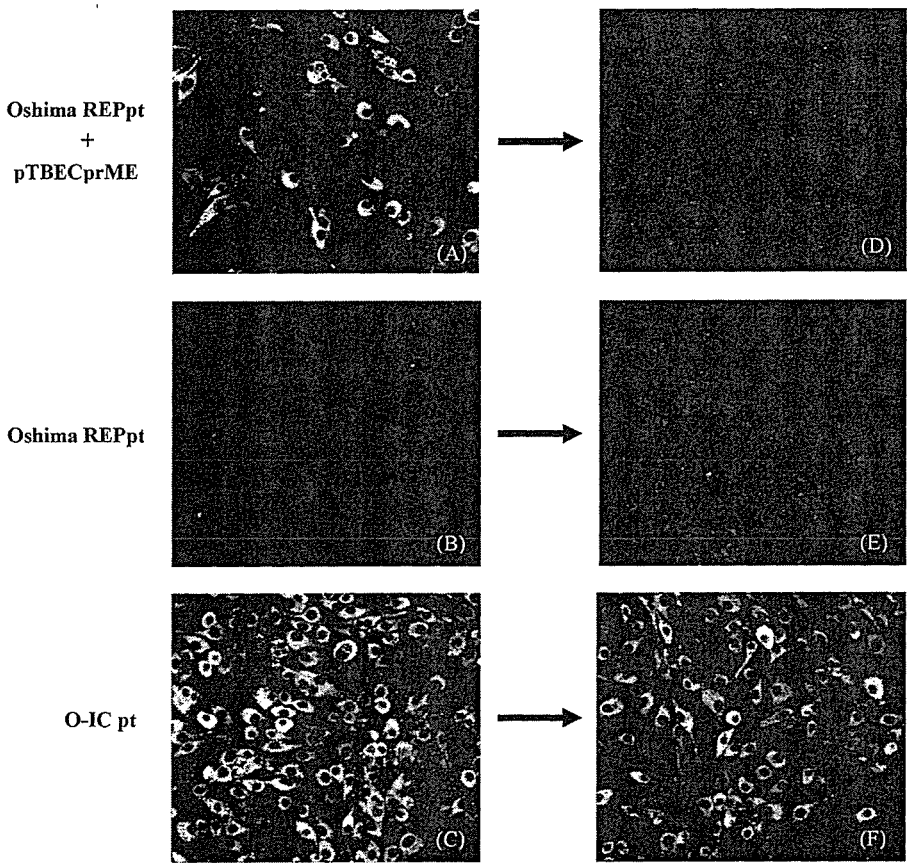

Fig. 3. Secretion of single-round infectious particles. Fresh BHK cells were infected with the culture supernatants of BHK cells transfected with in vitro-synthesized Oshima REPpt RNA and pTBECprME (A); Oshima REPpt alone (B); or O-IC pt RNA (C); and incubated for 24 h (A to C). Panels D to F show re-infection of fresh BHK-21 cells, as indicated by the arrows. Expression of the viral NS3 proteins was visualized by immunofluorescence using anti-NS3 antibodies.

the second transfection was examined. BHK cells were transfected with pTBECprME 24 h after electroporation with the Oshima REPpt RNA. The culture supernatant was harvested 24, 36, 48, or 60 h after the second transfection and used for the titration of VLPs, as described above. The VLP titer increased gradually with time and peaked 48 h after the second transfection. At 60 h after the second transfection, the titer was relatively reduced.

3.3. Characterization of single-round infectious particles

To analyze the secretion of VLPs from the cells that were transfected with pTBECprME and the Oshima REPpt replicon, the transfected cells and supernatant were subjected to Western blot analysis (Fig. 5). In the pTBECprME-transfected cells, E protein bands were detected by the E-specific antibodies, regardless of replicon RNA electroporation. When the C-prM-E viral structural proteins were expressed alone, the C-prM polyprotein bands were detected by prM-specific antibodies, due to the absence of the viral NS3 protease, as shown in Fig. 2. On the other hand, bands corresponding to prM were detected in the cells that expressed the viral structural proteins with the Oshima REPpt replicon RNA (Fig. 5; cell lysate, lane 1), which indicates that the C-prM junctions were partially cleaved by viral NS3 protease that was derived from the expression of the TBE replicon RNA. Moreover, E protein secretion was observed in the culture supernatants of cells that were transfected with pTBECprME and Oshima REPpt replicon, whereas E protein was not secreted from cells that were transfected with pTBECprME alone (Fig. 5; supernatant). This means that viral envelope proteins are incorporated into VLPs and secreted into the culture supernatant following appropriate cleavage of the C-prM junctions.

To confirm that the infectious particles were actually packaged by TBE structural proteins, a virus neutralization test was performed. Prior to infection of the BHK cells, the VLPs (1000 IU) were incubated with the anti-TBE E protein monoclonal antibody 1H4 (at 1:100 dilution), which has neutralizing activity against the TBE virus; this led to loss of infectivity, as revealed by IFA (Fig. 6). To elucidate the physical structures of the VLPs, equilibrium density gradient centrifugation in a 10–50% sucrose gradient was performed (Fig. 7). The VLPs that were secreted from the cells transfected with pTBECprME and Oshima REPpt RNA were precipitated with PEG, subjected to centrifugation in the sucrose gradient, and the fractions were collected and assayed by ELISA for the

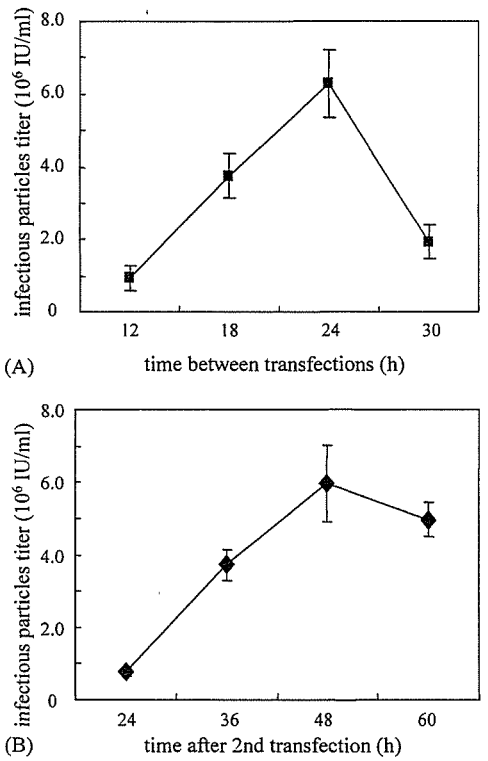

Fig. 4. Optimization of conditions for packaging of TBE replicon RNA. (A) Culture supernatants of cells that were transfected with replicon RNA and pTBECprME were collected 48 h after the second transfection, either at different time intervals between the RNA and plasmid transfections. (B) BHK cells were transfected with replicon RNA and pTBECprME at 24 h interval and the supernatant samples were collected at different times after the second transfection. The collected samples were precipitated with PEG, suspended in PBS that contained RNase A, and then used for the infection of the BHK cells. The infectious titers were calculated by counting the IFA-positive cells using anti-NS3 antibodies, as described in Section 2.

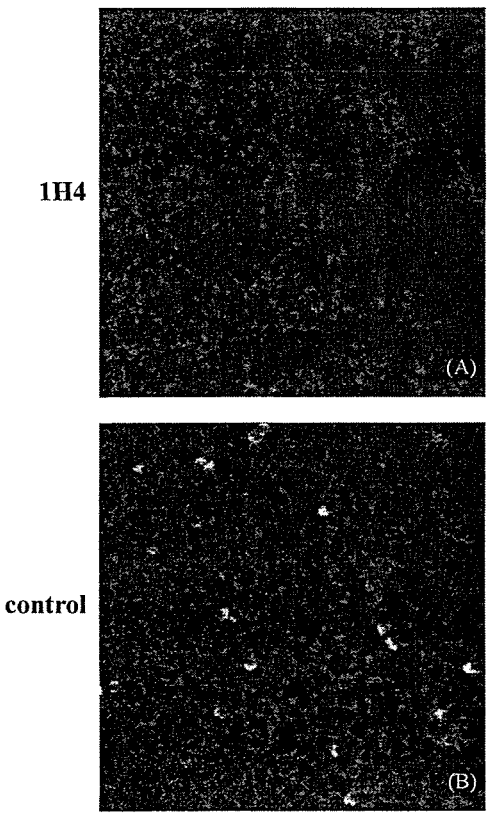

Fig. 6. Inhibition of replicon particle infectivity by virus-neutralizing antibody. Prior to infection, the virus particles were incubated with (A) or without (B) monoclonal antibody 1H4, which has neutralizing activity against the TBE virus. At 24 h post-infection, the cells were stained by IFA using anti-NS3 antibodies.

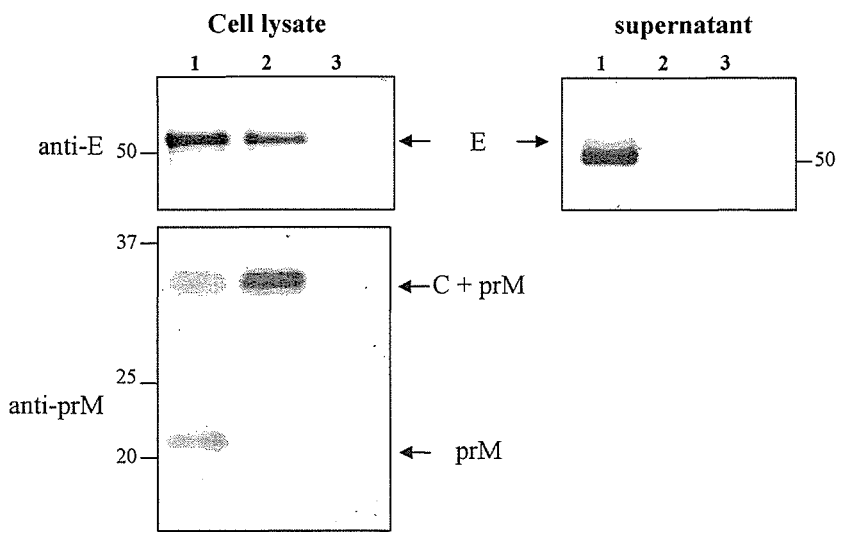

Fig. 5. Cleavage of the C-prM junctions by replicon NS3 protease, and secretion of viral proteins into the culture supernatant. BHK cells were transfected with Oshima REPpt RNA and pTBECprME (lane 1), pTBECprME (lane 2), or Oshima REPpt RNA (lane 3). At 24 h post-transfection with the pTBECprME plasmid, the cells and PEG-precipitated supernatant samples were separated by 12% SDS-PAGE under non-reducing condition and subjected to Western blotting. The viral proteins were visualized using the anti-E (upper panel) and anti-prM (lower panel) rabbit polyclonal antibodies. The positions of the individual proteins are marked, and the molecular size (in kDa) is indicated at the side.

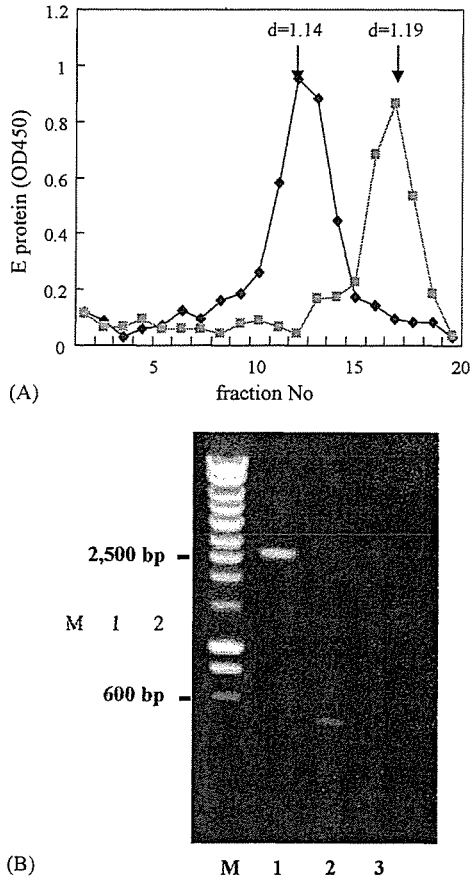

Fig. 7. (A) Sedimentation analysis of particles that were harvested from the culture supernatants of BHK cells that were transfected with in vitro-synthesized Oshima REPpt RNA and pTBECprME. The particles were pelleted with PEG and loaded onto a 10–50% sucrose gradient (squares). The E proteins in each collected fraction were analyzed by ELISA. Control gradients, which were loaded with purified RSPs (diamonds), were analyzed in parallel. The densities of the peak fractions are indicated by arrows. (B) RT-PCR analysis with TBE-specific primers located between the 5′-UTR and the NS1 regions of RNA that was extracted from the infectious particles. RNA was extracted from fractions 16–18 (in panel A), and subjected to RT-PCR (lane 2). PEG-precipitated supernatants of cells that were transfected with O-IC-pt (lane 1) and Oshima REPpt RNA (lane 3) were used as controls.

detection of TBE virus E proteins. The highest OD_{450} values for the E proteins were detected in fractions 16–18 (density of approximately 1.186 g/cm^3), which closely resemble the characteristics of the authentic TBE virus, as compared to the recombinant subviral particles (RSPs) (fractions 11–13; 1.14 g/cm^3), which were secreted from the pCAGprME-transfected cells. Furthermore, infectivity measured by IFA was observed only for fractions 16 to 18 ($\sim 6.5 \times 10^6$ IU/ml; data not shown). The RNA extracted from the VLPs in fractions 16–18 was reverse-transcribed and PCR-amplified using TBE-specific primers for the region between the 5′-UTR and NS1 gene. As shown in Fig. 7B, while a DNA fragment of 2500 bp (5′-UTR-NS1) was observed as the RT-PCR product of the authentic TBE virus (lane 1), a DNA fragment of the expected size (500 bp) was detected as the RT-PCR product of the VLP (lane 2), which indicates deletion of the region that encodes the viral structural gene and the absence of recombination with the mRNA of the viral structural gene provided by pTBECprME. No RT-PCR product was obtained from RNA that was extracted from the PEG-precipitated supernatant of cells that were transfected with Oshima REPpt RNA alone. In addition, no DNA fragment was amplified from the VLPs by RT-PCR for the partial prM and E genes, which indicates that the mRNA of the viral structural gene from pTBECprME was not incorporated into the particles. Combined with the data about single-round infectivity shown in Fig. 3, these data suggest that the replicon RNA was packaged into single-round infectious particles by *trans*-expressed viral structural proteins, without the regeneration of infectious viruses.

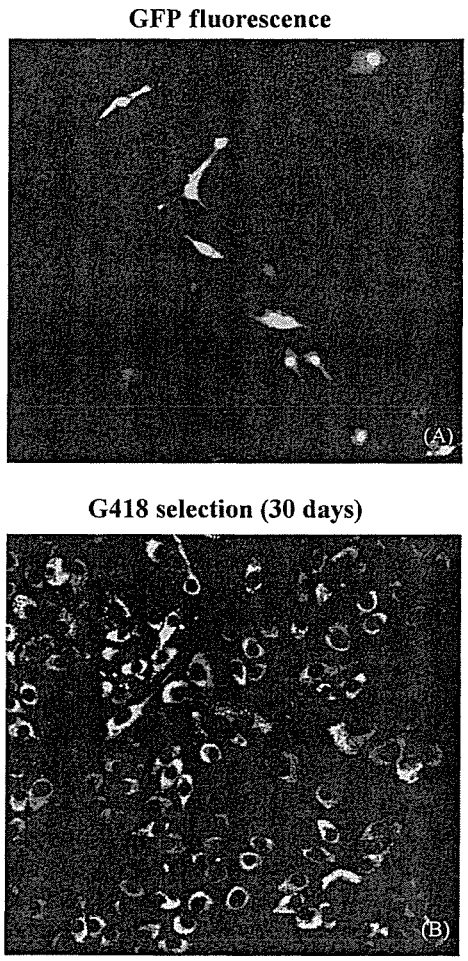

Fig. 8. Packaging of recombinant TBE replicon RNA that expresses a heterologous gene. (A) Culture supernatants from BHK cells that were sequentially transfected with Oshima REP-GFP RNA and pTBECprME were precipitated with PEG and used to infect fresh BHK cells. GFP fluorescence was observed at 60 h post-infection. (B) BHK cells in which the Oshima REP-NEO replicon RNA replicated persistently were transfected with pTBECprME. At 48 h post-transfection, the culture supernatants were precipitated with PEG and used to infect fresh BHK cells. The infected cells were cultured in the presence of G418 for 30 days, after which time the cells were stained by IFA using anti-NS3 antibodies.

3.4. Expression and delivery of heterologous gene by TBE replicon-based pseudoinfectious particles

In our previous study, a heterologous gene was inserted into the 3′-UTR of the TBE virus replicon [32]. Heterologous gene products were expressed in the replicon RNA-transfected cells, and none of the cells exhibited CPE. Therefore, the application of the replicon packaging system for gene delivery was examined. BHK cells were transfected with the Oshima REP-GFP replicon RNA, which expresses the GFP gene, and pTBECprME. At 48 h post-transfection with the pTBECprME plasmid, the culture supernatant was harvested, precipitated by PEG, and used to infect BHK cells. At 60 h post-infection, GFP expression in the infected cells was observed by confocal microscopy (Fig. 8A), which indicated that Oshima REP-GFP RNA is packaged into VLPs by *trans*-complementation of viral structural proteins, and that it acts as a vector for the expression of GFP as a heterologous gene. BHKrepTBE cells, which persistently harbor the TBE virus replicon, have been established previously by transfection of Oshima REP-NEO (containing the Neo gene in the 3′-UTR), followed by G418 selection. The pTBECprME plasmid was transfected into BHKrepTBE cells, and at 48 h post-transfection, the supernatant was precipitated with PEG and used to infect fresh BHK cells. At this point, the infectious titer had increased to 7.2×10^7 IU/ml, and the infected cells grew in the presence of G418 for at least 30 days (five passages), and produced viral non-structural proteins (Fig. 8B). These data indicate for the first time the application of the TBE replicon as a gene delivery vector.

4. Discussion

Subgenomic replicon vectors of positive-stranded RNA viruses have great potential in gene expression and vaccine applications [20,21,40]. Their advantages over other viral vector systems are following: (i) high level expression of encoded heterologous genes due to amplification of replicon RNA; (ii) cytoplasmic replication of replicon, which exclude the possibility of chromosomal integration; (iii) inability of replicon RNA to escape from transfected cells, which limits the spread of vaccine vectors; (iv) easy manipulations due to the relatively small genome size of replicon. In our previous study, we showed that the heterologous GFP gene and Neo gene with the IRES sequence could be inserted into the 3′-UTR of the TBE virus replicon and expressed without any apparent CPE [32]. To apply this TBE replicon to a gene delivery vector, we examined the packaging of replicon RNA into particles by sequential transfection with a plasmid that expresses the TBE virus structural proteins (C-prM-E). We then applied this packaging system to heterologous gene delivery vectors by packaging replicons that carried inserted heterologous genes, such as GFP and Neo. This is the first report about the application of a tick-borne flavivirus replicon packaging system to gene delivery. By using replicon encoding viral antigen and tumor specific antigen, TBE VLPs can be expected to induce protective antiviral and anticancer immunity.

Currently available flavivirus replicon-based vectors have certain advantages, such as their ability to replicate efficiently in a broad range of host cells without any apparent CPE and their genetic stability without spontaneous recombination [20,21]. It has been reported that alphavirus expression vectors have potential problems in terms of their cytopathic effects on host cells. In contrast, TBE replicons have no significant cytopathic effects. Thus, TBE replicon-based vectors facilitate longer-lasting expression of heterologous genes. Furthermore, no spontaneous regeneration of infectious viruses has been observed in the TBE replicon packaging system. In studies with alphaviruses, such as SFV and SIN, co-packaging of replicon RNA with helper RNA produced homologous recombination between the two RNA species, which led to regeneration of the infectious virus [41,42]. In our system, although the C/prM/E mRNA and replicon RNA contain overlapping elements of the viral genome, which raises the possibility of homologous recombination, neither co-packaging nor recombination between the two RNAs was observed in any of the experiments. These features of non-cytopathogenicity and genetic stability are important for the applicability of this tool to more-sensitive host systems.

In our packaging system, the titers of infectious particles reached 6.2×10^6 IU/ml following sequential transfection of pTBECprME and Oshima REPpt replicon RNA into BHK cells; 7.2×10^7 IU/ml VLPs were obtained by transfection of pTBECprME into BHKrepTBE cells when the cells were seeded into 24-well tissue culture plates. These titers are higher than those observed in the Gehrke study, in which particles were prepared by electroporation of the replicon into CHO-ME cells that continuously expressed the TBE virus prM/E proteins [43]. These titers are still below the maximum titers of alphavirus vectors [42,44], but are almost equivalent to those obtained with three-component alphavirus systems, using alphavirus packaging cell lines that have been designed to reduce the regeneration of infectious particles [45–48].

In addition to the vaccine and gene delivery systems, this TBE replicon packaging system can be applied to the study of genome RNA packaging and the rapid mapping of packaging signal(s) in TBE virus RNA and genomic RNA-binding domain(s) in the C protein. Compared to a full-length infectious clone, this packaging system has the advantage that it can separate replication events from assembly and genome RNA packaging events. Furthermore, while C and prM/E have been expressed separately in another packaging system [43], in our system, C/prM/E are provided from a single polyprotein, which is expressed by a plasmid that encodes all the viral structural protein genes. Thus, processing of the viral structural proteins is carried out as for the native virus, followed by the sequential events of genome packaging and particle assembly. Another advantage of this system is that particles cannot be secreted unless the viral structural proteins

are expressed with the replicon. It has been reported that co-expression of prM and E in the absence of C protein leads to the secretion of RSPs [49–51]. Therefore, in the Gehrke study, free RSPs were mixed with VLPs in the culture supernatant of replicon-transfected CHO cells that expressed continuously the prM/E proteins. However, when protein C/prM/E are expressed as a single polyprotein, as is the case in our system, the junction between C and prM remains uncleaved, due to the absence of the NS3 protease, which results in the viral proteins being retained intracellularly (Fig. 5). Moreover, in our system, the C-prM junction is cleaved only when the C/prM/E proteins are expressed with the replicon, which leads to the secretion of VLPs that are minimally contaminated with free RSPs (Fig. 7A). Accordingly, it is now possible to introduce mutations into the TBE replicon RNA and into genes for the structural proteins, and to investigate their effects on genome packaging and the release of virus particles.

In summary, we have established a new packaging system for TBE replicon RNA that uses complementation in *trans* of viral structural proteins. This system can be applied to the development of non-cytopathic gene delivery vectors, and it facilitates further studies into the molecular mechanism of TBE virus genome packaging.

Acknowledgments

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Role of the N-linked glycans of the prM and E envelope proteins in tick-borne encephalitis virus particle secretion

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Abstract

The tick-borne encephalitis (TBE) virus has two membrane glycoproteins (prM and E), which each has one N-linked glycan. Constructs that express prM and E proteins of TBE virus have been shown to produce virus-like particles (VLPs), which have surface properties that are similar to those of infectious viruses. To reveal the function of glycosylation of the TBE virus prM and E proteins in the secretion of VLPs, we expressed glycosylation-mutated prM and E proteins and compared the secretion levels and biological properties of the VLPs. In the prM protein glycosylation-deficient mutant, the level of secreted E protein was reduced to 60% of the wild-type level. On the other hand, in the E or prM-E protein glycosylation-deficient mutant, the level of secreted E protein was reduced to 10% of the wild-type level. Furthermore, the mutant which was glycosylated at positions 66 and 154 in protein E, the level of secreted E protein was four-fold higher than that of the wild-type. However, in the mutant which was glycosylated at position 66 only, E protein secretion was reduced to only 10% of the wild-type level. These data suggest that the glycan associated with the N-linked glycosylation site at position 154 in protein E plays an important role in VLP secretion.

Keywords: Tick-borne encephalitis virus; Mutagenesis; Glycosylation sites; Secretion

1. Introduction

The tick-borne encephalitis (TBE) virus, which is a member of the genus Flavivirus in the family *Flaviviridae*, causes fatal encephalitis in humans [6]. TBE virus is a single-stranded positive-polarity enveloped RNA virus. The RNA genome of TBE virus is about 11 kb in length. A single large open reading frame encodes three structural proteins [the core (C) protein, the membrane (prM) protein, and the envelope (E) protein], along with seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) [2].

Two viral membrane proteins, glycoprotein E (molecular mass of 52 kDa) and protein M (molecular mass of 7–8 kDa) are synthesized as part of a polyprotein precursor that is co- and post-translationally cleaved into the individual peptide chains [15]. Protein E mediates virus entry into the cell via receptor-mediated endocytosis, and it carries the major antigenic epitopes that promote a protective immune response [2,15]. Constructs that express prM and the full-length E proteins of several flaviviruses, including TBE virus, have been shown to produce virus-like particles (VLPs) [1,20,28,34]. VLPs are smaller than virions (30 nm versus 50 nm in diameter), and have surface properties and fusion activities that are similar to those of infectious viruses [28]. A previous study has shown that flavivirus VLPs are excellent

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immunogens, and since they resemble inactivated viruses in terms of antibody induction and protection against viral challenge, VLPs represent excellent candidates for a recombinant vaccine against infections with flaviviruses, such as TBEV [14,20].

The glycoproteins that are associated with the virus envelope mediate receptor binding and fusion. Therefore, these glycoproteins are assumed to be important determinants of virulence and pathogenicity. N-linked glycan binding to the viral glycoprotein increases the efficiency of folding and transport of glycoproteins by mediating interactions with intracellular animal lectins [11–13]. In certain types of viruses that bud into the ER lumen, it has been observed that interference with glycosylation results in low levels of virus growth, budding, secretion and pathogenicity [4,5,9,11,23,24,26,32].

The E protein has zero to two N-linked glycosylation consensus sequences, depending on the virus [2,27]. It has been suggested that the carbohydrate side chain may stabilize the dimer contacts between two E molecules [22]. Another study has revealed that the N-linked glycan does not play a major role in either the antigenic structure of the E protein of TBE virus or in viral infection [10,31]. Recently, Lorenz et al. [23] have shown that inhibition of N-linked glycosylation of the E protein or glucose trimming of the carbohydrate side chain of E results in a significant decrease in the secretion of VLPs, which suggests a critical role for this glycan in one or more assembly and/or secretion steps. However, whether or not the carbohydrate side-chain of the E protein is involved in flavivirus maturation remains unclear, since the E protein is differentially glycosylated in other flaviviruses, as in some strains of Kunjin virus and West Nile virus (nonglycosylated E protein) and dengue virus (two glycosylation sites) [2].

The M protein is synthesized as the precursor protein prM (molecular mass of 25 kDa). The interaction between prM and E is important for the later processing steps in viral protein folding [22]. It has been suggested that prM holds E in an inactive conformation to prevent low-pH rearrangements during transportation through the acidic compartments of the trans-Golgi network [16,35]. Shortly before the virus is released from the cell, the pr portion is cleaved from prM by the cellular protease furin, which produces mature virions that consist of E and M molecules [15,29]. The prM protein also contains one N-linked glycosylation consensus sequence [2]. However, there is little information on the role of the carbohydrate side chain of the flavivirus prM protein.

In this study, we introduced amino acid mutations into the glycosylation sites of the prM and E proteins that were expressed on vectors and, thus, we generated glycosylation-mutated prM and E proteins. We compared the synthesis, secretion levels, and biological properties of the VLPs, and investigated the function of glycosylation of TBE virus prM and E proteins in the secretion of TBE viral particles.

2. Materials and methods

2.1. Construction of plasmids

The production of recombinant plasmids that express the prM and E proteins of TBE virus strain Oshima 5–10 (pCAG-prME) has been described previously [30,34]. In particular, we used plasmids that expressed prM and E proteins with amino acid mutation(s) that eliminated or added glycosylation sites. The constructed mutants of pCAG-prME are listed in Table 1.

These mutations were introduced into pCAG-prME using the overlapping PCR method [17]. Briefly, the forward (X-F) and reverse (X-R) primers (in which X is the location of the glycosylation site) (Fig. 1) were designed so that the generated PCR products would include the appropriate glycosylation site and specific restriction enzymes sites. The following primers (with the corresponding restriction enzymes in parentheses) were used: prM-F, 5′-CCTACAGCTCCTGGGCAACG-3′; prM-R, 5′-GTGTCCCCTTCCTGTGAGAT-3′ (*Xho*I and *Bgl*II); E-154 (E)-F, 5′-AGTGGCACAGTGTGCAAGAG-3′; E-R, 5′-GCACACTGTGTATGTAAGAC-3′ (*Bsp*1407I and *Bst*1107I); E-Dengue (ED)-F, 5′-TTACCTGGAGTATGGGCGGT-3′; and ED-R, 5′-GCCGTCGGTAGGTGTTCTGA-3′ (*Bgl*II and *Bsp*1407I).

Table 1
Constructed pCAGprME mutant vectors and predicted glycosylation patterns

Name	Mutation			Glycosylation		
	prM	E-dengue	E-154	prM	E-dengue	E-154
pCAGprME-wt	Asn Gly Thr	Ser Asp Thr	Asn Glu Thr	+	–	+
pCAGprME-dMg	**Gln** Gly Thr	Ser Asp Thr	Asn Glu Thr	–	–	+
pCAGprME-dEg	Asn Gly Thr	Ser Asp Thr	**Gln** Glu Thr	+	–	–
pCAGprME-dMEg	**Gln** Gly Thr	Ser Asp Thr	**Gln** Glu Thr	–	–	–
pCAGprME-dMgA	Asn Gly **Ala**	Ser Asp Thr	Asn Glu Thr	–	–	+
pCAGprME-dEgA	Asn Gly Thr	Ser Asp Thr	Asn Glu **Ala**	+	–	–
pCAGprME-dMEgA	Asn Gly **Ala**	Ser Asp Thr	Asn Glu **Ala**	–	–	–
pCAGprME-aEg	Asn Gly Thr	**Asn** Asp Thr	Asn Glu Thr	+	+	+
pCAGprME-tEg	Asn Gly Thr	**Asn** Asp Thr	**Gln** Glu Thr	+	+	–
pCAGprME-aEgQ	Asn Gly Thr	**Gln** Asp Thr	Asn Glu Thr	+	–	+
pCAGprME-tEgQ	Asn Gly Thr	**Gln** Asp Thr	**Gln** Glu Thr	+	–	–

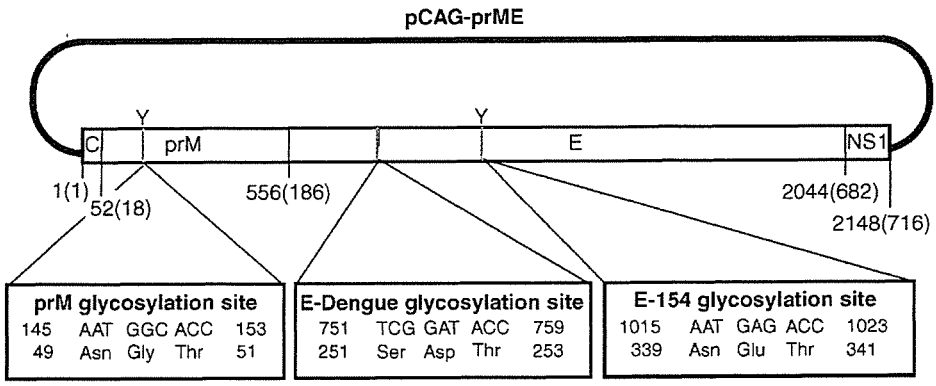

Fig. 1. Schematic representation of the recombinant TBE virus plasmid pCAGprME. The Y symbol shows the positions of the predicted glycans on the TBE virus. The amino acid sequences of the prM and E glycosylation sites are expanded at the bottom of the figure. Glycosylation mutations were introduced at the indicated sites in the prM and E proteins, replacing the amino acids shown in Table 1.

Mutants were generated with reverse and forward primers that contained the desired mutation. The first PCR product was generated with the F primer shown above, together with a primer that contained the mutations, i.e., X-gY-R, in which X is the location of the glycosylation site (Fig. 1) and Y is the substituted amino acid. The second PCR product was generated with the R primer shown above, together with a primer that contained the complementary strand mutations (X-gY-F). The primer sequences, in which the mutated nucleotides are underlined, were as follows: prM-gQ-F, 5′-TGCGTGTGGAACAAGGCACC-3′; prM-gQ-R, 5′-CACAGGTGCCTTGTTCCACA-3′; prM-gA-F, 5′-GCGTGTGGAAAATGGCGCCT-3′; prM-gA-R, 5′-CACAGGCGCCATTTTCCACA-3′; E-gQ-F, 5′-ACGTCGCTGCTCAGGAGACT-3′; E-gQ-R, 5′-TGAGTCTCCTGAGCAGCGAC-3′; E-gA-F, 5′-ACGTCGCTGCTAATGAGGCT-3′; E-gA-R, 5′-TGAGCCTCATTAGCAGCGAC-3′; ED-gN-F, 5′-GCAAAGCTAAATGATACCAA-3′; ED-gN-R, 5′-TTGGTATCATTTAGCTTTGC-3′; ED-gQ-F, 5′-GCAAAGCTACAGGATACCAA-3′; and ED-gQ-R, 5′-TTGGTATCCTGTAGCTTTGC-3′.

A mixture of the two purified PCR products served as the template for the third PCR, which was performed with the F and R primers. This final product was gel-purified, digested with the appropriate restriction enzymes, and cloned into pCAGprME-wt, to generate the pCAGprME-mutants. The plasmids were sequenced using a fluorescence autosequencer (ABI PRISM 310 Genetic Analyzer), to confirm the presence of the desired mutation and the absence of spurious changes.

2.2. Antibodies

In order to detect the TBE virus E protein in immunoprecipitates, the immunofluorescence assay, Western blotting, and ELISA, anti-Langat virus mouse hyperimmune ascitic fluid [3], which was found to cross-react with the E protein of the TBE virus, and the mouse monoclonal antibodies (MAb) 1H4, 4H8 and 2F9 (against TBE virus Far-Eastern subtype strain Oshima 5–10) [18] and MAb 1C3, 7G7, 1B3 and 5D6 (against the European subtype strain Neuderfl) [10] were prepared.

To detect TBE virus prM protein in Western blots, a rabbit polyclonal antibody against the prM protein of Oshima 5–10 was prepared by immunization with recombinant proteins that were expressed in the *Escherichia coli* Origami(DE3)pLysS strain (Novagen, San Diego, CA) that was transformed with the pET43 plasmid (Novagen), in which the ER luminal region of prM protein was cloned (Yoshii et al., manuscript in preparation).

2.3. Cells and transfection

293T cells were grown at 37 °C in high glucose Dulbecco's Modified Eagle's Medium (DMEM; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) that contained 10% fetal calf serum (FCS; MP Biomedicals, Irvine, CA) and L-glutamine (Kanto Chemical Co., Tokyo, Japan), and penicillin/streptomycin.

The 293 T cells were grown to about 60–70% confluence in six-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) and transfected with pCAGprME-wt or glycosylation-mutated plasmids using TransIT-LT1 (Pan-Vera, Madison, WI), according to the manufacturer's recommendations. Briefly, 10 μl of TransIT-LT1 was added to 100 μl of serum-free Opti-MEM (Invitrogen, Carlsbad, CA) and incubated at room temperature for 15 min. Then, plasmid DNA was added to the diluted TransIT-LT1, and the mixture was incubated at room temperature for 15 min. The Trans IT-LT1/DNA complex mixture was added to each well, and the cells were grown at 37 °C in high glucose DMEM that contained 10% FCS, L-glutamine, and penicillin/streptomycin for the appropriate time period.

2.4. Immunoprecipitation, SDS-PAGE, immunoblotting and lectin-blotting

The cells transfected with 3 μg of plasmid were washed with PBS and lysed with the lysis buffer [10 mM Tris–HCl (pH 7.8), 150 mM NaCl, 600 mM KCl, 5 mM EDTA, 1%

aprotinin, 1 mM PMSF, 2% Triton X-100]. The lysates of the transfected cells were centrifuged at 16,000 × *g* for 30 min and harvested the supernatant. Supernatants were reacted sequentially with MAb 1H4 and protein-G-Sepharose (Amersham Pharmacia Biotech, Buckinghamshire, England). The protein-G-Sepharose beads were washed twice with phosphate-buffered saline (PBS; pH 7.6) containing 0.05% Tween-20, suspended in sodium dodecyl sulfate (SDS) buffer [62.5 mM Tris–HCl buffer (pH 6.8), 2% SDS, 10% glycerol and 0.005% bromophenol blue], boiled for 5 min, and centrifuged at 10,000 × *g*. Supernatants were harvested and electrophoresed in SDS-polyacrylamide gels.

The protein bands on the SDS-PAGE gels were transferred onto polyvinylidene difluoride membranes. The membranes were incubated with 10 mM Tris–HCl-buffered saline containing 0.1% Tween-20 (TBST; pH 8.0) and 1% gelatin. For immunoblotting, the membranes were reacted for 1 h with the anti-Langat virus mouse hyperimmune ascitic fluid (1:200) or the anti-TBEV prM protein rabbit polyclonal antibody (1:1500). After washing, the membranes were reacted with alkaline phosphatase (AP)-conjugated antibody to mouse IgG (1:5000; Zymed, South San Francisco, CA) for 1 h, and then washed. For lectin-blotting, the membranes were reacted for 1 h with 20 μg/ml biotinylated lectin concanavalin A (Con A; J-oil mills, Tokyo, Japan). After washing, the membranes were reacted with AP-conjugated streptavidin (1:1000; Sigma, St. Louis, MO) for 1 h, and then washed. Protein bands were visualized with 5-bromo-4-chloro-3-indolylphosphatase (BCIP) and nitro blue tetrazolium (NBT) system (Novagen).

2.5. Four-layer enzyme-linked immunosorbent assay (ELISA)

At 24 h post-transfection, the cells that were transfected with 3 μg pCAGprME-wt or the glycosylation-mutated plasmids and the culture supernatants were harvested, and quantitated by four-layer ELISA, as described previously [34]. Briefly, the samples were diluted two-fold with PBS containing 0.1% Tween-20 and 0.5% bovine serum albumin (BSA), and added to MAb 1H4-coated wells of 96-well microtiter ELISA plates that were blocked with 3% BSA. The TBE virus-specific antigens in the fractions were detected using biotinylated MAb 4H8 and HRP-conjugated streptavidin (Zymed). HRP activity was detected by adding 100 μl of *o*-phenylene-diamine dihydrochloride (Sigma) in the presence of 0.03% H_2O_2. The plates were read at optical density (OD) 450 nm on a microplate reader. The non-transfected cell lysate and supernatant at 24 h post-transfection were used as negative controls. We determined the positive reaction if OD reading with the sample was more than that of negative control.

2.6. Immunofluorescence assay (IFA)

IFA was carried out as described previously [30]. 293T cells transfected with 5 μg pCAGprME or mutants were cultured on eight-well chamber slides (Sigma). At 24 h post-transfection, the cells were subsequently washed with PBS, fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton-X in PBS for 10 min at room temperature, and used as the antigen slides. The antigen slides were incubated with MAb 1H4, 4H8, 2F9, 1C3, 7G7, 1B3 or 5D6 for 1 h at 37 °C and washed in PBS. Fluorescein isothiocyanate-conjugated antibody to mouse IgG (1:500; Zymed) was then added to the slides. After incubation for 1 h at 37 °C and washing, the slides were observed under a fluorescence microscope. The IFA titer was determined as the highest dilution of antibody showing a positive fluorescence reaction.

2.7. Immunofluorescence colocalization studies

For immunofluorescence colocalization studies, 293T cells that were transfected with 5 μg pCAGprME or mutants were cultured in eight-well chamber slides (Sigma). At 6 h post-transfection, the cells were washed, fixed, and permeabilized as described above. The samples were incubated with antibodies for 1 h at 37 °C and washed in PBS. The following antibodies were used: MAb 1H4 for the E protein; anti-calnexin rabbit polyclonal antibody (1:200; Stressgen, San Diego, CA) as markers for the ER; and anti-giantin rabbit polyclonal antiserum (1:1000; Covance Research Products, Denver, PA) as markers for the Golgi complex. After extensive washing, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated antibody against mouse IgG and Texas Red-conjugated antibody against rabbit IgG (1:200; Jackson Immunoresearch Laboratories, West Grove, PA) for 1 h at 37 °C. The slides were viewed under Olympus IX70 confocal microscope.

2.8. Buoyant density determination of virus-like particles (VLPs)

Supernatants of the transfected 293T cells were cleared by centrifugation at 16, 000 × g for 30 min at 4 °C. The particles were then pelleted by precipitation with polyethylene glycol (PEG), as described previously [19]. Briefly, PEG 8000 and NaCl were added to the cleared supernatant to yield final concentrations of 10% PEG and 1.9% NaCl (w/w). The solution was stirred slowly at 4 °C overnight. After centrifugation at 12,000 × *g* for 30 min, the pellets were resuspended in PBS. The VLPs were purified by overnight equilibrium density centrifugation in a 10–50% sucrose gradient at 22,000 × *g* at 4 °C. Fractions of 0.5 ml were collected from the tops of the tubes, and E protein was quantitated by four-layer ELISA, as described previously [34].

2.9. Statistical analysis

The differences of the intracellular and extracellular expression levels of the glycosylation-mutated and wild-type viral proteins were analyzed using the Mann–Whitney *U*-test. Statistical significance was accepted at $P < 0.05$.

3. Results

3.1. Characteristics of glycosylation-deleted viral proteins

We investigated, by interfering with N-glycan substitution, whether the carbohydrate side-chains of the prM and E proteins were critical for the assembly and secretion of VLPs. Initially, three mutant plasmids pCAGprME-dMg, -dEg, and -dMEg were generated based on pCAGprME (Table 1). Mutations that substituted the asparagines-encoding AAT parental sequence with a glutamine-encoding CAG or CAA were constructed in pCAGprME (Fig. 1). Thus, we generated plasmids in which the glycosylation consensus sequence prM or E was mutated in such a way that it would not be recognized by the oligosaccharyltransferase.

The recombinant prM and E proteins were expressed by transfection of pCAGprME, or mutants thereof, into 293T cells. To verify that these plasmid-transfected cells expressed proteins with the expected patterns of glycosylation, the viral glycoproteins were immunoprecipitated with the anti-E MAb 1H4 and analyzed by Western blotting. The E protein bands were detected by the anti-Langat ascitic fluid in all samples after precipitation, and prM co-precipitated with E and was detected by the anti-TBE prM protein antibody. These results suggest that heterodimerization of prM and E occurs naturally. The nonglycosylated prM and E protein bands migrated faster than the glycosylated proteins due to the lack of glycan (Fig. 2A: upper panel, lanes 2 and 3; lower panel, lanes 1 and 3). Using ConA, which is a type of lectin that binds specifically to high-mannose type N-linked glycans, only the glycosylated protein bands were visible in all the samples (Fig. 2B).

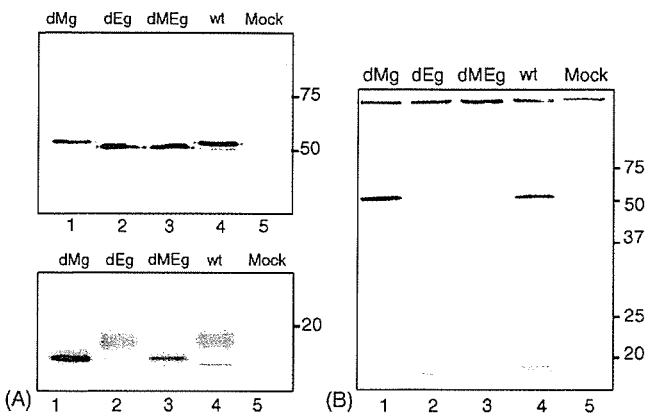

Fig. 2. Western blot analysis of glycosylation-deleted TBE virus proteins that were expressed in 293T cells. (A) The 293T cells were transfected with pCAGprME-wt or glycosylation-deleted mutant vectors (pCAGprME-dMg, -dEg, and -dMEg). At 24 h post-transfection, the cell lysates were immunoprecipitated with MAb 1H4, and used as samples for further analysis. The samples were subjected to Western blotting using the anti-Langat virus mouse hyperimmune ascitic fluid (upper panel) or the anti-TBEV prM protein rabbit polyclonal antibody (lower panel). (B) The samples from pCAGprME-wt and the glycosylation-deleted mutants were subjected to the lectin blot assay with Con A.

Several studies have established that single amino acid changes in critical determinants of the E protein are sufficient to cause inhibition of viral growth [8,25]. To examine that the amino acid substitution affected the characteristics of the recombinant proteins, such as charge and polarity, three additional pCAGprME derivatives (pCAGprME-dMgA, -dEgA, and -dMEgA) were generated (Table 1). A mutation that substituted the threonine-encoding ACC parental sequence with the alanine-encoding GCC was constructed in pCAGprME (Fig. 1). This mutation was designed to change the protein such that it would not be recognized by the oligosaccharyltransferase. The viral glycoproteins from pCAGprME-dMgA, -dEgA, and -dMEgA were immunoprecipitated and analyzed by Western blotting. pCAGprME-dMgA, -dEgA, and -dMEgA showed protein band mobilities that were similar to those of pCAGprME-dMg, -dEg, and -dMEg, respectively (data not shown). These results demonstrate that the constructed pCAGprME-mutant plasmids express prM and E proteins with the predicted glycosylation patterns.

3.2. Comparisons of the intracellular and extracellular expression levels of the glycosylation-deleted and wild-type viral proteins

To compare the intracellular and extracellular levels of viral proteins, 293T cells were transfected with 3 μg pCAGprME-wt or the glycosylation-mutated plasmids and incubated for 24 h. The cells and supernatants were harvested, and subjected to four-layer ELISA (Fig. 3). E proteins were detected in all the transfected cell lysates, and the samples from pCAGprME-wt and -dMEg showed similar expression levels of the E protein. However, the level of E protein in the sample from pCAGprME-dMg was higher than that in the sample from pCAGprME-wt, and the level of E protein in the sample from pCAGprME-dEg was 60% of that in the sample from pCAGprME-wt ($P < 0.05$; Fig. 3A).

The level of E protein in the supernatant of cells that were transfected with pCAGprME-dMg was about 60% of that in the supernatant of pCAGprME-wt-transfected cells ($P < 0.05$; Fig. 3B). On the other hand, the supernatants of pCAGprME-dEg and -dMEg showed dramatically reduced levels of the E protein ($P < 0.05$; Fig. 3B).

The cells and supernatants from cultures that were transfected with pCAGprME-dMgA, -dEgA, and -dMEgA showed intracellular and extracellular levels of protein E that were similar to those of cells and supernatants from cultures transfected with pCAGprME-dMg, -dEg, and -dMEg, respectively (data not shown). These results strongly suggest that the observed differences in the production levels of prM and E protein are due to glycan loss, rather than the amino acid substitutions per se.

3.3. Characteristics of the glycosylation-added and glycosylation-translocated viral proteins

As described above, the inhibition of E protein glycosylation had a major suppressive effect on the secretion of the

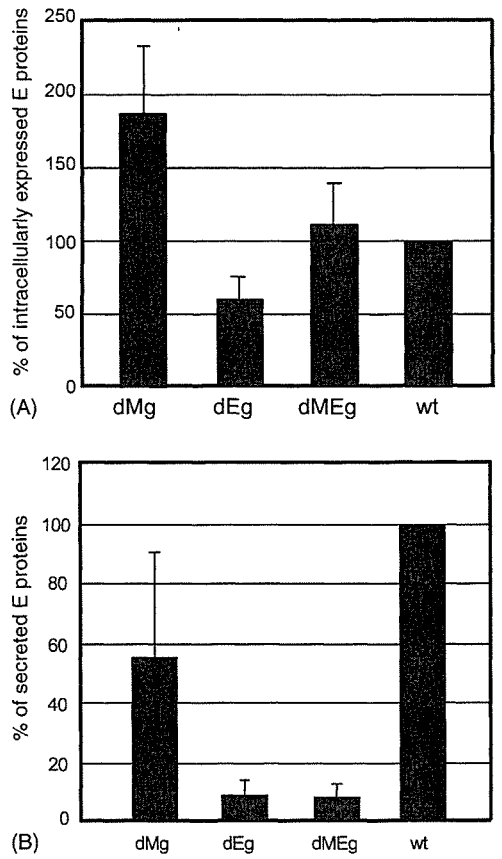

Fig. 3. Effects of nonglycosylated prM or E protein on intracellular expression (A) and extracellular secretion (B) of TBE vital proteins. The 293T cells were transfected with the appropriate glycosylation site-deleted mutant vector. At 24 h post-transfection, the cell lysates and cell culture supernatants were harvested. The levels of intracellularly expressed or secreted E protein were measured by four-layer ELISA. The data presented in this figure are from four independent experiments, and the percentages of intracellular and extracellular expression from the mutant vectors are calculated from the calibration curve for the data on the wild-type in each experiment.

prM and E proteins (Fig. 3B). In flaviviruses, the E protein has zero to two N-linked glycosylation consensus sequences, and the N-linked glycosylation consensus sequence at position 154 of the amino acid sequence of the E protein (E-154 glycosylation site; Fig. 1) is also present in other flaviviruses [2]. The E protein is differentially glycosylated in other flaviviruses, such as some strains of Kunjin virus and West Nile virus (nonglycosylated E protein) or dengue virus (two glycosylation sites) [2]. To investigate whether the position or number of glycans affected protein synthesis, additional pCAGprME mutant plasmids were prepared (Table 1). A mutation that substituted the TCG parental sequence (for serine) at position 66 of the amino acid sequence of the E protein (E-dengue glycosylation site; Fig. 1) with an AAT sequence (for asparagine) was constructed in the pCAGprME-wt and -dEg plasmids, thereby creating plasmid pCAGprME-aEg, which has two putative glycosylation sites in the E protein, and pCAGprME-tEg, which has only the E-dengue glycosylation site in the E protein plasmid (Table 1).

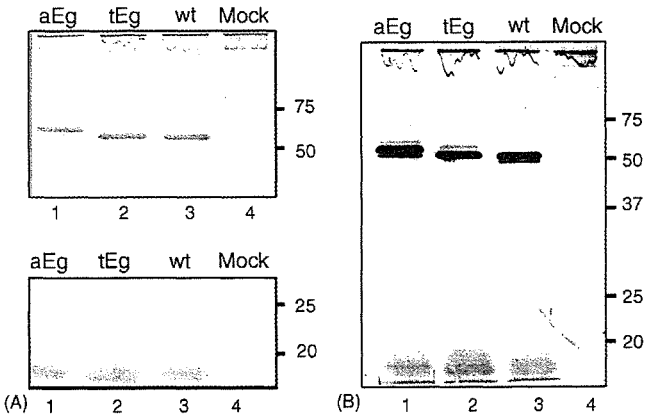

Fig. 4. Western blot analysis of TBE virus proteins from glycosylation-added or -translocated mutants that were expressed in 293T cells. (A) The 293T cells were transfected with pCAGprME-wt, -aEg, and -tEg. At 24 h post-transfection, the cell lysates were immunoprecipitated with MAb 1H4 and used as the samples in the subsequent analyses. The samples were detected in Western blots using the anti-Langat virus mouse hyperimmune ascitic fluid (upper panel) or the anti-TBEV prM protein rabbit polyclonal antibody (lower panel). (B) The samples from pCAGprME-wt and the glycosylation-added or -translocated mutants were detected in lectin blots with Con A.

To verify that the cells transfected with these plasmids expressed proteins with the expected glycosylation patterns, the viral glycoproteins were immunoprecipitated with the anti-E MAb 1H4 and analyzed by Western blotting. E protein bands were detected by the anti-Langat ascitic fluid in all of the samples after precipitation, and prM co-precipitated with E and was detected in the Western blot (Fig. 4A, lower panel, lanes 1–3). These results suggest that the heterodimerization of prM and E occurs naturally. The E protein band from pCAGprME-aEg migrated more slowly than did that of pCAGprME-wt, due to the addition of glycan (Fig. 4A, upper panel, lanes 1 and 3). On the other hand, the E protein band from pCAGprME-tEg migrated to the same extent as that of pCAGprME-wt (Fig. 4A, upper panel, lanes 2 and 3). Blotting with ConA showed similar results to blots using the anti-Langat ascitic fluid or the anti-TBE prM protein antibody (Fig. 4B).

To determine whether these amino acid substitutions in the E-dengue glycosylation site affected protein synthesis and secretion, control plasmids were also constructed. These plasmids included a mutation that substituted the TCG parental sequence (for serine) at position 66 of the amino acid sequence of the E protein with a CAG sequence (for glutamine) (pCAGprME-aEgQ and -tEgQ). These plasmids were designed to express glycosylated proteins that were similar to those of pCAGprME-wt and -dEg, as the introduced mutations would cause similar amino acids changes at the E-dengue glycosylation site, but would not be recognized by the oligosaccharyltransferase. The viral glycoproteins from pCAGprME-aEgQ and -tEgQ were immunoprecipitated and analyzed by Western blotting. The mobilities of the aEgQ bands were similar to that of pCAGprME-wt,

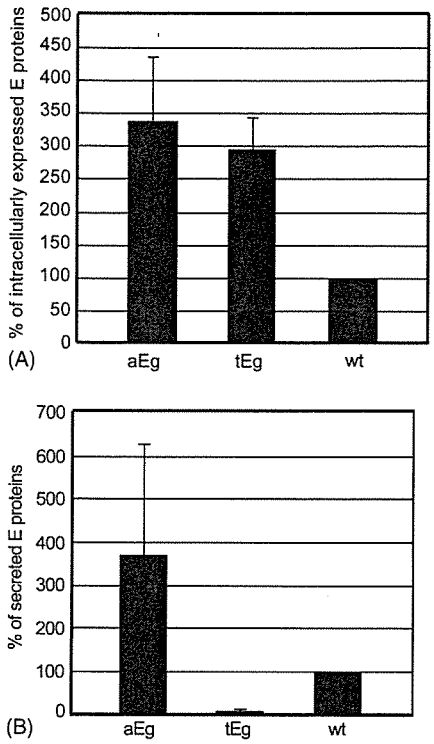

Fig. 5. Effects of the glycosylation site-added or -translocated E proteins on intracellular expression (A) and extracellular secretion (B) of TBE vital proteins. The 293T cells were transfected with the appropriate glycosylation site-added or -translocated mutant vector. At 24 h post-transfection, the cell lysates and cell culture supernatants were harvested. The levels of intracellularly expressed or secreted E protein were measured by four-layer ELISA. The data presented in this figure are from four independent experiments, and the mean percentages of intracellular expression and extracellular secretion are calculated for each mutant, as described in the legend to Fig. 3.

and the mobilities of the EgQ bands were similar to that of pCAGprME-dEg (data not shown). These results demonstrate that the constructed pCAGprME-mutant plasmids expressed prM and E proteins with the expected glycosylation patterns.

3.4. Comparison of the intracellular and extracellular expression levels of the glycosylation-added, glycosylation-translocated, and wild-type viral proteins

The intracellular and extracellular levels of viral protein were compared for pCAGprME-aEg, pCAGprME-tEg, and the wild-type, as described above. The levels of E protein in the cell lysates of both transfected mutants were three-fold higher than in those of pCAGprME-wt ($P < 0.05$; Fig. 5A). The levels of E protein in the supernatants of cells that were transfected with pCAGprME-aEg were about four-fold higher than in cells with pCAGprME-wt, whereas the corresponding level for pCAGprME-tEg was 90% lower than for pCAGprME-wt ($P < 0.05$; Fig. 5B).

The intracellular and extracellular expression levels of E protein in the samples from pCAGprME-aEgQ and -tEgQ were also compared. On the basis of glycosylation patterns, intracellular expression and extracellular secretion level of the pCAGprME-aEgQ and -tEgQ resembled pCAGprME-wt and -dEg, and differed from pCAGprME-aEg and -tEg (data not shown). These results suggest that the observed differences in the secretion levels of the prM and E proteins are due to either the loss or addition of glycan, rather than to the amino acid substitutions per se.

Table 2
Characterization of recombinant proteins in pCAGprME-wt and mutants transfected 293T cells by IFA test with various MAb

MAb	wt	dMg	dEg	dMEg	aEg	tEg
1H4	++	++	++	++	++	++
4H8	++	++	++	++	++	++
2F9	++	+	+	+	+	+
1C3	++	++	++	++	++	++
7G7	++	++	++	++	++	++
1B3	++	++	+	+	++	+
5D6	++	++	++	++	++	++

MAbs 1H4, 4H8 and 2F9 were prepared against the far-eastern subtype strain Oshima 5–10. MAbs 1C3, 7G7, 1B3 and 5D6 were prepared against the European subtype strain Neudoerf. The IFA titres are graded as + (200–2000) and ++ (>2000).

3.5. The antigenicity of glycosylation-mutated E proteins

The antigenicity of recombinant proteins in pCAGprME-transfected 293T cells was examined by IFA testing using various MAbs. Table 2 showed that most of MAbs reacted to all recombinant proteins in pCAGprME-wt or its mutants transfected 293T cells. However, MAbs 1B3 reacted lower with the recombinant proteins in pCAGprME-dEg, -dMEg and -tEg transfected 293T cells, implying that these mutants have conformational changes located in the epitopes for which these MAbs are specific.

3.6. Intracellular localization of glycosylation-mutated E proteins

To determine differences in the intracellular distribution of the prM and E proteins expressed from each plasmid, the transfected cells were fixed, permeabilized, and double-stained for the E protein and cellular marker antigens. The anti-calnexin antibody was used as a marker for the ER [11], and the anti-giantin antibody was used as a marker for the Golgi complex [21].

The distributions of the E protein overlapped almost completely with those of calnexin in the cells that were transfected with the respective plasmids (Fig. 6A). Some overlaps were also seen in the distributions of the E protein and giantin in the cells that were transfected with the pCAGprME-wt, -dMg and -aEg plasmids, respectively. However, in the cells that were transfected with pCAGprME-dEg, -dMEg, and -tEg, the distribution of E protein rarely overlapped with that of giantin (Fig. 6B). These data suggest that E protein that is not glycosylated at the E-154 glycosylation site localizes

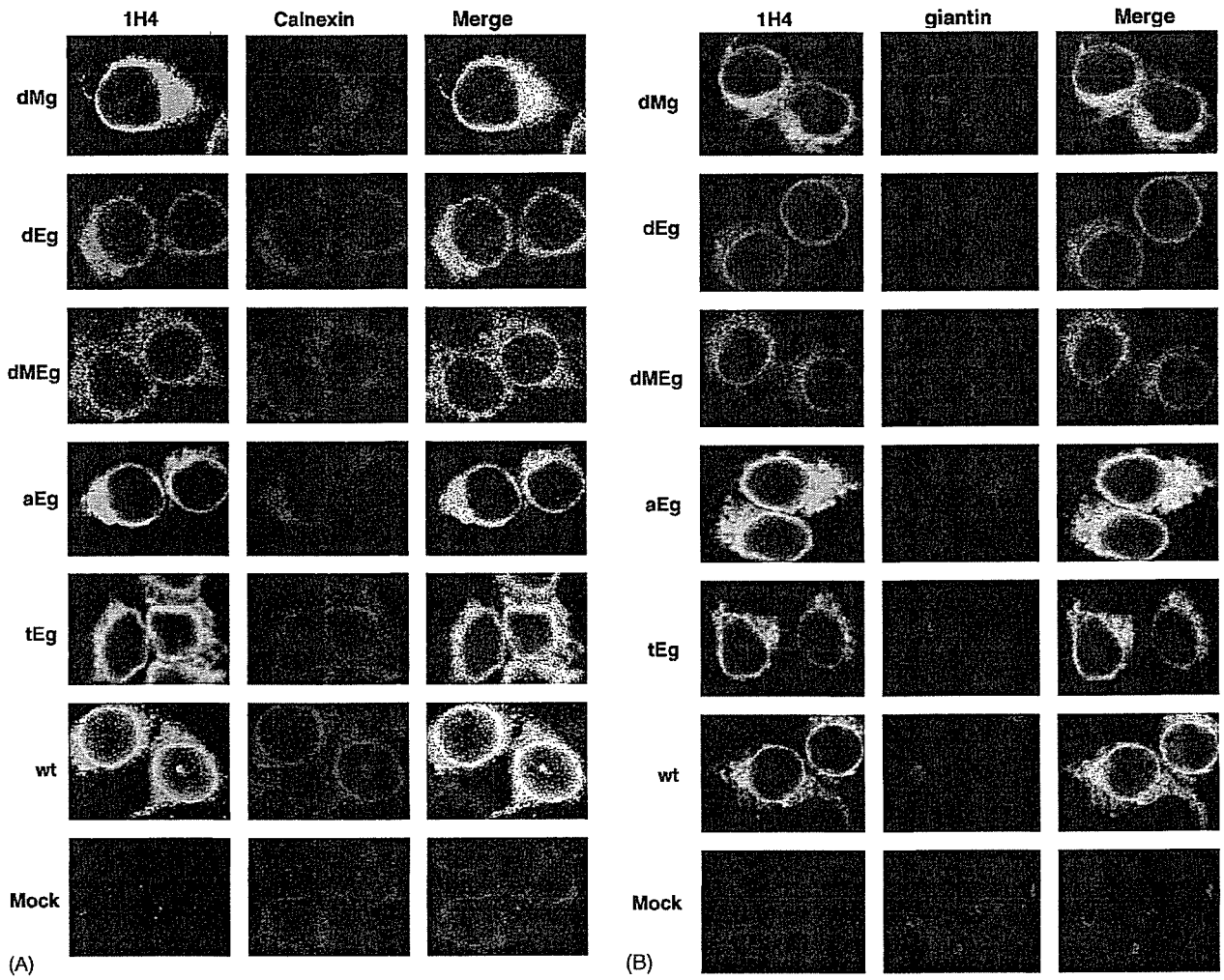

Fig. 6. Intracellular localization of the TBE virus envelope proteins. The 293T cells that were transfected with pCAGprME-wt or the glycosylation-mutated plasmids (pCAGprME-dMg, -dEg, -dMEg, -tEg, and -aEg) were fixed and subjected to indirect immunofluorescent co-staining with primary antibodies that recognize the TBE virus E protein, as well as antibodies against a cellular organelle marker protein. The proteins were then labeled with secondary antibodies that were conjugated to red or green light-emitting fluorophores. Cells with immunofluorescent staining of the ER by polyclonal anti-calnexin (A), with staining of the Golgi by the polyclonal anti-giantin antibody (B), and with staining by the anti-E protein MAb 1H4 (left column, respectively), are shown. Also shown is colocalization of the viral envelope proteins with the organelle markers, which are represented by the yellow regions within each cell in the merged images (right column, respectively).

almost always to the ER, and is rarely present in the Golgi complex.

3.7. Characteristics of glycosylation-mutated VLPs

A previous study showed that 293T cells that were transfected with pCAGprME produced VLPs [34]. To confirm that the protein expressed extracellularly by the pCAGprME mutants (pCAGprME-dMg and -aEg) formed VLPs, the culture media of the transfected 293T cells were purified by equilibrium density centrifugation, and each fraction was analyzed by ELISA, as described previously. The equilibrium banding profiles in sucrose density gradients indicated that the VLPs banded at a sucrose density of 1.13–1.14 g/cm^3, which is identical to the value reported previously for VLPs (data not shown) [28,34].

4. Discussion

N-linked glycan binding to viral glycoproteins is crucial for virus growth, budding, and secretion. In this study, we revealed, in detail, the function of glycosylation in the formation and secretion of TBE viral particles by comparing the synthesis and secretion levels, as well as the biological properties, of each glycosylation-mutated recombinant VLP.

In order to inhibit the glycosylation of the prM and E proteins, we substituted the amino acids within the glycosylation site with conservative amino acids substitutions that did not affect the characteristics of the residue. The advantage of this method is that we can demonstrate the effect of glycosylation on the secretion of VLPs without altering the functions of the cellular glycoproteins. In previous studies reporting similar experiments, glycosylation inhibitors, such as tunicamycin,

were used [7,22]. However, these drugs affect the cellular glycoproteins that participate in the production and secretion of VLPs. Besides, we showed that the cells and supernatant samples from the pCAGprME mutants, which contain various amino acid substitutions at the glycosylation site, had similar levels of E protein, and similar glycosylation patterns (data not shown). These results verify that the differences between the levels of prM and E proteins are caused not by the amino acid substitutions, but by the loss of glycan residues. Several studies have established that single amino acid changes in the critical determinants of the E protein are sufficient for viral growth inhibition [8,25]. However, an amino acid substitution that results in a significant change in biological characteristics is usually due to a non-conservative mutation.

The main finding of this study is that the glycan that occupies the E-154 glycosylation site plays an important role in the secretion of VLPs from mammalian cells. In the cells that were transfected with the mutant vectors, and which lacked the E-154 glycosylation site, the secretion levels of VLPs were very low (Figs. 3B and 5B). The E-154 glycosylation site is present in many flaviviruses, and previous studies have shown that a dengue virus mutant that had lost the E-154 glycosylation site grew slowly in mammalian cells [9]. Furthermore, another study revealed that a chimeric TBE and dengue virus mutant that had lost the E-154 glycosylation site displayed reduced neurovirulence in mice [26]. These studies and our data suggest that glycosylation of the E-154 glycosylation site has more pronounced effects on viral growth and VLP secretion than does glycosylation of the E-dengue or prM glycosylation sites.

The glycan at the E-dengue glycosylation site seems to facilitate the correct expression of the E protein, but does not participate in the secretion of VLPs (Figs. 4A, 5B and 6B). There is scant data on the relationship between the E-dengue glycosylation site and viral virulence. It has been suggested that the glycan at the E-dengue glycosylation site participates in the expression of the E protein, but not in the transportation of VLPs from the ER to the extracellular compartment. Moreover, it appears that glycosylation of the prM protein plays a minor role in the secretion of VLPs (Fig. 3B). A previous study has indicated that heterodimerization of prM and E proteins starts soon after synthesis, in a process that seems to be essential for the E protein to reach its final native conformation [22]. Our results suggest that, regardless of whether or not the prM protein is glycosylated, the heterodimerization of prM and E occurs normally (Fig. 2A). Thus, the glycan binding to the prM protein may play a less important role on the secretion of VLPs than that of E protein, although the lack of glycosylation of the prM protein may have a greater effect on the secretion of VLPs than the correct expression of E protein.

It is not entirely clear how the glycosylation of the prM and E proteins affects the secretion of VLPs. However, it is believed that intracellular animal lectins play important roles in the quality control and sorting of the glycoprotein along the secretory pathway, and that these lectins participate in the folding of the prM and E proteins, and in the transportation and the secretion of VLPs [11–13]. One of the functions of the intracellular animal lectins involves the selective transportation of the glycoprotein. The mannose lectin ERGIC-53, which is expressed in all cells of multicellular organisms, operates as a cargo receptor in the transport of glycoproteins from the ER to the Golgi [13]. Exit from the Golgi to endosomes may also involve traffic lectins. We have been unable to demonstrate directly the interaction between these lectins and the E protein. However, glycosylation at the E-154 glycosylation site seems to have an important impact on the transportation from the ER to the Golgi (Fig. 6B) and secretion of VLPs (Figs. 3B and 5B). It is possible that the glycan at the E-154 glycosylation site interacts with these lectins more strongly than do the other glycans that are associated with the prM and E proteins.

Another function of the intracellular lectins in glycoprotein secretion lies in aiding the efficient folding of the glycoprotein and the degradation of the misfolding glycoprotein. Calnexin and calreticulin are ER lectins that interact with partially glucose-trimmed glycoproteins in the ER and serve as important molecular chaperones. They promote the correct folding of their substrate proteins, and they are involved in the quality control and ER retention of incompletely folded and assembled proteins [11,12]. In flaviviruses and other viruses, there have been many reports that implicate calnexin and calreticulin in the correct folding of viral glycoproteins and in viral growth [4,23,24,32]. However, as determined by Western blotting under the nonreducing condition (Figs. 2 and 4), intrachain disulfide bond formation is normal in the mutant E proteins. It seems likely that inhibition of glycosylation causes a minor conformational change in the E protein, which would reduce the translocation and secretion of VLPs. On the other hands, other ER lectins and ubiquitin ligases that recognize the N-glycans interact with calnexin and calreticulin, and participate in translocation from ER into cytosol and degradation of glycoproteins accumulated in ER [12,33]. Intracellular expression level of the E protein from prM protein glycosylation-deficient vectors (pCAGprME-dMg and -dMEg) is more than that of the prM protein glycosylated vectors (pCAGprME-wt and -dEg) (Fig. 3A). Thus, the glycosylation of prM protein may play a role in transportation and degradation of prM and E protein accumulated in ER.

Previous studies have shown that flavivirus VLPs represent excellent candidates for a recombinant vaccine against flaviviruses, such as TBEV [14,20]. In our experiments, the levels of E protein in the supernatant samples from cells that were transfected with pCAGprME-aEg were four-fold higher than the wild-type (Fig. 5A), and the antigenicity of the E protein from pCAGprME-aEg was similar to that of the wild-type (Table 2). These results suggest that the addition of glycan to the E protein may ensure effective production of VLPs without changing VLP antigenicity, which encourages further study into the development of vaccines.

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Development of an enzyme-linked immunosorbent assay for serological diagnosis of tick-borne encephalitis using subviral particles

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Abstract

The similarity of symptoms produced by tick-borne encephalitis (TBE) and Japanese encephalitis (JE) and the high degree of cross-reactivity between TBE and JE viruses by serological tests make the development of a differential diagnostic test a priority. In this study, recombinant prM/E proteins of TBE virus strain Oshima 5–10 expressed in mammalian cells resulted in the release of subviral particles (SPs) into the culture medium. Using the SPs as antigens, enzyme-linked immunosorbent assay (ELISA) systems were developed to detect TBE virus-specific IgM and IgG antibodies, designated SP-IgG and SP-IgM ELISAs, respectively. Of 83 serum samples from encephalitis patients in Khabarovsk, Russia, which were positive with the neutralization test (NT), 82 were positive by the SP-IgG ELISA, for a sensitivity of 98.8%, which was higher than that of a commercial ELISA kit. All 12 NT-negative samples were also negative by the SP-IgG ELISA (specificity, 100%). Of 17 patient samples that were NT-positive, 16 (94.1%) were positive by the SP-IgM ELISA. Of 15 paired serum samples that yielded equivocal results by NT, 11 had positive results with the SP-IgM ELISA, indicating a diagnosis of TBE infection. The SP-IgG and SP-IgM ELISAs showed no cross-reactivity with antibodies to the JE virus. The results indicate that these ELISAs will be useful for the detection of TBE-specific antibodies.

Keywords: Tick-borne encephalitis virus; ELISA; Subviral particles

1. Introduction

Tick-borne encephalitis (TBE) virus belongs to the family Flaviviridae, genus Flavivirus, and causes fatal encephalitis in humans. There are three main genetic lineages of TBE virus; the European, Siberian, and Far Eastern subtypes. They cross-react each other (Hayasaka et al., 2001). Although there are various tests for detection antibodies for the serological diagnosis of TBE, neutralization tests (NTs) are used in areas where two or more flaviviruses are endemic because of their high degree of specificity for each virus. However, neutralization tests have some disadvantages, including the requirement for a high-level biocontainment facility to handle the live viruses, the need for advanced and skilled techniques, limitations on the number of samples that can be tested, and the time-consuming nature of the neutralization reaction. On the other hand, enzyme-linked immunosorbent assays (ELISA) based on inactivated TBE virus antigens are also used widely. Since many samples can be tested in a short time under ordinary laboratory conditions with ELISA, ELISA is a useful diagnostic method. However, the production process for the ELISA antigen requires purification and inactivation of the virus using skilled techniques in laboratories with biosafety facilities. It has also been reported that the standard TBE ELISAs can cross-react with antibodies to other flaviviruses (Dobler et al., 1996; Holzmann et al., 1996; Niedrig et al., 2001). Therefore, serological diagnostic methods which are safe, simple, and specific to TBE virus need to be developed.

The positive single-stranded RNA genome of the genus Flavivirus consists of about 11,000 nucleotides. It encodes three structural proteins, i.e., the core (C), precursor membrane (prM), and envelope (E) proteins, and seven nonstructural proteins, i.e.,

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NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Chambers et al., 1990). When recombinant prM and E proteins are co-expressed in mammalian cells, subviral particles (SPs) that do not contain nucleocapsids are released into the culture medium (Allison et al., 1995; Fonseca et al., 1994; Konishi et al., 1992). The recombinant prM and E proteins of the Oshima 5–10 strain of TBE virus can be used to produce SPs in such systems, as described previously (Yoshii et al., 2003). SPs are expected to be useful as antigens for the serological diagnosis of Flavivirus infections because they maintain the authentic characteristics of the viral antigens. The SPs of the European subtype TBE virus, expressed in insect cells using a recombinant baculovirus system, have been used to develop an IgM ELISA (Jaaskelainen et al., 2003).

In this study, ELISAs for the detection of anti-TBE IgG and IgM were developed using SPs of the Far Eastern subtype TBE virus expressed in mammalian cells as antigens. The ELISAs were evaluated using serum samples from patients with suspected TBE from Khabarovsk, Russia, and the results were compared with those obtained using the neutralization test and two commercial ELISA kits.

2. Materials and methods

2.1. Cells and virus strains

BHK-21 cells were cultured in Eagle's minimum essential medium containing 8% fetal bovine serum (FBS) and were used for the neutralization tests. The 293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% FBS and were used for the expression of recombinant proteins.

The Oshima 5–10 strain of TBE virus and the JaGAr-01 strain of Japanese encephalitis (JE) virus were used. The Oshima 5–10 strain was isolated from dogs in 1995 and was the Far Eastern subtype (Takashima et al., 1997). The JaGAr-01 strain was isolated from mosquitoes in Japan in 1959 (Matsuyama et al., 1960). The viruses were propagated by intracerebral inoculation of suckling mice.

2.2. Serum samples

All serum samples were heat-inactivated at 56 °C for 30 min and were stored at −40 °C. A total of 95 serum samples were collected from 43 patients in Khabarovsk, Russia, who were suspected of having TBE on the basis of clinical signs. Nine serum samples were single serum samples and 86 were paired samples from 34 patients. Acute samples were collected on from 1 to 17 days after onset of illness and convalescent samples were collected on from 5 to 72 days after onset of illness. The interval between collection of acute and convalescent samples were from 1 to 61 days. Ten serum samples were collected from patients with JE in Nepal and had been shown to have JE-specific IgM antibodies by using IgM ELISA (Akiba et al., 2001). They were all negative for TBE on the NT. Twenty-one negative control serum samples were obtained from individuals who were negative for TBE by the neutralization test.

2.3. Neutralization tests

These tests were carried out as described previously (Kariwa et al., 1995). Serum samples that produced a 50% reduction in focus formation of the Oshima 5–10 strain of TBE virus on BHK cells in 96-well plates as determined by immunohistochemical staining at a dilution of 1:20 or greater were judged to be neutralization test-positive. The patients who have greater than four-fold increase in the neutralizing titer in the convalescent phase compared with the acute phase of infection were diagnosed with TBE infection.

2.4. Plasmids and antigens

A plasmid encoding the prM and E proteins of the Oshima 5–10 strain (pCAGprME, Yoshii et al., 2003) was transfected into 293T cells as described previously (Yoshii et al., 2003). After a 48-h incubation at 37 °C, the culture medium was harvested and centrifuged at 12,000 rpm for 30 min. A one-third volume of polyethylene glycol (PEG) solution (40% w/v PEG8000, 7.6% w/v NaCl) was added to the collected culture medium supernatants. After gentle shaking at 4 °C for 2 h, the mixture was centrifuged at 10,000 rpm for 20 min. The pellets were dissolved in carbonate–bicarbonate buffer (Sigma Chemical Co., St. Louis, MO) to yield 1% of the original culture medium volume, and this was used as the positive antigen for the ELISAs. The negative antigen was prepared from the culture medium of nontransfected 293T cells.

2.5. SP-IgG ELISA

The monoclonal antibody (mAb) 1H4, which recognizes the E protein of the Oshima 5–10 strain of TBE virus (Komoro et al., 2000), was coated onto 96-well microplates (50 μl/well, 2 μg/ml in carbonate buffer). After overnight incubation at 4 °C, the plates were washed five times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST). A blocking solution (Block Ace diluted 1:4 in ddH_2O; Dai-Nippon, Osaka, Japan) was applied (200 μl/well), and the plates were incubated at 37 °C for 1 h. The plates were washed before adding the subviral particle antigen (50 μl/well, 1:10 dilution in PBST containing 0.3% bovine serum albumin) and incubating at 37 °C for 1 h. After washing, the serum samples were added (50 μl/well, 1:800 dilution in PBST containing 1% skim milk), and the plates were incubated at 37 °C for 1 h. Bound IgG antibodies were detected by adding 50 μl/well of alkaline-phosphatase-conjugated anti-human IgG goat IgG (1:2000 in PBST containing 0.3% bovine serum albumin; Sigma) and incubating at 37 °C for 1 h. The color reaction was developed by adding 100 μl/well of *p*-nitrophenyl phosphate and incubating at 37 °C for 90 min, and the absorbance at 405–620 nm was measured. The results for each serum sample were reported as the positive:negative ratio (P/N), that is, the ratio of the optical density (OD) with the positive antigen to the OD with the negative antigen.

2.6. SP-IgM Elisa

Anti-human IgM goat IgG (50 μl/well, 1:400 in carbonate buffer; ICN Biomedicals, Aurora, OH) was added to 96-well microplates. After overnight incubation at 4 °C, the plates were washed five times with PBST before adding 200 μl/well of blocking solution containing 3% bovine serum albumin in PBS and incubating at 37 °C for 1 h. After washing, the serum samples were added (50 μl/well, 1:100 dilution in PBST containing 1% skim milk), and the plates were incubated at 37 °C for 1 h before washing again. The subviral particle antigen was added (50 μl/well, 1:20 dilution in PBST containing 1% skim milk), and the plates were incubated at 37 °C for 1 h and washed again. The subviral particle antigen bound by the IgM antibodies was detected by the addition of biotinylated mAb 1H4 (50 μl/well, 0.25 μg/ml in PBST containing 0.3% bovine serum albumin) and incubation at 37 °C for 1 h, followed by washing, the addition of horseradish peroxidase–streptavidin (50 μl/well, 1:3000 in PBST containing 1% skim milk; Zymed, South San Francisco, CA), and incubation at 37 °C for 1 h. The color reaction was developed by the addition of 3,3′,5,5′-tetramethylbenzidine (100 μl/well; Sigma). The reaction was stopped after 15 min at room temperature by the addition of 100 μl of 0.5N H_2SO_4. The absorbance at 450 and 620 nm was measured using a plate reader. The P/N ratios were determined as described above for the SP-IgG ELISA.

2.7. Commercial ELISA

The commercial Immunozym FSME IgG and IgM kits (Progen Biotechnik, Heidelberg, Germany) were used for comparison with the SP-ELISA. In this commercial ELISA, the European subtype virus was used as antigens.

3. Results

3.1. SP-IgG ELISA

Ninety-five serum samples from patients with suspected TBE and 21 negative control samples were tested for the presence of anti-TBE IgG antibodies using the SP-IgG ELISA, and the sensitivity and specificity of the test were determined by comparison with the results of the neutralization test, using the corresponding cut-off values (Fig. 1). The sensitivity of the SP-IgG ELISA decreased with increasing cut-off values, while the specificity increased. The difference between the sensitivity and specificity was minimal when a cut-off value of 1.155 was used. At a cut-off value of 1.155, the sensitivity of the SP-IgG ELISA about 95 serum samples from patients suspected TBE was 98.8% (82/83) and the specificity was 100% (12/12) as compared with the neutralization results (Table 1). All of 21 samples which were negative by neutralization were negative on the SP-IgG ELISA. Only 57 of the 83 NT-positive samples were positive on the commercial IgG ELISA (Table 2), whereas five samples were negative and 21 samples were inconclusive because the results were close to the boundary values. The 12 samples that had neutralizing titers <1:20 were also negative on both the

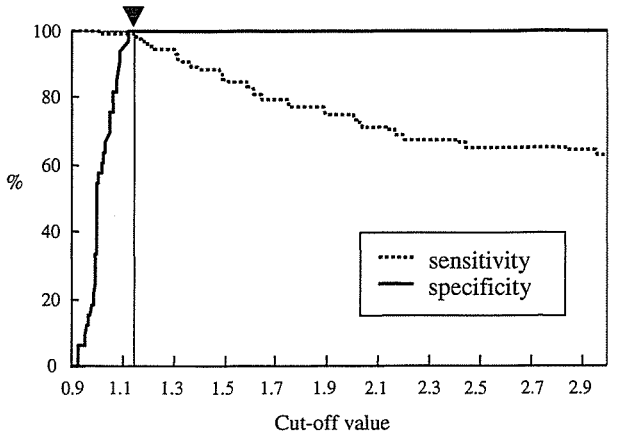

Fig. 1. Relationship between cut-off value, sensitivity, and specificity for the SP-IgG ELISA: 95 serum samples collected from 43 patients who were suspected TBE and 21 negative control serum samples were tested. The cut-off value was set as the point at which the difference in the sensitivity and specificity was minimal.

Table 1
Comparison of the results obtained by neutralization and SP-IgG ELISA (95 serum samples collected from 43 patients who were suspected TBE)

Neutralization test	SP-IgG ELISA		Total
	Positive	Negative	
Positive	82	1	83
Negative	0	12	12
Total	82	13	95

SP-IgG ELISA and the commercial IgG ELISA. IgG antibody was detected with SP-IgG ELISA in the samples from 1 to 72 days after onset of illness. On the other hand, the samples were positive with commercial IgG ELISA from 2 to 72 days.

3.2. SP-IgM ELISA

The distribution of the P/N ratios resulting from the analysis of the serum samples using the SP-IgM ELISA is shown in Fig. 2. The samples tested were clearly separated into two groups: one group of sera had P/N values between <1.0 and <1.25 and were presumed to be negative for TBE-specific IgM antibodies, and the other group had P/N values between <1.9 and <50 and were presumed to be positive for TBE-specific IgM antibodies. The mean of the maximum P/N ratio of the negative group (1.224)

Table 2
Comparison of the results obtained by the neutralization test and commercial IgG ELISA (95 serum samples collected from 43 patients who were suspected TBE)

Neutralization test	Commercial IgG ELISA			Total
	Positive	Negative	Inconclusive[a]	
Positive	57	5	21	83
Negative	0	12	0	12
Total	57	17	21	95

[a] Inconclusive results due to boundary values.

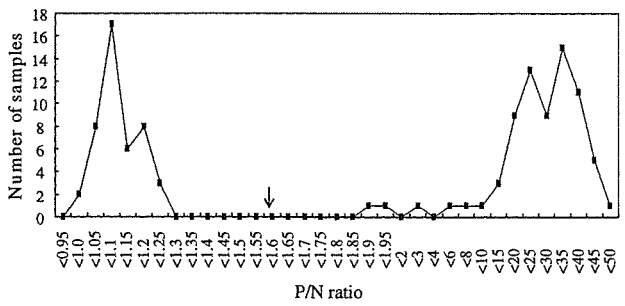

Fig. 2. The distribution of the P/N ratios for 95 serum samples tested with the SP-IgM ELISA: selected cut-off value.

and the minimum P/N ratio of the positive group (1.891) were selected as the cut-off value (i.e., P/N = 1.557).

Paired serum samples were collected from 34 of the patients suspected of having TBE; 17 of the 34 patients were diagnosed with TBE infection based on a greater than four-fold increase in the NT titer in the convalescent phase compared with the acute phase of infection. Two of the 34 patients had convalescent antibody titers that remained <1:20 and were thus found not to have been infected with TBE virus. Fifteen of the 34 patients did not have a conclusive diagnosis because the neutralizing titers of the paired sera did not increase significantly.

Table 3 shows the comparison of the testing results with the SP-IgM ELISA and the neutralization test for these paired serum samples from 34 patients. Of the 17 patients that tested positive for TBE infection by neutralization, 16 patients were positive for TBE-specific IgM antibodies on both the SP-IgM ELISA and the commercial IgM ELISA, and one patient were negative by both the SP-IgM ELISA and the commercial IgM ELISA. These serum samples of this one patient were collected on 2, 13 and 33 days after the onset of illness and neutralizing titers were 1:80, 80 and 320. These serum samples were all positive by both the SP-IgG ELISA and the commercial IgG ELISA. The two patients that were negative for TBE infection by neutralization also tested negative on both the SP-IgM ELISA and commercial IgM ELISA. Of the 15 patients that gave equivocal results by the neutralization test, 11 were positive for TBE-specific IgM antibodies by the SP-IgM ELISA.

Paired serum samples were not available for nine patients, of which seven were not diagnosed with TBE infection despite a high neutralizing titers (from 1:160 to 1:20,480) because the results for acute and convalescent phase sera could not be compared. Six of these seven patients were found to have TBE-specific IgM antibodies according to the SP-IgM ELISA. One patient whose neutralizing antibody titer was 1:160 was negative by the SP-IgM ELISA. The remaining two patient sera had neutralizing antibody titers <1:20 and were also negative by the SP-IgM ELISA.

Table 3
Comparison of the results obtained in by the neutralization test and SP-IgM ELISA (86 paired serum samples from 34 patients)

Neutralization test	SP-IgM ELISA		Total
	Positive	Negative	
Positive[a]	16	1	17
Negative[b]	0	2	2
Undetermined[c]	11	4	15
Total	27	7	34

[a] Neutralizing titers in the convalescent phase increased greater than four-fold compared with the acute phase.
[b] Neutralizing titers in both acute and convalescent samples were 1:<20.
[c] Undetermined due to no significant increase of neutralizing titers.

Table 4
Comparison of results obtained by commercial IgM ELISA and SP-IgM ELISA

Commercial IgM ELISA	SP-IgM ELISA		Total
	Positive	Negative	
Positive	52	0	52
Negative	13	23	36
Inconclusive[a]	7	0	7
Total	72	23	95

[a] Inconclusive results due to boundary values.

The results from the SP-IgM ELISA and the commercial IgM ELISA were compared (Table 4). Of the 95 serum samples tested, 52 were considered positive for TBE-specific IgM antibodies by both tests. Of the 36 samples that were negative with the commercial IgM ELISA, 13 were positive and 23 were negative by the SP-IgM ELISA; the 13 positive samples also had positive neutralizing antibody titers. Seven samples that gave inconclusive results by the commercial IgM ELISA were positive on the SP-IgM ELISA. IgM antibody was detected with SP-IgM ELISA in the samples from 1 to 72 days after onset of illness. The samples from 2 to 64 days were positive with commercial IgM ELISA. These results indicated that the sensitivity of the SP-IgM ELISA was better than that of the commercial IgM ELISA.

3.3. Cross-reactivity with Japanese encephalitis virus

Ten serum samples from patients with Japanese encephalitis virus infections, which were known to contain JE-specific IgM antibodies (Akiba et al., 2001), were tested to examine the

Table 5
Cross-reactivity of JE patient sera to TBE virus by commercial ELISAs and SP-ELISAs

Patient no.	Commercial ELISAs		SP-ELISAs	
	IgG	IgM	IgG	IgM
1	±[a]	±	–	–
2	–[b]	–	–	–
3	–	–	–	–
4	–	–	–	–
5	–	–	–	–
6	–	–	–	–
7	–	±	–	–
8	–	–	–	–
9	±	–	–	–
10	±	±	–	–

[a] ±: Boundary.
[b] –: Negative.