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HSP70 Copurifies with Zika Virus Particles

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ABSTRACT

Zika virus (ZIKV) has been identified as a cause of neurologic diseases in infants and Guillain-Barré Syndrome, and currently, no therapeutics or vaccines are approved. In this study, we sought to identify potential host proteins interacting with ZIKV particles to gain better insights into viral infectivity. Viral particles were purified through density-gradient centrifugation and subsequently, size-exclusion chromatography (SEC). Mass spectrometric analyses revealed viral envelope protein and HSP70 to comigrate in only one SEC fraction. Neither of these proteins were found in any other SEC fractions. We then performed neutralization assays and found that incubating viral particles with antibody against HSP70 indeed significantly reduced viral infectivity, while HSC70 antibody did not. Preincubating cells with recombinant HSP70 also decreased viral infectivity. Knockdown and inhibition of HSP70 also significantly diminished viral production. These results implicate HSP70 in the pathogenesis of ZIKV and identify HSP70 as a potential host therapeutic target against ZIKV infection.

1. Introduction

Zika virus (ZIKV) is a mosquito-borne single-stranded positive-sense enveloped RNA virus from the *Flaviviridae* family of viruses (Hamel et al., 2015). ZIKV is a teratogenic virus responsible for intrauterine growth restriction, microcephaly, and congenital eye disease, sensorineural hearing loss and the Guillain-Barré syndrome, characterized by immune-mediated demyelination of peripheral nerves (Tutiven et al., 2017). Transmission of the virus to humans occurs through the mosquito vectors *Aedes aegypti* and *Aedes albopictus* (Ioos et al., 2014). ZIKV has recently been the cause of major epidemics in South American countries especially Brazil, and due to the widespread presence of *Aedes*, ZIKV has rapidly become a global health threat prompting the World Health Organization to declare ZIKV as "Public Health Emergency of International Concern (PHEIC)" (Heymann et al., 2016). Despite the subsequent lift of the emergency state on November 8th of 2016 and a lack of robust epidemiological data, reports of ZIKV infection have been increasing with more countries reporting infections (Bogoch et al., 2016; Healthmap, 2017; WHO, 2016).

ZIKV was first identified in Uganda in 1947 from rhesus monkeys through a network that monitored yellow fever (Dick, 1952; Dick et al., 1952). It was later identified in humans in 1952 in Uganda and the

Abbreviations: AHI, allosteric HSP70 inhibitor; DAA, direct acting antiviral; DGC, density-gradient centrifugation; HCV, hepatitis C virus; HSP, heat shock protein; MOI, multiplicity of infection; MTase, methyltransferase; NBD, nucleotide binding domain; NCR, non-coding region; NS, nonstructural; PBS, phosphate-buffered saline; RdRP, RNA-dependent RNA polymerase; SAM, S-adenosyl-L-methionine; SBD, substrate binding domain; SEC, size-exclusion chromatography; TFA, trifluoroacetic acid; ZIKV, Zika virus

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United Republic of Tanzania (Bearcroft, 1956; Macnamara, 1954). The mode of transmission was identified to be a sylvatic cycle consisting of non-human primates and *Aedes* mosquitoes and occasionally involving human host. The recent outbreaks of ZIKV may have been the result of the adaptation the virus to human host resulting in a neuropathic strain of the virus (Faye et al., 2014). There are two genotypes of ZIKV, the African strain and the Asian strain, and these genotypes share 96% sequence identity, while the African strain and Dengue virus share 56% of their sequences (Baronti et al., 2014; Haddow et al., 2012; Lanciotti et al., 2008). The recent epidemics in Micronesia, Brazil, and French Polynesia have been caused by the ZIKV Asian genotype through an *Aedes* vector-human-*Aedes* vector mode of transmission cycle.

ZIKV possesses a single-stranded positive-sense RNA genome which is approximately 10.8 kb in length (Kuno and Chang, 2007). The protein-coding region is flanked by the 5′- and 3′-non-coding regions (NCRs). The organization of viral proteins in the genome is similar to other flaviviruses consisting of 5′NCR-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-2K-NS4B-NS5-3′NCR. The coding region is translated into a single polypeptide that is co- and post-translationally cleaved through both host and viral proteases into at least 10 peptides. The capsid, membrane and envelope proteins, which are the structural proteins, constitute the structural components of the viral particle, and the remaining proteins are non-structural (NS) involved in viral genome replication and assembly. NS1 is involved in viral replication and late in infection as a homodimeric protein (Song et al., 2016). It is also secreted as a hexameric lipoprotein particle which is involved in immunomodulation. NS2A is potentially involved in viral assembly (Saiz et al., 2016). NS2B acts as a cofactor of the NS3 protein which together function as the viral protease responsible for cleavage of the viral polyprotein and required for viral replication (Badshah et al., 2017). NS4A and NS4B are linked to microcephaly and act by deregulating Akt-mTOR signaling in fetal neural stem cells thereby inhibiting neurogenesis and inducing autophagy (Liang et al., 2016). NS5, the largest and most conserved NS protein, consists of an N-terminal S-adenosyl-L-methionine (SAM)-dependent methyltransferase (MTase) and a C-terminal RNA-dependent RNA polymerase (RdRP) involved in genome RNA capping and replication, respectively (Zhang et al., 2016).

Chaperones play important roles in the life cycle of many viruses including the *Flaviviridae* (Khachatoorian and French, 2016). In particular, we and others have shown the heat shock protein (HSP) 70 family of chaperones to play important roles in the hepatitis C virus (HCV) life cycle. Furthermore, other groups have reported the involvement of HSP70 family in Dengue virus and Japanese Encephalitis virus infection (Chuang et al., 2015; Howe et al., 2016; Taguwa et al., 2015; Ye et al., 2013). Two major members of this family include HSP70 and its constitutively expressed homolog, heat shock cognate (HSC) protein 70 In order to develop effective therapies against or prevent ZIKV infection, it is important to obtain a better understanding of the ZIKV life cycle and the interaction of viral proteins with host proteins. In this study, we purified viral particles to identify potential comigrating host proteins to better understand ZIKV infectivity. Here, we present a short report of our initial findings on the importance of HSP70 in ZIKV production.

2. Materials and methods

2.1. Cell culture

Human A549 and simian Vero cells were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C in Dulbecco's Modified Eagle Medium (Mediatech, 10–013-CM) supplemented with 10% fetal bovine serum (Omega Scientific, FB-01) and 2 mM L-Gln (Life Technologies, 25030). Mosquito C6/36 cells were similarly maintained at 28 °C.

2.2. Infectious virus production

C6/36 cells were seeded in twenty 10 cm plates with 10 ml medium and allowed to reach confluency. Subsequently, the cells were infected with ZIKV stock at a multiplicity of infection (MOI) of 0.1 in 5 ml of medium, and infection was allowed to proceed for 10 days at which point all the supernatants were combined and concentrated 15 fold utilizing Amicon Ultra-15 Centrifugal Filter Units (Millipore, UFC910024). The concentrated virus was aliquoted and stored in −80 °C. Viral titer was determined by plaque assays in Vero cells as described below in virus production assay.

2.3. Viral assays

All infection assays were performed at six different MOIs, 10, 1, 0.1, 0.01, 0.001, and 0.0001. The MOI of 0.01 typically results in a number of plaques suitable for counting and statistical significance.

2.3.1. virus production

Cells were seeded in 48 well plates and allowed to reach confluency in 48 h. Subsequently, the medium was removed, and the cells were washed gently with warm PBS. Cells were infected by addition of viral dilutions in Opti-MEM medium. After 5 h, the cells were washed with warm PBS and fresh growth medium was added. After 48 h, viral plaques were counted.

2.3.2. Neutralization

Viral particles were incubated with the indicated agents overnight at 4 °C with gentle mixing. Subsequently, the mixtures were washed by utilizing Amicon Ultra-0.5 ml Centrifugal Filters (Millipore, UFC510096). The cleared viral solutions were used to infect cells as described above in the virus production assay.

2.3.3. Protein incubation

Naïve A549 cells were incubated with recombinant proteins purified as described below. After 5 h, the cells were washed gently with warm PBS and infected for 5 h. Subsequently, cells were washed with warm PBS and after 48 h, viral plaques were counted.

2.4. RNA interference

siRNAs against HSC70 (Santa Cruz Biotech, sc-29349) and HSP70 (Thermo Scientific, M-005168-01-0005) as well as Control siRNA-A (Santa Cruz Biotech, sc-37007) and siGENOME Non-Targeting siRNA Pool #2 (Thermo Scientific, D-001206-14-05) were transfected into A549 cells using Lipofectamine 2000 Transfection Reagent (Life Technologies, 11668-019) according to manufacturer's instructions. All knockdowns were performed as previously described (Khachatoorian et al., 2014).

2.5. Production and purification of recombinant proteins

Recombinant proteins were produced and purified as described previously (Khachatoorian et al., 2014).

2.6. Antibodies

HSC70 (Santa Cruz Biotech, sc-7298), HSP70 (Santa Cruz Biotech, sc-66048), mouse IgG (Santa Cruz Biotech, sc-2025), Flavivirus Group Antigen Antibody, clone D1–4G2–4–15 (Millipore, MAB10216), Zika virus Envelope (GeneTex, GTX133314) and calnexin (Santa Cruz Biotech, sc-23954).

2.7. Density-gradient centrifugation

This centrifugation was performed as described previously (Parent et al., 2009) with a few modifications as follows. A549 cells were seeded in ten 15 cm plates and allowed to reach confluency in 48 h. Subsequently the cells were infected at an MOI of 0.1, and infection was