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The ubiquitin-proteasome system is essential for the productive entry of Japanese encephalitis virus

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ABSTRACT

The host-virus interaction during the cellular entry of Japanese encephalitis virus (JEV) is poorly characterized. The ubiquitin-proteasome system (UPS), the major intracellular proteolytic pathway, mediates diverse cellular processes, including endocytosis and signal transduction, which may be involved in the entry of virus. Here, we showed that the proteasome inhibitors, MG132 and lactacystin, impaired the productive entry of JEV by effectively interfering with viral intracellular trafficking at the stage between crossing cell membrane and the initial translation of the viral genome after uncoating. Using confocal microscopy, it was demonstrated that a proportion of the internalized virions were misdirected to lysosomes following treatment with MG132, resulting in non-productive entry. In addition, using specific siRNAs targeting ubiquitin, we verified that protein ubiquitination was involved in the entry of JEV. Overall, our study demonstrated the UPS is essential for the productive entry of JEV and might represent a potential antiviral target for JEV infection.

1. Introduction

Japanese encephalitis virus (JEV) is a mosquito-borne virus of the family *Flaviviridae* that causes viral encephalitis transmitted by *Culex* mosquito vectors (Le Flohic et al., 2013; Unni et al., 2011). The family *Flaviviridae* includes several other important pathogens, such as dengue virus (DENV), West Nile virus (WNV), yellow fever virus (YFV) and tick-borne encephalitis virus (TBEV). JEV is prevalent in most of Asia and northern of Australia (van den Hurk et al., 2009). Approximately 67,900 cases of JE occur annually, among which 20–30% are lethal and nearly 30–50% involve serious neurologic or psychiatric sequelae (Campbell et al., 2011; Yun and Lee, 2014). JEV is enveloped and approximately 50 nm in diameter, and it contains a positive single-stranded RNA genome. The ~11 kb genome encodes a polyprotein, which is cleaved into three structural proteins (capsid, precursor membrane and envelope) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) by viral and cellular proteases (Misra and Kalita, 2010).

Similar to the other members of the flavivirus family, such as DENV and WNV, JEV has been reported to undergo receptor-mediated, pH- and cholesterol-dependent internalization (Lee et al., 2008; Medigeshi et al., 2008; Nawa et al., 2007; Nawa et al., 2003). Different endocytosis pathways were utilized by JEV in different host cells (Chuang et al., 2015; Das et al., 2010; Yang et al., 2013; Zhu et al., 2012). After penetrating the plasma membrane by receptor mediated endocytosis, JEV particles are transported in the early endosomes, and the low-pH environment of the endosomes induces a conformational change of the envelope (E) protein, resulting in fusion of the endosomal and viral membranes (Kalia et al., 2013; Liu et al., 2015). Afterward, the capsid depolymerizes, leading to release of the viral genome (Smit et al., 2011). However, certain vital factors and pathways involved in intracellular trafficking and the precise mechanisms of JEV internalization are poorly characterized.

The ubiquitin-proteasome system (UPS), the major intracellular protein degradation machinery, plays important roles in many cellular processes such as the cell cycle, apoptosis, the host immune response, endocytosis, and signal transduction (Glickman and Ciechanover, 2002; Pickart, 2001). When ubiquitination is initiated, ubiquitin is activated in an ATP-dependent manner by an E1 ubiquitin-activating enzyme. The activated ubiquitin is then transferred to an E2 ubiquitin-conjugating enzyme. Finally, the ubiquitin conjugated to E2 is transferred to the substrate protein with the help of a specific E3 ubiquitin ligase (Gao and Luo, 2006). New ubiquitin molecules can be added to the first ubiquitin conjugated to the substrate protein, resulting in the formation of a poly-ubiquitin chain. Afterward, the ubiquitylated substrate protein is recognized and

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degraded by the 26S proteasome (Pickart, 2000), and the recycling of ubiquitin is processed by the deubiquitinating enzymes (Amerik and Hochstrasser, 2004).

The UPS had been reported to be exploited by many viruses to achieve successful infection (Amaya et al., 2015; Cheng et al., 2014; Greene et al., 2012; Lin et al., 2015; Raaben et al., 2010; Schneider et al., 2012; Teale et al., 2009). Regarding flaviviruses, the replication and egress of DENV was regulated by the UPS (Choy et al., 2015). In addition, 20 ubiquitination-related proteins that may be involved in WNV infection were identified in a genome-wide RNA interference (RNAi) screen. CBLL1, one of the identified ubiquitination-related proteins, had been determined to be involved in the cellular internalization of WNV. Furthermore, depletion of the free ubiquitin pool by the proteasome inhibitor MG132 was found to effectively block WNV entry (Krishnan et al., 2008). However, another report suggested a completely opposite conclusion, showing that the UPS did not participate in the internalization but enhanced the viral RNA translation and replication of WNV (Fernandez-Garcia et al., 2011). Either way, the potential roles of the UPS in JEV cellular internalization have yet to be investigated.

In our study, we demonstrated that JEV infection was impaired by proteasome inhibitors in HeLa and SH-SY5Y cells. Furthermore, viral entry appeared to require active ubiquitin-proteasome machinery. However, the inhibitors did not interfere with viral binding, penetration of the cellular membrane or initial translation of the viral genome. A time-frame experiment illustrated that the UPS was essential for the early stage of infection. It was also revealed that cellular trafficking of JEV particles was affected by the proteasome inhibitors. In addition, a proportion of the internalized virions were misdirected to the lysosome following treatment with a proteasome inhibitor, resulting in non-productive entry. We then focused on the role of ubiquitination in JEV infection. By knockdown of the expression of ubiquitin, ubiquitination was proved to be involved in JEV entry.

2. Materials and methods

2.1. Cell culture and virus

BHK-21 (baby hamster kidney cells), SH-SY5Y (human neuroblastoma cells) and HEK 293 T were grown in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT), and HeLa cells were cultured in minimum essential medium (MEM), both supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA); at 37 °C within 5% CO_2. The AT31 strain of JEV (kindly provided by T. Wakita, Tokyo Metropolitan Institute for Neuroscience) was propagated and titered by a plaque assay in BHK-21 cells. The pseudotype of vesicular stomatitis virus (VSVpp) was generated by a reverse genetics system, which was a gift from Hideki Tani (National Institute of Infectious Diseases, Japan) (Tani et al., 2010).

2.2. Reagents, antibodies and plasmids

MG132 (catalog no. S2619) was purchased from Selleck Chemicals. Lactacystin (catalog no. L6785) and bafilomycin A1 (catalog no. B1793) were obtained from Sigma-Aldrich. Mouse anti-E monoclonal antibody (catalog no. MAB8744) was purchased from Chemicon. Proteinase K (catalog no. V3021) was purchased from Promega. Mouse anti-ubiquitin (P4D1) monoclonal antibody was purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), rabbit anti-HA, and mouse anti-HA antibodies and the secondary antibody TRITC-conjugated goat anti-mouse IgG were purchased from Proteintech. Mouse and rabbit E-DIII antisera were obtained as described previously (Liu et al., 2015). Rabbit NS5 antiserum was kindly provided by C. J. Chen (Taichung Veterans General Hospital). pEGFP-LAMP1 and pCAGGS-CprME were obtained as described previously (Jin et al., 2013; Liu et al., 2015). An HA-tagged ubiquitin expression plasmid was kindly provided by Hanzhong Wang (Wuhan Institute of Virology, CAS, China). A JEV *Renilla* luciferase-reporting replicon, namely, SA14/U14163-Replicon, was kindly provided by Bo Zhang (Wuhan Institute of Virology, CAS, China).

2.3. Packaging of replicon recombinant viral particles (RVPs), replicon initial translation and luciferase assay

RVPs were generated as previously described (Liu et al., 2015). In brief, the SA14/U14163-Replicon cDNA plasmid was digested with XhoI (NEB, Beverly, MA) and purified using phenol-chloroform extraction. The linearized cDNAs were then subjected to *in vitro* transcription using a T7 mMessage mMachine kit (Ambion, Austin, TX). Next, 10 μg replicon RNA was electroporated into BHK-21 cells in a 4 mm electrophoresis cuvette at 850 V and 25 μF three times at 3-s intervals. After 24 h of incubation at 37 °C, pCAGGS-CprME was transfected into the electroporated cells using Lipofectamine 3000 reagent (Life Technologies, Grand Island, NY). The supernatants containing RVPs were collected after centrifugation at 48 h post-transfection. For replicon initial translation assay, BHK-21 cells were electroporated with 10 μg replicon RNA and 2 μg of mRNA containing a firefly luciferase gene for normalization of transfection. This control RNA was transcribed *in vitro* using the plasmid pTNT-Fluc which was generously provided by Dr. Mariano Garcia-Blanco of the Duke University Medical Center. The luciferase activities were measured at 2 h post-electroporation using the Dual-Luciferase Reporter assay kit (Promega, Madison, WI) according to the instructions. For the RVP infection assay, HeLa and SH-SY5Y cells in 24-well plates were infected with 100 μl RVPs (10^4 FFU) per well. After 24 h of incubation, the infected cells were lysed, and their luciferase activities were measured according to the instructions for the *Renilla* luciferase assay system.

2.4. Quantitative PCR (qPCR) and small interfering RNA (siRNA)

Total RNA was isolated using TRIzol reagent (Invitrogen, Grand Island, NY) according to the manufacturer's recommendations. The cDNAs from minus- and plus-strand genomic RNA were then reverse transcribed using the AT31 (9344 sense) and AT31 (9460 antisense) primers, respectively. Next, the generated cDNAs were amplified using Fast SYBR Green Master Mix (ABI, 4385612) and a quantitative real time-PCR system (ABI, StepOne). Following the amplification, a melting curve analysis was performed to confirm the specificity of the PCR products. The sequences of the primers for the qPCR were AT31 (9344 sense): 5′-AGCTTCTAGATGGTGAACACCGCA-3′ and AT31 (9460 antisense): 5′-TCACGTCCATCACGGTCTTTCCTT-3′. For RNAi, HeLa cells were grown to 30–50% confluence and then transfected with ubiquitin-siRNA using Lipofectamine RNAiMAX according to the manufacturer's instructions. The siRNAs were obtained from GenePharma. The oligonucleotides used were as follows: siRps27a-1 5′-CGUACUUUGUCUGACUACAAUdTdT-3′ and siRps27a-2 5′-GCACAAGAGAAAGAAGGUUAAdTdT-3′; siUba52-1 5′-CACCAACAACCUGCGUCCCAAdTdT-3′ and siUba52-2 5′-ACACCAUUGAGAAUGUCAAdTdT-3′ (Gatti et al., 2015). The sequence for the negative control siRNA was 5′-UUCUCCGAACGUGUCACGUdTdT-3′; this sequence was scrambled and lacked significant homology to the human genome.

2.5. Assays of viral binding and cell membrane penetration

HeLa cells were plated in a 24-well plate and incubated with JEV at MOI = 1 for 1 h at 4 °C. For the binding assay, the unattached viruses were washed away with cold PBS three times at 4 °C, and