Venezuelan Equine Encephalitis Virus Capsid Protein Inhibits Nuclear Import in Mammalian but Not in Mosquito Cells

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Venezuelan equine encephalitis virus (VEEV) represents a continuous public health threat in the United States. It has the ability to cause fatal disease in humans and in horses and other domestic animals. We recently demonstrated that replicating VEEV interferes with cellular transcription and uses this phenomenon as a means of downregulating a cellular antiviral response. VEEV capsid protein was found to play a critical role in this process, and its ~35-amino-acid-long peptide, fused with green fluorescent protein, functioned as efficiently as did the entire capsid. We detected a significant fraction of VEEV capsid associated with nuclear envelope, which suggested that this protein might regulate nucleocytoplasmic trafficking. In this study, we demonstrate that VEEV capsid and its N-terminal sequence efficiently inhibit multiple receptor-mediated nuclear import pathways but have no effect on the passive diffusion of small proteins. The capsid protein of the Old World alphavirus Sindbis virus and the VEEV capsid, with a previously defined frameshift mutation, were found to have no detectable effect on nuclear import. Importantly, the VEEV capsid did not noticeably interfere with nuclear import in mosquito cells, and this might play a critical role in the ability of the virus to develop a persistent, life-long infection in mosquito vectors. These findings demonstrate a new aspect of VEEV-host cell interactions, and the results of this study are likely applicable to other New World alphaviruses, such as eastern and western equine encephalitis viruses.

Venezuelan equine encephalitis virus (VEEV) is a member of the Alphavirus genus in the Togaviridae family. It circulates continuously in Central, South, and North America and has an ability to cause fatal disease in humans and horses. Along with some other New World alphaviruses, including eastern equine encephalitis virus (EEEV) and western equine encephalitis virus (WEEV), VEEV represents a serious public health threat in the United States (47, 60–62). Viruses in the VEEV complex are serologically classified into six distinct antigenic subtypes (58, 66, 67), with members of subtypes IAB and IC associated with major epidemics and equine epizootics. In humans, VEEV produces a greatly debilitating and sometimes fatal disease (51), with a high possibility of permanent neurological sequelae. During VEEV epizootics, equine mortality rate is low (<1%), neurological diseases, including disorientation, ataxia, mental depression, and behavioral changes, can be detected in up to 14% of infected individuals, especially children (22, 28). VEEV infection of mice leads to a biphasic disease with initial replication in lymphoid tissues, followed by viremia and penetration into the central nervous system (CNS), where the virus replicates until the death of the infected animal (15, 20, 21, 26). The result of the CNS infection is an acute encephalomyelitis that leads to a massive death of neuronal cells (9).

In spite of the continuous public health threat, the pathogenesis leading to invasion of the human CNS is poorly understood. The current experimental vaccine against VEEV infection (TC-83 strain) (4) is of poor efficacy and demonstrates a high rate of adverse reactions (4, 25). Over 8,000 humans have been vaccinated (2, 8, 43), and the cumulative data suggest that nearly 40% of vaccinated people develop a disease with some symptoms typical of those found in natural VEEV infection, including a febrile, systemic illness and other adverse effects (2).

No effective antivirals have been developed against this virus.

VEEV has a nonsegmented, positive-sense RNA genome that is approximately 11.5 kb long. The 5' two-thirds of the genome encodes four nonstructural proteins (nsP1 to nsP4) that form, together with cellular proteins, an enzyme complex required for viral replication (55). After the RNA entry into the cytoplasm, a nonstructural polyprotein is translated directly from the viral genome and utilized in the production of a full-length, negative-sense replicative RNA intermediate. This RNA is then used as a template for synthesis of positive-sense genomic RNA and transcription of a subgenomic 26S RNA. The latter, 4-kb-long RNA corresponds to the 3' one-third of the viral genome and is translated into a structural polyprotein that is co- and posttranslationally cleaved into the capsid and envelope glycoproteins E2 and E1 (46). Thus, the VEEV genome encodes a few proteins that have defined functions in RNA replication and virion formation. However, like some other viruses, if not all of them, VEEV is capable of interfering with the development of the cellular response induced by virus replication. Our previous studies demonstrated that one of the viral structural proteins, capsid (C_{VEEV}), not only is involved in the packaging of the viral RNA into the viral particles but also plays a critical role in the development of a cytopathic effect in tissue culture, downregulation of cellular transcription and,
thus, interference with activation of cellular genes (18). Moreover, a significant fraction of C_{VEE} is associated with the nuclear envelope, and its distribution is reminiscent of the localization of the nuclear pores (16). Taken together, the preliminary data indicated that VEEV capsid protein might be involved in the regulation of nucleocytoplasmic traffic and use this phenomenon as one of the means of inhibiting RNA polymerase I- and II-dependent cellular transcription.

In the present study, we further investigated the effect of C_{VEE} on nucleocytoplasmic transport. Our data demonstrate the following. (i) Both VEEV capsid and its active peptide (amino acids [aa] 33 to 68), which we identified in a previous study (16), block multiple nuclear import pathways, but they do not noticeably affect the passive diffusion of small proteins from the cytoplasm to the nucleus. (ii) This activity is specific for C_{VEE} but not for the capsid derived from the Old World alphavirus Sindbis virus (SINV). (iii) C_{VEE} expression does not noticeably affect nuclear import in the cells of mosquito origin. This inability to affect nuclear traffic provides one of the plausible explanations for the lack of profound cytopathic effect (CPE) in VEEV-infected arthropod cells and persistent, lifelong replication of VEEV in mosquito vectors without a noticeable effect on their biological functions.

**Materials and Methods**

**Cell cultures.** BHK-21 cells were obtained from Paul Olivo (Washington University, St. Louis, MO), NH3T3 cells were obtained from the American Type Culture Collection (Manassas, VA), HEK293 cells were provided by Robert Davey (University of Texas Medical Branch at Galveston). These cell lines were maintained at 37°C in alpha minimum essential medium (aMEM) supplemented with 10% fetal bovine serum (FBS) and vitamins. Mosquito C6/36 cells were obtained from Henry Huang (Washington University, St. Louis, MO). They were propagated in Dulbecco’s MEM supplemented with 10% heat-inactivated FBS and 10% tryptose phosphate broth.

**Plasmid constructs.** pVEErep/cTomatoplasm carried the VEEV replicon, in which two tdtomato genes (52) were fused in frame through a short linker, GHTGSGGSGSSG, which was previously applied for tandem cloning of other fluorescent proteins (52), and the entire cassette was cloned under control of the subgenomic promoter of the replicon. The tdtomato gene encodes a dimer of modified DsRed (52); thus, the entire cassette was designated 4xTomato to emphasize that this is a tetrameric protein. Further modifications of this expression cassette, which were aimed at fusing the 4xTomato with different nuclear localization signals (NLSs), were made by using PCR for cloning the sequences that were designed from the oligonucleotides. pVEErep/CVEE/GFP encoded the VEEV replicon, in which one of the subgenomic promoters controlled the expression of VEEV capsid, and the second promoter drove green fluorescent protein (GFP) expression. The expression of this marker was used for demonstrating the presence of the replicon in the cells. pVEErep/CsIN/GFP had the same design but encoded a SINV capsid protein (CsIN) under control of the subgenomic promoter. pVEErep/CsVEE/GFP contained a VEEV replicon having only one subgenomic promoter that drove the expression of the CsVEE-GFP fusion protein. To avoid cleavage, the last amino acid in CsVEE was deleted, and fusion was performed through the short linker peptide (Gly)4. pVEErep/CVEE/GFP and pVEErep/CVEE/IshRF-GFP had similar designs, but carried in the fusion protein only aa 1 to 68 of the VEEV capsid or CsVEE with previously identified framesshift mutations (18) affecting aa 58 to 85. The latter framesshift mutations made the capsid incapable of causing transcriptional shutoff and CPE development (18). pSRrep2v/Ubi-nP23/cherry HA encoded a SINV replicon that had a mutation in the nP23/nP3 cleavage site (53), and the subgenomic promoter controlled the expression of hemagglutinin (HA)-tagged VEEV nP23. This replicon was incapable of P23 processing; thus, the unprocessed SINV nP23 did not interfere with VEEV nP23 transport into the nucleus. Ubi was used to promote the expression of VEEV nP23 with an efficient start codon. pVEErep/Cherry contained a VEEV replicon that encoded one of the red fluorescent proteins, Cherry (52), under the control of the subgenomic promoter. All of the above-described VEEV and SINV replicon-based cassettes were synthesized by PCR-based techniques, and all of the heterologous genes were sequenced. All of the constructs that we used are presented in the corresponding figures. Sequences of the recombinant plasmids can be provided upon request.

**VEEV and SINV helpers used for replication packaging were described elsewhere (12, 56).**

**Viruses.** Vaccine strains VEEV TC-83 and SINV Toto1101 were rescued from the previously described infectious cDNA clones (42, 45). VEEV/nPS2/GFP was developed by random GFP insertion mutagenesis using the same strategy as we previously described for SINV (3). The GFP insertion was made after aa 3 of the nP2 gene in the VEEV TC-83 genome, and in contrast to similar SINV constructs (3), this additional sequence had only a minor effect on the efficiency of P23 cleavage (data not shown). The detailed description of this VEEV variant will be presented in a later publication. SIN/VEE chimeric virus encoded the SINV-derived 5’ and 3’ untranslated regions, the subgenomic promoter, and nPs, but the structural genes were VEEV TRD specific and had additional mutations, adapting this virus to efficient growth in BHK-21 cells (37).

**RNA transcriptions.** Plasmids were purified by centrifugation in CsCl gradients and linearized using the MluI restriction site located downstream of the poly(A) sequence of the VEEV replicons and helper-encoding plasmids. RNAs were synthesized by using SP6 RNA polymerase in the presence of a Cap analog as previously described (45). The yield and integrity of transcripts were analyzed by gel electrophoresis under nondenaturing conditions. RNA concentration was measured with a FluorChem imager (Alpha Innotech), and transcription reactions were used for electroporation without additional purification.

**Packaging of the replicons.** BHK-21 cells were coelectroporated with the in vitro-synthesized SINV replicon and BBdels/L2/C and BbdeDLS/G1 helper RNAs (12) or by VEEV replicon and HveC/C and HveC/G1 helper RNAs (56) by using previously described conditions (7). The indicated helper RNAs encode either capsid or viral glycoproteins and contain no packaging signals. They are packaged into viral particles very inefficiently. Packaged replicons were harvested at 24 to 30 h postelectroporation. Titer of the replicon-containing infectious virus particles were determined by infecting BHK-21 cells with serially diluted samples. Cells were further incubated for 16 h at 30°C in a CO2 incubator, and the numbers of infected cells were determined with an inverted fluorescent microscope by counting fluorescent virus particles that were detected in a mature virus particle. Titers of infectious VEEV TC-83 and VEEV/nPS2/GFP were determined using the standard plaque assay (31).

**Analysis of protein synthesis.** BHK-21 cells were seeded into six-well Costar plates at a concentration of 5 × 104 cells/well. After 4 h of incubation at 37°C in 5% CO2, they were infected at a multiplicity of infection (MOI) of 20 PFU/cell or 20 infectious units (i.u.) of viruses or packaged replicons, respectively, in 500 μl of aMEM supplemented with 1% FBS at room temperature for 1 h with continuous shaking. The medium was then replaced by a corresponding complete medium, and incubation continued at 37°C. At 8 h postinfection, the cells were washed three times with phosphate-buffered saline and then incubated for 30 min at 37°C in 0.8 ml of Dulbecco’s MEM medium lacking methionine, supplemented with 0.1% FBS and 20 μg/ml of [35S]methionine. After this incubation, cells were scraped into the medium, pelleted by centrifugation, and dissolved in 200 μl of standard protein loading buffer and loaded onto sodium dodecyl sulfate-10% polyacrylamide gels. After electrophoresis, gels were dried and autoradiographed.

**Microscopy.** BHK-21 cells were seeded on glass chamber slides (Nunc) and infected or coinfected by the packaged replicons for 1 h. Incubation was continued for 8 h at 37°C in a CO2 incubator. Cells were then fixed in phosphate-buffered saline and supplemented with 4% formaldehyde, and the distribution of the proteins was analyzed by using a Zeiss LSM510 META confocal microscope with a 63x/1.4 numerical aperture oil immersion Plan-Apochromat lens. For staining of VEEV nP2, cells were permeabilized with 0.5% Triton X-100 and stained with anti-VEEV nsP2 antibodies, kindly provided by Alphavax (Research Triangle Park, NC) (used at a 1:2,000 dilution). After being stained with Alexa Fluor 488-labeled secondary antibodies (Invitrogen), cells were analyzed via confocal microscopy. These goat anti-VEEV nP2 antibodies demonstrated considerable staining of the nuclei in the mock-infected cells. Therefore, before immunostaining, we were preabsorbed on the lysate of BHK-21 cells. For preabsorption of 1 μl of the antisera, 2 × 105 cells were used. Cells were pelleted by low-speed centrifugation, permeabilized with 0.5% Triton X-100, and additionally homogenized through a syringe needle. Preabsorption was performed for 1 h at room temperature with continuous shaking. Cell debris was then removed by centrifugation at 16,000 × g, and the supernatant was used for cell staining as described above. Staining with affinity-purified, rabbit anti-SINV nP2 (1:2,000) and anti-HA (1:200) (Covance) antibodies was performed using the same protocol, but the preabsorption step was omitted.
RESULTS

VEEV infection inhibits nuclear import. Our previous studies identified the presence of C_{VEE} on the nuclear membrane and suggested the possibility that this protein might affect nucleocytoplasmic trafficking and, thus, interfere with the expression of cellular genes (16). Therefore, in initial experiments, we developed an experimental system for analysis of inhibition of the nuclear import: we designed a VEEV replicon encoding a high-molecular-weight fluorescent protein that could not translocate into the nucleus by passive diffusion and whose presence in the nucleus could be achieved only by active, importin-dependent transport through the nuclear pores. It was previously believed that only the proteins having a molecular mass below 40 to 50 kDa could migrate into the nuclei by passive diffusion, which is mediated by different channels in the nuclear pore complex (NPC) (36). However, the recently published data indicated that the maximum size of the proteins that are capable of diffusing through the NPC could be larger than 60 kDa (59). Therefore, for this study, we designed a 4xTomato protein (see Materials and Methods for details), which had a molecular mass of 109.5 kDa, and cloned it into the VEEV replicon under control of the subgenomic promoter. Upon delivery into BHK-21 cells by electroporation, the expressed 4xTomato was detected only in the cytoplasm (Fig. 1A). In the following experiments, this protein was fused with a variety of NLS sequences. These modified genes were also cloned into the VEEV replicon (VEErep) under control of the subgenomic promoter (see Materials and Methods for details), and the replicons were packaged into infectious viral particles by using the previously described two-helper system (56).

The first tested expression cassette contained a triple simian...
in the cells indicated the presence of the CVEE-expressing VEEV capsid. GFP was used for two reasons: (i) its expression from other subgenomic promoter was driving the expression of the 10 kDa) under the control of the subgenomic promoter, and an active, so-called classical nuclear import pathway was inhibited only the receptor-mediated nuclear import pathways or blocks passive diffusion of small proteins as well. Inhibition of nucleocytoplasmic trafficking and distinguished whether it inhibited nuclear import. Moreover, based on the results of our previous studies (16, 18) and the results from another research group (1), it was indicated that VEEV nsPs do not block nuclear import.

VEEV capsid inhibits multiple nucleocytoplasmic transport pathways. The above-described experiments indicated that VEEV structural proteins function in the inhibition of nuclear import. Moreover, based on the results of our previous studies (16, 18) and the results from another research group (1), it was reasonable to expect that the VEEV capsid, but not the envelope glycoproteins, plays a critical role in this process. Therefore, in successive experiments, we tested whether CVEE affects nucleocytoplasmic trafficking and distinguished whether it inhibits the receptor-mediated nuclear import pathways or blocks passive diffusion of small proteins as well. Inhibition of the active, so-called classical nuclear import pathway was analyzed using the above-described VEEV replicon expressing the 4xTomato-3xNLS reporter protein (Fig. 2A). The second replicon encoded a small GFP (having a molecular mass of 27 kDa) under the control of the subgenomic promoter, and another subgenomic promoter was driving the expression of the VEEV capsid. GFP was used for two reasons: (i) its expression in the cells indicated the presence of the CVEE-expressing replicon, and (ii) the analysis of the intracellular distribution of the GFP provided information about whether passive diffusion of small proteins to the nucleus was affected by the expressed, tested proteins (59). The rationale for applying VEEV replicons to express capsid and its derivatives instead of using more traditional RNA polymerase II promoter-based expression cassettes was based on the fact that VEEV- and EEEV-derived capsids efficiently inhibit nuclear transport. Therefore, their expression from plasmid DNA-based cassettes could be problematic.

Both CVEE and 4xTomato-3xNLS-coding replicons were packaged into infectious viral particles, and BHK-21 cells were infected separately with VEERep/4xTomato-3xNLS and VEERep/CVEE/GFP or with both replicons together. In the VEERep/CVEE/GFP-infected cells (Fig. 2A), GFP was distributed in the cytoplasm and the nucleus, indicating that CVEE expression did not noticeably affect the passive diffusion of GFP and, most likely, other small proteins through the nuclear pores. As described above (Fig. 1A), the 4xTomato-3xNLS was concentrated in the nuclei of the cells infected with the VEERep/4xTomato-3xNLS-expressing replicons. However, when the same protein was produced in the cells coinfected with CVEE-encoding replicons, it was no longer transported into the nucleus and remained only in the cytoplasm (Fig. 2A, panel a). This was a strong indication that CVEE inhibits at least an importin-α/β-dependent nuclear import pathway, which is considered to be responsible for translocation into the nucleus of more than 50% of the proteins (30). It should be noted that CVEE expression from VEERep/CVEE/GFP and the expression of reporter protein 4xTomato-3xNLS and CVEE derivatives, fused with GFP (see this and the following sections), were additionally confirmed by metabolic labeling of the cell proteins with [35S]methionine, followed by an analysis of the cell extracts by gel electrophoresis (Fig. 3 and data not shown). The proteins of interest were readily detectable on gels and were expressed at levels comparable to those of capsids expressed by replicating viruses. To rule out the possibility that inhibition of nuclear import is a synergistic effect of VEEV nsP and capsid functions, we also expressed 4xTomato-3xNLS and VEEV capsid from the T7 promoter/encephalomyocarditis virus internal ribosome entry site-dependent cassettes in (i) a BHK-21 cell-derived cell line expressing T7 DNA-dependent RNA polymerase and (ii) BHK-21 cells infected with a vaccinia virus recombinant expressing the same enzyme (14). In both expression systems, the results were very similar to those described above: VEEV capsid efficiently inhibited nuclear import of 4xTomato-3xNLS (data not shown). Thus, the capsid protein itself can interfere with nuclear import.

However, the detected blockage of importin-α/β-dependent nuclear import does not necessarily indicate that the transport of all of the proteins is affected. Therefore, we additionally evaluated the function of two other, M9- and histone H2b-specific, pathways in the presence of CVEE. The M9 NLS, derived from hnRNPA1 (54), is recognized by transportin that mediates nuclear transport of the RNA-binding proteins. Histone H2b utilizes multiple nuclear import pathways, mediated by importin-α/β complex, transportin, importin 5, importin 7, importin 7/importin β complex, and importin 9 (35). The application of this reporter allows detection of alterations in nuclear import pathways other than those that are SV40 TAg NLS or M9 dependent.

Thus, we cloned both M9- and the entire mouse H2b histone-coding sequences into the carboxy terminus of the 4xTomato gene. VEERep/4xTomato-M9 and VEERep/4xTomato-H2b replicons were packaged into the infectious viral particles, and after infection of BHK-21 cells, both peptides demonstrated efficient functioning in the translocation of the 4xTomato into the nucleus (Fig. 2D). However, the M9 NLS was noticeably less efficient than were the triple SV40 TAg NLS and the H2b protein, and a significant fraction of the 4xTomato-M9 reporter remained in the cytoplasm (Fig. 2D). In the cells coinfected with the 4xTomato-encoding replicons and the replicon expressing CVEE, 4xTomato-M9 and 4xTomato-H2b proteins were not transported into the nucleus (Fig. 2B and C). These data indicate that CVEE is capable of inhibiting nuclear import mediated by at least three different types of NLSs and, most likely, its presence in the cells strongly affects the entire nuclear import.
SINV capsid expression does not affect nuclear import. Our previous study demonstrated that capsid protein of the Old World alphaviruses SINV and Semliki Forest virus (SFV) is incapable of inhibiting cellular transcription and causing CPE, in spite of being present in the nuclei (18). To further dissect the differences between the New World and the Old World alphavirus capsid functions in virus-host cell interactions, we cloned the SINV-specific capsid (CSIN) gene into the VEEV replicon and coinfected BHK-21 cells with packaged VEErep/4xTomato-3xNLS and VEErep/C_{SIN}/GFP (see Materials and Methods for details). (A) BHK-21 cells were coinfected with packaged VEErep/4xTomato-3xNLS and VEErep/C_{SIN}/GFP replicons. (a) Distribution of the 4xTomato-3xNLS protein; (b) distribution of GFP; (c) overlay of the two images. (B) BHK-21 cells were coinfected with VEErep/4xTomato-M9 and VEErep/C_{SIN}/GFP replicons. (a) Distribution of the 4xTomato-M9 protein; (b) distribution of GFP; (c) overlay of the two images. (C) BHK-21 cells were coinfected with VEErep/4xTomato-H2b and VEErep/C_{SIN}/GFP replicons. (a) Distribution of the 4xTomato-H2b protein; (b) distribution of GFP; (c) overlay of the two images. (D) Distribution of fluorescent proteins in BHK-21 cells infected with VEErep/C_{SIN}/GFP, VEErep/4xTomato-M9, or VEErep/4xTomato-H2b. Cells infected with VEErep/4xTomato-3xNLS are presented in Fig. 1. All of the images were acquired at 8 h postinfection. Bars correspond to 20 μm. The schematic representation of the replicons is shown for each panel.

FIG. 2. Expression of C_{SIN} affects nuclear import of the proteins with different NLSs. (A) BHK-21 cells were coinfected with packaged VEErep/4xTomato-3xNLS and VEErep/C_{SIN}/GFP (see Materials and Methods for details). (a) Distribution of the 4xTomato-3xNLS protein; (b) distribution of GFP; (c) overlay of the two images. (B) BHK-21 cells were coinfected with VEErep/4xTomato-M9 and VEErep/C_{SIN}/GFP replicons. (a) Distribution of the 4xTomato-M9 protein; (b) distribution of GFP; (c) overlay of the two images. (C) BHK-21 cells were coinfected with VEErep/4xTomato-H2b and VEErep/C_{SIN}/GFP replicons. (a) Distribution of the 4xTomato-H2b protein; (b) distribution of GFP; (c) overlay of the two images. (D) Distribution of fluorescent proteins in BHK-21 cells infected with VEErep/C_{SIN}/GFP, VEErep/4xTomato-M9, or VEErep/4xTomato-H2b. Cells infected with VEErep/4xTomato-3xNLS are presented in Fig. 1. All of the images were acquired at 8 h postinfection. Bars correspond to 20 μm. The schematic representation of the replicons is shown for each panel.
efficiently as the entire CVEE (16, 18). These data suggested that cumulative on the nuclear membrane and causes CPE as efficacious, correlates with the lack of CSIN-specific transcriptional shutoff at all. Thus, its inability to interfere with the nuclear import is undetectable in our experiment, or does not have this activity either causes only a minor effect on the nuclear import, which 3xNLS replicon alone (Fig. 1B). These experiments demonstrated the Old World alphavirus SINV-specific capsid peptide that has two short, distinct domains: aa 33 to 51, or entire subgenomic RNA replaced by SINV-specific counterparts were dramatically less cytopathic than were the parental VEEV and could not interfere with the cellular antiviral response (16, 18). These chimeric viruses either persisted in the cells, defective in the alpha/beta interferon response, or were cleared from the cells having functional alpha/beta interferon signaling (18). To further understand the differences between the New World and Old World alphavirus nsP2 activities in the modification of the nuclear function, we analyzed the distribution of VEEV nsP2, produced by different expression cassettes, and evaluated how CVEE interferes with the nuclear import of VEEV (and SINV) nsP2.

The recently published data suggested that VEEV nsP2 and its fragments can be detected in the nuclei (34). Therefore, in contrast to CVEE, the SINV-specific capsid had no noticeable effect on the translocation of 4xTomato-3xNLS into the nuclei (Fig. 4A). The latter protein was transported to the nucleus as efficiently as in the cells infected with the VEErep/4xTomato-3xNLS replicon alone (Fig. 1B). These experiments demonstrated that the Old World alphavirus SINV-specific capsid either causes only a minor effect on the nuclear import, which is undetectable in our experiment, or does not have this activity at all. Thus, its inability to interfere with the nuclear import correlates with the lack of CSIN-specific transcriptional shutoff (16, 18).

The VEEV capsid-specific N-terminal peptide plays a critical role in blocking nucleocytoplasmic transport. In the previous study, we identified an amino-terminal peptide in CVEE that plays a critical role in CPE development and induction of transcriptional shutoff. This peptide, located between aa 32 and 69 of CVEE, has two short, distinct domains: aa 33 to 51, which were previously shown to fold into an α-helix secondary structure (38, 39), and the downstream, positively charged amino acid sequence that likely contains a functional NLS. We have shown that the CVEE32-68 peptide, fused with GFP, accumulates on the nuclear membrane and causes CPE as efficiently as the entire CVEE (16, 18). These data suggested that this fragment might play a critical role in the CVEE-mediated inhibition of the nucleocytoplasmic traffic.

To test this hypothesis, we cloned the amino-terminal fragment of CVEE (aa 1 to 68) fused with GFP into the VEEV replicon, VEErep/CVEE1-68-GFP (Fig. 4B). The aa 1-to-68 sequence was applied instead of a minimal peptide to promote similar expression levels of this particular fusion protein and those in other cassettes. The second replicon encoded CVEE-GFP fusion, in which a short sequence in the CVEE (aa 58 to 85) was changed by two frameshift mutations (Fig. 4C). In our previous study (18), this CVEEfrsh-GFP fusion was found to be noncytopathic and incapable of inhibiting cellular transcription. The VEEV replicon, encoding the entire wild-type CVEE fused with GFP (Fig. 4D), was used as a positive control.

All of the replicons were packaged into infectious viral particles and used for coinfection of BHK-21 cells, together with the packaged VEErep/4xTomato-3xNLS. Expression of CVEE1-68-GFP inhibited translocation of 4xTomato-3xNLS to the nucleus (Fig. 4B), as did the expression of the control CVEE-GFP fusion (Fig. 4D). (CVEE1-68-GFP reproducibly formed small aggregates that might reflect its level of expression, which was ∼2-fold higher than those of other chimeric proteins.) However, in the cells coinfectected with CVEEfrsh-GFP-producing replicon and VEErep/4xTomato-3xNLS, transport of the 4xTomato-3xNLS to the nucleus was not altered (Fig. 4C), despite the fact that CVEE-GFP and CVEEfrsh-GFP were expressed at very similar rates and to similar levels; these levels were also very similar to those for other proteins presented in Fig. 3 (data not shown). Thus, the amino-terminal sequence of CVEE inhibits the classical nuclear import pathway. The previously described frameshift mutation in the CVEE peptide that makes this protein incapable of translocation into the nucleus and affects its ability to cause CPE and inhibiting cellular transcription also makes CVEE unable to block nuclear import.

VEEV nsP2 accumulates mainly in the cytoplasm. One of the nonstructural proteins, nsP2, of the Old World alphaviruses (SINV and SFV) is known to be present at a high concentration in the cell nuclei, where it functions in transcription inhibition (17, 48–50). In the case of SINV and, most likely, of SFV, this translocation is indirectly supported by the inability of the capsid protein to interfere with nuclear import. However, the newly described function of CVEE suggests to us that translocation of VEEV nsP2 into the nucleus might be problematic, and in contrast to its SIN-specific counterpart, this protein is likely to accumulate in the cell nuclei at a low concentration. Our previously published data indirectly supported the possibility that VEEV nsP2 does not play a critical role in modification of the nuclear function: (i) the VEEV (and EEEV) replicons were incapable of downregulating cellular transcription to a level incompatible with cell survival and persistently replicated in some of the cell lines of vertebrate origin (42), and (ii) the VEEV variants with either the capsid or entire subgenomic RNA replaced by SINV-specific counterparts were dramatically less cytopathic than were the parental VEEV and could not interfere with the cellular antiviral response (16, 18). These chimeric viruses either persisted in the cells, defective in the alpha/beta interferon response, or were cleared from the cells having functional alpha/beta interferon signaling (18). To further understand the differences between the New World and Old World alphavirus nsP2 activities in the modification of the nuclear function, we analyzed the distribution of VEEV nsP2, produced by different expression cassettes, and evaluated how CVEE interferes with the nuclear import of VEEV (and SINV) nsP2.

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order to generate definitive results, we applied multiple cell lines and a variety of expression constructs to analyze the intracellular distribution of this protein. In the initial experiments, we infected three different cell lines, BHK-21, HEK293, and NIH 3T3, with VEEV TC-83. The available VEEV nsP2-specific antibodies demonstrated significant staining of the nuclei in the mock-infected cells; therefore, they were preabsorbed in the lysate of the BHK-21 cells (see Materials and Methods for details). After the preabsorption, staining of the mock-infected cells was no longer detectable (Fig. 5D).

FIG. 4. Analysis of the 4xTomato-3xNLS distribution in cells expressing either CSIN or different variants of CVEE, fused with GFP. (A) BHK-21 cells were coinfected with packaged VEErep/4xTomato-3xNLS and VEErep/CSIN/GFP (see Materials and Methods for details). (a) Distribution of the 4xTomato-3xNLS protein; (b) distribution of GFP; (c) overlay of the two images. (B) BHK-21 cells were coinfected with VEErep/4xTomato-3xNLS and VEErep/CVEE1-68-GFP replicons. (a) Distribution of the 4xTomato-3xNLS protein; (b) distribution of CVEE1-68-GFP; (c) overlay of the two images. (C) BHK-21 cells were coinfected with VEErep/4xTomato-3xNLS and VEErep/CVEEfrsh-GFP replicons. (a) Distribution of the 4xTomato-3xNLS protein; (b) distribution of CVEEfrsh-GFP; (c) overlay of the two images. (D) BHK-21 cells were coinfected with VEErep/4xTomato-3xNLS and VEErep/CVEE-GFP replicons. (a) Distribution of the 4xTomato-3xNLS protein; (b) distribution of CVEE-GFP; (c) overlay of the two images. All of the images were acquired at 8 h postinfection. Bars correspond to 20 µm. The schematic representation of the replicons is shown for each panel.
By 8 h postinfection with VEEV TC-83, nsP2 was present in the nuclei of all of the tested cell lines at very low concentrations (Fig. 5A to C). At later times, we could find this protein at higher concentrations in the nuclei of a small percentage of the infected cells, which were already at very advanced stages of apoptosis. Thus, the experiments supported the hypothesis that in the presence of CVEE, VEEV nsP2 is unlikely to play a major role in cellular nuclei, because of its low presence in this compartment.

In the experiments that followed, we evaluated the effect of CVEE expression on the compartmentalization of nsP2 of different origins. BHK-21 cells were infected either (i) with the wild-type SINV, which, as we showed above, encodes capsid protein that does not affect nuclear import (Fig. 6A), or (ii) with SIN/VEE chimeric virus (37) that expresses all of the VEEV structural proteins (Fig. 6B). In the SINV-infected cells, SINV nsP2 was distributed both in the cytoplasm and in the nuclei (Fig. 6A), and in the SIN/VEE virus-infected cells, nsP2 was detected only in the cytoplasm (its concentration in this compartment was noticeably

FIG. 5. Distribution of VEEV nsP2 in the cells infected with VEEV TC-83. The nsP2 distribution in BHK-21 (A), NIH 3T3 (B), and HEK293 (C) cells is shown. Staining was performed at 8 h postinfection (see Materials and Methods for details). (D) Staining of the mock-infected cells with VEEV nsP2-specific antibodies. (Subpanels a) Staining with VEEV nsP2-specific antibodies; (b) nuclear staining with Sytox orange; (c) overlays of the images. Bars correspond to 20 µm.
higher than that in SINV-infected cells) (Fig. 6B). This was an indication that CVEE expression likely interferes with SINV nsP2 translocation to the nuclear compartment.

In another line of experiments, we designed an infectious VEEV TC-83 variant that contained a GFP insertion in the very amino terminus of nsP2, after aa 3 (Fig. 7). In spite of normal processing of the nonstructural polyprotein (data not shown) and productive virus replication, the nsP2/GFP, expressed by VEEV/nsP2GFP, was detected only in the cytoplasm of the infected cells (Fig. 7).

However, the above experiments could not provide a definitive answer to the question of whether VEEV nsP2 itself had not developed an ability for translocation into the nucleus or whether its cytoplasmic distribution was a result of CVEE function in blocking nuclear import. To distinguish between these possibilities, we infected BHK-21 cells with a VEEV replicon, VEErep/Cherry, that did not encode any structural proteins.

FIG. 6. SINV nsP2 distribution depends on the capsid protein, encoded by viral genome. Intracellular distribution of SINV nsP2 during SINV Toto 1101 replication (A) or replication of SINV/VEEV recombinant virus in BHK-21 cells (B). (C) Staining of the mock-infected BHK-21 cells. (Subpanels a) Staining with the SINV nsP2-specific antibodies; (b) nuclear staining with Sytox orange; (c) overlays of the images. Staining was performed at 8 h postinfection (see Materials and Methods for details). Images were acquired at 8 h postinfection. Bars correspond to 20 μm. In the schematic representations of viral genomes, SINV-specific sequences are indicated by open boxes and VEEV sequences are indicated by filled boxes.

FIG. 7. VEEV nsP2-GFP fusion protein distribution in the cells infected with VEEV/nsP2GFP. BHK-21 cells were infected with the chimeric virus at an MOI of 20 PFU/cell, and nsP2/GFP distribution was evaluated at 8 h postinfection with a confocal microscope. (a) Distribution of VEEV nsP2/GFP. (b) Overlay of nsP2/GFP and nuclear staining with Sytox orange. Bars correspond to 20 μm. The schematic representation of the viral genome is shown.
and stained the cells with VEEV nsP2-specific antibodies. Almost all of the nsP2 was detected in the cytoplasm of the replicon-infected cells (Fig. 8A). In another approach, we infected BHK-21 cells with a packaged SINV replicon that had a mutation in the nsP2/nsP3 cleavage cite and encoded the HA-tagged VEEV nsP2 under the control of the subgenomic promoter (Fig. 8B). This cleavage site mutant was incapable of P23 processing; thus, the unprocessed SINV nsP2 remained in the cytoplasm and could not interfere with VEEV nsP2 transport into the nucleus. In the cells infected with SINrep2V/nsP2\textsubscript{VEE}-HA, the HA-tagged nsP2 was also detected only in the cytoplasm (Fig. 8B). Taken together, the results of the experiments strongly suggested that (i) compared to SINV or SFV nsP2, the VEEV-specific counterpart had a dramatically reduced ability for translocation into the nucleus, and (ii) its presence in the nuclei at very low concentrations was not only the effect of CVEE functioning in modification of the nucleocytoplasmic traffic but also a result of the inability of VEEV nsP2 itself to import into the nucleus.

**CVEE does not affect nuclear import in mosquito cells.** The characteristic feature of alphavirus infection in the mosquito cells is the development of persistent, noncytopathic replication that mirrors the persistent infection in mosquito vectors. The antiviral response in arthropod cells is different from that in vertebrate cells and appears to be not as robust as that in cells of vertebrate origin. Moreover, inhibition of both nuclear import and cellular transcription, likely resulting in development of CPE, could be disadvantageous for persistent virus replication and would have a negative effect on the viability of infected mosquitoes and ultimately virus transmission in nature. Thus, we hypothesized that CVEE expression might have no effect on nuclear import in mosquito cells and experimentally tested this assumption.

Replicons, packaged into a VEEV TC-83-specific envelope, infected C\textsubscript{6}10 cells inefficiently (data not shown); therefore, we delivered VEErep/4xTomato-3xNLS, VEErep/C\textsubscript{VEE}-GFP, and VEErep/C\textsubscript{VEE}/GFP replicon genomes into these cells by electroporation. An evaluation of the 4xTomato-3xNLS distribution in the C\textsubscript{VEE}-GFP-expressing (Fig. 9A) and C\textsubscript{VEE}-expressing (Fig. 9B) cells strongly indicated that C\textsubscript{VEE} had no noticeable effect on the translocation of the 4xTomato-3xNLS protein into the cell nuclei. Regardless of the presence of C\textsubscript{VEE} and C\textsubscript{VEE}-GFP, the 4xTomato-3xNLS was efficiently transported into the nucleus. Thus, the failure to inhibit nuclear import correlates with the inability of the virus to cause CPE and appears to be one of the determinants of persistent replication in mosquito cells and mosquito vectors.

**DISCUSSION**

Replication of alphavirus-specific RNAs, translation of the viral proteins, and release of infectious particles do not require a nuclear function. Viral replication can proceed for a long time in the presence of ActD or even in anucleated cells (10). Nevertheless, the interaction of replicating virus with host cell nuclei plays a critical role in the infectious process and strongly...
determines pathogenesis on the molecular, cellular, and organism levels. As do other viruses, alphaviruses appear to induce an innate intracellular antiviral response (virus-induced cell response) that is mediated by cellular pathogen recognition receptors (27, 33). The latter response leads to the induction of cell signaling, initiating an antiviral state in the as-yet-uninfected cells and activation of an antiviral reaction in the already-infected cells. These processes interfere with productive viral replication and dissemination of the infection. Alphaviruses, in turn, developed mechanisms aimed at a strong modification of cell biology and/or the development of robust, rapid CPE. In these processes, the inhibition of cellular translational and transcriptional machineries appears to play a critical role (6, 13, 17–19). These modifications of the intracellular environment are likely to be multicomponent phenomena, determined by different viral nonstructural and structural proteins, and we have only recently begun to understand their mechanisms. The accumulated data strongly indicate that both the Old World and the New World alphaviruses are capable of downregulating cellular transcription but utilize very different mechanisms for achieving this goal (17, 18). The Old World alphavirus-specific nonstructural protein nsP2 is a key player in the inhibition of both cellular messenger and ribosomal RNA synthesis (17). This protein causes CPE development, either when being expressed in the context of nonstructural polyprotein or alone from different expression cassettes. On the other hand, the New World alphaviruses VEEV and EEEV use capsid protein, but not the nsP2, for causing the same phenomenon (1, 16–18). This structural protein is distributed both in the cytoplasm and in the nuclei of the infected cells and downregulates RNA polymerase I- and II-dependent transcription, and its expression ultimately leads to CPE development (1, 18). This function of VEEV capsid is determined by a short amino-terminal peptide capable of inhibiting transcription even when being fused to other proteins (16). Modifications of the active peptide in the VEEV genome made the virus dramatically less cytopathic and attenuated in vivo without having a strong effect on its replication rates (or had no effect at all for some of the constructs) (16).

The distinguishing features of the VEEV capsid-dependent transcriptional shutoff include its low rates of development and a lack of dependency on capsid-associated protease activity (16, 18). These data strongly indicated stoichiometric rather than enzymatic modes of capsid functioning. Moreover, a significant fraction of CVEE was found to be associated with the nuclear membrane, and its distribution was reminiscent of that of the NPCs (16). The CVEE32-68 peptide fused with GFP demonstrated a similar distribution and was also capable of blocking cellular transcription. Taken together, the results indicated that CVEE (and its active CVEE32-68 peptide) might be involved not only in the downregulation of cellular transcription but also in the modification of the NPC and the inhibition of nucleocytoplasmic traffic.

In the present work, we evaluated the function of several nuclear import pathways, mediated by different importins, and found that all of them were inhibited by the CVEE within 8 h postinfection. (Notably, for expression of this protein, we used the most adequate system, VEEV replicons, which express capsid in appropriate cellular compartments and at natural concentrations.) The CVEE1-68 peptide, fused with GFP, dem-
onstrated a very similar activity, and C\textsubscript{VEE}frsh-GFP, which does not inhibit cellular transcription and is undetectable in the cell nuclei and on the nuclear membrane, had no effect on the nucleocytoplasmic traffic. Thus, accumulation of C\textsubscript{VEE} in the nuclear envelope strongly correlated with the inhibition of nuclear import. We cannot completely rule out the possibility that not only the passive diffusion of the small proteins but also some of the nuclear import pathways, which we did not test in our study, continue to function during VEEV infection. However, we are certain that in infected cells, nucleocytoplasmic traffic is strongly modified, and a great fraction of the newly synthesized cellular proteins is not transported into the nucleus. Among the RNA viruses, blockage of nuclear import is not a new phenomenon. To date, inhibition of nuclear transport has been described for VSV, poliovirus, rhinovirus, and cardiovirus (23, 32, 40, 44). The VSV matrix protein interacts with the nucleoporin Nup98 and export receptor Rae 1 (11). Thus, M protein accumulates in the NPCs (41), in which it efficiently inhibits Rae 1-mediated mRNA nuclear export (41, 57) and lowers the rate of nuclear import through the importin α/β1-dependent pathway (40). Interestingly, VSV also efficiently inhibits cellular transcription (5), but the correlation between inhibition of nucleocytoplasmic traffic and downregulation of transcription has not been investigated. Two members of the Picornaviridae family, poliovirus and rhinovirus, employ different mechanisms and inhibit nuclear import by degrading the nucleoporins Nup62 and Nup153 (24) by virus-encoded proteases. The same proteases also process the transcription factors (29, 63–65). VEEV and, most likely, other New World alphaviruses employ a mechanism that appears to be based on an interaction of the capsid protein with NPC. Moreover, functioning of C\textsubscript{VEE}1-68-GFP fusion in inhibition of nuclear import suggests that capsid might function without proteolytic cleavage of the nucleoporins. However, the possibility of synergistic functioning of proteolytic and binding mechanisms needs further investigation.

To date, we cannot directly connect the transcriptional shut-off and inhibition of nucleocytoplasmic transport, because the reagents blocking specific nuclear import pathways are not available. These might be independent functions of C\textsubscript{VEE}, or it may be that this protein strongly downregulates nuclear import, and this event, in turn, leads to inhibition of synthesis of all of the cellular RNAs and contributes to CPE development. Further experiments are certainly needed to distinguish between hypotheses.

The Old World alphaviruses SINV and SFV produce capsid proteins that neither block nuclear import nor interfere with cellular transcription, and they do not induce CPE. Consequently, one of the characteristic features of such infection is the accumulation of large amounts of nsP2 in the cell nuclei. The newly described function of C\textsubscript{VEE} in the inhibition of the nucleocytoplasmic transport suggested that VEEV nsP2 had to be present in the cell nuclei at a lower concentration and is unlikely to play an important role in this compartment. Indeed, multiple lines of evidence, generated in our studies, support this hypothesis. Moreover, VEEV nsP2 itself was found to be inefficient in its translocation to the nucleus. It was detected almost exclusively in the cytoplasm not only in the virus-infected cells but also when produced by VEEV replicon or expressed from other vectors as GFP-nsP2 or nsP2-HA-tag fusions. Such VEEV nsP2 compartmentalization indirectly supports our previous finding that VEEV replicons, expressing no capsid, caused a less efficient CPE than did similar SINV- and SFV-based constructs and were capable of establishing a persistent replication in the mammalian cells (42). This was an indication that VEEV- and EEEV-specific nsP2 and other nsPs did not cause as strong a negative effect on cellular biology as did the nsPs of the Old World alphaviruses. Notably, the putative nsP2-specific NLS (which was described for SFV nsP2) is replaced in VEEV nsP2 by a different amino acid sequence (34). This peptide might still function as an NLS, and the mutations in this sequence affect virus and replicon RNA replication (34). However, as shown for SFV, mutations in this particular peptide could strongly affect the rates of the non-structural polyprotein processing and/or other nsP2 functions in RNA replication (48). Therefore, the existence of monopartite NLS in VEEV nsP2 remains questionable. Nevertheless, it is possible that this nonstructural protein, produced by replicating VEEV, has some function in the modification of the nucleocytoplasmic traffic because of its recently described interaction with karyopherin 1 (34).

Interestingly, in our study, C\textsubscript{VEE} was found to be incapable of blocking nuclear import in mosquito cells. Large, NLS-containing protein 4xTomato-3xNLS was transported into the cell nuclei regardless of the presence of C\textsubscript{VEE} in the same cells. Thus, the inability of VEEV capsid to interfere with nucleocytoplasmic trafficking provides a plausible explanation for the noncytopathic phenotype of VEEV in the mosquito cells. However, we strongly believe that this is not the only critical difference between virus replication activities in the cells of vertebrate and invertebrate origin.

In conclusion, we demonstrated the following. (i) C\textsubscript{VEE} efficiently inhibits nuclear import but does not affect passive diffusion of small proteins. (ii) The amino-terminal sequence of C\textsubscript{VEE} interferes with nuclear import as efficiently as does the entire C\textsubscript{VEE}. (iii) The capsid protein of the Old World alphavirus SINV or the previously defined frameshift mutant of C\textsubscript{VEE} (C\textsubscript{VEE}frsh), which is incapable of transcription inhibition, have no detectable effect on nucleocytoplasmic trafficking. (iv) We also speculate that inhibition of the NPC function is one of the critical mechanisms which the New World alphaviruses employ for the downregulation of cellular transcription and CPE development, and (v) we note that C\textsubscript{VEE} does not noticeably interfere with NPC-mediated nuclear import in the mosquito cells, and this might play a critical role in the ability of the virus to develop a persistent, lifelong infection in mosquito vectors.

These new findings demonstrate an additional aspect of VEEV-host cell interactions and viral replication. The results of this study are important for understanding New World alphavirus pathogenesis on the molecular level.

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