

Short report

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## The prM-independent packaging of pseudotyped Japanese encephalitis virus

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### Abstract

As noted in other flaviviruses, the envelope (E) protein of Japanese encephalitis virus (JEV) interacts with a cellular receptor and mediates membrane fusion to allow viral entry into target cells, thus eliciting neutralizing antibody response. The formation of the flavivirus prM/E complex is followed by the cleavage of precursor membrane (prM) and membrane (M) protein by a cellular signalase. To test the effect of prM in JEV biology, we constructed JEV-MuLV pseudotyped viruses that express the prM/E protein or E only. The infectivity and titers of JEV pseudotyped viruses were examined in several cell lines. We also analyzed the neutralizing capacities with anti-JEV sera from JEV-immunized mice. Even though prM is crucial for multiple stages of JEV biology, the JEV-pseudotyped viruses produced with prM/E or with E only showed similar infectivity and titers in several cell lines and similar neutralizing sensitivity. These results showed that JEV-MuLV pseudotyped viruses did not require prM for production of infectious pseudotyped viruses.

### Findings

Japanese encephalitis virus (JEV) is a serious mosquito-borne flavivirus that causes pandemic infectious disease of major public health importance in Asia. JEV is a member of the genus *Flavivirus* in the family *Flaviviridae*, which includes yellow fever virus, Dengue virus, West Nile virus, and St. Louis encephalitis virus [1,2].

The JEV single-stranded RNA genome ( $\approx$  11 kb) encodes three structural proteins – capsid (C), premembrane

(prM) or membrane (M), and envelope (E) protein – and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins [3-5].

The assembly of JEV in the endoplasmic reticulum is followed by modification of the two envelope proteins E and prM and virion export through the secretory pathway. PrM ( $\approx$  26 kDa) is a precursor of the membrane-anchored and it cleaved a soluble Pr peptide and virion associated M protein ( $\approx$  8 kDa) by trans-Golgi resident furin or

related enzyme [6], resulting in two different forms of virion: the intracellular E- and prM-containing form, and the extracellular E- and M-containing form [3,7].

The E protein plays a major role in virus assembly, adhesion, receptor binding and membrane fusion, hemagglutination inhibition (HI), and induction of neutralizing antibodies (Nabs) [8-10]. Therefore, the E protein is the principal target of neutralization by specific antibodies against JEV infection [4,11]. E proteins of JEV expressed in different viral vector systems such as vaccinia virus, sindbis virus, and baculovirus have elicited high levels of neutralizing antibodies against JEV infection and have been tested as second generation JEV vaccines in mice [7,12-14]. From these reports, it is unknown whether prM cleavage affects infectivity, E protein expression, or induction of neutralizing activity. A major function of prM was studied by blocking prM cleavage or by mutation of the conserved glycosylation motif of JEV prM [15]. Even though the direct role of prM during the viral replication was not elucidated [6], it has a crucial function in multiple stages of JEV biology.

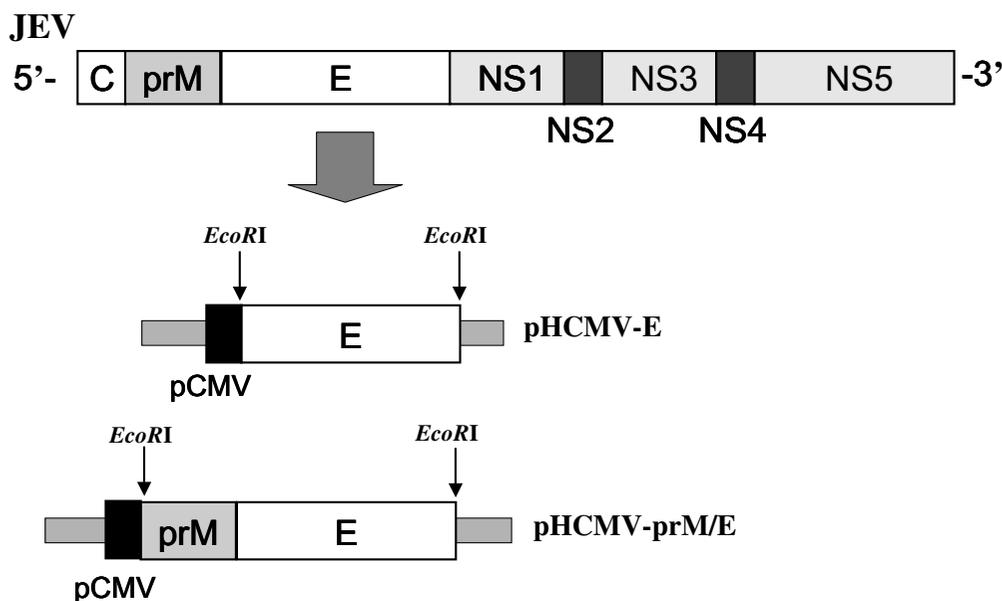
We generated pseudotyped viruses containing the prM/E or E protein of the current JEV vaccine strains Nakayama-NIH (NK) and Beijing-1 (BJ). The DNA fragments encoding the E and prM/E regions were amplified by polymerase chain reaction (PCR) from the cDNA of the NK strain and BJ strain kindly supplied from the Department of Vaccine, KFDA, Korea. PCRs were performed using one of the

two forward primers: for prM/E amplification, 5'-AATGA-GAATTCGACCATGTGGCTCGCAAGCTTGGC-3'; and for E amplification, 5'-GGTCGCGAATTCTGCAGGTTCAACT-GTCTGGGAGTG-3'. The reverse primer for both amplifications was 5'-ACCAATGTGCATGCTTAGCTCGAGAATTC-CATG-3'. Each primer had an *EcoRI* restriction site. To generate pHCMV-prM/E and pHCMV-E, the PCR products of prM/E and E were digested with *EcoRI* and subsequently cloned into pHCMV-G [16] digested with *EcoRI* (Figure 1).

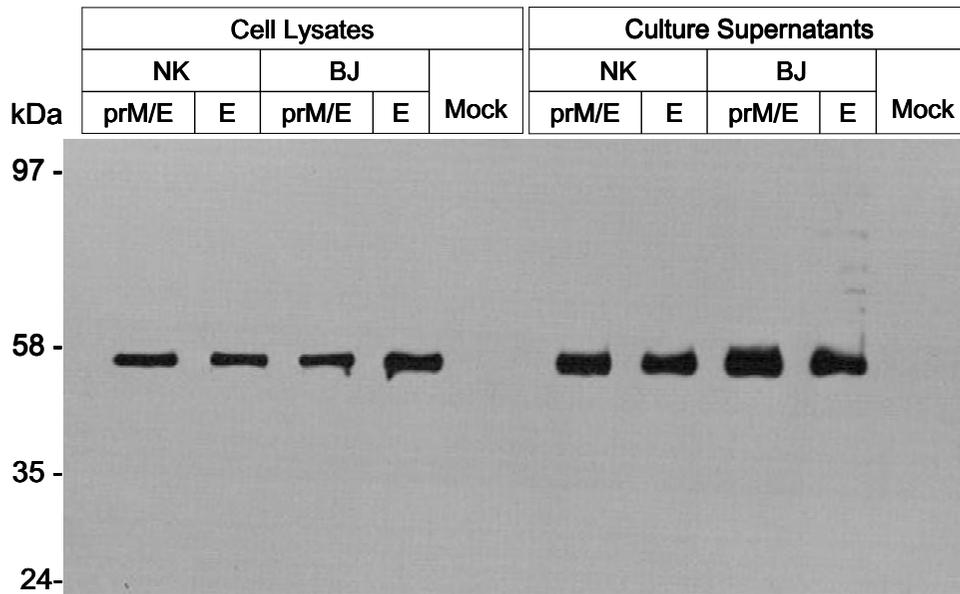
Pseudotyped viruses encoding prM/E or E of JEV NK and BJ strain were produced as previously described [17]. Briefly, TELCeB6 cells, a MuLV packaging cell line [18], were transfected with pHCMV-E or pHCMV-prM/E by a calcium phosphate method. After overnight incubation, the culture medium was replaced, and the cells were incubated for two additional days. The supernatants containing pseudotyped viruses were harvested by low speed centrifugation (1,500 × g, 5 min) to remove cell debris.

Figure 2 shows the expression of the E proteins from each pseudotyped JEV constructs by western blot analysis using anti-JEV (Nakayama) sera. The envelope genes of the NK and BK strains were expressed well in cell culture supernatants and lysates. Similar amounts of E proteins were expressed in NK and BJ transfected cells.

The JEV-pseudotyped viruses expressing prM-E and E were properly processed and released into the culture media



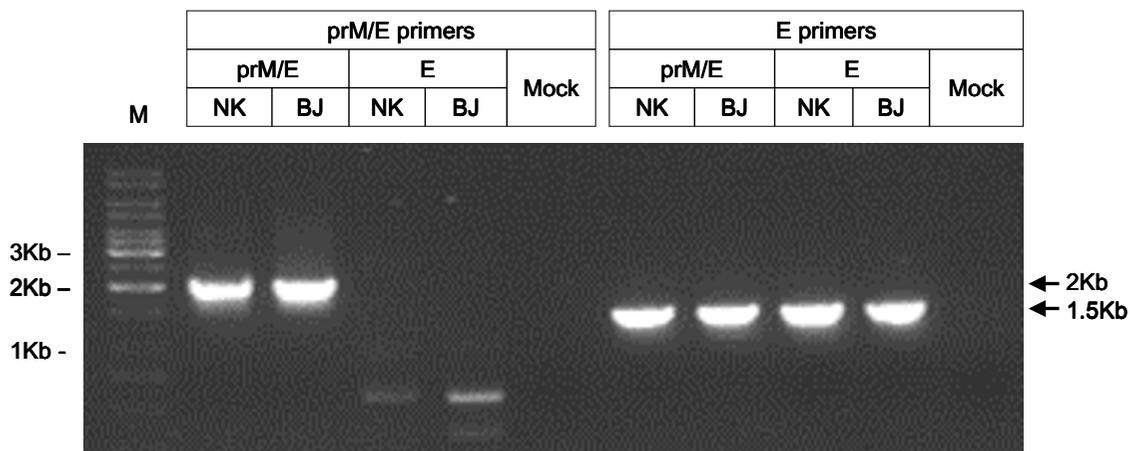
**Figure 1**  
**Construction of JEV pHCMV-prM/E and pHCMV-E.** Four plasmids were produced: pHCMV-prM/E (NK or BJ) and pHCMV-E (NK or BJ).



**Figure 2**  
**Expression of JEV envelope (E) protein.** Cell lysate and culture media of JEV-pseudotyped viruses were subjected to SDS-PAGE. JEV-E proteins were detected by western blotting using sera from mice immunized with JEV (Nakayama-NIH strain). The bands show the JEV-E protein (53 kDa) in cell lysates and culture supernatants. Non-transfected TELCeB6 cells were used as a negative control.

with similar levels of expression. However prM-E polyprotein bands were not detected despite many attempts. To check the expression of the *prM/Env* gene during pseudotyped virus formation, we performed reverse transcription (RT)-PCR. Figure 3 shows the amplified JEV *prM/Env*

(2062 bp), *Env* (1501 bp) transcripts in transfected TELCeB6 cells. We assumed that the cleavage of the prM protein is too rapid to detect with prM/E polyprotein and our sera from JEV-immunized mice did not contain the prM antibodies.



**Figure 3**  
**Expression of JEV *prM* and *Env* genes in TELCeB6 cells.** TELCeB6 cells were transfected with pHCMV-prM/E and pHCMV-E of each of NK and BJ strains. RT-PCR products of the indicated size were amplified from total RNA samples extracted from TELCeB6 cells. The products correspond to 2 Kb of *prM* (amplified by prM/E primer) and 1.5 Kb of *Env* (amplified by E primer). The molecular marker (Kb) is shown on the left.

To test the effect of prM on pseudotyped viral infectivity and viral titer, infection tests with the pseudotyped viruses were carried out in 96-well plates with Vero, PK15, CRFK, NIH3T3, HeLa, 293T, BHK-21, MDCK, and HOS cells. Four kinds of pseudotyped JEVs were added to the host cells and incubated at 37°C in a 5% CO<sub>2</sub> incubator for 36 hours. All infections were done in triplicate. After X-gal staining, JEV pseudotyped virus-infected cells appeared blue. This resulted from integration of the MuLV pseudovirus genome encoding β-galactosidase. These cells were counted as an infectious unit. All JEV pseudotyped viruses could infect PK15, CRFK, NIH3T3, HeLa, 293T, BHK-21, Vero, and MDCK cells, but not HOS cells (Table 1).

The titers of pseudotyped viruses were comparable to the infectivity of JEV in each host cell line. JEV pseudotyped virus with E or prM/E (both NK and BJ strain) could efficiently infect several cell lines, with typical titers between 1.14 × 10<sup>2</sup> and 1.36 × 10<sup>5</sup> infectious units (IFU)/mL. From Table 1, viruses pseudotyped with prM/E exhibited infectivity similar to those pseudotyped with E only. This showed that the infectivity and titer of JEV pseudotyped virus is not affected by prM deletion.

Neutralizing sensitivity was tested with JEV-immunized immune sera, which was supplied by the Catholic University of Korea, to check the effect of prM on pseudotyped JEV antigens. As previously described [17], neutralization assays were carried out with Vero cells in triplicate. Approximately 100 IFU/mL of pseudotyped viruses were incubated with 10-fold diluted sera from mice immunized with JEV for 1 h at 37°C, and the mixture was subsequently added to Vero cells. After 2 days of incubation, virus infection was monitored by X-gal staining as described above. The neutralizing sensitivity was expressed as virus reduction by neutralizing antibodies.

Figure 4 shows the reduction of pseudotyped JEVs by neutralization with homologous and heterologous sera. A

high neutralization was observed with 1:10 diluted serum. In contrast, no neutralizing activity was detected in the normal mouse serum (reduction was less than 30%). The neutralization by homologous sera was more complete than that by heterologous sera. However, there was no significant difference between pseudotyped JEV prM/Env and Env.

In summary, we generated pseudotyped JEVs that express prM/E or E proteins from two JEV vaccine strains NK and BJ. All four JEV pseudotyped viruses efficiently infected several cell lines and were neutralized by sera from JEV-immunized mice. The main purpose of generating JEV pseudotyped virus was to devise a safe and rapid assay system to assess neutralizing antibodies by avoiding the use of infectious, replication-competent JEVs in a Biosafety Level 3 laboratory. The titer of the four JEV pseudotyped viruses was greater than 10<sup>4</sup> IFU/mL. This confirmed the possibility of mass-producing viruses to conduct neutralization assays with Vero and CRFK cells. Even though a crucial function of prM in assembly and maturation of flaviviruses has been reported, the two types of JEV-MuLV pseudotyped virus that respectively express the E or prM/E proteins were found to have no significant difference in the level of transcription and the extent of protein expression, infectivity, titer, and neutralization sensitivity.

**Competing interests**

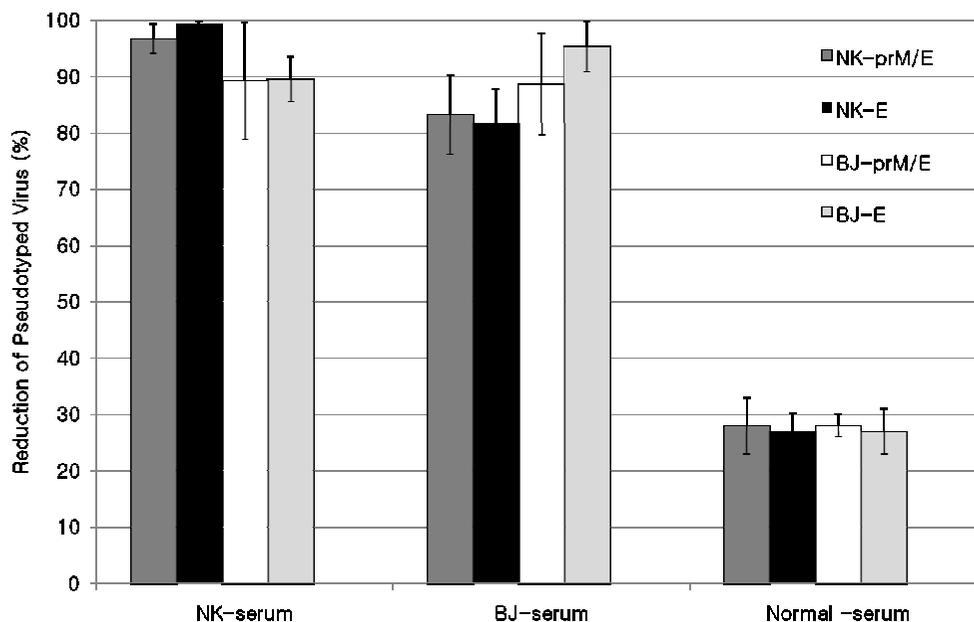
The authors declare that they have no competing interests.

**Authors' contributions**

HJL, JW, YRJ, YBK participated in the design of the study, HJL, JL, SHK performed the experiments, KIM and JHN provided key reagents, and HJL and YBK edited and approved the final manuscript.

**Table 1: Infectivity and titer of JEV pseudotyped viruses in host cells**

JEV pseudotyped virus	Infectious units/ml (IFU/ml)							
	Host cell							
	Vero	BHK-21	HeLa	CRFK	PK15	293T	MDBK	HOS
NK prM/E	4.09 *10 <sup>4</sup>	7.46 *10 <sup>3</sup>	4.08 *10 <sup>3</sup>	9.74 *10 <sup>4</sup>	1.33 *10 <sup>3</sup>	1.14 *10 <sup>2</sup>	2.08 *10 <sup>2</sup>	0
NK E	4.25 *10 <sup>4</sup>	7.58 *10 <sup>3</sup>	4.12 *10 <sup>3</sup>	1.20 *10 <sup>5</sup>	1.09 *10 <sup>3</sup>	1.32 *10 <sup>2</sup>	2.11 *10 <sup>2</sup>	0
BJ prM/E	4.12 *10 <sup>4</sup>	7.76 *10 <sup>3</sup>	4.35 *10 <sup>3</sup>	1.02 *10 <sup>5</sup>	1.82 *10 <sup>3</sup>	2.04 *10 <sup>2</sup>	2.08 *10 <sup>2</sup>	0
BJ E	4.83*10 <sup>4</sup>	7.91 *10 <sup>3</sup>	4.29 *10 <sup>3</sup>	1.36*10 <sup>5</sup>	2.90 *10 <sup>3</sup>	2.05 *10 <sup>2</sup>	2.23 *10 <sup>2</sup>	0



**Figure 4**

**Neutralizing assay with JEV pseudotyped viruses.** Four different JEV-pseudotyped viruses (NK-prM/E, dark grey; NK-E, black; BJ-prM/E, white; BJ-E, light grey) were incubated with sera from mice immunized with JEV-NK strain or JEV-BJ strain. Neutralizations were determined with 1:10 diluted serum.

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