Phosphorylation Sites in the Hypervariable Domain in Chikungunya Virus nsP3 Are Crucial for Viral Replication

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ABSTRACT Chikungunya virus (CHIKV, family *Togaviridae*) is a mosquito-transmitted alphavirus. The positive-sense RNA genome of CHIKV encodes four nonstructural proteins (nsP1 to nsP4) that are virus-specific subunits of the RNA replicase. Among nsP functions, those of nsP3 are the least understood. The C-terminal hypervariable domain (HVD) in nsP3 is disordered and serves as a platform for interactions with multiple host proteins. For Sindbis virus (SINV) and Semliki Forest virus (SFV), the nsP3 HVD has been shown to be phosphorylated. Deletion of phosphorylated regions has a mild effect on the growth of SFV and SINV in vertebrate cells. Using radiolabeling, we demonstrated that nsP3 in CHIKV and o'nyong-nyong virus is also phosphorylated. We showed that the phosphorylated residues in CHIKV nsP3 are not clustered at the beginning of the HVD. The substitution of 20 Ser/Thr residues located in the N-terminal half of the HVD or 26 Ser/Thr residues located in its C-terminal half with Ala residues reduced the activity of the CHIKV replicase and the infectivity of CHIKV in mammalian cells. Furthermore, the substitution of all 46 potentially phosphorylated residues resulted in the complete loss of viral RNA synthesis and infectivity. The mutations did not affect the interaction of the HVD in nsP3 with the host G3BP1 protein; interactions with CD2AP, BIN1, and FHL1 proteins were significantly reduced but not abolished. Thus, CHIKV differs from SFV and SINV both in the location of the phosphorylated residues in the HVD in nsP3 and, significantly, in their effect on replicase activity and virus infectivity.

IMPORTANCE CHIKV outbreaks have affected millions of people, creating a need for the development of antiviral approaches. nsP3 is a component of the CHIKV RNA replicase and is involved in interactions with host proteins and signaling cascades. Phosphorylation of the HVD in nsP3 is important for the virulent alphavirus phenotype. Here, we demonstrate that nsP3 in CHIKV is phosphorylated and that the phosphorylation sites in the HVD are distributed in a unique pattern. Furthermore, the abrogation of some of the phosphorylation sites results in the attenuation of CHIKV, while abolishing all the phosphorylation sites completely blocked its replicase activity. Thus, the phosphorylation of nsP3 and/or the phosphorylation sites in nsP3 have a major impact on CHIKV infectivity. Therefore, they represent promising targets for antiviral compounds and CHIKV attenuation. In addition, this new information offers valuable insight into the vast network of virus-host interactions.

KEYWORDS RNA replication, alphavirus, chikungunya, nsP3, protein phosphorylation

Chikungunya virus (CHIKV) is a mosquito-transmitted alphavirus (family *Togaviridae*). It is a member of the Semliki Forest virus (SFV) complex and is closely related to the o'nyong-nyong virus (ONNV). Three different genotypes of CHIKV are known: the East/Central/South African (ECSA), Asian, and West African (WA) genotypes (1). CHIKV is a reemerging virus that has caused major outbreaks in recent decades (2). Symptoms of CHIKV infection include fever, rash, muscle and joint pain, and

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arthritis; the arthritic symptoms often become chronic and may last for years (3). There is no licensed vaccine or specific antiviral compound to treat CHIKV infection.

CHIKV has an ~12-kb positive-strand RNA genome with 5' cap and 3' poly(A) structures. The genome contains two open reading frames (ORFs). A long 5' ORF occupying approximately two-thirds of the CHIKV genome encodes the P123 and P1234 polyproteins, which are precursors for the nonstructural proteins (nsP1 to nsP4). In infected cells, P123 and P1234 are processed in a well-regulated manner, resulting first in the short-lived P123+nsP4 complex that is responsible for synthesizing negative-strand RNA (4). The synthesis of negative-strand RNA coincides with the formation of membrane-associated alphavirus replicase complexes termed spherules (5, 6). Subsequent processing of P123 results in the formation of a stable nsP1+nsP2+nsP3+nsP4 complex. This complex synthesizes positive-strand RNAs, including new genomic RNAs and subgenomic (SG) RNAs. SG RNAs translate structural proteins encoded by the second ORF in the CHIKV genome (4). All nsPs are required for alphavirus genome replication and SG RNA synthesis. Their functions are facilitated by a large number of host proteins that interact with nsPs and/or other components of the viral replicase complex (7–14). In addition to participation in viral RNA synthesis, all nsPs have other functions that are often associated with the suppression of host cell transcription/translation and/or suppression of host antiviral responses (15–21).

Alphavirus nsP3 consists of three domains: the N-terminal macrodomain, the central alphavirus unique domain (AUD), and the C-terminal hypervariable domain (HVD). The macrodomain binds ADP-ribose and is able to hydrolyze ADP-ribose groups from mono(ADP-ribosyl)ated proteins (22); these functions have been shown to be critical for the initiation of CHIKV replication (23). The AUD of the related Sindbis virus (SINV) has been shown to bind Zn²⁺ ions (24). The CHIKV AUD has multiple roles, which are often exhibited in a species- and cell type-specific manner, in the replication and transcription of the virus genome (25). The HVD has been shown to be intrinsically disordered (12) and serves as a platform for binding different host factors (10, 12). Interactions with host factors are required for different steps of virus infection (26), and host factor(s) disruption results in a range of effects, from mild attenuation of the virus (10, 12, 13) to complete absence of viral RNA replication (9, 12, 21). It has also been shown that the motifs in the HVD mediate interactions with the phosphatidylinositol 3-kinase (PI3K)-Akt-mTOR cascade, directing proviral metabolic changes in infected cells (27, 28).

Phosphorylation is the most common posttranslational modification of proteins. To date, nsP3 is the only alphavirus replicase protein shown to be phosphorylated. Although phosphorylation is assumed to be a common modification in nsP3, it has been directly demonstrated only for a few alphaviruses, namely, SINV (29), SFV (30), and Venezuelan equine encephalitis virus (VEEV) (31). Deletion analysis results revealed that most, if not all, of these phosphorylation sites are located in the HVD in the SINV nsP3. Highly phosphorylated nsP3 is not required for SINV replication in vertebrate cells, and the lack of nsP3 phosphorylation diminishes, but does not block, SINV replication in mosquito cells (32). Subsequently, it was shown that temperature-sensitive mutations in the macrodomain and AUD have an impact on SINV nsP3 phosphorylation and that reduced nsP3 phosphorylation typically correlates with reduced negative- and/or positive-strand RNA synthesis (33). For SFV, the phosphorylation sites were mapped to Ser and Thr residues located at the beginning of the HVD sequence (34). Similar to SINV, the deletion of the phosphorylated region had only a mild effect on SFV replication *in vitro*; however, this deletion attenuated SFV *in vivo* (35). Similar results were also obtained for VEEV, in which all 53 potential phosphorylation sites in the HVD in nsP3 were eliminated by the substitution of Ser and Thr residues with Gly, Ala, Glu, or Gln residues. These modifications resulted in only a mild reduction in VEEV replication in mammalian cells; the effect was more prominent in mosquito cells (36). In contrast, the elimination of phosphorylation sites in the macrodomain and AUD resulted in severe defects in VEEV multiplication and RNA synthesis. Therefore, the

passage of viruses harboring such substitutions resulted in the reversion to the wild-type (WT) virus (31). Thus, these data suggest that the impact of nsP3 phosphorylation depends on the virus and/or phosphorylation site. However, phosphorylation of the HVD is largely dispensable for *in vitro* replication in vertebrate cells.

Phosphorylation of CHIKV nsP3 has been assumed but never directly demonstrated. Detailed analysis of the effects of host factors interacting with the HVD in CHIKV nsP3 has demonstrated that compared to other alphaviruses, CHIKV exhibits unique characteristics (9, 12), making it plausible that the same may apply for the phosphorylation of the HVD. Indeed, swapping the nsP3 parts corresponding to the phosphorylated region in the SFV nsP3 between CHIKV and SFV resulted in a drastic reduction in viral replicase activity and in strongly attenuated viruses that survived by acquiring adaptive mutations (28). This finding indicates that similarly located regions in nsP3 proteins of two closely related alphaviruses have dissimilar functions. Here, we demonstrate that the nsP3 proteins in CHIKV and ONNV are phosphoproteins. In contrast to those in SFV nsP3, the phosphorylation sites in CHIKV nsP3 do not form a distinct cluster; instead, they are scattered throughout the HVD. Replacement of Ser and Thr residues with Ala residues in the C-terminal half of the HVD significantly reduced the activity of CHIKV replicase and slightly diminished infectious virus rescue in mammalian cells. Replacement of Ser and Thr residues with Ala residues in the N-terminal half of the HVD had a stronger effect on replicase activity, and virus harboring these corresponding mutations survived by acquiring an adaptive C173Y substitution in the AUD. Combining these mutant regions completely abolished both the infectivity of the virus genome and the activity of the viral replicase. At the same time, these mutations did not affect the interaction of the HVD with the host G3BP1 protein. Interaction with BIN1, FHL1, and CD2AP proteins was reduced but not abolished. Thus, mutant HVD maintained interactions with host factors which are important for CHIKV infectivity and replication. Thus, in contrast to those in the cases of previously studied alphaviruses, the phosphorylation sites in nsP3 and/or their phosphorylation are crucial for CHIKV.

RESULTS

nsP3 in all CHIKV genotypes and in ONNV is a phosphoprotein. Analyses of nsP3 phosphorylation in CHIKV of the ECSA genotype (here designated CHIKV WT), CHIKV-Asian, and CHIKV-WA genotypes, as well as nsP3 in ONNV, were performed using radio-labeling. The WT SFV and its phosphorylation-negative mutant SFV^{Δ319–368} (35) were used as positive and negative controls, respectively. Labeling of infected BHK-21 cells was performed at the time when nsP3 synthesis activity was at a high level, between 4 and 6 h postinfection (hpi) for CHIKV and SFV and between 10 and 12 hpi for the slower-replicating ONNV. The level of nsP3 at the time corresponding to the end of labeling (6 hpi for CHIKV and SFV and 12 hpi for ONNV) was also confirmed using immunoblotting. Immunoprecipitation followed by autoradiography revealed that the nsP3 proteins in CHIKV WT, CHIKV-Asian, and CHIKV-WA are indeed phosphorylated. The same was observed for nsP3 in ONNV and SFV but not for nsP3 in SFV^{Δ319–368} (Fig. 1). Thus, regarding nsP3 phosphorylation, CHIKV and ONNV are similar to the previously studied alphaviruses. The observed phosphorylation signal varies in intensity, most likely because of differences in nsP3 amount (Fig. 1), resulting from different growth characteristics of analyzed viruses and/or because of differences between the polyclonal antibodies used in the experiments.

Deletions in the N-terminal region of the HVD in CHIKV nsP3 do not prevent phosphorylation. Five deletions affecting the region corresponding by position (but not by sequence) to the phosphorylation region in SFV nsP3 were previously made with the aim of attenuating CHIKV WT (37). All the viruses were viable and replicated to high titers (Fig. 2A). The labeling experiment revealed that the nsP3 proteins expressed by all of these mutants were phosphorylated (Fig. 2B). Thus, in contrast to those in the case of SFV, the phosphorylated residues in CHIKV nsP3 are not located exclusively at the beginning of the HVD.

The previously constructed mutants CHIKV^{Δ398–406} and CHIKV^{Δ398–413} (10) harbored

FIG 1 In CHIKV- and ONNV-infected BHK-21 cells, nsP3 is phosphorylated. BHK-21 cells were infected with CHIKV WT, CHIKV-Asian, CHIKV-WA, ONNV, SFV, or SFV Δ 319–368 at an MOI of 5; control cells were mock infected. (Top) At 6 hpi (12 hpi for ONNV), the cells were collected and lysed, and the proteins were separated using SDS-PAGE. The nsP3 proteins were detected using a mixture of polyclonal antibodies against nsP3 of CHIKV and nsP3 of SFV or using a polyclonal antibody against nsP3 of ONNV. Signals were visualized with the LI-COR Odyssey Fc imaging system. β -actin is shown as a loading control. (Bottom) At 2 hpi (8 hpi for ONNV), the cells were starved in PFM for 2 h and labeled by incubating with [32 P]phosphoric acid for 2 h. The labeled cells were collected and lysed, and nsP3 was immunoprecipitated with the appropriate polyclonal antibody. The precipitated proteins were separated using SDS-PAGE, and the signals were visualized using a Typhoon IP biomolecular imager. The experiment was repeated twice, and similar results were obtained each time.

small deletions (Fig. 2A) that cover the first of two SH3 domain-binding sites found in the HVD in CHIKV nsP3 (13). No potential phosphorylation sites were removed in CHIKV Δ 398–406 nsP3, and only two Thr residues were missing in the case of CHIKV Δ 398–413 nsP3. However, it was shown that, for SINV, mutations located outside the phosphorylated regions in nsP3 can also affect SINV phosphorylation (33). Therefore, we also performed an analysis of the phosphorylation of nsP3 encoded by CHIKV Δ 398–406 and CHIKV Δ 398–413. This assay revealed that the nsP3 proteins encoded by these mutants were highly phosphorylated; no obvious differences were observed between nsP3 in the mutants and nsP3 in CHIKV WT (Fig. 2B).

Elimination of potential phosphorylation sites in the nsP3 HVD renders CHIKV noninfectious. The CHIKV WT nsP3 HVD contains 46 Ser and Thr residues that are scattered throughout the HVD without any obvious clustering (Fig. 3A). Using larger deletions in the HVD in nsP3 increased the risk of removing other important functional motifs, which may result in a reduction/loss in virus infectivity. Conducting a functional analysis of the potential phosphorylation sites using one-by-one alanine substitutions was also not feasible. Therefore, to investigate the effects of eliminating the potential phosphorylation sites from large HVD regions, two mutants were constructed. In CHIKV Δ 5/T320–396A, 20 Ser and Thr residues located upstream of the first SH3 domain-binding site were substituted with Ala residues. The Ser320 residue, located on the boundary of the AUD and HVD, was included because it is known to be phosphorylated in SFV (34). In CHIKV Δ 5/T411–519A, 26 Ser and Thr residues located downstream of the first SH3 domain-binding site were substituted with Ala residues, and all the changes made to the mutants were made in combination in CHIKV Δ 5/T320–519A (Fig. 3A).

First, these mutations were introduced into pCMV-ICRES1, an infectious cDNA (icDNA) clone of CHIKV WT. Virus rescue efficiencies (for simplicity, here called infectivities) in BHK-21 cells transfected with pCMV-ICRES1, pCMV-ICRES1 Δ 5/T320–396A, pCMV-ICRES1 Δ 5/T411–519A, and pCMV-ICRES1 Δ 5/T320–519A were analyzed using an infectious center assay (ICA); virus replication was evaluated by determining the titers of P_0 stocks of the rescued viruses. Compared to that of pCMV-ICRES1, the infectivity of pCMV-ICRES1 Δ 5/T411–519A was reduced $\sim$ 10-fold. A similar reduction in infectivity was also observed for pCMV-ICRES1 Δ 5/T320–396A, and in addition, this mutant produced minute plaques observable only upon prolonged incubation of transfected cells. Thus, both large-scale mutations clearly affected CHIKV infectivity in vertebrate cells, and the effect caused by these mutations located in the N-terminal half of the HVD was

FIG 2 Small deletions in the HVD in CHIKV nsP3 do not affect its phosphorylation. (A) Schematic representation of the CHIKV genome and domain organization of nsP3. The locations of the deletions and swaps in nsP3 in the mutant viruses are shown. All CHIKV mutants were derived from CHIKV WT; SFV mutants were based on the SFV4 strain. Positions of amino acid residues flanking the deleted or swapped regions are shown. At the right of the drawings, titers (PFU/ml) of the P₀ stocks of the corresponding viruses are shown together with references to papers where the constructs were first described. (B) BHK-21 cells were infected with CHIKV WT, CHIKV $\Delta_{326-343}$, CHIKV $\Delta_{326-353}$, CHIKV $\Delta_{326-363}$, CHIKV $\Delta_{326-375}$, CHIKV $\Delta_{326-384}$, CHIKV $\Delta_{398-406}$, or CHIKV $\Delta_{398-413}$ at an MOI of 5; control cells were mock infected. The experiment was performed, and the data are presented as described for CHIKV in the legend of Fig. 1. The experiment was repeated twice with similar results obtained each time.

clearly more pronounced. Interestingly, CHIKV^{S/T320-396A} reached titers similar to those of CHIKV WT, while the titers of CHIKV^{S/T411-519A} were ~ 100 -fold lower (Fig. 3B). Sequencing of the nonstructural regions of three plaque-purified isolates of CHIKV^{S/T411-519A} did not reveal any reversions, pseudoreversions, or second-site mutations. Thus, the substitution of 26 Ser/Thr residues in the C-terminal half of the HVD affected the replication of CHIKV but not to the extent that would result in the selection of adaptive changes. In contrast, three plaque-purified isolates of CHIKV^{S/T320-396A} all contained identical nonsynonymous mutations, resulting in a change in the Cys173 residue in nsP3 to a Tyr residue (C173Y). It has been previously described that some mutations located in the AUD in nsP3 can compensate for defects introduced into the HVD in CHIKV nsP3 (12, 28). Therefore, the C173Y substitution was introduced into pCMV-ICRES1^{S/T320-396A}. ICA revealed that in contrast to pCMV-ICRES1^{S/T320-396A}, pCMV-ICRES1^{S/T320-396A+C173Y} formed large WT-like plaques, indicating that the second-site mutation compensated for the negative impact of the changes introduced into the N-terminal half of the HVD (Fig. 3B).

FIG 3 Substitution of Ser and Thr residues with Ala residues in the HVD in CHIKV nsP3 is detrimental to virus infectivity. (A) Location of potential phosphorylation sites in the HVD of CHIKV WT. Residues 301 to 530 in nsP3 are shown; the Ser and Thr (Continued on next page)

Finally, the results from the ICA performed using pCMV-ICRES1^{S/T320–519A} revealed no plaques, indicating either a lack of infectivity or the inability of CHIKV^{S/T320–519A} to produce plaques in BHK-21 cells. The complete absence of a capsid protein in the pCMV-ICRES1^{S/T320–519A}-transfected cells (Fig. 3C) strongly supports the former possibility. Thus, substitutions of Ser/Thr residues in the entire HVD resulted in a completely different phenotype from those acquired when the same substitutions were introduced into either half of the region. Interestingly, CHIKV tolerates the complete substitution of its HVD to that of SFV (28); consistent with this observation, a mutant virus, CHIKV^{SFV-HVD}, was efficiently rescued and replicated to a high titer (Fig. 2A).

Radiolabeling experiments performed with CHIKV^{S/T320–396A}, CHIKV^{S/T411–519A}, and CHIKV^{SFV-HVD}-infected cells revealed that nsP3 proteins of all three mutant viruses were phosphorylated (Fig. 3D). This finding confirms that phosphorylation sites are present in both halves of the CHIKV HVD and supports the hypothesis that nsP3 phosphorylation is needed for CHIKV infectivity.

Elimination of potential phosphorylation sites in the nsP3 HVD blocks viral replicase activity. To further analyze the molecular defect(s) behind the lack of infectivity of CHIKV^{S/T320–519A} and attenuation of CHIKV^{S/T320–396A} and CHIKV^{S/T411–519A}, a *trans*-replication assay was used. A *trans*-replication assay is more sensitive than an assay based on the use of infectious clones (38, 39) and enables an analysis of the effects of mutations in the absence of reversions or compensatory changes (40). The S/T320–396A, S/T320–396A+C173Y, S/T411–519A, and S/T320–519A mutations were transferred to the plasmid pCMV-P1234, the source of the replicase for the *trans*-replication assay. The pHSPoll-Fluc-Gluc plasmid was used as the source of a replication-competent template RNA (Fig. 4A). In the setup used, a spike in the activity of firefly luciferase (Fluc) serves as a measure of genomic transcript synthesis, while a spike in *Gaussia* luciferase (Gluc) activity reflects the synthesis of SG RNA by the viral replicase (39).

It was found that pCMV-P123^{S/T320–519A}4 was unable to enhance the expression of either reporter above background levels, indicating a complete lack of replicase activity (Fig. 4B). A less than 10-fold increase in Fluc expression was observed for pCMV-P123^{S/T320–396A}4. Gluc expression in the presence of pCMV-P123^{S/T320–396A}4 was >3,500-fold that of the background level but significantly lower than that observed for pCMV-P1234 (Fig. 4B). Thus, the replicase expressed by pCMV-P123^{S/T320–396A}4 was active but strongly attenuated. The increase in Fluc and Gluc activities in the presence of pCMV-P123^{S/T320–396A+C173Y}4 was significantly higher than that in the presence of pCMV-P123^{S/T320–396A}4; both enhancements were similar to those observed for WT pCMV-P1234 (Fig. 4B). This finding confirmed that C173Y is indeed a compensatory mutation. The activity of the replicase expressed by pCMV-P123^{S/T411–519A}4 was also significantly lower than that of the WT replicase. However, the differences were much smaller, less than 10-fold for genomic and only ~3-fold for SG RNA synthesis (Fig. 4B). Such a small decrease in replicase activity most likely enables the corresponding virus to survive without the need for reversion and adaptation, exactly as observed using sequencing of plaque-purified isolates. Combined, the data obtained for pCMV-P123^{S/T320–519A}4, pCMV-P123^{S/T320–396A}4, pCMV-P123^{S/T320–396A+C173Y}4, and pCMV-P123^{S/T411–519A}4, as well as those obtained using corresponding icDNA clones, strongly indicate that the observed lack of replicase activity (pCMV-P123^{S/T320–519A}4) and infectivity (pCMV-ICRES1^{S/T320–519A}) do not represent a simple combination of

FIG 3 Legend (Continued)

residues are shown in bold. Mutated regions incorporated into CHIKV^{S/T320–396A}, CHIKV^{S/T411–519A}, and CHIKV^{S/T320–519A} are indicated. The location of the secondary mutation C173Y in nsP3 is shown above the drawing. (B) Effects of the S/T320–396A, S/T320–396A+C173Y, S/T411–519A, and S/T320–519A mutations on virus rescue and on the titers of the P₀ stocks of rescued viruses. <2, values below the detectable limit; large, plaque diameter of >4 mm; *, minute plaques with diameters <1 mm were detectable only in the case of prolonged incubation. (C) Lysates from the cells transfected with pCMV-ICRES1 or pCMV-ICRES1^{S/T320–519A} and a lysate from the mock-transfected cells were subjected to immunoblot analysis with antibodies against the CHIKV capsid protein (left) and nsP3 (right). β -actin is shown as a loading control. (D) BHK-21 cells were infected with CHIKV WT, CHIKV^{S/T320–396A}, CHIKV^{S/T411–519A}, or CHIKV^{SFV-HVD} at an MOI of 5; control cells were mock infected. The experiment was performed, and the data are presented as described for CHIKV in the legend of Fig. 1. Experiments shown in panels B to D were repeated at least twice with similar results obtained each time.

FIG 4 Abrogation of the potential phosphorylation sites in the HVD in nsP3 inactivates the CHIKV RNA replicase function. (A) Organization of the plasmids used for the *trans*-replication assay. The locations of the mutations in pCMV-P1234^{GAA} and mutations that were introduced into pCMV-P123^{S/T320-396A}, pCMV-P123^{S/T320-396A+C173Y}, pCMV-P123^{S/T320-519A}, pCMV-P123^{S/T411-519A}, and pCMV-P123^{S/T411-519A} are shown. CMV, immediate early promoter of human cytomegalovirus; LI, leader sequence of the herpes simplex virus thymidine kinase gene; SV40Ter, simian virus 40 late polyadenylation region. The template RNA-expressing plasmid pHSPolI-Fluc-Gluc contains the following: HSPolI, a truncated promoter for human RNA polymerase I; CHIKV 5' UTR and partial 3' UTR; N77, a region encoding the 77 N-terminal amino acid residues of nsP1; SG, the CHIKV SG promoter; RZ, an antisense-strand ribozyme of the hepatitis delta virus; and MmTer, a terminator for RNA polymerase I in mice. (B) Results of the *trans*-replication assay. U2OS cells were cotransfected with pHSPolI-Fluc-Gluc and pCMV-P1234, pCMV-P123^{S/T320-396A}, pCMV-P123^{S/T320-396A+C173Y}, pCMV-P123^{S/T320-519A}, pCMV-P123^{S/T411-519A}, or pCMV-P1234^{GAA} (negative control). Cells were collected at 18 h posttransfection and lysed, and reporter activities were measured. Activity levels of Fluc (left) and Gluc (right) in the lysates from cells transfected with pCMV-P1234, pCMV-P123^{S/T320-396A}, pCMV-P123^{S/T320-396A+C173Y}, pCMV-P123^{S/T320-519A}, or pCMV-P123^{S/T411-519A} were measured and normalized to the levels of the cells transfected with pCMV-P1234^{GAA}. Each column represents an average of three independent experiments; the error bar shows the standard deviation. *, $P < 0.05$; **, $P < 0.01$; ns, not significant (Student's unpaired *t* test).

effects of S/T320–396A and S/T411–519A mutations. It is far more likely that the combination of these mutations caused qualitative differences. Thus, the replicase activity and, therefore, the infectivity of CHIKV are dependent not only on Ser/Thr residues in nsP3 but also most likely on their phosphorylation.

Mutations introduced into the HVD in CHIKV nsP3 alter the binding of cellular proteins known to interact with the HVD. The HVD in the CHIKV nsP3 is known to interact with multiple host proteins. The binding of G3BP1/2 to the HVD is crucial but

not sufficient for CHIKV replication; the HVD also needs to bind at least one more protein family in addition to G3BPs (12). Binding sites for the CD2AP, BIN1, and FHL1 proteins have been mapped; the binding of these proteins to the HVD in CHIKV nsP3 also induces allosteric changes in the downstream site located between G3BP1/2-binding motifs (12, 14). With the exception of the first SH3 domain-binding site on the border of the N- and C-terminal halves of the HVD, all these sites are located in the C-terminal half of the HVD (Fig. 5A), i.e., inside the region where Ser/Thr-to-Ala substitutions had relatively mild effects on the CHIKV replication (Fig. 3) and replicase activities (Fig. 4B). Nevertheless, it was of interest to analyze how the mutations introduced to CHIKV^{S/T320–519A}, CHIKV^{S/T320–396A}, and CHIKV^{S/T411–519A} affect the binding of these host factors. For this, plasmids expressing EGFP-HVD^{S/T320–519A}, EGFP-HVD^{S/T320–396A}, and EGFP-HVD^{S/T411–519A} fusion proteins were constructed (Fig. 5A) and used in pulldown experiments together with previously described WT EGFP-HVD-, EGFP-HVD^{P423A}-, and EGFP-HVD^{R428A}-expressing plasmids (10).

Consistent with previous findings (10), no CD2AP was pulled down using EGFP-HVD^{P423A} and EGFP-HVD^{R428A}. In contrast, CD2AP binding was observed for all fusion proteins harboring mutations in the potential phosphorylation sites. It was, however, noted that the HVD harboring S/T320–519A substitutions bind CD2AP with reduced efficiency. The same mutant also possessed slightly decreased ability to bind BIN1 (Fig. 5B). These effects were probably due to mutations in the SH3 domain-binding sites 1 and 2 (Fig. 5A). Consistent with the fact that G3BP1/2-binding sites were not affected (Fig. 5A), no obvious reduction in G3BP1 binding was observed for any of the mutants (Fig. 5B). Instead, the S/T320–519A and S/T411–519A substitutions clearly increased the binding of G3BP1 (Fig. 5B). This effect can be attributed to the weakening of CD2AP and BIN1 binding, as the binding of these proteins to SH3 domain-binding sites 1 and 2 has been proposed to affect an allosteric site located between G3BP1/2 binding motifs (Fig. 5A) and to regulate G3BP1/2 binding (13). The same allosteric site has been suggested to be affected by effects caused by the binding of FHL1 to its binding region (14). The S/T320–519A and S/T411–519A substitutions led to a major reduction in the binding of FHL1 but did not block it entirely (Fig. 5B). Taken together, these results show that the introduced substitutions did, directly or due to changes in phosphorylation, affect the ability of the HVD in CHIKV nsP3 to bind CD2AP, BIN1, G3BP1, and FHL1. However, even complete inhibition of BIN1 or CD2AP binding has little to no effect on CHIKV replication in BHK-21 cells (10, 12). Thus, altered/reduced/abolished binding of the analyzed host factors cannot explain the lethal phenotype of CHIKV S/T320–519A, which therefore must result from either the lack of phosphorylation and/or from the lack of binding of unknown host protein(s) or the inability of the mutated HVD to support the formation of correct complexes with multiple viral and host factors.

DISCUSSION

This study confirmed that nsP3 in CHIKV and ONNV is, similar to the nsP3 proteins in previously analyzed model alphaviruses, a phosphoprotein. However, several important differences were also revealed. It was found that in CHIKV nsP3, phosphorylated residues were scattered in both halves of the HVD. Elimination of potential phosphorylation sites in either half of the HVD resulted in the significant attenuation of the CHIKV in mammalian cell culture, and the same manipulation made in the entire HVD resulted in complete loss of infectivity. None of these effects had been observed in previously studied alphaviruses, highlighting the dangers of outcomes being associated with generalizations and emphasizing the need for detailed studies of each virus individually. Finally, it was clearly shown that the elimination of phosphorylation sites in the HVD inhibits CHIKV replicase activity, confirming a direct link between CHIKV RNA synthesis and nsP3 phosphorylation. Consistently, all the viable mutants of CHIKV exhibited nsP3 phosphorylation.

While it can be assumed that nsP3 in CHIKV^{S/T320–519A} lacks phosphorylation, we

FIG 5 Effects of substitutions of potential phosphorylation sites in the HVD in CHIKV nsP3 on the binding with CD2AP, BIN1, G3BP1, and FHL1. (A) Schematic presentation of expression constructs used for pulldown of host factors bound to the WT and mutant HVDs in CHIKV nsP3. EF1α, human EF1α promoter. The mutations in control constructs, which are unable to bind CD2AP (10), are shown under the drawing. The amino acid sequence of the HVD in CHIKV WT and positions of the alanine substitutions are shown. The binding site of FHL1 is shown in the box. Binding sites of other known host factors (14) are in bold and highlighted as follows: SH3 domain-binding sites (binding CD2AP/BIN1) are red, G3BP1/2 binding motifs are green, and an allosteric site is blue. (B) U2OS cells were transfected with pEGFP-HVD, pEGFP-HVD^{S/T320-396A}, pEGFP-HVD^{S/T320-519A}, pEGFP-HVD^{S/T411-519A}, pEGFP-HVD^{P423A}, and pEGFP-HVD^{R428A}. EGFP-binding beads were used to pull down proteins bound to the HVD that were detected with immunoblots using antibodies against CD2AP, BIN1, G3BP1, and FHL1. The relative amounts of immunoprecipitated proteins are shown below panels; the amount of protein immunoprecipitated from the lysate of cells transfected with pEGFP-HVD was set as 100. The lysate of mock-transfected U2OS cells was used as a control. The experiment was repeated twice with similar results obtained each time.

were unable to directly prove that this is the case. In the past, similar questions had been addressed by the use of transient expression and analysis of the corresponding recombinant proteins (34). Interestingly, revisiting data sets generated for the analyses of nsP3 interaction partners (10) revealed multiple peptides belonging to the HVD in nsP3; however, we could not reliably detect the presence of their phosphorylated

variants (data not shown). We cannot exclude that this outcome was due to the different methods used for sample preparations and mass spectrometry. However, it is also known that the SFV and CHIKV nsP3 proteins have a number of important differences. For example, they differ by the effects they have on the host cell; in turn, these effects depend on whether the nsP3 protein is bound to the plasma membrane (28). Furthermore, in infected cells, nsP3 forms at least two types of complexes that have different structures, compositions, and functions (41–43). Transient expression of recombinant proteins would yield information only for the complexes that are not involved in RNA replication. The only way to reproduce the complexes involved in RNA replication is to use replicating RNAs, which was attempted through the use of a *trans*-replicase. The results yielded clear-cut data indicating that no RNA synthesis occurs when the HVD in the CHIKV nsP3 lacks Ser/Thr residues. Thus, phosphorylation analysis of the corresponding mutant nsP3 in the context of the functional replicase complex could not be performed.

It is also not known whether the lack of phosphorylation was the only reason for the lack of RNA synthesis of the CHIKV harboring S/T320–519A mutations. It has been recently shown that to be able to replicate CHIKV must have the HVD that should be able to bind G3BPs and at least one other host factor (12); both of these requirements are fulfilled in CHIKV^{S/T320–519A} (Fig. 5B). It is possible that the substitution of 46 Ser/Thr residues with Ala residues resulted in the elimination of some currently unknown, yet important, interaction motif(s) in nsP3. In this regard, it is important that the mutations introduced into either half of the HVD attenuated the virus, and only their combination resulted in a lethal phenotype. Thus, the sites for any unknown host factor(s) must be present in both halves of the HVD. Nevertheless, this possibility does not contradict the hypothesis that these critical sites include phosphorylated Ser/Thr residues. It cannot be excluded that the observed lethal phenotype results from a change in the hydrophobic profile of the HVD. The use of hydrophilic residues instead of Ala residues would, however, generate other important problems. The use of Arg or Lys residues would generate opposite charges and possibly interfere with the interactions between the HVD and essential host factors. Negatively charged Glu and Asp residues can act as phosphomimetic residues, and their use could, therefore, mask the effect originating from the elimination of phosphorylation sites.

The molecular reasons for the RNA-negative phenotype of the CHIKV lacking Ser/Thr residues in the HVD are also intriguing. Mutations with similar effects have already been described in the macrodomain and AUD in the CHIKV (23, 25), highlighting the importance of all three nsP3 domains for RNA replication. For the HVD, however, it is unclear how this defect occurs, as this region, which is very flexible, tolerates both large insertions (40, 44) and significant deletions (Fig. 2). To the best of our knowledge, the only currently known mutations in the HVD of the CHIKV that result in loss of replicase activity are those blocking the interaction with G3BP1/2 proteins (9) or eliminating all other interaction sites for host proteins (12). The binding sites of the G3BP1/2 proteins are known (45) and were not affected by introduced substitutions; in fact, the introduced substitutions resulted in increased binding of G3BP1 (Fig. 5B). Furthermore, the HVD of CHIKV^{S/T320–519A} binds other known host factors, albeit with altered efficiencies (Fig. 5B). In addition, almost all known host factors bind to the C-terminal half of the HVD in CHIKV nsP3; nevertheless, Ser/Thr-to-Ala substitutions in this region had a relatively minor effect on CHIKV replicase activity and CHIKV infectivity. These data indicate the existence of an additional crucial factor or several factors that, in combination, are critical for CHIKV replication.

The crucial role of the phosphorylation sites in nsP3 for CHIKV infectivity has several important practical implications. Deletions at the beginning of the HVD in nsP3 do not eliminate its phosphorylation; however, they result in attenuated viruses, which are promising vaccine candidates (46). The attenuation observed for CHIKV^{S/T320–396A} and CHIKV^{S/T411–519A} indicates that the substitution of a large number of Ser/Thr residues

with Ala residues may also represent a promising approach for CHIKV attenuation that can be used for vaccine development. Dependence on nsP3 phosphorylation could be used to inhibit CHIKV replication. As cellular kinases represent one of the most prominent common nodes in the host-virus interaction (47), it can be envisioned that drugs designed against other viruses or indications may also possess anti-CHIKV activities. Thus far, such effects have been observed for sorafenib, a tyrosine kinase inhibitor (48). However, kinase inhibitors are a rapidly expanding group of drugs with many candidates in clinical development. Inhibitors of CK2, a Ser/Thr kinase, have shown promise as drugs for the treatment of human tumors (49, 50), and it is plausible that compounds with similar activities may have a use in the treatment of CHIKV infection.

MATERIALS AND METHODS

Cell culture. BHK-21 cells (ATCC CCL-10) were cultured in Glasgow's minimal essential medium (GMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; GE Healthcare), 2% tryptose phosphate broth, and 20 mM HEPES (pH 7.2). U2OS cells (ATCC HTB-96) were cultured in Iscove's modified Dulbecco's medium (IMDM, Gibco) supplemented with 10% FBS and 2 mM L-glutamine. All growth media contained 100 U/ml penicillin and 0.1 mg/ml streptomycin (PAA). Cells were maintained at 37°C in a humidified incubator with 5% CO₂.

Plasmids and viruses. All WT viruses and their mutants were rescued from plasmids containing corresponding icDNAs. In these plasmids, the cDNA of CHIKV of the ECSA genotype (strain LR 2006OPY1) was placed under the control of the immediate early promoter of the human cytomegalovirus (37), and the plasmid was designated pCMV-ICRES1. The cDNAs of CHIKV of the WA genotype (strain 37997), CHIKV of the Asian genotype (CNR20235 isolate), ONNV (Chad isolate), and SFV (strain SFV4) were under the control of the bacteriophage SP6 RNA polymerase promoter (51–53).

SFV^{Δ319–368}, CHIKV mutants with a deletion in the nsP3 region, and CHIKV^{SFV-HVD} have been previously described (10, 28, 35, 37). To obtain recombinant CHIKV harboring Ser/Thr-to-Ala substitutions, a fragment corresponding to the mutated HVD was obtained as a synthetic DNA (GenScript). icDNA clones of the CHIKV containing Ser/Thr-to-Ala substitutions in the entire HVD, in the region between amino acid residues 320 and 396 in nsP3, or in the region between amino acid residues 411 and 519 in nsP3 were obtained using standard cloning procedures and designated pCMV-ICRES1^{S/T320–519A}, pCMV-ICRES1^{S/T320–396A}, and pCMV-ICRES1^{S/T411–519A}, respectively. The codon for the Cys173 residue in nsP3 of pCMV-ICRES1^{S/T320–396A} was substituted with the codon for Tyr using a synthetic DNA fragment and standard cloning procedures; the resulting plasmid was designated pCMV-ICRES1^{S/T320–396A+C173Y}. These mutations were also introduced into pCMV-P1234, a previously described plasmid for the CHIKV trans-replication system (40); the resulting plasmids were designated pCMV-P123^{S/T320–519A}4, pCMV-P123^{S/T320–396A}4, pCMV-P123^{S/T411–519A}4, and pCMV-P123^{S/T320–396A+C173Y}4. The pCMV-P1234^{GAA} plasmid, which contains mutations resulting in the substitution of Asp^{466–467} to Ala^{466–467} in the catalytic center of nsP4, and pHSPoll-Fluc-Gluc, which is a plasmid encoding the template RNA, have been previously described (39, 40).

The pEGFP-HVD plasmids for the expression of enhanced green fluorescent protein (EGFP) fused with the HVD in CHIKV nsP3 and the plasmids pEGFP-HVD^{P423A} and EGFP-HVD^{R428A}, which harbor the P423A and R428A mutations, respectively, in the second SH3 domain-binding site, have been previously described (10). Similar plasmids containing the above-described Ser/Thr-to-Ala mutations in the HVD in CHIKV nsP3 were constructed using PCR and standard cloning procedures and designated pEGFP-HVD^{S/T320–519A}, pEGFP-HVD^{S/T320–396A}, and pEGFP-HVD^{S/T411–519A}.

Virological methods. A total of 1 μg of pCMV-ICRES1 and its mutant variants were used to transfect BHK-21 cells as previously described (54). To obtain stocks of CHIKV-Asian, CHIKV-WA, ONNV, SFV, and SFV^{Δ319–368}, the corresponding icDNA plasmids were linearized and transcribed using the mMessage mMachine SP6 transcription kit (Ambion); a total of 1 μg of each obtained RNA was used to transfect BHK-21 cells as previously described (55). The transfected cells were incubated at 37°C for 24 h before the P₀ virus stocks were collected.

Cells transfected with pCMV-ICRES1 or pCMV-ICRES1^{S/T320–519A} were collected at the same time as the corresponding virus stocks. The cells were washed with 1 ml of phosphate-buffered saline (PBS) and lysed by boiling in Laemmli sample buffer (100 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 200 mM dithiothreitol, and 0.2% bromophenol blue) for 10 min. The obtained lysates were analyzed by immunoblot analysis as described below.

Virus stocks were titrated by plaque assay. Briefly, serial virus stock dilutions in GMEM containing 0.2% bovine serum albumin were used to infect confluent layers of BHK-21 cells. After 1 h, the infection mixture was replaced with GMEM containing 2% FBS and 0.8% carboxymethyl cellulose (CMC). After 72 h of incubation at 37°C, the cells were stained with crystal violet, and plaques were counted.

Plaque-purified virus isolates were obtained as described previously (56). Briefly, BHK-21 cells on 6-well cell culture plates were infected with 20 PFU of viruses collected from cells transfected with pCMV-ICRES1^{S/T320–396A} or pCMV-ICRES1^{S/T411–519A}. After infection, cells were covered with a GMEM overlay containing 2% FBS and 0.9% Bacto agar. At 48 hpi, an overlay of 0.9% agar containing neutral red dye was added. Cells were incubated for another 8 h, after which plaques were picked and added into the growth medium of BHK-21 cells growing on 24-well plates. The cells were incubated at 37°C until the

cytopathic effect was observed; at this point, viral stocks were collected. For both viruses, genomic RNAs from three plaque-purified isolates were extracted using the RNeasy minikit (Qiagen). The obtained RNAs were reverse transcribed using a random hexamer primer and the First-Strand cDNA synthesis kit (Thermo Fisher Scientific). The whole nonstructural regions were PCR amplified to obtain sets of overlapping fragments that were analyzed using Sanger sequencing.

Pulldown of host proteins interacting with the HVD in CHIKV nsP3. U2OS cells grown on 60-mm plates were transfected with pEGFP-HVD, pEGFP-HVD^{P423A}, pEGFP-HVD^{R428A}, pEGFP-HVD^{S/T320-519A}, pEGFP-HVD^{S/T320-396A}, and pEGFP-HVD^{S/T411-519A} using Lipofectamine LTX reagent (Invitrogen). Cells were collected at 48 h posttransfection, and EGFP-bound proteins were pulled down as described previously (10). Briefly, cells were washed with PBS, trypsinized, and collected by centrifugation at $200 \times g$ for 10 min. The obtained pellet was lysed using ice-cold lysis buffer (150 mM NaCl, 20 mM HEPES [pH 7.2], 100 mM α -acetate, 2 mM $MgCl_2$, 0.1% Tween 20, 1% Triton X-100, and 1 tablet of Pierce protease inhibitor [Thermo Scientific] in 50 ml of buffer). After incubation on ice for 30 min, cell debris was removed by centrifugation for 10 min at 4°C and $15,000 \times g$. The supernatant was transferred into a new tube, EGFP-binding magnetic beads (GFP-Trap M; ChromoTek) equilibrated with lysis buffer were added, and samples were incubated with rotation for 1 h at 4°C. Next, the beads were washed four times with lysis buffer, and proteins bound to the beads were denatured by boiling in Laemmli sample buffer for 10 min. The obtained samples were used for immunoblot analysis.

Immunoblot analysis. To obtain samples from virus-infected cells, BHK-21 cells were infected with each virus at a multiplicity of infection (MOI) of 5. After 1 h, the infection mixtures were replaced with GMEM containing 2% FBS, and the cells were incubated at 37°C for 6 h (CHIKV, SFV, and mutants thereof) or for 12 h (ONNV). Then, the cells were washed with PBS and lysed by boiling in Laemmli sample buffer for 10 min.

The proteins from virus-infected cells or pulled down from cells transfected with pEGFP-HVD and its mutant variants were separated by SDS-PAGE in 10% gels. The CHIKV, ONNV, and SFV nsP3 proteins, as well as the CHIKV capsid protein, were detected using the corresponding rabbit polyclonal antibodies (all generated in-house); β -actin (catalog no. sc-47778; Santa Cruz Biotechnology) was used as a loading control. Mouse monoclonal antibodies (all from Santa Cruz Biotechnology) were used to detect G3BP1 [(H-10); catalog no. sc-365338], CD2AP [(B-4); catalog no. sc-25272], FHL1 [(H-4); catalog no. sc-374246], and BIN1 [amphiphysin II (2F11); catalog no. sc-23918]. The membranes were incubated with the appropriate secondary antibodies conjugated to fluorescent infrared dyes (LI-COR), and the signals were visualized with the LI-COR Odyssey Fc Imaging System. Band intensities were determined with the LI-COR Image Studio software and calculated as percentages of the corresponding band obtained for cells transfected with pEGFP-HVD that was set as 100.

Infectious center assay. An infectious center assay (ICA) was performed as previously described (39). Briefly, serial dilutions of BHK-21 cells transfected with 5 μ g of pCMV-ICRES1, pCMV-ICRES1^{S/T320-519A}, pCMV-ICRES1^{S/T320-396A}, pCMV-ICRES1^{S/T320-396A+C173Y}, or pCMV-ICRES1^{S/T411-519A} were seeded onto plated subconfluent BHK-21 cell cultures. The transfected cells were allowed to attach for 2 h and then covered with a GMEM overlay containing 2% FBS and 0.8% CMC. After 72 h of incubation at 37°C, the cells were stained with crystal violet, the plaques were counted, and the infectivity values were calculated (in PFU per 1 μ g of plasmid DNA).

nsP3 radiolabeling and immunoprecipitation. BHK-21 cells grown on 6-well plates were infected at an MOI of 5 for 1 h; the infection mixtures were then replaced with GMEM growth medium. At 2 hpi (CHIKV, SFV, and mutants thereof) or 8 hpi (ONNV), the cells were washed twice with phosphate-free Dulbecco's modified Eagle's medium (PFM; Thermo Fisher Scientific), starved in PFM for 2 h, and labeled for 2 h using [³²P]phosphoric acid (Hartmann Analytic) added at 100 μ Ci per well. The labeled cells were washed twice with PFM and once with PBS, collected, and lysed by boiling in 1% SDS for 10 min. The obtained lysates were clarified by centrifugation at $1,000 \times g$ for 10 min and diluted 1:10 with NET150 buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 5 mM EDTA, and 1% NP-40). The nsP3 proteins were bound to antibodies by incubating the cell lysates with appropriate antisera overnight at 4°C with end-over-end rotation. Then, 50 μ l of 50% suspension of protein A-Sepharose (GE Healthcare) was added to each sample, and samples were incubated for 1 h at 4°C with end-over-end rotation. Next, the samples were washed four times with NET400 buffer (50 mM Tris [pH 8.0], 400 mM NaCl, 5 mM EDTA, and 1% NP-40). The immunocomplexes bound to the protein A-Sepharose were collected by centrifugation at $3,000 \times g$. The supernatant was removed by aspiration, and the Sepharose pellet, resuspended in Laemmli sample buffer, was boiled for 10 min. The proteins were separated by SDS-PAGE in 10% gels, and the gels were vacuum dried and visualized using a Typhoon IP biomolecular imager (GE Healthcare).

Trans-replication assay. A trans-replication assay was conducted as previously described (40). Briefly, U2OS cells grown on 12-well plates were cotransfected with 1 μ g of pHSPoll-Fluc-Gluc and 1 μ g of pCMV-P1234, pCMV-P123^{S/T320-519A}, pCMV-P123^{S/T320-396A}, pCMV-P123^{S/T320-396A+C173Y}, pCMV-P123^{S/T411-519A}, or pCMV-P1234^{GAA} using Lipofectamine LTX with Plus reagent (Thermo Fisher Scientific). Eighteen hours posttransfection, the cells were collected and lysed, and the activities of the Fluc and Gluc reporters were measured using a dual-luciferase reporter assay system and a GloMax 20/20 luminometer (Promega).

Statistical analysis. Student's unpaired *t* tests were used to compare pairs of data sets to analyze the results of the trans-replication assay. The statistical analysis was performed with GraphPad Prism 8 software.

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We declare no conflicts of interest.

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