Construction and application of chimeric virus-like particles of tick-borne encephalitis virus and mosquito-borne Japanese encephalitis virus

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We have previously reported a system for packaging tick-borne encephalitis (TBE) virus subgenomic replicon RNAs into single-round infectious virus-like particles (VLPs) by using in trans expression of viral C/prM/E structural proteins. In this study, the trans-packaging system was applied to the generation of chimeric VLPs with mosquito-borne Japanese encephalitis (JE) virus. Although trans-expression of TBE virus C and JE virus prM/E proteins resulted in the secretion of VLPs, the expression of JE virus C/prM/E proteins did not lead to the secretion of VLPs, suggesting that homologous interaction between C and non-structural proteins or the genomic RNA is important for efficient assembly of infectious particles. Neutralization testing showed that the antigenic characteristics of the VLPs were similar to those of the native virus. Furthermore, the infectivities of the TBE virus- and JE virus-enveloped VLPs for the ISE6 tick cell line and C6/36 mosquito cell line were investigated. The VLPs were able to enter only those cells that were derived from the natural vectors for the respective viruses. TBE virus replicon RNA packaged in VLPs produced TBE virus non-structural proteins in tick cells, but could neither replicate nor produce viral proteins in mosquito cells. These findings indicate the importance of specific cellular factors for virus entry and replication during flavivirus infection of arthropods. These results demonstrate that chimeric VLPs are useful tools for the study of viral genome packaging and cellular factors involved in vector specificity, with the additional safety aspect that these chimeric VLPs can be used instead of full-length chimeric viruses.

INTRODUCTION

The genus Flavivirus (family Flaviviridae) contains important human pathogens, including tick-borne encephalitis (TBE) virus, Japanese encephalitis (JE) virus, yellow fever virus, dengue virus and West Nile (WN) virus. Flaviviruses can be divided into three phylogenetic and ecological groups: the tick-borne group, the mosquito-borne group and the no-known-vector group (Gaunt et al., 2001; Gould et al., 2003; Kuno et al., 1998). The extent of transmission of arthropod-borne viruses depends on both ecological and physiological parameters, of which vector competence is the most important factor. Vector competence is determined by extrinsic and intrinsic factors, such as the physiological ability of vector tissue to become infected and to maintain a particular infectious agent (Hardy et al., 1983; Kramer & Ebel, 2003; Nuttall & Labuda, 2003). The involvement of these factors in flavivirus infection is not well understood.

The flavivirus genome consists of a positive-polarity, single-stranded RNA of approximately 11 kb, which encodes three structural proteins, i.e. the core (C), premembrane (prM) and envelope (E) proteins, and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5), within a single long open reading frame (Chambers et al., 1990). The 5' and 3' untranslated regions (UTRs) have predicted secondary structures that are implicated in viral replication, translation and packaging of the genomes (Gritsun et al., 1997; Proutski et al., 1997; Rauscher et al., 1997). In the process of assembly of virus particles, viral structural proteins are inserted cotranslationally into the endoplasmic reticulum (ER) and processed by the NS2B–NS3 protease complex and signal peptidase, and the C proteins and genomic RNA, which form the icosahedral nucleocapsid, are encapsidated by budding into the luminal side of the ER (Mackenzie & Westaway, 2001). However, little is known about the details of the molecular mechanism of packaging.

The development of stable, infectious cDNA clones of flaviviruses has enabled the construction of chimeras of...
different flaviviruses (Caufour et al., 2001; Guirakho et al., 2001; Mathenge et al., 2004; Pletnev & Men, 1998; Pletnev et al., 1992, 2002). These chimeric viruses are useful tools for the study of viral replication cycles and for vaccine development. Moreover, they can be applied to the study of host factors that are involved in vector competence for flavivirus transmission in cases where chimeric viruses are generated from flaviviruses borne by different arthropods.

For several flaviviruses, such as TBE virus (Gehrke et al., 2003; Hayasaka et al., 2004), Kunjin (KUN) virus (Khromykh & Westaway, 1997), WN virus (Scholle et al., 2004; Shi et al., 2002), dengue virus (Pang et al., 2001) and yellow fever virus (Molenkamp et al., 2003), subgenomic replicons have recently been constructed by deleting genes for viral structural proteins. These replicons can replicate in cultured cells by virtue of functioning NS proteins, but they cannot produce progeny infectious viruses owing to the lack of viral structural proteins. In recent studies, the expression of viral structural proteins in cells harbouring replicon RNA has resulted in the secretion of particles, which have been designated virus-like particles (VLPs). VLPs are infectious and the replicon RNAs packaged in VLPs replicate in infected cells. However, as viral structural proteins are not encoded by the replicon, progeny viruses cannot be produced. Thus, this single-round infectivity feature of VLPs enables safe handling under biosafety level 2 (BSL-2) conditions. VLP systems were developed for TBE virus (Gehrke et al., 2003; Yoshii et al., 2005), KUN virus (Hayes et al., 2004; Khromykh et al., 1998) and WN virus (Scholle et al., 2004). The VLPs of flaviviruses are similar to the native virus in terms of their physical features and functional characteristics for infection. Therefore, VLPs can be substituted for native virions in investigations into the biological properties of flaviviruses.

Previously, we constructed a subgenomic replicon of Far-Eastern subtype TBE virus and developed the trans-packaging system for VLPs further (Hayasaka et al., 2004; Yoshii et al., 2005). In this study, the trans-packaging system was applied to the generation of chimeric VLPs between TBE virus and JE virus (tick- and mosquito-borne flaviviruses, respectively), to investigate the molecular mechanism of flavivirus packaging and the specificity of the competence of the natural host vectors for flavivirus infection.

**METHODS**

**Cells and viruses.** The baby hamster kidney (BHK)-21 cell line was grown at 37 °C in Eagle’s minimal essential medium (MEM) that was supplemented with 8 % fetal calf serum (FCS) and l-glutamine. C6/36 cells, which are derived from the mosquito *Aedes albopictus*, were grown at 28 °C in MEM with 10% FCS (Igarashi, 1978). The IS66 cell line from the tick *Ixodes scapularis* (kindly donated by Dr U. G. Munderloh, University of Minnesota, MN, USA) was grown at 34 °C in L-15B medium with 10% FCS, as described by Munderloh et al. (1994).

TBE virus strain Oshima 5-10 and JE virus strain Nakayama were used in this study (Mason et al., 1991; Takashima et al., 1997).

**Antibodies.** Rabbit polyclonal anti-prM, anti-E and anti-NS3 antibodies were generated by immunization with the recombinant prM, E and NS3 proteins, as described in our previous study (Yoshii et al., 2002). For the neutralization tests, we used the mouse anti-E monoclonal antibodies (mAbs) 1H4 and 4H8, which were prepared in our laboratory (Komoro et al., 2000). Mouse mAbs 10B4 (anti-JE virus E) and 13E7 (anti-JE virus prM), which were provided by Dr E. Konishi (Department of Health Sciences, Kobe University School of Medicine, Kobe, Japan), were used in Western blotting. The anti-TBE virus, anti-Langat virus and anti-JE virus mouse polyclonal antibodies were prepared from the sera of mice infected with TBE virus strain Oshima 5-10, Langat virus strain TP-21 and JE virus strain Nakayama, respectively.

**Plasmid construction.** Derivation of the recombinant plasmid pCTBECME, which expresses all of the viral structural proteins derived from the Oshima 5-10 strain of TBE virus, has been described previously (Yoshii et al., 2005). pcJEME, which is a pcDNA3-based plasmid that encodes the JE virus (Nakayama) genes for the signal sequence of prM and the prM and E proteins, was provided by Dr E. Konishi and Dr P. W. Mason (Department of Pathology, University of Texas Medical Branch, Galveston, TX, USA) (Konishi et al., 1998). For the construction of pcJECM, which encodes all of the viral structural proteins of the JE virus, viral RNA was extracted from a JE virus-inoculated suckling mouse brain, and RT-PCR was performed by using the primers BanHIJE5f (forward) and pcJEME1200r (reverse) (see Supplementary Table S1, available in JGV Online) as described previously (Takashima et al., 1997). The PCR products were digested with BamHI and BsoWI and inserted into the pcJEME plasmid.

For the construction of pcTBE-JEME, which encodes TBE virus C and the JE virus signal sequence of prM and the prM and E genes, these fragments were amplified by fusion PCR. First, the DNA fragment that encodes the region of the TBE virus gene from the 5’-UTR to the NS2B–NS3 cleavage site of protein C was amplified by using pcTBECSM as template with the INS2Bf and INS2Br primers. The PCR products were digested with BanHI and BsoWI and inserted into the pcJEME plasmid.

For the construction of pcTBENS2B/3, which expresses the TBE virus NS2B–NS3 polyprotein, PCR was carried out using the pcJEME plasmid. The DNA fragments were subsequently used as templates in a second round of PCR with the BanHIJE5f (forward) and pcJEME1200r (reverse) primers. The PCR products were digested with BamHI and BsoWI and inserted into the pcJEME plasmid.

For the construction of pcTBENS2B/3, which expresses the TBE virus NS2B–NS3 polyprotein, PCR was carried out using the XhoI NS2Bf and EcoRINS3r primers. The PCR products were digested with XhoI and EcoRI and inserted into the pcCAGGS plasmid (Niwa et al., 1991).

The TBE replicon RNA transcripts were prepared from the Oshima REPp plasmid, as described previously (Hayasaka et al., 2004).

**Preparation of VLPs.** TBE replicon RNA was transcribed from the Oshima REPp plasmid and electroporated into BHK-21 cells, as described previously (Hayasaka et al., 2004). After 24 h culture, the cells were transfected with the plasmid that expressed the flavivirus structural proteins, which was complexed with the TransIT-LT1 reagent (PanVera Corporation), as described previously (Yoshii et al., 2004). At 36 h post-transfection, the supernatant was harvested and cleared by centrifugation at 1000 g for 10 min. The particles in the supernatant were precipitated with 10% PEG (Mn=8000) and 1.9% NaCl for 2 h at 4 °C, and pelleted at 10 000 g for 20 min. The pellets were resuspended in PBS supplemented with RNase A (20 µg ml⁻¹).

**Infectivity assays.** Titration of the VLPs was carried out as described previously (Yoshii et al., 2005). Briefly, BHK-21 cells grown on
chamber slides were infected with serially diluted VLP solutions and incubated for 24 h at 37 °C. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. After blocking with 2% BSA, the cells were incubated with anti-TBE virus NS3 rabbit IgG antibodies for 1 h and then treated with fluorescein isothiocyanate-conjugated anti-mouse IgG antibodies (Jackson ImmunoResearch). The images were viewed and recorded by using confocal microscopy.

In the particle neutralization test, 100 IU (infectious units) of VLPs were incubated with a serial dilution of the antibodies (described above) prior to infection of BHK-21 cells for 1.5 h. Particle-infected cells were visualized as described above.

In the case of experimental infections of ISE6 or C6/36 cells, cells grown on 16-well chamber slides were infected with VLPs at an m.o. of 1 and incubated for 48 h. Infected cells were visualized as described above.

**SDS-PAGE and Western blotting.** Transfected cells and supernatants were electrophoresed in SDS/polyacrylamide gels. The protein bands on the gels after SDS-PAGE were transferred onto PVDF membranes and incubated with 1% gelatin in 25 mM TBS that contained 0.01% Tween 20 (TBST). After washing with TBST, the membranes were reacted with the anti-E and/or anti-prM antibodies and then treated with alkaline phosphatase-conjugated anti-mouse or anti-rabbit IgG (Promega).

**Infectivity to arthropod cells.** ISE6 or C6/36 cells were infected with infectious VLPs at an m.o. of 1. Following VLP absorption for 1 h, the supernatants were harvested and remaining VLPs in the supernatant were titrated. The cells were treated with 0.1 mg Pronase ml⁻¹ (Roche) for 40 min to prevent non-specific binding of VLPs to the cell surface, as described previously (Mizutani et al., 2003). The cell samples were harvested at 1, 2, 4 and 8 h post-infection. RNA was extracted from the cell and reverse transcription was carried out as described previously (Takahashi et al., 1997). PCR was carried out by using the following primers: for the region between the 5'-UTR and NS1 of TBE virus, TBE5'f and TBENS1r; for the I. scapularis β-actin gene, I-actin-F and I-actin-R; and for the A. albopictus β-actin gene, A-actin-F and A-actin-R (see Supplementary Table S1, available in JGV Online). The actin gene expression levels were used in control RT-PCR experiments to normalize the amount of cDNA used in each reaction.

**RESULTS**

**Packaging of TBE virus replicon RNA into single-round infectious particles that contain the JE virus envelope**

For expression of the flavivirus structural proteins used to package the TBE virus replicon RNA, the following four plasmid vectors, in which TBE virus and/or JE virus structural protein genes were cloned, were prepared (Fig. 1): pcTBECME, which encodes the TBE virus C/prM/E; pcJEME, which encodes the JE virus signal sequence of prM and the prM and E regions; pcJECE, which encodes the JE virus C/prM/E; and pcTBEC-JEME, which encodes the TBE virus C gene and the JE virus signal sequence of the prM-prM-E region.

For the RNA-packaging experiment, TBE virus replicon RNA was prepared from the Oshima REPpt plasmid, which was constructed from the TBE virus infectious cDNA O-IC plasmid (Hayasaka et al., 2004). In the BHK-21 cells that were infected with PEG-precipitated supernatant from pcTBEC-JEME- or pcTBECME- and replicon-transfected cells, the production of TBE virus NS3 proteins was detected by immunofluorescence assay (IFA) (Fig. 2a, d), which contrasted with the lack of viral protein production observed for cells that were infected with the supernatant of pcJEME- and replicon-transfected cells or that were transfected with replicon RNA alone (Fig. 2b, e). In the case of the supernatant from pcJECEME- and replicon-transfected cells, IFA-positive cells were rarely seen (Fig. 2c; representative field). A second passage, in which the supernatants of the infected BHK-21 cell cultures were transferred to fresh BHK-21 cells, resulted in infection in the case of the RNA transfection from full-length infectious cDNA O-IC (authentic virus particles; Fig. 2i), but not in the case of transfection with replicon Oshima REPpt and pcTBEC-JEME or pcTBECME (Fig. 2g, h). These data demonstrate that the particles (VLPs) secreted by pcTBEC-JEME- or pcTBECME- and TBE virus replicon Oshima REPpt-transfected cells have only single-round infectivity potential.

**C RNA homology requirement for the secretion of VLPs**

To analyse the secretion of VLPs from cells transfected with plasmids that express flavivirus structural proteins and the replicon, transfected cells and supernatant were subjected to Western blot analysis (Fig. 3a). In cells that were transfected with pcTBECME and Oshima REPpt, TBE virus prM and E protein bands were detected by the TBE virus prM- and E-specific antibodies. Moreover, similar intensities were detected by the JE virus prM- and...
E-specific antibodies for the JE virus prM and E protein bands in Oshima REPpt replicon-electroporated cells that were transfected sequentially with the pcTBEC-JEME, pcJECME and pcJEME plasmids. It has been reported previously that the C–prM junction is cleaved first by the NS3 protein on the cytoplasmic side, and then cleaved continuously by the ER signal peptidase on the ER-luminal side (Amberg et al., 1994; Lobigs, 1993; Sato et al., 1993).

Fig. 2. Secretion of chimeric particles with single-round infectivity. (a–f) Fresh BHK-21 cells were infected with the culture supernatants of cells that were transfected with in vitro-synthesized Oshima REPpt RNA and (a) pcTBEC-JEME, (b) pcJEME, (c) pcJECME or (d) pcTBECME, or (e) Oshima REP pt alone. As a control (f), RNA from the full-length TBE virus infectious cDNA O-IC pt was used. (g–i) Reinfection of fresh BHK-21 cells with the culture supernatants: (a)→(g), (d)→(h) and (f)→(i). Expression of viral proteins was visualized by immunofluorescence using anti-NS3 antibodies.
In this case, prM protein bands were detected as independent forms from the C proteins and no C–prM polyprotein band was detected, which indicates that the C–prM junctions were cleaved by viral NS3 protease derived from the expression of the TBE virus replicon RNA. On the other hand, viral protein secretion was observed in the culture supernatants of replicon-electroporated cells that were transfected sequentially with the pcTBECME, pcTBEC-JEME and pcJEME plasmids. However, low levels of E protein were secreted from cells that were transfected with Oshima REPpt RNA and pcJECME. Furthermore, the harvested supernatant was used to infect BHK-21 cells for titration of infectious VLPs (Fig. 3b). A relatively high titre of infectious VLPs (>10^6 IU ml^-1) was secreted from cells that were transfected with both the pcTBECME and pcTBEC-JEME plasmids, whereas few VLPs (250 IU ml^-1) were secreted from cells that were transfected with Oshima REPpt RNA and pcJECME. Semiquantitative RT-PCR showed that the secretion levels of replicon RNAs also corresponded to the infectious titre of VLPs from cells transfected with each plasmid (Fig. 3c). On the other hand, pcJEME plasmid transfection into replicon-electroporated cells resulted in no secretion of infectious VLPs, despite the secretion of viral structural proteins. This viral protein secretion was due to the secretion of subviral particles (SPs), which consist of a viral envelope without nucleocapsid or genomic RNA, as reported in our previous study (Konishi et al., 2001). These data indicate that the efficient packaging and incorporation of nucleocapsid require homologous interactions between flavivirus C proteins and non-structural proteins or genomic RNA, but not between C proteins and viral envelope proteins.

**Fig. 3.** Kinetics of VLP secretion. (a) Detection of flavivirus structural proteins. TBE virus replicon-electroporated BHK-21 cells were transfected sequentially with pcTBECME, pcTBEC-JEME, pcJECME, pcJEME or control plasmid. At 36 h post-transfection of the plasmid, cell lysate and supernatant samples were separated by SDS-PAGE under non-reducing conditions and subjected to Western blotting. Viral proteins were visualized by specific antibodies against the TBE virus prM/E (left panel) and JE virus prM/E (right panel) proteins. Positions of individual proteins are marked; molecular mass (in kDa) is indicated. (b) Titres (IU) contained in the culture fluids were determined by infectivity assay to BHK-21 cells. The data are means from four independent experiments (error bars indicate SEM). (c) Detection of replicon RNAs packaged in VLPs. Secreted VLPs were diluted serially and subjected to RT-PCR for the detection of TBE virus replicon RNA.
Secretion of subviral particles from cells that express chimeric structural proteins and NS2B–NS3 proteins

It is known that expression of the flavivirus envelope proteins prM and E leads to budding and secretion of SPs. To examine the mechanism involved in the differential secretion of infectious VLPs observed for pcTBEC-JEME and pcJECME transfection of replicon-electroporated cells, we investigated SP secretion from cells that expressed C–prM–E polyproteins with TBE virus NS2B–NS3 protease. The pcTBENS2B/3 plasmid, which expresses TBE virus NS2B–NS3 polyproteins, was prepared. The same amount of NS3 was expressed and similar cytoplasmic localization was observed in cells transfected with pcTBENS2B/3 compared with those transfected with the TBE virus replicon RNAs (Fig. 4).

Individual plasmids that express flavivirus C/prM/E structural proteins (pcTBECME, pcTBEC-JEME or pcJECME) were transfected alone or together with pcTBENS2B/3 into BHK-21 cells. When the C/prM/E viral structural proteins were expressed without NS2B–NS3 proteins, C–prM polyprotein bands larger than those seen for prM were detected by the prM-specific antibodies (Fig. 5; lower panel with cell lysate samples). When the
viral structural proteins were expressed with TBE virus NS2B–NS3 proteins, the prM protein bands were detected as separate forms from the C proteins and no C–prM polyprotein band was detected, which indicates that the C–prM junctions were cleaved by TBE virus NS3 protease, as observed for the co-expression of the TBE virus replicon RNA (Fig. 3). On the other hand, whilst no viral protein was secreted without NS2B–NS3 expression, viral protein secretion was observed in all supernatant samples of cells that expressed the flavivirus C/prM/E structural proteins together with TBE virus NS2B–NS3 proteins (Fig. 5; panel with supernatant samples), indicating that SPs are secreted by cleavage of the C–prM junction. This suggests that the low level of VLP secretion from cells that were transfected with pcJECME and TBE virus replicon RNA (Fig. 3) is not due to the budding property of the viral envelope prM/E proteins after processing by NS2B–NS3 protease, and that other factors, such as the mechanism for genome replication and packaging, are involved in the efficient secretion of infectious chimeric VLPs.

Neutralizing test for VLP infection of BHK-21 cells

To confirm the antigenic characteristics of the secreted VLPs, the reactivities of VLPs with anti-flavivirus antibodies were examined. The neutralization test for VLP infection of BHK-21 cells was performed by using the mouse mAbs and polyclonal antibodies against TBE virus, Langat virus (tick-borne flavivirus) and JE virus. As shown in Table 1, mAb 1H4, anti-TBE virus polyclonal antibodies and anti-Langat virus polyclonal antibodies neutralized TBE-envelope VLP infectivity at almost the same concentrations as they did native TBE virus infectivity. In addition, the anti-JE virus polyclonal antibodies neutralized JE-envelope VLP infectivity at almost the same concentration as they did JE virus infectivity. The cross-reactive mAb 4H8 showed similar reactivities for the VLPs and naive viruses. These data show that the envelope glycoproteins of VLPs have the same antigenicity as those of authentic virus particles.

Table 1. Neutralizing titres of antibodies against infectious particles of flaviviruses

Data are reciprocal numbers of the highest serum dilution that reduced the virus focal count by 50%.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>VLPs*</th>
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<tr>
<td></td>
<td>TBE-envelope</td>
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<tr>
<td>mAbs†</td>
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<tr>
<td>1H4</td>
<td>&gt;640</td>
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<td>4H8</td>
<td>40</td>
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<td>Polyclonal antibodies</td>
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<tr>
<td>Anti-TBE virus</td>
<td>160</td>
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<tr>
<td>Anti-Langat virus</td>
<td>&gt;640</td>
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<tr>
<td>Anti-JE virus</td>
<td>&lt;20</td>
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*VLPs were prepared from BHK-21 cells transfected with Oshima REPt replicon and pcTBEC-JEME (TBE-envelope VLPs) or pcJECME (JE-envelope VLPs).
†1H4, Specific for tick-borne flavivirus; 4H8, cross-reactive against tick-borne and mosquito-borne flavivirus.

Infectivities of TBE-envelope and JE-envelope VLPs for arthropod cells

The JE-envelope VLPs contain envelope proteins that are derived from the mosquito-borne JE virus and carry nucleocapsid and replicon RNA derived from the tick-borne TBE virus. Therefore, we examined the susceptibilities of cell lines derived from arthropods to infection with chimeric JE-envelope VLPs. ISE6 cells, derived from I. scapularis, and C6/36 cells, derived from A. albopictus, were infected with the TBE-envelope VLPs, JE-envelope VLPs, TBE virus or JE virus, and viral protein production was detected by IFA.

As shown in Fig. 6, the tick cell line ISE6 was susceptible to infection by the TBE virus and TBE-envelope VLPs and the viral proteins showed a cytoplasmic distribution. However, the ISE6 cells were not susceptible to infection by the JE virus or JE-envelope VLPs, although the JE-envelope VLPs contained replicon RNA derived from the TBE virus. On the other hand, the mosquito cell line C6/36 showed no signs of infection by the TBE virus or TBE-envelope VLPs. The C6/36 cells were positive for viral antigen after infection with the mosquito-borne JE virus. Interestingly, the C6/36 cells that were infected with JE-envelope VLPs were not positive by IFA.

In order to reveal the mechanism involved in the lack of susceptibility of tick and mosquito cells to JE-envelope VLPs, the internalization of VLPs was examined (Fig. 7). Following 1 h VLP absorption, replicon RNA was detected in ISE6 cells that were infected with TBE-envelope VLPs, whereas most of the JE-envelope VLPs remained in the supernatant and no replicon RNA was detected intracellularly. This indicates that the entry of JE-envelope VLPs into ISE6 cells is inefficient and, thus, no viral proteins are detected by IFA (Fig. 6). In contrast, the JE-envelope VLPs entered the C6/36 cells after 1 h VLP absorption, whereas the TBE-envelope VLPs remained in the supernatant (Fig. 7a). However, intracellular replicon RNA disappeared at 8 h post-infection (Fig. 7b). Taken together with the IFA results (Fig. 6), this suggests that JE-envelope VLPs can enter C6/36 mosquito cells, but cannot replicate therein, due to fact that their genomic replicon RNA is derived from the tick-borne TBE virus. Direct transfection of C6/36 cells with TBE virus full-length RNA or replicon RNA resulted in neither viral protein production nor virus particle secretion, as reported previously (Mandl et al., 1991).
DISCUSSION

In this study, we developed, for the first time, chimeric VLPs between mosquito-borne and tick-borne flaviviruses, using the trans-packaging system for TBE virus subgenomic replicons. In recent years, the development of reverse-genetic technologies has enabled the construction of flavivirus chimeras in various combinations (Caufour et al., 2001; Guirakhoo et al., 2000, 2001; Huang et al., 2000; Mathenge et al., 2004; Monath et al., 1999; Pletnev & Men, 1998; Pletnev et al., 1992, 2002). These chimeras may be useful tools for studying the biological features of flaviviruses and for vaccine development. However, several aspects of flavivirus chimeras require further study. The family Flaviviridae contains many important human pathogens that cause severe symptoms and high mortality (approx. 30%). Thus, recent studies of flavivirus chimeras have tended to use less pathogenic or vaccine strains of flavivirus for the development of live-attenuated vaccines. On the other hand, flavivirus VLPs have the same antigenicity and virus-entry characteristics as the wild-type virus, and the single-round infectivity of VLPs allows infection experiments to be conducted under BSL-2 conditions (Gehrke et al., 2003; Khromykh et al., 1998; Scholle et al., 2004; Yoshii et al., 2005). Therefore, our strategy is that chimeric VLPs can substitute for chimeric viruses in investigations of the biological properties of flaviviruses.

Chimeric VLPs that packaged the TBE replicon were recovered only following trans-expression of the TBE virus

Fig. 6. Susceptibility of ISE6 (tick) and C6/36 (mosquito) cell lines to infectious flavivirus particles. ISE6 (i–iv) and C6/36 (v–viii) cells were infected with TBE-envelope VLPs (i, v), JE-envelope VLPs (ii, vi), TBE virus (iii, vii) or JE virus (iv, viii), prepared from the culture supernatants of BHK-21 cell cultures. Viral protein production was visualized by immunofluorescence using specific antibodies, as described in Methods.

Fig. 7. (a) Internalization of TBE- or JE-envelope VLPs into arthropod cells. ISE6 and C6/36 cells were infected with VLPs. After 1 h absorption, the supernatants were harvested and remaining VLPs were titrated. Results are expressed as percentages of remaining VLPs compared with untreated controls. The data are means from three independent experiments (error bars indicate SEM). (b) Detection of replicon RNA from arthropod cells infected with TBE- or JE-envelope VLPs. ISE6 and C6/36 cells were infected with the VLPs. At each time point post-infection (p.i.), RNA samples were extracted and subjected to RT-PCR for the detection of TBE virus replicon RNA. Actin mRNA was used as an internal control.
C and JE virus prM/E proteins. In many studies of flavivirus chimeras, chimeric viruses have been recovered successfully by replacement of the viral envelope protein prM and E genes by those from other flaviviruses. These data indicate that the C proteins (or nucleocapsid) interact non-specifically with viral envelope proteins and that this interaction is not important for the assembly of virus particles. On the other hand, in experiments using trans-expression of the JE virus C/prM/E proteins, we failed to recover chimeric VLPs. Similar results have been shown previously in several studies of chimeric viruses, in which substitution of the C/prM/E protein genes of a tick-borne flavivirus with those of mosquito-borne flaviviruses resulted in inefficient recovery of chimeric virus (Chambers et al., 1999; Pletnev & Men, 1998; Pletnev et al., 1992) and, in a study by Harvey et al. (2004), packaging efficiency of dengue type 2 virus replicon RNAs by trans-expression of the KUN virus C/prM/E proteins was lower than that of KUN virus replicon RNA; the detailed mechanism underlying this finding has not been analysed.

It is known that the C terminus of the C protein is processed by the activity of the viral NS2B–NS3 protease, and that the N terminus of prM is subsequently cleaved by the cellular signal peptidase (Amberg & Rice, 1999; Amberg et al., 1994; Stocks & Lobigs, 1998; Yamshchikov & Companis, 1995; Yamshchikov et al., 1997). The processed prM protein then forms a heterodimer with the E protein, which is essential for the maturation and assembly of infectious particles (Allison et al., 1995; Konishi & Mason, 1993; Lorenz et al., 2002). In cells that expressed the JE virus C/prM/E structural proteins and TBE virus replicon or NS2B–NS3, prM proteins were detected in the form cleaved from the C protein, which indicates that the JE virus C–prM junctions are cleaved by the TBE virus NS2B–NS3 protease (Figs 3 and 5). However, in spite of the processing of the C–prM junctions, the expression of JE virus C/prM/E proteins in cells that harboured the TBE virus replicon resulted in a low level of secretion of viral proteins compared with cells that expressed the TBE virus C and JE virus prM/E proteins or the TBE virus C/prM/E proteins, which secreted VLPs that packaged the replicon RNA. The difference between the trans-expressed polyproteins is a reflection of whether the respective C proteins are homologous to the genomic replicon RNA. Thus, in addition to the processing of C–prM junctions, other factors related to the homology between the C protein and genomic RNA regulate the assembly of VLPs that package replicon RNA.

Efficient secretion of E, in the form of non-infectious SPs, was observed by the expression of JE virus C/prM/E proteins and TBE virus NS2B–NS3 proteins, but few infectious VLPs and fewer E proteins were secreted by the expression of JE virus C/prM/E proteins in TBE replicon-transfected cells. This indicated that some factor involved in the replication of replicon RNA regulated the process of viral particle assembly and secretion and retained viral structural proteins intracellularly. Recent studies of flavivirus non-structural proteins have revealed the involvement of NS2A and NS3 in the assembly and/or release of infectious virus particles (Khromykh et al., 2000; Kummerer & Rice, 2002; Liu et al., 2002) and it has been reported that the packaging of flavivirus genomic RNA into infectious particles is coupled to genome replication (Khromykh et al., 2001). From these studies, it has been concluded that homologous interaction between viral C and non-structural proteins or genomic RNA is important for the efficient assembly and secretion of infectious particles packaging genomic RNA. No signals or motifs in flavivirus RNA or C protein that determine the specificity of packaging have yet been defined. However, these findings can contribute to future studies of packaging signals and increase understanding of how flavivirus virions are assembled and secreted.

It has been shown that, in general, arthropod-borne flaviviruses infect either mosquito or tick cells (Lawrie et al., 2004). However, it is not known whether the ability of certain flaviviruses to infect certain cells but not others is due to viral entry into the cells or to replication and subsequent release from infected cells. The chimeric VLPs developed in our study have viral envelopes that are derived from mosquito-borne JE virus, and nucleocapsid (C protein plus subgenomic replicon RNA) derived from tick-borne TBE virus. Virus particles of flaviviruses enter cells by receptor-mediated endocytosis, the viral envelope fuses with the endosomal membrane and then the genomic RNAs are uncoated from the nucleocapsid and replicate (Heinz et al., 2004). Thus, our chimeric VLP system has the advantage that it can separate particle-internalization events from sequential viral-replication events. As shown in Figs 6 and 7, TBE virus and JE virus infected either ISE6 or C6/36 cells, and TBE-envelope VLPs infected only ISE6 cells. However, no TBE virus NS proteins were produced in either tick or mosquito cells that were infected with JE-envelope VLPs. RT-PCR revealed that the TBE- and JE-envelope VLPs could enter ISE6 or C6/36 cells, and that the TBE virus replicon RNAs could not replicate in C6/36 cells that were infected with JE-envelope VLPs. Similar results were reported in a study by Pletnev et al. (1992), in which a chimeric virus of TBE virus/dengue type 4 virus that contained prM/E protein genes from the TBE virus was restricted in its ability to enter C6/36 cells. It is not known whether JE virus genomic RNA can replicate in tick cells, but these data indicate that vector-specific factors in arthropod cells may be required for each step of virus entry and replication during flavivirus infection. Although the susceptibility of a tick- or mosquito-derived cell to a particular arbovirus does not always reflect vector association, it is a useful indicator.

In addition to their use in investigations of the biological properties of flaviviruses, chimeric VLPs can be applied to serological diagnosis as a substitute for neutralization testing, which uses infectious viruses. As shown in Table 1, infections with TBE- and JE-envelope VLPs were neutralized by mAbs and immune sera with titres similar to those
used to neutralize the TBE and JE viruses, indicating that the chimeric VLP system is an effective alternative to the use of native flaviviruses in neutralization tests. This chimeric VLP-based neutralization system does not require a high-level containment laboratory, as the subgenomic replicon RNA packaged in the VLPs does not have the genes that encode the viral structural proteins and thus is unable to produce infectious progeny viruses. Furthermore, by replacement of the prM/E genes with those from other flaviviruses in plasmids used for the trans-expression of viral structural proteins, the chimeric VLP system can be adjusted easily to develop neutralization tests for a variety of flaviviruses, as substitutes for tests involving the native viruses.

In summary, we have generated chimeric VLPs that comprise the JE virus envelope and TBE virus nucleocapsid, which contains the subgenomic replicon of the TBE virus. JE-envelope VLPs were secreted when the TBE virus C protein and JE virus prM/E proteins were expressed in TBE virus replicon-electroporated cells, but not when all of the structural proteins of JE virus were expressed. The TBE- and JE-envelope VLPs were neutralized by antibodies against flaviviruses with efficacies similar to those for the native TBE and JE viruses. The infectivities of the TBE- and JE-envelope VLPs for tick and mosquito cells suggest that vector host cell-specific factors are involved in each step of flavivirus entry and replication during arthropod infection.

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REFERENCES


