**N-linked glycan in tick-borne encephalitis virus envelope protein affects viral secretion in mammalian cells, but not in tick cells**

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Tick-borne encephalitis virus (TBEV) is a zoonotic disease agent that causes severe encephalitis in humans. The envelope protein E of TBEV has one N-linked glycosylation consensus sequence, but little is known about the biological function of the N-linked glycan. In this study, the function of protein E glycosylation was investigated using recombinant TBEV with or without the protein E N-linked glycan. Virion infectivity was not affected after removing the N-linked glycans using N-glycosidase F. In mammalian cells, loss of glycosylation affected the conformation of protein E during secretion, reducing the infectivity of secreted virions. Mice subcutaneously infected with TBEV lacking protein E glycosylation showed no signs of disease, and viral multiplication in peripheral organs was reduced relative to that with the parental virus. In contrast, loss of glycosylation did not affect the secretory process of infectious virions in tick cells. Furthermore, inhibition of transport to the Golgi apparatus affected TBEV secretion in mammalian cells, but not in tick cells, indicating that TBEV was secreted through an unidentified pathway after synthesis in endoplasmic reticulum in tick cells. These results increase our understanding of the molecular mechanisms of TBEV maturation.

**INTRODUCTION**

Tick-borne encephalitis virus (TBEV), a member of the genus *Flavivirus* within the family *Flaviviridae*, causes tick-borne encephalitis (TBE) in humans. TBE is endemic in Europe, Russia and Far-Eastern Asia, including Japan (Blaskovic *et al.*, 1967; Korenberg & Kovalevskii, 1999; Süss, 2011). TBEV can be divided into three subtypes: the Far-Eastern subtype (known as Russian spring–summer encephalitis virus), the European subtype and the Siberian subtype (Ecker *et al.*, 1999; Gritsun *et al.*, 1993, 1997; Wallner *et al.*, 1995). TBE remains a significant public health problem in these endemic regions.

*Flavivirus* virions are spherical with diameters of 40–50 nm and contain a nucleocapsid and envelope. The envelope has two viral proteins: the major envelope protein E and the small membrane protein prM/M. Both proteins are synthesized as part of a polyprotein precursor, which is co- and post-translationally cleaved into the individual proteins (Lindenbach *et al.*, 2007). Protein E has been well characterized and mediates viral entry via receptor-mediated endocytosis; in addition, it contains the major antigenic epitopes that generate protective immune responses (Heinz & Allison, 2003). The X-ray crystallographic structure of TBEV protein E ectodomain revealed that protein E forms head-to-tail homodimers that lie parallel to the viral envelope (Rey *et al.*, 1995). In low-pH conditions, as in endocytic vesicles, the homodimers dissociate, followed by the irreversible formation of homotrimeric (Allison *et al.*, 1995; Stiasny *et al.*, 2001, 2002).

In the majority of TBEV strains, as in other flaviruses, protein E contains a conserved N-linked glycosylation site. It has been reported that the deglycosylation of TBEV by endoglycosidase F does not impair infectivity (Winkler *et al.*, 1987), but the inhibition of N-linked glycosylation reduces the secretion of subviral particles from cells expressing the viral proteins prM and E (Goto *et al.*, 2005; Lorenz *et al.*, 2003). However, as functional analyses of the N-linked glycan on protein E have been limited to inhibitor treatment or subviral particle systems, little is known regarding the effects of glycosylation on the biological properties of infectious virions, including replication in the tick vector and pathogenicity in mammals.

In this study, we used an infectious TBEV cDNA clone to generate infectious virus containing protein E with or without the N-linked glycan and directly examined specific
Fig. 1. Construction of recombinant TBEV containing protein E with or without its N-linked glycan. (a) Schematic of recombinant TBEV. The symbol Y shows the predicted glycans on TBEV envelope proteins. The amino acid sequence of the protein E glycosylation site is expanded at the bottom of the figure for Oshima-IC-pt. In Oshima-IC-ΔEg, the glycosylation site of protein E contains a mutation (bold). (b) Confirmation of protein E glycosylation in recombinant viruses. BHK cells were infected with Oshima-IC-pt (pt) or Oshima-IC-ΔEg (ΔEg). At 48 h p.i., intracellular protein E was immunoprecipitated using anti-E antibodies. Precipitated protein E was detected using anti-E antibodies (left panel) or concanavalin A (right panel).

Fig. 2. Effect of protein E glycosylation on viral replication in BHK cells. (a) BHK cells were infected with Oshima-IC-pt or Oshima-IC-ΔEg at an m.o.i. of 0.01. At each time point, medium was harvested and virus titres were measured using plaque assays in BHK cells. (b) Plaques of Oshima-IC-pt and Oshima-IC-ΔEg in BHK cells at 4 days p.i.
phenotypic changes. Recombinant virus characteristics were examined in mammalian and tick cells as well as in a mouse model. The results suggest that glycosylation is critical for virus activity in mammals, but not in tick vectors.

RESULTS

Generation of glycosylation-deficient TBEV

TBEV protein E has one N-linked glycosylation site at amino acids 154–156. To examine the role of the N-linked glycan, recombinant TBEV expressing protein E that lacks the N-linked glycan, designated Oshima-IC-ΔEg, was constructed using an infectious cDNA clone of Oshima 5-10 TBEV strain. The asparagine at position 154 of protein E was mutated to glutamine to avoid recognition by oligosaccharyltransferase (Fig. 1a).

To confirm the absence of protein E glycosylation, the nucleotide sequences of the recovered Oshima-IC-ΔEg virus were examined. The introduced mutation was conserved even after 10 passages in baby hamster kidney (BHK) cells and no complementary mutation was found in the coding sequence of any structural protein.

BHK cells were infected with the Oshima-IC-parent (pt) or -ΔEg virus, and intracellular protein E was immunoprecipitated with anti-E monoclonal antibody and analysed on Western blots and lectin blots. As shown in Fig. 1(b), protein E was detected in immunoprecipitated eluates from cells infected with either Oshima-IC-pt or -ΔEg, but the band for protein E from Oshima-IC-ΔEg migrated faster than that from Oshima-IC-pt.

Fig. 3. Effect of protein E glycosylation on synthesis and secretion of protein E in BHK cells. BHK cells were infected with Oshima-IC-pt (pt) or Oshima-IC-ΔEg (ΔEg) at an m.o.i. of 0.01. At 48 h p.i., cell lysates and culture supernatants were harvested. Levels of intracellular and secreted protein E were measured by ELISA using (a) anti-E polyclonal antibodies, or (b) monoclonal antibodies recognizing conformational epitopes of protein E. The percentage of protein E was calculated from the calibration curve for the amount of pt in each experiment.
than that of protein E from Oshima-IC-pt (left panel). Protein E from Oshima-IC-pt was detected by concanavalin A, which binds specifically to high-mannose type N-linked glycans, whereas no band from cells infected with Oshima-IC-ΔEg was detected by concanavalin (Fig. 1b, right panel). These data indicate that, as expected, mutated protein E encoded by Oshima-IC-ΔEg virus was not glycosylated.

Characteristics of glycosylation-deficient TBEV in mammalian cells

To examine the effect of glycosylation of protein E on viral multiplication, BHK cells were infected with Oshima-IC-pt or -ΔEg at an m.o.i. of 0.01. Virus was harvested 12–72 h post-infection (p.i.) and the yield was quantified using a plaque assay. As shown in Fig. 2(a), a lower titre of infectious virus was secreted from cells infected with Oshima-IC-ΔEg compared with -pt. The plaque size in BHK cells was also smaller for Oshima-IC-ΔEg than for -pt. These data indicate that glycosylation of protein E affects viral multiplication in BHK cells.

To further characterize the role of glycosylated protein E, the secretion of viral particles was analysed. BHK cells were infected with Oshima-IC-pt or -ΔEg at an m.o.i. of 0.01. At 48 h p.i., cell lysates and culture supernatants were prepared and the levels of intracellular and secreted protein E were quantified. With the ELISA using polyclonal anti-E antibodies, the levels of intracellular and secreted protein E were similar between cells infected with Oshima-IC-pt and with -ΔEg (Fig. 3a). However, based on the ELISA using monoclonal antibodies specific for protein E conformational epitopes, low levels of protein E were detected in the culture supernatant of cells infected with Oshima-IC-ΔEg compared with -pt, while the levels of protein E in cell lysates were similar between the cells infected with Oshima-IC-pt and with -ΔEg (Fig. 3b). These data show that the lack of protein E glycosylation did not affect the production or secretion of protein E, but did affect the conformation of secreted protein E.

Next, we examined whether the N-linked glycan on protein E of secreted TBEV was involved in viral entry. A total of 100 p.f.u. of Oshima-IC-pt (pt) or Oshima-IC-ΔEg (ΔEg) was treated with serially diluted N-glycosidase F and the virus titres were determined using plaque assays in BHK cells. The p.f.u. of mock-treated virus was set at 100 %.

Although the N-linked glycan was not required for viral entry.

Characteristics of glycosylation-deficient TBEV in tick cells

The effect of the absence of protein E glycosylation was examined in the tick cell line ISE6. As was observed with BHK cells, protein E of Oshima-IC-pt was glycosylated but protein E of -ΔEg was not (Fig. 5a). In contrast to BHK cells, ISE6 cells infected with Oshima-IC-pt and with -ΔEg showed no difference in viral multiplication (Fig. 5b) or the amount of secreted protein E detected using anti-E monoclonal antibodies recognizing protein E conformational epitopes (Fig. 5c). To investigate whether the different incubation temperature between mammalian cells (37 °C) and tick cells (34 °C) affected the stability of the unglycosylated E protein, the virus multiplication was examined in BHK cells at 34 °C. As was observed with the incubation at 37 °C (Fig. 2a), a lower titre of infectious virus was secreted from cells infected with Oshima-IC-ΔEg compared with -pt in BHK cells at 34 °C (Fig. S1, available in JGV Online). These results indicate that the glycosylation of protein E was not important for viral multiplication or secretion in tick cells.

Flaviviruses are generally thought to bud into the endoplasmic reticulum of virus-infected cells, followed by transport in vesicles to the Golgi complex and release by exocytosis via the trans-Golgi network (Lindenbach et al., 2007; Mackenzie & Westaway, 2001). To analyse the differences in the roles of TBEV glycosylation in maturation and secretory processes between BHK and ISE6 cells, the effects of inhibitors of cellular secretory mechanisms were investigated in virus-infected cells. Tunicamycin was used to inhibit the glycosylation of newly synthesized
glycoproteins (Elbein, 1987). Tunicamycin treatment of BHK cells infected with Oshima-IC-pt or -ΔEg reduced the secreted virus titre; the reduction was the same for Oshima-IC-pt and -ΔEg (Fig. 6a). In ISE6 cells infected with Oshima-IC-pt or -ΔEg, tunicamycin treatment did not reduce the virus titres (Fig. 6b). This suggests that the glycosylation of newly synthesized glycoproteins, including protein E and other glycoproteins such as protein prM or NS1, is important for the maturation and secretion of TBEV in mammalian BHK cells, but not in tick ISE6 cells. The secretion of infectious virions was further analysed using brefeldin A (BFA), which interferes with anterograde transport from the endoplasmic reticulum to the Golgi apparatus (Fujiwara et al., 1988). BFA treatment of infected BHK cells significantly reduced the titres of secreted Oshima-IC-pt and -ΔEg, whereas BFA treatment of infected ISE6 cells did not reduce the virus titres (Fig. 6a, b). The glycosylation of the E proteins of Oshima-IC-pt was examined in ISE6 cells treated with tunicamycin or BFA (Fig. 6c). Tunicamycin treatment inhibited the glycosylation of the E proteins, whereas the E proteins were still glycosylated after BFA treatment. Thus, the E proteins were naturally glycosylated in ER after synthesis in ISE6 cells, but this was not necessary for virus secretion. Furthermore, the secretion of TBEV in tick ISE6 cells was independent of the traditional secretory pathway through the Golgi apparatus.

**Effect of glycosylation on virulence in mice**

The effect of glycosylation on pathogenicity was examined in a mouse model. Five-week-old female C57BL/6J mice were infected subcutaneously with Oshima-IC-pt or -ΔEg at 10⁵ p.f.u. mouse⁻¹ and monitored for 28 days (Fig. 7). All mice infected with Oshima-IC-pt showed general signs of illness such as hunched posture, ruffled fur and general malaise; one mouse died. However, no mice infected with Oshima-IC-ΔEg showed signs of illness or died.
To examine the correlation between disease development and viral replication in organs, viral loads in the blood, spleen and brain were compared between mice inoculated with 10^5 p.f.u. of Oshima-IC-pt and 10^5 p.f.u. of -ΔEg (Fig. 8). The levels of transient viraemia and multiplication in the spleen were lower in mice infected with Oshima-IC-D Eg than in those infected with -pt. In the brain, the virus was detected from 6 days p.i. in mice infected with Oshima-IC-pt, while with -ΔEg a low titre of virus was detected in only one mouse at 12 days p.i. These data indicate that the Oshima-IC-ΔEg virus cannot multiply efficiently in organs, leading to a loss of virulence in mice.

Similar high titres of neutralizing antibodies (>320) were observed in mice infected with Oshima-IC-pt or with -ΔEg at 12 days p.i. (data not shown), suggesting that lack of the N-linked glycan on protein E did not affect the induction of neutralizing antibodies.

**DISCUSSION**

N-linked glycans on viral glycoproteins play important roles in viral multiplication, immunogenicity and pathogenicity (Vigerust & Shepherd, 2007). In this study, we used an infectious TBEV cDNA clone to generate infectious virus with or without protein E N-linked glycan and investigated specific phenotypic changes in mammalian and tick cells.

The defect in protein E glycosylation reduced the secretion of infectious virions in mammalian cells. In studies of West Nile virus and dengue virus, a defect in glycosylation caused similar reductions in the release of infectious virions (Hanna et al., 2005; Lee et al., 2010; Li et al., 2006). Although the total level of secreted protein E remained constant, the conformational structure of protein E was affected by the lack of glycosylation, resulting in reduced virion infectivity. However, cleavage of the N-linked glycan after secretion did not affect virion infectivity in mammalian cells. These results indicate that glycosylation is important in retaining the conformational structure of protein E, which is necessary for virion infectivity during the intracellular secretory process in mammalian cells. In the endoplasmic reticulum, two homologous resident lectins (calnexin and calreticulin) bind N-linked core glycans and promote proper folding of glycoproteins (Ellgaard et al., 1999). It is known that the loss of glycosylation alters West Nile virus virion stability at mildly acidic pH (Beasley et al., 2005). Defects in protein E glycosylation may affect the proper folding and/or stability of virions, reducing the infectivity of TBEV in mammalian cells.
In the mouse model, protein E glycosylation affected TBEV pathogenicity. TBEV without protein E glycosylation did not multiply efficiently in peripheral organs, and eventually the virus could not enter the brain or cause disease in mice. Similarly, reduced neuroinvasiveness due to a defect in glycosylation was reported in West Nile virus studies (Beasley et al., 2005; Shirato et al., 2004). The mechanism of neuroinvasiveness of TBEV is unclear, but it has been reported that efficient viral multiplication in peripheral organs is required for TBEV entry into the brain (Mandl, 2005). Reduced infectivity of secreted virions owing to a defect in the glycosylation of protein E, as observed in cultured cells, is thought to reduce viral multiplication in peripheral organs and to reduce neuroinvasiveness.

TBEV with non-glycosylated protein E could efficiently induce neutralizing antibodies against TBEV without any clinical symptoms. Also, no revertant or compensatory mutation occurred during passaging. These data suggest that deletion of the protein E glycosylation site could attenuate TBEV.

The lack of protein E glycosylation did not affect the TBEV secretory process in tick cells, unlike in mammalian cells. Furthermore, the inhibition of transport from the endoplasmic reticulum to the Golgi apparatus did not affect TBEV multiplication in tick cells. In a previous report, nascent TBEV particles were observed inside vacuoles, and free nucleocapsids were seen in the cytosol or attached to the membrane of virus particle-containing vacuoles in tick cells, whereas viral particles appeared in the endoplasmic reticulum, Golgi apparatus and secretory pathway in mammalian cells (Senigl et al., 2006). Taken together, our data suggest that TBEV secretion in tick cells occurs through an unidentified mechanism different from the traditional secretory pathway through the Golgi apparatus.

Glycosylation-independent virus secretion was observed only in TBEV-infected tick cells. However, studies of mosquito-borne flavivirus have shown that glycosylation of protein E is important in both mammalian and mosquito cells (Hanna et al., 2005; Lee et al., 2010). The difference in virus maturation between arthropod vectors may be associated with the different ecology of tick-borne and mosquito-borne flaviviruses in their arthropod vectors. Unlike mosquito-borne flaviviruses, tick-borne flaviviruses establish and maintain a persistent infection across the various life-stages of the tick vector, through transstadial

**Fig. 7.** (a) Survival rate, (b) morbidity and (c) weight change following infection with TBEV. B6 mice were subcutaneously infected with $10^5$ p.f.u. of Oshima-IC-pt (filled circles) or Oshima-IC-$\Delta$Eg (open squares) and monitored for 28 days. Mouse morbidity was estimated based on >10% weight loss. The mean daily weight change was calculated based on the ratio of the daily weight to the weight at day 0. Error bars represent standard deviations.
and transovarial transmission in nature (Nuttall & Labuda, 2003). In persistent tick infections, viruses are thought to multiply regardless of the glycosylation of the E proteins. It is possible that because TBEV with glycosylated protein E has more effective transmission from tick vectors to mammals, it had a selective advantage during viral evolution.

In summary, we generated recombinant TBEV with or without glycosylated protein E. Deletion of the glycosylation site affected the maturation of TBEV infectious virions in mammalian cells and reduced TBEV virulence in mice. Our results suggest that TBEV is secreted in a glycosylation-independent manner in tick cells. Overall, these results increase our understanding of the molecular mechanism of TBEV maturation and can be applied to attenuate TBEV infection.

**METHODS**

**Cells.** BHK cells were grown at 37 °C in Eagle's MEM supplemented with 8% fetal bovine serum (FBS) and L-glutamine. The ISE6 cell line from *Ixodes scapularis* was grown at 34 °C in L-15B medium with 10% FBS and 5% tryptose phosphate broth.

**Virus.** TBEV Oshima-IC was prepared from infectious cDNA clones of Oshima 5-10 strain (accession no. AB062003) (Hayasaka et al., 2004), isolated in Hokkaido, Japan in 1995 (Takashima et al., 1997). Standard PCR mutagenesis techniques were used to construct the Oshima-IC-ΔEg virus, in which nucleotides for the glycosylation site in protein E were mutated as shown in Fig. 1(a).

RNA was transcribed from the Oshima-IC plasmid using a mMESSAGE mMACHINE SP6 kit (Life Technology) and was transfected into BHK cells using TransIT-mRNA (Mirus Bio) as described previously (Yoshii et al., 2004, 2011).

**Reagents.** N-glycosidase F (Roche) was used to cleave protein E N-linked glycan in infectious virions. A total of 100 TBEV p.f.u. were treated with serially diluted N-glycosidase F (10 μU ml⁻¹ to 1 U ml⁻¹) for 1 h at 37 °C and the virus was titrated.

The effects of tunicamycin (Sigma-Aldrich) and brefeldin A (Wako) on the secretion of viral particles were examined. At the indicated times p.i., virus-infected cells were treated with 2 μg ml⁻¹ of tunicamycin, or 2 μg ml⁻¹ of brefeldin A and the secreted virus was titrated after 12 h.

**Immunoprecipitation, SDS-PAGE, immunoblotting and lectin blotting.** BHK cells were infected with Oshima-IC-parent (pt) or -ΔEg. At 48 h p.i., the cells were lysed with 1% Triton X-100 in 10 mM TBS, incubated on ice for 20 min and centrifuged (16000 g, 20 min). The supernatant (excluding the nuclear fraction) was precleared on protein G-Sepharose beads (Amersham Pharmacia Biotech) for 2 h at 4 °C. The precleared lysates were precipitated with protein G-Sepharose beads with mouse monoclonal anti-E antibody

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**Fig. 8.** Virus replication in organs. Mice were subcutaneously infected with 10⁶ p.f.u. of Oshima-IC-pt (filled circles) or Oshima-IC-ΔEg (open squares). At the indicated days after infection, virus titres in (a) blood, (b) spleen and (c) brain were determined using plaque assays. Error bars represent standard deviations (n=3).
H4 (Komoro et al., 2000) for 2 h at 4 °C. Immune complexes were collected by centrifugation (10,000 g, 10 s) and washed four times with 1% Triton X-100 in 10 mM TBS. Protein samples were electrophoresed through 12% (w/v) polyacrylamide–SDS gels. 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. After a wash with 25 mM TBS containing 0.01% (v/v) Tween 20, the membranes were reacted with rabbit polyclonal anti-E antibodies (Yoshii et al., 2004) or biotinylated lectin concanavalin A (J-Oil Mills), followed by alkaline phosphatase-conjugated secondary antibody or streptavidin (Jackson ImmunoResearch), respectively. Protein bands were visualized using an alkaline phosphatase detection kit (Merck) according to the manufacturer’s protocol.

**ELISA.** The TBEV protein E was detected by sandwich-ELISA using a set of anti-E polyclonal antibodies or monoclonal antibodies recognizing conformational epitopes of protein E. Briefly, to prepare samples, virus-infected cells were lysed with 1% (v/v) Triton X-100 in 10 mM TBS and the supernatants were treated with 1% Triton X-100.

For ELISA using a set of anti-E polyclonal antibodies, Triton X-100-solubilized samples were added to 96-well microtitre plates coated with rabbit polyclonal anti-E antibodies. After blocking with 3% (w/v) BSA, protein E was detected by incubation with TBEV-infected mouse serum and horseradish peroxidase-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch).

For ELISA using monoclonal antibodies recognizing conformational epitopes of protein E, samples were added to wells coated with mouse monoclonal anti-E antibody H4, previously blocked with 3% (w/v) BSA. Protein E was detected by incubation with biotinylated monoclonal antibody (mAb) 4H8 and peroxidase-conjugated streptavidin (Sigma). Peroxidase activity was detected by adding o-phenylenediamine dihydrochloride (Sigma) in the presence of 0.03% (v/v) H2O2 and the absorbance was measured at 450 nm.

**Virulence in mouse.** Viruses were inoculated subcutaneously into 5-week-old female C57BL/6J mice (Charles River Laboratories). Morbidity was defined as >10% weight loss. The mice were monitored for 28 days p.i. to determine the survival curve and mortality rate. To analyse the virus distribution in tissues, the serum, brains and spleens were collected from mice 3, 6, 9 and 12 days p.i. The organs were weighed individually, homogenized and prepared as 10% suspensions in PBS (w/v) containing 10% FBS. The suspensions were clarified by centrifugation (1200 g for 5 min, 4 °C) and the supernatants were titrated using plaque assays in BHK cells.

**Titration and neutralization test.** For titration, cell monolayers prepared in 12-well plates were incubated with serial dilutions of the virus for 1 h, overlaid with minimal medium containing 2% FBS and 1.5% carboxymethyl cellulose, and incubated for 3 days. After incubation, the cells were fixed and stained with 0.25% crystal violet in 10% buffered formalin. Plaques were counted and expressed as p.f.u. ml⁻¹. For the neutralization test, serum samples that induced a 50% reduction in Oshima-IC-p7 plaque formation were examined.

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