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Minireview

Replication cycle of chikungunya: A re-emerging arbovirus

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ABSTRACT

Arboviruses (or arthropod-borne viruses), represent a threat for the new century. The 2005–2006 year unprecedented epidemics of chikungunya virus (CHIKV) in the French Reunion Island in the Indian Ocean, followed by several outbreaks in other parts of the world such as India, have attracted the attention of clinicians, scientists, and state authorities about the risks linked to this re-emerging mosquito-borne virus. CHIKV, which belongs to the *Alphaviruses* genus, was not previously regarded as a highly pathogenic arbovirus. However, this opinion was challenged by the death of several CHIKV-infected persons in Reunion Island. The epidemic episode began in December 2005 and four months later the seroprevalence survey report indicated that 236,000 persons, more than 30% of Reunion Island population, had been infected with CHIKV, among which 0.4–0.5% of cases were fatal. Since the epidemic peak, the infection case number has continued to increase to almost 40% of the population, with a total of more than 250 fatalities. Although information available on CHIKV is growing quite rapidly, we are still far from understanding the strategies required for the ecologic success of this virus, virus replication, its interactions with its vertebrate hosts and arthropod vectors, and its genetic evolution. In this paper, we summarize the current knowledge of CHIKV genomic organization, cell tropism, and the virus replication cycle, and evaluate the possibility to predict its future evolution. Such understanding may be applied in order to anticipate future epidemics and reduce the incidence by development and application of, for example, vaccination and antiviral therapy.

Introduction

Most of the emerging viral infections in humans, originate from known zoonosis. Pathogens have been engaged in long-standing and highly successful interactions with their hosts since their origins. Strategies have evolved that maximize invasion rate, ensuring timely replication and survival both within-host and between-host, thus facilitating reliable transmission to new susceptible hosts. It is therefore extremely important to better understand the evolutionary events that shape the genotype–phenotype of pathogens and reciprocally to determine how cells from human and non-human species resist to infection so that ultimately novel molecules may be designed to protect permissive cells from viral infection.

Viruses are exquisitely adapted to host parasitization and have selected host traits that reduce their impact on host life span and fecundity. Lessons from molecular clocks tell us that viruses already populated our planet a few billion years ago (Doolittle et al., 1996) whereas our first ancestor, *Sahelanthropus tchadensis*, lived in Africa about 7 million years ago and *Homo sapiens* emerged about 150,000 years ago, subsequently spreading throughout the world (Tishkoff et al., 2000). Consequently, since humans first evolved they have been exposed to viruses. Gathering information related to the ecological variations and the dynamics of viral–host co-evolution, is critical to better understand the underlying mechanisms of viral emergence or re-emergence that is responsible for serious diseases in human populations. Viral infectious diseases in humans constitute one of the main challenges that medical science must meet in the coming century. The vast majority of emerging and re-emerging viral infectious diseases responsible for devastating human illnesses, such as AIDS, hepatitis or hemorrhagic fevers, are caused by viruses with RNA genomes. Among these RNA viruses, arthropod-borne (arbo-)viruses, are especially important since many cause fatal diseases in humans and domestic animals. The *Alphaviruses* genus, that includes the group of viruses to which chikungunya virus (CHIKV) belongs, originated around 2000 to 3000 years ago (Weaver

Abbreviations: V, virus; CHIK, chikungunya; WEE, Western equine encephalitis; EEE, Eastern equine encephalitis; VEE, Venezuelan equine encephalitis; SF, Semliki Forest; BF, Barmah Forest; MID, Middelburg; NDU, Ndumu; ONN, O'nyong-nyong; RR, Ross River; SIN, Sindbis; DEN, dengue; WN, West Nile; C-ter, C-terminal; N-ter, N-terminal.

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et al., 1993). CHIKV, a virus transmitted to humans by infected mosquitoes, was originally isolated in 1952 from the serum of a febrile patient from the Makonde Plateau in Tanzania (Robinson, 1955; Ross, 1956). The CHIKV-associated symptoms observed 2–7 days following infection are characterized by chills and fever between 39 °C and 40 °C, headache (almost 70% of the patients), nausea/vomiting (about 60% of the patients), persistent myalgia/arthralgia (about 40% of patients; the poly-arthralgia is frequently very painful), and maculopapular rash (almost 60% of the patients). These symptoms are often clinically indistinguishable from those caused by dengue virus (DENV) which affects nearly 80 million people a year. Such similarity in clinical symptoms probably accounts for frequent misclassification and some underreporting of CHIKV infection in areas with endemic DENV (Carey, 1971). However, dual infections may occur in some cases (Myers and Carey, 1967). CHIKV-associated symptoms were also found to be very similar to those previously observed in Australia during unusual epidemic episodes caused by a related virus, the Ross River virus (RRV) (Nimmo, 1928; Weber, Oppel, and Raymon, 1946). CHIKV has also been isolated from Australian patients (Harnett and Bucens, 1990).

Since 2005 it has become clear that CHIKV infection of humans can also cause fatal disease in a small percentage of infected patients. Since the first identification of CHIKV-infected humans in 1952, outbreaks of CHIKV have occurred throughout African and Asian countries where it was responsible for illnesses in hundreds to thousands of individuals. Comparative observations of Asian and African episode of CHIKV have been reported (Nakao, 1972). In both Africa and Asia, the re-emergence was unpredictable, with intervals of 7 years to 20 years between consecutive epidemics. For example, cases of chikungunya in Indonesia were reported between 1973 and 1983. After a hiatus of 20 years, 24 distinct CHIKV outbreaks were reported during 2001 to 2003 (Berger, 2005).

The factors determining the risk for chikungunya epidemics are poorly understood. These factors are likely similar to those that determine the risk for RRV, and therefore RRV is a very informative model for *Alphavirus*-induced epidemics (Harley, Sleigh, and Ritchie, 2001). Epidemic determinants include adequate populations of reservoir hosts (potentially humans in some settings), vector mosquitoes, and appropriate climatic conditions for transmission. CHIKV is known for its wide geographic distribution (Strauss and Strauss, 1994). The CHIKV epidemic episode which developed in 2004 in the Indian Ocean, resulted in a crisis similar to that associated with the 1991 RRV epidemic outbreak in Australia (Harley, Sleigh, and Ritchie, 2001). This explosive outbreak of this little-known virus possibly makes this the first announcement that major viral threats exist in this region of Indian Ocean. This part of the world meets a number of criteria (ecological, geopolitical, climatical, social …), that are conducive to the emergence of other viral epidemic episodes. Re-emergence of CHIKV was also recorded in India during 2005–2006 (Arankalle et al., 2007). To understand what occurred in Reunion Island, it is important to better know the genetic nature of the virus, the ecology of vectors and reservoirs. We have recently reviewed the ecological and evolutionary perspective of CHIKV threat (Chevillon et al., 2008). A major challenge also concerns the characterization of the virus replication cycle. Efforts in virology basic research are required to better define the molecular interactions between the CHIKV and cells from different tissue origins. Such information is needed for understanding the pathophysiology of CHIKV-associated disease and developing drugs for future CHIKV therapy. The main objective of this review is to summarize the knowledge accumulated on the CHIKV replicative cycle and to discuss possible mechanisms of molecular crosstalk between the virus and host cell with respect to our most recent data and other data from the CHIKV literature, and also by extrapolating from publications related to other *Alphaviruses*.

CHIKV, a member of arboviruses

In the early 1940s, workers at the Rockefeller Foundation demonstrated that arboviruses could be discriminated by serological tests. Using hemagglutination inhibition and neutralization assays, it was suggested that Western equine encephalitis virus (WEEV), Eastern equine encephalitis virus (EEEV), Venezuelan equine encephalitis virus (VEEV) and Semliki Forest virus (SFV) are related but distinct. They were clustered in a group called A, whereas a second interrelated group B included dengue virus (DENV), Japanese encephalitis virus (JEV), Ntaya virus (NTAV), West Nile virus (WNV), and yellow fever virus (YFV) (Casals and Brown, 1954). Subsequently, electron microscopy, biochemical and genetic characterization of these viruses led to placement of both group A and group B arboviruses in a single virus family named *Togaviridae*. More recently, the classification was revisited based upon gene sequences, replication strategies, and structure of these viruses which resulted in the final assignment of the different arbovirus groups into *Flavivirus* or *Alphavirus* genus (Westaway et al., 1985). The *Flavivirus* genus includes more than 70 single-stranded RNA viruses divided into 8 serogroups (Chambers et al., 1990). The *Alphavirus* genus consists of 29 species of arboviruses that have been classified into 7 antigenic complexes: Barmah Forest (BF), Eastern equine encephalitis (EEE), Middelburg (MID), Ndumu (NDU), Semliki Forest (SF), Venezuelan equine encephalitis (VEE), and Western equine encephalitis (WEE). *Alphaviruses* have a wide geographic distribution (Strauss and Strauss, 1994). CHIKV is part of the SF group of Old World *Alphaviruses* that includes SFV, ONNV, and RRV. Most of the Old World *Alphaviruses* cause fever, rash and arthralgia while many of the New World *Alphaviruses* cause encephalitis. The seven antigenic complexes are indeed reflecting clades of viruses that share medically important characteristics.

The availability of partial or complete sequence data (Charrel, Zandotti, and de Lamballerie, 2006; George and Raju, 2000; Khan et al., 2002; Powers et al., 2001; Powers et al., 2000; Schuffenecker et al., 2006; Yadav et al., 2003) unsurprisingly indicated that genetic relationships are in agreement with the antigenically-based classification. Evolutionary relationships of *Alphaviruses* present some intriguing questions regarding the origin of the genus and subsequent species distribution and geographical expansion. For example, most of the New World viruses in the WEE antigenic group (WEEV, Highlands J virus, Fort Morgan virus) have emerged from an ancestral virus that results from a recombination between the E2 and E1 gene from a Sindbis-like virus and the remaining genome from an EEEV-like ancestor (Hahn et al., 1988; Weaver et al., 1997). On the basis of evolutionary rate estimation, it was calculated that this recombination may have occurred in the New World roughly 1300 to 1900 years ago, after the divergence of Aura virus from the ancestor of the Sindbis-related viruses (Weaver et al., 1997). Regarding dissemination, it has been proposed that *Alphaviruses* originated in the New World and were spread to the Old World twice, once to generate the SIN group, and once to create the SF group (Levinson, Strauss, and Strauss, 1990). Alternatively it has been suggested that the *Alphaviruses* originated either in the New World or the Old World and then were spread to the other hemisphere about 2000 to 3000 years ago (Weaver et al., 1993).

Among New World *Alphaviruses*, analysis of the South American group of EEEV suggested that Peru–Brazil EEEV have diverged from Argentina–Panama EEEV roughly 450 years ago (Weaver et al., 1994). Another surprising observation concerns the Middleburg virus (MIDV); MIDV which forms an independent cluster according to several phylogenetic analyses, falls into the group of CHIKV and SF-related viruses when phylogenetic trees are built from partial E1 nucleotide sequence (Powers et al., 2001) (Fig. 1). Evolutionary studies have also been reported for the SF group in Old World *Alphaviruses* that revealed some interesting genetic features relevant to evolution; an ancestor of the SFV, RRV, ONNV, and CHIKV groups

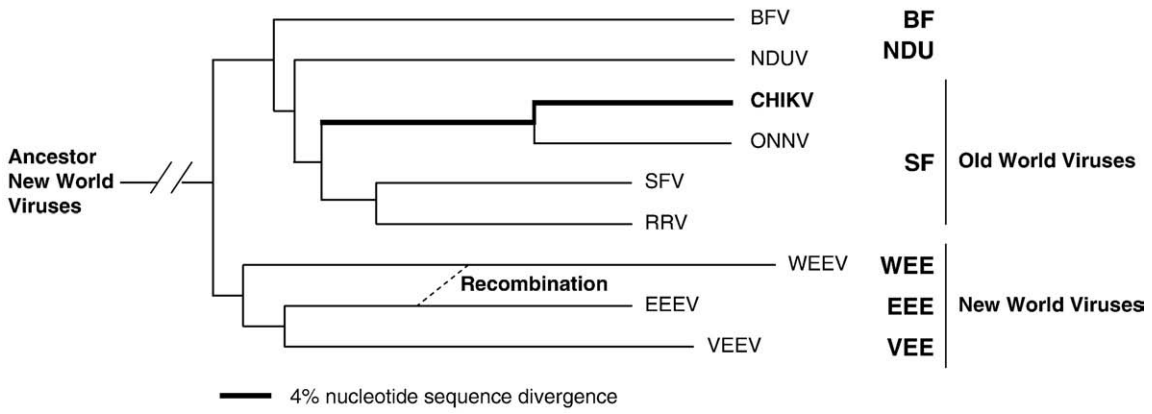

Fig. 1. A simplified phylogenetic tree of *Alphaviruses* assuming their New World origin, adapted from Powers et al. (2001). This figure illustrates the evolution of *Alphaviruses* generated from partial E1 envelope glycoprotein sequence and highlights the evolution of CHIKV. Only 6 of the 7 antigenic complexes are shown because MIDV, which forms an antigenic complex independent from the other using several phylogenetic analyses, fall into the group of CHIKV and other virus from the SF group when phylogenetic tree is deducted from partial E1 nucleotide sequence. Readers are invited to refer to the excellent paper by Powers et al. (2001) for more details. It is worth noting that WEEV arose by recombination between EEEV and the Sindbis virus (Hahn et al., 1988; Weaver et al., 1997). The recombinant virus contains the glycoprotein of the SINV-like parent but the nucleocapsid protein of the EEEV parent.

which originated from the New World was introduced in the Old World with complicity of the migratory animal reservoir, likely birds (Weaver et al., 1997), and next is expected to have spread in the Old World. It has been postulated that CHIKV evolved in Africa where it is maintained in a sylvatic cycle involving wild primates and *Aedes spp.* mosquitoes, and was subsequently introduced into Asia, where it was typically associated with *Aedes aegypti* mosquitoes (Powers et al., 2000). This virus is currently transmitted by *Aedes albopictus* in urban areas (Kumar et al., 2008).

Sequence comparisons in E1 indicated, for example, that the TA53 CHIKV isolate, collected from Tanzania in 1953, showed 98.5% and 97.5% nucleotide identity with the SA76 South African isolate collected in 1976 and the Ugandan UG82 strain isolated in 1982, respectively. A high identity (97.7%) was also observed between TA53 and the recent Reunion Island isolate OPY-1. In contrast TA53 and OPY-1 showed only 85.2% and 84.8% nucleotide identity in E1 with the 37997 West African strain. Using evolutionary rate estimation based on E1 nucleotide sequence comparison of CHIKV with different origins, it was predicted (Powers et al., 2000), that the Asian CHIKV genotype evolved from a hypothetical African ancestor approximately 50 to 430 years ago. Interestingly, the closely related virus ONNV is believed to have evolved from a CHIKV-like virus that adapted to *Anopheles* mosquito vector (Johnson, 1988), typically *A. funestus* and *A. gambiae*. Yet, other authors who performed phylogenetic E1 nucleotide sequence analysis hypothesized that CHIKV and ONNV are genetically distinct (Lanciotti et al., 1998; Powers et al., 2000). It is noteworthy that CHIKV, like RRV and BFV, uses the culicine mosquitoes *Aedes*, as their vector. An interesting biological difference has been recorded between CHIKV and ONNV, since CHIKV replicates in an *A. aegypti* cell line whereas ONNV does not (Chanas et al., 1979; Vanlandingham et al., 2005a). In addition, CHIKV also replicates in an *A. albopictus* C6/36 cell line (Igarashi, 1978; Pyke et al., 2004), which is also known to support RRV replication. Experiments with infectious clones of CHIKV and ONNV to produce chimeric viruses, demonstrated that infection of *A. gambiae* requires the entire ONNV structural gene sequences (Vanlandingham et al., 2005a; Vanlandingham et al., 2005b). It was postulated that the conserved repeated sequence elements in the 3′-non-translatable region of *Alphaviruses* may have a function in vector specificity (Khan et al., 2002). During the recent outbreak of CHIKV in the Indian Ocean territories, CHIKV samples were isolated from 127 patients from Reunion, Seychelles, Mauritus, Madagascar, and Mayotte islands and both complete nucleotide sequence of six selected isolates and partial E1 nucleotide sequence were used to build phylogenetic tree (Schuffenecker et al., 2006). This study demonstrated that the outbreak was initiated by a strain related to an East-African isolate. Sequence comparison between viruses isolated from Reunion Island and from the recent outbreak in the Kerala region in India indicated that the Indian isolate IND-06 shared 99.9% nucleotide identity with the reference Reunion Island isolates. Three unique substitutions were noted in the IND-06 isolate: two (T128K and T376M) in the nsP1 region and one (P23S) in the capsid protein (Arankalle et al., 2007). Accordingly, these two outbreaks can be attributed to the circulation of the same strain of CHIV.

CHIKV genomic organization

As a member of the *Alphavirus* genus, CHIKV is a small (about 60–70 nm-diameter), spherical, enveloped, positive-strand RNA virus (Higashi et al., 1967; Powers et al., 2001; Simizu et al., 1984; Strauss and Strauss, 1994). *Alphaviruses* are among the simplest membrane-enveloped viruses. Until recently, only two complete nucleotide sequences of CHIKV isolated from humans infected during the 1952 Tanzania outbreak were available corresponding to the strain Ross and the S27 CHIKV African prototype, respectively (Khan et al., 2002). Another complete nucleotide sequence was established from a CHIKV strain isolated in *A. furcifer* mosquito during the 1983 Senegal outbreak (accession no. AY726732). Additionally, several partial sequences (e.g., NCBI accession nos. L37661, AF490259, AF023283, AF192895, AF192907, U94597 (Powers et al., 2000; Yadav et al., 2003)), were also available from GenBank, including the 26S sequence of an Indian Strain M-713424 isolated in Madras (Yadav et al., 2003). The 2005–2006' Reunion outbreak of CHIKV was such a challenge for scientists that knowledge about new CHIKV isolates genomic sequences rapidly increased. Early 2006, the complete sequence of a CHIKV isolate from Reunion Island was made available through NCBI/GenBank accession no. DQ443544.1 (Charrel, Zandotti, and de Lamballerie, 2006). Next, complete sequences of six selected CHIKV isolates from Reunion and Seychelles islands were reported (Schuffenecker et al., 2006). Additionally, this last study also reported partial sequences of 119 CHIKV isolates from Reunion, Seychelles, Mauritius, Madagascar and Mayotte islands.

The genome of CHIKV is organized as follows: 5′ cap-nsP1-nsP2-nsP3-nsP4-(junction region)-C-E3-E2-6k-E1-poly(A)-3′ and is 11,805 nucleotides long, excluding the 5′ cap nucleotide, an I-poly(A) tract and the 3′ poly(A) tail (CHIKV S27 strain) (Fig. 2). The CHIKV genome resembles eukaryotic mRNAs in that it possesses 5′ cap structures and 3′ poly(A) tail. Although it has not been specifically investigated for CHIKV, it was documented with related *Alphaviruses* that the 5′ end

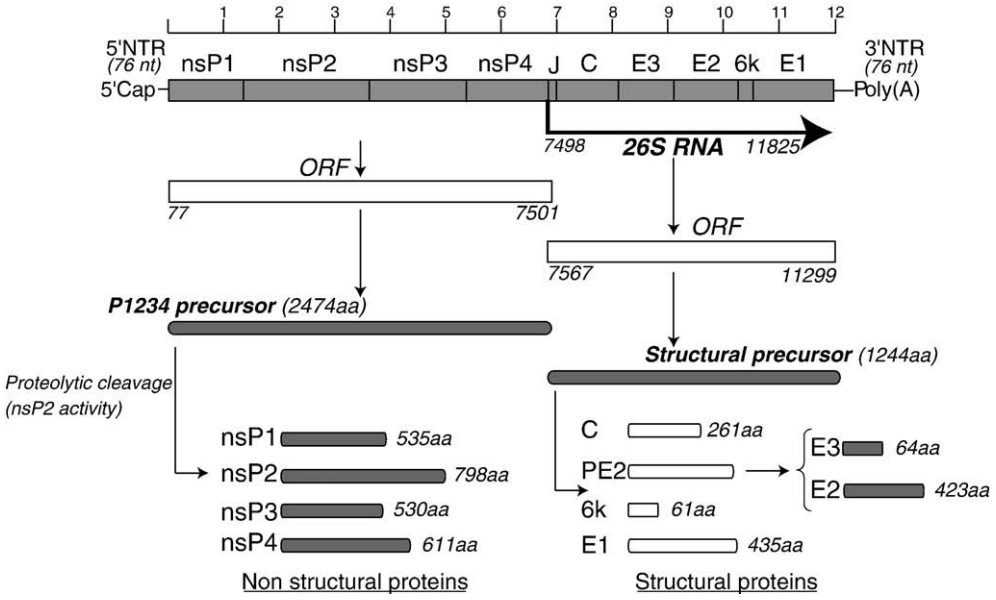

Fig. 2. Organization of the CHIKV genome and gene products. The CHIKV genome resembles eukaryotic mRNAs in that it possesses 5′ cap structures and 3′ poly(A) tail. The 5′ and 3′ proximal sequences of CHIKV genome carry non-translatable regions (NTR). The junction region (J) is also non-coding. A subgenomic positive-strand mRNA referred to as 26S RNA, is transcribed from a negative-stranded-RNA intermediate and serves as the mRNA for the synthesis of the viral structural proteins. The different non-structural proteins (nsP1–nsP4) and structural proteins (C, Capsid; E1, E2, E3, envelope; 6K) are generated after proteolytic cleavage of polyprotein precursors.

capped with a 7-methylguanosine. For CHIKV (e.g., S27 strain), the 5′-non-translatable regions (NTR) of CHIKV are composed of 76 nucleotides. The size of the 3′-NTR of *Alphaviruses* demonstrates an important heterogeneity. The 3′-NTR of CHIKV is composed of 526 nucleotides. By comparing the length of 3′-NTR of 23 *Alphaviruses* it becomes apparent that this part of the viral genome varies strongly, from 77 nt for Pixuna virus up to 609 nt for Bebaru virus (Pfeffer et al., 1998). Such variations largely depend on the number of repeated sequences which could account for sequence insertions into a smaller ancestral 3′-NTR or to deletions that may also have contributed to shape this region. Indeed, the 3′-NTR proximal sequences of *Alphavirus* genomes are enriched in A/T nucleotide (ranging from 58% for Babanki virus to 82% for Buggy Creek virus, two viruses that belong to the WEE group of viruses). Such feature is known to be critical for recombination and duplication events leading to homologous recombinations in positive-stranded viruses (Nagy and Bujarski, 1996; Pfeffer, Kinney, and Kaaden, 1998). The 3′-NTR of *Alphaviruses* are believed to contain several cis-acting conserved motifs, named conserved repeated sequence elements (RSEs), which regulate viral RNA synthesis (Ou, Strauss, and Strauss, 1983; Pfeffer, Kinney, and Kaaden, 1998). CHIKV 3′-NTR contains two copies of an incomplete RSE showing an identity with an RSE sequence found in ONNV and contains three RSEs at positions 11,382–11,416, 11,525–11,559, and 11,611–11,646 similar to RSE motives found in RRV and BFV but

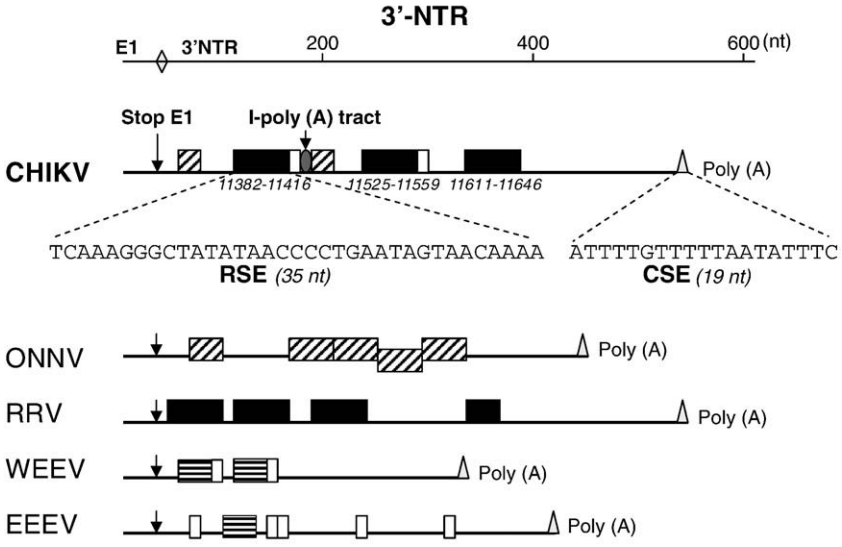

Fig. 3. Schematic representation of repeated sequence elements (RSE) and lengths of 3′-NTRs of CHIKV and related viruses (adapted from Pfeffer et al. (1998)). The downward-pointing arrow indicates the stop codon of the structural E1 glycoprotein gene region. Both the overall lengths of the 3′-NTR and repeats have been drawn to scale. Different types of shading indicate repeats of unique sequence. The open triangles preceding the poly(A) tail indicate the 19-nt that is a highly conserved sequence element (CSE), among *Alphaviruses*. The organization of the 3′-NTR in CHIKV revealed two copies of an incomplete RSE found in ONNV and three copies of RSE that represent the entire RRV-type RSE and repetitive sequences specific for CHIKV (slight sequence variations, below 10%, are found among the three RSE). The location of I-poly(A) in 3′-NTR of CHIKV is indicated (according to Khan et al. (2002)).

surprisingly different from RSE motifs found in ONNV (Khan et al., 2002; Pfeffer, Kinney, and Kaaden, 1998) (Fig. 3). Nucleotide sequence alignment revealed a four nucleotide difference and one nucleotide difference accompanied by one insertion in the second and third RSE motives, respectively. A deletion of RSEs in model *Alphaviruses* usually causes a reduced and delayed viral release in different cell types, suggesting a possible interaction with generally unknown cellular proteins involved in virus production, tissue specificity or both (Kuhn et al., 1991). For SINV, binding sites for cellular proteins have been identified in the 3′-NTR of the viral RNA (Pardigon, Lenches, and Strauss, 1993), opening new avenues for research. Interestingly, a 19-nt conserved sequence element directly adjacent to the poly(A) tract was found in all *Alphaviruses*, supporting the hypothesis that this region is a cis-acting sequence element during viral replication which possibly plays an essential role for virus growth, likely by acting as an essential initiation site for the viral replicase (Pfeffer, Kinney, and Kaaden, 1998; Strauss and Strauss, 1994). Yet it has also been claimed that deletions of the CSE do not prevent *Alphaviruses* replication (George and Raju, 2000). Finally, an internal poly(A) tract (I-poly(A)) has been described in the 3′-NTR of a highly passaged strains (Khan et al., 2002) while it does not exist in other CHIKV genomes (Pfeffer, Kinney, and Kaaden, 1998). The biological significance of this motif is unclear. Interestingly, three insertion/deletion events, two of which were observed in the 3′-NTR, were reported when nucleotide sequences from the S27 CHIKV strain and Reunion Island CHIKV isolates were compared (Schuffenecker et al., 2006). First the long I-poly(A) stretch observed in the S27 strain was replaced by a stretch of only 5 adenine nucleotides in Indian Ocean; second, a GAA codon was missing. The junction region of CHIKV which is also untranslated is composed of 68 nucleotides. Using SINV as a model, it has been shown that this region contains an internal promoter for transcription of the subgenomic mRNA, and the start site and 5′-non-translated leader sequence of the 26S mRNA (Grakoui et al., 1989).

The genome of the S27 CHIKV strain, similarly to the other *Alphaviruses*, contains two open reading frame (ORF) of 7424 nt and 3732 nt that encode non-structural and structural proteins of CHIKV, respectively (Fig. 2). A 1.13% sequence divergence (28 amino acid changes) was observed in non-structural proteins when compared with the 05.115 Reunion isolate with the highest diversity observed in nsP3 (2.26%) and the lowest in nsP2 (0.6%). One important difference reported by Schuffenecker et al. (2006), was the presence of an opal stop codon (UGA) in nsP3 instead of a CGA coding for an arginin in S27, the function of which is likely the regulation of the putative polymerase nsP4 protein expression by a read-through mechanism (Strauss et al., 1988). In addition, the capsid and E1 and E2 envelope glycoproteins showed 0.38%, 0.68% and 3.3% amino acid variation, respectively. Notably, with 14 amino acid changes, the envelope E2 protein showed the highest sequence variation.

CHIKV entry into human cells

Alphaviruses envelope glycoproteins function is attachment to cells. Despite the 3-D structure of CHIKV E1 glycoprotein has not been solved so far by crystallographic methods, the primary sequence is quite similar to the E1 fusion protein of SFV and it is likely that the 3-D folding of these related proteins is similar. Interestingly, the E1 protein of SFV revealed a remarkable fit to the available scaffold of Tick-borne encephalitis virus (TBEV, a flavivirus) (Lescar et al., 2001). Recently, a model for the CHIKV E1 structure became available (Schuffenecker et al., 2006). The E1 and E2 glycoproteins are expected to form heterodimers that associate as trimeric spikes on the viral surface.

Enveloped viruses utilize membrane-bound receptor(s) for entry into specific target host cells (Smith et al., 1995). The vertebrate host spectrum of arboviruses varies for each virus (Calisher, 1994; Chevillon et al., 2008; Higashi et al., 1967; Jupp and McIntosk, 1988; Rinaldo et al., 1975; Stim and Henderson, 1969). Within their mammalian hosts, arboviruses usually replicate in a wide variety of cells (Eaton and Hapel, 1976; Giovarelli et al., 1977; Heller, 1963; Murphy et al., 1973; Precious et al., 1974). The identity of some arbovirus receptors has been reported. The dendritic-cell-specific ICAM3-grabbing non-integrin (DC-SIGN or CD209) was found to serve as a receptor both for SINV (Klimstra et al., 2003) and for DENV (Kwan et al., 2005; Navarro-Sanchez et al., 2003; Tassaneetrithep et al., 2003). The laminin receptor and glycosaminoglycans have also been suggested to play a role for SINV entry (Lee et al., 2002; Wang et al., 1992). Interestingly, a laminin-like receptor in mosquito cells was suggested as the target for VEEV envelope glycoprotein (Strauss and Strauss, 1994). Although this review focuses on CHIKV tropism for human cells it is impossible to ignore that the virus transmission cycle requires infection of female mosquitoes via a viremic blood meal and, following a suitable extrinsic incubation period, transmission to another vertebrate host during subsequent feeding (Higgs and Beaty, 2004). However, despite able to replicate both in mosquitoes and higher vertebrate cells, neither mosquito cell surface receptor(s) nor human cell surface receptor(s) have been identified to date for CHIKV. This is an important question to be addressed. Determining to which cell types CHIKV can attach and productively infect are important issues required to understand the pathophysiology of CHIKV infection in humans. The ability of CHIKV to bind human cells and to replicate in cell cultures was only recently documented. In the absence of experimental evaluation of target cells, one could expect CHIKV to bind and infect a broad range of cell lines similarly to those infected by related *Alphaviruses*. To support this hypothesis, the related virus RRV was reported to persistently and productively infect a large variety of cells including human synovial cells (Cunningham and Fraser, 1985; Journeaux, Brown, and Aaskov, 1987), fibroblasts (Journeaux, Brown, and Aaskov, 1987), macrophages (Linn and Suhrbier, 1997; Linn et al., 1996) and CD4+ lymphocytes found in the mononuclear synovial effusion of patients with epidemic polyarthritis (Fraser and Becker, 1984). Similarly, VEEV can infect a broad array of human cell types (Kolokoltsov, Weaver, and Davey, 2005). It has been already documented that CHIKV can infect the epithelial FL cells that derive from the human amniotic membrane (Mantani et al., 1967) and human lung MRC-5 cells which are routinely used for CHIKV production (Edelman et al., 2000). However, the first extensive study of human cell lines and cellular subpopulations supporting virus replication *in vitro*, was published only two years ago (Sourisseau et al., 2007). This work demonstrated that binding of CHIKV and infectivity are limited to some cells lines and cellular subpopulations, indicating that CHIKV, like the other enveloped viruses, demonstrates a tropism for cells expressing an as yet unidentified surface receptor (s) that is not ubiquitously expressed on human cells. We have also further investigated CHIKV tropism for human cell types (Solignat et al., submitted for publication), by counting fluorescent cells that express the green fluorescent protein (GFP) following exposure to the strain 5′CHIKV-EGFP (Tsetsarkin et al., 2006) and by measuring the cytopathic effect of the virus in cell culture. We first evaluated whether or not blood-derived cells are sensitive to CHIKV. As summarized in Table 1, H9, a CD4+ T lymphoid cell line as well as the monocytoid U937 and TPH-1 cell lines and primary dendritic cells are resistant to CHIKV infection. In addition, primary peripheral blood mononuclear cells, including primary CD4+ T lymphocytes, primary CD14+ monocytes and dendritic cells were also reported to be refractory to CHIKV binding and infection (Sourisseau et al., 2007). Surprisingly, primary macrophages were highly sensitive to CHIKV and showed cytopathic effect following CHIKV infection (Fig. 4A). These data, together with other data from the literature (Sourisseau et al., 2007) support that primary macrophages are productively infected by CHIKV. Considering that the B-420 (EBV-transformed B cell line) was refractory to CHIKV (Sourisseau et al., 2007), these results suggest that, to the exception of macrophages and platelets,

Table 1
Summary of experiments aimed at investigating the sensitivity of different human cell lines to CHIKV.

Name	Cell type	Viral replication	Cytopathic effect		
			10^{-1}	10^{-2}	10^{-3}
THP-1	Monocytoid cell line	–	nd	nd	nd
U937	Monocytoid cell line	–	nd	nd	nd
Dendritic cells	Primary monocyte-derived cells	–	–	–	–
Macrophages	Primary monocyte-derived cells	+	+	+	+
H9	T-Lymphocyte-derived cells	–			
HUH7	Hepatocarcinoma cell line	++	++	+	–
SH-SY5Y	Neuroblastoma cell line	++	++	++	–
HeLa	Cervical carcinoma epithelial cell line	++	++	+	–
A549	Alveolar epithelial cell line	–	nd	nd	nd
HEK-293T	Kidney epithelial cell line	++	++	++	–

Viral replication was measured 24 h after exposure to the 5′CHIKV-EGFP virus at $25\times TCID_{50}$. Cells were then fixed using paraformaldehyde and GFP positive cells were counted under epifluorescence using a Leica microscope. ++: at least 80% of GFP cells; +: less than 40% of GFP cells. The cytopathic effect was evaluated by MTT assay 48 h post infection. Cells were infected with the indicated $TCID_{50}$ dilution (10^{-1} refers to $25\times TCID_{50}$). nd: not determined. All results are representative of 3 independent experiments. CHIKV used is the West African strain 5′CHIKV-EGFP that encodes a GFP protein (Vanlandingham et al., 2005b).

capable to bind CHIKV (Larke and Wheelock, 1970), most blood-derived cells lack CHIKV cell surface receptor(s). These results differ with respect to the tropism for lymphocytes reported for related viruses such as RRV and VEEV. CHIKV was also found to bind and infect a number of cells derived from other tissues. We have observed (Table 1) that the cervical carcinoma epithelial cell line HeLa, the kidney epithelial cell line HEK-293T, the hepatocarcinoma epithelial cell line HUH7, and the neuroblastoma cell line SH-SY5Y are infected by CHIKV and show cytopathic effects and syncytia formation after CHIKV exposure (Fig. 4B). In addition, BEAS-2B, Hs 789.Sk skin cells, dividing and non-dividing MRC-5 lung cells and endothelial cell lines (TrHBMEC and hCMEC/D3) were also susceptible to CHIKV (Sourisseau et al., 2007). In muscular tissues, CHIKV infects and replicates in skeletal muscle progenitor cells, designed as satellite cells, but not in muscle fibers (Ozden et al., 2007). More recently, it was reported that the syncytiotrophoblastic cell line BeWo is refractory to CHIKV (Couderc et al., 2008). In addition to this observation, we found that CHIKV is unable to replicate in the A549 alveolar epithelial cell line. Interestingly, the A549 alveolar epithelial cell line was reported to efficiently bind CHIKV. Given that a quite good correlation was reported between the efficiency of viral binding and sensitivity to infection (Sourisseau et al., 2007), this may suggest that a co-receptor is lacking at the surface of A549 cell line or that this particular cell type is protected by a restriction factor, tissue-specific, acting post-entry. It is not known whether mechanisms of restriction similar to other well-documented events of cellular resistance to viruses such as these generated by the tripartite motif proteins (TRIM/RBCC) and apolipoprotein B mRNA-editing enzymes (APOBEC) in HIV infected cells (Nisole et al., 2005; Zheng and Peterlin, 2005), exist for CHIKV, but it would not be surprising to find cells resistant to CHIKV by a restriction mechanism. This hypothesis is reinforced by the existence of a restriction mechanisms inhibiting EEEV replication after attachment, entry and uncoating in myeloid lineage cells (Gardner et al., 2008).

Host–CHIKV interactions and clathrin-dependent endocytosis

Cellular requirements for entry in target cells differ among *Alphaviruses*. Cholesterol represents one of the key constituents of small, dynamic, sterol- and sphingolipid-enriched domains on the plasma membrane which are called lipid rafts and which compartmentalize cellular processes. Functionality of lipid rafts is sensitive to cholesterol depleting agents as β-methyl cyclodextrin (mCD). We recently investigated the requirement for membrane cholesterol in CHIKV entry into mammalian HEK-293T cells. Cholesterol depletion of the target cell membrane significantly reduced (up to 65%) infection of the cells (Solignat et al., data submitted for publication) suggesting that cholesterol-enriched domains play an important role during entry of CHIKV particles in human cells. It is worth noting that New World *Alphaviruses* (i.e., VEEV) are apparently quite unsensitive to membrane cholesterol depletion during entry, whereas Old World *Alphaviruses* (i.e., SFV) entry into cells is apparently influenced by free membrane cholesterol and sphingolipids (Phalen and Kielian, 1991; Waarts et al., 2002). Such differences for cholesterol dependence were suggested to rely on sequence variations in E1 with implication of amino acid at position 226 (Lu, Cassese, and Kielian,

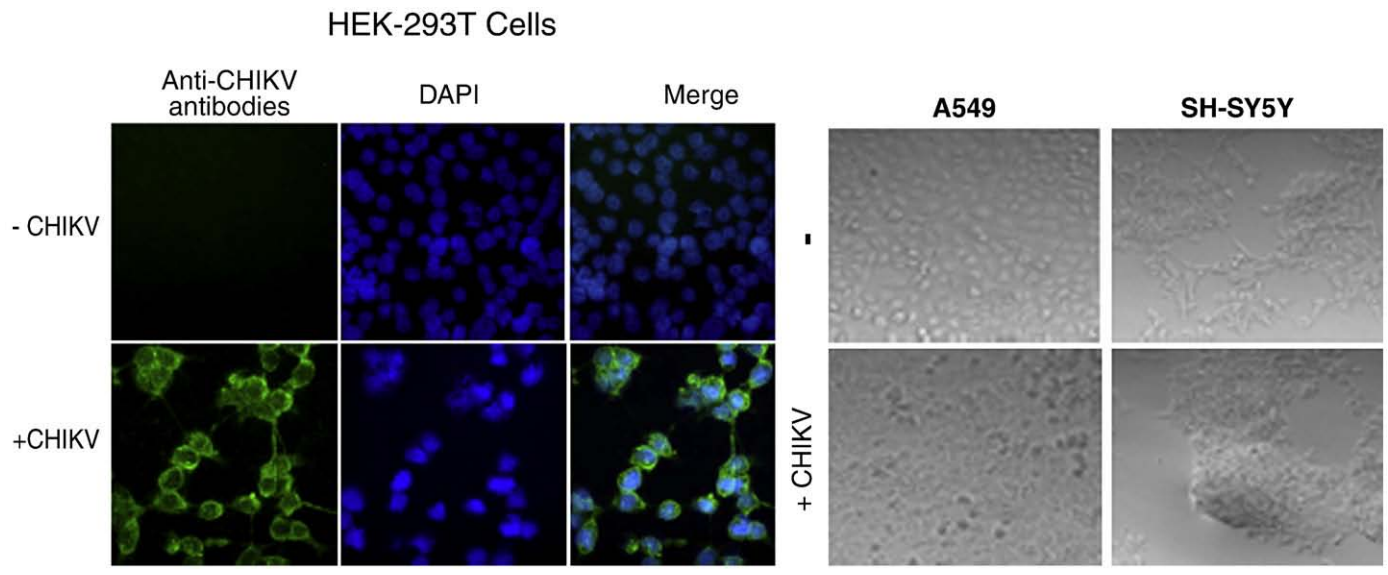

Fig. 4. Representative experiment of analysis of cells sensitivity to CHIKV. (A) Primary macrophages were exposed to $25\times TCID_{50}$ of 5′CHIKV-EGFP (lower panel). Infected cells were revealed by expression of green fluorescence 24 h after exposure to CHIKV. The upper panel corresponds to uninfected control primary macrophages. DAPI coloration is also shown. (B) Comparative analysis of CHIKV cytopathic effect into A549 and SH-SY5Y cell cultures. Cells were examined by phase contrast using a Leica microscope. SH-SY5Y exposed to CHIKV demonstrated syncytia formation and cell death after 48 h of culture (left, bottom panel) compared to control culture (left, upper panel). No cytopathic effect is evidenced in culture of A549 cells exposed to CHIKV.

1999) conferring cholesterol independence (Vashishtha et al., 1998). Recently, it was reported that the outbreak of CHIKV in Indian Ocean Islands, was initiated by a virus expressing a E1 glycoprotein with an A-226 amino acid but, as the epidemic progressed CHIKV isolated from the same geographic region expressed either a A-226 or a V-226 and eventually the new V-226 genotype largely dominated in infected humans (Schuffenecker et al., 2006). Tested in *A. albopictus* cells, this mutation was later found to modify the cholesterol dependence of the virus and to increase the fitness of CHIKV, causing a significant enhancement in the ability of the virus to disseminate into mosquito secondary organs and to be transmitted to suckling mice (Tsetsarkin et al., 2007).

Studies performed with SFV, were the first to establish the receptor-mediated endocytosis entry route (Helenius et al., 1980). Virus access into cells occurs through clathrin-dependent endocytosis vesicles (Kolokoltsov, Fleming, and Davey, 2006), and anti-clathrin antibodies experimentally introduced into the cell inhibited endocytosis of SFV (Doxsey et al., 1987). Clathrin-coated vesicules are then uncoated and form endosomes. We have recently explored CHIKV infection route in mammalian cells (M. Solignat, submitted for publication). We addressed the possible involvement of dynamin, a protein required for severing the clathrin-coated pit from the plasma membrane to create an autonomous clathrin-coated vesicle, for transport from the trans Golgi network, as well as for ligand uptake through the caveolae. We found that Dynasore, a potent inhibitor of dynamin GTPase activity significantly impairs CHIKV infection of epithelial HEK-293T cells. To further discriminate between clathrin and caveolae, we examined the effects of a series of well-characterized dominant negative mutants for cellular proteins associated with clathrin-mediated endocytosis or with the caveolar/raft pathway, on CHIKV infection. We found that a DN mutant of the Eps15 protein, known to impede the assembly of clathrin-coated pits without affecting clathrin-independent endocytic pathway, significantly reduces CHIKV infection. Together with observations by Sourisseau et al. (2007), who used RNA interference strategies, these results indicate that the clathrin-dependent endocytic pathway mediates CHIKV entry into human cells. In addition, using lysomotropic agents (chloroquine, monensin, ammonium chloride and Bafilomycine A1) (M. Solignat, submitted for publication) we found that preventing endosome acidification prior to virus exposure dramatically reduced CHIKV infection. Our results confirm and extend the data previously obtained by Sourisseau et al. (2007), who reported that treatment of HeLa cells with Bafilomycin-A1 or chloroquine, inhibited CHIKV replication cycle. Altogether these data indicate that CHIKV requires low endosomal pH to productively infected human cells. We finally questioned the nature of endocytic vesicle required for CHIKV transport through the host cell cytoplasm. Indeed, enveloped viruses that use endocytosis as a route of entry into host cells have different requirement for both early and late endosomes for entry and subsequent infection (Sieczkarski and Whittaker, 2003). The transport, sorting and maintenance of endosomal vesicles along the endocytic pathway are highly regulated by the Rab-family of GTPases. Rab5 associates to early endosomal vesicles, the function of which is mainly to recycle receptors, and is required for the transport of early endosomes along the microtubules. Rab7 is a marker for late endosomes, the main function of which is to deliver the ligand to lysosomes (Schmid et al., 1988) and its activity regulates sorting of cargo from early endosomes toward the late endosome/lysosome pathway. To gain insight into the route of endosomal delivery of CHIKV to human cells, HEK-293T cells expressing either wild type or dominant negative Rab5 or Rab7 proteins were challenged with CHIKV. For example, this strategy was used to demonstrate that early and late endosomes are required for VEEV entry into mosquito cells (Colpitts et al., 2007). Confocal microscopy analysis revealed that disrupting Rab5 function by expression of a dominant negative form of this proteins inhibited CHIKV antigens expression. In contrast, no significant modification in the level of CHIKV expressing cells was evidenced when cells overexpressed a dominant negative form of Rab7. Accordingly, entry of CHIKV in permissive human cells requires the integrity of the early endosome compartment while disruption of the late endosomal compartment has apparently only a very moderate impact on CHIKV infection.

Intracellular replication of CHIKV

Currently, there is no report in the literature describing experiments performed with CHIKV to explore this virus replicative cycle at steps taking place after CHIKV has been routed to the endosomes. Before experimental demonstration became available, general information regarding these steps of CHIKV replicative cycle can be deduced from data obtained with other *Alphaviruses* and strengthened by sequences comparison indicating that amino acid sequences expected to play a major functional role for an *Alphavirus* model are found in the corresponding CHIKV protein.

In *Alphaviruses* the fusion is mainly a function of E1. Inside the endosomes, it is generally admitted that the conformational reorganization of the E1–E2 envelope heterodimer, favoured by low pH, leads to an unstable state of E1 in which its previously buried fusion peptide is transiently directed toward the target membrane. The fusion peptide is located at the tip of the E1 molecule in domain II, close to amino acid 226. Such mechanisms may be conserved for CHIKV according to sequence conservation and similarities evidenced within the ribbon representation of the E1 molecule structure of CHIKV (Schuffenecker et al., 2006). Once delivered to the host cell cytoplasm, *Alphaviruses* capsid protein is able to bind large ribosomal subunit, and this binding reaction could possibly be active in disassembling nucleocapsids (Singh and Helenius, 1992). A binding element located at the C-ter end of the capsid sequence of SINV (Wengler and Wurkner, 1992) is conserved as a PGRRERMCMKIEND motif in CHIKV capsid protein (position 98 to 112 in the S27 strain)

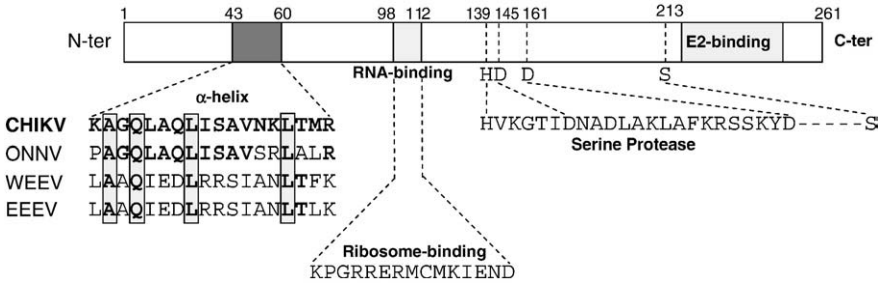

Fig. 5. Schematic representation of the capsid protein with the main interaction domains. Adapted after Perera et al. (2001). The conserved ribosome-binding sequence (amino acids 98 to 112) is indicated. The N-terminal domain α-helix is indicated and the corresponding amino acid sequence is shown for CHIKV and related viruses. The approximate location of the RNA-binding domain (Perera et al., 2001) and E2-binding domain (Hahn et al., 1988) are indicated. The amino acid residues (H139, D-145, D161, S213), that are likely to be involved in the serine protease activity are indicated.

(Fig. 5). *Alphaviruses* replication then proceeds along a two-step pathway. It takes place in the host cell cytoplasm and is associated with membranous structures of the cytoplasm that copurify with the mitochondrial fraction of infected cells (Gomatos et al., 1980). The composition of replication complex changes along infection (Shirako and Strauss, 1994). Early after infection, minus-strand RNAs (only detected at early stage of infection) and plus-strand (synthesized at a constant rate throughout the remainder of infection cycle) are both transcribed under control of non-structural proteins (nsPs). Non-structural proteins of CHIKV (e.g., S27 strain) are encoded by an open reading frame (ORF) of 7424 nucleotide initiated by an ATG at position 77/79 from the 5′ cap site of the 49S non-structural RNA and terminated by a TAG at position 7499/7501. This ORF encodes a polyprotein precursor of 2474 amino acids termed nsP123 that produces the different nsP, once proteolytically cleaved. According to data available for related *Alphaviruses*, after synthesis and maturation, the nsP123 precursor is expected to complex with the free nsP4 protein and host cell proteins to act as a minus-RNA strand replicase catalyzing the synthesis of the negative-strand RNA (Barton, Sawicki, and Sawicki, 1991). In CHIKV (e.g., S27 strain) the nsP4 protein is a 611 amino acids long protein that shows 91% identity with ONNV-nsP4. The presence of a RNA-dependent polymerase motif GDD, common to many RNA-dependent polymerases, near the C-ter at position 464–467 in nsP4 supports a similar function for CHIKV encoded nsP4. Together with nsP4, nsP1 is expected to catalyze the initiation (or continuation) of the negative-strand RNA synthesis. This protein is also involved in methylation and caping of the positive RNA (Mi et al., 1989). Indeed, CHIKV nsP1, a 535 amino acids long protein contains a consensus sequence (Q31-VTPNDHANARAFSHL-A47) at the N-ter region which is characteristic of *Alphaviruses*. The nsP3 protein also participates in the transcription of negative strands early in infection (Wang, Sawicki, and Sawicki, 1994). The nsP3 RNA replicase of CHIKV (e.g., S27 strain), is 530 amino acids long. It has two distinct domains, a N-ter which is conserved among *Alphavirus* (51% amino acid sequence identity at minimum), and a C-ter which varies significantly both in sequence and length among *Alphaviruses* (i.e., 134 amino acid in MIDV up to 246 amino acid for ONNV). Sequences comparison highlighted that the N-ter of nsP3 is related to the family of H2A histone/macro domain that are widely found in bacteria, plants and animals and conserved during evolution (Pehrson, 1998). A recent comparison of CHIKV and VEEV macro domains demonstrated a 57% sequence identity (Malet et al., 2009). Structure of CHIKV nsP3 defined by the same group with a 1.65 Å resolution, revealed that macro domain in nsP3 displays an ADP-ribose binding capacity. This RNA-binding property is enhanced by the presence of positively charged patches of amino acid at the surface of the protein. In addition this domain in CHIKV shares di-phosphoribose 1″-phosphate phosphatase activity with other macro domain-containing proteins. This activity is barely detected for SFV (Egloff et al., 2006). Despite the role of CHIKV macro domain remains elusive, the RNA-binding capacity of nsP3 could assist the recruitment of RNA by non-structural proteins. The non-conserved C-ter domain of nsP3 supports important deletions without apparent changes in the replication potential of the mutant virus, as demonstrated for VEEV (Davis et al., 1989). All *Alphavirus* nsP3 C-ter domains are rich in acidic residues as well as in serine and threonine. Although nsP3 of all *Alphaviruses* are globally negatively charged, it is worth noting that CHIKV and ONNV (−24 and −25 global charge, respectively), contain much more negatively charged amino acids than other members of the same group (−12 and −10 global charge for RRV and SFV, respectively). Using the SINV model, it has been found that nsP3 is heavily phosphorylated on serine or threonine residues, likely by several cellular kinase (Li et al., 1990). Phosphorylation of SINV nsP3 is likely required for efficient RNA synthesis and viral pathogenicity (Vihinen et al., 2001). Although there are several putative sites for phosphorylation within CHIKV nsP3 (S-320 and S-335 amino acids) which are also conserved in ONNV, suggesting that these residues probably control CHIKV nsP3 functions similarly to other alphaviral nsP3 proteins, there is so far no equivalent data available for CHIKV.

During replication, when the concentration of nsP123 in the cell is high enough to support an efficient bimolecular reaction, the precursor is further processed into mature proteins. In CHIKV, three cleavage sites similar to those present in other *Alphaviruses* are found in nsP123. To the exception of the nsP2/nsP3 cleavage site in WEEV and EEEV sequences, *Alphaviruses* nsP precursor cleavage sites are characterized as follows: the C-ter ($n-1$) residue is usually an amino acid with a small side chain (i.e., alanine, cysteine, glycine), the $n-2$ residue is invariably a glycine that is required for nsP2 catalytic activity and the $n-3$ is most frequently an alanine (Fig. 6). $n-2$ glycine and $n-3$ alanine residues are conserved in CHIKV sequence.

The fully cleaved precursor complexed together with host cell proteins act as a plus-strand RNA replicase to amplify the full-length subgenomic (26S) positive-strand mRNA using the negative-strand RNA as a template (Shirako and Strauss, 1994). Both steps are regulated by helicase and proteinase functions of nsP2 protein whose proteolytic cleavage plays a pivotal role in the viral replication process. The nsP2 of CHIKV (e.g., S27 strain) is a 798 amino acids long positively charged protein (net positive charge of +21). The degree of amino acid identity between nsP2 of CHIKV and other *Alphaviruses* ranges from 56% for WEEV up to 98% for ONNV. This protein contains a proteinase motif (CWA) at position 478–480 of nsP2 (Fig. 6). For SINV, the C481 residue is required for protease activity and forms the catalytic dyad together with W558 (Hardy and Strauss, 1989; Strauss and Strauss, 1994). In addition, the nucleoside triphosphate-binding motives (GVPGSGKS and DEAF) characteristic for *Alphaviruses* replicases are located in the N-ter of CHIKV nsP2 at position 186–193 and at position 252–255, respectively. Accordingly, although unproven experimentally, CHIKV nsP2 should retain both a protease

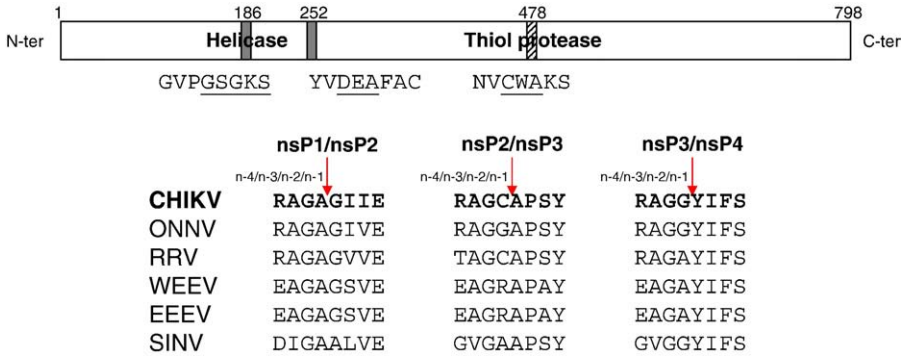

Fig. 6. Schematic representation of the non-structural protein nsP2, showing the location of the consensus sequences for the helicase and the proteinase, respectively. The sequence used for numbering is CHIKV sequence (Khan et al., 2002). The proteinase cleavage sites in the nsPs (nsP1/nsP2, nsP2/nsP3, and nsP3/nsP4), are shown in the lower panel (sequence of CHIKV is from Khan et al. (2002); the other sequences are from Strauss and Strauss (1994).

activity that catalyzes the cleavage of the precursor polypeptide to yield the mature nsP proteins and a RNA helicase function.

The subgenomic positive-strand mRNA of *Alphaviruses*, referred to as 26S RNA, serves as the mRNA for the synthesis of the viral structural proteins. For CHIKV, the ORF encoding the structural proteins is composed of 3735 nucleotides with an initiation at position 7567 and termination at position 11,299. This ORF encodes a polyprotein of 1244 residues from which the individual structural proteins are formed. This precursor is processed co-translationally and post-translationally into structural proteins C, PE2 and E1, and a small peptide termed 6K (that may act as a signal sequence for the translocation of E1). The capsid protein of CHIKV is 261 amino acids long. This protein of apparent molecular weight of 30 kDa, consists of an N-ter region of unknown function and poorly conserved among *Alphaviruses* except in a short region (43-KAGQLAQLISAVNKLTMR-60) predicted to play a role in the assembly of nucleocapsid cores, and of a C-ter region showing a conserved autoprotease domain (Fig. 5). For other *Alphaviruses* this later domain retains a serine protease activity acting in cis to release itself from the nascent polypeptide chain (Strauss and Strauss, 1990). The 3-D structure of the SINV capsid protein has been solved and it was found that the C-ter region folds homologous to that of chymotrypsin (Choi et al., 1991). It has been reported that residue S215 is the catalytic serine of the proteinase and that other important amino acids are H141, D147 and D161 (Hahn and Strauss, 1990). By comparing the SINV capsid sequence (264 amino acids) to the slightly shorter CHIKV capsid sequence (261 amino acids) (Khan et al., 2002), it can be identified as H139, D145, D161 and S213, suggesting that these conserved amino acid may be involved in CHIKV capsid autoprotease activity. Once the nucleocapsid protein is released from the nascent polypeptide chain, an N-terminal signal sequence leads to the insertion of glycoprotein PE2 into the endoplasmic reticulum. The signal sequence has a carbohydrate attachment site at an asparagine residue between residues 11 and 14 in all *Alphaviruses* examined (residue 12 in CHIKV S27 strain; PE2 start by: 1-SLAIPVMCLLAN-12), and it has been postulated that the addition of carbohydrate to this site is responsible for the retention of the signal sequence in PE2 (Garoff et al., 1990). With respect to observations made using *Alphaviruses* other than CHIKV (Strauss and Strauss, 1994), the cleavage between PE2 and 6K, catalyzed by signalase in the lumen of the ER, gives rise in CHIKV to a 6K protein of 61 amino acids long.

CHIKV assembly, budding, and maturation

Almost nothing is known regarding these steps of the virus replication cycle for CHIKV, and very few information can be extrapolated from sequence comparison between CHIKV envelope proteins and the corresponding proteins of other *Alphaviruses*. The E1 protein of CHIKV contains 435 amino acids. This protein of apparent molecular weight 44 kDa, has one possible glycosylation site (N-X-S/T, were X is any amino acid except proline), at position 141–143 (N-141, I-142, T-143, respectively). The E1 amino acid of CHIKV shows 88% identity to ONNV-E1. Interestingly, residue C-433 in the E1 of SFV which has been considered as a target for palmitoylation is conserved in CHIKV. Information gained from SFV indicates that after being transported to the trans Golgi, the PE2–E1 heterodimer moves to the cell surface. During this step, PE2 is cleaved by a cellular furin or furin-like proteinase to form E2 and E3 (de Curtis and Simons, 1988). The PE2 precursor has been clearly identified in the case of CHIKV as being a 65 kDa protein (Simizu et al., 1984). Quite recently, it has been reported that the PE2 of CHIKV can be cleaved by furin and the PC5A convertase. Inhibition of PE2 processing can be achieved by the furin inhibitor decanoyl-RVKR-chloromethyl ketone (Ozden et al., 2008). By SDS-PAGE analysis, it was shown that the E2 protein migrates faster than the E1 protein. Moreover, tunicamycine treatment of producer cells resulted in a higher electrophoretic mobility observed for PE2 and E1, supporting that both proteins are glycosylated (Konishi and Hotta, 1980; Simizu et al., 1984). The E2 protein contains 423 amino acids and shows 82% amino acid identity with ONNV E2 protein. This protein of apparent molecular weight 43 kDa has two possible glycosylation sites, at positions 263 and 345, Three putative palmitoylated cysteine residues are strongly conserved at the C-terminus of E2 for CHIKV and other *Alphaviruses* (Ivanova and Schlesinger, 1993; Schmidt et al., 1988) suggesting that these modifications are critical for protein function. Fatty acylation of the corresponding cysteine residues is expected to be responsible for translocation of E2 from the lumina to the cytoplasmic side of the endoplasmic reticulum, as described for SINV (Ivanova and Schlesinger, 1993). The E3 protein of CHIKV consists of 64 amino acids and shows an apparent molecular weight of 11 kDa. E3 of CHIKV, is not associated with virions but is released from infected cells (Simizu et al., 1984). In addition to the four conserved cysteine residues present in E3 proteins of *Alphaviruses*, E3 from CHIKV contains an additional cysteine/proline rich PPCIXCC sequence also present in E3 proteins from 50% of *Alphaviruses*, including that from ONNV (Parrott et al., 2009). The function of CHIKV E3 protein has not been studied so far, yet it could share disulfide isomerase activity recently described for SINV E3 protein that could be involved in proper folding and disulfide bound formation in viral envelope glycoprotein spike formation (Parrott et al., 2009).

Alphavirus virion assembly starts with nucleocapsid assembly in the cytoplasm. The nucleocapsid of *Alphaviruses* is characterized by an icosahedral symmetry (with T = 4 symmetry) and consists of the viral RNA encapsidated with 240 copies of the C protein. Nucleocapsids of related viruses are known to contain a putative coiled-coil α-helix important for core assembly that is conserved in CHIKV C protein (Perera et al., 2001) (Fig. 5). Deletion analysis of SINV capsid showed that the N-ter region extending from amino acid 76 to 107 is also essential for full-length progeny RNA-binding and packaging (Geigenmuller-Gnirke, Nitschko, and Schlesinger, 1993). From the same model, it has been shown that the RNA region between residues 746 and 1226 is specifically bound by the nucleocapsid (Strauss and Strauss, 1994; Weiss et al., 1989). Thus, the genomic RNA-binding region overlaps the region required for binding to the large ribosomal subunit. The capsid protein probably forms RNA-bound dimers initiated by interactions through residues 81–261 and next the coiled-coil interactions mediated through the α-helix stabilize the nucleic-acid bound dimers. The lateral interactions with other capsid proteins determine the structure of the icosahedral shell. A total of 120 copies of these dimers further oligomerize to form the nucleocapsid of CHIKV (Perera et al., 2001). Nucleocapsid complexes assembled in the cell cytoplasm are thought to diffuse freely to the plasma membrane.

Alphaviruses bud through the cell membrane. Virions acquire a lipid bilayer envelope containing the virus-encoded E1 and E2 glycoproteins. The mature envelope glycoprotein spikes are composed of trimers of E1–E2 (Ekstrom, Liljestrom, and Garoff, 1994). The envelope of *Alphaviruses* contains 240 copies of two virus-encoded glycoproteins, E1 and E2, organized into 80 trimers of E1–E2 heterotrimers (Fuller, 1987). During budding, the nucleocapsid appears to undergo a maturation event and requires binding to E2 for appropriate targeting to the cell membrane (Suomalainen, Liljestrom, and Garoff, 1992). Using RRV-SINV chimeras it was shown that these capsid-E2 interactions involved residues within the 33 amino acids of E2 cytoplasmic domain (RRV: 390-TARRKCLT-PYALTPGAVVPLTLGLLCCAPRANA-422) (Lopez et al., 1994); a similar sequence: RRRCITPYELTPGATVPFLLSLICCIRTAKA is found in CHIKV (Fig. 7). As previously discussed, WEEV arose by recombination between EEEV and the SINV (Hahn et al., 1988; Weaver et al., 1997). The recombinant virus contains the glycoprotein of the SINV-like parent but the nucleocapsid protein of the EEEV parent. Changes were evidenced in both capsid and E2 of WEEV likely as a consequence of a

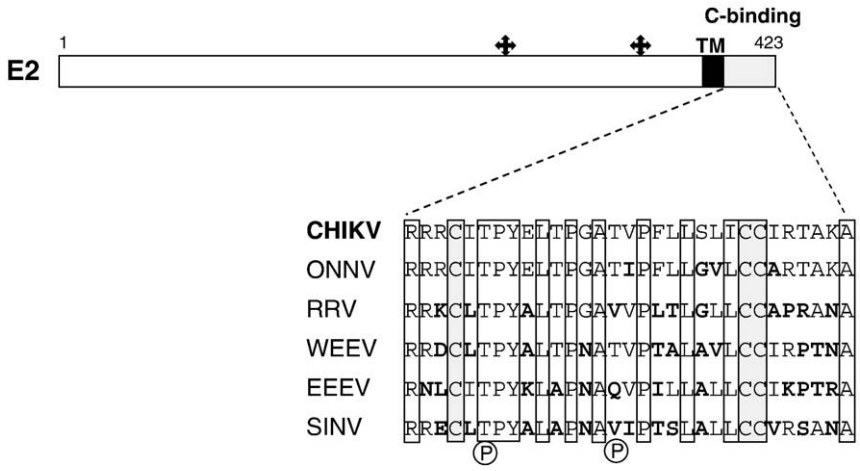

Fig. 7. Schematic representation of the E2 envelope glycoprotein. Adapted after Zhao et al. (1994). The amino acid sequences of the E2 cytoplasmic domain from different viruses are shown. The sequence of CHIKV is from Khan et al. (2002) (NCBI, accession no. AF369024). The Cysteine residues (C) are believed to be palmitoylated and this could serve to anchor the E2-tail at the surface of the inner membrane. The NetPhos sequence analysis (www.Cbs.dtu.dk/services/NetPhos/) predicts that threonines (T) are likely to be phosphorylated (Ⓟ) whereas the tyrosine (Y) is probably not. A stretch of highly conserved residues TP, is characteristic of an ERK2 phosphorylation site.

selective process to adapt these two proteins obtained from different parents to one another. In the C-ter cytoplasmic domain of E2, there are 8 amino acid differences between WEEV and SINV among which 4 are changed to the EEEV sequence.

The nucleocapsid, E1 and E2 glycoproteins are phosphorylated (Liu et al., 1996; Waite et al., 1974). At least some of these phosphorylations might play a role in the assembly and/or post-assembly steps since the use of protein kinase or phosphatase inhibitors interfere with viral maturation (Liu and Brown, 1993). In the presence of calmodulin/Ca2+ dependent protein kinase inhibitor (i.e., W7 inhibitor), there is an aberrant assembling of nucleocapsid units which form high ordered complexes into the cell cytoplasm but fail to participate to virus particles formation at the cell membrane (Liu and Brown, 1993). Finally, phosphorylation of T398 and Y400 residues is considered important for cellular subcompartment translocation and assembly of virions (Liu et al., 1996). To date, the final stages of the CHIKV replication cycle have not been investigated and most assumptions from other *Alphavirus* models remain to be tested. We have observed CHIKV budding in electron microscopy experiments. As shown in Fig. 8, HEK-293T human cells (Figs. 8A and B) chronically infected with CHIKV show evidence for budding of small viral particles at the surface of infected cells. It is worth noting that only a low percentage of particles show high electron density characteristic for maturation. A similar investigation performed using the

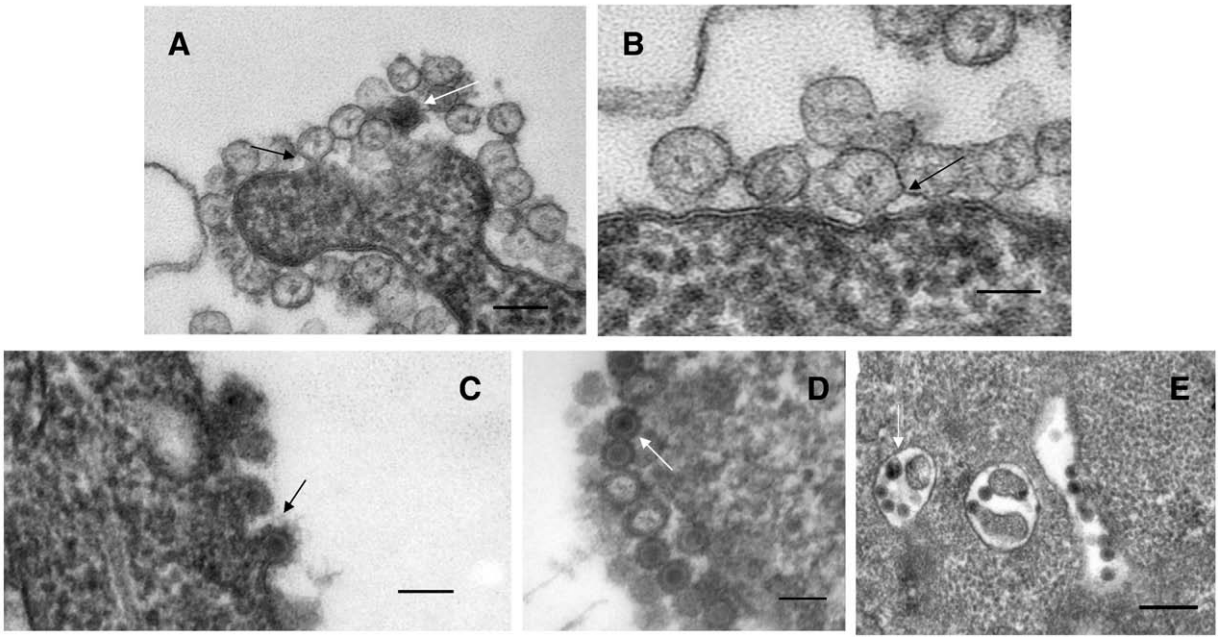

Fig. 8. HEK-293T cells (A and B) chronically infected with CHIKV were fixed with glutaraldehyde 48 h after infection and processed for thin-layer electron microscopy. Panel A, shows evidence of budding and the presence of an electron-dense mature particle (white arrow). Most of the particles (size ranging from 45 to 80 nm) are characterized by low density material bordered with a membrane bilayer (black arrow). The bar corresponds to 100 nm. Panel B illustrates the budding of larges particles with low density material at the cell membrane (black arrow). The bar corresponds to 100 nm. Similar experiments were performed using BHK-21 cells (C, D, and E) chronically infected with CHIKV. Panel C shows the presence of several electron-dense particles budding at the cell surface. The bar corresponds to 100 nm. Panel D, illustrated the massive production of particles from BHK-21 cells. The bar corresponds to 90 nm. Two different types of particles can be observed, viral particles of about 65 nm with an electron-dense capsid of 32 nm (white arrow) and particles of about 76 nm showing only a small electron-dense material in the middle very similar to the most frequent particles observed in CHIKV-infected HEK-293 T cells. Panel E illustrates one example of the presence of CHIKV particles within cellular vacuoles. Particles found within the endosomes appear to be electron-dense. The bar corresponds to 300 nm.

chronically infected BHK-21 cells (Figs. 8B and C) showed the budding of viral particles with electron-dense capsids and the production of higher proportions of mature particles. The mature CHIKV particles were also observed within endosomal vesicles (Fig. 8E), probably during the propagation of the virus to neighbouring cells. It has been suggested that the envelope of the mature *Alphavirus* particle must be derived from a segment of the plasma membrane from which all host cell proteins are excluded (Garoff and Simons, 1974; Strauss, 1978). This contrast with other enveloped RNA viruses which acquire envelope by budding at the cell surface, such as HIV for which the presence of cellular compound associated to virions has been well-documented. Cellular materials such as CD3, CD4, CD5, CD11a, CD18, CD25, CD30, CD43, CD54, CD63, CD71, HLA class I, and HLA-DR have been detected as integral components of cell-free HIV-1 virions or contaminants that are difficult to remove (Arthur et al., 1992; Benkirane et al., 1994; Briant et al., 1996; Hildreth and Orentas, 1989). It is therefore likely that further investigations using highly sensitive methods, would demonstrate the presence of cellular compounds associated to CHIK virions. Similarly, despite it was concluded from preliminary results, that no kinase activity was incorporated into *Alphaviruses* (Waite et al., 1974) low kinase activity has been found specifically associated with viral preparations of SINV and SFV ultrapurified by co-sedimentation in sucrose (Tan and Sokol, 1974). In addition, protein phosphatase activity is also incorporated into *Alphaviruses* particles (Tan and Sokol, 1974). In other enveloped RNA viruses, such as retrovirus HIV-1 and HTLV-1, we and others have demonstrated that cellular kinases, active during budding or packaged into the virus particle, play essential role in the replicative cycle of the virus (Cartier et al., 2003; Hemonnot et al., 2004; Hemonnot et al., 2006). The presence of cellular protein kinase in CHIKV particles would merit to be addressed.

According to this sum of sequence-derived information, a synthetic view of the possible CHIKV replicative cycle can be drawn as presented in Fig. 9.

Prospects for treatment of CHIKV infection

Experiments to improve our understanding of CHIKV genomic organization, cell tropism/cell surface receptor(s) binding, and the different steps of the virus replication cycle are ongoing. This understanding is an important prerequisite for evaluating models to predict future CHIKV evolution and then develop novel therapies and vaccination strategies to reduce disease incidence. This review summarizes the most recent knowledge about human host cells–CHIKV interactions. Based on the increasing sum of information recently gained on the CHIKV replication cycle, therapeutic strategies aiming at inhibiting replication and controlling propagation in infected humans should be soon evaluated in humans. Some of these promising strategies are discussed below.

Togaviruses are known to stimulate interferon production in the host cell and interferon impacts viral replication in return. It has been reported a long time ago, that priming of target cells with low doses of interferon before infection with CHIKV impacted on the virus replication cycle (Paucker and Boxaca, 1967) while treatment of infected cells with the interferon inhibiting agent actinomycin D, enhances CHIKV replication (Gifford and Heller, 1963; Heller, 1963). Very recent observations performed in a mouse model knocked out for IFNα/β receptors genes, demonstrated that the severity of CHIKV infection is critically dependent on the functionality of type-I IFN signalling (Couderc et al., 2008). Moreover, differences in TNF-signalling capacities were proposed as the basis for discrepancies observed for CHIKV sensitivity between adult and neonates mice. Despite the synergistic inhibition of *in vitro* CHIKV replication has been documented for α-interferon and ribavirin combination, Briolant et al. (2004) interferon treatment has not been evaluated *in vivo* against CHIKV, to the best of our knowledge. However, whatever the *in vivo* effect of interferon against CHIKV in humans, it would be difficult to use for treatment of CHIKV-infected patients in the case of an epidemic episode.

Additional therapeutic strategies may consist in boosting immune responses against specific CHIKV sequences. Indeed vaccination

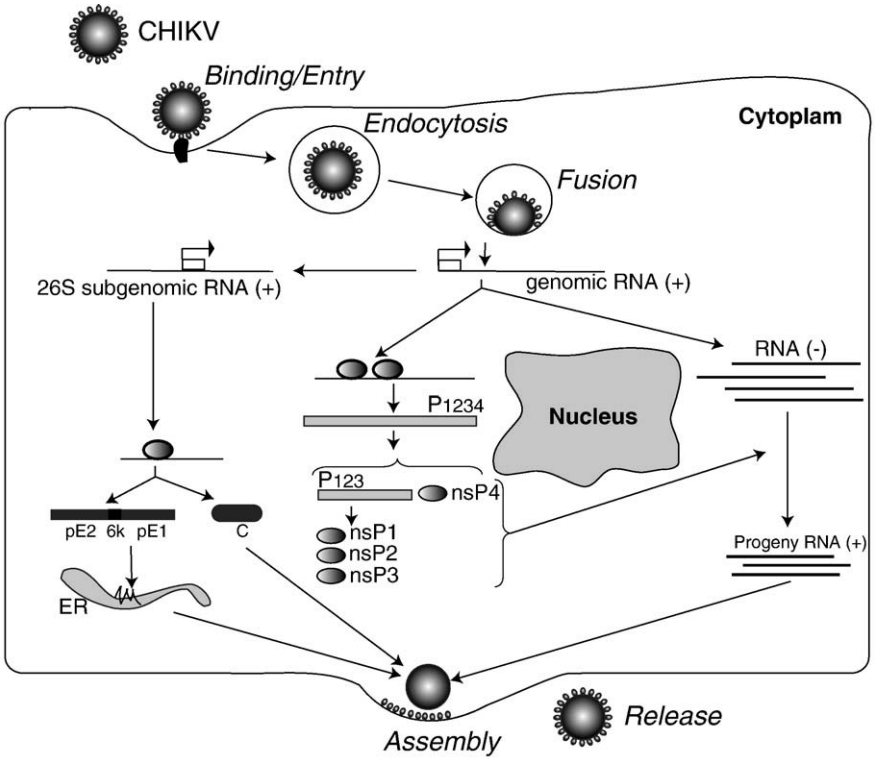

Fig. 9. Summary of the CHIKV replication cycle. The different steps of the CHIKV replication cycle are described in detail in this review.

strategies against CHIKV have been developed in the past 30 years. The most important mechanisms by which the immune system protects an animal from viral diseases are the neutralization of virus infectivity by the humoral response (neutralizing antibodies), and virus-infected cell lysis by the cellular response (cytoxic T cells). Although very few data has been accumulated regarding anti-CHIKV antibody response, special attention has been devoted, on antibodies able to neutralize *Alphaviruses*. In response to SINV infection, the neutralizing antibody response is more frequently directed against the glycoprotein E2 rather than against E1 (Strauss et al., 1991). Following infection with RRV, neutralizing epitopes in E2 have also been described (Vrati et al., 1988) supporting that neutralizing antibodies directed against E2 may apply to many *Alphaviruses*. It is not our purpose to review the development of vaccine against CHIKV in this review. However, it is worth noting that studies performed in order to design a protective vaccine against CHIKV provide indications that neutralizing epitopes are shared between different strains of CHIKV (Eckels, Harrison, and Hetrick, 1970; Edelman et al., 2000; Harrison et al., 1967, 1971; Kitaoka, 1967; Levitt et al., 1986; White et al., 1972). The capsid protein of CHIKV has been shown to represent a dominant target for CD8 lymphocytes in mouse (Linn et al., 1998). H-2Kd restricted *Alphavirus*-specific CTL that recognize the QYSGGRFTI sequence of CHIKV capsid protein (a region believed to be involved in binding to the surface glycoprotein E2), can clear a cytopathic CHIKV infection from a persistent and productively infected macrophage culture (Linn et al., 1998). If the target of anti-CHIKV CTL activity was located to a small region of the capsid protein in humans, this might restrict the ability of individuals to generate CTL only to certain persons with the appropriated HLA genotype. Pertaining to this point, it has been reported that patients who rapidly recovered from RRV disease have developed a strong anti-RRV CD8+ CTL response (Fraser et al., 1983).

Since enveloped viruses utilize membrane-bound proteins to mediate attachment and entry into specific target host cells, peptides that mimic portions of their envelope glycoproteins can likely inhibit infectivity. This has been previously demonstrated for several virus including HIV, DENV, and WNV (Hrobowski et al., 2005; Qureshi et al., 1990). Pertaining to this point, it is important to emphasize that cell surface receptors not only permit the enveloped virus to attach cells but can also serve to deliver aberrant signals to the target cell as demonstrated for several enveloped viruses including HIV and WNV (Briant et al., 1998; Glass et al., 2006; Lee et al., 2006; Roggero et al., 2001). Considering the requirement for envelope protein rearrangement during virus entry and the role played by disulfide bonds during this process, membrane impermeant sulfhydryl reagents capable of blocking thiol-disulfide bonds, were analysed for antiviral properties and partial inhibition of SFV and SINV was observed for the 5-5′-dithio-bis (2 nitrobenzoic acid), DTNB thiol-blocker (Glomb-Reinmund and Kielian, 1998).

We have previously discussed herein the fact that entry of CHIKV into the cell cytoplasm likely occurs by endocytosis in clathrin-coated vesicles followed by transfer to endosomes where the low pH leads to a conformational reorganization of the E1–E2 heterodimer such that the fusion domain in E1 is exposed, and the virus envelope fuses with the endosomal membrane. In the past, chloroquine (a quinoline that displays anti-inflammatory properties and is mainly known as the antimalarial Nivaquine) has been assayed for treatment of chronic CHIKV arthritis (Brighton, 1984). Due to its main effect—i.e., raising endosomal pH—the drug also has an exceptionally broad spectrum of antimicrobial activity allowing inhibition of viruses from different families which require a pH-dependent step for entry. Another possibility is that glycosylation inhibition (inhibition of biosynthesis of sialic acid moieties that are present on viral envelope glycoproteins) might represent an alternative mechanism for the antiviral effect of chloroquine (Savarino et al., 2006). The anti-CHIKV effect of chloroquine was confirmed by *in vitro* experiments performed by members of the Sentinelles France's national disease surveillance network on chikungunya (de Lamballerie and Canard, 2006). However, the double blind placebo-controlled clinical trial performed on the Reunion Island that included 27 CHIKV-infected patients who received Nivaquine at 10 mg/kg during three days followed by treatment at 5 mg/kg was not convincing enough to justify further investigations of the potential use of this molecule in managing acute CHIKV infection (De Lamballerie et al., 2008). A thorough understanding of the routes used by this virus to infect its target cells may help to design new antiviral strategies that break the human/vector infection cycle. One should expect that *in vitro*, *in silico*, *ex-vivo*, and *in vivo* screening of drugs directed against the different CHIKV molecules should rapidly provide candidates for treatment of patients. Systematic screening of FDA approved molecules to be adapted for new prescription either isolated or as multicomponent therapies (Borisy et al., 2003), may represent an interesting strategy. For example, nsP2 that belongs to the highly conserved superfamily of proteins containing nucleoside triphosphate-binding domains (Gorbalenya et al., 1989) may represent an attractive target. Vidalain et al. (2007) have recently reported interaction of nsP2 with cellular transcription factors using a 2-hybrid screening strategy. Based on these results, a bank of 35,000 chemical compounds is currently under screening to select inhibitors of CHIKV. Similarly, the recent characterization and structure resolution of CHIKV nsP3 macro domain (Malet et al., 2009) combined to the high degree of conservation of these motives in bacteria (Pehrson and Fuji, 1998) opens the way to the evaluation of the antiviral potential of available anti-bacterial compounds and subsequently to a rational structure-based optimization. Finally, several possibilities also exist that may consist in inhibiting CHIKV assembling and/or budding. Interestingly, peptides that mimic the E2 tail of *Alphaviruses*, inhibit virus production probably by preventing C-E2 interactions (Collier et al., 1992). More recently it was shown that decanoyl-RVKR-chloromethyl ketone, a furin inhibitor, blocked the cleavage of PE2 into E2 and E3 and thereby inhibited CHIKV spreading in myoblast cell cultures (Ozden et al., 2008).One can expect that among these research some will open new avenues for anti-CHIKV therapy.

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