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pH-dependent entry of chikungunya virus into *Aedes albopictus* cells

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ABSTRACT

Background: The chikungunya virus (CHIKV) recently caused explosive outbreaks in Indian Ocean islands and India. During these episodes, the virus was mainly spread to humans through the bite of the mosquito *Aedes albopictus*. Concomitantly to the description of symptoms of an unexpected severity in infants and elderly patients, a viral genome microevolution has been highlighted, in particular consisting in the acquisition of an A226V mutation in the gene encoding envelope glycoprotein E1, which was later found to confer an increased fitness for *A. albopictus*. We previously decrypted the entry pathway used by CHIKV to infect human epithelial cells and showed that these mechanisms are modulated by the E1-A226V mutation. In this report we investigated the conditions for CHIKV entry into mosquito cells and we assessed the consequence of E1 gene mutation on these parameters.

Principal findings: Our main findings indicate that CHIKV infection of *A. albopictus* cell lines is sensitive to Bafilomycin A1 and chloroquine and to membrane cholesterol depletion.

The E1-226V mutated LR-OPY1 isolate collected during the 2005 outbreak in La Réunion replicated more efficiently than the 37997 African reference strain in C6/36 cells. Moreover, the LR-OPY1 strain displayed greater membrane cholesterol dependence and was more sensitive to inhibition of endosomal pH acidification. Finally, using electron microscopy, we imaged CHIKV entry into C6/36 cells.

Conclusions: Our data support that CHIKV is endocyted into *A. albopictus* cells and requires membrane cholesterol as well as a low-pH environment for entry. These features are modulated in some extent by the A226V mutation in the E1 gene of the LR-OPY1 isolate. Altogether, our data provide information regarding the pathways used by CHIKV to infect *A. albopictus* cells.

1. Introduction

Chikungunya virus (CHIKV) is an *Alphavirus* transmitted to humans by blood-sucking mosquitoes. To ensure its ecological cycle, CHIKV replicates efficiently in both insects and vertebrate hosts. While CHIKV infection is highly cytopathic for mammalian cells (Ozden et al., 2007) and causes an acute febrile illness associated with severe, often debilitating, polyarthralgias in humans (Borgherini et al., 2007; Rulli et al., 2007), *Alphaviruses* replicate in mosquito cells without evident deleterious consequence and results in life-long persistent infection of insect vectors (Karpf and Brown, 1998). Accordingly, CHIKV-host interactions may significantly differ in those two models.

Until recently, CHIKV episodes were restricted to diffuse epidemics in Africa where it was propagated by a variety of mosquitoes from the *Aedes* family, including *Aedes furcifer*, *Aedes luteocephalus*, *Aedes taylori*, *Aedes africanus* (Diallo et al., 1999; Jupp and Kemp, 1996). Then, the virus was imported in Thailand and India where it has became an urban disease, transmitted largely by *A. aegypti* mosquitoes (for review, see (Chevillon et al., 2008)). During the past decade, important epidemiological outbreaks were reported, first in 2004, when CHIKV re-emerged in Kenya (Sergon et al., 2008) and subsequently in 2005, when the virus spread eastward, causing millions of disease cases throughout countries in and around the Indian Ocean, notably in the island of La Reunion and other neighboring islands of Seychelles, Madagascar, Mauritius and Mayotte (Beltrame et al., 2007; Chastel, 2005). Nowadays, Chikungunya virus continues spreading in India and Southeast Asia countries, including Indonesia, Malaysia, Thailand and Singapore (Pialoux et al., 2007; Ravi, 2006). A highlight of these recent episodes is that the virus is mainly vectored to humans by *Aedes albopictus* mosquitoes. In 2007, a smaller outbreak of Chikungunya disease developed in the Northern Eastern part of Italy, where the local transmission has been made possible by the enormous population of *A. albopictus* and the presence of a viremic patient coming from the Indian Ocean area (Rezza et al., 2007). Importation of CHIKV into Europe, with recent autochthonous cases in France (Gould et al., 2010), indicates that the virus is moving to

Abbreviations: CHIKV, chikungunya virus; SINV, sindbis virus; a.a., amino-acid; ECSA, East Central/South Africa.

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novel ecological niches and that Chikungunya infection may become a threat for population of temperate areas.

Molecular epidemiological studies revealed that CHIKV genomes circulating at different time points both in the Indian Ocean and in Asia belong to the same lineage and group of viruses namely the Eastern-Central-South African (ECSA) phylogroup (Schuffenecker et al., 2006). Evolutionary analysis pointed the emergence of a strain having an alanine to valine substitution at codon 226 (A226V) of the envelope 1 (E1) gene in Reunion island (Schuffenecker et al., 2006) and India (Kumar et al., 2008). This mutation was considered as a key evolutionary event that contributed to the transmission and spatial distribution of CHIKV in these regions. Indeed, E1-A226V enhances infectivity of CHIKV for *A. albopictus* and not *A. aegypti* mosquitoes (Tsetsarkin et al., 2007; Vazeille et al., 2007), thereby increasing the transmissibility of CHIKV by *A. albopictus*. Since infectivity of CHIKV pseudotypes was equally enhanced by the presence of this mutation, it was proposed to have a direct effect in the entry process, resulting in differences in cellular tropism (Salvador et al., 2009). These observations have important implications for the design of vector control strategies to fight against the virus in regions at risk of chikungunya fever.

The general picture for the entry of *Alphaviruses* into target cells involves binding of the viral E2 envelope glycoprotein to unknown cell surface receptor(s) and endocytosis of the virus-receptor complex into endosomes (for review see (Strauss and Strauss, 1994)). In endocytic organelles, the low pH environment acts as a cue to induce E1 glycoprotein structural rearrangements, to activate the fusion of the viral and intracytoplasmic host cell membranes and subsequent penetration of the viral genome into target cell cytoplasm (White et al., 1980). We previously evidenced that CHIKV uses a dynamin-dependent endocytic route and requires low endosomal pH and membrane cholesterol for entry into human cells (Bernard et al., 2010). At this time, the cellular pathways hijacked by CHIKV to infect mosquito cells remained largely unravelled. The present study was thus designed to provide some light on CHIKV entry mechanism in vector's cells. To achieve this goal, we took advantage of ultrastructural electron microscopy imaging and of endocytosis perturbators. We also focused on particular aspects that retained attention in the context of mosquito cells infection by *Alphaviruses*, specifically the requirement for low endosomal pH, that was extensively debated in the literature (Hernandez et al., 2001; Hunt et al., 2011; Paredes et al., 2004). This question was addressed through the use of chloroquine, a weak base, and Bafilomycin A1, a v-ATPase inhibitor. Since the E1-226V mutation confers an increased fitness to CHIKV in *A. albopictus* cells, we finally investigated the influence of the recent genome microevolution detected during the Reunion island and India outbreaks on entry routes used by CHIKV (Salvador et al., 2009; Tsetsarkin et al., 2007; Vazeille et al., 2007). We found that exposure to a low-pH environment is required for productive infection of *A. albopictus* cell cultures. Here, we confirm that the E1-A226V mutation present in the LR-OPY1 isolate decreases dependence for membrane cholesterol, Moreover, this mutation was associated with an increased sensitivity to lysomotropic agents. The results accumulated in this study indicate that entry of CHIKV is mediated at least in part, by identical routes in insect and human hosts. These pathways are modulated in some extent by recent genomic microevolution in E1 envelope glycoprotein gene.

2. Materials and methods

2.1. Cell lines and cultivation

The C6/36 mosquito cell line, derived from *A. albopictus*, was grown at 28 °C and 5% CO_2 in Minimal Essential Medium (MEM) (Sigma) complemented with 10% inactivated FCS and 1% antibiotics.

2.2. Production of viral stocks and titration

Full length green fluorescent protein (GFP)-expressing CHIKV subgenomic clones pCHIKic4 (37997 viruses), pCHIK-LRic (LR-OPY1 viruses) and pCHIK-LRic-226A (LR-OPY1$_{V226A}$ viruses) were kindly provided by S. Higgs (UTMB, Galveston) (Tsetsarkin et al., 2006, 2007). Each infectious clone was transcribed *in vitro* from the SP6 promoter using the mMESSAGE mMACHINE kit (Ambion) according to manufacturer's instructions. RNA was then electroporated into BHK-21 cells derived from hamster kidney fibroblasts (ATCC# CCL-10™). Briefly, 5.10^6 cells were washed in ice cold Phosphate Buffer Salin (PBS) and then electroporated with 0.5 μg of RNA with 2 pulses at 1.5 kV, 25 μF and αΩ. After two days, cell culture supernatant was harvested, filtered onto 0.22 μm filters, aliquoted and stored at −80 °C. Viral stocks were tittered using plaque assay formation performed on Vero cells, as previously reported (Bernard et al., 2010).

2.3. Virus entry assays and drug treatments

Cholesterol depletion was performed by cultivation of the cells for five passages into the appropriate medium supplemented with 10% cholesterol-depleted FCS. Serum depletion in cholesterol was obtained by incubating FCS with 2% CAB-O-Sil (Acros Organics) for 12 h at room temperature as previously described (Weinstein, 1979). Inhibition of endosome acidification was reached through the use of chloroquine and Bafilomycin A1 at concentrations of drugs previously determined to be efficient and without side effect on cell viability. For controls, the cells were treated with the corresponding amount of appropriate dilution solvent. For infection assays, the cells (3.10^5) were seeded into 24 well plates 24 h before infection. Then, the cells were incubated with drugs as indicated and challenged with CHIKV (m.o.i. of 5) as specified in the Section 3. Infection level was monitored after an additional 16 h in culture, except when specified in the text.

2.4. Flow cytometry

Detection of CHIKV infected cells was performed by flow cytometry analysis. Twenty-four hours after the viral challenge, the cells were extensively washed, trypsinized and fixed with a 4% paraformaldehyde solution for 20 min at 4 °C. After additional washings, the cells were resuspended in PBS and GFP expression was monitored directly by flow cytometry. Fluorescence intensity was recorded (20,000 events) on a COULTER EPICS XL flow cytometer (Beckman Coulter). Student *t*-tests, calculated using the VassarStats Website (http://faculty.vassar.edu/lowry/VassarStats.html), were used to determine differences between infection levels and were.

2.5. Electron microscopy

Cells were allowed to bind viruses for 30 min at 4 °C, and then shifted to 28 °C for 10 min to promote viral entry. Then, the cells were processed for thin-layer electron microscopy as described previously (Brun et al., 2008). Briefly, the cells were fixed for 1 h at 4 °C in a solution containing 2.5% glutaraldehyde in 0.1 M cacodylate pH 7.4. The cells were then rinsed three times in cacodylate buffer and post-fixed with 1% OsO_4. After an additional washing, the cells were incubated for 30 min in 0.5% tannic acid. Dehydratation was obtained with a graded series of ethanol solutions (from 25 to 100%) before embedding in Epok resin at 60 °C for 48 h. Ultrathin sections were cut on a Reichert OMU2 microtome and then examined under a Hitachi H7100 transmission electron microscope.