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Establishment of tracking system for West Nile virus entry and evidence of microtubule involvement in particle transport

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A B S T R A C T

West Nile virus (WNV) is one of flaviviruses and has emerged recently in the United States as a significant cause of viral encephalitis. Although cellular entry of WNV is important for viral pathogenesis, its mechanisms have not been elucidated fully. To explore the entry mechanisms, a virus-particle tracking system in live cells by using fluorescently labeled subviral particles (SVPs) and time-lapse epifluorescence microscopy was established. This study revealed that, following cellular entry, SVP movements could be divided into two phases: early (slow movement) and late (fast movement) phase. Moreover, fast viral particle movements at the late phase correlated with SVP–microtubule association.

1. Introduction

West Nile virus (WNV) is a flavivirus transmitted by Culex mosquitoes to vertebrate hosts. In humans, infection can result in febrile illness, meningitis, and encephalitis. WNV is found throughout Africa, Europe, Central Asia (Kramer et al., 2007), and most recently in North America. Since the outbreak in New York in 1999 (Marfin and Gubler, 2001), WNV has spread across the United States. From 1999 to 2006, 23,500 human cases were reported, of which 904 cases (4%) were fatal (Sejvar, 2007).

Cellular entry is a requisite step in the life cycle of WNV. WNV is thought to enter host cells *via* clathrin-dependent endocytosis (Chu and Ng, 2004; Krishnan et al., 2007). Following entry, the virus colocalizes with both early and late endosomes, and Rab5, an early endosome molecule, is required for WNV entry (Chu and Ng, 2004; Krishnan et al., 2007). After WNV particles are transported to late endosomes, the nucleocapsid is released to the cytoplasm *via* envelope-fusion (Stiasny and Heinz, 2006). Furthermore much evidence on entry mechanisms of other flaviviruses such as Japanese encephalitis and Dengue viruses give us much effective hints, but the details of entry mechanisms between other flaviviruses and West Nile virus are different, though basically they exploit common cellular components. For example, Dengue virus exploits DC-SIGN to bind cell surface, while West Nile can DC-SIGNR much more efficiently than DC-SIGN (Davis et al., 2006). Therefore, in this study, a virus-particle tracking system in living cells using subviral particles (SVPs) labeled with a fluorescent dye was established and used to observe particle movements in viral entry.

The effect of nocodazole on particle movements in viral entry was investigated, because nocodazole treatment delayed the neuroinvasion of WNV probably due to impairment of axonal retrograde transport of WNV (Hunsperger and Roehrig, 2009), and the results showed that nocodazole drastically inhibited the particle movements in WNV entry steps.

2. Materials and methods

2.1. Cells

293T and 293T-SVPs cells were maintained in DMEM supplemented with P/S and 10% FBS in 5% CO_2 at 37 °C without or with 10 μg/ml of blasticidin S-HCl (Invitrogen, Carlsbad, USA),

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respectively. Vero cells were maintained in EMEM supplemented with P/S and 10% FBS in 5% CO_2 at 37 °C. C6/36 cells were maintained in EMEM supplemented with P/S and 10% FBS in 5% CO_2 at 28 °C.

2.2. Antibodies

The following antibodies were used: anti-West Nile virus envelope (E) protein mouse monoclonal antibody (clone 3.91D, Chemicon, Temecula, USA), anti-prM rabbit polyclonal antibody (Imgenex, San Diego, USA), anti-mouse IgG antibody and anti-rabbit IgG antibody conjugated with horseradish peroxidase (Biosource, Camarillo, USA), Alexa Fluor 488 goat anti-mouse IgG antibody (Invitrogen), and mouse IgG3 isotype control (Beckman Coulter, Brea, USA).

2.3. Plasmids

A lentiviral vector CSII-CMV-MCS-IRES2-Bsd and the packaging plasmids (pCAG-HIVgp and pCMV-VSV-G-RSV-Rev) were gifts from Dr. Miyoshi (Riken, Tsukuba, Japan). pWNRepII-REN, which bears the cDNA sequence of WNV replicon RNA containing the Renilla luciferase gene was a gift from Dr. Doms (University of Pennsylvania, Philadelphia, USA) (Pierson et al., 2006). The prM-E fragment from the West Nile virus New York strain (6-LP) (Shirato et al., 2004) was amplified by PCR from pCAGGS-C-prM-E, which was a gift from Dr. Takashima (Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan) as a template, and subcloned into the CSII-CMV-MCS-IRES2-Bsd vector digested with Xho I-Not I, and the resulting plasmid was named CSII-CMV-MCS-IRES2-Bsd-SVPs. The PCR fragment was verified by sequencing.

2.4. WNV-E Sandwich ELISA

A sandwich ELISA using anti-E monoclonal antibody (clone 402) was performed to detect SVPs as described previously (Takahashi et al., 2009). Anti-E monoclonal antibodies diluted in 50 mM carbonate buffer (pH 9.5) were absorbed onto an ELISA plate at 4 °C overnight, After the wells were washed four times with PBS containing 0.05% (w/v) Tween-20 (PBS-T), samples diluted in PBS-T containing 0.1% (w/v) BSA were added to the wells and incubated at 37 °C for 1 h. Following four washes, an anti-E monoclonal antibody (clone 402) conjugated with HRP was diluted in PBS-T containing 10% FBS and added to the wells. After incubation at 37 °C for 1 h, the wells were washed and the signal was detected with o-phenylenediamine dihydrochloride and a multi-well plate reader (Bio-Rad, Hercules, USA).

2.5. Immunoblot analysis

Samples were separated by SDS-PAGE, transferred onto PVDF filters (Millipore, Billerica, USA), and incubated with primary and then secondary antibodies. Positive signals were detected by ECL western blotting reagents (GE Healthcare Life Science, Pittsburgh, USA) and quantified using a Lumino Image Analyzer (Fuji Film, Tokyo, Japan).

2.6. Establishment of the 293T-SVPs cell line

293T cells were transfected with CSII-CMV-MCS-IRES2-Bsd-SVPs and the packaging plasmids (pCAG-HIVgp and pCMV-VSV-G-RSV-Rev) using lipofectamine 2000 (Invitrogen). After 48 h, pseudotyped lentiviruses bearing prM and E genes were harvested from the supernatant. 293T were infected with the pseudotyped lentiviruses and selected with blasticidin S-HCl at 10 μg/ml for one week. The cells were then subjected to a limited dilution in a 96-well plate, and high expression clones were selected by immunoblotting with anti-E antibody. The selected clones were designated 293T-SVPs.

2.7. Synthesis and labeling of SVPs

293T-SVPs cells were seeded on culture dishes and labeled with DiD (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt, Invitrogen) at 5 μM for 6 h. After washing out the dye, fresh medium was added and the cells were incubated for one day. Supernatant containing DiD-labeled SVPs (SVPs-DiD) was concentrated with Amicon Ultra-15 (100 kDa, Millipore) and purified on a 20–60% (w/v) continuous sucrose gradient by ultracentrifugation in a Beckman SW41 rotor at 4 °C for 14 h at 250,000 × *g*. In parallel, supernatants from non-labeled 293T-SVPs cells and DiD-labeled 293T cells were processed as controls. After fractionation, the fractions were evaluated by a WNV-E sandwich ELISA, and the sucrose fractions of approximately 40–45% (w/w) were used for further experiments.

2.8. Immunostaining of SVPs

Purified SVPs and SVPs-DiD were absorbed onto cover slips at 4 °C overnight and stained with an anti-E monoclonal antibody or normal IgG at room temperature for 2 h. After washing three times, samples were incubated with an Alexa-488 conjugated anti-mouse IgG antibody at room temperature for 1 h, followed by observation with a confocal microscope (Olympus, Tokyo, Japan).

2.9. Electron microscopy

Purified SVPs and SVPs-DiD were applied to copper grids (400-mesh) covered by carbon-coated collodion film and stained with 2% phosphotungstic acid (pH 7.0). The samples were observed under a transmission electron microscope (Hitachi High-Technologies, Tokyo, Japan).

2.10. Hemagglutination (HA) assay

SVPs and SVPs-DiD were subjected to a serial 2-fold dilution in PBS containing 0.2% BSA (pH 8.0) and then mixed with pigeon red blood cells in 0.2 M PBS. The HA assay was performed at pH 6.5 and incubated for 1 h at 37 °C, followed by megascopic observation.

2.11. Fusion assay

The fusion assay was performed as described previously (Guirakhoo et al., 1991) with some modifications. C6/36 cell monolayers were grown in 96-well plates. The cells were precooled with cold Opti-MEM I medium for 15 min at 4 °C and incubated for 1 h at 4 °C with SVPs or SVPs-DiD diluted with Opti-MEM I medium. Then, the inoculum was removed, and prewarmed fusion buffer (40 °C) was added. The fusion buffer consisted of EMEM without sodium bicarbonate, buffered with 20 mM MES-NaOH at pH 6.0, or 20 mM HEPES-NaOH at pH 7.4 as a control buffer. Following 2 min incubation, the fusion buffer was replaced by Opti-MEM I medium and the cells were further incubated for 2 h at 40 °C. After incubation, the cells were fixed with 3% paraformaldehyde (PFA) and stained with DAPI. The numbers of nuclei and cells in three microscopic visual fields (magnification 200-fold) were counted and the ratios of the numbers of nuclei and cells (nuclei/cells) were calculated.

2.12. Tracking analyses of SVPs-DiD

Time-lapse imaging was performed at 37 °C by epifluorescence microscopy (Leica, Buffalo Grove, USA), immediately after the