Capsid Protein of Eastern Equine Encephalitis Virus Inhibits Host Cell Gene Expression

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Eastern equine encephalitis virus (EEEV), a mosquito-borne member of the Alphavirus genus of the Togaviridae family, is among the deadliest of the mosquito-borne viruses. In humans, the fatality rate following symptomatic infection approaches 80%, and many survivors develop crippling sequelae, such as mental retardation, convulsions, and paralysis (18, 37).

EEEV possesses a single-stranded, positive-sense RNA genome of approximately 11.7 kb. The four viral nonstructural proteins (nsP1-4) are produced from the full-length genomic RNA as a polyprotein and carry out functions important for viral RNA synthesis and polyprotein processing. A second polyprotein, translated from the 26S subgenomic mRNA, gives rise to the three major structural proteins, namely, the capsid and the two envelope proteins, E1 and E2. The envelope proteins are involved in receptor recognition, virus attachment, penetration, and membrane fusion during viral entry. These proteins also cooperate with the capsid during viral assembly and release. Based on observations made with the capsids of other alphaviruses, the EEEV capsid is expected to encapsulate the viral genomic RNA, to interact with the viral glycoproteins during assembly, and as a function to release itself from the nascent structural polypeptide chain (54, 63, 65).

The alpha/beta interferon (IFN-α/β) system plays a major role in early innate immune responses and constitutes the first line of defense against viral infection. Production of IFN-α/β is mediated by the activation of transcription factors, such as NF-κB, ATF-2/c-Jun, and interferon regulatory factors (IRFs). IFN-α/β released from cells binds to the IFN-α/β receptor, activating a Jak-STAT signaling cascade that leads to the phosphorylation and activation of STAT-1 and STAT-2 transcription factors. Activation (phosphorylation) of STAT-1 and STAT-2 leads to their heterodimerization, translocation to the nucleus, and association with p48/IRF-9 to form a complex that binds DNA sequences containing interferon-stimulated response elements and promotes the induction of specific gene expression (5, 29). IFN-α/β regulates the transcription of numerous genes, a number of which encode antiviral proteins, and leads to the induction of an antiviral state in cells.

Due to the importance of the IFN response as an antiviral defense, many viruses have developed mechanisms to inhibit this response by means of blocking or inhibiting IFN production, IFN-mediated signaling, and/or the activity of IFN-induced gene products (3, 12, 26). For example, the influenza A virus NS1 protein has been shown to suppress the initiation of the IFN response (23, 49, 53, 66, 67, 74). In another example, the highly virulent Ebola virus carries two IFN antagonist proteins: VP35 blocks IFN production, and VP24 blocks IFN signaling. The ability of these proteins to inhibit different steps in the IFN response likely plays a role in the extreme virulence of Ebola virus (6, 11, 32, 33, 59).

Among the positive-strand RNA viruses, some members of
the Flaviviridae family have been shown to carry proteins that block the IFN response. For example, the hepatitis C virus NS3-4A protease inhibits signals that lead to IFN-α/β expression (9, 20, 42, 43). In addition, other flaviviruses inhibit IFN-induced Jak-STAT signaling. For example, the dengue virus nonstructural protein NS4B strongly inhibits IFN signaling, whereas NS4A and NS2A partially block IFN signaling (14, 38, 50, 51). In contrast, the flaviviruses Japanese encephalitis virus and Langat virus use the NS5 protein to block IFN signaling, suggesting that viruses from the same family that cause different spectra of human disease may use different mechanisms to overcome the IFN response (7, 44, 45).

Studies with alphasviruses have demonstrated the importance of the IFN system in controlling infection and disease (1, 22, 30, 60, 71, 73). Mice with a defect in the IFN-α/β response succumb faster to infections with several alphasviruses than do wild-type mice (30, 60, 73). However, it remains unknown whether alphasviruses, specifically EEEV, carry proteins that inhibit the interferon response.

Previous studies with Sindbis virus (SINV), an Old World alphasivirus, suggest that nsP2 is important for the suppression of the antiviral response, including the production of IFN-α/β in infected cells (22). Whether the same applies to the New World encephalitic alphasviruses (i.e., EEEV and Venezuelan equine encephalitis virus), which cause a different and more severe spectrum of clinical disease in humans than that of the Old World viruses (65), has not been determined. Because of the evolutionary, genetic, and biological differences between the New World and Old World alphasviruses, we hypothesized that the mechanisms by which Old and New World alphasviruses evade the innate immune response might also differ. We therefore sought to identify specific EEEV proteins with IFN antagonist properties, anticipating that such factors will play an important role in EEEV pathogenesis. Our results indicate that the EEEV capsid functions as a potent IFN antagonist protein by globally inhibiting host cell gene expression. Mechanistically, we demonstrate that the capsid protein inhibits the expression of RNA polymerase II-transcribed genes and also promotes phosphorylation of the eukaryotic initiation factor 2α (eIF2α) subunit, suggesting capsid-mediated effects upon translation as well.

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**MATERIALS AND METHODS**

**Cells and viruses.** 293T and A549 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). BRST7 cells expressing the T7 RNA polymerase were maintained in DMEM-10% FBS with 400 μg/ml of G418. A Newcastle disease virus expressing an uninduced, empty vector-transfected control. To determine whether the EEEV proteins affect IFN signaling, Vero cells were transfected with 1 μg of plasmid DNA together with an IFN-β promoter-chloramphenicol acetyltransferase (CAT) reporter plasmid and the indicated firefly luciferase plasmids, using the calcium phosphate mammalian transfection method (Stratagene, La Jolla, CA). One day after transfection, the cells were infected with Sendai virus at an MOI of ~8 and incubated for 1 h at 37°C in 5% CO₂. After incubation, the virus inoculum was removed, and 2 ml of DMEM-BGM was added to the cells. The cells were then incubated at 37°C for 4 h. The virus inoculum was removed, and 0.5 ml of DMEM-10% FBS was added to the cells. The infected cells were incubated for 20 h at 37°C in 5% CO₂ prior to detection of green fluorescence by microscopy.

**Reporter gene assays.** Reporter assays were performed as previously described (11). To determine whether the EEEV proteins block IFN induction, 293T cells (~1 × 10⁶ cells) were transfected with 1 μg of expression plasmid DNA together with an IFN-β promoter-chloramphenicol acetyltransferase (CAT) reporter plasmid and the indicated firefly luciferase plasmids, using the calcium phosphate mammalian transfection method (Stratagene, La Jolla, CA). One day after transfection, the cells were infected with Sendai virus at an MOI of ~8 and incubated for 1 h at 37°C in 5% CO₂. After incubation, the virus inoculum was removed, and 2 ml of DMEM-BGM was added to the cells. The cells were then incubated at 37°C for 4 h. The virus inoculum was removed, and 0.5 ml of DMEM-10% FBS was added to the cells. The infected cells were incubated for 20 h at 37°C in 5% CO₂ prior to detection of green fluorescence by microscopy.

**Transfection of the EEEV capsid with reporter plasmids.** 293T cells were transfected with 1 μg of a GFP or firefly luciferase pCAGGS mammalian expression plasmid (whose expression is driven by the RNA polymerase II promoter) in the presence or absence of the plasmid encoding the capsid protein. Twenty-four hours later, cells transfected with the firefly luciferase plasmid were lysed with reporter lysis buffer following the manufacturer’s protocol, and the firefly luciferase activity was measured in the cell lysates. Cells transfected with...
a GFP plasmid were observed under a fluorescence microscope for a GFP signal. In another set of experiments, BSRT7 cells were transfected with 1 μg of a firefly luciferase pCAGGS plasmid (under the control of an RNA polymerase II promoter) or 1 μg of a firefly luciferase pMI plasmid (under the control of the T7 promoter) in the presence or absence of the plasmid carrying the capsid gene. Twenty-four hours later, cells were lysed and luciferase activity was measured as described above.

Detection of phosphorylated eIF2α. Cell lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane, blocked for 1 h in 5% nonfat dry milk dissolved in Tris-buffered saline, and then probed with antibodies against phospho-eIF2α (serine 51) (Bio-source, Camarillo, CA), eIF2α (Biosource), or α-tubulin (Sigma, St. Louis, Missouri). Secondary antibodies conjugated to horseradish peroxidase and a chemiluminescence detection system from Perkin-Elmer (Wellesley, MA) were used to visualize the proteins. To determine the levels of eIF2α phosphorylation in EEEV-infected cells, 293T cells were infected with EEEV strain FL93-939 (MOI = 3), and supernatants and cell lysates were obtained at 5 and 24 h postinfection (p.i.). Cell lysates were then assayed for eIF2α as described above, and the supernatants were assayed for virus titer by a plaque assay in Vero cells as previously described (1).

Quantitative RT-PCR. Quantitative RT-PCR was performed by using a previously published SYBR green protocol with an ABI7900 HT thermal cycler (75). Briefly, 293T cells were transfected with 1 μg of PR8 NS1 plasmid together with 0, 0.25, 0.5, or 1 μg of the EEEV capsid plasmid. One day after transfection, cells and RNA were extracted simultaneously from the cells by using a PLAGEN (Ambion) following the manufacturer's protocol. cDNAs were created using M-MLV reverse transcriptase and oligo(dT)20 primers. qRT-PCR was performed with DNA-free (Ambion) following the manufacturer's protocol, and the values were used to calculate the transcript copy number by using the following formula: transcript copy number = 2.500 × 1.93[HKG] × [TGI], where 2.500 is an empirical estimate of the number of actin mRNAs/cell (39), 1.93 reflects a typical reaction amplification efficiency of 93%, HKG is the housekeeping gene sample’s median Ct value, and TGI is the target Ct value. Three housekeeping genes were used for global normalization in each experiment (actin, Rps11, and tubulin genes). For Western blot analysis, rabbit NS1 polyclonal antibody (Biosource), or α-tubulin (Sigma, St. Louis, Missouri) were used.

Selection of stable cell lines containing EEEV replicons. BHK-21 cells were electroporated with 5 μg of in vitro-synthesized RNA. Twenty-four hours after electroporation, the medium was replaced with DMEM-10% FBS containing 12.5 μg/ml of puromycin, and surviving cells were individually selected and expanded under puromycin selection. RNAs were extracted from the stable cell lines, and PCR and sequencing analyses were performed to identify potential changes within the replicons.

RESULTS

Expression of EEEV capsid protein counteracts the antiviral effects of the IFN response. To identify EEEV genes that inhibit the IFN-α/β response, we employed a previously described assay that measures the ability of individual viral proteins to complement the replication of NDV-GFP (51, 55). Briefly, A549 cells were transfected with an empty expression plasmid or a plasmid encoding Ebola virus VP35, Nipah virus W, or individual EEEV proteins. At 1 day posttransfection, cells were infected with NDV expressing GFP, and 1 day later, the cells were analyzed for GFP expression. As previously reported, transfection of empty plasmid induced the production of IFN, and therefore the replication of NDV-GFP was inhibited. In contrast, cells transfected with plasmids encoding the known IFN antagonist proteins Ebola virus VP35 and Nipah virus W supported NDV-GFP replication (55). Interestingly, when plasmids encoding the EEEV proteins were transfected, only the capsid protein was able to rescue NDV-GFP replication (Fig. 1). To further address the possibility that one of the nonstructural proteins might also influence the IFN-α/β response, the IFN response was also examined in cells stably transfected with EEEV replicons. However, in these cell lines, we did not detect either inhibition of IFN-β promoter activation or inhibition of IFN-induced gene expression (data not shown), further supporting the view that the capsid is the primary inhibitor of IFN responses in EEEV-infected cells.

EEEV capsid is a general inhibitor of gene expression. Next, reporter gene-based assays were conducted with the goal of determining whether the capsid inhibits IFN-α/β production and/or the cellular response to IFN-α/β. 293T cells or Vero cells were transfected with an IFN-β promoter-CAT reporter plasmid or an interferon-responsive ISG54 promoter-CAT reporter plasmid together with a constitutively expressed simian virus 40 promoter-luciferase reporter plasmid which served as a control for transfection efficiency. The 293T cells were then infected with Sendai virus to activate the IFN-β promoter, and the Vero cells were treated with 1,000 units/ml IFN-β to activate the ISG54 promoter. The results showed that transfection of the capsid plasmid inhibited the expression of both Sendai virus- and IFN-β-induced reporter genes (Fig. 2A and C). However, when the results of the reporter assay were normalized to the luciferase controls, we observed that the capsid protein also down-regulated the activity of the transfection control protein (firefly luciferase), suggesting that capsid may generally inhibit gene expression (Fig. 2B and D). To determine whether the inhibition of the reporters was due to a global inhibition of gene expression, we transfected the capsid together with either luciferase plasmid alone or with a GFP expression plasmid. The results demonstrated that transfection of the capsid inhibited the expression of both luciferase and GFP (Fig. 2E and F, respectively). These results suggest that expression of the capsid protein broadly inhibits the expression of multiple genes expressed from a variety of promoters.

To further characterize the inhibition of gene expression, the capsid was coexpressed in BSRT7 cells (which stably express T7 RNA polymerase) with a plasmid carrying the firefly luciferase gene under the control of either an RNA polymerase II promoter or a T7 promoter. A dose-dependent inhibition of luciferase activity was observed only in cells transfected with the RNA polymerase II-driven promoter plasmid, but this inhibition was not observed when the luciferase gene was under the control of the T7 promoter (Fig. 3). These data suggest that capsid inhibition is selective and can target RNA polymerase II gene expression. The insensitivity of the T7 promoter plasmid also suggests that the inhibition of gene expression in reporter gene assays was not due to toxic effects of the capsid.

Capsid protein induces phosphorylation of the α subunit of eIF2. The previous results suggest that the EEEV capsid protein targets cellular mRNA synthesis. However, some alphaviruses are known to activate protein kinase R (PKR) and consequently induce the phosphorylation of the α subunit of the translation initiation factor eIF2 (16). Therefore, to determine whether the capsid might induce the phosphorylation of eIF2α,
293T cells were transfected with a luciferase reporter plasmid in the absence or presence of a plasmid encoding the capsid protein, the entire EEEV structural polyprotein, or the LaCrosse virus (LCV) NSs protein, previously described as a general inhibitor of host cell transcription (58). Twelve or 24 h after transfection, cells were lysed and assayed for the presence of phosphorylated eIF2α. Phosphorylation of eIF2α was evident at 1 day posttransfection in cells expressing the EEEV capsid, EEEV structural proteins (including capsid), and LCV NSs but was strongest in cells where the capsid alone was expressed (Fig. 4A). The ability of the capsid to strongly induce eIF2α phosphorylation may contribute to the inhibition of gene expression noted above. How the capsid stimulates eIF2α phosphorylation remains to be determined. However, it is notable that LCV NSs, which like the EEEV capsid inhibits mRNA expression (see below), also promotes eIF2α phosphorylation. To determine whether capsid-induced eIF2α phosphorylation might be relevant to EEEV infection, 293T cells were infected with EEEV (MOI = 3), and supernatants and cell lysates were collected at 5 and 24 h p.i. Phosphorylation of eIF2α was more evident at 24 h p.i. than at 5 h p.i. (Fig. 4B and data not shown) and also correlated with higher virus titers than those at the 5-h time point (8.6 ± 0.04 log_{10} versus 5.3 ± 0.07 log_{10}, respectively).

Capsid protein inhibits host mRNA accumulation. Although inhibition of protein synthesis through the PKR pathway is a hallmark of alphavirus infection, a second described mechanism for the shutdown of host protein synthesis by Old World alphaviruses is the inhibition of host cell transcription (27, 28). Therefore, we evaluated whether the capsid also affects RNA levels by transfecting an influenza A/PR/8/34 (H1N1) virus NS1 protein expression plasmid under the control of the RNA polymerase II promoter. We selected the influenza virus NS1 plasmid because of the availability of an established quantitative RT-PCR assay for the NS1 gene and of antibodies for the detection of the NS1 protein by Western blotting. For this experiment, cells were transfected with the NS1 protein expression plasmid in the absence or presence of increasing amounts of capsid expression plasmid, and relative NS1 mRNA copy numbers were determined by quantitative RT-PCR. In the presence of the capsid gene, the PR8 NS1 mRNA copy number was decreased >7-fold compared to the control copy number (Fig. 5A), and this reduction in RNA levels correlated with a substantial reduction in NS1 protein levels (Fig. 5B). In conclusion, these results suggest that the capsid inhibits protein synthesis by inhibiting host cell transcription.
FIG. 2. EEEV capsid is a general inhibitor of gene expression. (A and B) Vero cells were cotransfected with the ISG54 promoter-CAT reporter plasmid, a constitutively expressed luciferase reporter plasmid, and empty vector or a plasmid expressing EEEV capsid or Nipah virus V protein. At 24 hours posttransfection, the cells were mock treated or treated with 1,000 U human IFN-β/ml for 24 h. Cells were harvested and then assayed for CAT and luciferase activities. (A) Nonnormalized CAT activity, with the untreated empty vector control value set to 1.
The N terminus of the EEEV capsid mediates the inhibition of gene expression. To map the specific area of the capsid gene required for the inhibition of protein synthesis, the coding regions for the N-terminal (aa 1 to 126) and C-terminal (aa 127 to 261) regions of the capsid protein were cloned into a mammalian expression plasmid and transfected into 293T cells along with a GFP or luciferase reporter plasmid. A decrease in GFP and luciferase expression was observed only in cells transfected with the N-terminal half of the capsid protein, whereas the C-terminal half, which contains the protease active site, did not produce a significant decrease in the expression of the reporter genes (Fig. 6A and B). Consistent with this observation, only the N-terminal half of the capsid was able to induce the phosphorylation of eIF2α (Fig. 6C). To determine whether the effects of the capsid extend to endogenous gene expression, empty vector or plasmids encoding full-length capsid, the N terminus, and the C terminus were transfected into 293T cells, and the relative mRNA copy numbers of the ISG54 and STAT-1 genes were measured by quantitative RT-PCR after IFN treatment. In the presence of the full-length capsid or the capsid N terminus, the relative copy numbers of ISG54 and STAT-1 decreased two- and fourfold, respectively, compared to those of the control, whereas the effect of overexpressing the C-terminal half of the capsid had less of an effect on mRNA accumulation (Fig. 6D). It should be noted that these experiments, which rely upon transient transfection and leave some percentage of cells untransfected, likely underestimate the effects of capsid on endogenous gene expression. When the expression of each of the capsid constructs was analyzed by Western blotting (for the HA tag), the carboxy-terminal half was expressed at much higher levels than either the full-length capsid or the amino-terminal half, possibly reflecting the ability of the latter two constructs to inhibit gene expression (data not shown).
tion, further supporting our findings that expression of the capsid gene from the replicon RNA a few weeks after selection with puromycin was applied. Sequence analysis of the capsid gene revealed a partial or complete deletion of the capsid gene as a direct fusion to the luciferase gene such that expression of endogenous cellular mRNAs and proteins. Two replicons also expressed the capsid protease activity would cleave and separate the capsid gene from luciferase (Fig. 7A). BHK cells were electroporated with replicon RNA without in vitro-transcribed replicon RNA, and cell lysates were used for Western blot analyses of NS1, HA capsid, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control (B). The data represent means ± standard errors among samples from two independently transfected wells. The experiment was repeated twice and gave consistent results.

An EEEV replicon harboring the capsid gene inhibits protein synthesis. We finally turned to EEEV replicons to determine whether an untagged EEEV capsid expressed in the context of EEEV nonstructural proteins would inhibit the expression of endogenous cellular mRNAs and proteins. Two EEEV replicons harboring the EEEV nonstructural protein gene region, a luciferase gene, and the puromycin resistance gene were constructed. One of the replicons also expressed the capsid gene as a direct fusion to the luciferase gene such that capsid protease activity would cleave and separate the capsid from luciferase (Fig. 7A). BHK cells were electroporated with equal amounts of in vitro-transcribed replicon RNA, and 24 h after electroporation, selection with puromycin was applied. Interestingly, cells electroporated with replicon RNA without the capsid gene formed colonies more efficiently than did replicon-infected cells with the capsid gene (data not shown). Given that the amino terminus of the capsid is required to inhibit host cell gene expression, these data suggest that, ultimately, capsid expression from replicons is toxic to host cells, presumably because of its ability to inhibit gene expression.

**DISCUSSION**

Several viral mechanisms for the inhibition of the IFN response have been reported. However, to date, the mechanisms by which New World encephalitic alphaviruses inhibit the IFN response have not been described. Most of our knowledge about alphaviruses comes from studies with SINV and Semliki Forest virus (SFV), which cause a different spectrum of clinical disease in humans than the New World encephalitic viruses. Specifically, studies with SINV suggested that the nsP2 protein is involved in inhibiting the IFN response by interfering with host protein synthesis at the levels of transcription and translation (22, 24, 27). During our attempts to study viral proteins involved in the inhibition of the IFN response by the New World encephalitic viruses, specifically EEEV, we identified the capsid protein as a potent IFN antagonist. Interestingly, overexpression of the EEEV capsid protein did not show significant inhibition of the antiviral IFN response, suggesting that the strategies used by the Old World and New World alphaviruses to overcome the IFN response are different. Similar to previous reports for nsP2 of SINV (24, 27), the inhibition of the IFN response by EEEV capsid was mediated by a global shutdown of host protein synthesis.

Previous studies have defined roles for alphavirus capsid proteins in proteolytic cleavage of the structural polyprotein, RNA encapsidation, nucleocapsid formation, and binding to ribosomes to facilitate the uncoating process during viral replication (25, 35, 40, 46, 56, 62, 64, 68, 69, 72). In addition, it was reported that the capsid of SFV induces phosphorylation of PKR, leading to inhibition of protein synthesis (16). Therefore, we first investigated whether the EEEV capsid might induce phosphorylation of the α subunit of eIF2, which would suggest an activation of PKR and a means of globally inhibiting protein synthesis. Overexpression of the EEEV capsid gene in 293T cells induced the phosphorylation of eIF2α. However, attempts to establish persistent replication of an EEEV replicon harboring the capsid gene in PKR−/− mouse embryo fibroblasts were unsuccessful (data not shown), suggesting that the absence of PKR does not relieve the toxic effects of the capsid-expressing replicon. Therefore, it seems possible that other mechanisms are involved in the inhibition of host protein synthesis. Thus, we sought to determine whether the capsid gene also inhibits the accumulation of host cell mRNAs, which would be a function analogous to that of the Old World alphavirus nsP2 proteins (24, 27). Cotransfection of the capsid gene inhibited the expression of reporter genes driven by RNA polymerase II but not by the T7 reporter construct, suggesting a mechanism whereby the capsid targets some aspect of RNA polymerase II synthesis or induces host cell RNA degradation. This result also suggests that capsid-induced inhibition of reporter gene expression is not simply due to toxic effects of capsid overexpression.
FIG. 6. The N terminus of the EEEV capsid mediates the inhibition of gene expression. (A) 293T cells were cotransfected with a plasmid expressing the GFP reporter and either empty vector or a plasmid expressing the N terminus (aa 1 to 126) or the C terminus (aa 127 to 261) of the EEEV capsid. At 24 h posttransfection, cells were examined by microscopy for GFP expression. (B) The experiment was performed as described for panel A, except that a luciferase reporter plasmid was used and the cells were harvested and assayed for luciferase activity. (C) 293T cells were cotransfected with a luciferase reporter plasmid and empty vector (vector) or a plasmid expressing EEEV full-length capsid or the N terminus (aa 1 to 126) or C terminus (aa 127 to 261) of the capsid. Twenty-four hours after transfection, the cells were harvested and Western blotting was performed to detect the phosphorylation of eIF2α, total eIF2α, and α-tubulin as a loading control. (D) 293T cells were transfected with empty plasmid or with a plasmid encoding the full-length capsid or the N-terminal or C-terminal half of the capsid. At 24 h posttransfection, the cells were treated with human IFN-β, and 12 h after treatment, RNAs were extracted from the cells for quantitative real-time RT-PCR of STAT-1 and ISG54 mRNAs. The data represent the means ± standard errors among samples from three wells in two experiments.
To further determine the effects of untagged capsid in the context of viral replication, we evaluated host protein synthesis of EEEV replicons, with and without the capsid gene. As previously reported for other EEEV replicons (57), no adaptive viral mutations were identified in our EEEV replicon-bearing cells that did not carry capsid. However, only the EEEV replicon without the capsid gene was readily able to establish persistent replication in IFN-deficient cells, whereas capsid-containing replicons that did yield foci contained a complete or partial deletion of the N-terminal half of the capsid. This observation is reminiscent of previous findings with SINV replicons, in which adaptive mutations in nsP2 that reduced the cytopathic effect caused by this protein were required to establish persistent replication (21, 22, 24). These data support the conclusion that EEEV capsid is a general inhibitor of host cell gene expression. They also suggest that the capsid may contribute to cell death during the course of EEEV replication.

Our results demonstrate that the expression of EEEV capsid in cells is sufficient to counteract the antiviral effects of IFN. They therefore suggest a novel mechanism by which EEEV inhibits the IFN response, through capsid-mediated suppression of host cell gene expression, and suggest an additional and important function for the capsid protein in viral pathogenesis. These results also identify the N-terminal region of the capsid as responsible for this unique function, providing evidence that this function is protease independent. In this regard, EEEV appears to evade IFN responses in a manner different from hepatitis C virus, where the NS3-4A protease cleaves IPS-1 (13, 34, 42, 48) and TRIF (43) to inhibit activation of IRF3. Instead, this mechanism is similar to that proposed for the Old World alphavirus SINV, where nsP2 acts as a general inhibitor of gene expression (24, 27). However, further studies are necessary to map specific residues involved in the inhibition of host gene expression. The N terminus of the EEEV capsid is positively charged (rich in lysine and arginine residues) and also rich in proline and is believed to be important for protein-protein interactions and encapsidation of viral RNA (65). Whether these positively charged residues play a role in the capsid-mediated inhibition of the cellular response, perhaps by specifically binding host proteins or host RNAs or by localizing the capsid protein in specific cell compartments, needs to be determined. Interestingly, the capsid protein of SFV (an Old World alphavirus) has been suggested to have two nuclear localization signals (17), and computer-based analysis has identified potential nuclear localization signals within the N-terminal half of the EEEV capsid. Further experiments should explore whether the EEEV capsid localizes to the nucleus and/or sites of mRNA translation and whether localization is
important for capsid inhibition of gene expression. Further studies should also be carried out to evaluate the importance of eIF2α phosphorylation in capsid inhibition of protein expression, perhaps by using cells expressing a nonphosphorylated form of eIF2α. Such experiments will clarify whether or not the inhibition of host cell mRNA accumulation and eIF2α phosphorylation separately contribute to capsid-mediated inhibition of gene expression or whether phosphorylation of eIF2α occurs as a consequence of the inhibition of host cell gene expression. It will also be of interest to determine whether capsid that enters the host cell with incoming viral particles may contribute to suppression of host cell gene expression.

The inhibition of protein synthesis as a way to inhibit the IFN response by alphaviruses is a mechanism shared with several other arboviruses, including members of the Bunyaviridae family (70). LCV and Bunyamwera and Rift Valley fever viruses have previously been shown to inhibit the IFN response by mediating inhibition of host protein synthesis (8, 10, 36, 70). Mechanistically, they interfere with RNA polymerase II transcription (8, 41, 70). Interestingly, vesicular stomatitis virus, which is also transmitted by insects, also inhibits IFN responses by globally targeting host cell gene expression (2, 15, 19).

These observations contrast with those made recently for arthropod-borne flaviviruses, such as West Nile and dengue viruses, which target specific components of the IFN induction or signaling pathway (7, 31, 47, 50, 51). Further experiments are needed to define the exact mechanism by which New World alphaviruses inhibit cellular transcription and the full consequences of this inhibition for viral replication.

Our results raise interesting questions regarding Old World and New World alphavirus pathogenesis and the different means of inhibiting gene expression. It will be of interest to determine whether the capsid protein is used to inhibit host protein synthesis by all New World alphaviruses or whether this property correlates with a given virus’s ability to cause encephalitis. Future studies using capsid and nsP2 constructs derived from other alphaviruses, including Venezuelan encephalitis virus and Western equine encephalitis virus, are necessary to address this question. This study also provides important information about the specific mechanisms used by EEEV to evade the host response, and more importantly, we present evidence of a novel mechanism for the capsid protein in viral pathogenesis. This information may provide insights that facilitate the development of effective measures to control EEEV infection.

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