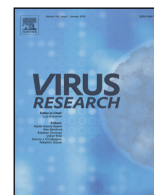
Virus Research 229 (2017) 9–16

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres



Introducing a cleavable signal peptide enhances the packaging efficiency of lentiviral vectors pseudotyped with Japanese encephalitis virus envelope proteins

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ARTICLE INFO

Article history:
Received 13 June 2016
Received in revised form 6 November 2016
Accepted 7 December 2016
Available online 16 December 2016

Keywords:
Signal peptide
Packaging efficiency
Japanese encephalitis virus
Pseudotyped virus

ABSTRACT

Research into the properties of Japanese encephalitis virus (JEV) has been facilitated by use of pseudotyped viruses. The signal peptide is a key determinant for membrane targeting and membrane insertion, which could affect packaging of pseudotyped viruses. In this study, we generated three lentiviral vectors pseudotyped with JEV envelope proteins that co-express either a strong signal peptide from vesicular stomatitis virus (VSV)-G (VSVMEpv) or a weak signal peptide of JEV (SPMEpv), or a virus without a signal peptide in front of the JEV prM/E (MEpv). Western blot demonstrated that JEV E protein and HIV p24 were present in the same particles of the three pseudotyped JEV-E based lentiviral vectors. Electron microscopy revealed that the three pseudotyped JEV-E based lentiviral vectors were 120–180 nm in diameter. Real-time quantitative reverse transcriptase polymerase chain reaction showed that the titer of VSVMEpv was 17-fold higher than that of MEpv, while the titer of SPMEpv was six-fold higher than that of MEpv. Inclusion of a signal peptide enhanced packaging efficiency of pseudotyped JEV-E based lentiviral vectors. With a strong signal peptide helping they generate a higher number of viral particles. Green fluorescent protein and luciferase expression showed that the transduction titer or relative fluorescence units of VSVMEpv, SPMEpv and MEpv were not significantly different. We suggest that the signal peptide does not influence the infectivity of pseudotyped JEV-E based lentiviral vectors. In addition, our findings indicated that pseudotyped JEVs show preferential tropism for BHK-21 cells, supporting the mimic function displayed by parental JEV. Therefore, our study provided a cost-effective method to generate pseudotyped JEV-E based lentiviral vectors, which may represent a valid model to investigate some of the infectious properties of JEV.

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1. Introduction

Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus that causes viral encephalitis and death in humans (Huang et al., 2014), and reproductive failure in sows and boars (Lindahl et al., 2012). The M protein of JEV is synthesized as the precursor membrane (prM) protein in infected cells, which is then cleaved to M by a cellular protease, furin, during virion maturation (Li et al., 2008). The E protein is the main pathogenic factor and is responsible for several important processes including virus attachment, fusion, penetration, cell tropism, virulence and attenuation (Misra and Kalita, 2010; Smit et al., 2011; Stiasny et al., 2011). There are several studies that have evaluated JEV infection, however, the endocytic pathway utilized by JEV is still unclear, as is the receptor for JEV on neuronal cells (Kalia et al., 2013; Yang et al., 2013; Zhu et al., 2012). Thus, an appropriate system to explore mechanisms of viral entry needs to be developed.

Pseudotype formation based on the vesicular stomatitis virus (VSV) bearing foreign viral envelope proteins is a powerful tool for analyzing mechanisms of viral neutralization and entry (Jakubczak et al., 2001; Wang et al., 2014). These systems have been used to study infection with viruses that are difficult to handle because of their high-level pathogenicity for humans (Zhang et al., 2010). In addition, because pseudotyped viral particles can only complete a single infection, the systems allow us to focus on the entry

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http://dx.doi.org/10.1016/j.virusres.2016.12.007
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mechanisms of particular viral envelope proteins without considering the impact of viral replication. Examples of pseudotyped flaviviruses include: glycoproteins from hepatitis C virus (Bartosch et al., 2003; Garrone et al., 2011), dengue virus (Hu et al., 2007) and JEV (Lee et al., 2009). In the case of JEV pseudotyped virus expressing E or prM/E can efficiently infect several cell lines, with typical titers between 1.14×10^2 and 1.36×10^5 infectious units (IFU)/mL. However, a better approach to enhance the titers of pseudotyped JEV is still needed. Inefficiencies may be related to the construction of pseudotyped vectors or their transduction efficiency. The latter can be highly variable and is mainly dependent upon culture conditions and cell type (8, 19). For the former, signal peptides are key determinants for membrane targeting and the insertion of secretory and membrane proteins (Lindemann et al., 2001), which could be involved in the packaging of pseudotyped viruses (Caporale et al., 2009).

Taking this into consideration, we constructed three plasmid vectors that expressed either a strong signal peptide of VSV-G, a weak signal peptide of JEV, or one without signal peptide in front of the JEV prM/E protein. Signal peptide function was evaluated by comparing the expression of lentivirus-based pseudotype reporter viruses for JEV. Moreover, we showed that pseudotyped JEV-E based lentiviral vectors, had similar cell tropism to wild-type JEV had, which also appears to enter cells through a pH-dependent mechanism. We discuss the usefulness of these particles for understanding the mechanism of entry of JEV and their potential uses in simple diagnostic assays.

2. Materials and methods

2.1. Cells, plasmids and viruses

The HEK-293T and BHK-21 cell lines were used. Cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), penicillin–streptomycin (Gibco/Brl Div., Grand Island, NY, USA), and 2 mM L-glutamine at 37 °C in a humidified atmosphere of 5% CO_2. The plasmids were derived from eukaryotic expression vector pcDNA3.1, HIV vector system including pCDH-CMV-Luc–copGFP, pMD2.g, psPAX2.1 (Invitrogen, Carlsbad, CA), and pAP-1-Luc (Stratagene, La Jolla, CA, USA). The SCYA201201 strain of JEV (Genbank: KM658163) was isolated from pig cerebrospinal fluid in 2012 (Sichuan, China), and is maintained in our laboratory.

2.2. Predicting signal peptides

All predictions made by the SignalP 4.1 server can optionally be accompanied by a posterior label (location) probability plot. Note that the posterior probability plot is not a prediction in itself. The pattern of the plot might even deviate from the prediction, which would be a sign of uncertainty in the prediction (Kall et al., 2004; Kall et al., 2007).

2.3. Plasmid construction

Truncations were made using a reverse transcriptase polymerase chain reaction (RT-PCR) to amplify the prM signal peptide with *ME* gene and ME alone from cDNAs of JEV wild-type strain SCYA201201 (Genbank: KM658163) at the desired lengths. Restriction enzyme cleavage sequences for EcoRI in the 5′ primers and BamHI in the 3′ primers allowed cloning into the transfer vector pcDNA3.1, generating pcDNA3.1-SPME and pcDNA3.1-ME (Fig. 2). To acquire a strong signal peptide envelop vector (Tessier et al., 1991), a 54-amino-acid signal peptide from VSV G was amplified from plasmid pMD2.g and combined with the ME gene using overlapping PCR with a forward primer designed to have an EcoRI at the 5′ end and a reverse primer with a BamHI sites at the 5′ end. The resulting VSV/ME fragment was sub-cloned into pcDNA3.1 to generate the plasmid pcDNA3.1-VSVME. To achieve a double labeling lentiviral vector, the luciferase (*Luc*) gene was amplified from pAP-1-Luc and sub-cloned into pCDH-CMV–copGFP to generate the plasmid pCDH-CMV-Luc–copGFP. Primers used in this study are shown in Table 1.

2.4. Generation and concentration of human immunodeficiency virus (HIV)–JEV pseudotype particles

HIV–JEV pseudotypes were generated as previously described (Kutner et al., 2009; Witting et al., 2013). HEK-293T cells at 70% confluence were co-transfected with envelop expression plasmid (pcDNA3.1-VSVME, pcDNA3.1-SPME, pcDNA3.1-ME or pMD2.g), HIV vector system pCDH-CMV-Luc–copGFP and psPAX2.1, using Lipofectamine 3000 (Invitrogen). Lipid–DNA complexes were removed 4–6 h later and replaced with DMEM supplemented with 10% FBS. At 48 h post-transfection, the culture medium was collected, filtered with a 0.45 μm cellulose acetate syringe filter (Millipore) to remove debris, and mixed with 5 × PEG8000 NaCl buffer (Genomeditech, Shanghai, China) at 4 °C for 6 h. After incubation, the mixture was clarified by centrifugation at 4 °C for 15 min at 4000 *g* in a microcentrifuge. The supernatant was removed and the pellet re-suspended in one hundredth of the original volume. Aliquots of virus were stored at −80 °C. All calculations of lentiviral genomic RNA and mRNA expression refer to concentrated supernatants.

2.5. Transmission electron microscopy (TEM)

At 48 h post-transfection, HEK-293T cells were washed and detached with phosphate-buffered saline (PBS), fixed for 3 h in 1% glutaraldehyde, washed, fixed for 30 min in 1% osmium tetroxide, dehydrated in ethanol, and embedded in epoxy resin. Sections of 50–70 nm were stained with saturated uranyl acetate, and examined by TEM (Morgan and Rose, 1968).

2.6. Identification of protein expression by western blotting

Western blot was performed as described previously (Kahl et al., 2004). Concentrated pseudotyped JEV-E based lentiviral vector particles were subjected to 12% SDS–PAGE electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore). Western blot was performed using standard methods and anti-JEV E-protein mouse monoclonal antibody (Abcam) diluted 1:200 as the primary antibody, and a 1:5000 dilution of horseradish peroxidase (HRP)-labeled sheep anti-mouse IgG antibody as the secondary antibody. The bands were visualized using an Immun-Star WesternC detection system (Bio-Rad). The same method was used to identify expression of HIV p24 protein, except that we replaced the primary antibody with a 1:200 dilution of anti-p24 rabbit monoclonal antibody (Abcam) and the secondary antibody with a 1:5000 dilution of HRP-labeled goat anti-rabbit IgG antibody (Sangon Biotech). Densinometric scanning and quantification of the bands present on the western blots was carried out using Image J for Windows (NIH) with three replications (Abràmoff and Ram, 2004).

2.7. Real-time RT-PCR

To determine RNA titers in pseudotyped JEV-E based lentiviral vector particles, viral RNA was isolated from the concentrated particles using the Qiagen RNeasy Minikit, total RNA was reverse

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