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Host translation shutoff mediated by non-structural protein 2 is a critical factor in the antiviral state resistance of Venezuelan equine encephalitis virus

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ABSTRACT

Most previous studies of interferon-alpha/beta (IFN-α/β) response antagonism by alphaviruses have focused upon interruption of IFN-α/β induction and/or receptor signaling cascades. Infection of mice with Venezuelan equine encephalitis alphavirus (VEEV) or Sindbis virus (SINV) induces serum IFN-α/β, that elicits a systemic antiviral state in uninfected cells successfully controlling SINV but not VEEV replication. Furthermore, VEEV replication is more resistant than that of SINV to a pre-existing antiviral state *in vitro*. While host macromolecular shutoff is proposed as a major antagonist of IFN-α/β induction, the underlying mechanisms of alphavirus resistance to a pre-existing antiviral state are not fully defined, nor is the mechanism for the greater resistance of VEEV. Here, we have separated viral transcription and translation shutoff with multiple alphaviruses, identified the viral proteins that induce each activity, and demonstrated that VEEV nonstructural protein 2-induced translation shutoff is likely a critical factor in enhanced antiviral state resistance of this alphavirus.

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1. Introduction

The *Alphavirus* genus of the *Togaviridae* family of viruses consists of positive-sense single-stranded RNA viruses broadly classified into arthritogenic (*e.g.* Sindbis virus (SINV) and chikungunya virus (CHIKV)) and encephalitic (*e.g.* Venezuelan and eastern equine encephalitis viruses (VEEV, EEEV)) disease-causing groups. Members of this genus are responsible for millions of annual infections and ongoing epidemic outbreaks in several parts of the world, such as the current CHIKV epidemic in the Indian Ocean region (Schwartz and Albert, 2010) which has recently spread to the Caribbean, United States and Central and South America (Van Bortel et al., 2014; Vega-Rua et al., 2014; Weaver, 2014). Infection with arthritogenic alphaviruses causes a febrile illness, which can lead to arthralgia/arthritis lasing for months or years after infection (Gardner et al., 2012). In contrast, encephalitic alphavirus infection results in prodromal disease of varying duration and severity which can progress to fatal encephalitis in a significant number of cases depending upon the virus (Ryman and Klimstra, 2008).

Alphavirus replication and disease severity in mouse models is dependent on their resistance to or avoidance of the antiviral state generated following IFN-α/β induction, and it has been proposed that human disease severity is also associated with resistance to or avoidance of the antiviral effects of IFN (Ryman and Klimstra, 2008; Suhrbier et al., 2012; Yin et al., 2009). Infection of mice with VEEV elicits the highest levels of induced systemic IFN-α/β while significantly lower levels are observed following SINV infection (Ryman and Klimstra, 2008), and little to no IFN is induced by EEEV infection (Gardner et al., 2008). For CHIKV, robust IFN induction is observed in the serum of infected patients (Wauquier et al., 2011; Chow et al., 2011), and infected non-human primates (Messaoudi et al., 2013), whereas little IFN is detected in the serum of infected mice (Ryman and Klimstra, 2008). However, non-hematopoietic cells are the primary source of IFN during CHIKV infection (Schilte et al., 2010). Mice with functional IFN-α/β responses efficiently control SINV (Byrnes et al., 2000; Ryman et al., 2000; Ryman et al., 2002) and CHIKV infection (Schwartz and Albert, 2010; Gardner et al., 2012). In contrast infection with VEEV

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(Davis et al., 1994; Anishchenko et al., 2004) or EEEV (Aguilar et al., 2005; Davis et al., 1989) is usually fatal. While the severity of EEEV infection is linked to its avoidance of replication in myeloid lineage cells and consequent suppression of IFN and other innate immune responses (Trobaugh et al., 2014; Gardner et al., 2011), mortality and disease progression observed following VEEV infection is proposed to reflect greater resistance to the antiviral state induced by IFN (Yin et al., 2009).

IFN signaling upregulates hundreds of Interferon stimulated genes (ISG's), many of which possess antiviral activities (de Veer et al., 2001; Sarkar and Sen, 2004), of which several have been shown to inhibit alphavirus replication (Lenschow et al., 2005; Hyde et al., 2014; Bick et al., 2003). Notably, in conditions where replication of other alphaviruses is highly restricted by IFN-α/β priming, successful replication of VEEV can be observed (Yin et al., 2009; Perri et al., 2003). The resistance of VEEV to many antiviral effectors which comprise the antiviral state in IFN-primed cells suggests a global mechanism that overcomes their inhibitory activities, rather than resistance to the activity of each ISG individually. To suppress the induction of cell stress responses, alphaviruses have been shown to block host cell transcription (Frolova et al., 2002; Aguilar et al., 2007) and translation (Yin et al., 2009; Gorchakov et al., 2005), and it is possible that the induction of one or more such processes during infection of IFN-primed cells by VEEV is able to suppress the pre-existing antiviral state. The Old world alphaviruses mediate host transcription and translation shutoff through an activity of the nonstructural protein nsP2 (Yin et al., 2009; Gorchakov et al., 2005; Fros et al., 2013), while the capsid protein of New world alphaviruses shuts off host cell transcription (Aguilar et al., 2007; Garmashova et al., 2007). The viral protein involved in host translation arrest during New World alphavirus infection has not been determined conclusively.

Most previous studies exploring the mechanisms of alphavirus mediated IFN-α/β antagonism were performed in unprimed cells, cells treated with IFN-α/β post infection, or cells over-expressing individual ISGs such as Interferon-inducible protein with tetratricopeptide repeat 1 (IFIT1) (Hyde et al., 2014; Frolov et al., 2012; Atasheva et al., 2010; Reynaud et al., 2015). However, rapid induction of serum IFN-α/β in mice after VEEV and SINV infection upregulates an antiviral state in most cells at sites where the infection has not progressed, causing the of majority cells infected by these viruses *in vivo* to be primed to resist infection. Thus, previous *in vitro* work in unprimed cells primarily represents the few cells initially infected after inoculation of mice. The interaction of VEEV and SINV with a pre-established antiviral state was explored in recent studies (Ryman and Klimstra, 2008; Yin et al., 2009), which demonstrated that VEEV was far more resistant to a pre-existing antiviral state than SINV.

Previous studies have also focused on the effect of a generalized shutoff, or when specific, virus-induced transcription shutoff on induction of IFN-α/β responses (Fros et al., 2013; Frolov et al., 2012), while the role of translation shutoff in antiviral state antagonism has not been emphasized. For SINV, both transcription and translation shutoff are induced by the same protein (Gorchakov et al., 2005), and the relative contribution of these functions in resisting the antiviral state is difficult to explore. Similarly, most previous work with VEEV or EEEV has implicated capsid induced transcription shutoff to play a major role in suppression of IFN-α/β induction, despite the temporally deferred synthesis of this viral protein during infection (Atasheva et al., 2010; Atasheva et al., 2015). Induction of host translation shutoff by VEEV has been localized to the nonstructural protein region of the genome, which is translated before the capsid region during infection (Yin et al., 2009), suggesting a role for this activity in the antiviral state resistance of VEEV.

Here we have examined possible mechanisms underlying resistance of VEEV to an IFN-α/β induced, pre-established antiviral state and identified/confirmed the proteins that mediate host transcription and translation shutoff with CHIKV, SINV, VEEV and EEEV through individual protein expression. In *in vitro* testing, VEEV was more resistant than SINV, CHIKV and EEEV to the global antiviral state in mouse and human cells, and this resistance became evident at a point after initial translation of the incoming virus genome. Furthermore, a panel of mutant viruses deficient in host macromolecular synthesis shutoff demonstrated that sensitivity to the antiviral state was correlated with slower rates of this activity. Using a plasmid expression system to study host macromolecular synthesis shutoff independent of virus replication rates, we found that expression of VEEV, CHIKV or SINV nsP2, or VEEV or EEEV capsid expression, but not control nsPs or GFP was sufficient to block host translation, with VEEV and EEEV capsid translation blockade likely secondary to transcription shutoff. VEEV and EEEV nsP2 did not inhibit transcription. VEEV or EEEV capsid and CHIKV or SINV nsP2 expression directly inhibited host transcription. EEEV nsP2 also failed to block host translation revealing a stark difference between VEEV and EEEV. Importantly, in the absence of transcription shutoff, host translation in IFN-primed cells was inhibited more efficiently by VEEV nsP2 than that of SINV nsP2. Furthermore, when VEEV nsP2 was expressed in IFN-primed cells, levels of ISG's were lower, and replication of an unrelated IFN-sensitive virus (yellow fever virus 17−D) was enhanced over IFN-primed control cells. Overall, we conclude that VEEV nsP2-induced host translation shutoff early after infection downregulates the antiviral state by decreasing levels of ISG's and creating an environment more permissive for viral replication.

2. Materials and methods

2.1. Cell culture

Neuro 2a, Vero and Huh7 cells (acquired from American Type Culture Collection (ATCC)) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 200mM L-glutamine (L-glut; Sigma, 10,000 units/mL penicillin (Sigma), and 10 mg/mL streptomycin (Sigma). Tetracycline-inducible murine embryonic fibroblasts (MEF; Clontech) were maintained in the above medium supplemented additionally with 50 mg/mL G418. The generation of, and target gene induction from, tetracycline-inducible murine embryonic fibroblasts overexpressing GFP, IFIT1 and Interferon-stimulated gene 20 (ISG20) has been previously described (Zhang et al., 2007). BHK-21 cells (ATCC) were maintained in RPMI supplemented with 10% donor bovine serum (DBS), 10% tryptose phosphate broth (TPB), and supplements as above. All cells were grown at 37 °C with 5% CO_2.

2.2. Viruses and replicons

Construction of cDNA clones for VEEV ZPC738 (Anishchenko et al., 2004), EEEV FL93–939 (Aguilar et al., 2008), SINV TR339 (Klimstra et al., 1998) and CHIK-LR (Tsetsarkin et al., 2006) has been previously described. Mutant VEEV viruses P713G, P713S, P713K and Q739L were generated by site-directed mutagenesis using appropriate overlapping primers and Quikchange kit and according to manufacturer's guidelines (Agilent). VEEV mutants CD and CD/nsP2 739L were generated similarly by deleting amino acids 64–68 from capsid using site-directed mutagenesis. Yellow fever virus (YFV) vaccine strain 17-D was used to construct a reporter virus expressing Nano-luciferase (nLuc) (Hall et al., 2012) by inserting the nLuc gene followed by the *Thosea asigna* virus (Tav) 2A-like protease in frame between Capsid and prM, as previously described for alphaviruses (Thomas et al., 2003). Viruses were

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