Inhibition of Chikungunya Virus Infection in Cultured Human Muscle Cells by Furin Inhibitors

IMPAIRMENT OF THE MATURATION OF THE E2 SURFACE GLYCOPROTEIN*

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Chikungunya virus (CHIKV) is a mosquito-transmitted Alphavirus that causes in humans an acute infection characterized by polyarthralgia, fever, myalgia, and headache. Since 2005 this virus has been responsible for an epidemic outbreak of unprecedented magnitude. By analogy with other alphaviruses, it is thought that cellular proteases are able to process the viral precursor protein E3E2 to produce the receptor-anchored E2 protein that associates as a heterodimer with E1. Destabilization of the heterodimer by exposure to low pH allows viral fusion and infection. We show that among a large panel of proprotein convertases, membranous furin but also PC5B can process E3E2 from African CHIKV strains at the HRQRRL64 § ST site, whereas a CHIKV strain of Asian origin is cleaved at RRQRRL64 § SI by membranous and soluble furin, PC5A, PC5B, and PACE4 but not by PC7 or SKI-1. Using fluorogenic model peptides and recombinant convertases, we observed that the Asian strain E3E2 model peptide is cleaved most efficiently by furin and PC5A. This cleavage was also observed in CHIKV-infected cells and could be blocked by furin inhibitor decanoyl-RVKR-chloromethyl ketone. This inhibitor was compared with chloroquine for its ability to inhibit CHIKV spreading in myoblast cell cultures, a cell-type previously described as a natural target of this virus. Our results demonstrate the role of furin-like proteases in the processing of CHIKV particles and point out new approaches to inhibit this infection.

Chikungunya virus (CHIKV)† is a mosquito-transmitted Alphavirus belonging to the family Togaviridae, which was first reported in 1952 in Tanganyika. It is responsible for an acute infection of abrupt onset characterized by high fever, polyarthralgia, myalgia, headache, chills, and rash (1). The symptoms are generally of short duration (1 week), and recovery is often complete, although some patients have recurrent episodes for several weeks after infection (1, 2). CHIKV is endemic in Africa, India, and Southeast Asia and is transmitted by Aedes mosquitoes through an urban or sylvatic transmission cycle. In 2006 an outbreak of CHIKV fever occurred in numerous islands of the Indian Ocean (the Comoros, Mauritius, Seychelles, Madagascar, La Réunion, etc.) before jumping to India where an estimated 1.4 million cases have been reported (3–5). More recently, imported infections have been described in Europe, and around 200 endemic cases have been reported in Italy (6). Clinically, this CHIKV epidemic was accompanied by more severe symptoms than previous outbreaks, with reports of severe polyarthralgia and myalgia, complications, and deaths. In muscle biopsies from two infected adults we could identify cellular targets for CHIKV as muscle satellite cells (the muscle progenitor cells) and confirmed in vitro the susceptibility of these cells to CHIKV infection (7). Up until now no other cell targets have been identified in humans except for a recent report showing that fibroblast cells were infected in a fatal neonatal case (8). Other cell types such as human epithelial and endothelial cells, primary fibroblasts, and to a lesser extent, monocyte-derived macrophages, were shown in vitro to be sus-

* The abbreviations used are: CHIKV, Chikungunya virus; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; SARS, severe acute respiratory syndrome virus; PC, proprotein convertase; dec-RVKR-cmk, deca-noyl-RVKR-chloromethyl ketone; wt, wild type; Abz, 2-aminobenzoxyc acid; Tyx, 3-nitrotyrosine; Mes, 4-morpholineethanesulfonic acid; Fmoc, fluore- nyl 9-methoxy carbonyl; m.o.i., multiplicity of infection; Q, quenched.

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Processing of Chikunguya Virus Envelope Proteins by Furin

TABLE 1
Oligonucleotides used to clone E3E2 and perform site-directed mutagenesis

| Primer name | Primer sequence |
|-------------|-----------------|
| E3-For      | 5’-GGGGAACACCTTTTGACGAAAAAGTGGCATATCTTGGATCCAGATGGAATGCTGGTCCATAC-3’ |
| E2-Rev      | 5’-GGGGAACACCTTTTGACGAAAAAGTGGCATATCTTGGATCCAGATGGAATGCTGGTCCATAC-3’ |
| H60R-For    | 5’-ATGGTTCTCTCCCCCGCCCGAGCACAGCA-3’ |
| H60R-Rev    | 5’-TTTGCCCTGTGGGCGCCGCGAGACAC-3’ |
| R63A, R64A-For | 5’-TTTCCCTCCGGCGCAAGCCACCGGACAACTTCAATG-3’ |
| R63A, R64A-Rev | 5’-AAATGGATTTTCTTGTTCGTCCCCTGGCGCGTGGAGAA-3’ |

**Experimental Procedures**

**Opening Reading Frame Cloning and Plasmid Constructs**—E3E2-encoding sequence from CHIKV wild-type strain 05.115 (GenBank accession number AM258990) was amplified by reverse transcription-PCR (Titan One tube; Roche Applied Science) from purified genomic RNA and cloned using a recombination-based cloning system (Gateway system; Invitrogen) into pDONR207 (Invitrogen). PCR primers E3-For and E2-Rev (Table 1) were used to amplify and clone the E3E2 sequence. 5’ extremities of E3-For and E2-Rev primers contained attB1.1 and attB2.1 sites, respectively. These sequences allowed recombination of PCR products into pDONR207 following the manufacturer’s recommendations (BP cloning reaction, Invitrogen). Constructs were transformed and amplified in Escherichia coli DH5α strain. To mutate E3E2 wild-type sequence and introduce H60R or R63A and R64A substitutions, two-step PCRs were carried out. Two PCR fragments overlapping at the editing site were amplified from E3E2 wild-type sequence using either forward or reverse mutagenic primers (Table 1) in combination with appropriate E3-For or E2-Rev primers. Then the PCR fragments were mixed, and a ligating PCR was performed using only the attB-containing primers.

A previously reported (26) derivative of the mammalian expression vector pIRE2-EGFP (Clontech) that contains a V5 epitope downstream of the multicloning site (pIRE2-EGFP+V5) was made compatible with the Gateway® technology using the Gateway Vector Conversion system (Invitrogen). Briefly, pIRE2-EGFP+V5 was linearized with BstEl and blunted before cloning the Gateway cassette frame B. Wild-type E3E2 sequence or corresponding mutants were shuffled from pDONR207 in this vector by in vitro recombination following the manufacturer’s recommendations (LR cloning reaction, Invitrogen). The P2′ T66I mutant was obtained by PCR as described earlier (26).

**Drug and Peptide**—The peptide dec-RVKR-cmk; Bachem) was dissolved at 5 mM in DMSO and used at concentrations of 50 or 100 μM. Chloroquine was purchased from Sigma-Aldrich and used at concentrations of 1 or 2 μM.

**Proprotein Convertases, Cells, and Metabolic Labeling**—For radiolabeling experiments, HEK293 cells were transiently cotransfected with 1 μg of a pIRE2 recombinant CDNA of CHIKV E3E2 tagged at the C terminus with a V5 epitope together with either 1 μg of empty vector or a similar vector coding for the convertase furin, a soluble form of furin (sFurin, lacking the transmembrane domain), PACE4, PC5A, PC5B, PC7, or SKI-1, similar to the SARS-CoV construct previously.

cceptible to infection and allowed viral production with a cytotoxic effect (9).

Treatment of CHIKV-induced fever is only symptomatic, including use of paracetamol or non-steroid anti-inflammatory drugs (2). To date only a limited open pilot study has been carried out with chloroquine as a treatment of chronic Chikungunya arthritis on 10 patients, with improvement in symptoms such as morning stiffness (10) and another where a single patient did not respond to chloroquine (11). More recently, an unsuccessful clinical trial with chloroquine has been performed during the CHIKV outbreak in La Réunion but with chloroquine doses that are thought to be below the efficient concentrations (12). However, a recent paper demonstrated an effect of chloroquine on CHIKV infection in vitro in HeLa cells (9), in agreement with previous reports dealing with the inhibitory effect of chloroquine on other Alphavirus infections in vitro (13–15).

Besides chloroquine, infection by alphaviruses can be inhibited in vitro by blocking the intracellular cleavage of viral envelope glycoproteins. Alphavirus envelope glycoproteins are initially produced first as precursors (E3E2 or p62) and during virion maturation further cleaved at short multibasic motifs. Among cellular proteases, the basic amino acid–specific furin or furin-like proprotein convertases (PCs) have been shown to be involved in such a Ca2+-dependent processing, resulting in the cleavage of surface glycoproteins at the C terminus of the consensus sequence (K/R)X(K/R)R ↓ (16–18). In the trans–Golgi network (TGN) or in a post-TGN compartment, E3E2 is cleaved close to its N terminus to form E2 and a residual peptide E3 (19). Amino acid changes within the cleavage site have been shown to induce defects in viral production due to the presence of uncleaved E3E2 precursor, as demonstrated for Sindbis virus (20–22). Such a block in infection could also be produced for Semliki Forest virus (23), and exposure of the virus to very low pH restores infectivity (24). In the present paper we show that among a large panel of PCs, furin, but also other furin-like proteases, can process CHIKV E3E2 in vitro and in vivo. Furin can best process synthetic peptides mimicking the cleavage site. We also investigated the inhibitory effect of an irreversible furin-inhibiting peptide decanoyl-RVKR-chloromethyl ketone (dec-RVKR-cmk) (25) on CHIKV infection of a natural cellular target, i.e. human muscle satellite cells. We demonstrate that dec-RVKR-cmk is able to induce a stronger inhibition of viral infection than chloroquine when added just after infection. Moreover, combination of both drugs induces an additive effect, which is enhanced when drugs are added before infection. Besides demonstrating a new way to inhibit CHIKV infection in natural cellular targets, these data demonstrate for the first time the role of furin-like proteases in the processing of CHIKV particles.
reported (27). In the case of the furin co-transfection, the cells were either incubated with or without 10 μM chloroquine (Sigma) for 24 h before transfection. The cells were then labeled with [35S]Cys + Met for 4 h, and the cell lysates in radioimmune precipitation assay buffer were immunoprecipitated with a V5-specific monoclonal antibody (mAb-V5). The immunoprecipitates were resolved on an 8% SDS-PAGE gel, dried, and autoradiographed (28).

**Virus and Infection of Myoblasts**—CHIKV was obtained from a clinical sample from La Reunion outbreak in 2005 as previously described (29). Virus was propagated twice in insect C6/36 cells, and supernatants were frozen at −80 °C and titrated (see below). Muscle cells were infected at a multiplicity of infection (m.o.i.) of 10 to 10−2 according to the experiments. Infection was performed for 2 h at 37 °C in serum-free medium before washing the cells twice with culture medium.

For viral infections, immortalized cultures of human myoblasts were used. Immortalized cultured myoblasts consisted of a human myogenic cell line, called LHCN-M2, that had previously been obtained by introduction of human telomerase and cyclin-dependent protein kinase 4 (30). Cells were cultured in T25 flasks until confluence (7–10 days) before plating. Cells were cultivated in Ham’s F-10 medium supplemented with 50 μg/ml gentamycin and 20% fetal calf serum and trypsinized when confluence was reached before plating under the same conditions as above. Viral samples were titrated as TCID50/ml on Vero cells, as previously described (9).

**Immunofluorescence**—After infection/treatment of muscle cell cultures, samples were fixed at 15 h post-infection in 4% paraformaldehyde and processed for immunofluorescence using mouse hyperimmune ascitic fluids raised against CHIKV as previously described (7, 29).

**Western Blotting**—Muscle cell cultures were infected (m.o.i. 0.1) for 2 h and washed with medium, and dec-RVKR-cmk or chloroquine (100 and 2 μM, respectively) was then added separately or together. Supernatants were harvested 16 h later, and lysis was performed in 0.5% Nonidet P-40, 20 mM Tris-HCl, pH 8, 120 mM NaCl, and 1 mM EDTA supplemented with the Complete Protease Inhibitor Mixture from Roche Applied Science. CHIKV E2 protein was detected in protein extracts using previously described anti-E2 monoclonal antibody (31) and standard immunoblotting techniques.

**Peptide Synthesis and Kinetic Parameters**—All intramolecularly quenched fluorogenic (IQF) peptides were synthesized by Fmoc-based solid phase chemistry using automated multipeptide synthesizer instrument (Intavis, Germany). For efficient incorporation of two unnatural amino acids, namely Abz (2-aminobenzoic acid, a fluorescent moiety) and Txv (3-nitrotyrosine, a fluorescence quench group), at the N and C termini of the peptide chain, a double coupling step was used. The crude peptides were purified by reverse phase high-performance liquid chromatography and fully characterized by mass spectrometry (32). Enzyme digestions were conducted for 3 h with 20 μg of each peptide and recombinant soluble furin (New England Biolabs) and PC5A (enzyme activity ~0.1–0.15 unit, where 1 unit represents 1 pmol of free amidomethylcoumarin released per min from 100 μM t-butoxycarbonyl-RVRR-amidomethylcoumarin substrate (33)). To determine the site of peptide cleavage, each crude digest was examined by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry as described (32). The kinetic parameters $K_m$ and $V_{max}$ for each digestion were measured from intensity of fluorescence released as described earlier. Typically, recombinant furin or PC5A (2 ml, activity ~0.1–0.15 units) was incubated for 4 h with increasing concentrations (1–20 mM) of each intramolecularly quenched fluorogenic peptide in 100 ml of buffer consisting of 25 mM Tris + 25 mM Mes + 2.5 mM CaCl$_2$, pH 7.4, in a 96-well microtiter plate at 37 °C. The rate of hydrolysis was obtained from the changes in fluorescence readings, and the values were transformed into fmol/h of peptide cleaved by using standard curve and quenching corrections (32). Data were collected in duplicate, and each value is the mean of three independent experiments. $V_{max}$ and $K_m$ were calculated as described (32).

**RESULTS**

**In Vitro Study of the Processing of the Furin-like Motif by the PCs**—Comparison of different alphaviruses indicated the presence of a proprotein convertase motif in CHIKV E3E2 glycoprotein (Fig. 1) (17). Isolates collected during the recent outbreak in La Réunion island (e.g. strain 05.115) exhibited a HRQRR$_{64}$ motif at the E3E2 junction (29) like other CHIKV strains originating from Africa (527, 37997, and LR2006 OPIY1) and two Asian CHIKV strains (Nagpur and AF15561) were aligned to corresponding sequences from O’Nyong-nyong, Semliki Forest, Ross River, and Sindbis viruses. E3E2 cleavage site is indicated by a bold arrow. Amino acid conservation rate is indicated by a histogram. The RQRR$_{64}$ motif conserved between CHIKV strains is delimited by a square box.

**FIGURE 1.** CHIKV E3E2 junction exhibits a furin site. Proprotein E3E2 sequences from four African CHIKV strains (05.115, S27, 37997, and LR2006 OPIY1) and two Asian CHIKV strains (Nagpur and AF15561) were aligned to corresponding sequences from O’Nyong-nyong, Semliki Forest, Ross River, and Sindbis viruses. E3E2 cleavage site is indicated by a bold arrow. Amino acid conservation rate is indicated by a histogram. The RQRR$_{64}$ motif conserved between CHIKV strains is delimited by a square box.
Essing of various viral glycoproteins (17). For this, we selected two intramolecularly quenched fluorogenic peptides, one 14-mer (long) and the other 11-mer (short), that contain the same physiologically relevant RRQRR64SI cleavage site that characterizes CHIKV strains originating from Asia (Table 2). We also included two mutant derivatives where the important P1 Arg64 was substituted by Ala and a short form where the P2P1 Arg-Arg64 was replaced by Ala-Ala64 (Table 2). When incubated with recombinant furin or PC5A, both wild-type (wt) peptides were efficiently cleaved by furin and PC5A at the correct physiological site. As shown by MALDI-TOF mass spectrometry, A and B, the crude 3-h digest of Q-Asiatic-CHIKV-short wt peptide with furin exhibited two strong peaks at m/z 1075 and 741 due to the N-terminal fragment Abz-SPRRQRRSIKD-Tyx-A (calculated \( M_r = 1074 \)) and C-terminal fragment SIKD-Tyx-A-CONH2 (calculated \( M_r = 739 \)), respectively, with complete disappearance of the peak at m/z 1798 of the undigested peptide. This clearly suggested a cleavage at R64S by the PCs. An additional peak at m/z 897 was also observed due to the formation of the fragment RSIKD-Tyx-A-CONH2 (calculated \( M_r = 896 \)). This indicated another cleavage at QR63264RS site. When the mutant R64A peptide was incubated with PCs, again cleavage was observed but exclusively digested at QR63264AS, leading to peaks at m/z 920 and 811 for the N- and C-terminal fragments Abz-SPRRQRASIKD-Tyx-A-CONH2 (calculated \( M_r = 918 \)) and ASIKD-Tyx-A-CONH2 (calculated \( M_r = 810 \)), respectively, as shown for furin cleavage (Fig. 2, C and D). So far it is not clear whether both these secondary cleavage sites

| Peptide name       | Amino acid sequence                          | \( M_r \) |
|--------------------|----------------------------------------------|---------|
| Q-Asiatic-CHIKV-long wt | Fmoc-Abz-\(^{55}\)LTCSPRRQRRSIKD68-Tyx-A | 2236    |
| Q-Asiatic-CHIKV-long mut | Abz-\(^{55}\)LTCSPRRQRAASIKD68-Tyx-A | 2028    |
| Q-Asiatic-CHIKV-short wt | Abz-\(^{58}\)SPRRQRRSIKD68-Tyx-A | 1797    |
| Q-Asiatic-CHIKV-short mut | Abz-\(^{58}\)SPRRQRAASIKD68-Tyx-A | 1712    |
| Q-Asiatic-CHIKV-short double mut | Abz-\(^{58}\)SPRRQAAASIKD68-Tyx-A | 1627    |

FIGURE 2. E3E2 junction cleavage by convertases. A and B, MALDI-TOF mass spectra of a 3-h furin digest of Q-Asiatic-CHIKV-short wt peptide. Abz-SPRRQRRSIKD-Tyx-A-CONH2 (\( M_r = 1798 \)) was cleaved by furin into Abz-SPRRQR-CONH2 (NT1, \( M_r = 1074 \)) and SIKD-Tyx-A-CONH2 (CT1, \( M_r = 739 \)) that were detected by mass spectrometry. C and D, in contrast to wt peptide, Q-Asiatic-CHIKV-short mutant peptide Abz-SPRRQRAASIKD-Tyx-A-CONH2 (\( M_r = 1712 \)) was cleaved by furin into Abz-SPRRQR-CONH2 (NT2, \( M_r = 918 \)) and ASIKD-Tyx-A-CONH2 (CT2, \( M_r = 811 \)).
are relevant to CHIKV infection. The minute peak at m/z 1712 was due to the undigested peptide. But unlike the wt peptide, no additional cleavages were observed in this case, as expected. It may be further pointed out that when a P1P2 Ala double mutant peptide (Abz-319SPRRQAASIK-328-Tyx-A) was similarly treated with the PCs, no cleavage was detectable in the mass spectra even after 18 h of incubation (not shown).

Measurement of the kinetic parameters $V_{\text{max}}$ and $K_m$ for cleavages of short wt and mutant peptides (Table 3) by furin and PC5A suggested that these PCs are potential host candidate enzymes for maturation of the Asiatic CHIKV E3E2. Mass spectrometric and $V_{\text{max}}/K_m$ values showed that these PCs cleave the E3E2 protein at the physiological site and that furin is ~3-fold more efficient than PC5A in cleaving the wt peptide. Data with mutant peptides further confirmed the above conclusion and also indicated that besides the major cleavage at Arg64, there is an additional cleavage at Arg63 as well. With the R64A mutant peptide we noted that the efficiency of cleavage by furin and PC5A is enhanced but still keeps the ~3-fold faster rate with furin versus PC5A. In all cases the Michaelis-Menten curves showing the various cleavages confirmed that furin and PC5A are efficient PCs for the processing of the Asiatic CHIKV E3E2 surface glycoprotein (not shown).

**Furin-like Convertases and the Processing of E3E2 Protein**—We tested the ability of different proprotein convertases (the soluble PACE4 and PC5A, and membrane-bound furin, PC5B, PC7, and SKI-1) to cleave E3E2 proprotein when transiently expressed in HEK293 human cells. Results from Fig. 3 indicate that among the different proteases, the membrane-bound convertases furin (63%) and to a lesser extent PC5B (10%) (35), were able to process E3E2 from a CHIKV isolate bound convertases furin (63%) and to a lesser extent PC5B, PC7, and SKI-1) to cleave E3E2 proprotein when transiently expressed in HEK293 human cells. Results from Fig. 3 indicate that among the different proteases, the membrane-bound convertases furin (63%) and to a lesser extent PC5B (10%) (35), were able to process E3E2 from a CHIKV isolate from La Réunion island (strain 05.115; Schuffeneker 2006) into (10%) (35), were able to process E3E2 from a CHIKV isolate from La Réunion island (strain 05.115; Schuffeneker 2006) into.

### Table 3: Kinetic parameters for *in vitro* digestion of various intramolecularly quenched fluorogenic peptides by furin and PC5A

| Enzyme          | $V_{\text{max}}/K_m$ | $V_{\text{max}}/K_m$ | $V_{\text{max}}/K_m$ | $V_{\text{max}}/K_m$ |
|-----------------|----------------------|----------------------|----------------------|----------------------|
|                 | $\text{nmol} \cdot \text{h}^{-1}$ | $\text{mol} \cdot \text{h}^{-1}$ | $\text{mol} \cdot \text{h}^{-1}$ | $\text{mol} \cdot \text{h}^{-1}$ |
| Q-Asiatic-CHIKV-short wt peptide | Furin 35.02 ± 2.55 | 0.122 ± 0.024 | 286.55 | 2.08 |
|                 | PC5A 33.04 ± 3.20 | 0.30 ± 0.059 | 111.96 | 0.81 |
| Q-Asiatic-CHIKV-short mut peptide | Furin 51.98 ± 4.58 | 0.056 ± 0.008 | 1447.70 | 27.7 |
|                 | PC5A 9.38 ± 1.41 | 0.185 ± 0.007 | 507.097 | 9.7 |

### Figure 3. Ex vivo processing of CHIKV E3E2 glycoprotein precursor by furin and furin-like convertases.

A. E3E2 precursor sequence was tagged with V5 at its C terminus as indicated. SP corresponds to E3E2 signal peptide. E2 transmembrane domain is indicated by a black box. B. CHIKV E3E2 tagged at the C terminus with a V5 epitope was transiently co-expressed in HEK293 cells with either furin, a soluble form of furin (sFurin), PACE4, PC5A, PC5B, PC7, or SKI-1. In the case of the furin, cells were either incubated with or without 10 mM chloroquine (CQ) for 2 h before transfection. Cells were subsequently labeled with [35S]Cys + Met for 4 h, and cell lysates were immunoprecipitated with a V5-specific monoclonal antibody (mAb-V5). The immunoprecipitates were resolved on an 8% SDS-PAGE gels, dried, and autoradiographed. The quantitation of % processing was obtained from the ratio of the level of E2 to unprocessed E3E2.

### Figure 4. E3E2 cleavage is inhibited by furin inhibitor dec-RVKR-cmk.

LHCN-M2 cells were infected for 2 h with CHIKV (m.o.i. = 0.1), then cultured for 15 h in the presence of 2 mM chloroquine (CQ), 100 mM furin inhibitor dec-RVKR-cmk (FI), or both combined (FI + CQ). Total cell lysate and viral particles purified from ultracentrifuged supernatant were analyzed by Western blot for E3E2 and E2 expression.

**Processing of E3E2 by Furin and Furin-like Convertases during CHIKV Infection**—To demonstrate E3E2 cleavage by furin and furin-like convertases during CHIKV replication, we used a relevant infection system based on muscle cell progenitors, a cell type previously identified as a major target of CHIKV *in vivo* (7). The immortalized myoblast cell line LCHN-M2 was...
infected with CHIKV (strain 05.115) at 0.1 m.o.i. and cultured for 15 h in the presence of 2 mM chloroquine or 100 μM furin inhibitor dec-RVKR-cmk (25, 27). At these concentrations of chloroquine and dec-RVKR-cmk, no cytopathic effects were detectable in cell cultures as evidenced by 4',6-diamidino-2-phenylindole staining. Western blot analysis of E3E2 processing in infected myoblast cells revealed that a post-infection treatment with dec-RVKR-cmk significantly reduced the processing of E3E2 into E2 (Fig. 4). Moreover, viral particles secreted by dec-RVKR-cmk-treated cells were highly immature, with a large fraction of E3E2 being unprocessed. This demonstrated the critical role of furin and furin-like enzymes in the processing of CHIKV E2 glycoprotein in infected myoblasts. Chloroquine alone had essentially no visible effect on the processing, although the combination of both drugs marginally reduced cleavage above levels previously observed with the furin inhibitor alone. Altogether with the results reported in Fig. 3, we concluded that chloroquine weakly affected proprotein convertase activity and E3E2 processing in this system.

CHIKV Spreading from Cell to Cell Depends on Furin and Furin-like Activity—We then tested the ability of dec-RVKR-cmk or chloroquine to inhibit CHIKV infection and cell-to-cell spreading of the virus. In a first set of experiments myoblasts were infected with 0.05 m.o.i., then treated with dec-RVKR-cmk or chloroquine. Infection was performed for 2 h, and after washes drugs were added at concentrations of 50 or 100 μM, respectively. Assessment of the level of infection by immunofluorescence revealed that whereas ~30% of control cells are infected, a significant ~6-fold inhibition of infection was found when cells were treated post-infection with 100 μM dec-RVKR-cmk and to a lesser extent (~3-fold) with 2 μM chloroquine (Fig. 5, A and D). Assessment of the extent of infection by counting immunoreactive cells/focus in a minimum of 10 microscopic fields. D, significant microscopic fields obtained by immunofluorescence using CHIKV-specific antibodies are shown.
chloroquine ranging from 1 to 2 μM (Fig. 5, A and D). Moreover, a drastic reduction of viral production (at 16 h post-infection) was obtained with 100 μM dec-RVKR-cmk (Table 4) and to a much lower extent with chloroquine. Interestingly, inhibition was not correlated with a reduction in the number of fluorescent foci of cells (Fig. 5B) but only with a reduction in the number of positive cells per fluorescent focus (Fig. 5C). Thus, dec-RVKR-cmk or chloroquine only affected viral spreading but not CHIKV replication itself once it entered target cells. This suggested that immature viral particles produced by dec-RVKR-cmk-treated cells are impaired for viral spread into neighboring cells. Chloroquine also inhibited viral particle maturation to some extent (Fig. 3B) but mainly inhibited viral entry into target cells. Indeed, chloroquine is an alkalinizing agent known to block the pH-dependent fusion of viruses in acidic endosomes (9). Interestingly, combination of both dec-RVKR-cmk and chloroquine treatments at optimal doses (i.e. 100 and 2 μM, respectively) showed additive inhibitory activities both on virus spreading and virus titers (Fig. 6).

Chloroquine and dec-RVKR-cmk Block CHIKV Entry in Muscle Cell Progenitors—To improve the efficiency of CHIKV inhibition and to test whether furin-inhibiting agents could also act on the early steps of CHIKV infection, we treated the cells before infection with either dec-RVKR-cmk or chloroquine or both and maintained the drugs during and after infection. As shown in Fig. 7, A and C, a dramatic decrease in the number of infected cells and viral titers was observed when drugs were added before the viral inoculum. A significant inhibition was observed with an m.o.i. of 0.01, but analysis of viral yields indicated that inhibition could be seen even for higher m.o.i. ranging from 0.1 to 1 (Fig. 7D). An additional effect could be observed when both drugs were combined for pretreatment. In contrast to what was observed when the drugs were added only after viral infection, inhibition of CHIKV could also be seen by counting the number of fluorescent foci (Fig. 7A). This suggested that dec-RVKR-cmk, in addition to inhibiting maturation of viral particles, could also block early steps of myoblast infection by CHIKV. To test this hypothesis, experiments described above were repeated, but infection and viral titers were determined under single replication cycle conditions (i.e. 7 h post-infection). Both dec-RVKR-cmk and chloroquine efficiently blocked CHIKV...
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A

Untreated

FI

CQ

FI + CQ

B

% infected cells

Untreated

FI

CQ

FI + CQ

FIGURE 8. Inhibition of CHIKV entry or early replication steps by dec-RVKR-cmk. LHCN-M2 cells were cultured for 4 h before infection, for 2 h during infection with CHIKV (m.o.i. = 0.1), and for 7 h post-infection with 2 μM chloroquine (CQ), 100 μM dec-RVKR-cmk (FI) or both combined (FI + CQ). Cells were subsequently fixed in paraformaldehyde and processed for immunofluorescence with CHIKV-specific antibodies. A, significant microscopic fields obtained by immunofluorescence using CHIKV-specific antibodies are shown. B, results were expressed as the percentage of immunoreactive (infected) cells.

entry and/or early replication steps as assessed by the percentage of cells expressing CHIKV antigens (Fig. 8, A and B). But how dec-RVKR-cmk inhibited entry or early replication steps of CHIKV will require further investigations. CHIKV particles were fully matured in the inoculum used to infect myoblasts (data not shown) and thereby did not require further trans-processing and maturation by myoblast convertases. Thus, our data suggest that furin-like inhibitors could also affect an early step of CHIKV entry or replication cycle that is independent of E3E2 cleavage.

DISCUSSION

Since 2005 an epidemic outbreak of CHIKV fever, a mosquito-borne viral disease, spectacularly swept through La Réunion island (more than 250,000 cases) and then to India with an estimated 1.4 million cases. Phylogenetic analysis based on partial E1 sequences from African and Asian isolates revealed the existence of three distinct CHIKV phylo-groups, one containing all isolates from West Africa, one containing isolates from Asia, and one corresponding to Eastern, Central, and Southern African isolates (39). The recent epidemic of CHIKV was initiated by a strain related to East African isolates from which viral variants have evolved following a traceable microevolution history (29). In this report we demonstrate that CHIKV glycoprotein contains a functional proprotein convertase cleavage site at the E3E2 junction that is processed by furin and furin-like enzymes to achieve CHIKV maturation.

Sequence comparisons between CHIKV isolates from different phylo groups revealed slight variations at the E3E2 junction. CHIKV isolates from La Réunion island exhibit a HRQRR64 SI motif at E3E2 junction like other isolates of African origin. In contrast, CHIKV strains of the Asian phylo group exhibit a RRQRR64 SI motif. Although only furin (and to some extent PC5B) can process E3E2 from African CHIKV strains, Asian strains can be cleaved by a much larger spectrum of convertases including membranous and soluble furin, PC5A and PC5B, and PACE4. We also showed that this phenotype correlates with a lower susceptibility to E3E2 cleavage inhibition by chloroquine. His60 at P5 position from the cleavage site is the critical residue that determines the spectrum of furin and furin-like convertases that process E3E2 glycoprotein. Unfortunately, this mutation cannot be related for the moment with any evident clinical characteristics. In addition, we could not access to CHIKV strains of Asian origin to compare susceptibility to furin inhibitors or chloroquine in mammalian and mosquito infection models. However, our observations suggest that single amino acid variations within the processing site of Alphavirus glycoproteins could deeply impact their susceptibility to convertases and eventually modify their spreading and tropism.

In that context, single amino acid substitution/deletion around the hemagglutinin furin-like cleavage site resulting from large scale vaccination against the flu virus were also found to affect the furin cleavage in the emerging vaccine-modified Fujian H5N1 strain (40). Processing of viral glycoproteins by host cellular convertases to achieve maturation is a characteristic shared by many viruses from various families, including human immunodeficiency virus, severe acute respiratory syndrome virus (SARS), Ebola, respiratory syncytial virus, and others (27, 41–43). Among alphaviruses, E3E2 glycoprotein of Semliki Forest virus, a virus closely related to CHIKV, was previously shown to be processed by furin and related convertases (18). In this respect, our observations confirm the critical role of furin in the proper maturation of Alphavirus glycoproteins. But we also demonstrated that furin inhibitor dec-RVKR-cmk can significantly reduce the processing of E3E2 CHIKV glycoproteins in infected myoblast cultures. Human muscle satellite cells, which have been reported as natural target cells for CHIKV, were expressed as the percentage of immunoreactive (infected) cells.

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inhibition of E3E2 cleavage in infected cells, although some additive effect could be observed when both drugs were combined. dec-RVKR-cmk had previously been shown to be active in reducing the glycoprotein cleavage and viral spread of SARS and respiratory syncytial virus (27, 41). Our report provides for the first time such an efficient approach to harness CHIKV replication in vitro based on the inhibition of E3E2 glycoprotein processing.

Up until now the treatment of CHIKV fever has been only symptomatic, with administration of nonsteroidal anti-inflammatory drugs. Numerous reports showing the antiviral activity of chloroquine in vitro, in particular the inhibition CHIKV-related virus Mayaro (14), motivated a clinical trial in La Réunion Island to test in vivo the efficiency of this molecule. In conclusive results have been obtained because the low doses used were likely inappropriate, as recently suggested (12). As mentioned above, chloroquine had limited inhibitory effects on furin activity and the cleavage of CHIKV E3E2 precursor, although it efficiently blocked virus entry in pretreated cells (Fig. 7, A and B) and spreading from infected to non-infected cells (Fig. 5, B and C). This suggested that chloroquine inhibits viral entry at the early stage. In agreement with this idea, a recent paper showed that chloroquine inhibits CHIKV infection in HeLa cells when added 1 h before, but less efficiently when added after infection (9). When added extracellularly, chloroquine enters in acidic, low pH organelles, such as endosomes, Golgi vesicles, and lysosomes where it is protonated and concentrated. Chloroquine can affect virus entry in many ways, inhibiting pH-dependent fusion of viral particles or altering the glycosylation of cellular receptors (38). We showed that pre-treated cells are resistant to infection by CHIKV, suggesting that chloroquine essentially affects either the pH-dependent fusion or the complex glycosylation of a cellular receptor. Although fusion of alphaviruses is clearly pH-dependent, we cannot discriminate between these two hypotheses with our current knowledge of the system. In conclusion, our results indicate that dec-RVKR-cmk inhibits the processing of viral glycoproteins, whereas chloroquine targets viral entry with minor effects on E3E2 cleavage. Admittedly, we cannot exclude that in addition, chloroquine also affects the glycosylation of viral envelope proteins and budding of viral particles as previously reported for other viruses (14, 44).

The additive effect of dec-RVKR-cmk and chloroquine also support the idea that these two drugs act by essentially distinct mechanisms. The combinatorial action of chloroquine and dec-RVKR-cmk led to an almost total suppression of viral spread and yield. To our knowledge this is the first report of an additive inhibitory effect of chloroquine and furin inhibitor on viral infection and a combinatorial activity that had only been reported previously for preventing anthrax toxin-mediated killing of macrophages (45). This observation begs the re-evaluation of some of the therapeutic strategies that were based on chloroquine treatment alone in the acute phase of CHIKV fever.

Finally, our most surprising result is the strong inhibition of CHIKV entry when pretreating the cells with convertase inhibitor dec-RVKR-cmk (Fig. 7B). This was unexpected since our CHIKV inoculum produced on mosquito cells corresponds to fully matured viral particles (not shown), which do not require further processing by cellular convertases. Consequently, pre-treatment of muscle cells with dec-RVKR-cmk should not have affected virus entry and the number of infected foci. Our hypothesis to explain this observation is that dec-RVKR-cmk treatment could alter the cleavage of proteins involved in CHIKV endocytosis or early replication steps. Furin inhibition could impair maturation of cellular proteins, including the CHIKV receptor itself, or viral accessory factors as was reported for the human immunodeficiency virus protein Vpr (46).

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