at position 67 (aspartate to alanine), prM and E proteins were not co-immunoprecipitated, indicating that this mutation critically affected the prM–E interaction.

Effect of mutations in the conserved region on intracellular localization

To evaluate whether the mutations in the conserved region affected the intracellular distribution of viral proteins, cells

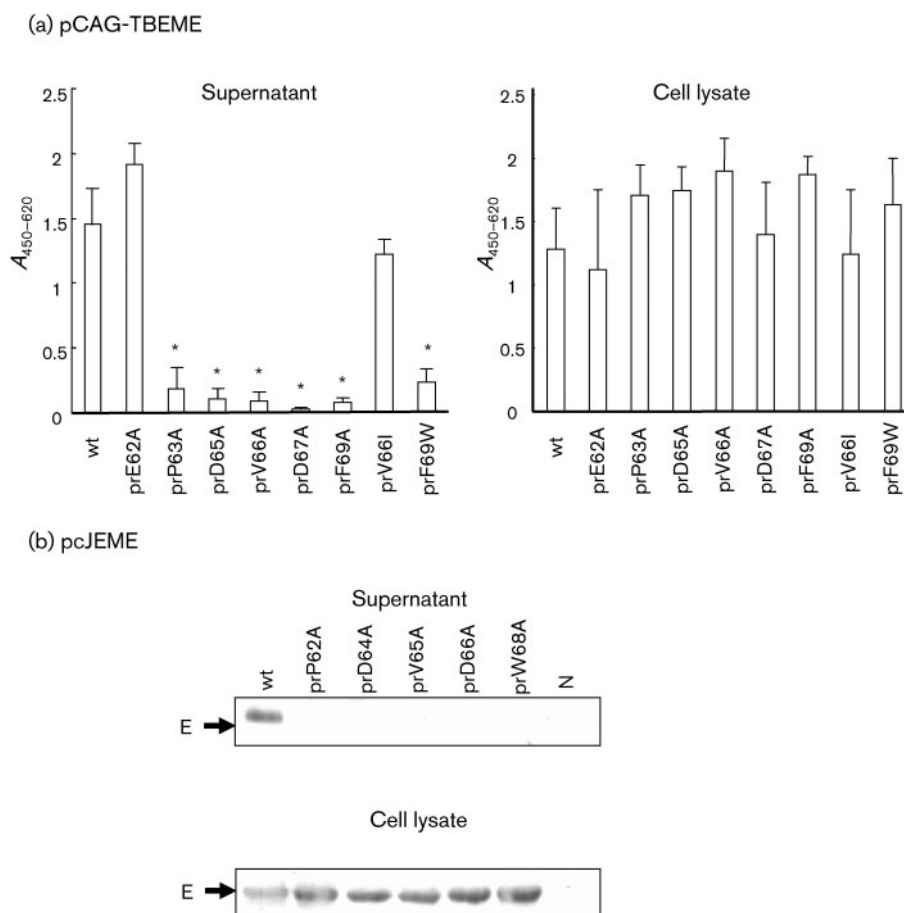


Fig. 1. Mutations in the conserved region of prM reduce the secretion of SPs. PrM and E proteins were expressed in 293T cells by transfection of wild-type (wt) pCAG-TBEME (a) or pcJEME (b) with or without a mutation in the conserved region in prM. After a 24 h incubation, E protein in the solubilized sample was detected by ELISA using TBEV E-specific mAb (a) or Western blotting using JEV E-specific mAb (b). Mean absorbance values ($\pm$ SD) were calculated from triplicate experiments. Asterisks indicate statistically significant differences compared with the wild-type (wt) plasmid (Student's *t*-test, $P < 0.05$). N, Non-transfected control.

transfected with each pCAG-TBEME plasmid were double-stained for TBEV proteins and cellular marker antigens. The distribution of viral proteins in the ER was not affected by the mutations in prM (Fig. 3a). However, whilst the distribution of viral proteins in the Golgi complex was observed in the wild-type-transfected cells, co-localization of viral proteins and Golgi markers was low with the prM protein containing mutations (Fig. 3b). Co-localization of prM and E proteins was similar in all pCAG-TBEME-transfected cells (see Supplementary Fig. S1, available in JGV Online). These data suggested that the mutation in the conserved region of prM caused a defect in the transport of the viral protein to the Golgi complex.

Ultrastructural analysis of particle assembly by prM mutations

To examine whether the mutations in prM affected the assembly of SPs, electron microscope analysis of

pCAG-TBEME-transfected cells was performed. As shown in Fig. 4, spherical SPs were observed in the lumen of membrane-bound vesicles in wild-type-transfected cells (Fig. 4a, b). In contrast, filamentous structures were observed in the lumen of membrane-bound vesicles in cells transfected with pCAG-TBEME containing the prP63A, prD65A, prV66A or prF69A mutation (Fig. 4c–f). The filamentous structures were 30–40 nm in width and 0.1 to >1.0 μ m in length and had constrictions every 50–100 nm. In prD67A-transfected cells, no SPs or filamentous structures were detected (Fig. 4g). These results indicated that the conserved region of prM has important roles in virion assembly.

Effect of mutations in the conserved region on viral multiplication

To confirm the effect of mutations in prM on viral multiplication, alanine substitutions affecting the secretion

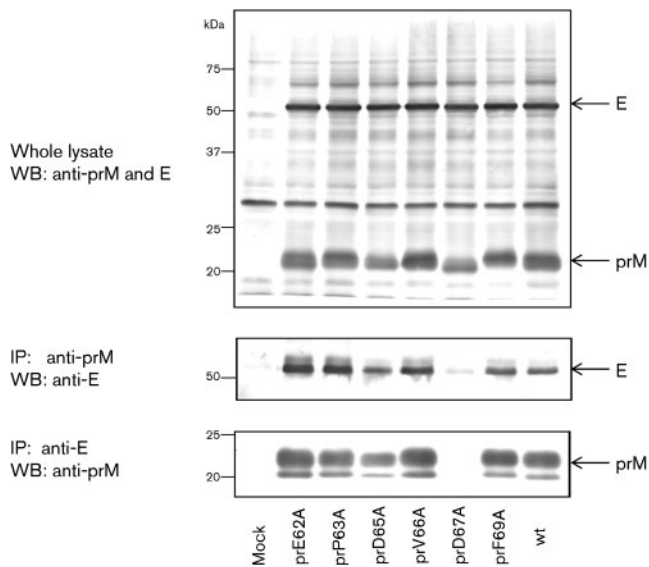


Fig. 2. Effect of mutations in the conserved region of prM on prM–E interaction. The prM protein, with or without (wt) a mutation in the conserved region, was expressed in 293T cells by transfection of the pCAG-TBEME plasmid containing various mutations. After 24 h incubation, cells were harvested, and whole-cell lysate and post-nuclear supernatant immunoprecipitated (IP) with mouse anti-E or anti-prM antibodies were analysed by Western blotting (WB). Protein bands were detected by anti-E and anti-prM rabbit polyclonal antibodies. The positions of the individual proteins are marked and molecular size is indicated (kDa).

and assembly of SPs were engineered in the full-length infectious cDNA of TBEV (Oshima-IC) (Table 2). Baby hamster kidney (BHK-21) cells were transfected with *in vitro*-transcribed mRNA from Oshima-IC, and the culture supernatant was harvested at 8–120 h post-transfection. As presented in Fig. 5(a), cells transfected with Oshima-IC containing prM mutations produced fewer infectious virus particles than cells transfected with wild-type Oshima-IC. The secreted E protein was quantified by ELISA and compared with the infectious virus titre to examine whether the lower level of infectious virus resulting from the presence of prM mutations was due to a reduction in virion secretion or to loss of infectivity of the secreted virion (Fig. 5b). Virus titres were reduced by the prM mutations at a rate similar to that of viral protein secretion, indicating that the infectivity of secreted virions was unaffected by the prM mutations. These results were consistent with the low level of secretion of SPs by prM mutations observed in Fig. 1. Cells transfected with Oshima-IC prD67A produced fewer virus particles than those transfected with Oshima-IC containing the other prM mutations, indicating that this mutation affects the virus multiplication associated with the loss of the prM–E interaction and particle assembly, as observed in the SP experiment.

To investigate the possibility of the appearance of reversion or second-site mutations, each virus was passaged ten times

(over a period of 30 days) and the nucleotide sequences of their prM and E genes were determined. Reversion to wild-type sequence was observed in Oshima-IC prP63A at passage 9 and in prD67A at passage 2. No reversion or second-site mutation appeared in Oshima-IC prD65A, prV66A or prF69A, and their growth rates were still slower than wild-type virus. These data confirmed the importance of the conserved region in prM in viral multiplication.

Molecular modelling of the conserved region on trimeric spikes of immature virions

To identify potential amino acid residues that could interact with the conserved regions, the crystal structure of the pr peptide and the E protein was superimposed on the pseudo-atomic structure of the trimeric spikes of three prM–E heterodimers in immature virions. This structure was then refined by simulated annealing with molecular dynamics calculation (Fig. 6 and Supplementary Table S1, available in JGV Online). This highlighted amino acid residues that could potentially interact with the conserved regions of the prM proteins (prM-1, prM-2 and prM-3) within the trimeric spike of the prM–E heterodimers: (i) the fusion peptide of E-2 with the conserved region of prM-1; (ii) arginine-16, lysine-19, methionine-39 and threonine-81 of prM-3 with the conserved region of prM-2; and (iii) arginine-16, lysine-19, methionine-39 and threonine-81 of prM-2 with the conserved region of prM-3. As shown in Fig. 6, whilst the conserved region of prM-1 is located between the fusion loop of E-1 and E-2, the conserved regions of prM-2 and prM-3 were suggested to interact with similar regions of each other, indicating the dimeric association between prM-2 and prM-3. These results suggest that the conserved region in prM could serve as an important domain for the association between heterodimers in the formation of a spike.

DISCUSSION

In flaviviruses, the prM and E proteins play important roles in the assembly and secretion of virions. Many studies have demonstrated that the co-expression of prM and E proteins in cells leads to the formation of SPs that are structurally and functionally similar to native virions (Allison *et al.*, 1995b; Fonseca *et al.*, 1994; Mason *et al.*, 1991; Op De Beeck *et al.*, 2003; Yamshchikov & Compans, 1993). These studies indicated that critical determinants for the assembly and secretion of the virions were present in the prM and/or E proteins. During the assembly process, prM proteins are assumed to coat E proteins. It has been shown that the furin cleavage sequence between the pr region and the M protein is important for maturation of infectious virions prior to their release from cells (Elshuber *et al.*, 2003; Stadler *et al.*, 1997) and that the transmembrane domain of the prM protein serves as a retention signal for the membrane of the ER (Op De Beeck *et al.*, 2003, 2004). In several reports, single point mutations in prM were found

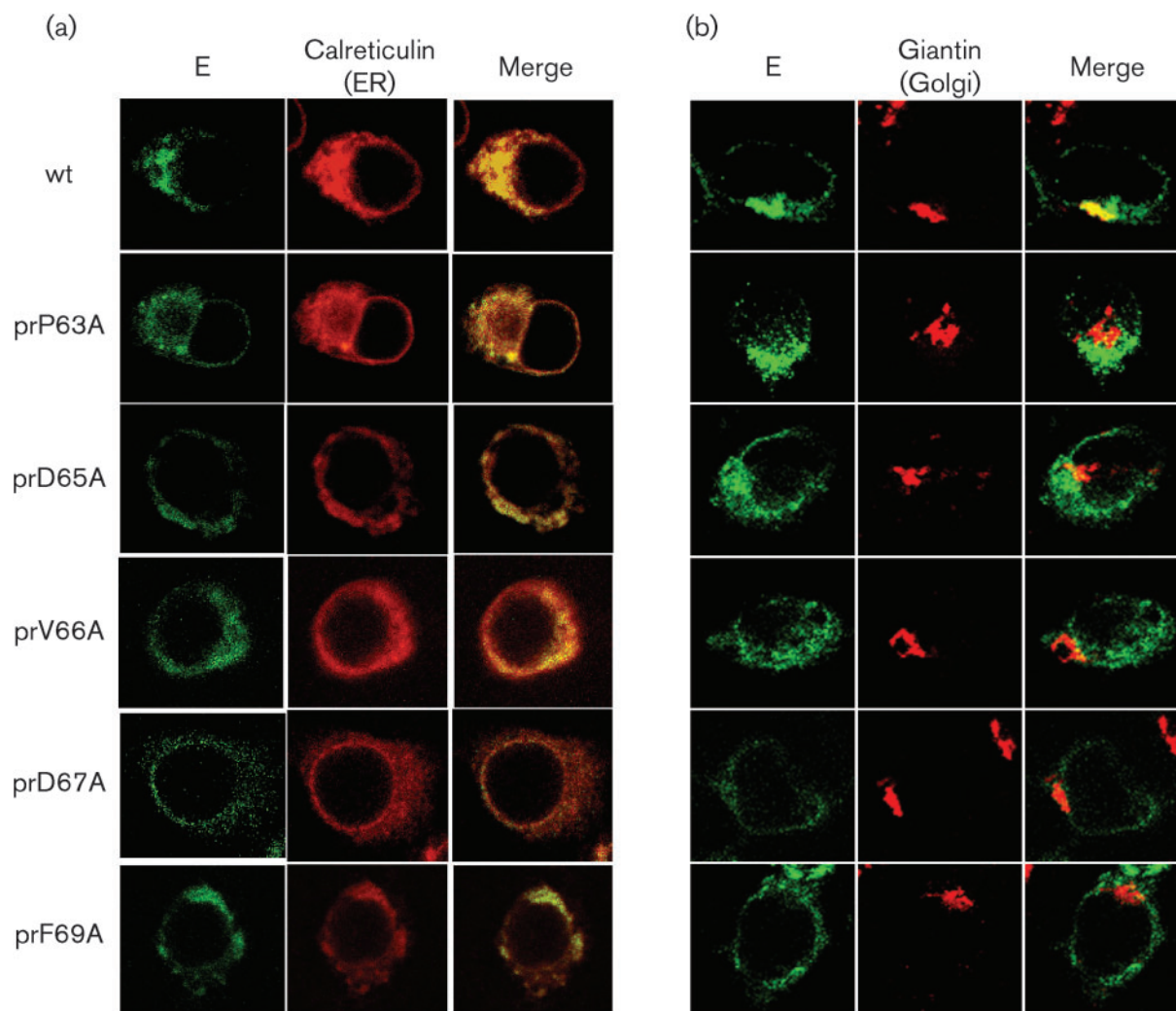


Fig. 3. Intracellular localization of expressed E proteins. 293T cells transfected with pCAG-TBEME wild-type (wt) or various mutant plasmids were fixed and subjected to dual staining with antibodies against calreticulin (an ER marker) (a) or giantin (a Golgi marker) (b) and anti-E antibodies. Co-localization of the E protein with the ER or Golgi marker proteins is depicted in the merged images.

to affect viral particle formation and secretion without preventing prM–E heterodimerization (Pryor *et al.*, 2004; Tan *et al.*, 2009). Another study showed that coordinated cleavage between the C protein and prM promoted the uptake of nucleocapsids into budding flavivirus membranes (Lobigs & Lee, 2004). However, little is known about the functional residues of the prM protein involved in the assembly and secretion process. In this study, we demonstrated for the first time that the conserved region of the prM ectodomain in flaviviruses has critical functions in the assembly and secretion process.

Multiple-sequence analysis revealed that the conserved region in prM is present in a large number of flaviviruses, including tick-borne, mosquito-borne and no-known-vector flaviviruses (Table 1). As shown in our previous study, a proline-to-serine mutation at position 63 of the

prM of TBEV reduces the secretion of virus particles (Yoshii *et al.*, 2004) and we considered that the conserved region plays a crucial role in the assembly and secretion of virus particles. It was revealed that the secretion of SPs was impaired by a single mutation in the conserved region of both TBEV and JEV (Fig. 1). This indicated that the conserved region in the prM protein is a critical and common molecular determinant for the assembly and secretion of flaviviruses.

The interaction between the prM and E proteins is important during the early events of virus particle maturation and assembly. Previous studies have shown that the E protein requires co-synthesis of prM to achieve structural conformation, whilst prM folds independently of other viral components (Guirakhoo *et al.*, 1992; Lorenz *et al.*, 2002). PrM–E heterodimerization is a crucial process

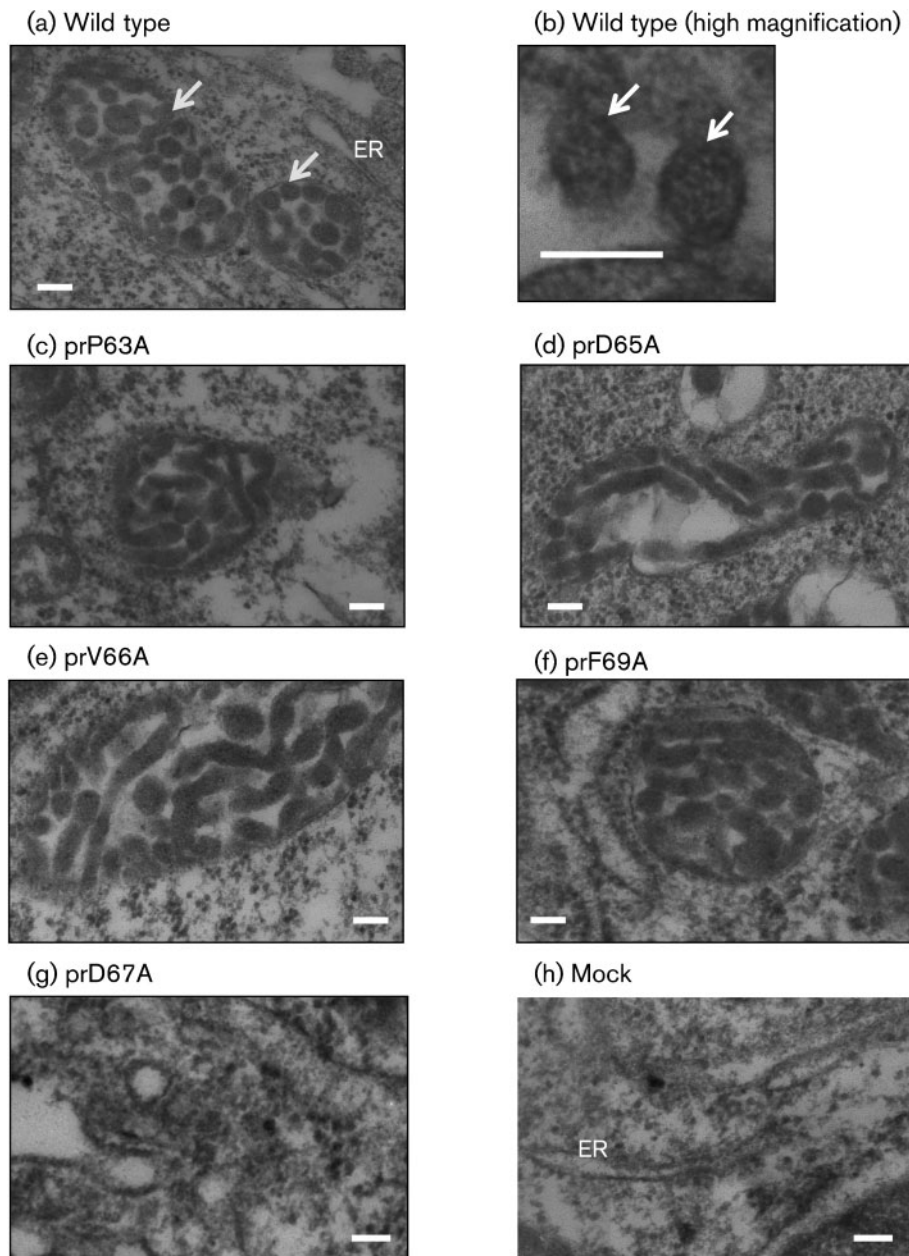


Fig. 4. Electron micrographs of 293T cells transfected with pCAG-TBEME containing various mutations. (a, b) Spherical SPs (arrows; diameter 20–30 nm) were observed in the lumen of membrane-bound vesicles in wild-type-transfected cells. (c–f) Filamentous structures were observed in the lumen of membrane-bound vesicles in cells transfected with pCAG-TBEME containing the prP63A (c), prD65A (d), prV66A (e) or prF69A (f) mutation. (g, h) No particulate and filamentous structures were observed in prD67A-transfected cells (g), similar to mock-transfected control cells (h). Bars, 50 nm.

for sequential virus particle formation. In this study, co-immunoprecipitation experiments demonstrated that prM–E heterodimerization was not impaired by the mutations in the conserved region of the prM protein except for the mutation at position 67 (Fig. 2). This mutation impaired the interaction between the prM and E protein, leading to the failure of further steps in the viral particle assembly process. These data suggested that the

reduction in SP secretion by the prM mutations, except for position 67, was due to later steps in the viral particle assembly process and secretion, and not to disruption of prM–E heterodimerization in the early events of virus particle assembly.

Previous reports have shown that flavivirus particles bud into the ER lumen, followed by transport through the

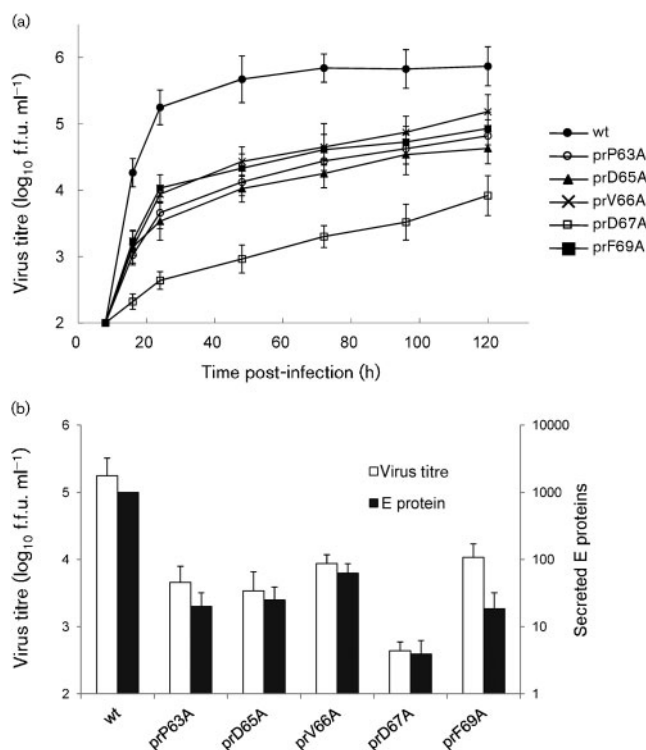


Fig. 5. Effect of mutations in the conserved region of prM on virus particle secretion. (a) Growth curve of the Oshima-IC virus wild-type (wt) and prP63A, prD65A, prV66A, prD67A and prF69A mutants. A monolayer of BHK-21 cells was infected with the individual viruses at an m.o.i. of 0.01. At each time point, the culture medium was harvested and virus titres were determined by focus-forming assay in BHK cells. (b) Comparison of the titre of infectious virus and secreted E proteins. After 24 h incubation, the relative amount of secreted E protein was quantified by ELISA and compared with the virus titre. The amount of E protein from the Oshima-IC wild-type virus was set at 1000. Data are means $\pm$ SD from triplicate experiments.

secretory pathway (Mackenzie & Westaway, 2001). In this study, the expressed viral proteins with the prM mutations were not transported to the Golgi complex, and accumulated in the ER (Fig. 3). Furthermore, ultrastructural analysis revealed long filamentous structures, rather than spherical SPs, in the lumen of the ER resulting from the prM mutations (Fig. 4). The width of the filamentous structures was approximately the same as the diameter of the SPs, and constrictions were observed every 50–100 nm. It was suggested that they were derived from abnormal budding in which a particle failed to pinch off, resulting in the bud growing into a tubular structure. Filamentous structures may not undergo transport through the secretory pathway due to their abnormal budding. It is also possible that they are derived from cellular membrane components induced by prM mutations. In any case, the mutations in the conserved region of prM clearly affected the budding process of the virus particle, which must have

occurred after the prM–E heterodimerization. No SPs or filamentous structures were observed with the mutation at position 67. This could be attributed to the disruption or the prM–E heterodimerization in the early events of virus particle assembly, as described above. In a study of dengue virus, this negatively charged residue was shown to interact with the positively charged surface of domain II of the E protein (Li *et al.*, 2008).

The budding mechanism of flaviviruses is considered to be distinct from that of other enveloped viruses. In many enveloped viruses, important cytoplasmic domains directing virus budding have been identified, such as late-domain motifs (Bieniasz, 2006; Kail *et al.*, 1991; Morita & Sundquist, 2004; Whitt *et al.*, 1989; Zhao *et al.*, 1994). These domains interact with cellular factors resulting in efficient budding. However, the cytoplasmic loops of the flavivirus prM and E proteins consist of only a few amino acid residues between their two transmembrane regions. Thus, it is considered that the ER-luminal regions of the prM and/or E proteins play critical roles in the assembly of flaviviruses. The ER membrane does not normally form vesicles that bud into the lumen. It is possible that prM–E heterodimers, alone or with cellular factors in the ER lumen, assemble laterally and induce membrane curvature into an isometric lattice, as reported in the budding of membrane transport vesicles (Keen *et al.*, 1979; Wieland & Harter, 1999). The conserved region in prM may have important roles in this process, which were impaired by the mutations, leading to the abnormal budding.

The conserved region in prM could be involved in the oligomerization of the prM–E heterodimers. Immature virions of flaviviruses contain 60 trimeric spikes and each spike consists of three prM–E heterodimers (Zhang *et al.*, 2003). The asymmetrical spike has been considered a single assembly unit because the association between heterodimers within a spike is sufficiently strong, compared with the association between spikes. The dominant contacts between the prM–E heterodimers within a spike are between pre-peptides at the extremities of the spikes. Based on the crystal structure of the pr peptide of dengue virus 2 (Li *et al.*, 2008), the conserved region of prM covers the fusion peptide of the E protein in each heterodimer and aspartate-63 and -65 were involved in the complementary electrostatic patches. Our structural model for an asymmetrical trimer of prM–E heterodimers indicates that the conserved region in prM could serve as an important domain for the association between heterodimers in the formation of a spike (Fig. 6 and Supplementary Table S1). The mutations in the region might affect the association between heterodimers and cause perturbation of oligomerization of prM–E heterodimers, resulting in the inefficient induction of membrane curvature and/or pinch-off.

In summary, we demonstrated that the conserved region in prM is a critical determinant in flavivirus assembly and secretion. Mutations in this region, except for aspartate-67,

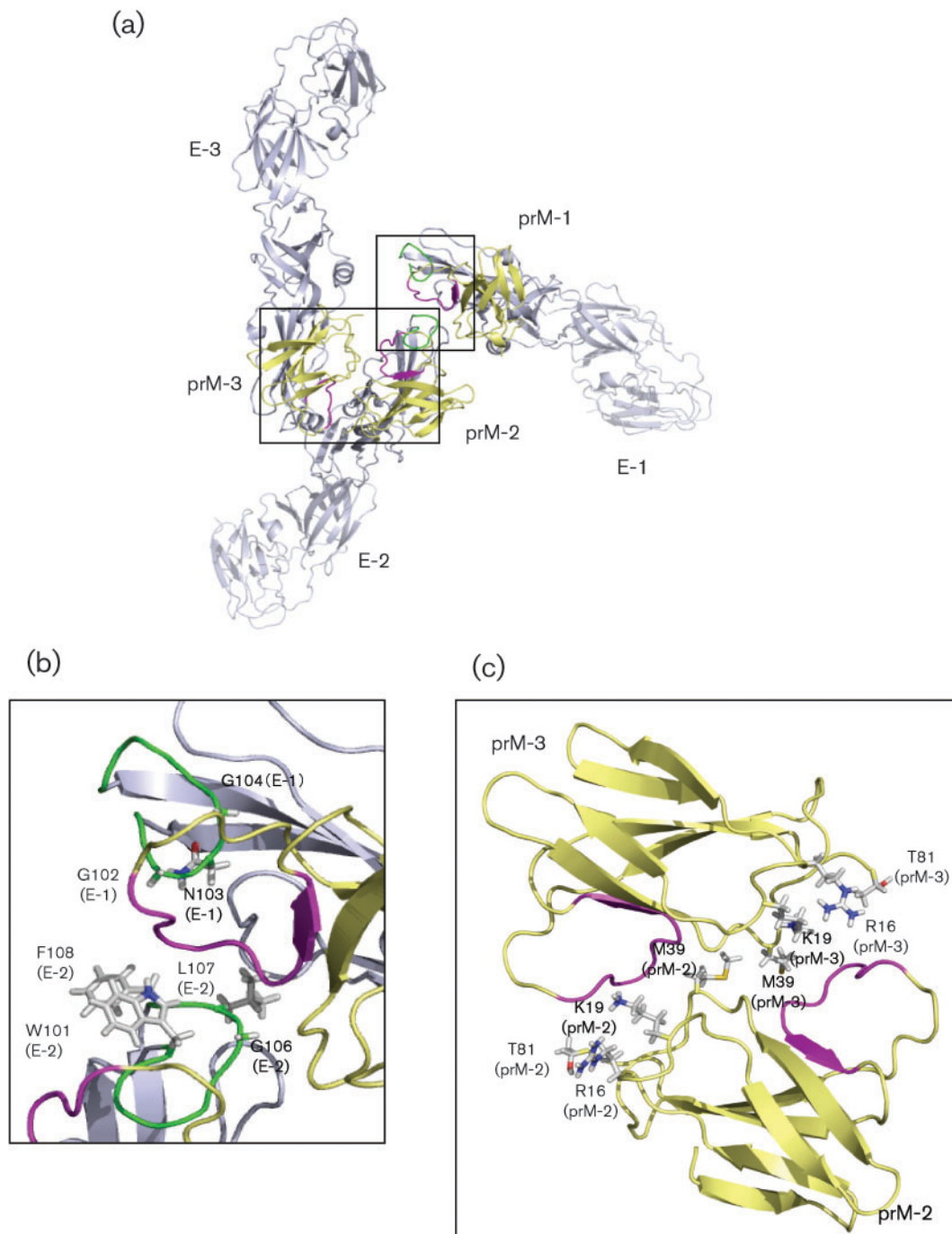


Fig. 6. Structural model for an asymmetrical trimer of prM-E heterodimers. (a) Overall structure of an asymmetrical trimer of prM-E heterodimers. (b) Close-up view of the binding interface between prM-1 and E-1 or E-2. (c) Close-up view of the binding interface between prM-2 and prM-3. E and prM molecules are coloured blue-white and yellow, respectively. The fusion peptide of the E protein and the conserved region of prM protein are shown in green and magenta, respectively. Residues represented by stick models show the amino acid position within 4.0 Å of the conserved region of prM. This figure was prepared using PyMOL (DeLano Scientific; DeLano, 2002).

did not affect the interaction between the prM and E proteins but impaired the budding step, which is independent of previously known functions of the prM

protein. These results contribute to the further understanding of prM function, revealing the molecular mechanism of flavivirus assembly and secretion.

METHODS

Cells. BHK-21 cells were grown at 37 °C in minimum essential medium supplemented with 8 % (v/v) FCS and L-glutamine. Human embryonic kidney 293T cells were cultured at 37 °C in Dulbecco's modified Eagle's medium, containing 10 % (v/v) FCS, L-glutamine and penicillin/streptomycin.

Antibodies. For the detection of TBEV prM and E proteins by ELISA, immunoprecipitation and immunofluorescence experiments, mouse anti-E mAbs 1H4 and 4H8, prepared in our laboratory, were used (Komoro *et al.*, 2000). Rabbit polyclonal anti-prM and anti-E antibodies were prepared by immunization with recombinant prM and E proteins, as described previously (Yoshii *et al.*, 2004). Mouse anti-E mAb 10B4 was kindly provided by Dr Konishi (Kobe University Graduate School of Medicine, Kobe, Japan) for the detection of JEV E protein by Western blotting. For the immunofluorescence co-localization studies, anti-calreticulin rabbit polyclonal antiserum (Affinity BioReagents) or anti-giantin rabbit polyclonal antiserum (Covance Research Products) was used. FITC-conjugated anti-mouse IgG antibodies and Texas Red-conjugated anti-rabbit IgG antibodies (Jackson ImmunoResearch) were used as secondary antibodies in the immunofluorescence assays, and alkaline phosphatase-conjugated anti-rabbit and anti-mouse IgG antibodies (Jackson ImmunoResearch) were used in the Western blot analysis.

Plasmids. Plasmid pCAG-TBEME, a pCAGGS-based plasmid encoding the TBEV (Oshima 5-10 strain) signal sequence of the prM, and the prM and E genes, was constructed as described previously (Yoshii *et al.*, 2003).

Plasmid pcJEME, a pCDNA3-based plasmid that encodes the JEV (Nakayama strain) signal sequence of prM, and the prM and E genes, was kindly provided by Dr Konishi and by Dr Mason (Department of Pathology, University of Texas Medical Branch, Galveston, TX, USA) (Konishi *et al.*, 1998),

The TBEV infectious cDNA clone used, Oshima-IC, was constructed previously (Hayasaka *et al.*, 2004).

All mutations within the conserved sequence of the prM protein were engineered in the pCAG-TBEME, pcJEME and Oshima-IC full-length plasmid constructs using standard PCR mutagenesis techniques (Table 2).

Expression of viral prM and E proteins. 293T cells were transfected with each plasmid complexed to TransIT-LT1 reagent (PanVera) in Opti-MEM (Invitrogen), as described previously (Yoshii *et al.*, 2003). After a 24 h incubation, the cells and supernatant were harvested and used for further experiments.

ELISA. The E protein of TBEV was detected by ELISA, as described previously (Yoshii *et al.*, 2003). Briefly, transfected cells were lysed with 1 % (v/v) Triton X-100 in 10 mM Tris-buffered saline (TBS) and the supernatants were treated with 1 % Triton X-100. Triton X-100-solubilized samples were added to mAb 1H4-coated wells of 96-well microtitre ELISA plates, previously blocked with 3 % (w/v) BSA. The E protein was detected by incubation with biotinylated mAb 4H8 and HRP-conjugated streptavidin (Sigma). HRP activity was detected by adding o-phenylenediamine dihydrochloride (Sigma) in the presence of 0.03 % (v/v) H₂O₂ and absorbance was measured at 450–620 nm.

Immunoprecipitation. 293T cells were transfected with pCAG-TBEME containing the various mutations, as described above. After a 24 h incubation, the cells were lysed with Triton X-100 in 10 mM TBS, incubated on ice for 20 min and centrifuged (16 000 g, 20 min). The supernatant, which excluded the nuclear fraction, was pre-cleared on protein G-Sepharose beads (Amersham Pharmacia Biotech) for

2 h at 4 °C. Pre-cleared lysates were combined with protein G-Sepharose beads with mAb 1H4 and precipitated by incubation for 2 h at 4 °C. Immune complexes were pelleted (10 000 g, 10 s) and washed four times with 1 % Triton X-100 in 10 mM TBS. Subsequently, the precipitated materials were solubilized and analysed by SDS-PAGE and Western blotting.

SDS-PAGE and Western blotting. Protein samples were separated by SDS-PAGE [12 % (w/v) acrylamide]. The protein bands were transferred onto PVDF membranes and incubated with 1 % (w/v) gelatin in 25 mM TBS containing 0.01 % (v/v) Tween 20 (TBST). After washing with TBST, the membranes were reacted with an antibody against the viral protein of TBEV or JEV, followed by alkaline phosphatase-conjugated secondary antibody. Protein bands were visualized using an alkaline phosphatase detection reagent kit (Novagen), according to the manufacturer's protocol.

Immunofluorescence assay. 293T cells were grown on eight-well chamber slides (Nalge Nunc International) and transfected with pCAG-TBEME containing the various mutations. After 8 h incubation, cells were rinsed with PBS, fixed with 4 % (w/v) paraformaldehyde for 10 min and permeabilized with 0.2 % (v/v) Triton X-100 for 4 min at room temperature. After blocking with 2 % (w/v) BSA, the cells were incubated for 1 h with mouse mAb 1H4 and antibodies that recognize marker proteins of various cellular organelles or anti-prM antibody. After extensive washing, the cells were incubated with fluorescently labelled conjugated secondary antibodies. The cells were washed three times with PBS, followed by mounting of the coverslips on glass slides. Images were viewed and collected with an Olympus IX70 confocal microscope.

Electron microscopy. 293T cells were transfected with pCAG-TBEME containing the various mutations. After a 24 h incubation, the cells were harvested and centrifuged (1000 g, 5 min). The pellets were fixed with 3 % (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 3 h and rinsed three times with 0.1 M phosphate buffer. After post-fixation in a 1 % (w/v) osmium tetroxide solution for 1.5 h, the pellets were dehydrated through a series of graded ethanols and embedded in Epon 812 via QY1 (Nishin EM). Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined under a JEM 1210 transmission electron microscope (JEOL) at an acceleration voltage of 80 kV.

Virus recovery and titration. Infectious RNA was transcribed from the Oshima-IC plasmid using an mMESSAGE mMACHINE SP6 kit (Ambion) and electroporated into BHK-21 cells, as described previously (Hayasaka *et al.*, 2004). At 1–5 days post-electroporation, the secreted virus was recovered and the infectious virus titre was assayed using a focus count assay, as described previously (Takashima *et al.*, 1997). Briefly, monolayers of BHK-21 cells were grown in 96-well plates and inoculated with serial dilutions of the virus. After 38 h incubation, virus foci were visualized by immunofluorescent staining using anti-TBEV E antibodies.

Passaging experiments. Passages of the virus on BHK-21 cells were performed by transferring aliquots (200 µl) of cell culture supernatants (at 3 days post-infection) cleared of cell debris and insoluble material by low-speed centrifugation to fresh BHK-21 cells grown in 24-well culture plates.

Molecular modelling. The model structure for an asymmetrical trimer of prM–E heterodimers was generated by superimposing the crystal structure of dengue 2 virus (Protein Data Bank code: 3C6E) onto the pseudo-atomic structure determined by cryo-electron microscopy (Protein Data Bank code: 3C6D). This structure was refined by molecular dynamics simulations followed by energy minimizations. First, the system was gradually heated from 50 to

1000 K for 13 ps of simulation and then cooled to 300 K for 10 ps. A harmonic constraint of $10 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$ was applied on the C α atoms of the protein during the simulation. The time step was set to 1 fs. After molecular dynamics simulations, the energy minimization was conducted without positional constraints using 10 000 steps of steepest descent, followed by conjugate gradient minimization, until the root mean square gradient was $\leq 0.01 \text{ kcal mol}^{-1} \text{ \AA}$. All molecular dynamics and energy minimization calculations were performed using Discovery Studio 2.5 (Accelrys, <http://accelrys.com/products/discovery-studio/>) with a CHARMM force field with a generalized Born implicit solvent model.

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