Chapter 5

Dynamics of Chikungunya Virus Cell Entry Unraveled by Single Virus Tracking in Living Cells

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Abstract

Chikungunya virus (CHIKV) is a rapidly emerging mosquito-borne human pathogen causing major outbreaks in Africa, Asia and within the Americas. The cell entry pathway hijacked by CHIKV to infect a cell has been studied before using cell entry inhibitor molecules. The majority of studies conclude that CHIKV enters cells via clathrin-mediated endocytosis. Other studies however show that CHIKV enters cells independently of clathrin. Here, we applied live-cell microscopy and monitored the cell entry behaviour of single CHIKV particles in living cells transfected with fluorescent marker proteins. This approach allowed us to receive a detailed insight in the dynamic events that occur during CHIKV cell entry. We observed that almost all particles fused within 20 minutes post-addition to the cells. The vast majority of particles that fused first co-localized with clathrin. The time from initial co-localization with clathrin till the moment of membrane fusion was on average 1.7 minutes, highlighting the fast nature of the cell entry process of CHIKV. Furthermore, these results also show that the virus spends a profound time searching for a receptor. Membrane fusion was predominantly observed from within Rab5-positive endosomes and often occurred within 40 seconds post-delivery to endosomes. Furthermore, we confirmed that a valine at position E1-226 enhances the cholesterol-dependent membrane fusion properties of CHIKV. To conclude, our work confirms that CHIKV cell entry occurs via clathrin-mediated endocytosis and shows that fusion occurs from within acidic early endosomes.

Importance

Since its re-emergence in 2004 chikungunya (CHIKV) has rapidly spread around the world, leading to millions of infections. CHIKV often causes chikungunya fever, a self-limiting febrile illness with severe arthralgia. Currently, there is no vaccine or specific antiviral treatment available against CHIKV. A potential antiviral strategy is to interfere with the cell entry process of the virus. However, contradicting results were published with regard to the cell entry pathway used by CHIKV. Here, we applied a novel technology to visualize the entry behaviour of single CHIKV particles in living cells. Our results show that CHIKV cell entry is extremely rapid and occurs via clathrin-mediated endocytosis. Membrane fusion is seen from within acidic early endosomes. Furthermore, the membrane fusion capacity of CHIKV is strongly promoted by cholesterol in the target membrane. Taken together, this study provides an exquisite insight in the cell entry process of CHIKV.
Introduction

Chikungunya virus (CHIKV) is a human arboviral pathogen, which was first isolated from a febrile patient in East Africa in 1952 [1]. Since then, numerous small CHIKV outbreaks were reported in Africa and Asia at irregular intervals. In 2004, the virus re-emerged and spread rapidly around the world [1,2]. At the end of 2013, the first autochthonous case of CHIKV was reported in the Americas [3]. Within 1.5 year, the virus has spread over 44 countries within Central America and caused more than one million infections [3]. CHIKV often leads to Chikungunya fever (CHIKF) which is characterized by high fever, headache, overall weakness and joint pain [4]. CHIKF is mostly self-limiting yet symptoms can be severe and disabling; up to 80% of the patients experience recurrent joint pains for months to years after infection [5–7]. There is no vaccine and no specific antiviral treatment available to prevent or treat infection [2,4].

CHIKV is an alphavirus, belonging to the *Togaviridae* family, which also includes Semliki Forest Virus (SFV), Sindbis virus (SINV), Ross River virus (RRV), and Venezuelan equine encephalitis virus (VEEV). Alphavirus cell entry and membrane fusion is facilitated by the viral glycoproteins E1 and E2. Of these proteins, E2 is responsible for receptor binding and E1 facilitates the low-pH dependent membrane fusion process [8,9]. Multiple receptors have been identified that facilitate SFV, SINV, RRV and VEEV cell entry but none of these receptors appear to be crucial [10–16]. The identified receptors predominantly act as attachment factors to capture the virus. Upon virus-receptor interaction, the virus is internalized via clathrin-mediated endocytosis (CME) [9,17,18]. After internalization the virus is transported to Rab5-positive early endosomes where membrane fusion predominantly occurs [9,19,20]. For VEEV, however, infection of mosquito cells was described to also depend on Rab7-positive late endosomes [18,21]. In addition, liposomal membrane fusion studies showed that besides low pH also target membrane cholesterol and sphingomyelin are required for SFV and SINV fusion [22–25].

Whereas the cell entry pathway of SFV, SINV, and VEEV is well studied, relatively little data is published on CHIKV cell entry. To date, prohibitin, phosphatidylserine-mediated virus entry-enhancing receptors, and glycosaminoglycans have been described to function as receptors for CHIKV but infection can also occur in the absence of these proteins [26]. Thus, also for CHIKV receptors mainly act to facilitate the initial virus-cell contact, and based on the literature it is conceivable that alphaviruses do not require a specific receptor for cell entry. Controversial reports have been published on the route of cell entry. Initially, dynamin was found to be important for CHIKV cell entry. Dynamin is important in numerous cell entry pathways like clathrin-mediated endocytosis, caveolar endocytosis [20], and phagocytosis [27]. CHIKV infection was also found to depend on EPS15 [28], a mediator of both clathrin-dependent [29] and clathrin-independent cell entry pathways [30]. Specific inhibitors like siRNAs against the clathrin heavy chain did not
interfere with CHIKV infection in HEK239T cells [28], which suggests that CHIKV
infects cells via a clathrin-independent pathway. On the other hand, however, siRNAs
against clathrin did inhibit CHIKV infection in human umbilical vein endothelial
cells (HUVEC), U-2 OS cell, primary human umbilical vein endothelial cells [31]
and the mosquito cell line C6/36 [32]. Together, this indicates that CHIKV cell entry
mainly occurs via CME.

The intracellular trafficking behavior after CHIKV entry is poorly
understood although few reports describe the function of early and late endosomes
within infection. For example, Bernard et al. found that integrity of Rab7-positive
endosomes is not required for CHIKV infection of HEK293T cells [28], which
suggests that CHIKV fuses from within early endosomes. In the mosquito cell line
C6/36, however, CHIKV infection was dependent on both Rab5 and Rab7-positive
endosomes [32], indicating that fusion might also occur from Rab5/Rab7-positive
maturing endosomes and Rab7-positive late endosomes. Furthermore, and in line
with other alphaviruses, low-pH-dependent CHIKV fusion is strongly promoted by
target membrane cholesterol and sphingomyelin [33,34].

Here, we dissected the cell entry pathway of CHIKV and elucidated the
dynamics involved in virus-cell binding, internalization, trafficking to endosomes,
and membrane fusion. Also, the site of membrane fusion is unraveled. Cell entry
of CHIKV was visualized by single particle tracking of fluorescently labeled virions
in living cells expressing fluorescent marker proteins. This approach allowed us to
receive an exquisite insight in the dynamic virus-host interactions that occur until
the moment of membrane fusion.

Results

Characteristics of DiD-labeled chikungunya virus

To visualize the dynamics involved in CHIKV cell entry and membrane
fusion, we labeled the virus with the lipophilic fluorescent probe DiD. This probe was
chosen because of its self-quenching properties at high surface density, as previously
described [36]. DiD was incorporated into CHIKV particles such that its fluorescence
was largely quenched but still allowed detection of single virus particles. Membrane
fusion is measured as a sudden increase in fluorescence intensity due to dilution of
the probe in the target cell membrane.

For this study, the well-characterized synthetic CHIKV strain LS3 was used
[35]. The amino acid sequence of LS3 E1 and E2 is identical to that of the clinically
relevant LR2006-OPY1 CHIKV strain [35], and therefore it is expected that these
viruses will have the same cell entry behavior. The probe DiD was added at a
concentration of 2 nmol per 2 × 10^{11} virus particles. At this ratio, a uniformly labeled
virus preparation was seen (Fig. 1A). Fig. 1B shows the total fluorescence intensity
of individual CHIKV particles derived from three distinct labeling procedures.
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Approximately 80% of the spots had a fluorescence intensity below 40 arbitrary units. Next, the labeling efficiency was assessed. To this end, the number of DiD-labeled particles were counted in 25 random image areas. In parallel, qPCR was used to determine the GCP titer. In line with our previous work on DENV, approximately 2% of the total number of particles present in solution are visualized under the conditions of the experiment (based on three individual experiments) [36]. Thereafter, viral infectivity was calculated by determining the number of GCPs divided by PFUs in five independent experiments. The GCP/PFU-ratio was 579 [95% CI: 237;920] for unlabeled virus and 1156 [95% CI: 467;1845] for DiD-labeled virus (Fig. 1C). Although the GCP/PFU-ratio increased two-fold upon DiD labeling, the increase was not statistically significant suggesting that the overall infectivity of the virus preparations is comparable. Taken together, at these labeling conditions, a uniform labeling is achieved that visualizes 2% of the total number of CHIKV particles present in solution. For further experiments, only particles with a fluorescence intensity lower than 40 a.u. were used as these likely represent single virus particles.

Rapid chikungunya virus cell entry and fusion in BS-C-1 cells

The cell entry behavior of CHIKV was studied in the kidney epithelial cell line BS-C-1, as epithelial cells are thought to contribute to viremia during natural infection [1,39]. Indeed, CHIKV infection of BS-C-1 cells at an MOI of 1 resulted in $2.6 \times 10^6$ PFUs/ml progeny virions at 24 h p.i., demonstrating that these cells are permissive to CHIKV. Moreover, the BS-C-1 cells are relatively flat which allows us to capture complete viral trajectories from virus-cell binding until the moment of membrane fusion. Fig. 2A (no treatment) shows a cell entry trajectory of a single CHIKV particle. Initially, the DiD signal is low but constant over time. At 1044 s p.i., a sudden and dramatic increase in fluorescence is seen which is indicative for the moment of membrane fusion (Fig. 2A & 2B – no treatment). After fusion the DiD-
signal remains high for a profound period of time (up to 30 min); the DiD-labeled compartment being highly dynamic and DiD-labeled structures frequently pinch off to be transported elsewhere, indicating the highly dynamic nature of endocytic vesicles. A movie showing the DiD signal over time till the moment of membrane fusion is added as supplemental data (Movie S1).

To ensure that the increase in fluorescence indeed reflects membrane fusion, DiD-labeled CHIKV particles were added to BS-C-1 cells treated with NH₄Cl. NH₄Cl is known to neutralize the endosomal pH thereby inhibiting the membrane fusion capacity of CHIKV [28,40,41]. Indeed, the infectivity of CHIKV was more than 4 logs reduced in the presence of NH₄Cl when compared to the positive control (5.1×10² PFU/ml for NH₄Cl-treated cells versus 1.2 × 10⁷ PFU/ml for non-treated cells at an MOI of ~5, respectively). In 4 independent tracking experiments, a total of 42 single virus trajectories were recorded in NH₄Cl-treated cells. These cells showed no toxicity.
as measured by a MTT assay (Fig. S2A) and endosomal acidification was efficiently blocked (Fig. S3A). As expected, no changes in fluorescence intensity were seen. An example of a trajectory is given in Fig. 2A and the fluorescence intensity of one representative CHIKV-DiD particle in a NH₄Cl-treated cell is plotted in Fig. 2B (NH₄Cl).

As a second control, we inactivated the membrane fusion properties of CHIKV through DEPC treatment. DEPC treatment of viral particles is known to covalently modify histidines on viral glycoproteins, thereby abolishing viral fusion without changing the protein structure [42,43]. DiD-labeled CHIKV was treated with 2 mM DEPC for 30 min at room temperature. Direct titration of DEPC-treated CHIKV by plaque assay revealed that the virus was completely non-infectious. In subsequent tracking experiments, 8 DEPC-treated single CHIKV-DiD particles were recorded in 3 independent experiments. Yet again, the fluorescence intensity remained constant for the duration of the experiment. An example is shown in Fig. 2A and 2B (DEPC-treated). Together these results indicate that a sudden dramatic increase in fluorescence indeed reflects the moment of membrane fusion.

Next, the fusion kinetics of 113 single CHIKV particles recorded in 93 individual single particle tracking experiments were analyzed. We found that CHIKV cell entry is a very rapid process, with the first fusion events occurring within 2 min p.i.. Half of all investigated fusion events occurred within the first 9 min after infection and more than 95% of all fusion events occurred within 22 min p.i. (Fig. 2C).

**Chikungunya entry occurs via clathrin-mediated endocytosis**

We next aimed to receive a more detailed insight in the dynamic virus-host interactions that occur during virus entry. Alphaviruses are generally considered to enter cells via CME [9,17,18]. For CHIKV, however, conflicting reports were published and therefore we first investigated whether CHIKV cell entry is indeed mediated by clathrin. For this purpose, CME was perturbed with two widely used small compound inhibitors: chlorpromazine and Pitstop2. For both compounds, cytotoxicity was tested using the MTT assay and transferrin was used to confirm whether the compound was biologically active [44,45]. Unfortunately, chlorpromazine was found to be toxic at concentrations higher than 30 µM (Fig. S2B). At 30 µM chlorpromazine, only a minor reduction (30%) in transferrin uptake was seen (Fig. S3B) and therefore this compound was unsuitable for further use. In contrast, transferrin uptake was almost completely abolished (>95%) at a concentration of 25 µM Pitstop2 (Fig. S3C) and no signs of cytotoxicity were seen (Fig. S2C & S2D).

BS-C-1 cells were pretreated with Pitstop2 and infection was allowed for 18 h in the presence of the inhibitor. Indeed, a significant drop in infection was found upon treatment of the cells with 25 µM Pitstop2 (Fig. 3A). Yet, since residual infectivity was seen we next employed a more direct microscopic fusion assay using DiD-labeled CHIKV. If viral entry is affected by Pitstop2, a decrease in membrane fusion activity should be detected. DiD-labeled CHIKV was allowed to enter and fuse within cells for 30 min, after which unbound particles were washed away. Next, random microscopic
Figure 3. CHIKV infects cells via clathrin-mediated endocytosis.

(A) Flow cytometry analysis of CHIKV infection in the presence of Pitstop2. BS-C-1 cells were pre-incubated with Pitstop2 for 15 min, after which cells were infected with CHIKV LS3-GFP (MOI 1) and incubated for an additional 16 h. Data represents 2 individual experiments in triplicate, with a P-value of 0.0277 (25 µM). Error bars represent standard error of the mean (SEM).

(B) CHIKV cell entry and membrane fusion in the presence of inhibitors. The percentage of fusion is normalized to a non-treated positive control (p.c.). P-values are 0.0004 (Pitstop2), 0.0002 (NH₄Cl) and <0.0001 (DEPC). Data is from at least three individual experiments. Error bars represent SEM.

(C) Flow cytometry analysis of CHIKV infection in anti-CHC siRNA transfected HeLa cells. Cells were transfected with anti-CHC siRNAs, incubated for 72 h and infected with CHIKV LS3-GFP (MOI 5). The percentage of infection...
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images were taken and the fluorescence intensity was quantified. First, the fusion assay was validated using NH\textsubscript{4}Cl-treated cells and DEPC-treated virus as described above. **Fig. S4** shows representative image of both treatment conditions and the non-treated virus control. Quantification of the total fluorescence intensity is depicted in **Fig. 3B**. Indeed, NH\textsubscript{4}Cl-treatment severely hampered the membrane fusion capacity of the virus. Furthermore, almost no fusion was observed using DEPC-treated virus. Importantly, a dramatic inhibition of CHIKV fusion was seen in Pitstop2-treated cells (**Fig. 3B**), which suggests that CHIKV indeed enters BS-C-1 cells via CME. To further confirm CHIKV cell entry via CME, we tried to knock-down the clathrin heavy chain (CHC) using siRNAs. BS-C-1 cells are however difficult to transfect at high efficiency and although we tried multiple transfection conditions and agents we failed to efficiently inhibit transferrin uptake in BS-C-1 cells (example given in **Fig. S3D**). We then used HeLa cells, cells that are easy to transfect and permissive for CHIKV [39]. Indeed, transferrin uptake was efficiently inhibited in anti-CHC siRNA transfected HeLa cells (**Fig. S3E**). Next, CHIKV infectivity was assessed in CHC siRNA-transfected HeLa cells by flow cytometry. The number of infected cells was reduced up to 90% when compared to cells transfected with a non-targeting siRNA control (**Fig. 3C**). We also attempted to use HEK293T cells but despite multiple efforts these cells de-attached from the plate upon transfection thereby preventing further experiments. Taken together, the above results show that CHIKV enters BS-C-1 and Hela cells via CME.

To reveal the dynamics of CHIKV entry via CME we next transfected BS-C-1 cells with clathrin-YFP and simultaneously tracked the clathrin and CHIKV-DiD signal in living cells. The vast majority of particles (17 of 19 fusion-positive CHIKV particles) colocalized to clathrin prior to fusion. An example of CME of CHIKV is shown in the filmstrip in **Fig. 3D**. Furthermore, 2 movies are added to the supplementary data (**Movies S2A & S2B**). The average duration of colocalization between CHIKV and clathrin was 48.1 s (SEM ± 10.6, n=15; 2 particles were excluded as the duration of colocalization could not be estimated) (**Fig. 3E**), which is in line with kinetics of clathrin-coated pit formation and internalization as described by Schelhaas et al. [46]. We also determined the time between the disappearance of the clathrin signal and the moment of fusion. On average fusion occurred 51.9 s after clathrin colocalization (SEM ± 13.0, n=16; 1 particle was excluded as the exact time of clathrin disappearance could not be determined). Moreover, approximately 95% of the fusion events occurred within 90 s after colocalization, which indicates that CHIKV fuses rapidly after entry (**Fig. 3F**).
Based upon the kinetics described above, it can be estimated that CHIKV entry (from initial clathrin co-localization till membrane fusion) is completed within roughly 1 min and 40 s. When solely assessing the fusion kinetics, it was found that approximately 50% of all fusion events occurred within the first 9 min p.i. (Fig. 2C). The large difference between these time points indicates that most CHIKV particles spend a profound time searching for a cellular attachment factor.

**Chikungunya fusion mainly occurs from within early endosomes**

We next addressed whether CHIKV fuses from within early or late endosomal compartments. First, we tracked DiD-labeled CHIKV particles in BS-C-1 cells prior transfected with Rab5-GFP, a marker for early endosomes. Fig. 4A shows a filmstrip of a single CHIKV particle fusing from within a Rab5-positive compartment. At 98 and 118 s p.i. the virus is seen in close proximity with Rab5-positive structures, however no colocalization is seen. Colocalization is visible at 135 s p.i. and continues till the moment of membrane fusion at 265 s p.i. Two movies showing a CHIKV fusion event in colocalization with Rab5 (Movies S3A and S3B) are available as supplementary data.

A total of 39 fusion events was recorded in Rab5-transfected cells. Of these fusion events 37 (95%) occurred in co-localization with Rab5 (Fig. 4B). Next, tracking was performed in cells transfected with Rab7-GFP, a specific marker for late endosomes. A total of 23 fusion events were recorded, of which only 4 (17%) were in colocalization with a Rab7-positive structure (Fig. 4B). Together, these results show that CHIKV predominantly fuses from within Rab5-positive early endosomes. The remaining particles fuse from within Rab5/Rab7-positive maturing endosomes or Rab7-positive late endosomes.

Further analysis of the CHIKV trajectories in Rab5-transfected cells revealed that CHIKV resided on average 37.6 s (SEM ± 7.8, n=30) in early endosomes before fusing. Interestingly, though, approximately 40% of all particles fused almost immediately (within 10 s) after colocalization (Fig. 4C). This finding might reflect the maturation state of the endosome at the time the virus is delivered, as the pH gradually drops during endosomal maturation [47,48].

To further confirm that fusion occurs from within early endosomes, we assayed the number of infected cells upon transfection with the dominant negative mutant Rab5-S34N. Rab5-S34N caused a significant reduction (25%) in infection compared to the wild-type control (Fig. 4D, left panel), yet this reduction was not as pronounced as expected based on our single particle tracking results. This might again be related to the overall low transfection efficiency and thus expression of the Rab5 constructs in BS-C-1 cells. Indeed, if we arbitrarily changed the gating to only include cells with a high GFP expression (Fig. S5A and S5B), the inhibition of infection was more pronounced (up to 49%, Fig. S5C). To further proof that fusion occurs from within Rab5-positive early endosomes we also assessed the effect in HeLa cells. As expected, the effect of Rab5-S34N was more pronounced in HeLa cells than in BS-C-1 cells (Fig. 4D).
Figure 4. CHIKV interacts with Rab5-positive endosomes at the time of fusion.
(A) Time series of a cell expressing Rab5-GFP (green) upon infection with DiD-labeled CHIKV particles (red). Arrows indicate the position of the virus in each panel. Scale bar represents 2 µm. (B) Bar diagram showing the percentage of fusion events in Rab5-positive and Rab7-positive structures. In total, 39 fusion events were analyzed in Rab5-GFP expressing cells, and 23 fusion events in Rab7-GFP expressing cells. (C) Dot plot demonstrating how long CHIKV resides in Rab5-positive organelles prior to fusion. A total of 30 individual fusion events were analyzed and each dot represents 1 fusion event. (D) CHIKV infectivity in cells expressing wt-Rab5-GFP or dominant-negative Rab5-GFP. Infection was quantified and normalized to the wt-Rab5-GFP control. For both cell lines two individual experiments were carried out in triplicate. P-values are 0.0160 (BS-C-1) and < 0.0001 (HeLa). Error bars represent SEM. (E) pH-dependent membrane fusion properties of LS3 at as determined by a bulk fusion assay. At least 3 measurements were performed per pH-value. Error bars represent the standard deviation (SD).
The pH values within early endosomes typically range from pH 6.8 till 5.5 [48,49]. Earlier reports showed that the pH threshold for fusion is strain-specific [34] yet all strains reported thus far fused at early endosomal pH values. To confirm that CHIKV-LS3 is also able to fuse at an early endosomal pH, we assessed the pH-dependent membrane fusion properties of CHIKV-LS3 by use of a bulk fusion assay [24,25,34]. Here, pyrene-labeled CHIKV is mixed with liposomes consisting of PC/PE/SPM/Chol and fusion is triggered by the addition of a low pH-buffer. Upon fusion, the pyrene phospholipids are diluted into the liposomes resulting in a decrease in fluorescence intensity, which can be monitored continuously. Fig. 4E shows the total fusion extent as function of the pH. No fusion was seen at a neutral pH (7.4). CHIKV fusion was first observed at pH 5.9 and maximal fusion was seen at pH values lower than pH 5.5. Thus, the threshold of CHIKV fusion was pH 5.9, which indicates that CHIKV-LS3 fusion can indeed occur from within early endosomes.

A valine at position 226 in the E1 protein increases the cholesterol dependency of CHIKV fusion

As cholesterol has been found to be important in alphavirus fusion and infection [24,25,50,51], we subsequently determined the effect of cholesterol on CHIKV cell entry and fusion. The E1-226 residue is an important determinant of cholesterol dependency of alphavirus infection [52,53]. For CHIKV, an increased cholesterol dependence in infection was seen once E1 had a valine residue instead of an alanine residue at position 226 [28,54]. To study the effect of the E1-A226V mutation on membrane fusion, we mutated the infectious clone of LS3 (E1-226V) to create a virus with an alanine at this position (LS3-226A). Both strains exhibited similar growth kinetics in Vero cells (data not shown). The membrane fusion activity of both strains was first evaluated in the microscopic fusion assay with cells that were cholesterol-depleted with various concentrations of methyl-beta-cyclodextrin (7.5, 5, 2.5 mM). At these conditions, no cytotoxicity was seen in the MTT assay (Fig. S2E). At identical infection conditions, the fluorescence intensities measured for LS3-226A were 1.6 times higher than the intensities measured for LS3-226V, which suggests that LS3-226A fuses more efficiently with the host cell membrane than LS3-226V. Hence, all experimental conditions were normalized to the positive control (no inhibitor) of the corresponding virus strain. There was a significant difference (two-way ANOVA, P < 0.001) in cholesterol dependency between both LS3 strains. Whereas cholesterol depletion had no effect on LS3-226A entry and/or fusion, depletion of cholesterol greatly inhibited the entry and/or fusion extent of LS3-226V (Fig. 5A). Importantly, upon replenishment of membrane cholesterol the extent of LS3-226V fusion was restored to control levels (Fig. 5A).

To more specifically investigate whether cholesterol has an effect on membrane fusion we next employed the bulk fusion assay. Viral fusion was measured using liposomes with increasing concentrations of cholesterol. The bulk fusion assay revealed that the presence of cholesterol in the target membrane promotes the
membrane fusion capacity of both LS3 strains (Fig. 5B). Yet, LS3-226A fusion was also observed in the absence of cholesterol in the target membrane, indicating cholesterol is not strictly required for fusion. In contrast, LS3-226V did not fuse with liposomes when the cholesterol concentrations were ≤ 11 mol%. Together, these results confirm that the E1-A226V mutation indeed increases cholesterol dependency of CHIKV fusion.

**Figure 5. CHIKV infection and membrane fusion is promoted by target membrane cholesterol** (A) Effect of cholesterol depletion on cell entry and fusion of LS3-226A and LS3-226V. BS-C-1 cells were depleted from cholesterol using methyl-beta-cyclodextrin (7.5, 5, 2.5 mM, for 1h). Thereafter, the cells were infected with either LS3-226A or LS3-226V (MOI 20) and the extent of membrane fusion was measured at 30 min post-infection. As a control, cells were replenished with water soluble cholesterol (200 µg/ml, for 30 min) and infected. P= 0.0008, two-way ANOVA. Data are from at least 3 individual experiments. Error bars represent SEM. (B) Bulk fusion assay data showing the cholesterol-dependent membrane fusion properties of LS3-226A and LS3-226V. Open squares represent LS3-226A, whereas filled squares represent LS3-226V. At least 3 measurements per data-point were performed. Error bars represent SD.

**Discussion**

This paper describes the mechanistic and kinetic events that occur during CHIKV cell entry in BS-C-1 cells. We show that almost all particles that fused first co-localized with clathrin structures. The time of co-localization is consistent with the reported overall lifespan of clathrin-coated structures [46]. CHIKV particles predominantly fuse from within Rab5-positive early endosomes. The cell entry process of CHIKV is extremely fast as 50% of the particles fused within 1.7 minutes after co-localization with clathrin. Furthermore, 40% of the particles fused instantly upon delivery to acidic endosomes. Finally, we showed that a valine at position 226 in E1 increased CHIKV cholesterol dependency of fusion when compared to a virus with an alanine at this position.

The observation that CHIKV enters cells via CME is in line with two earlier reports on CHIKV cell entry [31,32]. In these studies a significant inhibition in viral
infectivity was seen in cells treated with drugs or siRNAs that have been proposed to interfere with CME. In contrast however, another study showed that siRNAs against the clathrin heavy chain did not interfere with infection and a clathrin-independent but EPS15-dependent CHIKV pathway was proposed [28]. This suggests that CHIKV entry is strain- and/or target cell-dependent. The receptor utilized by the virus to infect cells may be an important factor herein, especially as CHIKV has been proposed to interact with multiple receptors [55–58]. Another possibility is that CHIKV has the capacity to hijack multiple entry pathways. In this case, inhibitor studies may not identify the dominant cell entry pathway of the virus as under inhibiting conditions the virus will enter through another pathway. In BS-C-1 cells, residual infectivity was seen in cells treated with the CME inhibitor Pitstop-2 while transferrin uptake was abolished. Residual infectivity was also found in anti-CHC siRNA transfected HeLa cells, albeit to a lesser extent. In fact, in all of the above studies residual infectivity was seen, indicating that CHIKV indeed has the capacity to infect cells via multiple pathways. An advantage of the approach used in this study is that we were able to monitor CHIKV cell entry in the absence of inhibitors. The results presented in this paper clearly show that 89% of the particles that fused first co-localized with clathrin. Collectively, and in line with other alphaviruses, we conclude that CME is the major cell entry pathway of CHIKV in cells.

Almost all viruses that are internalized by the cell via endocytosis are first delivered to Rab5-positive early endosomes before being targeted to Rab5/Rab7-positive maturing endosomes, Rab7-positive late endosomes and lysosomes [20]. Previous studies on alphaviruses showed that SFV fused from within early endosomes and VEEV was found to fuse from maturing and/or late endosomes [18,21,59,60]. Our single particle tracking data shows that CHIKV predominantly fuses from within early endosomes. Approximately 40% of the particles fused within 10 s after co-localization with Rab5, demonstrating that CHIKV almost instantly fuses with the endosomal membrane. The time that CHIKV resides within endosomes is probably controlled by the pH of the endosomal lumen. Instant fusion may occur when the particles are delivered to endosomes were the pH is lower than the pH threshold for fusion. We show here that the pH threshold for CHIKV-LS3 fusion is 5.9 although strain-specific pH-dependent properties exist [34]. The short time that CHIKV resides in endosomes is in sharp contrast with dengue virus particles that spend minutes in late endosomal compartments prior to fusion. For dengue negatively charged lipids are required to complete the fusion process [61] and this dependency likely prevents fusion from within early endosomes. Our results are in line with an earlier study of Bernard et al. who showed that CHIKV infection in HEK-293T cells is dependent on the integrity of early endosomes, but not on late endosomes [28]. In mosquito cells both early and late endosomes are required for CHIKV infection, suggesting that in these cells CHIKV is trafficked to maturing/late endosomes before fusion [32]. This discrepancy may be related to the different virus strains used but could also be related to potential differences in the endosomal pH between cells [62].
To our knowledge, this is the first study that describes the kinetics of CHIKV entry at the single particle level. The first fusion events were detected 2 min p.i., and 50% of all fusion events observed occurred within 9 min p.i. This nicely corresponds with data described for SFV [63]. In the latter study, the authors added NH₄Cl at different time-points p.i. and assessed the number of infected cells by flow cytometry. Addition of NH₄Cl at 2-3 min p.i. did not prevent SFV infection and 50% of the total infection extent was observed if NH₄Cl was added at 6 min p.i., indicating that both SFV and CHIKV poses very rapid entry kinetics. Besides assessing the overall time to fusion, we here also assessed the kinetics of the major steps in CHIKV entry. Half of the particles fused within 1.7 minutes after co-localization with clathrin, demonstrating that virus particles spend a profound time searching for a receptor. This also shows that once the virus co-localizes with clathrin the entry process is extremely fast.

Lastly, we confirmed that position 226 in the CHIKV E1 protein is an important determinant of the cholesterol-dependent membrane fusion properties of the virus. The E1-A226V mutation was first reported in a clinical CHIKV strain during the 2005/2006 outbreak on La Réunion and was associated with CHIKV vector specificity [54,64], pH threshold of fusion in cell fusion assays [65,66] and the host immune response towards the virus [67,68]. Furthermore, the E1-A226V mutation was linked to increased cholesterol dependency of CHIKV infection [28,54,66]. We confirm that the E1-A226V mutation influences the pH threshold for fusion using a direct bulk fusion assay and demonstrate that higher concentrations of cholesterol are needed within the target membrane to facilitate efficient fusion. Our results are in agreement with reports published on other alphaviruses [25,50,53].

Studying the cell entry pathway at the single particle level is important as it provides detailed and quantitative information in the dynamic events that are involved in CHIKV cell entry. Furthermore, by measuring fusion instead of infection as a read out of CHIKV cell entry, we avoid artifacts of inhibitors exerting their effects not only during entry but also at later stages of infection. Therefore, this study provides a better understanding of the CHIKV cell entry process, and the entry of alphaviruses in general.

Materials and Methods

Cells

Green monkey kidney BS-C-1 cells (ATCC CCL-26) were maintained in DMEM (Gibco) supplemented with 10% FBS (Lonza), 25 mM HEPES, penicillin (100 U/ml), and streptomycin (100 U/ml). Green monkey kidney Vero-WHO (ATCC CCL-81) cells were cultured in DMEM (Gibco) supplemented with 5% FBS (Lonza), penicillin (100 U/ml), and streptomycin (100 U/ml). Baby hamster kidney cells (BHK-21, ATCC CCL-10) cells were cultured in RPMI (Gibco) supplemented
with 10% FBS (Lonza), penicillin (100 U/ml), and streptomycin (100 U/ml). Finally, human adenocarcinoma HeLa cells (ATCC CCL-2) were cultured in DMEM (Gibco) supplemented with 10% FBS (Lonza), penicillin (100 U/ml), and streptomycin (100 U/ml). All cells were maintained at 37 °C under 5% CO₂.

**Virus production, purification, and labeling**

The CHIKV strains Leiden Synthetic strain (LS3) and GFP-encoding reporter virus (LS3-GFP) were described previously [35]. CHIKV strain LS3-226A is identical to LS3 except it has an alanine at position 226 in the E1 protein instead of a valine. The LS3-226A clone was generated by quick-change mutagenesis and standard cloning techniques (details available upon request). Infectious virus was produced essentially as described by Scholte *et al.* [35] except that BHK-21 cells were transfected with RNA transcripts by electroporation with a Gene Pulser Xcell™ system (BioRad) with the settings 1.5 kV, 25 µF, and 200 Ω. At 24 h post-transfection, the medium was harvested and used to inoculate Vero-WHO cultures at an MOI of 0.01 to produce large CHIKV working stocks.

For the production of purified CHIKV preparations, monolayers of BHK-21 were inoculated with CHIKV LS3 at MOI 4. At 24 h post-infection (p.i.) supernatant was harvested and cleared from cell debris by low speed centrifugation. CHIKV particles were subsequently pelleted by ultracentrifugation in a Beckman type 19 rotor at 54,000 × g for 2.5 h at 4°C. The virus pellet was resuspended overnight in HNE buffer (5 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.4) and purified by ultracentrifugation on a sucrose density gradient (20 to 50%, wt/vol in HNE) in a Beckman SW41 rotor at 50,000 × g for 18 h at 4°C. The 40% to 45% section containing the virus was harvested, aliquoted and stored at -80°C.

For the microscopy studies, CHIKV was labeled with the lipophilic fluorescent probe 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt (DiD, LifeTechnologies), essentially as previously described for DENV [36]. To this end, 2 × 10¹¹ genome containing particles (GCPs) of purified CHIKV were mixed with 2 nmol DiD in dimethylsulfoxide (DMSO; final concentration of 2%) in an end volume of 50-60 µl. The mixture was incubated for 30 min at room temperature in the dark. Next, unincorporated dye was removed by size exclusion chromatography on a Sephadex G-50 fine (Pharmacia) columns. DiD-labeled CHIKV was stored at 4°C in the dark and was used within 2 days.

The number of individual DiD-labeled virus particles was estimated by fluorescence microscopy as described previously by Van der Schaar *et al.* [36]. DiD-labeled viruses were detected by epi-fluorescence microscopy in a Leica Biosystem 6000B instrument using a 635 nm helium-neon laser. Analysis was done using the ‘particle analyzer’ plugin of ImageJ.

As a control, CHIKV was treated with diethylpyrocarbonate (DEPC) (Sigma Aldrich). DEPC was freshly dissolved in cold ethanol to obtain a 1 M stock and diluted in PBS to an end concentration of 2 mM prior to usage. Then, 5 × 10⁸ GCPs
of DiD-labeled CHIKV LS3 were diluted in 2 mM DEPC and treated for 30 min at room temperature in the dark.

Virus for the bulk fusion assay was labeled biosynthetically with pyrene, as described before [34]. Briefly, BHK-21 cells were cultured in the presence of 15 μg/ml of 1-pyrenehexadecanoic acid (Invitrogen) 48 h prior to infection. BHK-21 cells were infected at MOI 4 and at 24 h p.i. virus was harvested and purified as described above.

**Virus quantification**

The infectious virus titer was determined by standard plaque assay. The number of plaque forming units (PFUs) were determined on Vero-WHO cells at 37°C. Plaques were counted two days after infection. Furthermore, reverse transcriptase quantitative PCR (RT-qPCR) was used to determine the number of GCPs, as described previously [34].

**Pharmacological inhibitors, siRNAs, plasmids**

Chlorpromazine, methyl-beta-cyclodextrin and cholesterol (water soluble) were all purchased at Sigma Aldrich. Ammonium chloride (NH₄Cl) was obtained from Merck and Pitstop2 from Abcam. All chemicals were dissolved and stored according to manufacturer’s instructions. The cytotoxicity of the compounds was tested using a standard MTT assay. MTT was purchased at Sigma and used at a final concentration of 0.45 mg/ml. Compounds were considered toxic and were therefore excluded from further analysis when the cell viability was lower than 75% of the non-treated control.

ON-TARGETplus SMARTpool siRNAs against human CTLC (clathrin heavy chain, CHC; L-004001-01), the ON-TARGETplus Non-targeting Pool (D-001810-10) and the transfection agent DharmaFECT2 (T-2002-01) were purchased from Dharmacon. 3 out of 4 siRNAs of the ON-TARGETplus SMARTpool against human CTLC were predicted to be 100% complementary to the Green Monkey CTLC gene. The clathrin-LCa-eYFP plasmid was a kind gift from Xiaowei Zhuang (Harvard University, Cambridge, Massachusetts, USA). The Rab5-wt-GFP plasmid and its dominant negative mutant Rab5-S34N-GFP was generously provided by Dr. P. van der Sluijs (University Medical Center, Utrecht, The Netherlands). The pGL-wt-Rab7 plasmid, containing a GFP reporter gene, was obtained from Gary R. Whittaker (Cornell University, College of Veterinary Medicine, NY, USA).

The inhibitory effect of the agents was checked by use of controls. Transferrin-AF633 (3 μg/ml, LifeTechnologies) was used as a control for chlorpromazine, Pitstop2, and siRNAs. LysotrackerGreen (5 μM, LifeTechnologies) staining was used as a control for NH₄Cl.
Microscopic fusion assay

The membrane fusion capacity of CHIKV was estimated by a microscopy-based fusion assay, as described before [37]. Briefly, BS-C-1 cells were seeded into 8-well Lab-Tek II chambered coverglass slides (Nunc) to obtain a sub-confluent monolayer the next day. Cells were washed three times with serum-free, phenol red-free MEM (Gibco), after which phenol red-free MEM supplemented with 1% Glucose was added. DiD-labeled CHIKV was added to the cells at MOI 20. Cells were subsequently incubated at 37°C for 30 min to allow viral fusion. Next, unbound virus was removed by washing three times with serum-free, phenol red-free MEM, and fresh phenol red-free MEM was added. Microscopic analysis was done using the Leica Biosystems 6000B instrument by randomly selecting fields using differential interference contrast (DIC) settings. A total of 20 random snapshots were taken per experiment in both the DIC and DiD channels. Snapshots were analyzed using the ‘particle analyzer’ plugin of ImageJ. The total area of fluorescent spots was quantified in arbitrary units (a.u.) for each snapshot and averaged per experiment.

To study the route of entry, several endocytic inhibitors were used. The microscopy-based fusion assay was performed as described above with the exception that the cells were pre-treated with the inhibitor of interest. NH₄Cl (50 mM) was added 1 h prior to the start of the experiment and Pitstop2 (25 µM) 15 min in advance. CHIKV was added to the cells in the presence of the inhibitor. Both inhibitors were diluted in phenol red-free MEM supplemented with 1% Glucose.

Single particle tracking of DiD-labeled CHIKV

Single particle tracking experiments were performed as described previously for DENV [37]. Unless indicated otherwise, 1.25 × 10⁶ BS-C-1 cells were transfected with 5 µg of plasmid DNA by electroporation using a Gene Pulser Xcell™ system (BioRad) and a Square Wave pulse (100 V, 25 ms). Subsequently, BS-C-1 cells were seeded into 8-well Lab-Tek II chambered coverglass slides (Nunc) to obtain 50 - 70% confluency at the day of tracking.

Directly before the experiment, cells were washed three times with phenol red-free MEM and phenol red-free MEM supplemented with 1% Glucose was added to the cells. GLOX, a glucose oxidase solution was added to prevent phototoxicity [37]. Cells were mounted onto the Leica Biosystems 6000B microscope and kept at 37°C throughout the whole experiment. DiD-labeled CHIKV was added in situ and image series were recorded at 1 frame per second for 25 to 30 minutes. To localize the nucleus and plasma membrane of the cell, DIC snapshots were taken before and after the imaging. Image analysis and processing was done by ImageJ and Imaris x64 7.6.1. Particles smaller than 40 a.u. were considered individual particles and selected for further analysis. To avoid the chance of misinterpretation, particles that fused in close proximity of the nucleus were excluded from tracking behavior analysis as cells are thicker within this region and movement in Z-axis cannot be detected with our microscope (Fig. S1).
Flow cytometry analysis to determine the number of infected cells

Flow cytometry analysis was used to assess the effect of the pharmacological inhibitor Pitstop2, siRNAs against CTLC, and the Rab5 DNM on CHIKV infection. For the inhibitor studies, Pitstop2 (25, 12.5, 6.25 µM) was diluted in BS-C-1 medium containing 2% FBS. Cells were pre-incubated with Pitstop2 for 15 min after which CHIKV LS3-GFP was added to the cells at an MOI of 1 and infection was allowed for 1.5 hours at 37°C. Then, BS-C-1 medium containing 10% FBS was added and incubation was continued overnight in the presence of the inhibitor. At 18 h p.i. cells were fixed with 4% PFA and analyzed by flow cytometry.

For the siRNA experiments, siRNA was transfected into cells according to the manufacturer’s protocol. At 72 h post-transfection, HeLa cells were infected with CHIKV LS3-GFP at MOI 5. At 18 h p.i., cells were fixed with 4% PFA and analyzed by flow cytometry.

For the DMN Rab5 infection studies, BS-C-1 and HeLa cells were transfected with either Rab5-GFP or DN Rab5-GFP using lipofectamine3000 according to manufacturer’s instructions. At 24 h post-transfection, HeLa cells were infected with CHIKV LS3 at MOI 5. BS-C-1 cells were infected at 48 h post-transfection with CHIKV LS3 at MOI 1. At 18 h p.i., cells were fixed with 4% PFA, stained with rabbit anti-E2-stem (1:1000), obtained from dr. G. Pijlman (Wageningen University, Wageningen, The Netherlands) and chicken anti-rabbit AF647 (1:300, LifeTechnologies), and analyzed by flow cytometry.

Liposomal bulk fusion assay

Liposomes (large unilamellar vesicles) of 200 nm were prepared by freeze-thaw extrusion as described before [24,34]. Unless otherwise specified, liposomes consisted of phosphatidylcholine (PC) from egg yolk, phosphatidylethanolamine (PE) prepared from transphosphatidylation of egg PC, sphingomyelin (SPM) from porcine brain, and cholesterol from ovine wool (all from Avanti Polar Lipids, Alabaster, AL) in a molar ratio of 1:1:1:1.5. Fusion of pyrene-labeled CHIKV with liposomes was monitored in a Fluorolog 3-22 fluorometer (BFi Optilas, Alphen aan den Rijn, The Netherlands) as described before [24,34,38]. Briefly, $4 \times 10^{10}$ pyrene-labeled CHIKV particles were mixed with $6 \times 10^{10}$ liposomes in a total volume of 665 µl HNE buffer at 37°C and continuous stirring. Fusion was triggered through the addition of 35 µl of 0.1 M MES with 0.2 M acetic acid pretitrated with NaOH to achieve the desired pH. Excitation and emission wavelengths were 345 and 480 nm, respectively. The fusion scale was set such that 0% fusion corresponded to the initial excimer fluorescence and 100% of fusion to the signal obtained after adding 35 µl of 0.2 M octaethyleneglycol monododecyl ether (C12E8; Sigma-Aldrich, Steinheim, Germany) which causes an infinite dilution of the probe. The extent of fusion was determined using the average fluorescent signal between 50 and 60 s after the pH drop.
Cholesterol depletion assay

BS-C-1 cells were depleted of cholesterol by using various concentrations (7.5, 5, 2.5 mM) of methyl-beta-cyclodextrin. Cells were plated in 8-wells LabTek II chambered coverglass slides as described above for the microscopic CHIKV fusion assay. Next, methyl-beta-cyclodextrin in phenol red-free MEM was added to the cells and incubation was continued for 1 h at 37°C. Then, cells were washed three times with phenol red-free MEM and the experiment was continued as a standard fusion assay. Depletion of cholesterol was reversed by adding medium containing water soluble cholesterol (200 µg/ml) to the cells. In this case, following the treatment with methyl-beta-cyclodextrin, cells were washed three times and incubated for 30 min at 37°C in the presence of cholesterol. Subsequently, the cells were again washed three times to remove free cholesterol and the experiment was continued as a standard fusion assay.

Statistics

Unless indicated otherwise statistical analysis was done using the two-tailed student’s t-test in GraphPad Prism 5 software. P-values lower than 0.05 (P < 0.05) were considered to be statistically significant.

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Supplemental Information

Video S1. CHIKV fusion event.
DiD-labeled CHIKV particle (red) resides in the left down corner before travelling towards the right upper corner and fusing. Fusion is seen as a sudden burst in fluorescence intensity. Movie shows the same particle as in Fig. 1A (no treatment). Image recording was performed at 1 frame/s. Virtual time is shown in the right down corner.

Video S2. CHIKV/clathrin colocalization.
(S2A) DiD-labeled CHIKV particle (red) co-localizes with clathrin-YFP; then the clathrin signal disappears and the virus is visible till membrane fusion. Clathrin-YFP is green and co-localization is visible as yellow. (S2B) and (S2C) give two other examples except for clarity the movie is stopped after clathrin dissociation. Image recording was performed at 1 frame/s. For all movies the virtual time is shown in the right down corner.

Video S3. CHIKV fusion upon Rab5 co-localization.
(S3A) DiD-labeled CHIKV particle that travels towards a Rab5-positive endosome and fuses within 2 s after co-localization. (S3B) DiD-labeled CHIKV particle that travels towards a Rab5-positive endosome and fuses within 21 s after co-localization (virtual time). For both movies virtual time is shown in the right down corner.

Figure S1. A representative DIC image of a BS-C-1 cell.
In the DIC image the cell membrane is indicated with a white line. The cytoplasm and the nucleus are indicated with the numbers I and II, respectively. Particles that fused above or in the direct vicinity (< 2 µm) of the nucleus (II) were excluded from tracking behavior analysis. Scale bar: 25 µm.
Figure S2. Cell viability assays.
MTT assays of BS-C-1 cells upon treatment with NH₄Cl (A), Chlorpromazine (B), Pitstop2 (C,D), and methyl-beta-cyclodextrin (E). (A,B,C,E) cells were incubated with the inhibitor for a similar period as for the microscopic cell entry and fusion assay. In (D) cells were incubated overnight in presence of the inhibitor to mimic conditions for flow cytometry.

Figure S3. Cargo control assays.
LysotrackerGreen and Transferrin, were used as cargo controls for NH₄Cl (A) and Chlorpromazine (B), Pitstop2 (C) and anti-CHC siRNAs (D,E), respectively. (A-D) BS-C-1 cells. (E) HeLa cells. Cargo controls were analyzed by microscopy. Total fluorescence intensity was analyzed by the ‘Particle Analyzer’ plugin in ImageJ and normalized to the non-treated control or non-targeting siRNA control. Error bars represent SEM.
Figure S4. CHIKV microscopic entry/fusion assay.
Representative images of cells infected with DiD-labeled CHIKV. Top, non-treated positive control; middle, DEPC treated DiD-labeled CHIKV (2 mM DEPC for 30 min); bottom, BS-C-1 cells prior treated with 50 mM NH₄Cl for 1 h. One representative image per condition (DIC, DiD, overlay) is shown. Intensity is equally enhanced for visual purposes. Fusion events appear as bright red spots in the DiD channel. Scale bar: 25 µm.
Figure S5. Flow cytometry gating strategy for determining infection in Rab5-GFP-expressing cells.
(A) Standard gating (I) strategy. (B) Step-wise change of the GFP-gating to only include cells with a high GFP expression (II-IV). (C) The percentage inhibition of infection found for Rab5-S34N compared to wild type Rab5 in the different GFP-gating strategies. The experiment is repeated twice in triplicate. Error bars represent SEM.