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.

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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	+	–	–

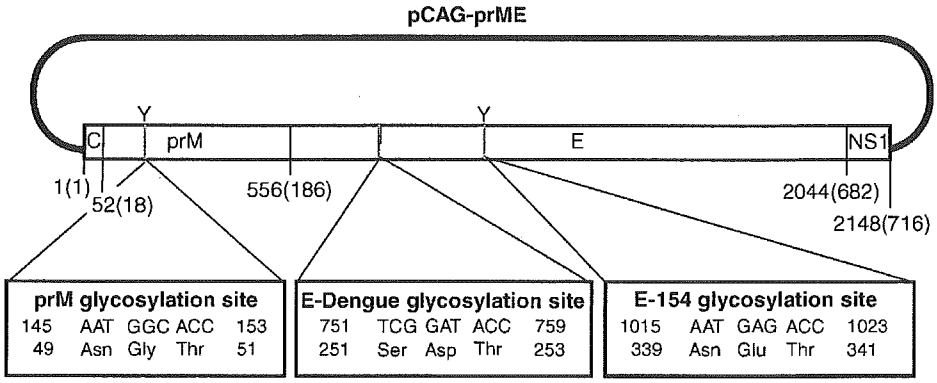

Fig. 1. Schematic representation of the recombinant TBE virus plasmid pCAGprME. The Y symbol shows the positions of the predicted glycans on the TBE virus. The amino acid sequences of the prM and E glycosylation sites are expanded at the bottom of the figure. Glycosylation mutations were introduced at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

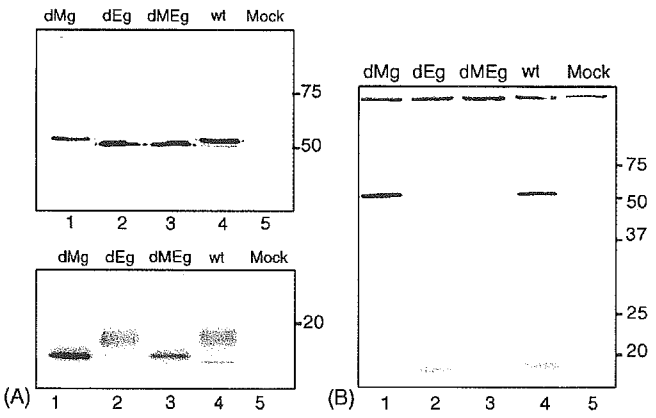

Fig. 2. Western blot analysis of glycosylation-deleted TBE virus proteins that were expressed in 293T cells. (A) The 293T cells were transfected with pCAGprME-wt or glycosylation-deleted mutant vectors (pCAGprME-dMg, -dEg, and -dMEg). At 24 h post-transfection, the cell lysates were immunoprecipitated 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

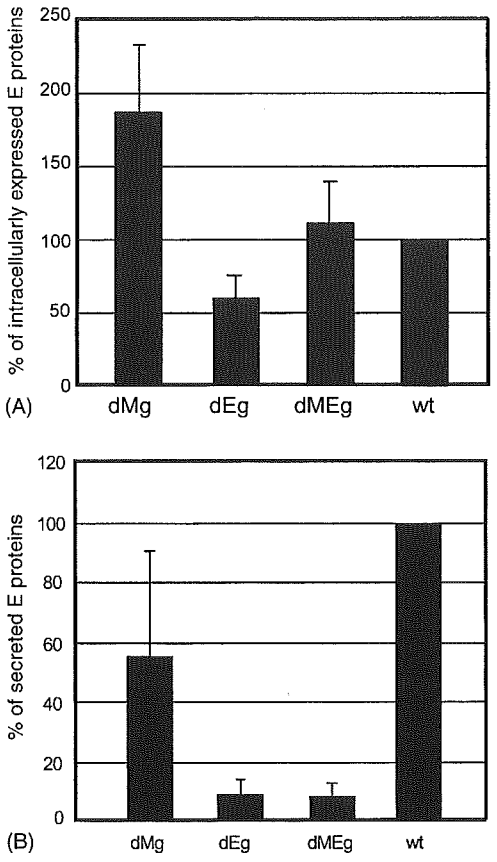

Fig. 3. Effects of nonglycosylated prM or E protein on intracellular expression (A) and extracellular secretion (B) of TBE vital proteins. The 293T cells were transfected with the appropriate glycosylation site-deleted mutant vector. At 24 h post-transfection, the cell lysates and cell culture supernatants 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).

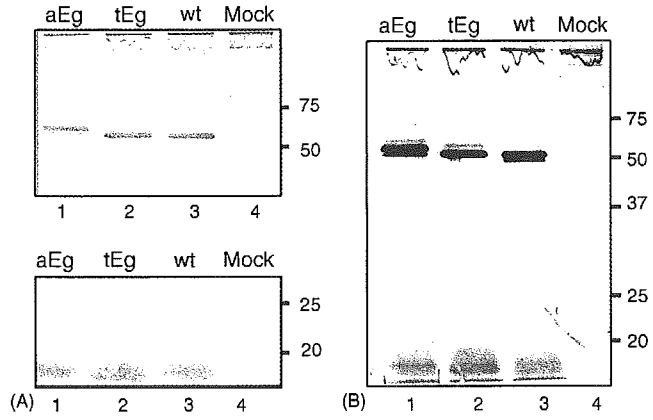

Fig. 4. Western blot analysis of TBE virus proteins from glycosylation-added or -translocated mutants that were expressed in 293T cells. (A) The 293T cells were transfected with pCAGprME-wt, -aEg, and -tEg. At 24 h post-transfection, the cell lysates were immunoprecipitated with MAb 1H4 and used as the 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,

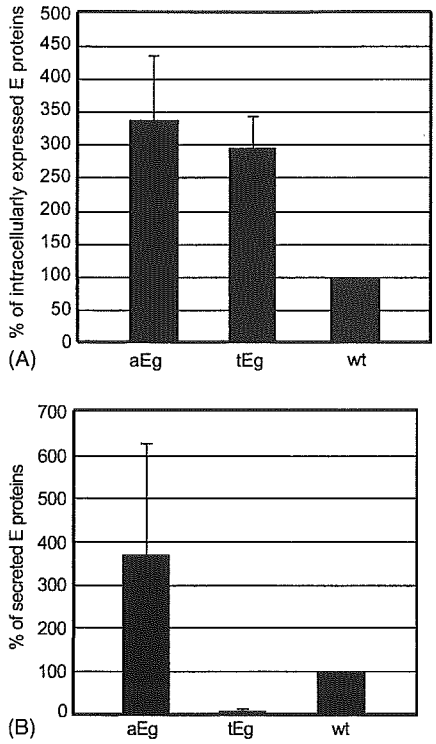

Fig. 5. Effects of the glycosylation site-added or -translocated E proteins on intracellular expression (A) and extracellular secretion (B) of TBE vital proteins. The 293T cells were transfected with the appropriate glycosylation site-added or -translocated mutant vector. At 24 h post-transfection, the 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

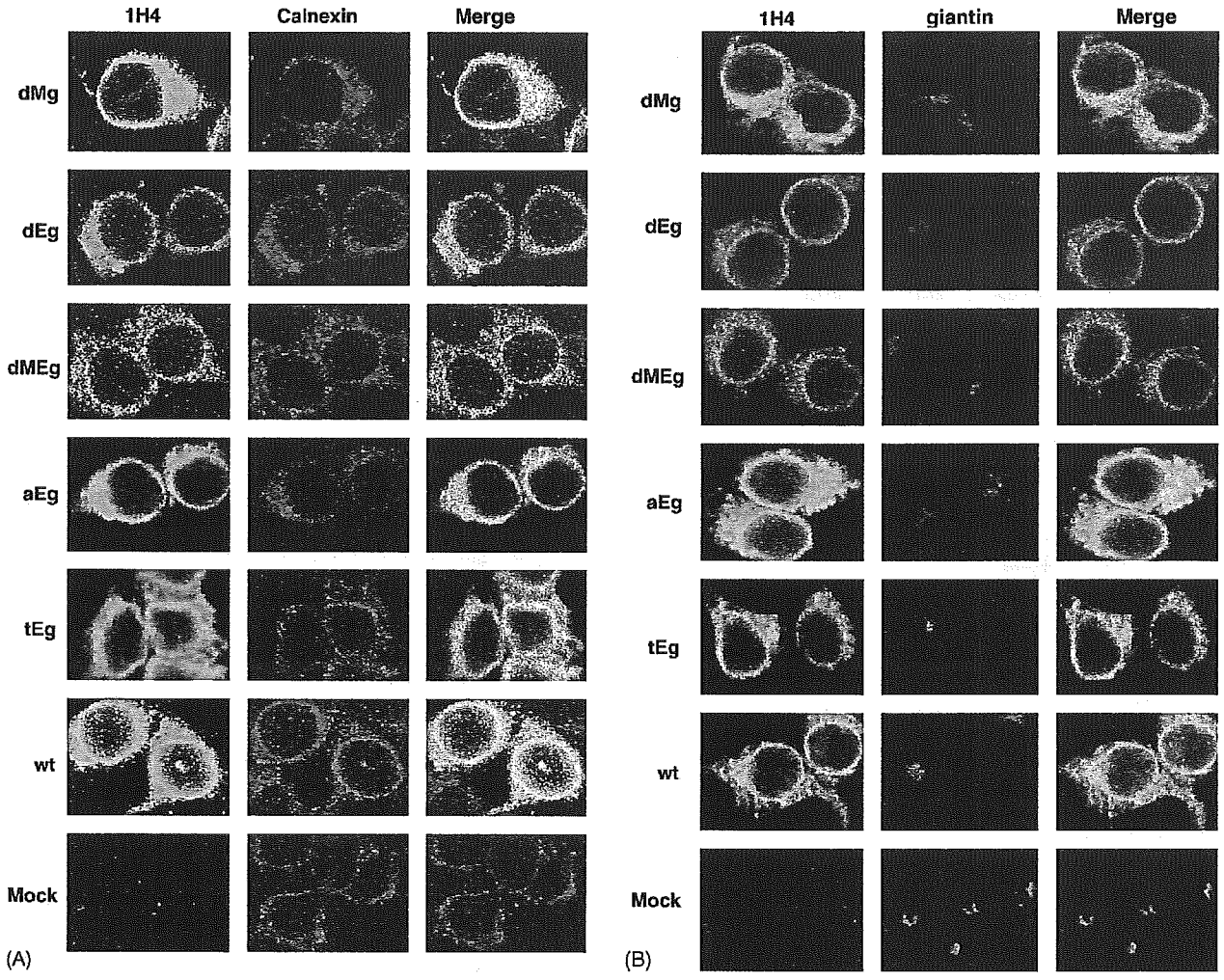

Fig. 6. Intracellular localization of the TBE virus envelope proteins. The 293T cells that were transfected with pCAGprME-wt or the glycosylation-mutated plasmids (pCAGprME-dMg, -dEg, -dMEg, -tEg, and -aEg) were fixed and subjected to indirect immunofluorescent co-staining with primary antibodies that 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 fourfold 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.

Acknowledgments

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Nucleocapsid protein of cell culture-adapted Seoul virus strain 80-39: Analysis of its encoding sequence, expression in yeast and immuno-reactivity

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Abstract. Seoul virus (SEOV) is a hantavirus causing a mild to moderate form of hemorrhagic fever with renal syndrome that is distributed mainly in Asia. The nucleocapsid (N) protein-encoding sequence of SEOV (strain 80-39) was RT-PCR-amplified and cloned into a yeast expression vector containing a galactose-inducible promoter. A survey of the pattern of synonymous codon preferences for a total of 22 N protein-encoding hantavirus genes including 13 of SEOV strains revealed that there is minor variation in codon usage by the same gene in different viral genomes. Introduction of the expression plasmid into yeast *Saccharomyces cerevisiae* resulted in the high-level expression of a hexahistidine-tagged N protein derivative. The nickel-chelation chromatography purified, yeast-expressed SEOV N protein reacted in the immunoblot with a SEOV-specific monoclonal antibody and certain HTNV- and PUUV-cross-reactive monoclonal antibodies. The immunization of a rabbit with the recombinant N protein resulted in the induction of a high-titered antibody response. In ELISA studies, the N protein was able to detect antibodies in sera of experimentally infected laboratory rats and in human anti-hantavirus-positive sera or serum pools of patients from different geographical origin. The yeast-expressed SEOV N protein represents a promising antigen for development of diagnostic tools in serology, sero prevalence studies and vaccine development.

Key words: codon usage, ELISA, hantavirus, monoclonal antibodies, nucleocapsid protein, Seoul virus, yeast expression

Introduction

Hantaviruses represent a separate genus *Hantavirus* in the family *Bunyaviridae*. They can cause two types of diseases in humans, "Hantavirus Cardiopulmonary Syndrome" (HCPS) in the Americas and "Hemorrhagic Fever with Renal Syndrome" (HFRS) mainly on the Eurasian continent [for reviews see [1,2]]. The prototype virus of the genus *Hantavirus*, the Hantaan virus (HTNV), carried by the striped field mouse (*Apodemus agrarius*), was identified as the causative agent of Korean Hemorrhagic Fever (KHF), a severe form

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of HFRS, with a case fatality ratio of up to 10% due to severe bleeding, shock and renal failure [3,4]. Milder clinical courses of usually urban HFRS with a case fatality ratio of about 1% are caused by infections with the closely related Seoul virus (SEOV) carried by different species of the genus *Rattus*, i.e. *R. rattus*, *R. norvegicus* and *R. losea* [4–7].

In general, hantaviruses are transmitted from persistently infected rodent reservoir hosts to humans by inhalation of virus-contaminated aerosols originating from rodent excreta. In fact, data from experimental infection of urban rats (*R. norvegicus*) and investigations in an enzootic focus of SEOV-infected rats suggested the important role of virus-contaminated urine in the transmission of SEOV [8]. An additional transmission mode in male rats may be associated with aggression and wounding that is caused by increased testosterone levels [9].

SEOV infections have been reported from different Asian countries, i.e. Korea, China, Japan, (Far East) Russia, Indonesia and Cambodia [6,10–15]. However, SEOV is the only "cosmopolitan" hantavirus known so far and represents an example of global anthropogenic expansion of an infectious agent [16]. This is supported by the detection of human SEOV infections in other countries outside Asia, e.g. in the USA, Baltimore area [17] and Brazil [18], and circulation of SEOV in rat populations from Australia, USA, Germany, Northern Ireland, Indonesia and Greece [19–24]. Furthermore, infections by rat-borne hantaviruses have been reported in medical research institutions where laboratory rat colonies were handled [25]. In contrast, recent studies in Central Europe failed to detect human SEOV infections [26].

In general, diagnostics of human hantavirus infections is based on serological methods, i.e. immunofluorescence, ELISA and immunoblot tests. As an alternative to serological tests based on virus antigen or virus-infected cells, highly specific and sensitive tests were developed on the basis of heterologously expressed nucleocapsid (N) proteins. To prevent specificity problems associated with *E. coli*-expressed recombinant N (rN) protein [27], we have recently generated rN proteins of different hantaviruses, namely HTNV, different strains of Puumala virus (PUUV), Sin nombre virus (SNV), Andes virus (ANDV) and Dobrava virus strains associated to *Apodemus agrarius* (strain Slovakia; DOBV-Slk) and *A. flavicollis* (strain Slovenia; DOBV-Slo) in yeast *Saccharomyces cerevisiae* for use in diagnostic assays [28,29, J. Schmidt et al., submitted for publication].

The objective of the present study was the yeast expression and characterization of SEOV strain 80-39 rN protein for a potential use in diagnostic assays and vaccine applications. To prove if this rN protein can be taken in serological investigations as a representative of the SEOV species, the N protein-encoding sequence of Korean strain derivative 80-39-Berlin (80-39-B), cell culture-passaged in the biosafety level 3 laboratory in Berlin, was determined and its phylogenetic relationship to other SEOV strains was analyzed, most importantly to exclude a potential influence of nucleotide and resulting amino acid sequence alterations due to cell culture passaging. The purified SEOV rN protein was used to study its immuno-reactivity using monoclonal antibodies, sera of experimentally SEOV-infected rats, rabbits immunized with hantavirus rN proteins and human sera or serum pools from HFRS and HCPS patients.

Materials and Methods

Rat Sera und Tissue Samples

The generation of serial follow-up serum samples from four experimentally SEOV (strain SR-11)-infected Wistar rats has been described previously [30]. As negative control, sera from five wild-trapped rats from Japan, previously demonstrated to be non-infected [31], were used. In addition sera from 11 brown rats (*R. norvegicus*) trapped in Westphalia, Germany, were included in the study.

Total DNA from liver tissue samples of two wild-trapped brown rats from Germany was used for mitochondrial (mt) DNA characterization.

DNA Isolation, PCR Amplification and Determination of Rat mt DNA Sequences

Total rat DNA was isolated using the QIAamp DNA Mini Kit according to the protocol of the manufacturer (QIAGEN, Hilden, Germany). The 12S rDNA sequence was PCR-amplified using the

primers L1091 (5′-GGGATTAGATACCCCAC-TAT-3′) and H1478 (5′-TGACTGCAGAGGGT-GACGGGCGGTGTGT-3′) from the isolated rat DNA and sequenced using the ABI Prism Big Dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol on ABI 377 DNA sequencers.

RNA Isolation, RT-PCR, Cloning and Sequencing of the SEOV N Protein-encoding Sequence

Vero E6 cells were infected with SEOV (strain 80-39) [5]. Infected cells as well as cell culture supernatant were treated with Trizol reagent (Gibco BRL, Eggenstein, Germany). The suspensions were used to isolate viral RNA with the RNeasy Kit (QIAGEN), according to the recommendations of the manufacturer. After reverse transcription of the RNA by RevertAid H Minus M-MuLV Reverse Transcriptase (MBI Fermentas, St. Leon-Rot, Germany) with the Seoul-S-RT Primer 5′-TAGTAGTAGACTCCCT AAA-GAGCTA-3′, the N protein-encoding sequence (aa 2-429) of the cDNA copy was PCR-amplified using Pfu DNA Polymerase (Stratagene, Amsterdam, The Netherlands) with the SEOV-N Forward Primer 5′-AAGCTAGCGCAACTATG GAAGAAATCCAGAGAG-3′ and the SEOV-N Reverse Primer 5′-AAGCTAGCTTATAATTTC ATAGGTTCCTGGTTTGA-3′, containing unique sites for *Nhe*I. The PCR amplification products were purified and subsequently inserted into the plasmid pSTBLUE-1 with the Perfectly Blunt Cloning Kit (Novagen, Schwalbach, Germany), according to the recommendations of the manufacturer. Plasmids were purified using the Spin Mini Kit (QIAGEN). DNA sequencing of three plasmids was performed using an ABI Prism Big Dye terminator cycle sequencing kit (Applied Biosystems). The N protein-encoding sequences obtained for three pSTBLUE-1/SEOV-N plasmids were identical.

Codon Use Analyses

The DnaSP 4.0 program [32] was used to calculate codon usage tables as well as the G + C content at the second (G + C2), third (G + C3), and all coding positions (G + Cc) for each N protein-encoding sequence analysed. The same program was also used to calculate different codon bias measures: the Relative Synonymous Codon Usage (RSCU; [33]) values, the Effective Number of Codons (ENC; [34]), the Codon Bias Index (CBI; [35], and the Scaled Chi square (SChi2; [36]) values. To examine the similarities of a set of genes in terms of their similarity of codon usage, we followed McInerney's approach implemented in the GCUA program [37]. A distance matrix was produced from all the N protein-encoding sequences based on the average differences in their RSCU values. The analysis was continued with a tree-like representation of this RSCU distance matrix produced using PAUP* and comparing that to the ML phylogeny of the sequences based on a substitution model.

Phylogenetic Analysis of Nucleotide and Amino acid Sequences

For phylogenetic analysis, the sequences were aligned on amino acid level and then reverse-translated to nucleotide sequences using DAMBE software [38]. DotPlot analysis implemented in BioEdit software package [39] was used to check the reliability of the alignment. We carried out two types of phylogenetic analysis to investigate evolutionary relationships using PAUP* 4.0b10 [40]: (i) neighbour-joining (NJ) based on LogDet/paralinear distances which were designed to deal with unequal base frequencies in each pairwise sequence comparison – thus it allows base compositions to vary over the tree [41] and (ii) maximum-likelihood (ML) analysis. Before the ML analyses, we used likelihood ratio tests and the computer application MrModeltest v1.0b. [42] to determine the best-suited model of sequence evolution. The best-fit model selected by MrModeltest v1.0b. for the N protein-encoding sequence data set and then used to reconstruct phylogenetic trees was the general time-reversible model [43] with an allowance for invariant sites and a gamma shape for among-site rate variation under the hierarchical likelihood ratio test method. Heuristic ML searches were performed with 10 replicates of random sequence addition and TBR branch swapping. Non-parametric bootstrap analyses with 100 pseudo-replicates were performed to obtain estimates of support for each node of the ML tree; NJ bootstraps employed 1000 iterations.

Generation of the Yeast Expression Plasmid

The SEOV-N-encoding sequence was isolated from pSTBLUE-1/SEOV-N as an *Nhe*I fragment and inserted into *Xba*I-linearized, CIP-dephosphorylated plasmid pFX7-His. This plasmid represents a derivative of pFX7 that contains a Gal/Pyk hybrid promoter, a formaldehyde resistance gene and in front of the unique *Xba*I insertion site an MHHHHHH-coding sequence [28,29]. The DNA sequence in the joining region of pFX7-His plasmid and the SEOV N-encoding insert in the expression plasmid pFX7-His-SEOV-N was confirmed by DNA sequencing using primer pyk5 (5′-TTCTTTTTCATCCTTTGG-3′).

Expression and Purification of SEOV rN protein

The recombinant plasmid pFX7-His-SEOV-N was introduced into competent yeast *S. cerevisiae* cells of the haploid strain AH22 derivate 214 (*ura3 leu2 his4*). Expression and purification of the His-tagged rN protein was performed according to a protocol described recently for rN proteins of other hantaviruses [28,29]. Briefly, yeast cells were grown in formaldehyde-containing YEPD medium. Synthesis of the SEOV rN protein was induced by adding galactose. Yeast cells were pelleted by centrifugation and disrupted using glass beads. The His-tagged SEOV rN protein, enriched by centrifugation steps, was finally purified using nickel-chelate resin according to the protocol of the manufacturer (QIAGEN). The rN proteins of PUUV strains Vranica/Hällnäs (PUUV-Vra), Kazan (PUUV-Kaz) and Sotkamo (PUUV-Sot), HTNV (strain Fojnica), SNV and ANDV were purified according to the same protocol.

Human Sera and Serum pools

An anti-SEOV human serum and two anti-HTNV human sera originated from the Republic of Korea. The anti-DOBV and anti-PUUV serum pools were each generated from four routine diagnostic serum samples of German HFRS patients regarded as positive for anti-DOBV-IgG and anti-PUUV-IgG, respectively, by in-house mAb-capture ELISA and Western blot tests based on yeast-expressed DOBV and PUUV rN proteins [Meisel et al., unpublished data] and chemiluminescence focus reduction neutralization assays [44]. The anti-SNV pool contained 15 routine diagnostic serum samples of HCPS patients from the US found to be anti-SNV-IgG positive by SNV strip blot assay [45] and our in-house SNV-IgG ELISA and Western blot tests using yeast-expressed SNV rN protein (J. Schmidt et al., submitted for publication]. For preparation of an anti-ANDV pool 56 anti-ANDV-IgG-positive serum samples derived from Argentinean and Chilean HCPS patients were used which were confirmed to be reactive by in-house SNV- or ANDV-IgG ELISA and Western blot tests [46,47, J. Schmidt et al., submitted for publication]. As a control a negative serum pool was generated from German serum samples of our routine diagnostics found to be negative for anti-HTNV-, anti-DOBV-, anti-PUUV-, anti-SNV- and anti-ANDV-IgG by our in-house ELISA tests based on the corresponding yeast-expressed rN proteins (29, Schmidt et al., submitted for publication].

Immunization of Rabbits with Yeast-expressed Hantavirus N Proteins

Three rabbits were immunized subcutaneously three times with an interval of 2–4 weeks with 100 μg of one of the purified His-tagged, yeast-expressed rN proteins of SEOV, SNV or ANDV dissolved in PBS. Blood samples were taken 1 week after booster immunization and up to 3 weeks later. After clotting of the blood and centrifugation the sera were stored at −20°C until use. The generation of HTNV- and PUUV-Sot-rN protein-specific rabbit sera followed the same protocol as has been described recently [29].

SDS-Polyacrylamide Gel Electrophoresis (SDS–PAGE) and Western Blot

SDS–PAGE and Western blot analysis were performed as described recently [29]. Aliquots of 2 μg of each purified rN protein of SEOV, ANDV, SNV, PUUV-Vra, PUUV-Sot, PUUV-Kaz and HTNV were run on a 12.5% SDS polyacrylamide gel and electro-blotted to nitrocellulose membrane. After blocking, the membranes were incubated overnight at room temperature (RT) with a panel of N-specific monoclonal antibodies (mAbs)

(diluted 1/50 up to 1/1000) raised against SEOV (mAb R31; purchased from Progen Biotechnik GmbH, Heidelberg, Germany), HTNV (mAb B5D9, [48]; from Progen Biotechnik GmbH; mAbs E5/G6, Eco2, [49]), PUUV (mAb A1C5, [48]; from Progen Biotechnik GmbH; mAbs 1C12, 5E1, 3G5, 5A3, 2E12, 4C3, [50]), SNV (mAb 5F1/F7) and ANDV (mAb 5C2/E10; both from Immunological and Biochemical Testsystems GmbH, Reutlingen, Germany). A His-tag-specific mAb was purchased from Amersham Pharmacia Biotech (Freiburg, Germany). The filters were washed three times and incubated for 4 h at RT with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Sigma, Deisenhofen, Germany) diluted 1/250 in serum dilution buffer. Finally the filters were stained with 4-chloro-1-naphthol substrate prepared according to the protocol of the manufacturer (Sigma).

ELISA

The ELISA investigations were performed essentially as described recently [29]. Briefly, polystyrene microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight with 2 µg/ml SEOV rN protein (or the rN proteins of HTNV, PUUV-Vra, PUUV-Sot, ANDV and SNV) diluted in carbonate buffer. After blocking with 3% bovine serum albumin (BSA) in PBS containing 0.05% Tween 20 100 µl of 1/200 diluted rat or rabbit sera, or 1/400 diluted human serum in 1% BSA in PBS containing 0.05% Tween 20 were added. After an incubation of 2 h to each well 100 µl HRP-conjugated goat anti-rat IgG (Sigma; dilution of 1/5000) or anti-rabbit IgG (Sigma; dilution of 1/3000) or anti-human IgG (DakoCytomation, Hamburg, Germany; diluted 1/6000) were added, and the plates were incubated again at 37°C for 1 h. After 10 min of incubation with 100 µl of 3,3′,5,5′-tetramethylbenzidine (TMB) (Biorad, Hercules, CA) at RT the reaction was stopped by addition of 100 µl of 1 M H_2SO_4. Finally, the optical density (OD) values were measured at 450 nm (reference 620 nm).

The final OD value for each serum sample was calculated as the difference of the OD values measured in antigen-containing and antigen-free wells. These final OD values for serum dilutions of 1/400 or 1/200 were regarded as positive if the mean OD exceeded the mean OD + 3 standard deviations (SD) obtained with negative control samples. The endpoint titer for each serum sample was defined as the serum dilution where the OD value is three-times higher as the background OD value that is measured in highly diluted sera and does not decrease with further dilution of the serum. In our experiments the background OD varied between 0.01 and 0.1.

Results and Discussion

Sequence Analysis and Phylogeny of the N Protein-encoding Sequence of SEOV Strain 80-39

Comparison of the nucleotide sequence of the N protein-encoding open reading frame (ORF) from 80-39-Berlin (80-39-B), a cell culture-passaged Korean SEOV strain used herein, to the recently published corresponding sequence of SEOV strain 80-39 (AY273791) demonstrated only two silent nucleotide exchanges G15A (codon position 5) and G1275A (codon position 425). This results in a sequence identity of 99.6% at the nucleotide and 100% at the amino acid level (data not shown). As observed for all SEOV and HTNV sequences, in front of the N-ORF of SEOV 80-39-B two very close, in-frame ATG codons were found (data not shown). As expected for a Murinae-adapted virus, the potential second ORF on the S segment – present in almost all Arvicolinae- and Sigmodontinae-adapted hantaviruses – was found to lack a translation initiation codon and to be interrupted by numerous stop codons (data not shown). This very high identity of two cell culture-adapted lineages of SEOV strain 80-39 might suggest strong selection constraints in the Vero E6 cell culture. This is in line with the observation of a high genetic stability of SEOV maintained under a natural environment and sequence identities of SEOV strains isolated from various districts of Eastern Asia [51,52].

The nucleotide sequence diversity of SEOV 80-39-B to N protein-encoding sequences of other SEOV strains ranged from 1.3% for the most related (American) strain Tchoupitoulas (GenBank accession number AF329389) to 12.5% for the most ancestral SEOV strain Gou3 from China (GenBank accession number AB027522; see Fig. 1). As expected, the level of amino acid

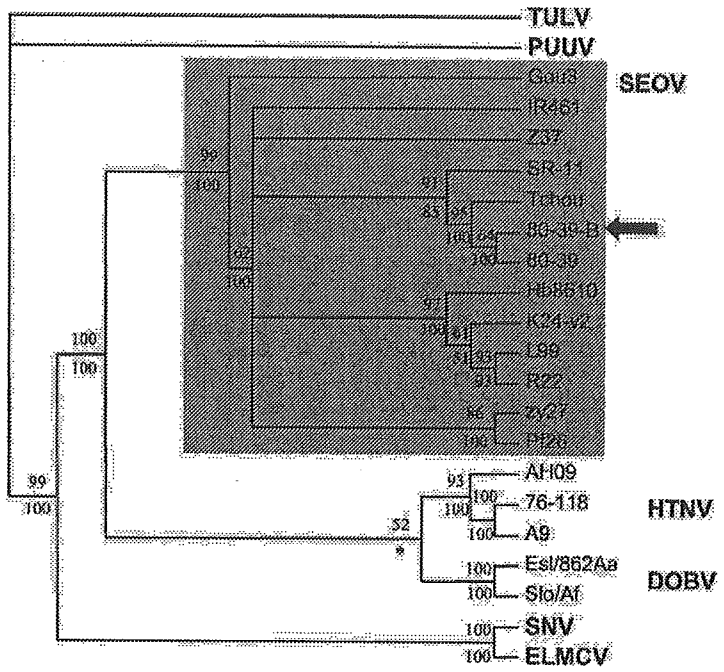

Fig. 1. Topologies for a hantavirus phylogeny based on nucleocapsid (N) protein-encoding gene sequences. Maximum-likelihood (ML) strict consensus tree based on GTR + Γ + I model ($\alpha = 1.2286$, proportion of invariable sites = 0.2900). Neighbour-joining (NJ) based on LogDet/paralinear distances recovered the same topology. Values above branches refer to ML bootstrap proportions from 100 iterations, values below branches represent NJ bootstrap proportions from 1000 iterations. Asterisk indicates that the node was not supported by the reconstruction method. The SEOV branches are marked by light-grey background; the obtained sequence is indicated by an arrow. The following analyzed SEOV S segment sequences are included (accession numbers put in parentheses): SEOV strains 80-39 (AY273791), Tchoupitoulas (Tchou; AF329389), SR-11 (M34882), IR461 (AF329388), L99 (AF488708), R22 (AF488707), K24-v2 (AF288655), Hb8610 (AF288643), zy27 (AF406965), Pf26 (AY006465), Z37 (AF187082), Gou3 (AB027522). As outgroup the following sequences were used: HTNV strains 76-118 (M14626), A9 (AF329390), AH09 (AF285264); DOBV strains Esl/862Aa (AJ269550) and Slo/Af (L41916); SNV strain NM H10 (L25784), El Moro Canyon hantavirus (ELMCV) strain RM-97 (U11427), TULV strain Moravia/5302v/95 (Z69991), and PUUV strain Vranica/Hällnäs (U14137).

divergence between the SEOV strains was much lower (0.3% to SR-11 – 1.4% for IR461). The divergence of the SEOV sequence 80-39-B to sequences of the most closely related hantavirus species HTNV and DOBV is much higher ranging from about 25–27% at the nucleotide level to 15–18% at the amino acid level. The diversity reaches a highest value of about 40% at both the nucleotide and amino acid level for the most distantly related Arvicolinae- and Sigmodontinae-adapted viruses SNV, ELMCV, PUUV and TULV used as outgroup viruses (data not shown).

The results of various codon bias measures and the G+C content at different codon positions are listed in Table 1. ENC (Effective Number of Codons) is a very simple, but effective way of measuring codon use bias [34]. ENC ranges from 20, if only one codon is used for each amino acid, to 61, if all codons are used equally. ENC values of 35 or less are considered biased and genes with low ENC values are restricted in the use of synonymous codons compared with genes with high ENC values. In this study, a total of 22 N protein-encoding hantavirus sequences including 13 from SEOV strains have ENC values that are indicative of more random codon usage (ranging from 47.2% to 54.5%). Interspecific comparison of ENC values also revealed that there is minor variation in codon usage by the same gene in the different hantavirus genomes. Codon Bias Index (CBI) is a measure of the deviation from the equal use of synonymous codons [35]. CBI values range from 0 (uniform use

Table 1. Various measures of codon usage bias and GC content at different codon positions of each hantavirus N protein-encoding gene examined

Virus	Strain	ENC	CBI	SChi2	G+C2	G+C3s	G+Cc
SEOV	80-39-B	49.012	0.322	0.332	0.399	0.414	0.459
	80-39	49.058	0.318	0.330	0.399	0.419	0.461
	Tchou	48.912	0.324	0.325	0.399	0.414	0.459
	SR-11	48.865	0.322	0.330	0.401	0.421	0.462
	IR461	47.205	0.349	0.364	0.394	0.415	0.455
	L99	48.387	0.319	0.320	0.399	0.414	0.458
	R22	48.574	0.317	0.314	0.396	0.409	0.456
	K24-v2	48.681	0.322	0.322	0.396	0.416	0.459
	Hb8610	48.845	0.322	0.309	0.401	0.421	0.461
	zy27	48.187	0.346	0.340	0.401	0.419	0.462
	pf26	48.680	0.339	0.323	0.399	0.415	0.460
	Z37	47.826	0.328	0.344	0.401	0.421	0.462
	Gou3	50.579	0.351	0.329	0.401	0.428	0.468
HTNV	76-118	49.623	0.331	0.344	0.392	0.407	0.451
	A9	49.376	0.324	0.324	0.389	0.385	0.442
	AH09	51.511	0.300	0.294	0.392	0.417	0.450
DOBV	Esl/862Aa	54.480	0.281	0.228	0.382	0.396	0.446
	Slo/Af	50.725	0.286	0.273	0.380	0.432	0.458
SNV	NM H10	53.153	0.292	0.247	0.394	0.363	0.435
ELMCV	RM-97	48.977	0.353	0.323	0.380	0.350	0.425
TULV	Moravia/5302v/95	53.065	0.279	0.249	0.377	0.391	0.436
PUUV	Vranica/Hällnäs	48.307	0.334	0.323	0.358	0.341	0.421

ENC, effective number of codons [34]; CBI, codon bias index [35]; SChi2, Scaled Chi Square values [36]. (G+C2), (G+C3), (G+Cc), G+C content at the second, third and all coding positions, respectively, for each N protein-encoding sequence analysed. For details see Materials and Methods section.

of synonymous codons) to 1 (maximum codon bias). The CBI values listed in Table 1 are indicative of random codon usage and display a relatively low degree of variation in codon usage. The same is true for the SChi2 values (Table 1), another measure of potential codon bias based on the difference between the observed number of codons and those expected from equal usage of codons [36]. We also found that the percentage of GC-nucleotides of each of the 22 homologous genes examined in this study were very similar (ranging from 42.1% for PUUV to 46.8% for SEOV strain Gou3).

The reconstruction of the phylogenetic relationships of SEOV strains by maximum likelihood (ML) methods demonstrated that they represent a monophyletic group (Fig. 1). In line with previous data [52], the *R. rattus*-associated SEOV strain Gou3 represented the most ancestral strain, as evidenced also by phylogenetic investigation of M segment sequences (data not shown). As expected from the high-level of sequence diversity of Arvicolinae- and Sigmodontinae-adapted viruses SNV, ELMCV, PUUV and TULV (see above) these viruses form clearly separated branches in the ML tree. The same topology was inferred by using the NJ algorithm based on the LogDet/paralinear distances suitable for tree reconstruction where base compositions vary significantly between sequences (Fig. 1). The relationships in a tree based on codon usage similarities (data not shown) do not coincide with the relationships by virtue of similarity of nucleotide substitutions and therefore, we can be confident that our hypothesis of phylogenetic relationships is not being constructed because of problematic codon bias and nucleotide composition effects [53].

Expression in Yeast, Purification and Antigenic Characterization of SEOV rN Protein

As expected, the main portion of the yeast-expressed rN protein of SEOV strain 80-39-B was recovered from the nickel-chelation chromatography column in buffer E elution fractions (pH 4.5). The analysis

of these fractions in 12.5% SDS polyacrylamide gels revealed the presence of a protein band of the expected molecular mass of about 49 kDa (data not shown). The yield of the purified protein of about 0.9 mg/g wet weight corresponds to those values observed for rN proteins of PUUV-Vra [28] and other hantaviruses [29]. Analysis in the SDS-PAGE and immunoblot using a His-tag specific mAb demonstrated the absence of degradation products suggesting a highly pure and stable rN protein (data not shown).

As expected, the SEOV-specific mAb R31 reacted with the yeast-expressed SEOV rN protein. In addition, this mAb reacted also with rN protein of HTNV, but failed to recognize rN antigens of SNV, ANDV and PUUV strains (data not shown). In line with previous data [49], the antigenic similarity of rN proteins of SEOV and HTNV was also confirmed by the reactivity of HTNV-specific mAbs E5/G6 and Eco2 with the SEOV rN protein. The SEOV rN protein was also detected by the highly cross-reactive mAbs 1C12, 5A3 and 4C3 raised against PUUV confirming earlier data [54]. In contrast it did not react with the HTNV-specific mAb B5D9, the PUUV-specific mAbs 5E1, 3G5, 2E12 and AlC5, SNV-specific mAb 5F1/F7 and ANDV-specific mAb 5C2/E10 (data not shown). The discrepancies between the reactivity of mAb 2E12 with HTNV and SEOV in immunofluorescence assays [54] and its failure to react in Western blots with rN proteins of HTNV [29] and SEOV (this paper) is most likely caused by a discontinous nature of its epitope [50].

Cross-reactivity of Rabbit Sera Raised Against rN Proteins of SEOV and other Hantaviruses

The immunization of a rabbit with purified SEOV rN protein resulted in the induction of a high-titered SEOV-specific antibody response (Table 2). Similarly, rabbits immunized with yeast-expressed rN proteins of SNV and ANDV developed high titers of homologous antibodies (Table 2). The observed strong antibody response is in line with the immunogenicity of yeast-expressed rN proteins of other hantaviruses observed in rabbits [29, this paper] and mice [55].

As expected, all rabbit sera were found to react not only strongly with the homologous, but also with heterologous rN proteins. In general, the reciprocal endpoint titer for the homologous rN antigen was found to be the highest (Table 2; given in bold). The titer of N-specific antibodies in the SEOV rN-immunized rabbit was only slightly lower to the rN protein of the closely related HTNV, but much lower to those of PUUV-Sot, SNV and ANDV. Similarly, the anti-HTNV rN rabbit serum [29] reacted with equal titers to SEOV and HTNV rN protein (Table 2).

Analysis of the Kinetics of the Homologous and Cross-reactive Antibody Response of Experimentally SEOV-infected rats

In the first follow-up serum sample (taken 7 days after infection) of all four experimentally SEOV-infected rats [30] high-titered SEOV-N-specific antibodies were detected (Fig. 2). This is in line with data obtained for these rats using a recently developed ELISA based on *E. coli*-expressed HTNV rN protein [31].

In general, the reciprocal endpoint titers for all four rats at all time points for SEOV were higher than those for HTNV and PUUV-Vra. The level of cross-reactivity to HTNV rN protein was found to be increased during the time of observation reaching the highest endpoint titer in the latest follow-up serum sample. The level of cross-reactivity to PUUV-Vra rN protein was much lower (Fig. 2A, B and D) or even totally non-detectable (Fig. 2C). At 7 days post-infection in 3 of 4 rats HTNV and PUUV-Vra rN proteins failed to detect hantavirus-specific antibodies. These data are in line with observations demonstrating the necessity of a homologous N antigen for a highly sensitive detection of hantavirus-specific antibodies in humans, especially during the early phase of infection (46,56, J. Schmidt et al., submitted for publication]. As reported for human sera [57, J. Schmidt et al., submitted for publication], the level of cross-reactivity was more pronounced for rat sera taken during the later phase of infection.

Potential use of Yeast-expressed SEOV rN Protein for Diagnostic Purposes and Seroprevalence Studies

In general, the reciprocal endpoint titers of anti-SEOV, -HTNV, -DOBV, -SNV and -ANDV positive human sera or serum pools for the