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N-glycosylation of the premembrane protein of Japanese encephalitis virus is critical for folding of the envelope protein and assembly of virus-like particles

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Summary. – Premembrane (prM) and envelope (E) proteins, the major structural proteins of Japanese encephalitis virus (JEV) each contain single potential N-glycosylation site. In this study, the role of N-glycosylation of these proteins on their folding and activity were investigated. Three mutant prM and/or E (prM-E) genes lacking N-glycosylation sites were generated by site-directed mutagenesis. The effects of the N-glycan on folding, secretion and cytotoxicity of mutant proteins were determined by comparison with their wild type (wt) counterparts. Removal of N-glycan from the prM protein resulted in a complete misfolding of the E protein and failure to form virus-like particles (VLPs). A similar removal of N-glycan from the E protein led to a low efficiency of its folding and VLPs formation. The secretion and cytotoxicity of the E protein was also markedly impaired in case the glycosylation sites in the prM or E or both proteins were removed. These results suggest that the N-glycosylation of the prM protein is critical to the folding of the E protein, which makes it pivotal in the cytotoxicity of JEV particles and their production.

Keywords: Japanese encephalitis virus; premembrane protein; envelope protein; N-glycosylation; protein folding; virus-like particles; secretion; cytotoxicity

Introduction

Japanese encephalitis virus (JEV) is a mosquito-transmitted, zoonotic flavivirus that causes 50,000 cases and 10,000 deaths every year (Barrett, 2008). JEV has three structural proteins, i.e., capsid, membrane or premembrane (prM), and envelope (E) protein (Chambers *et al*., 1990). E protein is the main structural protein on the flavivirus surface and plays a major role in viral binding, entry, tissue tropism, neurovirulence, neuroinvasiveness, and immune stimulation (Allison *et al*., 1995; Alka *et al*., 2007; Chen *et al*., 2004; Kroeger and McMinn, 2002; Liu *et al*., 2004; Ni and Barrett, 1998). prM protein interacts with E protein to form prM-E heterodimer, which is important for flavivirus maturation (Heinz and Allison, 2000; Mackenzie and Westaway, 2001). Co-expression of prM and E protein in mammalian cells is known to produce virus-like particles (VLPs) (Allison *et al*. 1995; Wu *et al*., 2006). E protein requires prM protein co-expression to acquire its native conformation, suggesting a chaperone-like role for prM protein in the folding of E protein (Konishi and Mason, 1993).

prM and E proteins are N-glycosylated in most flaviviruses, although the amount and location of glycosylation sites are variable in different viruses or strains (Heinz and Allison, 2000). N-glycosylation of E protein is known to be important in viral propagation, infectivity, secretion, and neuroinvasion (Beasley *et al*., 2005; Bryant *et al*., 2007; Lee *et al*., 2010; Li *et al*., 2006; Shirato *et al*., 2004; Vorndam *et al*., 1993). However, little attention had been paid to the roles of the N-glycosylation in prM protein until Kim *et al.* (2008) reported that glycosyla-

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Abbreviations: CE = conformational epitope; E = envelope; ER = endocytoplasmic reticulum; JEV = Japanese encephalitis virus; LE = linear epitope; MAb(s) = monoclonal antibody(ies) prM = premembrane; VLPs = virus-like particles

tion of JEV prM protein was associated with viral release and pathogenesis. But how the N-glycan in prM protein affects the viral release and pathogenesis is still unclear.

In this study, we attempted to assess the role of N-glycans in JEV prM and E proteins by constructing mutants of the corresponding genes with deleted N-glycosylation sites and determining their effects on protein folding, cytotoxicity and secretion, and virus particle production. The results showed that a removal of the N-glycan from the prM protein led to a complete misfolding of the E protein and failure to produce VLPs, while a removal of the N-glycan from the E protein resulted just in its partial misfolding. In addition, a removal of the N-glycan(s) from the prM or E or both proteins affected the secretion and cytotoxicity of the E protein.

Materials and Methods

Cells and viruses. HEK-293FT and BHK-21 cells were grown in DMEM containing 10% FBS. Human JEV P3 virulent strain was propagated in BHK-21 cells.

Antibodies. Monoclonal antibodies (MAbs) that recognized prM were kindly provided by R. H. Hua (HarBin Veterinary Research Institute, Harbin, China). E protein linear epitope (LE) MAb (1:200) was generated in our laboratory (Li *et al.*, 2010). The E protein conformational epitope (CE) MAb (1:10) was purchased from Thermo Scientific (USA). A rabbit polyclonal antibody to calnexin (ER marker) was purchased from Abcam Ltd (1:100, China). The CY3-conjugated anti-mouse IgG (1:50), FITC-conjugated anti-rabbit IgG (1:50), and HRP-conjugated anti-rabbit (1:5,000) or mouse IgG (1:5,000) were used for the indirect immunofluorescence, capture ELISAs and western blot analysis (Boster Biological Technology, China).

Plasmid constructs. The prM-E and E genes were amplified from viral RNA using RT-PCR and subcloned into pcDNA3.1 (+) under the control of HCMV promoter, which resulted in recombinant plasmids pCprME and pCE. In the plasmid pCprME, the N-linked glycosylation sites were eliminated by site-directed mutagenesis, by substituting the asparagine (N) with glutamine (Q), and then resulted in recombinant plasmids pCN15Q, pCN154Q, and pCN15/154Q (Fig. 1a).

Transfection of cells. HEK-293FT cells were plated onto 12-well plates containing glass coverslips. Cells were transfected using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer′s protocol.

Confocal microscopy. The expression and subcellular localization of E protein were identified by confocal microscopy as previously done with some modifications (Shi X *et al.*, 2005). Briefly, cells transfected with mutated or wt prM-E gene were fixed with 4% paraformaldehyde. The cells were incubated with polyclonal antibody to calnexin and linear epitope MAbs to E protein. After washing, the cells were incubated with CY3-conjugated anti-mouse IgG and FITC-conjugated anti-rabbit IgG. Localization of fluorescence-labeled proteins was examined by using a Zeiss LSM confocal microscope (Carl Zeiss, Inc., Germany).

Indirect immunofluorescence assay. The folding of E protein was identified by indirect immunofluorescence assay with LE or CE MAbs of E protein. The immunofluorescence was performed as previously described (Qi WB *et al.*, 2008). Briefly, cells were fixed with 4% paraformaldehyde 48 hrs post-transfection. The LE and CE MAbs of E protein were added to cells individually. The cells were then probed with CY3-conjugated anti-mouse IgG.

Western blot analysis. Cells transfected with pCprME, pCN15Q, pCN154Q, and pCN15/154Q were collected 48 hrs post-transfection. After separation by SDS-PAGE, the proteins were transferred to a PVDF membrane (Millipore, USA). Blots were then treated with HRP-conjugated anti-mouse IgG and finally developed using an enhanced chemiluminescent substrate SuperSignal West Pico (Thermo Scientific, USA).

ELISA. The antigen capture ELISA for JEV detection was developed by a previous study (Mei *et al.*, 2012). Briefly, supernatants of the transfected HEK-293 cells were diluted 1:5 and added to the plates. Rabbit anti-JEV serum was added to each well and incubated for 30 min. The wells were washed three times with PBS and incubated with HRP-conjugated anti-rabbit antibody. The peroxidase substrates were added to each well. Absorbance at 450 nm was read by an MRX Revelation plate reader (BioTek, China).

Flow cytometry. The HEK-293FT cells were transfected with pCprME, pCN15Q, pCN154Q, pCN15/154Q and pcDNA3.1(+) in 12-well plates. Cells were harvested 48 hrs post-transfection by being digested with trypsin (0.25%) and then stained using an annexin V-FITC Apoptosis detection kit (KeyGEN, China), according to the manufacturer′s instructions. Early apoptotic, late apoptotic, and necrotic cells were quantified by flow cytometry using a Becton Dickinson FACSCalibur (Becton Dickinson, USA).

Electron microscopy. The HEK-293FT cells were plated in a 10-cm dish and transfected with the plasmids pCprME, pCN15Q, pCN154Q, and pCN15/154Q. Cells were fixed with 2.5% glutaraldehyde 48 hrs post-transfection then dehydrated, placed in EPON resin (Electron Microscopy Sciences, England), thin-sectioned, placed on a copper grid, post-stained, and examined using a transmission electron microscope (FEI Tecnai G^2 20 TWIN, Fei company, USA).

Results

Expression and subcellular localization of prM and E proteins

The prM-E gene of JEV was amplified from viral RNA by RT-PCR, and subcloned into pcDNA3.1 (+) and resulted in pCprME. The N-linked glycosylation sites were eliminated by site-directed mutagenesis in the plasmid pCprME, by substituting asparagine (N) with glutamine (Q), which resulted in pCN15Q, pCN154Q, and pCN15/154Q mutants. Plasmids were then individually transfected into HEK-293FT cells. Forty-eight hours post-transfection, cells were digested with trypsin and collected by centrifugation. The status of protein

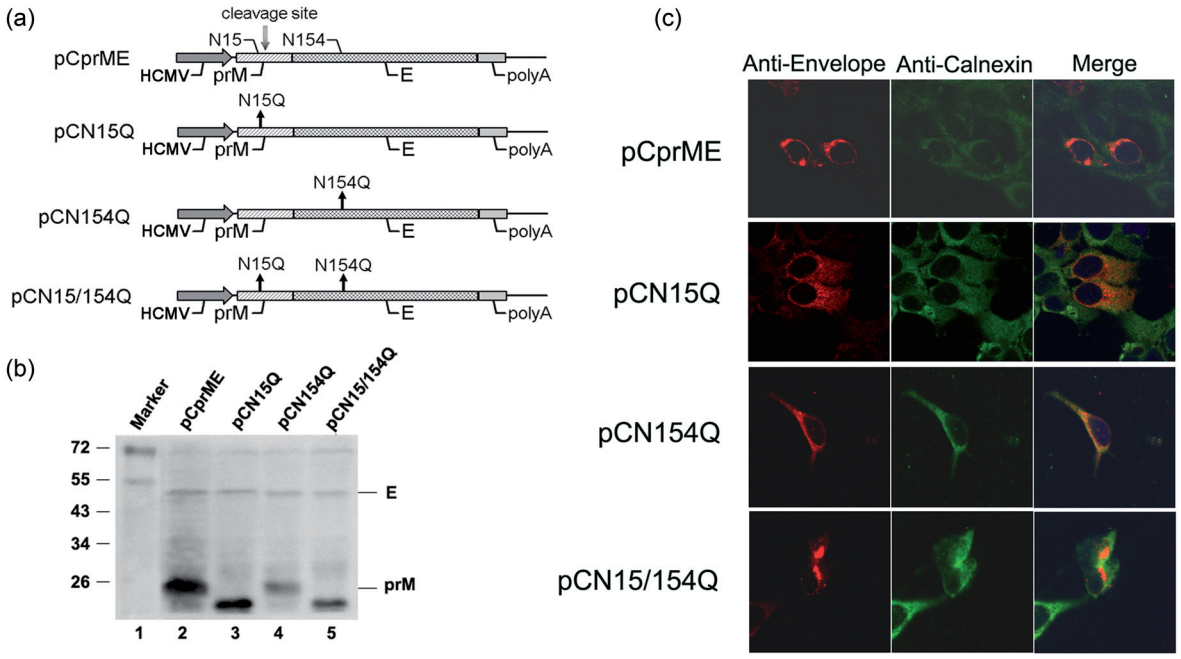

Fig. 1

Expression and subcellular localization of prM-E protein in cells

(a) Scheme of plasmid constructs with mutants of prM and/or E gene at N-glycosylation sites (pCN15Q, pCN154Q, and pCN15/154Q) and wt genes (pCprME, control). (b) Western blot analysis of prM and E proteins expressed in HEK cells transfected with individual plasmid constructs. (c) Immunofluorescence assay of subcellular localization of prM-E protein in HEK cells transfected with individual plasmid construct.

glycosylation was identified by western blot analysis. As shown in Fig. 1b, the molecular weight values of the glycosylated prM and E proteins were estimated at about 20 and 53 kDa (Fig. 1b, lane 2), respectively. Mutation of the N-linked glycosylation site in prM (N15) (Fig. 1b, lanes 3 and 5) or E (N154) (Fig. 1b, lanes 4 and 5) resulted in increased electrophoretic mobility when compared with the wt prM (Fig. 1b, lanes 2 and 4) or E proteins (Fig. 1b, lanes 2 and 3), indicating that sites on both prM and E were modified by N-glycan attachment. It is also suggested that cleavage of prM and E proteins was not impaired when the N-linked glycosylation sites on prM and/or E were removed. Likewise, the localization of the E protein was not affected by deletion of N-glycans in either the prM or the E, because the wt and mutational proteins were all found to colocalize with ER (Fig. 1c).

Effect of N-glycosylation of prM and E proteins on E protein folding

The effect of N-glycosylation on E protein folding was assessed by immunofluorescence with MAbs that recognize CE and LE of E. The LE MAb could recognize all expressed forms of E protein, whereas the CE MAb only reacted with the folded E protein. As shown in Fig. 2, the LE MAb could bind to E protein expressed from cells transfected with each of the plasmids, with or without mutation of the glycosylation sites (Fig. 2a,c,e,g,k), indicating that all the E proteins were expressed in tranfected cells. In contrast, the CE MAb only recognized E protein from pCprME-transfected group with high efficiency (Fig. 2b), to a much lesser extent than the E protein from pCN154Q-transfected group (Fig. 2f). No fluorescence was observed in cells transfected with pCN15Q (Fig. 2d) or pCN15/154Q (Fig. 2h) in the CE MAb-stained groups. Furthermore, the CE MAb did not recognize the E protein expressed in cells transfected with pCE (E gene in pCDNA3.1) (Fig. 2l), suggesting that the folding of E protein required the co-expression of prM protein, which was consistent with a previous study (Konishi and Mason, 1993). All these data indicate that E protein does not fold correctly when the N-linked glycosylation is absent from the prM protein, while the efficiency of E protein folding is reduced when the glycosylation site is deleted.

Effect of N-glycosylation of prM and E proteins on protein secretion and VLPs formation

To investigate the roles of N-linked glycosylation sites on VLPs formation, plasmids were transfected into cells.

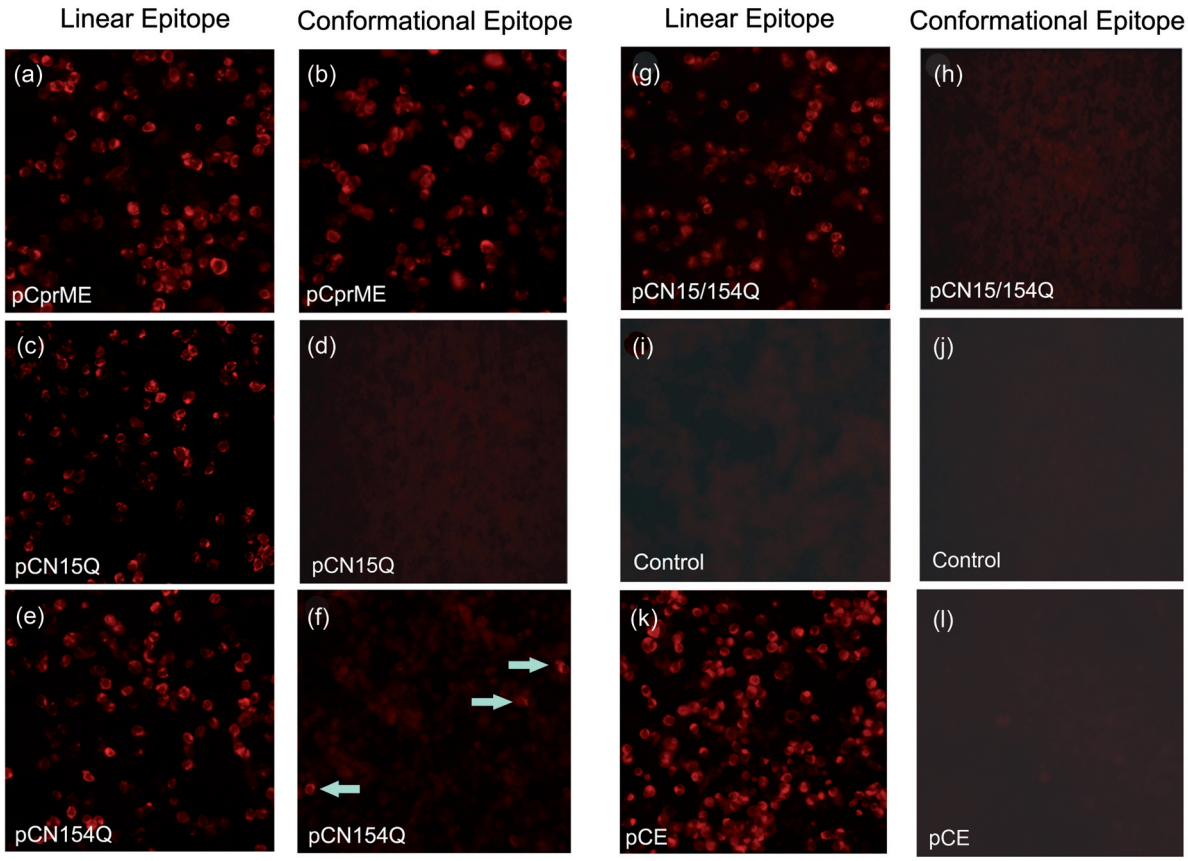

Fig. 2

Effect of N-glycosylation on E protein folding

Immunofluorescence assay of conformational and linear epitopes on the E protein expressed in HEK cells transfected with individual plasmid constructs (a-l). MAbs specific to CE and LE of the E protein, were employed respectively.

Cell sections were observed under a transmission electron microscope. VLPs formation was found in pCprME- and pCN154Q-transfected cells (Fig. 3a). The diameter of the VLPs was estimated to be 80–100 nm, which was consistent with previous reports (Allison *et al.*, 1995). VLPs were not found in pCN15Q- or pCN15/154Q-transfected cells in which the N-linked glycosylation site of prM was eliminated (data not shown). To confirm the role of N-linked glycosylation in protein secretion, a capture ELISA was used to measure the E protein in the medium of transfected cells. In pCprME-transfected cells, the amount of E protein in the supernatant was similar to 10^4 PFU/ml of JEV, suggesting that there was a large amount of E protein in the medium. The values measured at A_{450} of the supernatant from cells transfected with pCN15Q, pCN154Q, or pCN15/154Q were the same as the negative control (Fig. 3b), indicating that there was none or a very low amount of the E protein in the supernatant. These results suggest that prM-E protein can't be secreted from cells when any of the N-linked glycosylation sites in prM-E are deleted.

Effect of N-glycosylation of prM and E proteins on cytotoxicity

The effect of N-glycosylation on cytotoxicity of prM-E protein was assayed by an annexin V-FITC Apoptosis detection kit. The number of apoptotic cells was quantified by flow cytometry. As shown in Fig. 4, 9.38% of the cells transfected with pCprME showed to be in late apoptotic or necrotic stage, which was significantly higher than that in pCN15Q (2.00%), pCN154Q (3.74%), pCN15/154Q (1.30%), or pcDNA3.1 (0.76%) transfected groups. When the N-linked glycosylation sites of prM or E was ablated, the number of apoptotic cells was greatly decreased, suggesting that the loss of glycosylation might lead to lower cytotoxicity.

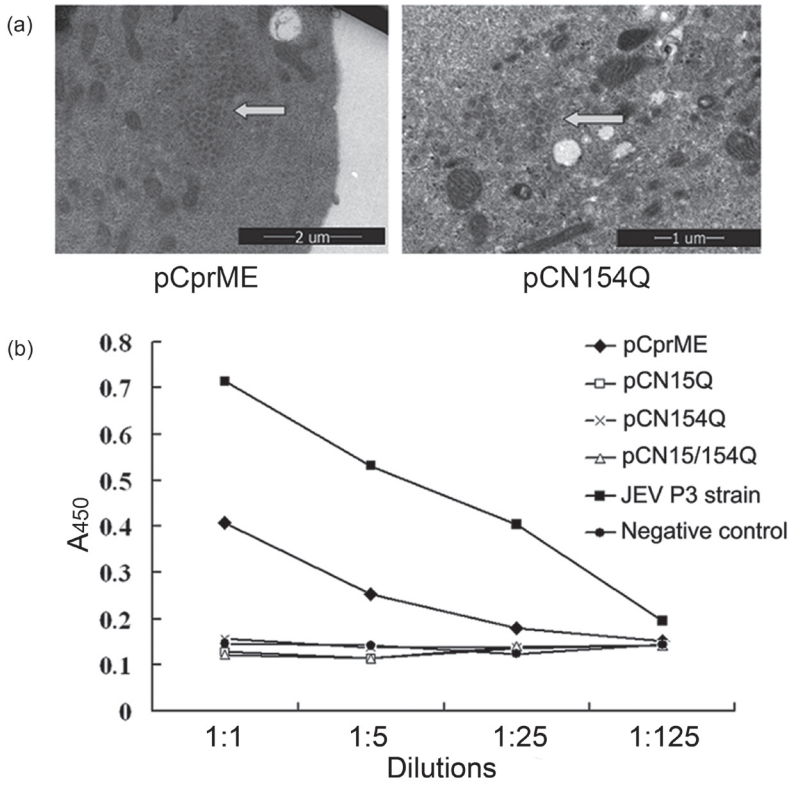

Fig. 3

Effect of N-glycosylation on secretion of the prM-E protein and VLP formation

(a) VLPs (arrows) observed by electron microscopy in HEK cells transfected with individual plasmid constructs. (b) ELISA of prM-E protein in supernatant from HEK cells transfected with individual plasmid constructs. Supernatant dilutions shown on abscissa.

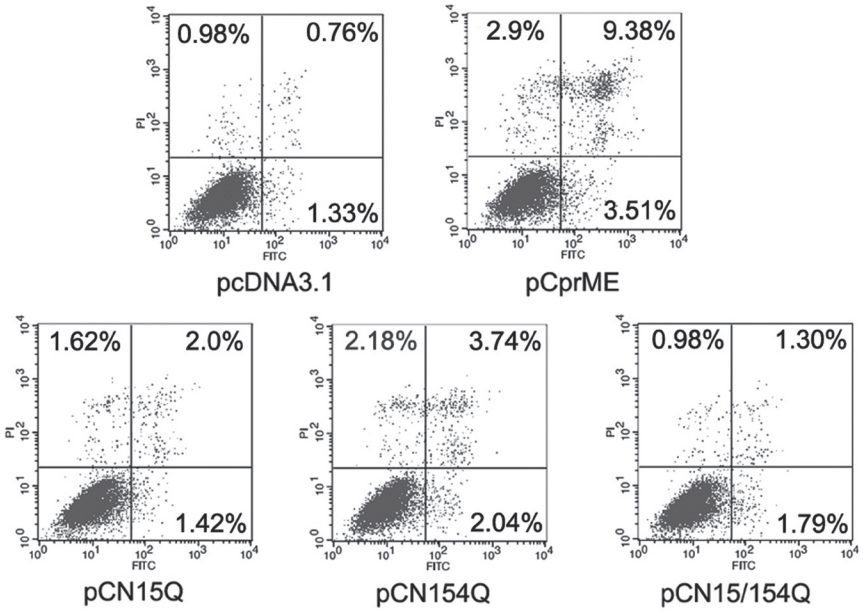

Fig. 4

Detection of apoptotic cells by annexin V and propidium iodid double-staining

Discussion

The envelope (E) is the main structural protein of JEV and it plays important role in infectivity and virulence. E proteins in the family *Flaviviridae* are N-glycosylated, but the site and number of sugar chains differ in flavivirus E proteins (Chambers *et al.*, 1990). The glycosylation site in E protein is known to be critical for viral propagation, infectivity, virulence, and neuroinvasion (Beasley *et al.*, 2005; Bryant *et al.*, 2007; Lee *et al.*, 2010; Li *et al.*, 2006; Shirato *et al.*, 2004). How the glycosylation site in E is affecting the phenotypes of JEV is not clear. In our study the N-glycan in E was found to be an important help factor for the folding of itself as well as assembly and secretion of the viral particles. However, it might not be essential for protein folding and assembly since some of the E proteins could still acquire its native conformation bearing the N-glycosylation site mutant. Viral-like particles were also found in the absence of N-glycosylation in E protein. Thus, it is easy to understand why the N-linked glycosylation sites are so variable in flavivirus E proteins.

prM is known to be an important E chaperone in flaviviruses (Konishi and Mason, 1993; Lorenz *et al.*, 2002). No signal was detected by the E protein CE MAb in the pCE expression cells, which demonstrated that the E protein CE MAb could be a suitable tool for structural E protein detection. The prM protein is also a glycoprotein and its glycosylation site is highly conserved in flaviviruses, suggesting that the glycan may have important role in flaviviruses. Most previous studies have indicated important roles of N-glycosylations sites in E protein, but few have focused on prM N-glycosylation. Kim *et al.* (2008) generated a recombinant JEV with N-glycosylation mutant in prM by reverse genetics and they found that JEV secretion was reduced at the stage of virus release, rather than assembly. However, the glycan in prM protein was located in the precursor (pr) section, which was cleaved in the trans-Golgi network during assembly. Thus, the glycan in prM protein should have a function before viral secretion and it may have effects at the stages of protein expression, location, processing, folding, or assembly. In our experiment, the E proteins were incorrectly folded when the N-linked glycosylation site in prM protein was removed, while the assembly of VLPs was also suppressed. These results showed that the reduction in protein secretion might be due to protein misfolding. Thus, the glycan in prM protein may have more important roles than that in E protein, where it is critical for the folding of E and assembly of VLPs. It is hypothesized that the N-linked glycosylation of prM is critical to keep its own steric configuration. If prM protein can′t fold natively, the chaperone-like role will be disabled and E protein will be misfolded.

Previous studies have shown that high level expression of VLPs could result in fusion, apoptosis, and death in some JEV susceptible cells (Kojima *et al.*, 2003; Konishi *et al.*, 2001). In this study, apoptosis induced by JEV VLPs was found in pCprME transfected 293FT cells, which suggested that the VLPs were also toxic for this cell line. In the glycosylation site mutant groups, apoptosis was reduced if VLPs formation was suppressed, indicating that the cytotoxicity of prM-E protein depended on its correct folding and assembly. JEV infection is also known to lead to ER stress and an unfolded protein response, which triggers p38-dependent apoptosis (Su *et al.*, 2002). In this study, when we removed the N-linked glycosylations of prM or E, we could still observe massive production of proteins and accumulation of mutant proteins in cells. But the apoptosis induced by unfolded protein response was not observed.

In summary, our study demonstrates that N-glycans in prM protein plays critical role in the folding of E protein, and consequently impacts the assembly, secretion and cytotoxicity of JEV particles.

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