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

Running title: Roles of N-linked glycan in TBEV envelope protein

The Content Category: Animal Viruses-Positive-strand RNA

Kentaro Yoshii, Natsumi Yanagihara, Mariko Ishizuka, Mizuki Sakai, Hiroaki Kariwa

Laboratory of Public Health, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan

Corresponding author: Kentaro Yoshii, Ph. D., D.V.M.

Postal address: Laboratory of Public Health, Graduate School of Veterinary Medicine, Hokkaido University, kita-18 nishi-9, kita-ku, Sapporo, Hokkaido 060-0818, Japan

Tel/fax: +81-11-706-5213

E-mail: kyoshii@vetmed.hokudai.ac.jp

Word count for summary: 188

Word count for text: 3992

Abbreviations

Baby hamster kidney: BHK

brefeldin A: BFA

fetal bovine serum: FBS

minimal essential medium: MEM

monoclonal antibody: MAb

parent: pt

Tick-borne encephalitis: TBE

Tick-borne encephalitis virus: TBEV

Summary

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-East Asia, including Japan (Blaskovic *et al.*, 1967; Korenberg & Kovalevskii, 1999; Suss, 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; Gritsun *et al.*, 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 homotrimers (Allison *et al.*, 1995; Stiasny *et al.*, 2001; Stiasny *et al.*, 2002).

In the majority of TBEV strains, as in other flaviviruses, 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 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 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 analyzed on Western blots and lectin blots. As shown in Fig. 1b, 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 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 a multiplicity of infection of 0.01. Virus was harvested 12 to 72 h post-infection, and the yield was quantified using a plaque assay. As shown in Fig. 2a, a lower titer of infectious virus was secreted from cells infected with Oshima-IC-ΔEg compared with Oshima-IC-pt. The plaque size in BHK cells was also smaller for Oshima-IC-ΔEg than for Oshima-IC-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 analyzed. BHK cells were infected with Oshima-IC-pt or -ΔEg at a multiplicity of infection of 0.01. At 48 h post-infection, 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 Oshima-IC-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 Oshima-IC-pt, while the levels of protein E in cell lysates were similar between the cells infected with Oshima-IC-pt and with Oshima-IC-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 pfu of Oshima-IC-pt or -ΔEg was treated with serially diluted N-glycosidase F, which cleaves all types of asparagine-bound glycans, and the infectivity of the resultant virus was analyzed using a plaque assay (Fig. 4). There was no reduction of the virus titer of Oshima-IC-pt or –ΔEg even after treatment with 1 U ml^{-1} of N-glycosidase F, indicating that the cleavage of N-linked glycans on secreted TBEV does not directly affect viral entry processes such as receptor binding and membrane fusion.

These results demonstrate that the conformational structure of protein E during secretion was affected by the lack of N-linked glycosylation and this reduced virion infectivity, 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, and protein E of Oshima-IC-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 titer of infectious virus was secreted from cells infected with Oshima-IC-ΔEg compared with Oshima-IC-pt in BHK cells at 34°C (supplementary figure 1). 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 ER 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 analyze 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 titer, and 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 titer of Oshima-IC-pt or ΔEg (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 analyzed 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 titers of secreted Oshima-IC-pt and –ΔEg, whereas BFA treatment of infected ISE6 cells did not reduce the virus titers (Fig. 6a and 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 while The E proteins were still glycosylated after BFA treatment. Thus, the E proteins were naturally glycosylated in ER after synthesis in ISE6 cells, but it 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.

The effect of the glycosylation on the 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 10^5 pfu/mouse 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

177 infected with Oshima-IC-ΔEg showed signs of illness or died.

178 To examine the correlation between disease development and viral replication in organs, viral
179 loads in the blood, spleen, and brain were compared between mice inoculated with 10^5 pfu of
180 Oshima-IC-pt and 10^5 pfu of Oshima-IC-ΔEg (Fig. 8). The levels of transient viremia and
181 multiplication in the spleen were lower in mice infected with Oshima-IC-ΔEg than in those infected
182 with Oshima-IC-pt. In the brain, the virus was detected from 6 days post-infection in mice infected
183 with Oshima-IC-pt, while a low titer of virus was detected in only one mouse at 12 days
184 post-infection with Oshima-IC-ΔEg. These data indicate that the Oshima-IC-ΔEg virus cannot
185 multiply efficiently in organs, leading to a loss of virulence in mice.
186 Similar high titers of neutralizing antibodies (>320) were observed in mice infected with
187 Oshima-IC-pt or with Oshima-IC-ΔEg at 12 days post-infection (data not shown), suggesting that
188 lack of the N-linked glycan on protein E did not affect the induction of neutralizing antibodies.
189
190 **Discussion**
191
192 N-linked glycans on viral glycoproteins play important roles in viral multiplication,
193 immunogenicity, and pathogenicity (Vigerust & Shepherd, 2007). In this study, we used an infectious
194 TBEV cDNA clone to generate infectious virus with or without protein E N-linked glycan and
195 investigated specific phenotypic changes in mammalian and tick cells.
196 The defect in protein E glycosylation reduced the secretion of infectious virions in mammalian
197 cells. In studies of West Nile virus and dengue virus, a defect in glycosylation caused similar
198 reductions in the release of infectious virions (Hanna *et al.*, 2005; Lee *et al.*, 2010; Li *et al.*, 2006).
199 Although the total level of secreted protein E remained constant, the conformational structure of
200 protein E was affected by the lack of glycosylation, resulting in reduced virion infectivity. However,
201 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 pHs (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 a West Nile virus study (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 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

Baby hamster kidney (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.

252

253 ***Virus***

254 TBEV Oshima-IC was prepared from infectious cDNA clones of Oshima 5-10 strain (accession
255 No. AB062003) (Hayasaka *et al.*, 2004), isolated in Hokkaido, Japan in 1995 (Takashima *et al.*,
256 1997). Standard PCR mutagenesis techniques were used to construct the Oshima-IC-ΔEg virus, in
257 which nucleotides for the glycosylation site in protein E were mutated as shown in Fig. 1a.

258 RNA was transcribed from the Oshima-IC plasmid using a mMESSAGE mMACHINE SP6 kit
259 (Life Technology. Carlsbad, CA, USA) and was transfected into BHK cells using TransIT-mRNA
260 (Mirus Bio, Madison, WI, USA), as described previously (Yoshii *et al.*, 2011; Yoshii *et al.*, 2004).

261

262 ***Reagents***

263 N-glycosidase F (Roche, Basel, Switzerland) was used to cleave protein E N-linked glycan in
264 infectious virions. A total of 100 TBEV plaque forming units (pfu) were treated with serially diluted
265 N-glycosidase F (10 μU ml^{-1} to 1 U ml^{-1}) for 1 h at 37°C, and the virus was titrated.

266 The effects of tunicamycin (Sigma-Aldrich, St. Louis, MO, USA), and brefeldin A (Wako, Osaka,
267 Japan) on the secretion of viral particles were examined. At the indicated times post-infection,
268 virus-infected cells were treated with 2 μg ml^{-1} of tunicamycin, or 2 μg ml^{-1} of brefeldin A, and the
269 secreted virus was titrated after 12 h.

270

271 ***Immunoprecipitation, SDS-PAGE, immunoblotting and lectin-blotting***

272 BHK cells were infected with Oshima-IC-parent (pt) or ΔEg. At 48 h post-infection, the cells
273 were lysed with 1% Triton X-100 in 10 mM TBS, incubated on ice for 20 min, and centrifuged
274 (16,000 × g, 20 min). The supernatant (excluding the nuclear fraction) was precleared on protein
275 G-Sepharose beads (Amersham Pharmacia Biotech) for 2 h at 4°C. The precleared lysates were
276 precipitated with protein G-Sepharose beads with mouse monoclonal anti-E antibody 1H4 (Komoro

277 *et al.*, 2000) for 2 h at 4°C. Immune complexes were collected by centrifugation (10,000 × g, 10 s)
278 and washed four times with 1% Triton X-100 in 10 mM TBS. Protein samples were electrophoresed
279 through 12% (w/v) polyacrylamide-SDS gels. Protein bands were transferred onto PVDF membranes
280 and incubated with 1% (w/v) gelatin in 25 mM TBS containing 0.01% (v/v) Tween 20. After a wash
281 with 25 mM TBS containing 0.01% (v/v) Tween 20, the membranes were reacted with rabbit
282 polyclonal anti-E antibodies (Yoshii *et al.*, 2004) or biotinylated lectin concanavalin A (J-Oil Mills,
283 Tokyo, Japan), followed by alkaline phosphatase-conjugated secondary antibody or streptavidin
284 (Jackson ImmunoReserach, West Grove, PA, USA), respectively. Protein bands were visualized
285 using an alkaline phosphatase detection kit (Merck, Darmstadt, Germany) according to the
286 manufacturer's protocol.
287
288 ***ELISA***
289 The TBEV protein E was detected by sandwich-ELISA using a set of anti-E polyclonal
290 antibodies or monoclonal antibodies recognizing conformational epitopes of protein E. Briefly, to
291 prepare samples, virus-infected cells were lysed with 1% (v/v) Triton X-100 in 10 mM TBS, and the
292 supernatants were treated with 1% Triton X-100.
293 For ELISA using a set of anti-E polyclonal antibodies, Triton X-100-solubilized samples were
294 added to 96-well microtiter plates coated with rabbit polyclonal anti-E antibodies. After blocking
295 with 3% (w/v) bovine serum albumin, protein E was detected by incubation with TBEV-infected
296 mouse serum and horseradish peroxidase-conjugated anti-mouse IgG antibody (Jackson
297 ImmunoResearch).
298 For ELISA using monoclonal antibodies recognizing conformational epitopes of protein E,
299 samples were added to wells coated with mouse monoclonal anti-E antibody 1H4, previously
300 blocked with 3% (w/v) bovine serum albumin. Protein E was detected by incubation with
301 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) H_2O_2, 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 International, Inc., Wilmington, MA, USA). Morbidity was defined as >10% weight loss. The mice were monitored for 28 days post-infection determine the survival curve and mortality rate. To analyze the virus distribution in tissues, the serum, brains, and spleens were collected from mice 3, 6, 9, and 12 days post-infection. The organs were weighed individually, homogenized, and prepared as 10% suspensions in PBS (w/v) containing 10% FBS. The suspensions were clarified by centrifugation (4,000 rpm 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 5 days. After incubation, the cells were fixed and stained with 0.25% crystal violet in 10% buffered formalin. Plaques were counted and expressed as pfu ml^{-1}. For the neutralization test, serum samples that induced a 50% reduction in Oshima-IC-pt plaque formation were examined.

Acknowledgement

This work was supported by Grants-in-Aid for Scientific Research (22780268 and 21405035)

and the Global COE Program from the Ministry of Education, Culture, Sports, Sciences and Technology of Japan, and Health Sciences Grants for Research on Emerging and Re-emerging Infectious Disease from the Ministry of Health, Labour and Welfare of Japan.

Author Disclosure Statement

No competing financial interests exist in this paper.

References

Allison, S. L., Schalich, J., Stiasny, K., Mandl, C. W., Kunz, C. & Heinz, F. X. (1995). Oligomeric rearrangement of tick-borne encephalitis virus envelope proteins induced by an acidic pH. *J Virol* **69**, 695-700.

Beasley, D. W., Whiteman, M. C., Zhang, S., Huang, C. Y., Schneider, B. S., Smith, D. R., Gromowski, G. D., Higgs, S., Kinney, R. M. & Barrett, A. D. (2005). Envelope protein glycosylation status influences mouse neuroinvasion phenotype of genetic lineage 1 West Nile virus strains. *J Virol* **79**, 8339-8347.

Blaskovic, D., Pucekova, G., Kubinyi, L., Stupalova, S. & Oravcova, V. (1967). An epidemiological study of tick-borne encephalitis in the Tribec region: 1953-63. *Bull World Health Organ* **36**, Suppl 1:89-94.

Ecker, M., Allison, S. L., Meixner, T. & Heinz, F. X. (1999). Sequence analysis and genetic classification of tick-borne encephalitis viruses from Europe and Asia. *The Journal of general virology* **80**, 179-185.

Elbein, A. D. (1987). Inhibitors of the biosynthesis and processing of N-linked oligosaccharide chains. *Annual review of biochemistry* **56**, 497-534.

Ellgaard, L., Molinari, M. & Helenius, A. (1999). Setting the standards: quality control in the secretory pathway. *Science* **286**, 1882-1888.

Fujiwara, T., Oda, K., Yokota, S., Takatsuki, A. & Ikehara, Y. (1988). Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. *The Journal of biological chemistry* **263**, 18545-18552.

Goto, A., Yoshii, K., Obara, M., Ueki, T., Mizutani, T., Kariwa, H. & Takashima, I. (2005). Role of the N-linked glycans of the prM and E envelope proteins in tick-borne encephalitis virus particle secretion. *Vaccine* **23**, 3043-3052.

Gritsun, T. S., Frolova, T. V., Pogodina, V. V., Lashkevich, V. A., Venugopal, K. & Gould, E. A. (1993). Nucleotide and deduced amino acid sequence of the envelope gene of the Vasilchenko strain of TBE virus; comparison with other flaviviruses. *Virus research* **27**, 201-209.

Gritsun, T. S., Venugopal, K., Zanotto, P. M., Mikhailov, M. V., Sall, A. A., Holmes, E. C., Polkinghorne, I., Frolova, T. V., Pogodina, V. V., Lashkevich, V. A. & Gould, E. A. (1997). Complete sequence of two tick-borne flaviviruses isolated from Siberia and the UK: analysis and significance of the 5' and 3'-UTRs. *Virus research* **49**, 27-39.

Hanna, S. L., Pierson, T. C., Sanchez, M. D., Ahmed, A. A., Murtadha, M. M. & Doms, R. W. (2005). N-linked glycosylation of west nile virus envelope proteins influences particle assembly and infectivity. *J Virol* **79**, 13262-13274.

Hayasaka, D., Gritsun, T. S., Yoshii, K., Ueki, T., Goto, A., Mizutani, T., Kariwa, H., Iwasaki, T., Gould, E. A. & Takashima, I. (2004). Amino acid changes responsible for attenuation of virus neurovirulence in an infectious cDNA clone of the Oshima strain of tick-borne encephalitis virus. *The Journal of general virology* **85**, 1007-1018.

Heinz, F. X. & Allison, S. L. (2003). Flavivirus structure and membrane fusion. *Advances in virus research* **59**, 63-97.

Komoro, K., Hayasaka, D., Mizutani, T., Kariwa, H. & Takashima, I. (2000). Characterization of monoclonal antibodies against Hokkaido strain tick-borne encephalitis virus. *Microbiology and immunology* **44**, 533-536.

Korenberg, E. I. & Kovalevskii, Y. V. (1999). Main features of tick-borne encephalitis eco-epidemiology in Russia. *Zentralbl Bakteriol* **289**, 525-539.

Lee, E., Leang, S. K., Davidson, A. & Lobigs, M. (2010). Both E protein glycans adversely affect dengue virus infectivity but are beneficial for virion release. *J Virol* **84**, 5171-5180.

Li, J., Bhuvanakantham, R., Howe, J. & Ng, M. L. (2006). The glycosylation site in the envelope protein of West Nile virus (Sarafend) plays an important role in replication and maturation processes. *The Journal of general virology* **87**, 613-622.

Lindenbach, B. D., Thiel, H. J. & Rice, C. M. (2007). *Flaviviridae:* The viruses and their replication. In *Fields virology*, 5th edn, pp. 1101-1152. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott Williams & Wilkins.

Lorenz, I. C., Kartenbeck, J., Mezzacasa, A., Allison, S. L., Heinz, F. X. & Helenius, A. (2003). Intracellular assembly and secretion of recombinant subviral particles from tick-borne encephalitis virus. *J Virol* **77**, 4370-4382.

Mackenzie, J. M. & Westaway, E. G. (2001). Assembly and maturation of the flavivirus Kunjin virus appear to occur in the rough endoplasmic reticulum and along the secretory pathway, respectively. *J Virol* **75**, 10787-10799.

Mandl, C. W. (2005). Steps of the tick-borne encephalitis virus replication cycle that affect neuropathogenesis. *Virus research* **111**, 161-174.

Nuttall, P. A. & Labuda, M. (2003). Dynamics of infection in tick vectors and at the tick-host interface. *Advances in virus research* **60**, 233-272.

Rey, F. A., Heinz, F. X., Mandl, C., Kunz, C. & Harrison, S. C. (1995). The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution. *Nature* **375**, 291-298.

Senigl, F., Grubhoffer, L. & Kopecky, J. (2006). Differences in maturation of tick-borne encephalitis virus in mammalian and tick cell line. *Intervirology* **49**, 239-248.

Shirato, K., Miyoshi, H., Goto, A., Ako, Y., Ueki, T., Kariwa, H. & Takashima, I. (2004). Viral envelope protein glycosylation is a molecular determinant of the neuroinvasiveness of the New York strain of West Nile virus. *The Journal of general virology* **85**, 3637-3645.

Stiasny, K., Allison, S. L., Mandl, C. W. & Heinz, F. X. (2001). Role of metastability and acidic pH in membrane fusion by tick-borne encephalitis virus. *J Virol* **75**, 7392-7398.

Stiasny, K., Allison, S. L., Schalich, J. & Heinz, F. X. (2002). Membrane interactions of the tick-borne encephalitis virus fusion protein E at low pH. *J Virol* **76**, 3784-3790.

Suss, J. (2011). Tick-borne encephalitis 2010: epidemiology, risk areas, and virus strains in Europe and Asia-an overview. *Ticks and tick-borne diseases* **2**, 2-15.

Takashima, I., Morita, K., Chiba, M., Hayasaka, D., Sato, T., Takezawa, C., Igarashi, A., Kariwa, H., Yoshimatsu, K., Arikawa, J. & Hashimoto, N. (1997). A case of tick-borne encephalitis in Japan and isolation of the the virus. *Journal of clinical microbiology* **35**, 1943-1947.

Vigerust, D. J. & Shepherd, V. L. (2007). Virus glycosylation: role in virulence and immune interactions. *Trends in microbiology* **15**, 211-218.

Wallner, G., Mandl, C. W., Kunz, C. & Heinz, F. X. (1995). The flavivirus 3'-noncoding region: extensive size heterogeneity independent of evolutionary relationships among strains of tick-borne encephalitis virus. *Virology* **213**, 169-178.

Winkler, G., Heinz, F. X. & Kunz, C. (1987). Studies on the glycosylation of flavivirus E proteins and the role of carbohydrate in antigenic structure. *Virology* **159**, 237-243.

Yoshii, K., Igarashi, M., Ito, K., Kariwa, H., Holbrook, M. R. & Takashima, I. (2011). Construction of an infectious cDNA clone for Omsk hemorrhagic fever virus, and characterization of mutations in NS2A and NS5. *Virus research* **155**, 61-68.

Yoshii, K., Konno, A., Goto, A., Nio, J., Obara, M., Ueki, T., Hayasaka, D., Mizutani, T., Kariwa, H. & Takashima, I. (2004). Single point mutation in tick-borne encephalitis virus prM protein induces a reduction of virus particle secretion. *The Journal of general virology* **85**, 3049-3058.

Figure legends

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 post-infection, 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 ΔEg at a multiplicity of infection of 0.01. At each time point, medium was harvested, and virus titers were measured using plaque assays in BHK cells.

(b) Plaques of Oshima-IC-pt and ΔEg in BHK cells at 4 days post-infection.

Fig. 3. Effect of protein E glycosylation on synthesis and secretion of protein E in BHK cells.

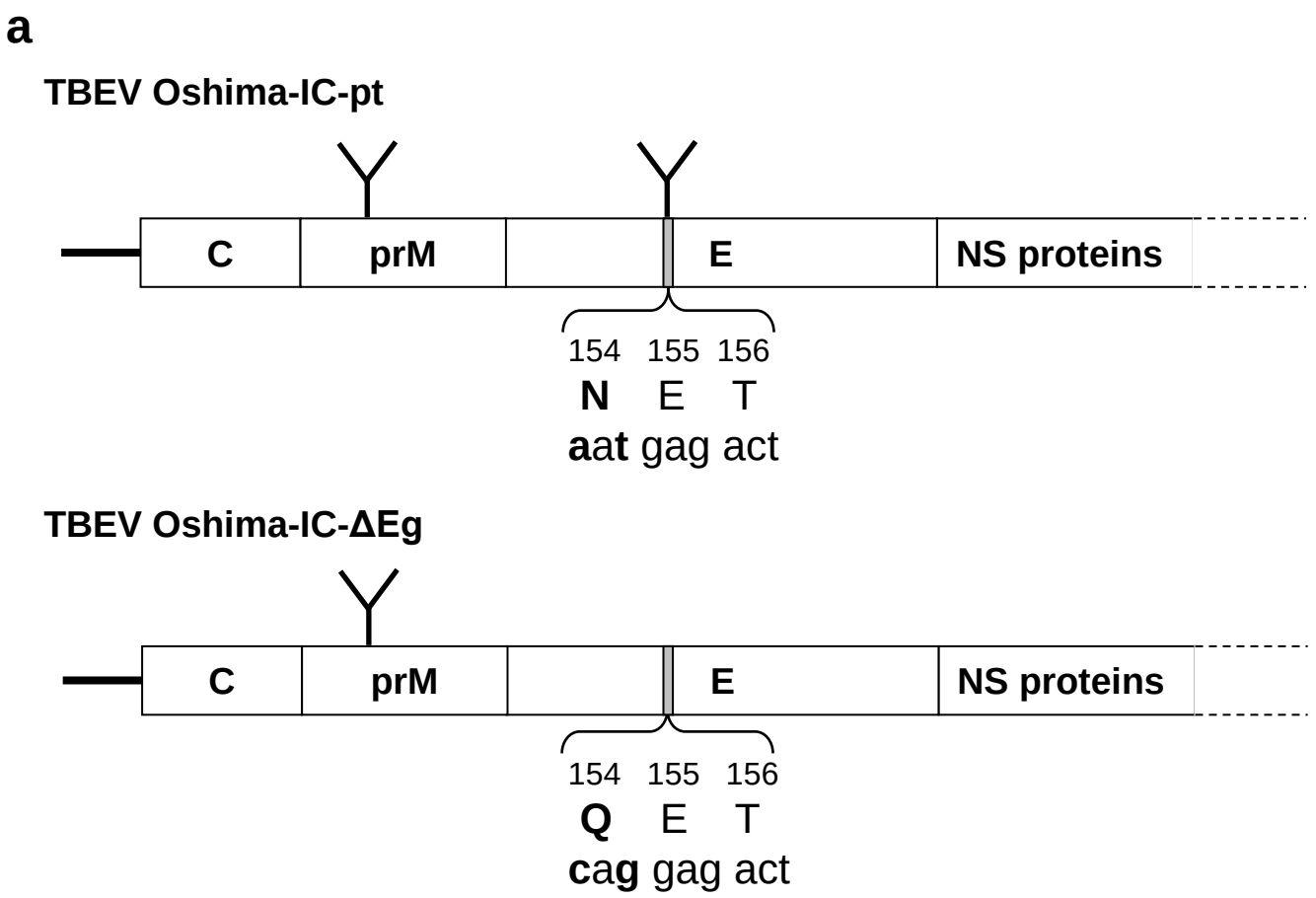
BHK cells were infected with Oshima-IC-pt (pt) or Oshima-IC-ΔEg (ΔEg) at a multiplicity of infection of 0.01. At 48 h post-infection, cell lysates and culture supernatants were harvested. Levels of intracellular and secreted protein E were measured by ELISA using anti-E polyclonal (a) or monoclonal antibodies recognizing conformational epitopes of protein E (b). The percentage of protein E was calculated from the calibration curve for the amount of pt in each experiment.

Fig. 4. Infectivity of TBEV after cleavage of the N-linked glycan on the virion.

453 A total of 100 pfu of Oshima-IC-pt (pt) or Oshima-IC-ΔEg (ΔEg) was treated with serially diluted
454 N-glycosidase F, and the virus titers were determined using plaque assays in BHK cells. The pfu of
455 mock-treated virus was set at 100%.
456
457 **Fig. 5. Effect of protein E glycosylation on viral replication in ISE6 cells.**
458 ISE6 cells were infected with Oshima-IC-pt or Oshima-IC-ΔEg at a multiplicity of infection of 0.01.
459 (a) Intracellular protein E was immunoprecipitated using anti-E antibodies. Precipitated protein E
460 was detected using anti-E antibodies (upper panel) or concanavalin A (lower panel). (b) At each time
461 point, the medium was harvested, and virus titers were determined using plaque assays in BHK cells.
462 (c) At 48 h post-infection, cell lysates and culture supernatants were harvested. The levels of
463 intracellular and secreted protein E were measured by ELISA using an anti-E monoclonal antibody
464 recognizing conformational epitopes of protein E. The percentage of protein E was calculated from
465 the calibration curve for the amount of pt in each experiment.
466
467 **Fig. 6. The effect of inhibitors on the secretion of infectious virus.**
468 BHK (a) and ISE6 (b) cells were infected with Oshima-IC-pt or Oshima-IC-ΔEg at a multiplicity of
469 infection of 0.01. At 24 h post-infection for BHK and 72 h for ISE6 cells, the medium was replaced
470 with fresh medium containing 2 μg ml^{-1} of tunicamycin (Tuni), 2 μg ml^{-1} of brefeldin A (BFA), or
471 DMSO (Mock). After 12 h, the medium was harvested, and virus titers were determined using plaque
472 assays in BHK cells. (c) After the treatment of tunicamycin (Tuni), brefeldin A (BFA) or DMSO
473 (Mock), Intracellular protein E in ISE6 cells infected with Oshima-IC-pt was immunoprecipitated
474 using anti-E antibodies. Precipitated protein E was detected using anti-E antibodies (left panel) or
475 concanavalin A (right panel). NC: uninfected negative control cell.
476
477 **Fig. 7. Survival rate (a), morbidity (b), and weight change (c) following infection with TBEV.**

478 B6 mice were subcutaneously infected with 10^5 pfu of Oshima-IC-pt (closed circles) or
479 Oshima-IC-ΔEg (open squares) and monitored for 28 days. Mouse morbidity was estimated based on
480 >10% weight loss. The average daily weight change was calculated based on the ratio of the daily
481 weight to the weight at day 0. Error bars represent standard deviations.
482
483 **Fig. 8. Virus replication in organs.**
484 Mice were subcutaneously infected with 10^5 pfu of Oshima-IC-pt (closed circles) or Oshima-IC-ΔEg
485 (open squares). At the indicated days after infection, virus titers in blood **(a)**, spleen **(b)**, and brain **(c)**
486 were determined using plaque assays. Error bars represent standard deviations (n = 3).
487

Figure 1



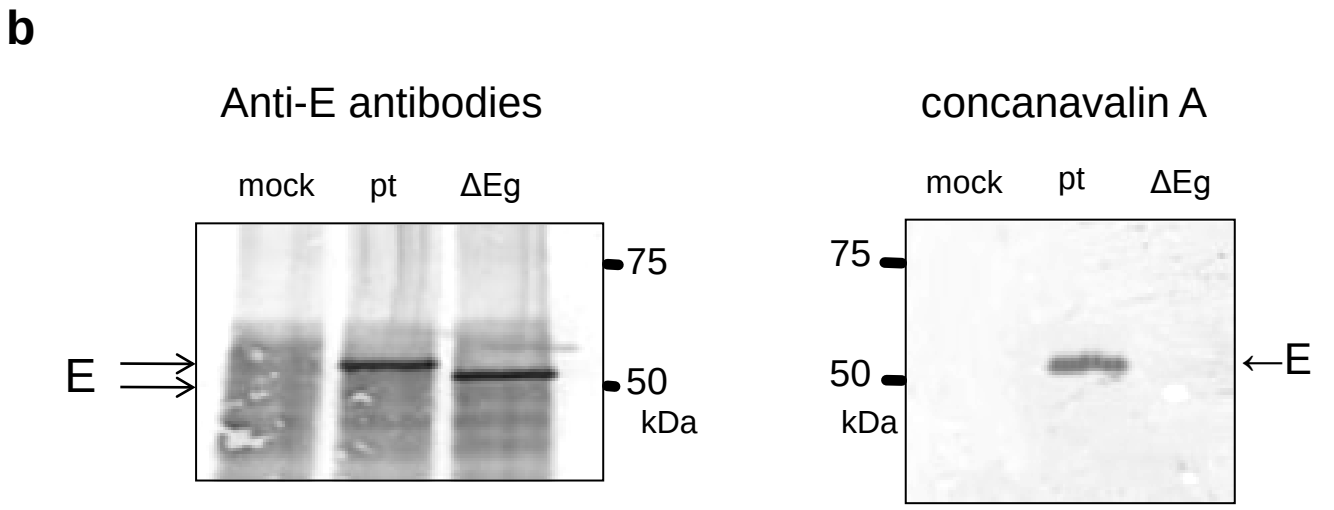


Fig.1. Construction of recombinant TBEV containing protein E with or without its N-linked glycan.

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 post-infection, 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).

Figure 2

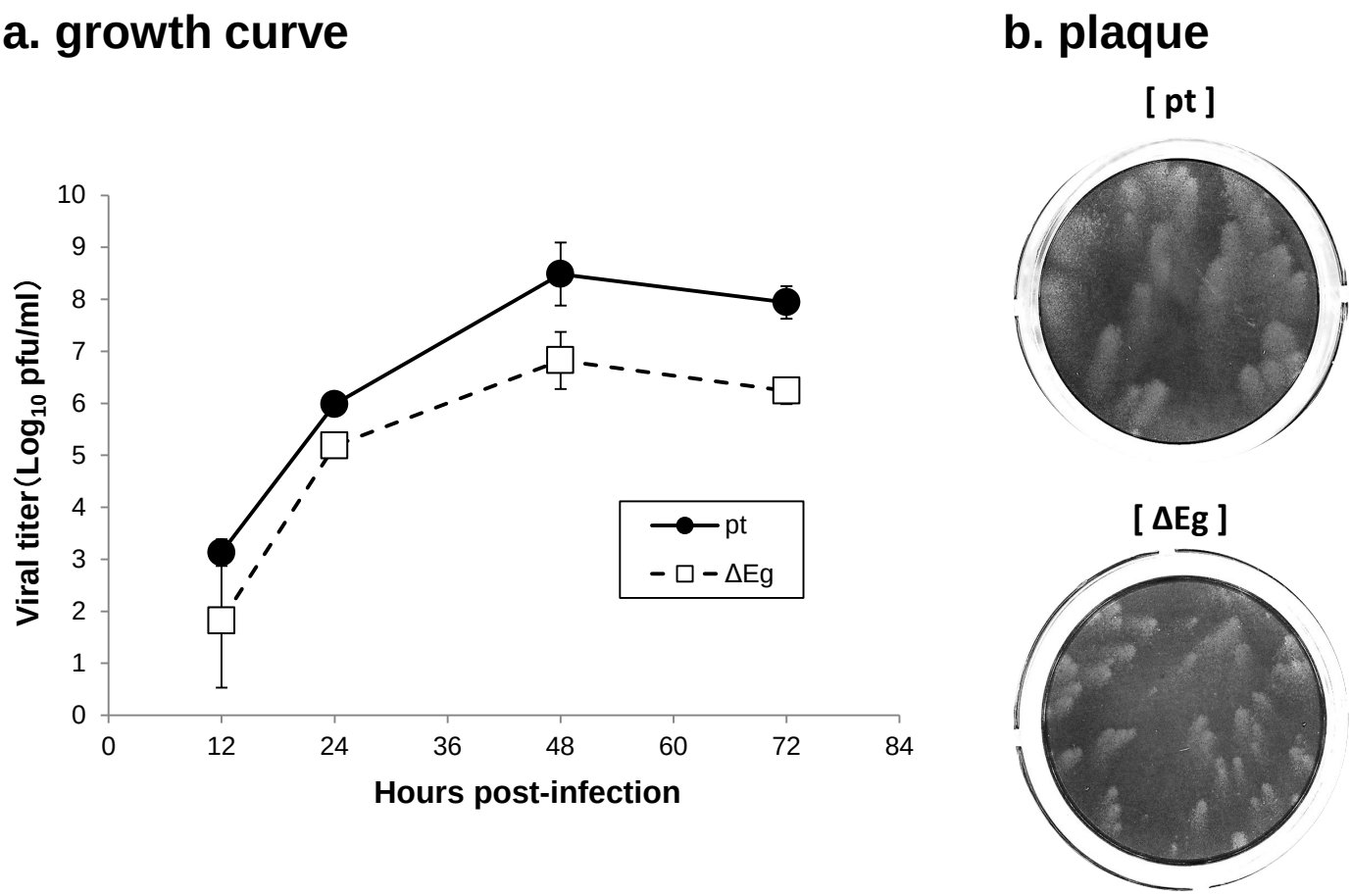


Fig. 2. Effect of protein E glycosylation on viral replication in BHK cells.
(a) BHK cells were infected with Oshima-IC-pt or ΔEg at a multiplicity of infection of 0.01. At each time point, medium was harvested, and virus titers were measured using plaque assays in BHK cells. (b) Plaques of Oshima-IC-pt and ΔEg in BHK cells at 4 days post-infection.

Figure 3

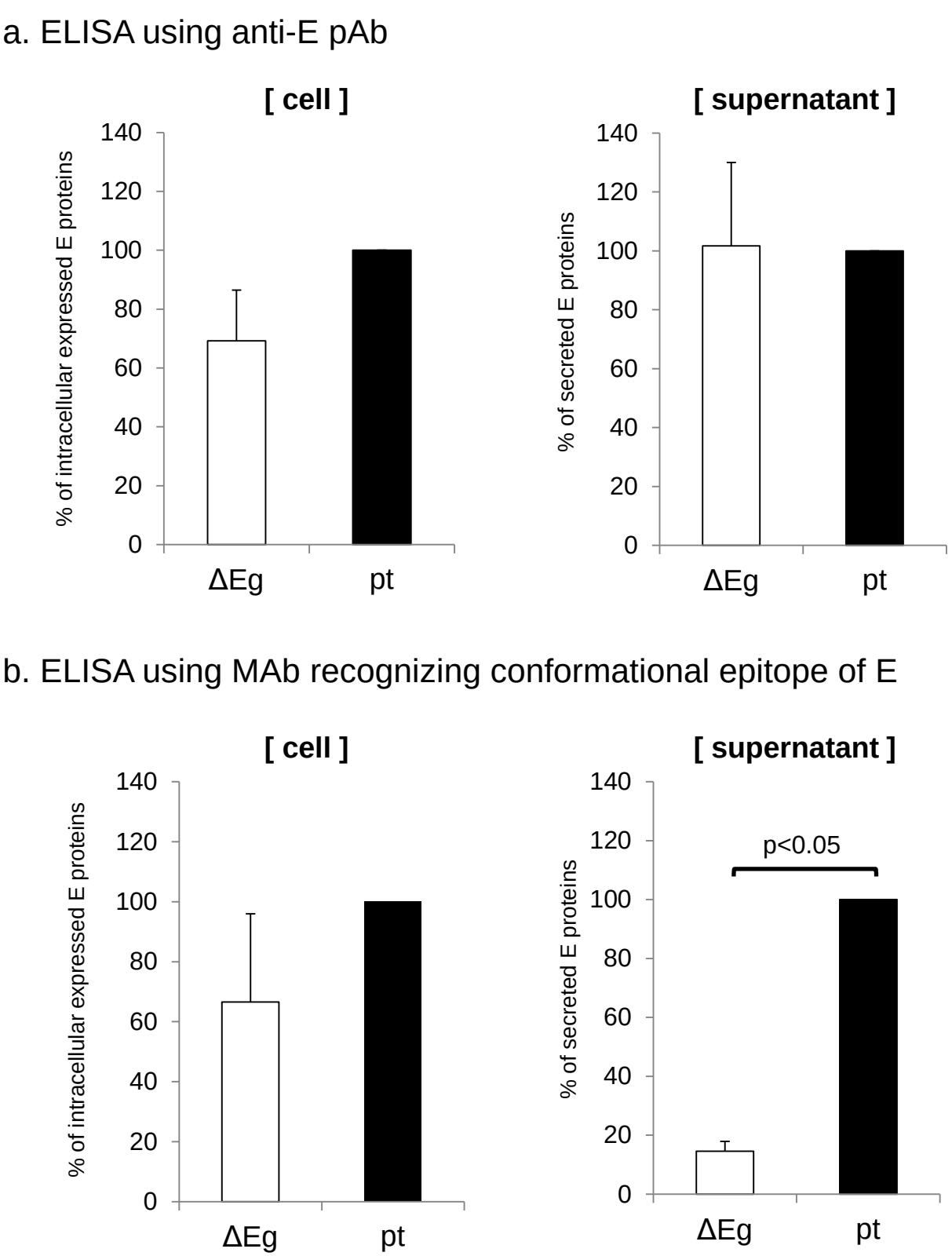


Fig. 3. Effect of protein E glycosylation on synthesis and secretion of protein E in BHK cells. BHK cells were infected with Oshima-IC-pt (pt) or Oshima-IC-ΔEg (ΔEg) at a multiplicity of infection of 0.01. At 48 h post-infection, cell lysates and culture supernatants were harvested. Levels of intracellular and secreted protein E were measured by ELISA using anti-E polyclonal (a) or monoclonal antibodies recognizing conformational epitopes of protein E (b). The percentage of protein E was calculated from the calibration curve for the amount of pt in each experiment.

Figure 4

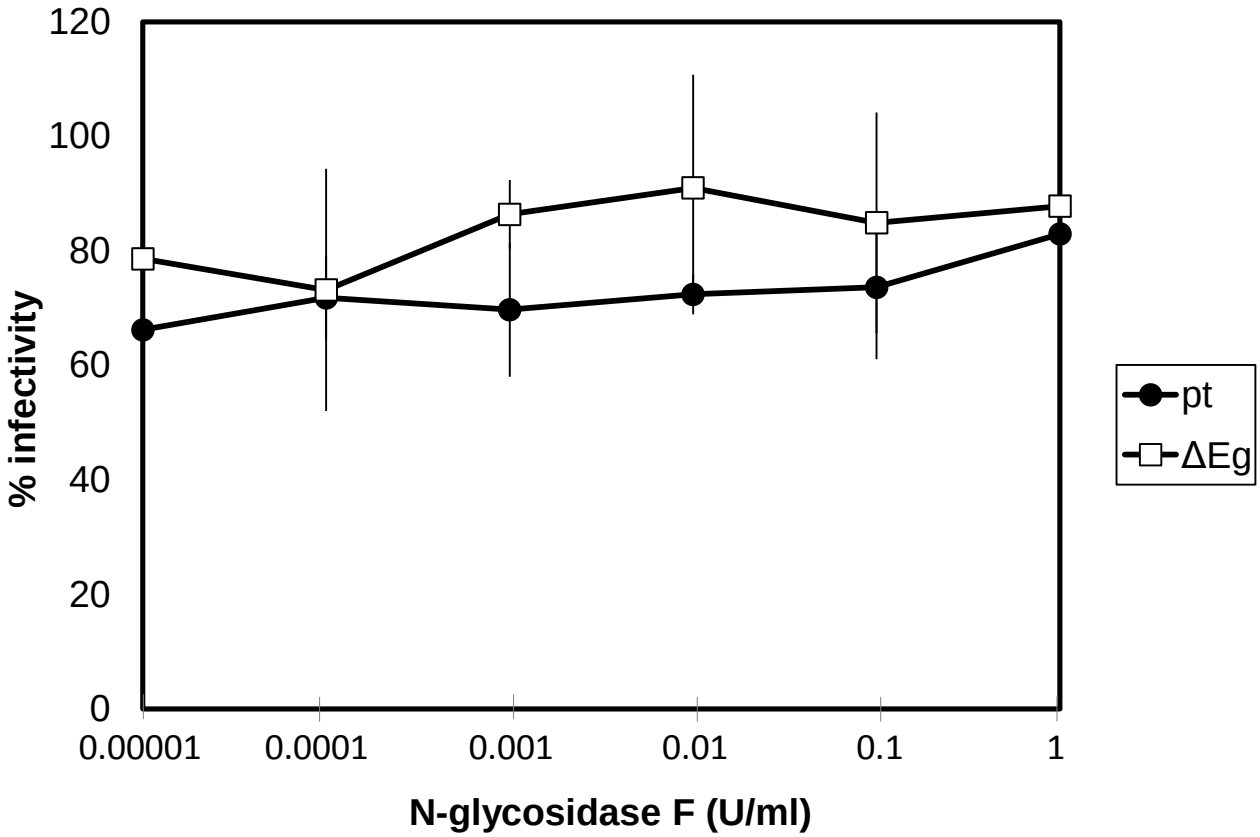


Fig. 4. Infectivity of TBEV after cleavage of the N-linked glycan on the virion.
A total of 100 pfu of Oshima-IC-pt (pt) or Oshima-IC-ΔEg (ΔEg) was treated with serially diluted N-glycosidase F, and the virus titers were determined using plaque assays in BHK cells. The pfu of mock-treated virus was set at 100%.

Figure 5

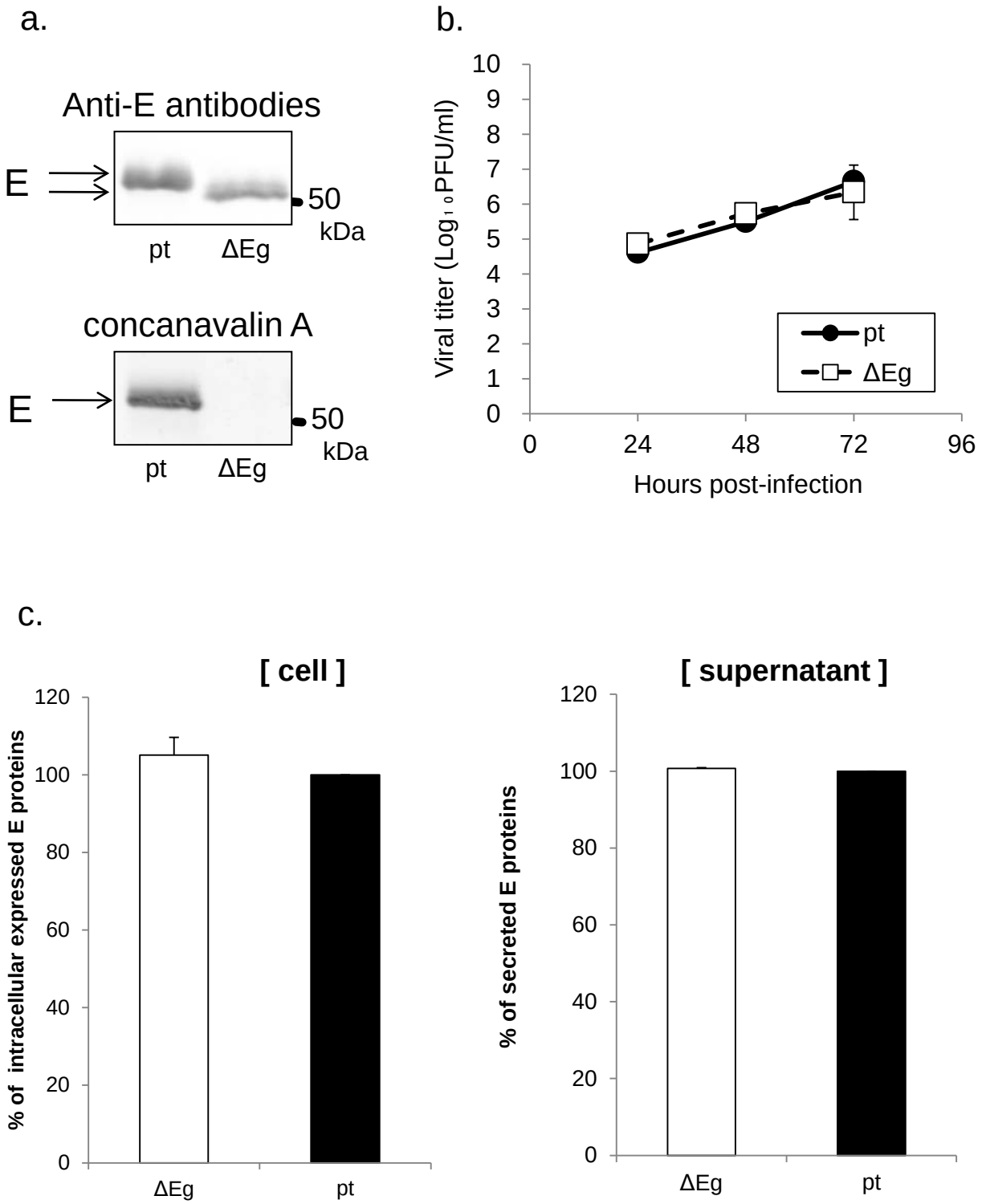


Fig. 5. Effect of protein E glycosylation on viral replication in ISE6 cells.
ISE6 cells were infected with Oshima-IC-pt or Oshima-IC-ΔEg at a multiplicity of infection of 0.01. (a) Intracellular protein E was immunoprecipitated using anti-E antibodies. Precipitated protein E was detected using anti-E antibodies (upper panel) or concanavalin A (lower panel). (b) At each time point, the medium was harvested, and virus titers were determined using plaque assays in BHK cells. (c) At 48 h post-infection, cell lysates and culture supernatants were harvested. The levels of intracellular and secreted protein E were measured by ELISA using an anti-E monoclonal antibody 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.

Figure 6

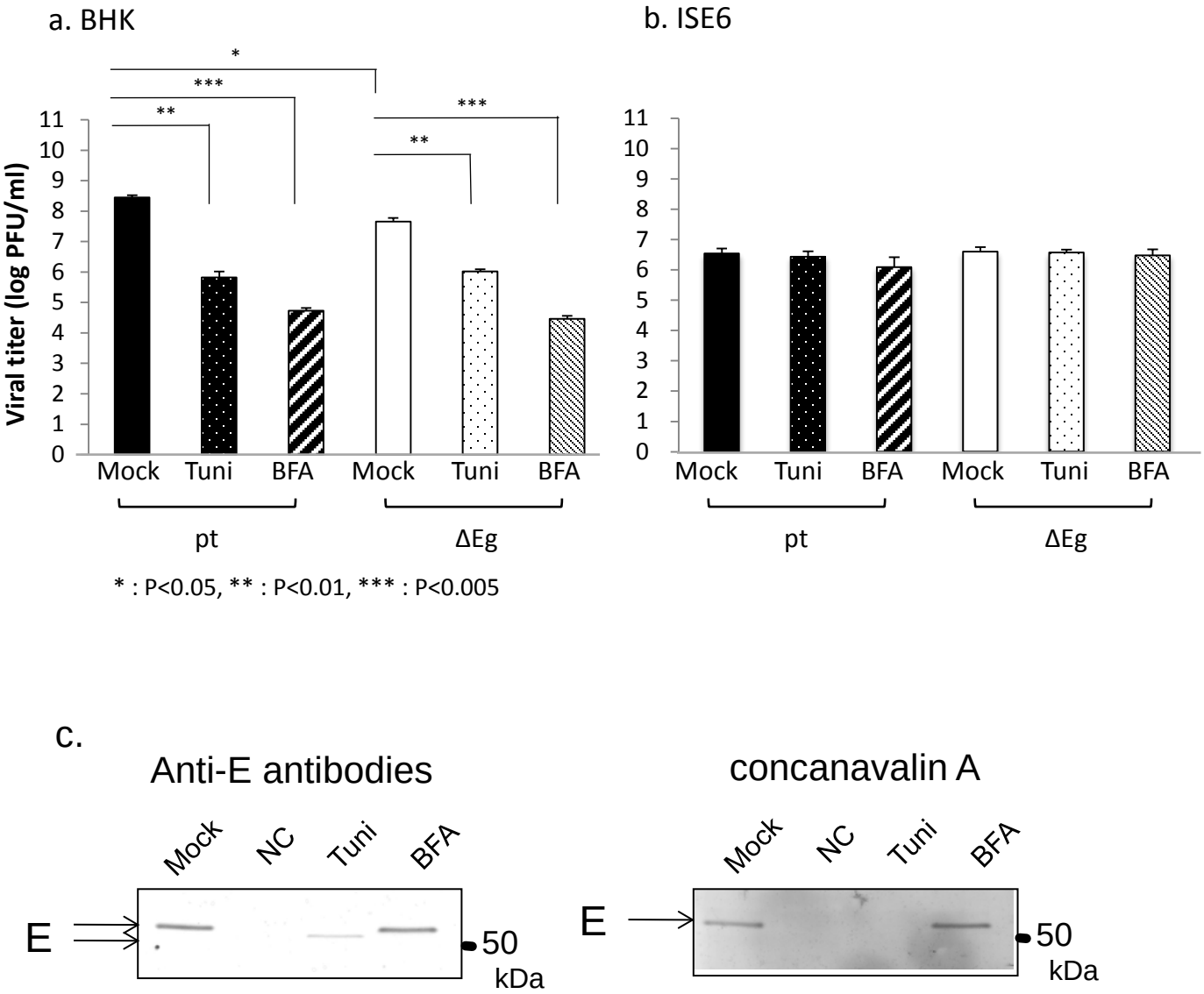


Fig. 6. The effect of inhibitors on the secretion of infectious virus.
BHK (a) and ISE6 (b) cells were infected with Oshima-IC-pt or Oshima-IC-ΔEg at a multiplicity of infection of 0.01. At 24 h post-infection for BHK and 72 h for ISE6 cells, the medium was replaced with fresh medium containing 2 μg ml^{-1} of tunicamycin (Tuni), 2 μg ml^{-1} of brefeldin A (BFA), or DMSO (Mock). After 12 h, the medium was harvested, and virus titers were determined using plaque assays in BHK cells. (c) After the treatment of tunicamycin (Tuni), brefeldin A (BFA) or DMSO (Mock), Intracellular protein E in ISE6 cells infected with Oshima-IC-pt was immunoprecipitated using anti-E antibodies. Precipitated protein E was detected using anti-E antibodies (left panel) or concanavalin A (right panel). NC: uninfected negative control cell.

Figure 7

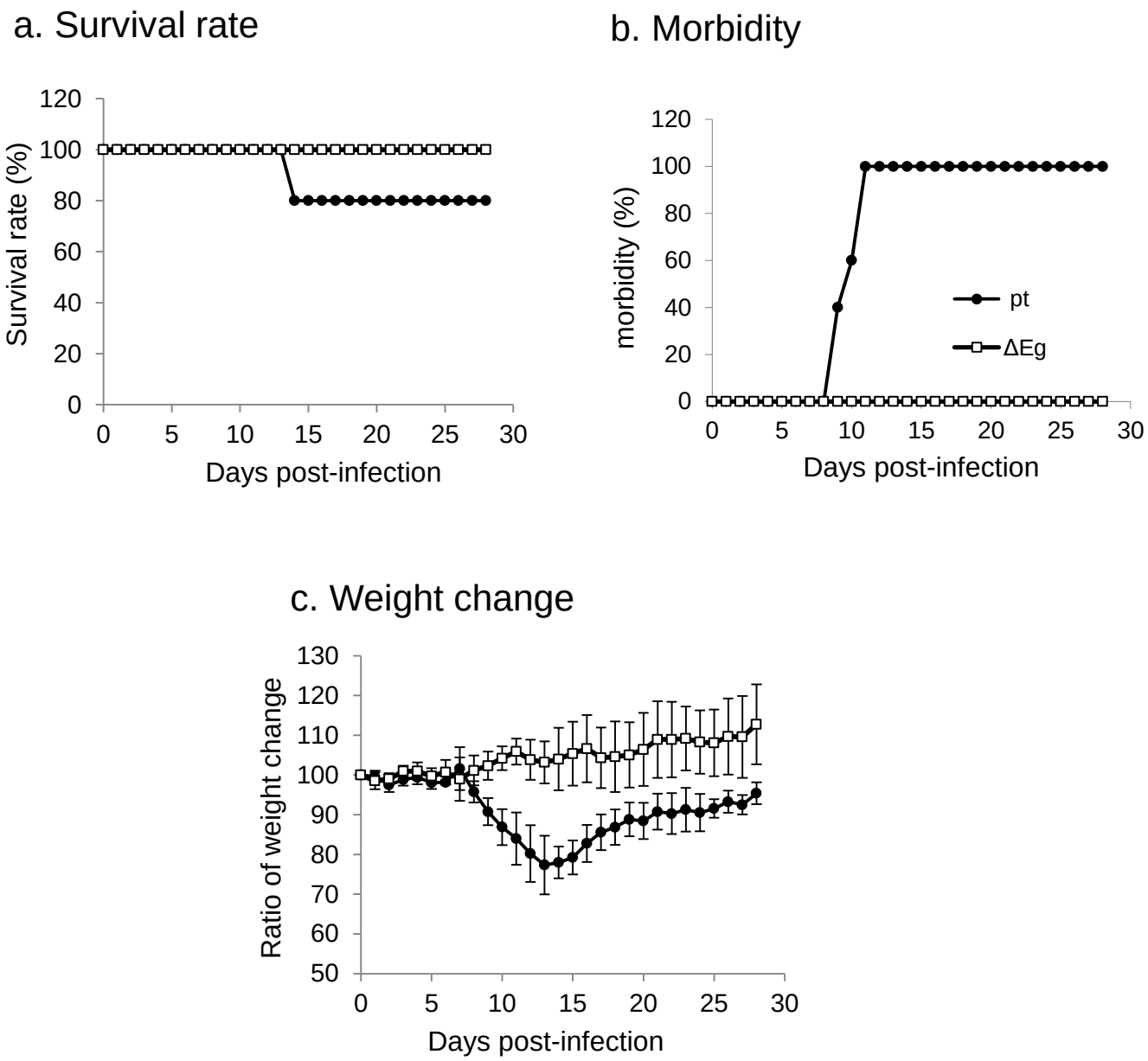


Fig. 7. Survival rate (a), morbidity (b), and weight change (c) following infection with TBEV. B6 mice were subcutaneously infected with 10^5 pfu of Oshima-IC-pt (closed circles) or Oshima-IC-ΔEg (open squares) and monitored for 28 days. Mouse morbidity was estimated based on >10% weight loss. The average daily weight change was calculated based on the ratio of the daily weight to the weight at day 0. Error bars represent standard deviations.

Figure 8

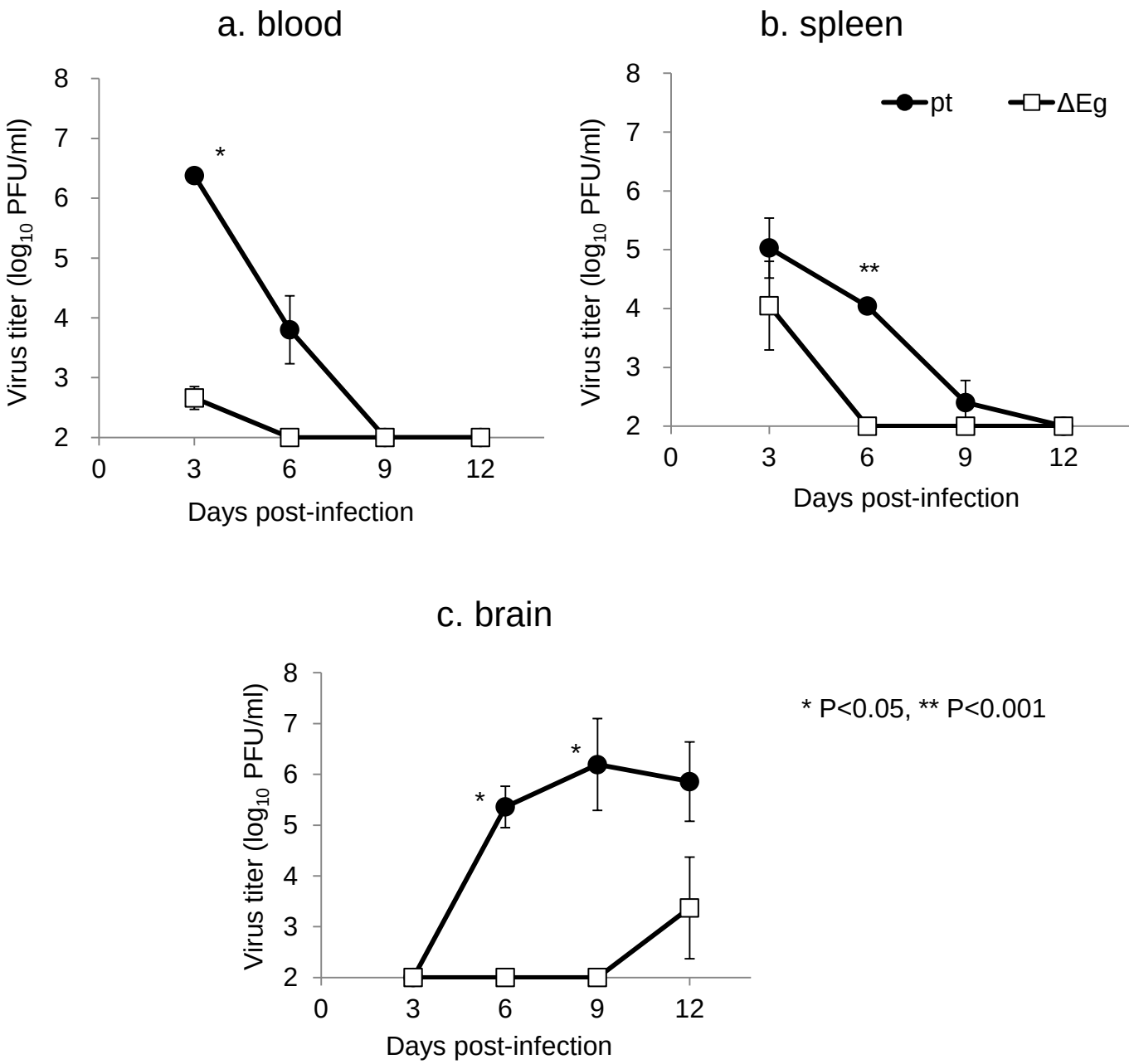


Fig. 8. Virus replication in organs.
Mice were subcutaneously infected with 10^5 pfu of Oshima-IC-pt (closed circles) or Oshima-IC-ΔEg (open squares). At the indicated days after infection, virus titers in blood **(a)**, spleen **(b)**, and brain **(c)** were determined using plaque assays. Error bars represent standard deviations (n = 3).