

AN ALPHAVIRUS NSP1 DETERMINANT MODULATES TYPE I INTERFERON INDUCTION

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ABSTRACT

CATHERINE C. CRUZ: An Alphavirus nsP1 determinant modulates Type I Interferon induction

(Under the direction of Mark T. Heise, PhD.)

Alphaviruses are mosquito-transmitted viruses that can cause severe disease in both animals and humans. The host type I Interferon (IFN) response plays a critical role in limiting alphavirus infections. However, the specific interactions of type I IFN with that of the alphaviruses remain largely uncharacterized. Therefore, to gain insight into these early interactions, this study addresses the role of genetic virulence determinants in modulating the host type I IFN response. These analyses are based upon previous detailed mapping studies that identified a virulence determinant within the nsP1/nsP2 cleavage domain of the mouse adapted, neurovirulent Sindbis AR86 alphavirus that is critical for AR86 pathogenesis *in vivo*. We demonstrate that a Threonine to Isoleucine change at this position, leads to a mutant virus that robustly induces IFN independently of effects on viral replication or viral mediated shutoff of host macromolecular syntheses. Furthermore, we demonstrate that the modulation in type I IFN induction is not specific to the Sindbis AR86 virus, as a similar mutation within the Ross River Virus, also leads to robust type I IFN responses, suggesting that the nsP1/nsP2 determinant may be fundamentally important for all alphaviruses. Additional work has established that the nsP1/nsP2 determinant modulates type I IFN induction through the IPS-1 signaling pathway, primarily mediated by the RIG-I and PKR receptors. As RIG-I

and PKR recognize specific RNA ligands, such as free 5' triphosphates on uncapped viral RNAs, we next determined whether the nsP1/nsP2 determinant would disrupt the viral capping apparatus. We found increased synthesis of uncapped viral 26S subgenomic RNAs made within infected cells containing the nsP1/nsP2 mutation. Furthermore, the uncapped 26S RNAs differentially activated RIG-I which was phosphatase dependent. Therefore, altogether, our data presents a novel mechanism in which a genetic determinant specifically enhances the viral capping apparatus in order to evade the host type I IFN receptors. And disruption of the capping apparatus leads to the synthesis of uncapped RNAs that are efficiently recognized by the RIG-I and PKR host sensors to induce IFN to aid in the clearance of the viral infection.

For the Cruz family

To my mom and dad; Claire and Danny,

To my brother and sister; Craig and Neri,

And to my adorable little monsters; Daniel and Lauren.

*This is for your love, support, constant encouragements,
and never-ending faith in me.*

*I admire your unwavering courage to explore, your relentless pursuit of your
dreams, your willingness to share, and your simple enjoyment of life and of those
around you. I thank you for instilling in me these same values, and most importantly,
for teaching me to never give up. They have shaped me into the person that I am
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TABLE OF CONTENTS

LIST OF FIGURES.....	ix
LIST OF ABBREVIATIONS.....	xi
Chapter	
1. INTRODUCTION.....	1
ALPHAVIRUS DISEASE AND PATHOGENESIS.....	2
Alphavirus epidemiology.....	2
Animal Models of Alphavirus-Induced Disease.....	3
MOLECULAR BIOLOGY OF ALPHAVIRUS.....	6
Alphavirus particle structure and cell entry.....	6
Genome organization and replication.....	7
Virus assembly and budding.....	11
ALPHAVIRUS INTERACTIONS WITH THE INNATE IMMUNE SYSTEM.....	12
The Type I Interferon Signaling Pathway.....	12
Pattern Recognition Receptors.....	14
Interferon stimulated genes to limit alphavirus replication.....	22
Alphavirus modulation on IFN induction.....	25
DISSERTATION OBJECTIVES.....	30

REFERENCES.....	32
2. MODULATION OF TYPE I IFN INDUCTION BY A VIRULENCE DETERMINANT WITHIN THE ALPHAVIRUS NSP1 PROTEIN.....	46
ABSTRACT.....	47
INTRODUCTION.....	48
RESULTS.....	51
DISCUSSION.....	59
MATERIALS AND METHODS.....	63
ACKNOWLEDGMENTS.....	67
REFERENCES.....	68
3. A ROSS RIVER VIRUS NSP1 MUTATION LEADS TO ENHANCED SYNTHESIS OF UNCAPPED 26S RNAS TO INDUCE TYPE I IFN.....	86
ABSTRACT.....	87
INTRODUCTION.....	88
RESULTS.....	94
DISCUSSION.....	103
MATERIALS AND METHODS.....	108
ACKNOWLEDGMENTS.....	115
REFERENCES.....	116
4. DISCUSSION AND FUTURE DIRECTIONS.....	141
REFERENCES.....	154

LIST OF FIGURES

Figure 2.1:	The mutant SIN T538I virus induces more type I interferon.....	74
Figure 2.2:	The Sindbis T538I mutant virus shuts off host RNA transcription and protein translation with similar kinetics to the wild type AR86.	76
Figure 2.3:	Characterizations of the RRV A532V mutant virus.....	78
Figure 2.4:	The RRV A532V mutant virus induces more type I IFN than the wild type.	80
Figure 2.5:	The RRV mutant virus induces more IFN than the wildtype RRV in the absence of the Type I IFN $\alpha\beta$ Receptor.....	82
Figure 2.6:	Characterization of virus-induced shutoff by the RRV A532V mutant.	83
Figure 2.7:	The RRV A532V mutant robustly induces IRF-3 phosphorylation compared to wild type virus.....	85
Figure 3.1:	The IPS-1 molecule is critical for RRV A532V mediated IFN induction.	123
Figure 3.2:	The RRV A532V mediated type I IFN induction is independent of TICAM-1.	125
Figure 3.3:	The RRV A532V mediated IFN induction is dependent upon RIG-I and not MDA-5.	127
Figure 3.4:	PKR contributes to the RRV A532V IFN induction.....	129
Figure 3.5:	The RRV A532V mutant displays increased full length minus, plus strand, and 26S RNA synthesis.....	131
Figure 3.6:	The RRV A532V viral RNAs more robustly activate IFN induction.	133
Figure 3.7:	The RRV A532V full length viral RNAs do not differently activate IFN induction.	135
Figure 3.8:	The A532V viral 26S RNA is a better activator IFN inducer.....	136
Figure 3.9:	The RRV A532V virus makes uncapped 26S RNA.....	138

Supplemental Figure 3-1: GFP expression from the A532V encoded 26S RNAs is lower than those of the wild type RRV.....	139
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LIST OF ABBREVIATIONS

A532V	Ross River Virus Valine mutant
Ala	Alanine
AR86	Sindbis AR86 virus
BHK	Baby Hamster Kidney
CARD	Caspase recruitment domain
cDNA	Complementary DNA
CIP	Calf Intestinal Alkaline Phosphatase
CSE	Conserved sequence element
DC	Dendritic cell
DCS	Donor Calf Serum
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
EEEV	Eastern Equine Encephalitis Virus
EMCV	Encephalomyocarditis virus
ER	Endoplasmic reticulum
FBS	Fetal Bovine Serum
GFP	Green fluorescent protein
HEC-1B	Human endometrial carcinoma
HEK	Human embryonic kidney
hpi	Hours post infection
i.c.	Intracranial
IFA	Immunofluorescent Assay

IFN	Interferon
IFN $\alpha\beta$	Type I Interferon
IFNR	Interferon α receptor
IKK	IkappaB kinases
Ile	Isoleucine
IP	Immunoprecipitation
IPS-1	IFN-promoter stimulator 1
IRF-3	Interferon regulatory factor-3
IRF-7	Interferon regulatory factor-7
ISG	Interferon stimulated gene
ISGF3	Interferon stimulated gene factor 3
ISRE	Interferon stimulated response element
IU	International units
JAK	Janus tyrosine kinase
kB	Kilobase
LGP2	Laboratory of genetics and physiology 2
LPS	Lipopolysaccharide
MDA-5	Melanoma-differentiation-associated gene 5
MEFS	Mouse embryonic fibroblasts
ml	Milliliter
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MTT	3-[4,5-Dimethylthylthiazol-2yl]-2,5-Diphenyltetrazolium

MyD88	Myeloid differentiation primary response gene 88
NF- κ B	Nuclear factor kappa beta
NsP	Nonstructural polyprotein
NsP1	Nonstructural protein 1
NsP2	Nonstructural protein 2
NsP3	Nonstructural protein 3
NsP4	Nonstructural protein 4
NsPs	Nonstructural proteins
OAS	2'-5'-oligoadenylate synthetase
P1234	Nonstructural polyprotein precursor
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PFU	Plaque forming unit
PKR	Double stranded RNA-activated protein kinase R
Poly A	Polyadenylate
Poly IC	Polyinosine-polycytidylic acid
PPO	2, 5-diphenyloxazole
PRRs	Pattern recognition receptors
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative real-time polymerase chain reaction
RD	Repressor domain

RIG-I	Retinoic acid inducible gene-I
RLRs	RIG-I-Like receptors
RNA	Ribonucleic acid
RPM	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RRV	Ross River virus
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SFV	Semliki Forest virus
ssRNA	Single stranded ribonucleic acid
STAT	Signal transducer and activator of transcription
T538I	Sindbis AR86 Isoleucine mutant
TBK1	TANK Binding Kinase 1
Thr	Threonine
Ticam-1	TIR domain-containing adaptor molecule 1
TLR	Toll-like receptor
TMG	2,2,7-trimethylguanosine
UTR	Untranslated region
UV	Ultraviolet light
Val	Valine
VEEV	Venezuelean Equine Encephalitis virus
WEEV	Western Equine Encephalitis virus

CHAPTER ONE

INTRODUCTION

ALPHAVIRUS DISEASE AND PATHOGENESIS

Alphavirus epidemiology

Alphaviruses are mosquito-borne viruses from the family *Togaviridae*. Currently, there are more than 29 members of the *Alphavirus* genus that are widely distributed around the world (10, 27). They are classified into two major groups according to their geographical location and designated as “New World viruses” which are found primarily in North and South America, and the “Old World viruses” which are found in Europe, Asia, Australia, and Africa. The New World viruses include Venezuelan equine encephalitis virus (VEEV), Western equine encephalitis virus (WEEV), and Eastern equine encephalitis virus (EEEV) and are often associated with fever and severe encephalitis that are sometimes fatal (5, 27). The Old World viruses include Sindbis virus (SIN), Semliki Forest Virus (SFV), Ross River Virus (RRV), O’nyong-Nyong virus (ONN), and Chikungunya virus (CHIK). Old World alphavirus diseases are more associated with fever, rash, and polyarthrititis/myositis (68, 69). Although Old World alphaviruses infections are generally not fatal, the arthritic symptoms can be quite debilitating, often lasting from several weeks to years (111). Importantly, viruses in this group, such as CHIK, ONN, and RRV are capable of causing large-scale outbreaks affecting tens of thousands to millions of individuals, and should be considered significant emerging disease threats. The successful re-emergence of these viruses are influenced by many factors such as increased populations, a decrease in herd immunity, and the expansion of transmission vectors (111).

Alphaviruses exist in an enzootic life cycle, cycling between mosquito vectors and small mammals or birds that serve as amplification hosts. Generally, the vector species and animal reservoirs used are believed to play a role in the distribution of certain members of alphaviruses (27). For instance, Sindbis viruses that use birds as their amplification host, are found to be more widely distributed than other viruses, such as RRV and VEEV, which more predominately uses mammalian hosts, thus limiting their geographical spread. Occasionally, alphaviruses will crossover from the mosquito into larger animals, such as humans and horses, to cause large scale epidemic diseases. The potential for crossovers have caused serious global health concerns as outbreaks in the human populations have inflicted thousands to millions of people (30, 63, 114). The latest CHIK virus outbreak occurred in 2005, in the island of La Reunion, infecting more than two hundred thousand people (106). Following this outbreak, more CHIK outbreaks were reported in the nearby Indian subcontinent and Africa as well as sporadic introduction into the Western Hemisphere (63, 74, 78, 111). Additionally, a small outbreak in Italy occurred in 2007 as an infected traveler from India arrived in a remote northern region, spreading the infection (3, 81). These incidents illustrate the growing threat that alphaviruses can emerge in new areas, since infected travelers can rapidly move into non-endemic areas, thereby perpetuating the outbreak.

Animal Models of Alphavirus-Induced Disease

Mouse models have vastly increased our understanding of alphavirus-induced diseases, defining the viral and host factors needed to sustain or combat

viral infections. Use of these models has given insight into the replication patterns within mammalian hosts. First, alphaviruses infections occur through the bite of an infected mosquito on the dermal layer of the skin, where alphaviruses will replicate in the residing Langerhans or dendritic cells (DCs). The infected DCs then migrate to the draining lymph nodes (DLNs) where further viral replication takes place. As replication increases, a high titer viremia in the blood stream occurs, facilitating spread into other organs and tissues. In some cases, a high viremia also facilitates virus invasion of the central nervous system (5).

Several groups have developed mouse models that closely mirror human disease to study alphavirus pathogenesis (23, 33, 69). These studies have led to the discovery of both genetic and host factors that are necessary for alphavirus to cause disease (68, 113). The Sindbis group viruses are the most extensively studied. In mice, most Sindbis viruses display an age dependent restriction of pathogenesis (27). For instance, in neonatal mice, Sindbis virus infections result in lethal disease characterized by increased pro-inflammatory cytokine expression and high viremia in serum, muscles, brain, and in the spinal cord. However, as the age of the mice increases, the replication of the virus is more restricted resulting in less severe or no disease. More importantly, the majority of Sindbis viruses are avirulent in adult mice. However, a few Sindbis strains are still pathogenic in adult mice causing neurovirulent disease. Examples are the neuroadapted Sindbis virus (NSV) (107) and Sindbis AR86 (32, 98). Detailed mapping studies have identified the genetic determinants responsible for the adult mouse neurovirulence of these viruses. Suthar et al. (2005) performed comparative analyses between Sindbis

AR86 and a closely related Sindbis virus, GirdwoodS.A. GirdwoodS.A. and AR86 differ only by 22 single amino acid changes as well as a 18 amino acid sequence in the nsP3 protein that is only present in GirdwoodS.A. Interestingly, in mice that are greater than 14 days old, GirdwoodS.A. virus was avirulent even when administered in high doses. AR86, on the other hand, retains its neurovirulence regardless of the age of the animal. Using their mouse models, Suthar et al. (2005) further defined AR86 adult mouse neurovirulence determinants, identifying four major virulence determinants in the viral genome. The virulence determinants were a Threonine at nsP1 position 538, an 18 amino acid deletion at nsP3 386, a Cysteine at nsP3 537, and a Serine at E2 position 243 (104). All four determinants were found to be essential for adult mouse neurovirulence. Interestingly, the nsP1 determinant was found to be absolutely critical as a single codon change from a Threonine to an Isoleucine severely attenuated this virus (32, 104). Furthermore, Heise et al. (2000) demonstrated that insertion of a Threonine into other avirulent Sindbis viruses was able to partially rescue virulence (32). The exact role of this determinant in affecting mouse neurovirulence is currently not understood. However, possible interactions with host factors will be highlighted in this dissertation. The determinants for other neurovirulent Sindbis viruses have also been identified. Studies with NSV identified a Histidine codon, position 55, in the E2 glycoprotein that plays a major role in the neurovirulence of this virus (107). The neurovirulence of a related virus, Semliki Forest virus strain SFV4, was mapped to the genetic determinants within the nonstructural proteins (108). Although the exact mechanisms for many of these virulence determinants to act *in vivo* are not clearly understood, these studies

provide insight into the genetic determinants that are located within the genome to impact the competence of an alphavirus to establish disease.

In addition to revealing genetic determinants that impact alphavirus pathogenesis, mouse models have also identified host factors that are necessary for controlling alphavirus replication (52, 86). Studies using mice that are deficient for one or more molecules within the Interferon (IFN) pathway highlight the role for IFNs to control and inhibit alphavirus replication (84, 113). The complete removal of the IFN system within mice generally results in lethal disease, even upon infection of avirulent viruses. Therefore, an intact IFN system is a critical part of the host innate immune system to combat these viruses. In the latter part of this chapter, we will further discuss the roles of the Type I IFN and the interactions with the alphavirus family.

MOLECULAR BIOLOGY OF ALPHAVIRUS

Alphavirus particle structure and cell entry

Alphaviruses are enveloped particles enclosing one copy of a single stranded positive sense genome. The genome is encapsidated by the viral capsid proteins forming a nucleocapsid core structure. The nucleocapsid core is further surrounded by a lipid bilayer derived from the host cell plasma membrane. The viral glycoproteins, E1 and E2, are embedded within the outer lipid shell. The E1 and E2 glycoproteins form heterodimers and assemble into 80 trimeric spikes forming an icosahedral lattice structure (T=4 symmetry) at the virus surface (40). The

glycoproteins primarily mediate entry of the virus into a host's cell where the E2 glycoprotein is responsible for receptor attachment and the E1 glycoprotein mediates fusion of the virus with the cell membranes. Although it is thought that E1 may also play a role in receptor attachment. Currently, the process of alphavirus entry into a host cell is still being debated. Though, there are two main hypotheses. One hypothesis is that the virus utilizes receptor-mediated endocytosis, although the exact alphavirus receptor(s) remains elusive. In this pathway, the alphavirus E2/E1 glycoprotein engages a receptor. Then, the virion is internalized within a cellular endosome. As the pH within the endosome lowers, the E2/E1 glycoproteins undergo a conformational change exposing a fusion peptide on the E1 glycoprotein. The fusion peptide inserts into the plasma membrane, creating a fusion pore. The viral nucleocapsid core is then inserted into the host cytoplasm. However, recent evidence counteracts this theory by demonstrating that a low pH environment is not necessary for fusion (72). Thus, an alternative pathway for alphavirus entry has been proposed to occur at the cell surface facilitating fusion of the virus membrane with that of the cell membrane. Consequently, a fusion pore is created allowing insertion of the nucleocapsid core containing the viral genome. Upon entry, the host cellular translational machinery will translate the viral genome that initiates viral replication.

Genome organization and replication

The alphavirus genome consists of a single stranded positive sense RNA that is approximately 11-12Kb long. The genome contains a 5'- methylguanylate cap

structure and a 3'-polyadenylate (poly-A) tail similar to those found on host cellular mRNAs. The genome encodes two open reading frames where the 5' 2/3 encodes for the nonstructural proteins and the 3' end encodes for the structural proteins. The structural proteins are tightly regulated by an internal 26S subgenomic promoter that transcribes short 26S RNAs that are identical to the 3' end of the viral genome. The 26S RNAs, like those of the full-length genomes, are also capped and polyadenylated. The 26S RNAs are made in 5-fold molar excess to that of the full length genomic RNAs for translation of the structural proteins that are required for virion assembly (51). Additionally, the alphavirus genome also encodes regulatory segments. These segments consist of four conserved sequence elements (CSE) found throughout the alphavirus genomes. The most highly conserved CSE is found in a 19-nucleotide region at the 3' untranslated region (UTR), immediately preceding the poly (A) tail. The 3' CSE serves as a strong promoter to facilitate negative sense RNA synthesis (29). The other CSEs are found at the 5' UTR and at the junction across the subgenomic promoter to facilitate synthesis of the full-length positive sense and 26S RNAs. Furthermore, mapping studies have demonstrated that the CSEs recruit both host and viral factors necessary for viral replication (46, 101).

There are four nonstructural proteins encoded by the virus genome. The major function of the nonstructural proteins is to replicate and transcribe the viral genomes although some individual nsP proteins can play additional roles in an infected cell (50, 82). NsP1 is a palmitoylated protein that is tightly associated with the cytoplasmic surface of the plasma membranes, endosomes, and lysosomes, thereby anchoring the replication complex to the cell membranes (49, 99). NsP1 is

also a guanine-7-methyltransferase that exhibits guanylyltransferase activities and is involved in capping the full-length positive sense and 26S RNAs. NsP2 is a multifunctional protein that encodes for several distinct functions such as; a papain-like protease that mediates the cleavage of the nonstructural proteins, a nucleoside triphosphatase (NTPase), which is essential for viral replication, an RNA helicase, as well as a 5'-triphosphatase that facilitates the capping of the viral RNA. The nsP2 protein also contains nuclear localization signals and ~50% can be found in the nucleus (67). The nsP3 protein is a phosphoprotein that is important for viral replication, however, its exact function is unknown (110). And finally, the nsP4 protein is the RNA-dependent RNA polymerase that mediates synthesis of the viral RNAs.

Upon infection by alphavirus, the viral genome is immediately translated to produce two nonstructural polyproteins, P123 and P1234. The P123 polyprotein is generated by an opal termination codon encoded between the nsP3 and nsP4 gene. Translational readthrough of the opal codon occurs with 10-20% efficiency, generating the P1234 polyprotein (34). The nonstructural polyproteins are then cleaved into their mature nonstructural proteins by the viral nsP2 protease. There are three conserved cleavage domains that reside between the nsP1/2, nsP2/3, and nsP3/4 polyprotein. Each cleavage domain is conserved among the alphaviruses and differentially recognized by the nsP2 protease (101). The coordinated sequential cleavage of the nonstructural polyproteins that proceeds next is well organized to tightly regulate the synthesis of the viral genomes. First, nsP4 is cleaved from the P1234 polyprotein through nsP2 *cis* cleavage. Then, P123

associates with mature nsP4, to form a viral replication complex that binds to the conserved 19-nucleotide sequence at the 3'-UTR of the positive sense genome. The P123-nsP4 complex will then mediate full-length negative sense RNA synthesis, using the positive sense RNA as its template. As P123 polyproteins accumulate, the nsP2 protease will then mediate *trans* cleavage of the polyproteins. The first *trans* cleavage occurs between P1/2, thus creating mature nsP1, polyprotein intermediate P23, and nsP4. These proteins, in association, continue to make negative sense RNAs. However, they are also capable of binding to the minus sense RNA at the 5'-CSE, to begin generating full-length positive sense RNAs. Additionally, 26S RNA synthesis also occurs, though the 26S RNA levels are lower than that of the full-length positive sense RNAs (51, 59). Finally, *trans* cleavage of P23 occurs last, generating mature nsP1, nsP2, nsP3, and nsP4 proteins. The mature nsPs continue to synthesize full length positive sense RNAs, but in addition, will also bind to the internal subgenomic promoter to mediate full synthesis of short 26S RNAs. As mentioned before, a five-fold ratio of 26S RNA is made to the full-length positive sense RNA. The 26S RNAs encode for the structural proteins that are then translated by the host cellular translational machinery. The final cleavage between P23 is thought to end negative strand RNA synthesis, thus switching completely to full-length RNA and 26S RNA synthesis (51). In agreement with this model, minus RNA strand synthesis usually occurs early and then ceases about 4-7 hours post-infection (59). Since the mature nonstructural proteins are cleaved into their mature forms and do not undergo further cleavage, synthesis of full length positive sense and 26S RNAs occurs throughout the remainder of the virus life cycle.

Virus assembly and budding

The structural proteins are translated from the 26S RNAs as a single polyprotein. The polyprotein encodes, in this order, for the capsid, the glycoproteins (E3, E2, and E1), and the 6K protein. The capsid protein possesses serine protease activity and is immediately cleaved from the polyprotein by autoproteolytic cleavage (64). Cleavage of the capsid protein exposes a signal sequence within the PE2 polyprotein (E3/E2 precursor) that directs the polyprotein into Endoplasmic Reticulum (ER) and the Golgi (40, 56). A second signal sequence within the 6K protein allows continuation of the translocation of the E1/6K polyprotein into the ER (65). As the polyprotein translocates across the ER, it is further processed to release the E1 and 6K proteins. The PE2 is cleaved last by host furin proteases to generate E3 and E2 glycoproteins. As the structural proteins translocate across the ER and Golgi, they are post-translationally modified. High mannose and complex carbohydrates are added to the glycoproteins as the proteins as the structural proteins are translocated to the cell surface. Meanwhile, the previously released capsid proteins will immediately bind to the full-length positive sense genomes to form the nucleocapsid core. This binding is specific for the genomic RNA as the capsid protein recognizes a specific packaging genome located within the nsP1 or nsP2 coding sequences (14, 112). As the 26S RNAs do not contain these regions, they are not encapsidated. The nucleocapsid core consists of ~240 capsid proteins surrounding a single genome, thus creating a stable complex. The nucleocapsid

core then binds to the viral glycoproteins where the virus particle finishes assembly and buds from the plasma membrane.

ALPHAVIRUS INTERACTIONS WITH THE INNATE IMMUNE SYSTEM

The Type I Interferon Signaling Pathway

Type I Interferons (IFNs) are an important component of the host's innate immunity, acting as the first line of defense against invading pathogens. Interferons were initially discovered as a cytokine that interfered with Influenza A virus infection indicating antiviral properties (38). Since their discovery, a vast number of studies have furthered our understanding of how IFNs act. It is now well established that IFNs induce expression of antiviral genes that act to limit viral replication and spread through mechanisms such as host-mediated shutoff of protein translation, viral nucleic acid degradation, and release of cytokines to shape the downstream adaptive immune response to clear the virus (87, 103). Furthermore, it has been clearly demonstrated that the host Type I IFN is essential for mounting a robust response against virus infection as mice that have a genetic defect for type I IFN signaling are highly susceptible to many virus infections (87). Therefore, the overall importance of Type I IFN to the host is critical.

The Type I IFNs in humans comprise of 7 subtypes, IFN- α , IFN- β , IFN- κ , IFN- ϵ , IFN- ω , IFN- τ and IFN- δ , although IFN- $\alpha\beta$ are the most well studied. It has been demonstrated that many different cell types can secrete IFN- $\alpha\beta$ cytokines, though the recognition receptors that mediate these responses differ. In humans, there are a total of 13 IFN- α subclasses (14 in mice) and only one IFN- β . Upon recognition of

a viral infection, the type I IFN system will respond to the virus by activating a signaling cascade leading to IFN- β transcription. Virus recognition is mediated by the host pattern recognition receptors (PRRs). Host PRRs recognize specific virus motifs present on the virus particles or generated during viral replication. These motifs are referred to as pathogen-associated molecular patterns (PAMPS) and include viral glycoproteins and nucleic acids. Upon binding of a PRR to its PAMP, specific signaling pathways that consist of various co-adaptor and scaffold proteins are activated. The different PRR signaling pathways ultimately converge to activate the IKK and TBK1/IKK ϵ kinases, which in turn, activates the transcription factors, nuclear factor kappa B (NF- κ B) and Interferon Regulatory Factor 3 (IRF-3), respectively. NF- κ B is normally kept in an inactive state by the I κ B protein. Upon IKK activation, IKK phosphorylates I κ B, resulting in I κ B polyubiquitylation and degradation, thus releasing NF- κ B. NF- κ B will then translocate to the nucleus to upregulate pro-inflammatory cytokine expression and IFN induction (83). IRF-3 activation occurs a little differently. IRF-3 is ubiquitously expressed within a cell shuttling in and out of the nucleus. However, activation of the TBK1 and IKK ϵ kinases phosphorylates the C-terminal end of IRF-3. Phosphorylated IRF-3 will then dimerize, translocate to the nucleus, and associate with co-activator CBP/p300 to bind to IRF-3 specific promoters. The binding of an enhancosome made up of IRF-3/CBP/p300, NF- κ B, and the ATF-2/c-Jun transcription factors, specifically activates the IFN-B promoter upregulating IFN- β mRNA transcription (35).

The IFN- β cytokine is then secreted from the cell where it will bind to the IFN- α Receptor complex (IFNAR1 and IFNAR2) that is present on nearby adjacent cells

or on its own cell membrane. This in turn, activates the Janus activated kinase (JAK) and Signal transducer and activator (STAT) pathways. Activation of the JAK-STAT signaling pathway results in the phosphorylation of the STAT-1 and STAT-2 proteins, and in association with IRF-9 (forming the ISGF3 complex), translocates to the nucleus to bind to several Interferon stimulated response elements (ISREs) in DNA to initiate transcription of IFN stimulated genes (ISGs). There are approximately more than 300 ISGs that are upregulated by type I IFNs. However, only a few have been characterized for their antiviral activities. One ISG encodes for IRF-7. IRF-7 is a transcription factor with similar structural homology to IRF-3 that acts as a positive feedback loop for the type I IFN response. IRF-7 is phosphorylated by the TBK1/IKK ϵ kinases, resulting in IRF-7 homodimerization and translocation into the nucleus to stimulate IFN- α transcription. Additionally, phosphorylated IRF-7 can bind to phosphorylated IRF-3, forming heterodimers that can bind to IFN- β promoter, thus amplifying the type I IFN response. Therefore, the type IFN response can be divided into 2 distinct phases: 1) An induction phase mediated by pattern recognition receptors and 2) an amplification phase that is dependent upon IFN receptor signaling through the JAK/STAT pathway. Although, it should be noted that some PRRs directly stimulate IRF-7 activation to induce type I IFN.

Pattern Recognition Receptors

The IFN induction phase is mediated by Pattern Recognition Receptors (PRRs) that can recognize different pathogen-associated molecular patterns

(PAMPs) expressed by viruses. The PRRs that recognize viruses include the Toll-Like Receptors (TLRs), the retinoic acid inducible gene (RIG-I)-Like Receptors (RLRs), as well as the double stranded RNA dependent Protein Kinase R and 2'-5'-oligoadenylate synthetases (OASs). TLRs are transmembrane receptors with leucine-rich repeats that recognize viral PAMPs at the cell surface or within endosomal compartments. Each TLR is specific for a particular PAMP recognition, such as lipids, proteins, and nucleic acids. Examples of viral PAMPs and their corresponding TLR are dsRNA (TLR3), ssRNA (TLR7 and TLR8), and 2'-deoxyribose (cytidine-phosphate- guanosine) (CpG) DNA (TLR9). Although, TLR4 is thought to mainly recognize bacterial lipopolysaccharides, reports have demonstrated a critical role for TLR4 in inhibition of respiratory syncytial virus (48), suggesting that TLR4 does play a role in fighting viral infections. The TLRs use four TIR-domain-containing adaptor molecules to activate distinct downstream activation pathways to induce IFN $\alpha\beta$ and pro-inflammatory cytokine responses via NF κ -B activation. The four co-adaptors are MyD88, TIRAP (also known as Mal), TICAM-1 (also known as TRIF), and TRAM. All the TLRs, with the exception of TLR3, utilize the MyD88 dependent signaling pathway to activate NF- κ B. In addition, TLR7, 8, and 9 can activate IRF-7 to mediate IFN- α induction. TLR3, however, solely uses the TICAM-1 molecule to induce IRF-3 activation and inflammatory cytokines production. TLR4, on the other hand, uses all four co-adaptors activating both the TICAM-1 and MyD88 dependent signaling pathways. However, TLR4 mediated type I IFN induction is strictly dependent upon TICAM-1 signaling, whereas induction of the pro-inflammatory response requires both TICAM-1 and MyD88 pathways (36).

The RIG-I-Like Receptors comprise of three RNA helicases, RIG-I, melanoma differentiation associated gene 5 (MDA-5) and the laboratory of genetics and physiology 2 (LGP2). All three RNA helicases reside exclusively in the cytosol of a cell and encode a C-terminal DExD-box RNA helicase and ATPase dependent domain (88). RIG-I and MDA-5, however, encode two N-terminal caspase recruitment domains (CARD). The CARD domains facilitate the recruitment of the IFN-promoter stimulator (IPS-1; also known as MAVS, VISA, and Cardif) (44, 66, 94, 116) co-adaptor that is present on the mitochondrial membranes. IPS-1 also encodes a CARD domain, thus homotypic CARD-CARD interactions with RIG-I or MDA-5 then lead to the recruitment of additional signaling factors to mediate the downstream activation of the IKK and TBK1/IKK ϵ kinases, thus activating both NF- κ B and IRF-3 (39, 73, 77, 123). RIG-I and LGP2 also encode a repressor domain (RD) at the C-terminal end of the protein, whereas MDA-5 does not, indicating that the RNA helicases may have different regulatory mechanisms (89). It has been proposed that RIG-I normally remains in an auto-inhibitory state that is regulated by the RD domain. Upon binding of the RNA substrate to its C-terminal end (RD), RIG-I undergoes a conformational change exposing its CARD domains, thus leading to RIG-I activation. Further, the RD domain of RIG-I has also been linked to the distinct binding specificities for RIG-I. Therefore, the presence of an RD domain may explain why RIG-I and MDA-5 can mediate IFN induction to distinct viruses. The LGP2 helicase also encodes the RD domain, although it lacks the N-terminal CARD domains that would allow LGP2 to interact with IPS-1. The LGP2 protein can form complexes with both RIG-I and MDA-5. Interestingly, the LGP2 RD domain

inhibits RIG-I mediated IFN signaling and not that of MDA-5. Thus, similar to RIG-I, LGP2 can also recognize the same RNA ligands, however, with greater affinity. Therefore, it is believed that LGP2 acts as a negative regulator to RIG-I inhibiting its IFN signaling as a negative feedback mechanism. However, LGP2 has also been demonstrated to be a positive regulator of EMVC mediated IFN induction, a pathway that is strictly mediated by MDA-5 (118). Therefore, the exact role(s) of LGP2 has not been clearly defined.

Although both MDA-5 and RIG-I activate the same signaling pathways, it is clear that the RNA helicases discriminate between viruses, therefore diversifying the recognition of the IFN response. Several studies using RIG-I, MDA-5, or IPS-1 genetically deficient mice have reported the exclusive role for either RIG-I or MDA-5 to recognize specific viruses (41, 47). Examples of viruses that require RIG-I to mediate IFN induction include Influenza, Sendai virus, and Hepatitis C virus (43, 102). MDA-5, on the other hand, induces IFN to encephalomyocarditis virus (EMCV) and to synthetic poly IC RNA substrates (22). However, in addition to recognizing different RNA viruses, it was further demonstrated that RIG-I and MDA-5 could overlap in their virus recognition (37, 42, 57). For example, both RIG-I and MDA-5 can induce IFN responses to West Nile Virus, Measles virus, and Reoviruses (13, 37, 42). The exact mechanism(s) underlying virus specificity is hypothesized that different viral RNA structure motifs exist between these viruses, thus allowing RIG-I and MDA-5 to discriminate between RNA ligands. In the case with Reoviruses, different RNA ligands are exposed during virus replication to activate both RIG-I and MDA-5. In support of this theory, it was determined that the reovirus

genome consists of ten dsRNA segments of varying sizes where the long dsRNA segments mainly activated MDA-5 and the short dsRNA segments (less than 2Kb) activated RIG-I (42). These studies therefore suggest that a length dependent discrimination is employed by the two RNA helicases to recognize various viruses. However, a length dependent recognition by RIG-I does not explain RIG-I recognition of viruses with long single stranded genomes (such as West Nile Virus whose genome is ~11Kb). Therefore, it is hypothesized that in addition to short dsRNAs, other RIG-I RNA ligands must exist (88, 103). Therefore, a number of intensive studies have been initiated to identify the natural RNA ligand to RIG-I. Thus far, several RNA ligands with different structural motifs have been proposed, although there are conflicting reports. Therefore, this area of research is still heavily debated. Some examples of RIG-I ligands are free 5'-triphosphates, 3'-monophosphates, as well as 5'-monophosphates present on either ssRNA or dsRNA (60, 75). Additionally, viral RNA ligands may also encode nucleotide sequence specificities that facilitate RIG-I binding such as poly U/A tracts found within the 3'UTR of flaviviruses (90, 109). It is important to note though, that many of these studies utilized chemically synthesized RNAs with modified phosphates in their analysis. Therefore, a major argument presented within the field is that these RNA substrates do not reflect the natural viral RNAs made within an infected cell. Thus, several studies have identified potential RIG-I ligands by extracting viral RNAs made within infected cells and transfecting those RNAs into RIG-I deficient or wild type cells (75, 79). Using this approach, Rehwinkel et al. (2010) found that Influenza viral genomic RNAs were the major activators of RIG-I and that RIG-I activation required

the presence of a 5' triphosphate. Furthermore, the authors were able to demonstrate that the viral RNA transcripts and the RNase L cleaved RNAs were minor contributors to RIG-I activation. Therefore, 5'-triphosphates present on dsRNAs and ssRNAs are proposed to be the natural RIG-I ligands. This hypothesis is further supported by the fact that 5'-triphosphates are naturally generated during virus replication in the cytoplasm of infected cell. Host mRNAs are normally post-transcriptionally modified within the nucleus to contain a 5' cap structure or a 5'-monophosphate before they are exported into the cytoplasm. Therefore, the 5'-triphosphates on host mRNAs are never exposed to RIG-I. In support of this, Rehwinkel et al. (2010) also demonstrates that Influenza viral RNA transcripts, which are modified with an attached 5'-cap, do not activate RIG-I. Accordingly, the RNA helicases are able to discriminate self from non-self RNAs. The RNA substrates for MDA-5 have also been extensively studied and are found to be long dsRNA and ssRNA that consist of large secondary structures (22, 76). However, activation of MDA5 can occur in the presence or absence of phosphates.

Another set of PRRs that play roles in mediating IFN induction to viruses are the dsRNA dependent PKR and RNase L proteins. PKR and RNase L are Interferon stimulated genes though both proteins are constitutively expressed at basal levels in most tissues. PKR encodes two dsRNA binding domain as well as a regulatory kinase domain. PKR can recognize dsRNA that are longer than 30 nucleotides regardless of the nucleotide sequence (71). Although it has been demonstrated that PKR can recognize shorter RNAs, such as 16 nucleotide ssRNAs that form stem loop structures (70). However, it was found that the presence of a 5'-triphosphate

on the stemloop RNA was necessary for PKR recognition. As PKR is highly upregulated after IFN- $\alpha\beta$ induction, it is generally thought to participate as an antiviral factor during the IFN amplification phase. However, recent evidence has suggested that PKR can act directly as a PRR to recognize West Nile Virus (21) inducing IFN- β synthesis possibly through an NF- κ B pathway that is independent of RIG-I signaling. PKR has also been demonstrated to induce IFN to an Vaccinia Virus E3L mutant (119). The Vaccinia virus E3L protein is a known inhibitor of IRF-3 and IRF-7 phosphorylation. Thus, the authors used the E3L mutant to circumvent the viral IFN antagonist properties to address the PRRs that mediate IFN induction. The authors found that PKR could recognize vaccinia virus dsRNA intermediates to induce IRF-3 phosphorylation. Furthermore, they found that IRF-3 phosphorylation was dependent upon the IPS-1 signaling pathway. However, the roles for PKR to cooperatively signal IFN induction with the RLR helicases have not been defined. Therefore, the exact mechanism for PKR to mediate IRF-3 activation via the RLR-IPS-signaling pathway remains unknown.

Recent evidence suggests that another ISG, RNase L, can also play a role in the IFN induction pathway. RNase L is an endoribonuclease that cleaves single stranded regions of RNAs into small RNA cleavage products. Studies that were performed in RNase L deficient mouse embryonic fibroblast cells demonstrated a reduction in the IFN- β response to transfected poly IC ligands or upon infection with Sendai virus suggesting a small role to modulate IFN responses. The authors further demonstrated that the small cleavage products produced from functionally RNase L were capable of inducing IFN- β production. Also, IFN induction was

dependent upon an intact IPS-1 signaling pathway. Interestingly, the small RNA cleavage products were less than 200 nucleotides long, contained 3'-monophosphate rather than 5'-triphosphates, and signal IFN- β induction through both the MDA-5 and RIG-I RNA helicases (60). Therefore, RNase L is a PRR that is activated by viral dsRNA replicates and indirectly induces IFN production by amplifying the responses of the RLRS.

Alphaviruses are potent IFN inducers *in vivo* (15). However, the pattern recognition receptors that mediate these responses are not clear. Several studies using Sindbis and Chikungunya viruses have demonstrated that the IPS-1 signaling pathway is important for alphaviruses to mediate IFN induction (6, 93). However, these studies also report conflicting evidence for which upstream PRR is responsible for recognizing their viruses. Burke et al. (2009) demonstrated that both MDA-5 and PKR, but not RIG-I, contribute to IFN induction upon Sindbis infection of primary mouse embryonic fibroblast cells (6). It should be noted, though, that these studies were performed using a non-cytopathic Sindbis NsP2 mutant (39nc) to characterize their results. Therefore, we cannot exclude the possibility that recognition of the wild type Sindbis virus would be mediated through different receptor combinations. Schilte et al. (2010), on the other hand, demonstrates that wild type Chikungunya infections of similar fibroblast cells are mediated by both RIG-I and MDA-5 (93). Therefore, it can be inferred that multiple receptors may contribute to the overall recognition of alphaviruses or that different members of the alphavirus family differentially activate IFN induction. Regardless of the PRR that is responsible for mediating IFN induction, activation of the type I IFN leads to upregulation of genes

capable of inhibiting alphavirus replication, and thus making type I IFNs absolutely essential for alphavirus control.

Interferon stimulated genes to limit alphavirus replication

As discussed previously, IFN- $\alpha\beta$ induction results in the stimulation of hundreds of ISGs. These ISGs encode for numerous proteins that act as PRRs to detect viral replication, transcription factors to facilitate IFN production and amplification, and anti-viral effectors that limit viral spread. Thus far, the functions of only a few anti-viral effectors have been characterized to actively inhibit virus infections. There are four major anti-viral effectors that have been the most extensively studied. Some have also been demonstrated to play a role in inhibiting alphavirus infections *in vivo* (87). They are the IFN-stimulated protein of 15kDa (ISG15), dsRNA dependent PKR protein, the GTPase myxovirus resistance 1 (Mx1), and the endoribonuclease L (RNase L). Therefore, examples of ISGs and how they exert anti-viral activities are discussed next.

ISG15 is a ubiquitin homologue that regulates the expression of more than 100 target proteins. These target proteins participate in various cellular pathways such as RNA splicing, cell cycle regulation, and cell cytoskeleton organization (121). ISG15 is conjugated to target proteins by three conjugation enzymes, an E1-like ubiquitin-activating enzyme (UBE1L), and E2 ubiquitin conjugating enzyme (UBCH8), and an E3 ubiquitin ligase (HERC5). The attachment of the ISG15 molecules is similar to that for ubiquitin, thus ISG attachment is referred to as ISGylation. However, unlike ubiquitylation, ISGylation does not lead to the

degradation of the target protein. Instead, ISGylation modifies a protein's function similarly to the activating effects found by the K63-linked ubiquitin attachment. As an example, ISGylation of IRF-3 prevents virus mediated IRF-3 degradation, thus ensuring robust IFN- β production. Additionally, recent reports that ISG15 conjugation to the NS1 protein of Influenza A viruses also leads to an inhibition of virus replication. Therefore indicating a specific targeting of a viral IFN antagonist (122). ISG15 also plays an important role *in vivo* to several viruses, including Sindbis viruses (52, 53). Mice that are deficient for the Interferon α receptor (IFNAR) are readily susceptible to Sindbis virus infections and will succumb to disease. However, using a recombinant Sindbis virus to express the ISG15 gene from a duplicate 26S promoter will protect the IFNAR-/- deficient mice from the Sindbis infections. Furthermore, a similar ISG15 rescue in ISG15-/- mice also displayed a protective phenotype against a lethal Sindbis infection (53). Although ISG15 is clearly important for Sindbis infection, the exact mechanisms to protect these mice *in vivo* are not fully understood.

PKR is a member of a kinase family that regulates protein translation in response to cellular stress. PKR is normally kept in an inactive state within the cell by its N-terminal kinase domain. Upon binding to its viral ligand, PKR undergoes autophosphorylation and dimerization, leading to PKR activation. Active PKR dimers will then bind and phosphorylate the subunit of the eukaryotic translation initiation factor 2 (eIF2 α) at serine 51, resulting in a halt in global protein translation. This process then leads to a decrease in the viral proteins that are necessary for viral replication and assembly. PKR has been demonstrated to mediate protection

to several viruses (1). Mice deficient for both the PKR and RNase L gene were highly susceptible to a lethal strain of West Nile Virus, corresponding with enhanced viral titer in the draining lymph nodes and sera, as well as earlier viral entry into the central nervous system (92). Furthermore, PKR is required for the IFN-mediated resistance to Vesicular stomatitis virus (100). PKR also mediates partial protection against Sindbis viruses by restricting early viral replication in the draining lymph nodes (DLN) of infected mice and in *in vitro* dendritic cell cultures (86). Interestingly, although PKR exhibited some antiviral effects, unlike previous results with IFNAR deficient mice, PKR was not absolutely required to mediate full protection of mice from Sindbis viruses. These studies suggest that an alternative, IFN $\alpha\beta$ dependent pathways are responsible for restricting Sindbis viruses *in vivo*. Therefore, studies to identify alternative ISGs that inhibit alphaviruses are being pursued.

There are several encoded ISGs that have been identified to inhibit Sindbis replication. Ryman et al. (2005) used Affymetrix microarray technology to identify approximately 44 potential genes that are candidates for IFN-induced antiviral ISGs that act to inhibit Sindbis viruses, independently of the PKR and RNase L pathways (85). Some of the identified genes are ISG15, the zinc finger antiviral protein (ZAP), and viperin (120). The ZAP protein was originally isolated as an antiviral protein that inhibited Moloney murine leukemia virus (MMLV) (16). Recently, ZAP was also demonstrated to inhibit multiple members of the alphavirus family, including Sindbis, RRV, SFV, and VEEV (2). Although, ZAP inhibition of Sindbis virus was the strongest of the alphaviruses tested. The ZAP protein was found to block translation of the incoming Sindbis genomic RNAs, leading to a failure to produce the nsP

replication complex. Altogether, ZAP functions to prevent Sindbis replication and the accumulation of viral RNAs within the cytosol of a cell. Viperin has been less extensively studied. Viperin was first identified as an antiviral molecule that could inhibit human cytomegalovirus, possibly through inhibition of viral structural protein synthesis (8). However, Zhang et al. (2007) found that viperin also partially protects mice upon Sindbis infections, although the exact mechanism mediating protection has not been identified (120). Therefore, it is unclear exactly how many anti-viral ISGs mediate inhibition of alphaviruses or the precise mechanisms that facilitate this inhibition. It is, however, clear that the type I IFN system employs many arms to fully combat alphaviruses, making IFN absolutely essential to control these viruses.

Alphavirus modulation on IFN induction

The Type I IFN response is rapid, robust, and quite effective in inhibiting virus replication and spread. This is highlighted by the fact that mice with a genetic defect for the various IFN molecules either rapidly succumb to virus infections or display increased viral replication (84, 91, 92, 100, 105). Furthermore, avirulent viruses that are normally cleared in mice with an intact IFN response are more virulent in mice without one (105, 113). Although despite the fact that the IFN response is critical to inhibit many different virus infections, a number of viruses seem to persist even in the presence of intact IFN response. Furthermore, evidence suggests that virulent viruses, such as Venezuelan Equine Encephalitis virus (VEEV) seem to be more resistant to IFN $\alpha\beta$ activities than their avirulent counterparts (17, 113). In support of this, exogenous priming of the IFN system with IFN $\alpha\beta$ proteins or with a poly IC

inducer, failed to protect mice against a virulent VEEV (26), suggesting a mechanism for VEEV to evade IFN responses. Therefore, given the importance of the type I IFN response to control virus replication, it is not surprising that viruses have evolved mechanisms to counteract these actions. These anti-IFN strategies can range from non-specific mechanism(s) such as virus induced shutoff of the host macromolecular synthesis to specific inhibition of host molecules that directly interfere with IFN signaling or the anti-viral ISGs or to masking of the viral ligands to evade and IFN response. Several studies have suggested mechanism(s) for alphavirus to actively evade or antagonize the host type I IFN system.

A major mechanism for alphaviruses to evade the host type I IFN response is thought to be virus mediated shutoff of the host RNA and protein synthesis (15, 24, 25). As an alphavirus enters a cell, the viral genome is translated to immediately begin the viral replication life cycle. The replication of the viral genome is fairly robust and occurs rapidly after infection. To aid in this progress, a generalized mechanism of host shutoff is employed by the virus. A few hours post-infection, the cellular transcription and translation machinery is redirected to synthesize only the viral RNAs and proteins. The exact mechanism of the host shutoff has not been clearly identified although; depending on the alphavirus used, several studies have implicated a role for the nonstructural and structural proteins to act within the nucleus of a cell. These studies have led to the general concept that Old World alphaviruses (Sindbis and Semliki Forest Virus) mediate transcription shutoff via the viral nsP2 protein whereas the New World viruses (Venezuelan equine encephalitis and Eastern equine encephalitis viruses) utilize the capsid structural proteins (18,

20). In support of these hypotheses, studies have demonstrated that both the nsP2 and capsid proteins can localize to the nucleus. Furthermore, mutations that disrupts nsP2 and capsid nuclear localization, results in viruses that are unable to shutoff synthesis of cellular RNAs (18, 25). It has been theorized that a generalized host shutoff mechanism, in addition to enhancing viral replication, also acts as non-specific inhibition of the IFN $\alpha\beta$ responses. In support of this, alphaviruses that are defective for their ability to completely shutoff host cellular synthesis are also robust IFN inducers (24). Therefore, the actions of host shutoff serves two purposes, 1) to aid in efficient viral replication and 2) to inhibit IFN $\alpha\beta$ induction as well as their targeted ISGs. However, recent reports have suggested that in addition to a generalized host shutoff, alphaviruses also employ specific IFN antagonists to counteract the early IFN $\alpha\beta$ responses (4, 97, 117). Therefore, the alphaviruses are likely to employ multiple mechanisms that antagonize the IFN response.

Many viruses have also evolved to specifically antagonize the IFN response by encoding specific molecules that directly inhibit steps within the IFN pathway. These include molecules that target the initial IFN induction phase, inhibit molecules within the IFN signaling phase (amplification), or to directly inhibit ISG functions. For example, Hepatitis C virus (HCV) encodes a viral protease, NS3/4A, which specifically cleaves the IPS-1 signaling molecule from the mitochondrial membranes. IPS-1 is absolutely essential for the RIG-I and MDA-5 RNA helicases to induce an IFN response. Therefore, viral mediated cleavage of IPS-1 efficiently downregulates IFN β induction upon HCV infection (11, 54). Ebola viruses, on the other hand, can inhibit IFN $\alpha\beta$ signaling through inhibiting the JAK-STAT pathway. Ebola viruses

encode a viral protein, VP24, which blocks the nuclear accumulation of phosphorylated STAT-1 (61, 80). Therefore, phosphorylated STAT-1 will not bind to its ISG promoters, thus effectively preventing a cell from upregulating anti-viral genes. Though alphaviruses were thought to not employ specific mechanisms to antagonize IFN induction/signaling, instead relying on the nonspecific shutoff mechanisms as described above, recent reports have also identified that alphaviruses can mediate specific IFN inhibition independently of its non-specific, global shutoff of the host macromolecular syntheses (4, 6, 117). Simmons et al. (2009) demonstrates that Venezuelan equine encephalitis virus (VEEV) can directly block STAT-1 phosphorylation, and subsequent nuclear localization, when stimulated with IFN- β (97). Additionally, Yin et al. (2009) also demonstrates a similar STAT antagonism with Sindbis viruses (117). Interestingly, the adult mouse neurovirulent Sindbis AR86, as described earlier, also inhibits STAT-1 activation and mutation of a single virulence determinant located at nsP1 position 538 (32, 34, 104) disrupts this inhibition (Simmons et al., manuscript in progress). Importantly, these studies provide a direct link of a genetic determinant that modulates pathogenesis *in vivo*, to antagonism of STAT activation, suggesting that viral modulation of these pathways contributes to viral virulence.

In addition to encoding specific molecules to directly antagonize IFN signaling, alphaviruses may employ other mechanisms to avoid type I IFN induction (12, 28). Transmission of mosquito-borne viruses requires a successful transition from the mosquito into vertebrate hosts by targeting specific cell types that allow the virus to efficiently replicate and spread throughout the host. In order to achieve this,

mosquito-borne viruses need to overcome many barriers of the host's innate defense, including the type I IFN response. Studies have demonstrated that mosquito borne viruses target Langerhans cells or myeloid dendritic cells (DCs) that are present beneath the skin's dermal surface (55, 58, 115). Additive post-translational modification to the structural proteins, such as glycosylation, enhances virus-DC interactions due to glycoprotein interactions with the mannose binding C-type lectin receptors, such as DC-SIGN, that are present on these cells (9, 45). Shabman et al. (2007) discovered, that in addition to promoting DC infections, mosquito derived Ross River Virus (RRV) was also a poor IFN inducer (95). In contrast, mammalian derived RRV viruses were robust IFN inducers. These studies further defined that the mammalian derived viruses, added complex N-linked glycans to the E2 glycoprotein, whereas mosquito derived viruses added high mannose N-linked glycans and that the presence of the complex glycans were responsible for mediating IFN induction (96). Further, the presence of the high mannose glycans did not actively antagonize the IFN response, as the mosquito-derived viruses were unable to suppress the IFN induction mediated by the mammalian virus. Therefore, though it is unclear whether complex N-linked glycans actively promote type I IFN induction or whether high mannose N-linked glycans allow the virus to avoid recognition, perhaps by retargeting the virus during entry to avoid contact with PRRs, these studies do demonstrate that post translational modifications to the virus can significantly impact recognition by the host. Therefore, additional studies are required to fully evaluate the combined effects of glycan mediated IFN modulation in

combination with nonspecific and specific effects on IFN induction in the context of alphavirus infection.

DISSERTATION OBJECTIVES

Alphaviruses are vector borne viruses that can cause massive outbreaks of human disease (30, 62, 63, 114). The viral interactions with the host type I IFN system play an important role in determining the outcome of virus infection due to the IFN's potent antiviral activities and its ability to shape the downstream adaptive immune response. However, many viruses have evolved mechanisms to antagonize or evade the type I IFN response. The viral antagonistic mechanisms range from non-specific effects such as shutoff of host cellular processes to specific inhibition of molecules within the IFN signaling pathway (18, 19, 31, 80).

Additionally, simple evasion mechanisms of the host IFN system, such as masking of viral RNA ligands, are also employed by many viruses to avoid IFN detection (7, 28). Therefore, understanding the processes in which a host cell recognizes incoming virus to induce type I IFNs, and how viruses subvert these processes, is important for improving vaccines and developing therapeutics against them.

Previous reports from our lab have identified a single determinant within the viral nsP1 protein that plays an important role in Sindbis AR86 neurovirulence in adult mice (32, 104). Therefore, the goals of this dissertation are to further understand the role of the nsP1 determinant to interact with the host immune system, specifically the type I IFN system. The dissertation objectives are as follows:

- 1) Does the Sindbis AR86 nsP1 determinant modulate IFN induction and is it specific to this virus?
- 2) What are the IFN induction pathways that are involved in alphavirus recognition and how does the nsP1 determinant modulate these pathways?

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CHAPTER TWO

MODULATION OF TYPE I IFN INDUCTION BY A VIRULENCE DETERMINANT WITHIN THE ALPHAVIRUS NSP1 PROTEIN

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ABSTRACT

Alphaviruses are mosquito-borne viruses that cause serious human and animal diseases. Previous studies demonstrated that a determinant within the nsP1/nsP2 cleavage domain of the virulent Sindbis AR86 virus played a key role in regulating adult mouse virulence without adversely affecting viral replication. Additional characterization of this determinant demonstrated that a virus with the attenuating mutation induced more type I IFN production both *in vivo* and *in vitro*. Interestingly, this phenotype was not specific to the Sindbis AR86 virus, as a similar mutation in a distantly related alphavirus, Ross River Virus (RRV), also led to enhanced IFN induction. This effect was independent of virus-induced host shutoff, since IRF-3 phosphorylation, which occurs independently of *de novo* host transcription/translation, was induced more robustly in cells infected with the mutant viruses. Altogether, these results demonstrate that critical determinants within the nsP1/nsP2 cleavage domain play an important role in regulating alphavirus induced IFN responses.

Keywords: Alphaviruses, Sindbis, Ross River, nsP1 mutants, type I IFN induction.

INTRODUCTION

Alphaviruses are mosquito-borne viruses capable of infecting humans and causing various diseases ranging from acute encephalitis to long-term virus-induced arthritis. The Sindbis-group viruses are among the most studied and well characterized of the alphaviruses, having been used to identify viral and host factors that contribute to virus-induced disease in mice (27, 35, 42). The majority of the Sindbis virus strains are avirulent in adult mice, however, there are a few exceptions, including the neurovirulent neuroadapted Sindbis virus (NSV) (44) and AR86 strains (17, 40). Genetic studies have mapped virulence determinants of these neurovirulent viruses to both the structural and nonstructural proteins. Detailed genetic mapping studies using chimeric viruses that encode regions of the adult mouse virulent AR86 and the closely related avirulent GirdwoodS.A identified four major neurovirulence determinants in the AR86 genome including, a Threonine at nsP1 position 538, an 18-amino acid deletion at nsP3 386, a Cysteine at nsP3 position 537, and a Serine at E2 position 243 (42). While all four determinants were found to be essential for adult mouse virulence, the determinant within nsP1 was found to be critical; as a single coding change of this determinant from a Thr to Ile severely attenuated the virus, while introduction of a Thr at this position into non-virulent viruses partially rescued virulence (17). Currently, the exact mechanism by which this determinant affects viral virulence is not understood.

The alphavirus nonstructural proteins form the viral replication complex that mediates cytoplasmic viral RNA synthesis within infected cells. Nonstructural polyprotein processing is intimately linked to viral RNA synthesis (21, 38). Upon

viral entry, the virion-associated RNA is immediately translated to form the replication complex consisting of the nonstructural precursor, nsP123, and nsP4, which together mediate negative strand RNA synthesis (41). Later in the course of infection, the polyprotein precursor nsP123 is further processed by nsP2 to produce mature nsP1, nsP2, and nsP3. The mature nonstructural proteins, together with nsP4, form the replication complex to synthesize full length genomic RNA. The mature nsPs also bind to an internal subgenomic promoter to synthesize smaller subgenomic RNAs that encode the viral structural proteins. Previous studies using mutants that lack the ability to efficiently process the nonstructural polyprotein precursor nsP123 showed dysregulation of viral RNA synthesis (21). Interestingly, the wild type AR86 Thr to Ile change at nsP1 position 538, is located in the P3 position of the conserved nsP1/2 cleavage recognition domain (38). The presence of an attenuating Ile at this position results in increased kinetics of nonstructural polyprotein processing as well as earlier induction of 26S subgenomic RNA synthesis. Furthermore, this mutation did not affect full length negative or positive strand RNA synthesis, though it did result in increased virus production (18). Whether differential nonstructural protein processing or the increased 26S RNA is important for the attenuated phenotype of this virus *in vivo* has yet to be determined.

In addition to mediating viral replication, the nonstructural proteins interact with host factors to alter environmental conditions that favor viral replication (10, 26). The nonstructural proteins of Old World alphaviruses, such as Sindbis and Semliki Forest virus, have been shown to mediate shutoff of host transcription and translation (11). While the exact mechanism of virus-mediated host shutoff is not

completely understood, it has been proposed that mature nsP2 is required (14, 15). Alphaviruses are thought to down regulate host mRNA and protein synthesis in order to limit competition for the cellular machinery to maximize viral replication while simultaneously limiting induction of type I IFN and related IFN stimulated genes (ISGs).

Sindbis viruses are highly sensitive to the effects of type I IFN (9). Type I IFN receptor deficient mice exhibited enhanced susceptibility to Sindbis virus marked by increased virulence, broadened tissue tropism, and uncontrolled virus replication (34), demonstrating that type I IFN plays an important role in controlling Sindbis virus infection. Therefore, it is likely that Sindbis virus utilizes several mechanisms to limit and/or suppress type I IFN induction. One likely mechanism involves generalized suppression of host macromolecular synthesis. In support of this, Sindbis virus mutants that are defective for host shutoff are more potent inducers of the type I IFN response (14, 15). However, host shutoff independent mechanisms likely exist, as a mutation in the nsP2 protein of Semliki Forest Virus, a related alphavirus, affects type I IFN induction independently of generalized host shutoff (6).

In this study, we demonstrate an important role for a determinant at the P3 cleavage position between nsP1 and nsP2 in regulating type I IFN induction. Mutation of this position in two distantly related alphaviruses, the AR86 strain of Sindbis and Ross River Virus, resulted in enhanced type I IFN induction and IRF-3 activation in comparison to the wild type viruses. Furthermore, the P3 mutation had little effect on kinetics of virus mediated host transcription and translation shutoff. Taken together, these results suggest that the P3 determinant, which regulates

AR86 virulence, plays a major role in regulating type I IFN induction that is independent of virus mediated host shutoff.

RESULTS

An attenuating Isoleucine mutation at nsP1 position 538 (T538I) in the Sindbis virus AR86 backbone leads to increased type I IFN induction *in vivo* and *in vitro*.

A virulence determinant located within the nsP1/nsP2 cleavage domain (nsP1 538) of the Sindbis virus, AR86, regulates viral nonstructural polyprotein processing and subgenomic RNA synthesis (18). Given that the presence of the attenuating Ile mutation led to earlier induction of viral 26S RNA synthesis, which could alter type I IFN induction, we assessed whether the attenuated mutant virus exhibited any differences in type I IFN induction from that of the wild type virus both *in vivo* and *in vitro*. To evaluate whether the viruses exhibited differential IFN induction *in vivo*, six-week old CD-1 mice were mock infected or infected with the wild type AR86 (previously referenced as s300 (42)) or mutant T538I (nsP1 538 Ile previously referenced as s340) viruses by the intracranial route (i.c.) and bled between 9 and 18 hours post infection. Serum type I interferon responses were measured using an interferon bioassay on L929 cells. As shown in figure 2.1A, serum from mice infected with the mutant T538I virus exhibited robust type I interferon levels compared to mice infected with the wild type AR86 virus at both 12 and 18 hours post infection as detectable by bioassay. Similar results were observed in C57BL/6 mice (data not shown). Importantly, wild type AR86 did not

induce detectable type I interferon up to 48 hours post-infection (data not shown), indicating that the wild type virus was not simply delayed in the induction of type I interferon. To further assess whether the mutant virus was a more potent inducer of type I IFN, L929 cells were infected with either the wild type AR86 virus or the T538I mutant and type I IFN levels in the supernatant were evaluated. As shown in figure 2.1B, similar to the *in vivo* results, the attenuated mutant was a more potent inducer of type I IFN than the wild type virus. These results were not restricted to L929 cells, since the mutant virus was also a more potent IFN inducer in human cell lines such as A549 cells (data not shown). Overall, these results suggest that the determinant at nsP1 position 538 plays a major role in regulating type I IFN induction by the virus.

Previous studies with Sindbis viruses have suggested that virus-mediated host cellular macromolecular shutoff is linked to IFN induction. Some Sindbis virus mutants that are potent IFN inducers are also defective in their ability to shutoff host RNA synthesis (14), raising the possibility that the nsP1 mutation in AR86 affected type I IFN induction through effects on host macromolecular synthesis. Therefore, we assessed the kinetics of transcriptional and translational shutoff between the wild type and mutant AR86 viruses. These studies utilized L929 cells, since the T538I mutant exhibited enhanced type I IFN induction in this cell type (figure 2.1B). Unfortunately, we were unable to achieve 100% infection at low MOIs. Therefore, the MOI was increased to 50 to increase the percent of infected cells and to limit the confounding effect of nonproductively infected cells in the culture continuing transcription/translation and complicating the analysis of virus-induced shutoff of

these processes. As shown in figure 2.2A, both the wild type and the mutant viruses exhibited similar kinetics of host transcription shutoff at 5 hours post-infection as observed by the loss of host mRNA, 28S rRNA, and 18S rRNA. By 8hpi, both viruses have efficiently turned off host transcription. Interestingly, in Neuro2A cells, the wild type Sindbis AR86 virus exhibited less efficient shutoff of host mRNA, 28S rRNA and 18S rRNA as compared to the mutant T538I virus suggesting that the T538I mutant may actually shutoff transcription more efficiently than the wild type virus (data not shown). Additionally, the wild type and mutant Sindbis viruses were equally efficient at inhibiting host protein translation (figures 2.2B and 2.2C). As early as 4 hours post-infection, both viruses exhibit greater than 25% shutoff of host protein synthesis and by 8 hours post-infection greater than 75% of host protein synthesis shutoff was observed (figure 2.2C). Most importantly, when comparing the β -actin band, the mutant T538I virus displayed similar kinetics of host protein shutoff as compared to wild type AR86 virus (figure 2.2B). Similar observations were also made in Neuro2A and BHK-21 cells (data not shown). These results suggest that the Sindbis virus mutant induced enhanced type I IFN production in the absence of a detectable defect in generalized host cell shutoff.

Mutation of the nsP1 determinant in Ross River Virus results in enhanced type I IFN induction by the mutant virus.

To determine if the effect of the amino acid within the nsP1/nsP2 cleavage domain on type I IFN induction was specific to AR86, similar mutations were introduced in the distantly related alphavirus, Ross River Virus (RRV) (figure 2.3A).

RRV encodes an Alanine codon at the same position within the nsP1/2 cleavage site and we examined whether substitution with an Isoleucine (A532I) or Valine (A532V) would result in a viable virus with similar effects on type I IFN induction as seen with the Sindbis T538I mutant (figure 2.1). Substitution of an Isoleucine in RRV resulted in a significantly attenuated virus that grew poorly in BHK-21 cells (data not shown). In contrast, substitution of an Alanine to Valine resulted in a viable virus that grew to levels comparable to wild type RRV, though the Valine mutant displayed a smaller plaque phenotype on BHK-21 cells (data not shown). Furthermore, the RRV A532V mutant had a similar RNA specific infectivity and particle to PFU ratio to those observed with the wild type virus (data not shown). In both single (figure 2.3B) and multi-step (figure 2.3C) growth curves in BHK-21 cells, the A532V mutant exhibited a significant and reproducible reduction in viral yield at intermediate times (7-16hpi), although the endpoint yield was identical to wild type RRV. Therefore, the A532V mutant is viable, though it may exhibit a slight replication defect as compared to the wild type virus.

In order to assess the impact of the A532V mutation on type I IFN induction, L929 cells were infected with either the wild type RRV or the RRV A532V mutant, and type I IFN induction in the supernatant was measured by bioassay. As shown in figure 2.4A, the A532V mutant exhibited enhanced IFN induction (~90 fold) in comparison to wild type RRV at 24 hours post-infection. Similar results were found by qRT-PCR analysis for IFN-beta mRNA transcripts at early times post-infection (figure 2.4B and 2.4C). Altogether, these data, along with the earlier findings with the Sindbis AR86 viruses, highlight the importance of the determinant within the

nsP1/nsP2 cleavage site in modulating type I IFN responses by at least two alphaviruses.

The A532V mutation affects type I IFN induction.

Recent work from our group and others has demonstrated that another alphavirus, Venezuelan equine encephalitis virus (VEEV) can antagonize STAT1 activation following treatment with type I IFN (39, 46). Although, to date we have found no evidence that wild type RRV can antagonize STAT1 activation (Simmons and Heise, unpublished), we were interested in determining whether the A532V mutation affected the early induction of type I IFN or the amplification phase of the type I IFN response, which is dependent upon type I IFN signaling and STAT1 activation. Thus, we compared the wild type and mutant A532V RRV viruses for their ability to induce type I IFN in primary MEFs derived from wild type mice or mice deficient in the type I IFN $\alpha\beta$ receptor (IFNR), which are therefore unable to mount an amplified type I IFN response. As shown in figure 2.5A, similar to the results from the L929 cells, the mutant virus was a more potent type I IFN inducer than wild type RRV in wild type MEFs. In IFNR deficient MEFs, the overall type I IFN induction was reduced compared to wild type cells, however the mutant virus still induced significantly more type I IFN than the wild type virus (figure 2.5B). Therefore, though we cannot rule out an effect of the A532V mutation on type I IFN signaling and subsequent amplification of the IFN response, these results strongly suggest that the nsP1 mutation does have an effect on the inductive phase of the type I IFN response.

The RRV A532V exhibits a mild defect in virus-induced shutoff of host RNA and protein synthesis.

The Sindbis T538I mutant exhibited little defect in virus-induced shutoff, therefore we determined whether this was also the case with the RRV A532V mutant. RRV or A532V were evaluated for their ability to shut off host protein and RNA synthesis in a manner similar to that described above for AR86. L929 cells were infected with the RRV viruses at an MOI of 50 to enhance the percentage of infected cells in the culture. At this MOI, the percentage of infected cells was greater than 80% for both viruses as demonstrated by an Immunofluorescent Assay (IFA) (data not shown). Importantly, the RRV A532V mutant still induced an enhanced IFN response compared to the wild type virus at this MOI (figure 2.6D). Both wild type RRV and the mutant A532V viruses inhibited cellular RNA transcription as early as 5 hours post-infection as indicated by the decline in 18S rRNAs as compared to mock infected cells (figure 2.6A). By 8 hours post-infection, both viruses efficiently turned off 18S rRNA synthesis. Similar results were also obtained in BHK cells though interestingly, in BHK cells, the A532V mutant displayed a slight delay in transcriptional shutoff at early times post-infection compared to the wild type RRV virus (data not shown). Therefore, though the A532V mutant is capable of shutting off host RNA synthesis we cannot rule out the possibility that the A532V mutant virus may be slightly delayed in its ability to turn off cellular RNA synthesis. Analysis of virus induced inhibition of host protein synthesis (figure 2.6B) demonstrated that both viruses inhibit cellular protein synthesis in L929 cells with similar kinetics as

indicated by the loss of the β -actin band (figure 2.6C). Similarly to the transcriptional shutoff data, in BHK cells, the A532V mutant was slightly delayed in inhibiting protein translation in comparison to the wild type virus (data not shown). Therefore, though the mutant RRV is ultimately able to shut off host cell RNA and protein synthesis, based on these results, we cannot rule out the possibility that a slight defect in shutoff might contribute to the enhanced type I IFN induction by the A532V RRV mutant.

The A532V mutant virus is a strong inducer of IRF-3 phosphorylation than the wild type virus.

The results with the Sindbis T538I mutant suggest that host shutoff defects do not contribute to the enhanced type I IFN induction by the mutant virus. Furthermore, though we could not detect a defect in the RRV A532V mutant's ability to shutoff host transcription or translation in L929 cells, the delayed kinetics of shutoff by the A532V mutant in BHK cells left open the possibility of shutoff dependent effects for the RRV mutant. Therefore, to more clearly address this issue, we assessed the RRV viruses for their ability to activate IRF-3, a step that is independent of *de novo* host RNA and protein synthesis and therefore not susceptible to shutoff mediated effects (32). IRF-3 is an essential transcription factor that is immediately activated by several pathogen-associated molecular pattern receptors to induce early transcription of IFN- β and IFN- α genes. Activation is initiated upon IRF-3 phosphorylation resulting in protein dimerization and nuclear translocation. Therefore, we assessed whether the mutant RRV virus exhibited

differential IRF-3 activation compared to the wild type RRV in L929 cells, which exhibited high levels of type I IFN induction by the RRV A532V mutant (figure 2.4A). L929 cells were transfected with poly IC as a positive control (16), which resulted in robust IRF-3 phosphorylation as indicated by the shift in the IRF-3 protein band in comparison to the mock treated cells (figure 2.7A). Additionally, phosphatase treatment of cell lysates confirmed that the upper band was indeed a phosphorylated protein (data not shown). Analysis of the virally infected cells demonstrated that the RRV A532V mutant virus induced IRF-3 phosphorylation at 10 hours post-infection; however, the wild type RRV virus did not display IRF-3 phosphorylation until as late as 12 hours post-infection. Also, there was substantially more IRF-3 phosphorylation at 12 hours post-infection in cells infected with the A532V mutant virus (figure 2.7B), consistent with our previous data that the RRV A532V mutant induces more IFN than wild type RRV. Similar to the results with RRV, the AR86 T538I mutant induced faster and more robust IRF-3 activation than the wild type virus in HEC-1B cells, human endometrial carcinoma cells, that have been well characterized in IRF-3 activation studies and do not respond to IFN treatment (8, 43)(data not shown). Altogether, these results suggest that both the AR86 and RRV mutants affect IFN induction through a mechanism that is independent of virus-induced host shutoff. However, given that the RRV A532V mutant did exhibit delayed shutoff kinetics in BHK cells, we cannot rule out a synergistic role for shutoff dependent and independent effects in enhancing type I IFN induction by the RRV A532V mutant.

DISCUSSION

Type I interferon is an essential component of the host response to viral infection, because it directly activates antiviral systems and modulates antiviral activities of other components of the host innate and adaptive immune systems. However, a number of viruses have evolved mechanisms to antagonize or evade type I IFN induction. These mechanisms range from nonspecific effects, such as rapid shutoff of host cell macromolecular synthesis (1, 14), masking viral RNA from host cell sensory proteins (7, 25), to specific inhibition of host cell dsRNA sensors or signaling molecules that link these sensor molecules to transcription factors that regulate type I interferon transcription (4, 5, 22, 23). Studies with Sindbis virus suggest that host cell shutoff plays a major role in regulating viral type I interferon induction (14), though studies with Semliki Forest virus (SFV) also indicate that alphaviruses can modulate type I IFN responses independently of host shutoff (6). In this report, we present evidence that an attenuating mutation at nsP1 position 538 (T538I) in the neurovirulent strain of Sindbis virus, AR86, modulates type I interferon induction without affecting this virus's ability to shut off host cell macromolecular synthesis. Additionally, this determinant has been shown to play a key role in regulating viral neurovirulence (17), and it is likely that the enhanced type I IFN induction by the nsP1 538 mutant virus *in vivo* (figure 2.1A) is at least partially responsible for its attenuating effect on AR86. Furthermore, the importance of this determinant in regulating type I IFN induction by alphaviruses is underscored by our finding that an analogous mutation in another alphavirus, Ross River Virus, exerts a similar effect on type I IFN induction (figure 2.4 and 2.5).

Using a genetically related Sindbis virus, Gorchakov et al. (2005) demonstrated that viruses with mutations resulting in defects in host translation or transcription shutoff induced more type I interferon (14). However, we found either no difference or a very mild defect in host shutoff with the mutant Sindbis and RRV viruses, as well as differences in the kinetics of virus induced IRF-3 activation, which is independent of host shutoff. This finding strongly suggests that these viruses can affect type I IFN induction independently of effects on host shutoff. It is also important to note that previous work with an SFV nsP mutant, which also demonstrated a role for the nonstructural proteins in modulating type I IFN induction independently of host shutoff effects, did not observe a differential effect on IRF-3 activation (6), which raises the possibility that the determinants in our AR86 and RRV mutants may affect interferon induction through different mechanisms than those observed with SFV. Furthermore, previous work from our lab and others have demonstrated a role for VEE to inhibit STAT-1 activation (39, 46), a necessary step in the IFN signaling pathway to amplify the type I IFN response indicating that these viruses may employ additional mechanisms to antagonize this pathway. However, our analysis in IFN $\alpha\beta$ Receptor deficient MEFS, coupled with preliminary data that wild type RRV does not inhibit STAT-1 activation, further suggests that our determinant is affecting the initial IFN induction step.

The nsP1 virulence determinant described here might affect type I IFN induction through several different mechanisms. First, the nsP1 mutant may induce more type I IFN by producing earlier and/or increased quantities of a viral ligand, for efficient recognition by the host sensors. Previous studies examining very early

times post-infection demonstrated that the AR86 mutant virus initiated 26S RNA synthesis more quickly than the wild type virus, though both viruses exhibited equivalent levels of 26S RNA synthesis by 4-6 hours post-infection (18). Although, we did not observe this kinetic difference in our L929 RNA labeling assays, this likely reflects the time-points analyzed in the current studies. Our earlier results raise the possibility that early or more abundant 26S RNA synthesis by the mutant virus might be linked to the difference in type I IFN induction. Preliminary analysis indicates that the RRV mutant may also produce more viral RNA than the wild type virus (data not shown). In this model, the wild type virus could delay 26S promoter induction until later times when virus-induced host shutoff efficiently antagonizes type I interferon induction. The attenuating mutation at nsP1, however, up-regulates 26S RNA synthesis prior to the time of effective virus-induced shutoff (18). This model suggests that the subgenomic RNA may be a potential target for host cell sensors, however; this does not rule out a role for multiple viral RNA species, such as the full length positive or negative sense RNA, or dsRNA complexes to activate IFN induction. Further investigations will be required to address the exact viral ligand required as well as the pathway that is mediating this response and whether this mechanism holds true for both viruses. Second, the nsP1 mutant may induce more type I IFN by altering the structure of one or more viral RNAs and thereby producing a stronger ligand. Gitlin et al. (2006) has reported that RIG-I plays an important role in responding to Sindbis virus infection (13), while PKR has also been shown to contribute to type I IFN induction during flavivirus infection (12). Both RIG-I and PKR interact with 5'-triphosphates on uncapped RNAs (19, 30, 31), though both

proteins can also recognize other RNA ligands (29, 36). Both nsP1 and nsP2 proteins are involved in capping viral RNAs and though the C-terminus of nsP1 has not been implicated in capping activity, it is possible that the determinant at nsP1 is altering the efficiency of capping viral genomic or subgenomic RNAs (2, 3, 19, 24, 33, 45). In this model, the nsP1 mutants are providing the host sensor, RIG-I, with viral ligands that contain free 5'-triphosphates that lead to stronger IFN- β induction. Finally, though there is no direct evidence to suggest that the wild type and T538I mutant Sindbis AR86 viruses or RRV and the A532V RRV mutant differ in their ability to actively suppress type I IFN induction, it is possible that the mutation is modulating type I IFN induction through different mechanisms in the context of each virus. Regardless of the ultimate mechanism responsible for the differential type I IFN induction phenotype, these results further support the idea that determinants in the viral nonstructural region of alphaviruses play a major role in regulating type I IFN induction, with a concomitant impact on viral virulence.

In summary, we have demonstrated that wild type AR86 virus was a poor inducer of type I interferon, while the mutant T538I virus containing an attenuating Ile codon at nsP1 position 538 induced a robust type I interferon response *in vitro* and *in vivo*. An analogous determinant exhibited a similar effect in another, distantly related alphavirus (RRV), suggesting that this determinant plays an important role in regulating type I IFN induction by multiple alphaviruses. The altered interferon induction was independent of virus-induced host translation or transcription shutoff, suggesting that the determinant within the nsP1/nsP2 cleavage site acts through a more specific mechanism to modulate the host type I interferon response.

MATERIALS AND METHODS:

Viruses and Cell Culture: The AR86 molecular clones pS300 (wild type nsP1 538 Thr) and pS340 (mutant nsP1 538 Ile) were described previously (17). The wild type Ross River Virus molecular clone, pRR64 (20), was generously provided by Dr. Richard Kuhn (Purdue University). The Ross River Virus mutant, pRR64-A532V, was generated by introducing a single nucleotide change (thymine to guanine) at nucleotide position 1670, by PCR mutagenesis resulting in a single codon change from an Alanine (wild type) to Valine (A532V) at nsP1 position 532.

Viral stocks were generated by *in vitro* transcription as previously described (18, 28). Briefly, cDNA plasmids were linearized and used as templates for full length RNA transcripts generated by SP6-specific mMessage mMachine *in vitro* transcription kits (Ambion). Transcripts were electroporated into BHK-21 cells using a Bio-Rad electroporator. Supernatants were harvested 24 hours later, centrifuged for 20 min at 3,000 RPM, and frozen in 0.5 ml aliquots. Alternatively, electroporated supernatants were pelleted through a 20% (w/v) sucrose/phosphate buffered saline (PBS) cushion at $72,000 \times g$ by ultracentrifugation (four hours) to concentrate viral stocks. The pelleted viruses were resuspended in PBS and 0.1ml aliquots were frozen at -80°C . Viral titers were determined by standard plaque assays on BHK-21 cells.

BHK-21 cells were maintained in alpha minimum essential medium (Gibco) supplemented with 10% bovine calf serum (BioWhittaker), 10% tryptose phosphate broth, and 0.29 mg/ml of L-glutamine. L929 mouse fibroblast cells were maintained in alpha minimum essential medium (Gibco) supplemented with 10% bovine calf

serum, 10% tryptose phosphate broth, and 0.29 mg/ml of L-glutamine. Neuro2A mouse neuroblastoma cells (N2A) were grown in MEM containing nonessential amino acids and 10% fetal bovine serum (HyClone). HEC-1B cells (ATCC) were grown in MEM containing 10% fetal bovine serum, 0.29 mg of L-glutamine per ml, and penicillin/streptomycin. Primary Mouse Embryonic Fibroblast (MEFS) cells were generated from 13-15 day old Sv/129 wild type or IFN $\alpha\beta$ receptor deficient (IFNR $^{-/-}$) embryos and maintained in DMEM/F12 media containing 10% Fetal Bovine Serum, 10% tryptose phosphate broth, 0.29 mg/ml of L-glutamine, and 50ug/ml of gentamicin (Gibco).

Animal studies: Specific pathogen-free six-week old female CD-1 mice were obtained from Charles River Breeding Laboratories (Raleigh, NC), while C57Bl/6J mice were bred in house. Animal housing and care were in accordance with all University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee guidelines. In all studies, six- to eight-week-old mice (groups of 3 to 6 animals/study) were anesthetized with Ketamine supplemented with Xylazine (Barber Med.) prior to intracranial (i.c.) inoculation with a standard dose of 10^3 PFU of virus in diluent [phosphate-buffered saline (PBS, pH 7.4)], supplemented with 1% donor calf serum (DCS, Gibco)). Mock infected mice received diluent alone. At the indicated timepoints, mice were bled from the tail vein and sera were frozen at -80°C until analyzed for type I interferon by bioassay (see below).

Type I IFN bioassays: Total amounts of type I IFN were measured by using an interferon bioassay as previously described (37). Briefly, L929 cells were seeded in 96 well plates. Samples, including the standards, were acidified to a pH \leq 2.0 for 24 hours, then neutralized to pH of 7.4, UV treated for 10 minutes to inactivate residual virus, and added to cells in a titration of serial two-fold dilutions. After overnight incubation, 50ul of a 4×10^6 PFU/ml stock of encephalomyocarditis virus (EMCV) was added to each well. Twenty-four hours post-infection, cell viability was determined using 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay, and the absorbance was read on a microplate reader at 570nm. The IFN in each sample was compared to an IFN standard (from Chemicon or R&D Systems that has been normalized to the National Institutes of Health reference IFN) present in each plate and converted to international units (IU/ml).

Quantitative real-time PCR analysis: L929 cells were seeded in 6-well dishes and either mock infected or infected with RRV wild type and A532V RRV mutant viruses at an MOI of 5. At the indicated time points, total RNA was isolated using the Trizol (Invitrogen) and the PureLink RNA Mini Kit protocol (Invitrogen). Total RNA was reverse transcribed using random primers and SuperScript III Reverse Transcriptase Kit (Invitrogen). cDNAs were then probed for IFN-beta and 18S rRNA message using TaqMan real-time PCR with primer probe sets (Applied Biosystems) and analyzed on the Prism 7000 machine (Applied Biosystems).

Analysis of Protein Synthesis: L929, BHK-21, or Neuro2A cells were either mock infected or infected with the Sindbis AR86 or RRV viruses at an MOI of 50 and 10 (BHK and N2A). At various times post-infection, the media was removed and cells were starved with Minimum Essential Eagle Medium with Earle's salts lacking methionine and cysteine (MP biomedical) for 1 hour at 37°C. The starvation media was then replaced with media supplemented with 33μCi of ³⁵S-methionine and ³⁵S-cysteine (Amersham Pro-mix) and incubated at 37°C for one hour. Following each labeling period, cells were lysed in NP-40 Lysis buffer containing protease inhibitors (Roche). Equal cell lysates were analyzed on a 10% sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE). Gels were fixed in buffer containing 10% acetic acid and 40% methanol. The gels were then dried, exposed to a phosphoimaging screen, and scanned using a Storm Phosphoimager (GE Healthcare). β-actin bands were quantified using ImageQuant software (GE Healthcare), and samples are represented as comparison to mock infected cells.

Analysis of Host RNA Synthesis: L929, BHK, or Neuro2A cells were either mock infected or infected with the Sindbis AR86 or the RRV viruses at an MOI of 50 and 10 (BHK and N2A). After 1 hour incubation, 2ml of media were added back to cells. At various times post-infection (2, 5, 8, and 16hpi), the media were replaced with media supplemented with 20uCi/ml [3H]-uridine (in the presence or absence of actinomycin D (1ug/ml)). The cells were labeled for a total of 3 hours, then washed with 1XPBS and lysed in Trizol (Invitrogen). RNA was extracted and equal volumes were denatured in glyoxal and dimethyl sulfoxide (DMSO) for 1 hour at 50°C. The

RNA was analyzed on a 0.8% agarose NaPO₄ gel. The agarose gel was washed twice in methanol followed by overnight incubation in 2.5% 2, 5-diphenyloxazole (PPO) in methanol. The gel was washed three times in water to precipitate the PPO, dried, and exposed to film.

SDS-PAGE and Western Blot Analysis: Protein extracts were resolved on 10% SDS-PAGE followed by transfer to a PVDF membrane. Anti-IRF-3 C-20 antibody (Santa Cruz) was used for detection of phosphorylated IRF-3 in L929 cells. For re-probing of the membranes, anti- β -actin (Sigma) antibodies were used. Membranes were washed and incubated in the appropriate anti-rabbit and anti-goat secondary antibodies.

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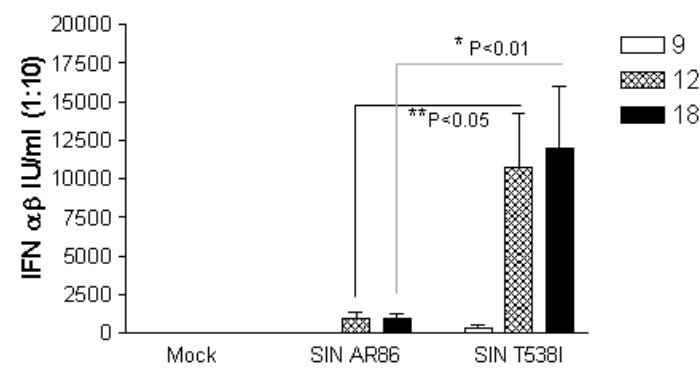
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FIGURE 2.1

A.



B.

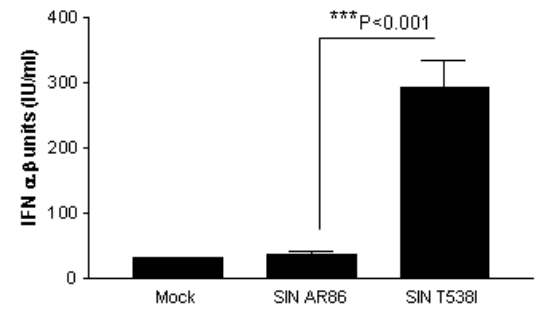


Figure 2.1. The mutant SIN T538I virus induces more type I interferon. A)

Groups of six-week old (n=3) CD-1 mice were infected with diluent alone, wild type Sindbis AR86, or Sindbis T538I mutant at 1×10^3 pfu via the intracranial (i.c.) route. Serum was harvested at the indicated time points (hours) and diluted (1:10) into media. B) L929 cells were either mock infected or infected with wild type AR86 or T538I mutant at an MOI of 5.0 and supernatants were harvested at 18 hours post-infection. Serum and supernatants were subjected to an interferon bioassay on L929 cells. Each bar represents the average of triplicate samples and the p values were determined by ANOVA statistical analysis. Error bars represent the standard error of the mean. The limit of detection in each bioassay was 31IU/ml.

FIGURE 2.2

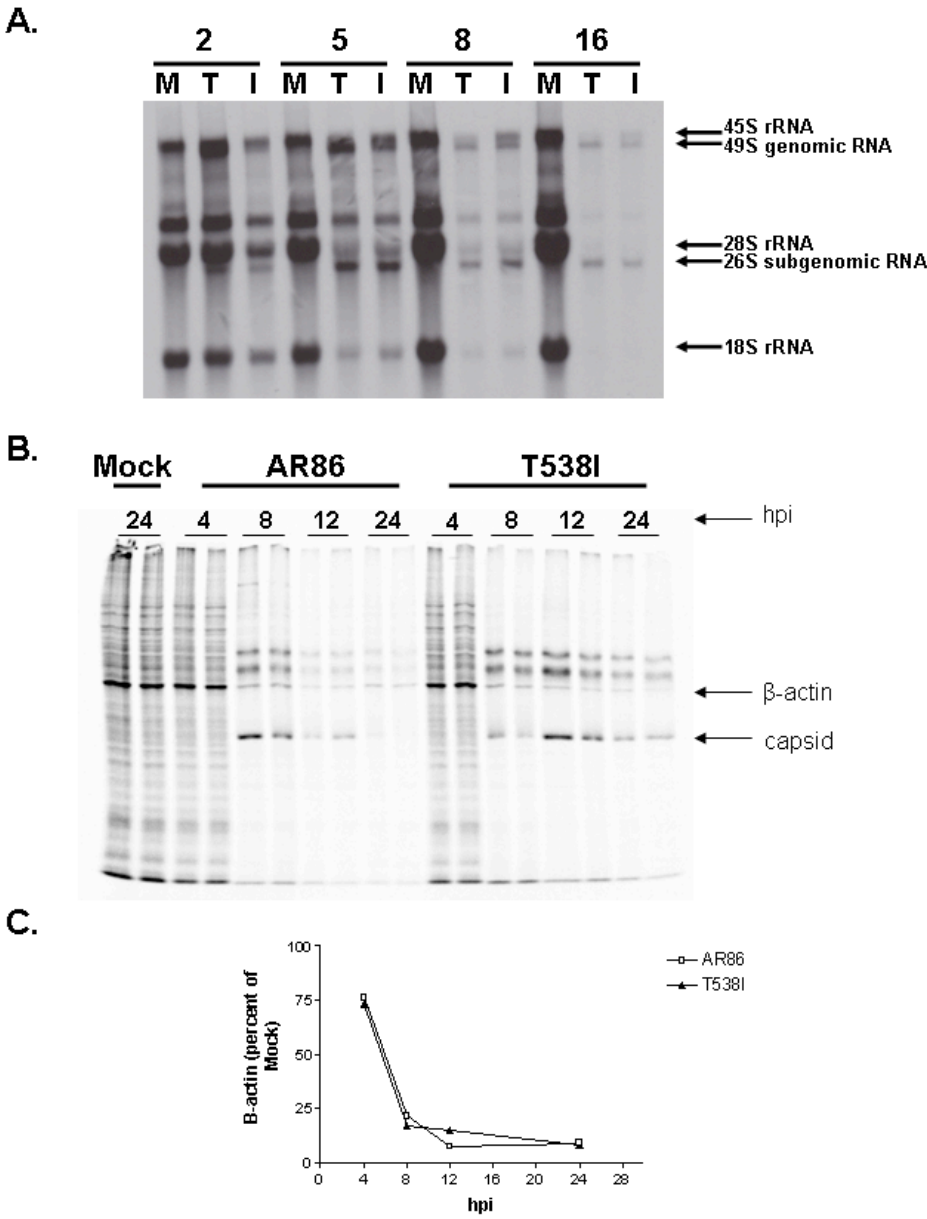
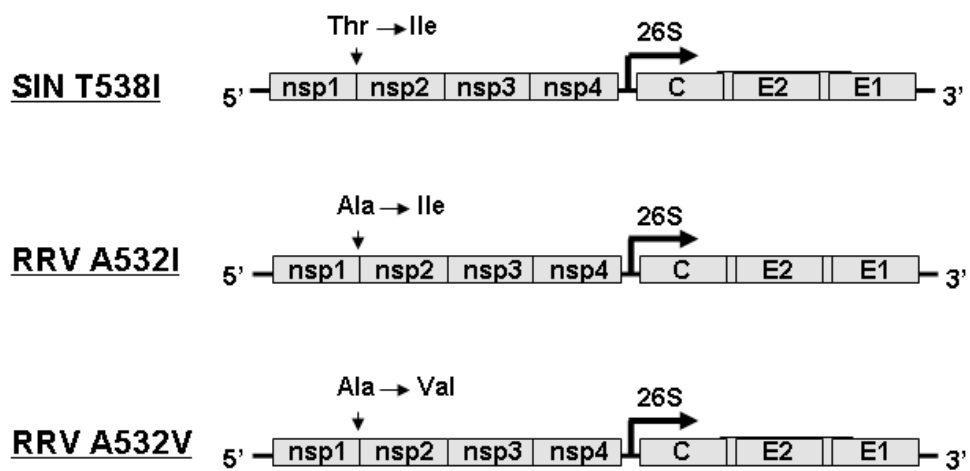


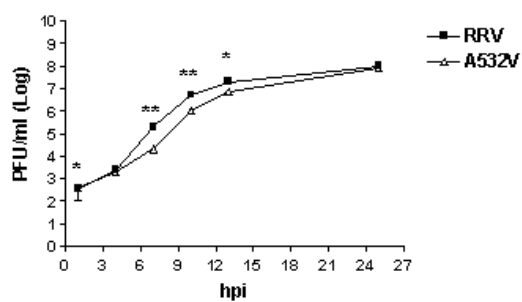
Figure 2.2: The Sindbis T538I mutant virus shuts off host RNA transcription and protein translation with similar kinetics to the wild type AR86. L929 cells were mock infected (M) or infected with wild type Sindbis AR86 (T) or mutant T538I (I) at an MOI of 50. A) To analyze host RNA synthesis, at various times post-infection (2, 5, 8, and 16 hours), the media was replaced with media containing 20 μ Ci/ml of 3H-Uridine and cells were labeled for a total of 3 hours. Total RNA was harvested and analyzed by agarose gel electrophoresis as previously described. B) To analyze host protein synthesis, L929 cells were labeled with 35S Met/Cys for 1 hour at the hours indicated and cell lysates (in duplicates) were analyzed by SDS-PAGE. C) Residual host cell protein synthesis in figure 2.2B was evaluated by measuring the amount of radioactivity detected in the protein band corresponding to actin (as marked by the arrow) and normalized to the amount of radioactivity detected in the same protein band in mock infected cells (AR86-□, T538I-▲). The data shown are representative of two independent experiments.

FIGURE 2.3

A.



B.



C.

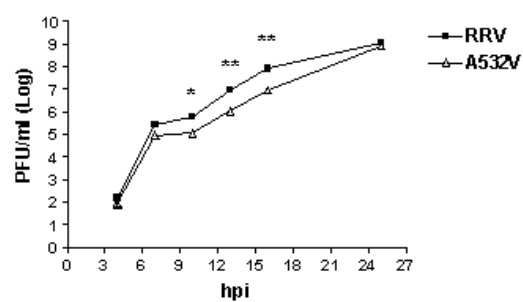


Figure 2.3. Characterizations of the RRV A532V mutant virus. A) Schematic diagram of the single amino acid substitutions in the Sindbis AR86 (T538I) and Ross River Virus (A532I and A532V) mutants. B) Single step growth curve. BHK cells were infected at an MOI of 5 with either RRV (■) or A532V (Δ) viruses. Supernatants at 1, 4, 7, 10, 13, and 25hpi were analyzed by plaque assay. C) Multi-step growth curve. BHK-21 cells were infected at an MOI of 0.01 with either RRV (■) or A532V (Δ) viruses. Supernatants at 4, 7, 10, 13, 16, and 25hpi were analyzed by plaque assays. Each data point represents the average of triplicate samples and significance was determined by a 2-factor ANOVA statistical analysis (* $p < 0.05$, ** $p < 0.01$). Error bars represent the standard error of the mean.

FIGURE 2.4

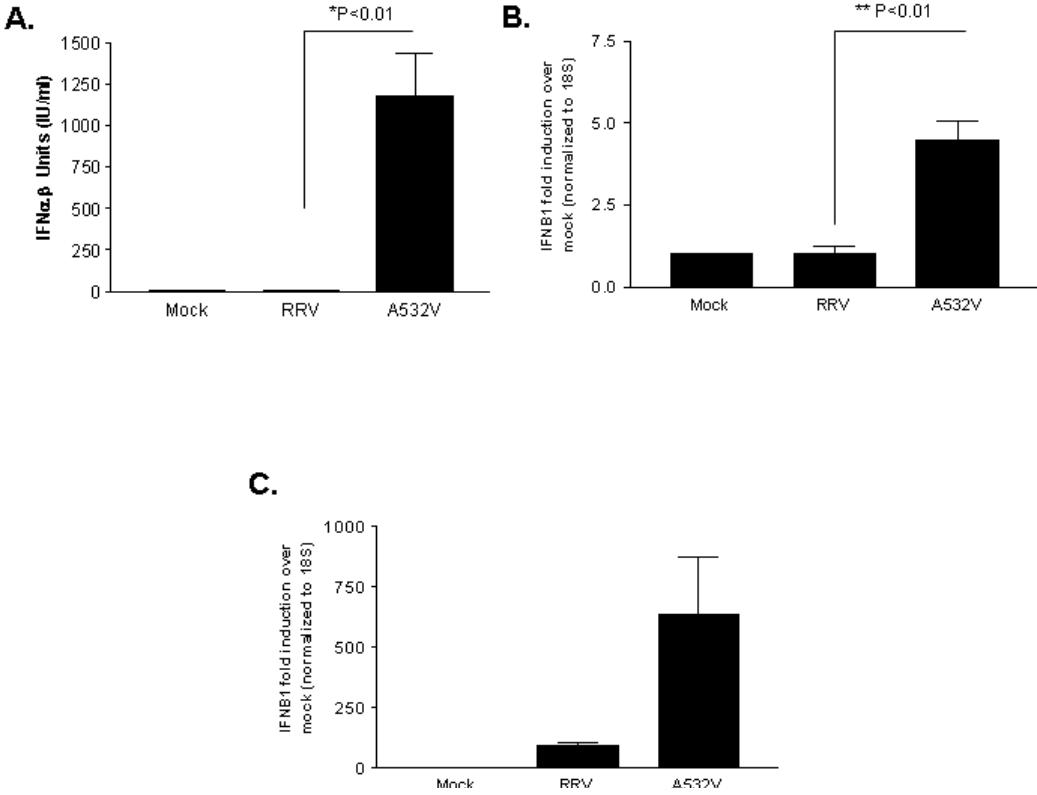


Figure 2.4. The RRV A532V mutant virus induces more type I IFN than the wild type. L929 cells were infected with RRV and A532V viruses at an MOI of 5. A) Type I IFN bioassays were performed on the supernatants harvested at 24 hpi. The limit of detection for the bioassay is 7 IU/ml. B) and C) Total RNA was extracted at 6 hpi (B) or 12 hpi (C) and analyzed by quantitative real time PCR (Applied Biosystems) for IFN-beta message transcripts. The data are represented as the fold induction over Mock infected cells and have been normalized to 18S rRNA. Each bar above represents the average of triplicate samples and the p values were determined by ANOVA statistical analysis. Error bars represent the standard error of the mean.

FIGURE 2.5

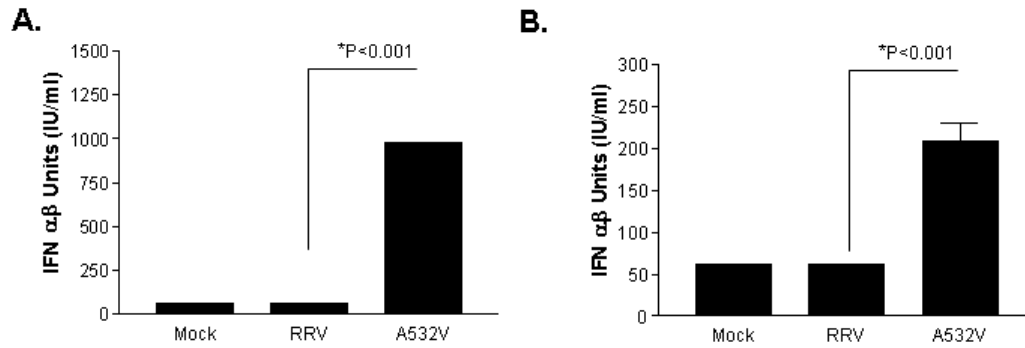


Figure 2.5. The RRV mutant virus induces more IFN than the wild type RRV in the absence of the Type I IFN $\alpha\beta$ Receptor. A) Sv/129 MEFS and B) Sv/129 IFNR^{-/-} MEFS were infected with RRV and A532V viruses at an MOI of 5. Type I IFN bioassays were performed on the supernatants harvested at 24 hpi. Each bar represents the average of triplicate samples and the p values were determined by ANOVA statistical analysis. Error bars represent the standard error of the mean and the limit of detection for each bioassay is 61 IU/ml).

FIGURE 2.6

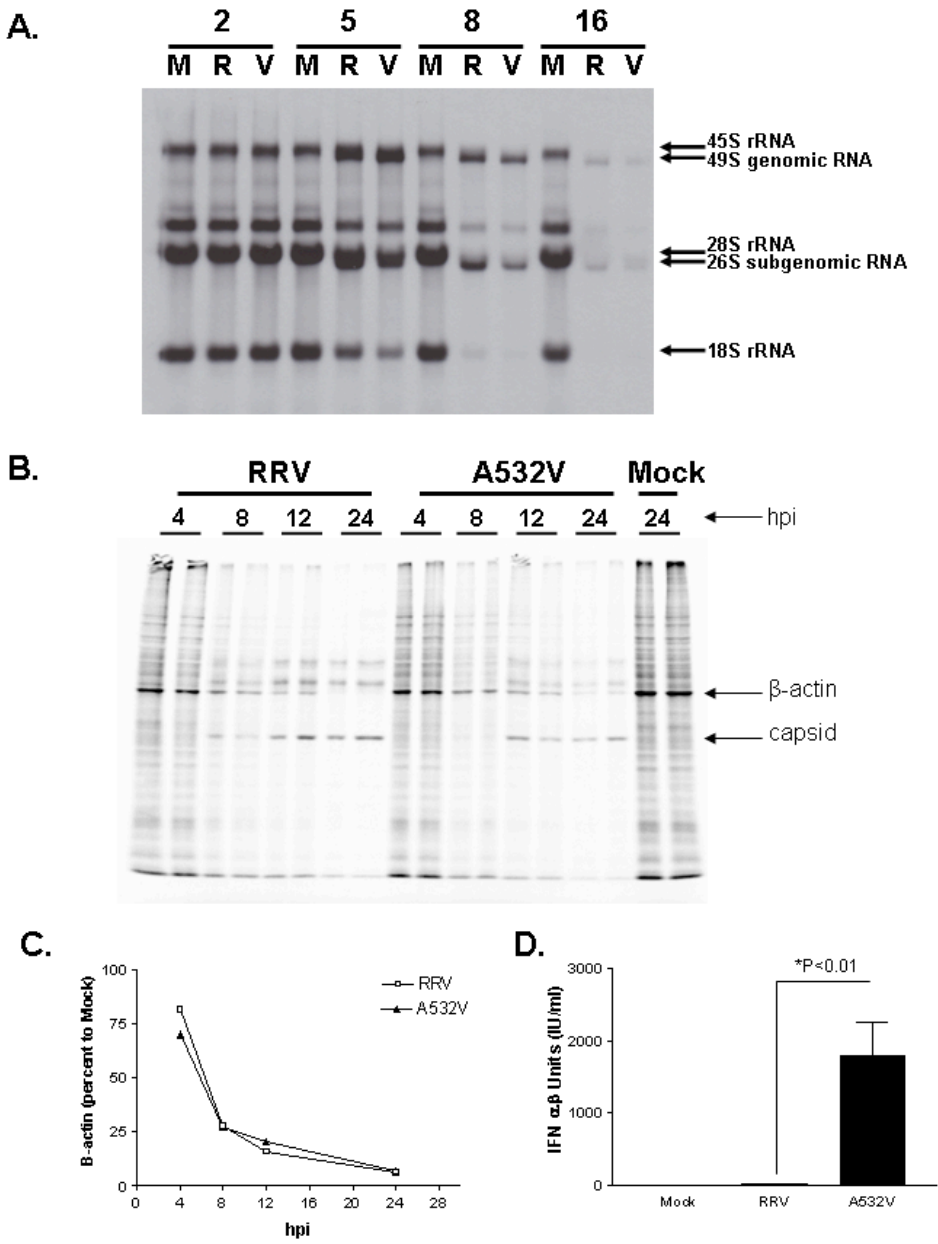


Figure 2.6. Characterization of virus-induced shutoff by the RRV A532V

mutant. L929 cells were either mock infected (M) or infected at an MOI of 50 with RRV (R) or A532V (V). A) At various times post-infection (2, 5, 8, and 16 hrs), cells were labeled with 20 μ Ci/ml of 3H-Uridine (as previously described) for analysis of host RNA synthesis. B) L929 cells were labeled with 35S Met/Cys at the indicated times post-infection for 1 hour to monitor host protein synthesis. Total cell lysates (duplicates) were analyzed by SDS-PAGE as described in the materials and methods. C) The kinetics of cellular β -actin protein synthesis was evaluated by measuring the amount of radioactivity detected by densitometry in the β -actin protein band in the infected cells and normalized to the mock infected cells at 24hpi. D) L929 cells were either mock infected or infected with RRV or A532V mutant viruses at an MOI (50). Supernatants at 24hpi were analyzed for IFN by bioassay as previously described. The limit of detection in this assay is 2 IU/ml and each bar represents the average of triplicate samples. The p-values were determined by ANOVA statistical analysis and error bars represent the standard error of the mean

FIGURE 2.7

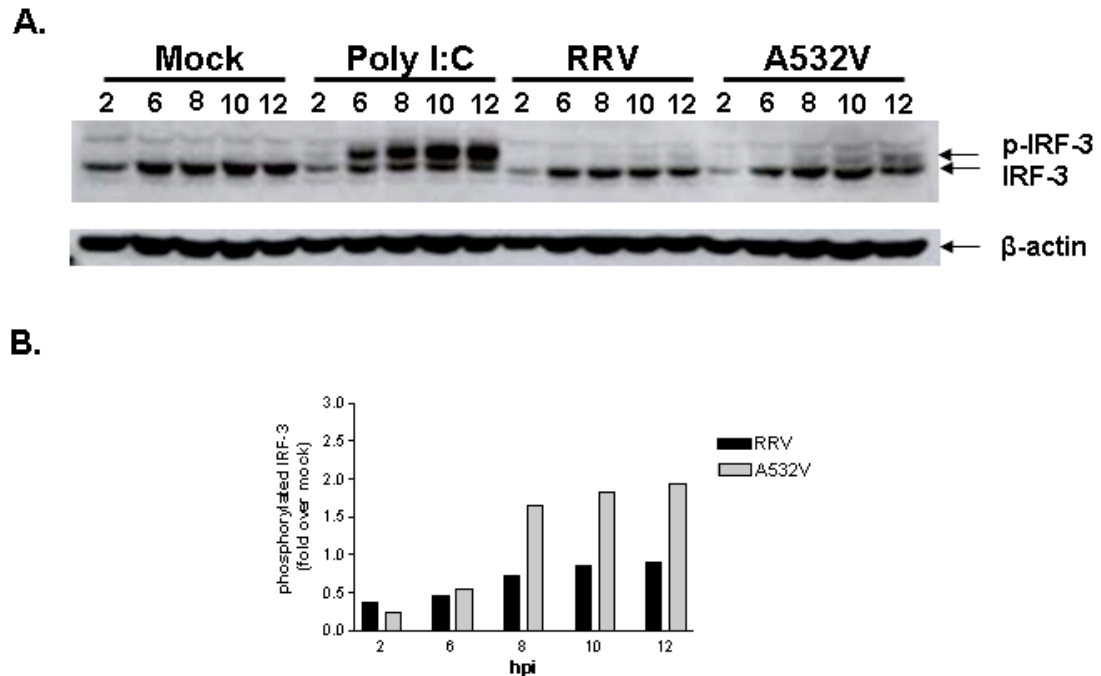


Figure 2.7. The RRV A532V mutant robustly induces IRF-3 phosphorylation

compared to wild type virus. A) L929 cells were either mock infected, infected with RRV and A532V viruses at an MOI of 5, or transfected with 1ug of poly I:C with Lipofectamine 2000 (Invitrogen). Cells were lysed at the indicated hours in NP40 lysis buffers containing protease and phosphatase inhibitors. 20ug of total protein was analyzed by SDS-PAGE and probed with an anti-IRF-3 antibody (Santa Cruz, C-20) for phosphorylated murine IRF-3 (p-IRF-3). The membranes were then re-probed with an anti- β -actin antibody (Sigma). B) The phosphorylated IRF-3 bands in (A) were quantified using ImageQuant 5.0 and represented as fold over mock. The data shown are representative of three independent experiments.

CHAPTER THREE

A ROSS RIVER VIRUS NSP1 MUTATION LEADS TO ENHANCED SYNTHESIS OF UNCAPPED 26S RNAs TO INDUCE TYPE I IFN.

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ABSTRACT

The Type I Interferons are potent mediators of the innate immunity that play an important role to restrict alphavirus pathogenesis *in vivo*. It is well known that pattern recognition receptors, such as the RIG-I-Like RNA helicases, initiate early and rapid IFN induction to clear various virus infections by directly shaping the downstream adaptive immunity. The exact mechanism(s) of IFN induction in response to alphavirus infections, however, have not been fully characterized. In this report, we demonstrate that a determinant located within the conserved nsP1/nsP2 cleavage site of Ross River Virus (RRV) leads to an overall increase in viral RNA synthesis, thus driving excess IFN induction independently of host macromolecular shutoff. We further demonstrate in various deficient embryonic fibroblast cells that the RRV mutant drives excess IFN induction through IPS-1 signaling and is dependent upon RIG-I and PKR, but not MDA-5. Furthermore, we are able to identify that the RRV mutant synthesizes uncapped 26S RNAs, exposing 5'-triphosphates that induce robust IRF-3 and RIG-I activation. Therefore, our results demonstrate that a novel mutation within the RRV nonstructural proteins may interfere with the viral capping apparatus, thus generating increased amounts of uncapped 26S RNAs to mediate IFN induction through a RIG-I and/or PKR dependent pathway.

INTRODUCTION

The Type I Interferon (IFN) system is a highly coordinated component of the host innate immunity, acting early in viral infection to prevent replication and spread. The antiviral strategies employed include activation of IFN stimulated genes (ISGs), such as the protein kinase R (PKR) or the 2'-5'-oligoadenylate synthetases (OASs)/RNase L, to act directly on limiting virus replication or to the production of pro-inflammatory cytokines and chemokines that selectively direct the downstream adaptive response (42, 51). Type I IFN induction is initiated by host sensing of various pattern-associated molecular patterns (PAMPs), such as double-stranded RNA (dsRNA) and single-stranded (ssRNA), by pattern recognition receptors (PRRs) during viral infection (2, 43, 54). Therefore, an important emphasis has been placed on identifying the host sensors as well as the activating viral ligands to further our understanding to combat these viruses. The known PRRs to recognize viral infections include the Toll-Like Receptors (TLRs), the RIG-I (retinoic acid-inducible gene I)-Like RNA Helicases (RLHs), as well as the PKR and 2'-5'-OAS/RNase L proteins. The TLRs are transmembrane receptors that act at the cell surface or within endosomal compartments, recognizing various PAMPs such as lipopolysaccharides, CpG DNA, and viral RNA substrates to induce IFN and pro-inflammatory cytokines (26). TLR3 is the only known TLR to not utilize the MyD88 co-adaptor, instead using the TIR domain-containing adaptor molecule 1 (Ticam-1, also known as TRIF) to induce IFN in response to dsRNA (59). The RIG-I-Like RNA helicases, on the other hand, are cytosolic sensors that recognize both dsRNA and ssRNA (25, 39). RIG-I and MDA-5 (melanoma differentiation-associated antigen-5)

RNA helicases have been extensively studied and plays a direct role in IFN induction to several RNA viruses, such as Flaviviruses (9), Picornaviruses (16), and Influenza viruses (39). The specific viral ligands to activate the RLHs, however, are less clear.

RIG-I and MDA-5 are DExD/H box RNA helicases that encode two N-terminal Caspase Recruitment Domains (CARDS) and a C-terminal helicase/ATPase domain (60). Upon activation, RIG-I and MDA-5 interact with the CARD domains of the IFN-promoter stimulator 1 (IPS-1) (also known as MAVS/VISA/Cardif) present on the mitochondrial membranes (27, 31, 47, 57). The CARD-CARD interactions then lead to recruitment of other signaling factors for the activation of the downstream TBK1/IKK- ϵ and IKK kinases to induce IRF-3 and NF- κ B mediated IFN induction (24). RIG-I and MDA-5 can discriminate between different RNA ligands, thus diversifying the host's response to various different viruses. MDA-5 primarily recognizes long dsRNA segments as well as ssRNAs that form large secondary structures (25, 38). The natural ligand for RIG-I is still being debated. Recent reports have identified the presence of a 5'-triphosphate moiety on ssRNA and dsRNAs to activate RIG-I (23, 37, 39). Free 5'-triphosphates within the cytosol of a cell are generally made by virus replication. Therefore, many viruses encode different mechanisms to shield the 5'-triphosphates from the host sensors. Some examples are; expression of a viral antagonist, such as influenza NS1, to sequester the RNA from RIG-I (39) or to block RIG-I activation (12), removal of the 5'-triphosphate, as found in Borna disease virus, to avoid RIG-I detection (19), or through the covalent attachment of a viral peptide, such as Picornavirus VPg, that

prevents RIG-I recognition (59). Another mechanism is through the simple addition of a 7-methyl-guanosine cap, like those found on host mRNAs (23). Viral RNA capping, thus serves as a dual function to protect RNAs from RIG-I sensing as well as to facilitate translation of viral proteins. Although, the requirement of free 5'-triphosphates to activate RIG-I have been found in studies utilizing *in vitro* transcribed RNAs, as well as viral RNAs directly isolated from infected cells, other synthetic RNAs without 5'-triphosphates have also been found to activate RIG-I. These studies include short dsRNA (poly I:C), 3'-monophosphates generated by RNase L RNA cleavage, 5'-monophosphates, as well as poly U/A tracts found in the 3'-UTR of several flaviviruses (25, 30, 44, 53, 55). In addition to the RIG-I-Like helicases, the PKR and 2'-5'-OAS/RNaseL ISGs also reside in the cytosol, recognizing dsRNA to mediate an antiviral signaling cascade. Several studies have reported additional roles to induce Type I IFN. PKR primarily recognizes dsRNA, although reports have suggested that it can recognize 5'-triphosphates on ssRNAs (34). PKR can induce Type I IFN to several viruses, including West Nile Virus (15) and Vaccinia virus (62). Although, the exact IFN induction pathways have not been clearly defined, it has been reported that PKR can signal through the IPS-1 pathway, thus connecting PKR to the RLRs (62). Furthermore, upon activation, RNase L cleaves ssRNA and dsRNA that feedback into the RIG-I IFN induction pathway, thus amplifying the IFN response (30). Altogether, the host employs numerous PRRs, with distinct RNA specificities, that ultimately converge to robustly induce IFN to combat the wide range of viruses it encounters.

Alphaviruses are single stranded positive sense RNA viruses that encode an 11-12Kb genome. The RNA genomes encode a 5' cap structure and a Poly-adenylate tail, thus mimicking host mRNAs for translation of its viral proteins (50). The genome consists of two open reading frames that encode for the nonstructural proteins as well as the structural proteins. The structural proteins are further encoded under an internal 26S subgenomic promoter. The nonstructural proteins make up the replication complex of this virus. NsP1 is a methyltransferase that displays guanylyl-transferase activities to mediate viral RNA capping (1). NsP2 is a multi-functional protein serving as the viral protease to mediate cleavage of the nonstructural polyprotein (28). In addition to its role as a protease, NsP2 is also a 5' triphosphatase that removes the 5' γ -phosphate from newly synthesized RNAs before the NsP1 protein attaches a 7-methyl-guanine cap (56). NsP3 is a phosphoprotein, however, its function has not been clearly defined (7). And nsP4 is the viral RNA-dependent-RNA polymerase (40). Upon infection, the viral genome is translated to generate the nonstructural polyprotein, NsP1234, where nsP4 is immediately cleaved from the polyprotein by the Nsp2 protease. Then, the NsP123 polyprotein forms a complex with nsP4 to mediate minus strand RNA synthesis (48). After accumulation of minus sense RNA, the NsP123 polyprotein is further processed to the mature nsP1, nsP2, and nsP3 proteins (29). The fully processed nsPs bind to the minus strand RNAs, switching to synthesis of the full length plus sense RNAs. The mature nsPs also bind to the internal 26S subgenomic promoter to mediate the synthesis of the viral structural proteins. The structural proteins then

encapsidate the full-length plus sense RNAs, forming new virion particles that are released from the cell.

Alphaviruses are potent type I IFN inducers (11). The mechanisms of IFN induction or the IFN pathways that mediate this response, however, are unclear. It has been previously reported that a generalized mechanism of virus mediated host macromolecular shutoff counteracts the IFN responses to both Sindbis and Venezuelan Equine Encephalitis Virus (11, 14). These studies demonstrate that alphavirus mutants that are defective for host macromolecular shutoff, are also robust IFN inducers. However, recent studies suggest that in addition to host shutoff, there may be specific viral antagonists that act early to delay IFN induction until virus mediated shutoff is firmly established (4, 6, 49). The efforts to identify these early IFN interactions are hampered though, due to how little is relatively known about the pattern recognition pathways that are mediating these responses. Recent studies with Sindbis and chikungunya viruses have demonstrated IFN induction is mediated through an IPS-1 dependent pathway, although the upstream RNA helicases seem to display different virus specificities. Sindbis IFN induction seems dependent upon MDA-5 and PKR signaling, but not RIG-I (5). Chikungunya, on the other hand, signals through both RIG-I and MDA-5 (45). The RNA sensor differences may be due directly to the virus strain used, thus complicating efforts to characterize a model for alphavirus sensors and the viral ligands that mediate these responses.

We have previously identified a virulence determinant within the neurovirulent Sindbis AR86 virus (20, 52). The determinant is located within the conserved

nsP1/2 cleavage domain, affecting both processing of the nonstructural polyprotein as well as disregulating viral RNA synthesis (21). The determinant is not specific to the Sindbis viruses as a similar mutation within another alphavirus, Ross River Virus, also leads to a mutant that displays increased RNA synthesis and induces IFN independently of virus mediated shutoff of host macromolecular syntheses (6). In this report, we analyzed the Pattern Recognition Receptor pathways that are involved in recognizing the RRV A532V mutant virus. Thus far, we have identified that the IPS-1 signaling pathway is crucial for IFN induction as mouse embryonic fibroblast cells (MEFS) that are deficient for the IPS-1 molecule fail to induce IFN by both viruses. Analysis of MEFS that are deficient for either the MDA-5 or RIG-I RNA helicases, or PKR, demonstrated that the majority of the IFN induction by the mutant virus is dependent upon both RIG-I and PKR, but independent of MDA-5. Analysis of potential IFN inducing ligands demonstrated that the mutant viruses exhibited increased viral RNA synthesis leading to greater quantities of full-length genomic and subgenomic 26S RNAs within an infected cell, suggesting that disregulated viral RNA synthesis is at least partly responsible for this mutation's effect on type I IFN induction. We have further determined that the viral 26S subgenomic RNAs from infected cells can activate IRF-3 and RIG-I and that activation was dependent upon phosphatase treatment. Based upon these results, we found that the 26S RNAs made within the RRV mutant A532V are not efficiently capped, thereby exposing 5'-triphosphates that lead to RIG-I activation. Altogether, these results link a novel mechanism of IFN induction directly to a disruption of the RNA synthetic processes

by making 26S uncapped RNAs, which in turns drives excess IFN induction through the RIG-I and PKR sensors.

RESULTS

The RRV A532V mutant induces IFN via IPS-1 dependent signaling, but independently of TICAM-1.

Previous studies identified a mutation at the P3 position of the nsP1/nsP2 cleavage domain that affects type I IFN induction by both Sindbis virus and Ross River virus (RRV) (6). In the case of RRV, a virus where the P3 Alanine was changed to Valine (A532V) induced up to 100 fold higher levels of type I IFN than the wild type RRV. This effect was independent of virus induced host macromolecular shutoff and also acted at the level of type I IFN induction, since the mutant virus exhibited enhanced IFN induction in type I IFN receptor deficient cells that are incapable of amplifying type I IFN induction through IFN receptor signaling (6). Therefore, in order to more fully understand the mechanism by which the A532V mutation affects type I IFN induction, we set out to further define both the host pathways that recognize the mutant virus and the viral ligand(s) responsible for the enhanced IFN induction, with the goal of determining whether the mutant was simply a better inducer of the same pathways that recognize the wild type virus or was activating a completely different viral sensor than the wild type virus.

Several alphaviruses, including Sindbis virus (5, 16), Semliki Forest virus (3), and Chikungunya virus (45) have been shown to induce type I IFN in fibroblasts via the intracellular RNA sensors, RIG-I and MDA-5, that signal through the adaptor

molecule IPS-1. Therefore, IPS-1 deficient MEFs were tested for their ability to respond to the RRV A532V-gfp mutant. Wild type and IPS-1 deficient MEFS were infected with the wild type RRV-gfp (figure 3.1A) and A532V-gfp (figure 3.1B) mutant viruses and type I IFN induction assessed. As indicated in figure 3.1C, the mutant RRV A532V-gfp virus induced greater than ~50 fold IFN in comparison to RRV-gfp. However, no IFN induction is observed in the IPS-1 deficient MEFS when infected by either virus. These studies suggest that IPS-1 is essential for induction by both the wild type virus, as well as the excess IFN induction by the A532V mutant. To further confirm the bioassay results, we analyzed IFN- β mRNA levels following infection of wild type versus IPS-1 deficient MEFs with either virus. While the mutant virus induced more IFN- β mRNA at 6 hours post-infection than the wild type virus in C57Bl/6 MEFS, neither virus elicited IFN- β mRNA in the IPS-1 deficient MEFS (figure 3.1D). In contrast to IPS-1's essential role in mediating type I IFN induction by both the wild type and mutant viruses, we observed no role for the adaptor molecule TRIF/Ticam-1 (22) in this process (figure 3.2), suggesting that neither virus induces type I IFN through TLR3 in murine fibroblasts.

MDA-5 and RIG-I contribute to the recognition of wild type RRV, but the excess type I IFN induction by the A532V mutant is RIG-I dependent and MDA-5 independent.

Given the essential role that IPS-1 plays in baseline type I IFN induction by the wild type RRV and the excess type I IFN induction by the mutant virus, we next assessed relative contribution of RIG-I and MDA-5 in recognizing both the wild type

and mutant viruses. RIG-I or MDA-5 deficient MEFS (along with their wild type controls) were infected with the RRV-gfp and RRV A532V-gfp mutant viruses. As demonstrated in figure 3.3A, MDA-5 contributes to the IFN induction by wild type RRV, as the overall IFN induction by RRV-gfp in MDA-5 deficient MEFs was significantly lower than that observed in wild type MEFS. However, the MDA-5 deficient MEFS still retained the ability to respond to the A532V mutant as measured by bioassay, indicating that while MDA-5 does contribute to the overall recognition of RRV, it is not required for the excess type I IFN induction by the A532V mutant. Therefore, we analyzed the IFN induction profile by both RRV-gfp and A532V-gfp viruses in RIG-I deficient MEFS. As shown in figure 3.3B, RIG-I also contributes to type I IFN induction against the wild type virus, suggesting that both RIG-I and MDA-5 contribute to viral recognition and type I IFN induction by the wild type RRV. In contrast to the MDA-5 deficient cells, the A532V mutant was a poor inducer of type I IFN in RIG-I deficient cells, suggesting that RIG-I plays a major role in driving IFN induction in response to the A532V mutant. However, while IPS-1 deficiency completely ablated type I IFN induction by both the wild type and mutant viruses, the mutant virus still elicited higher levels of type I IFN than the wild type virus in RIG-I deficient cells, suggesting that either MDA-5 might contribute to the residual excess type I IFN induction by the mutant virus in the absence of RIG-I, or another sensor was contributing to the type I IFN induction by the A532V mutant.

PKR also contributes to RRV A532V IFN induction.

Though RIG-I and MDA-5 are thought to be the major RNA sensors that signal via the IPS-1 adaptor, recent work has shown that PKR can also contribute to type I IFN responses against both alphaviruses (5, 41) and flaviviruses (15), and PKR dependent type I IFN induction is also IPS-1 dependent (62). Furthermore, RNase L has been shown to enhance RIG-I dependent type I IFN induction by degradation of viral and host RNAs to produce more RIG-I ligand (30). Therefore we initially tested whether MEFs that were doubly deficient in PKR and RNase L (63) exhibited any defect in their response to either wild type RRV or the A532V mutant. As demonstrated in figure 3.4A, the excess IFN induction observed in the wild type MEFs by the A532V mutant is dependent upon PKR and/or RNase L. To determine whether this effect reflected a role for either PKR or RNase L, or a role for both proteins, we assessed IFN induction following infection of PKR or RNase L single knockout MEFs with either the wild type or A532V mutant RRVs. As demonstrated in figure 3.4B, PKR, but not RNase L, contributed to the IFN induction by the A532V mutant, since the A532V mutant continued to elicit high levels of type I IFN in RNase L deficient MEFs, but reduced type I IFN induction compared to wild type virus in PKR deficient cells. Importantly, similar to our results with RIG-I deficient MEFs, though PKR deficiency resulted in a significant reduction in type I IFN induction by the mutant virus, the A532V-gfp virus still induced more type I IFN than the wild type virus in the PKR deficient cells. These results suggest that both PKR and RIG-I contribute to the high level of type I IFN induction by the A532V mutant, and that these sensor molecules may synergize to drive high levels of type I IFN induction by the mutant virus.

The RRV A532V mutant displays increases synthesis of viral RNAs within infected cells.

Both RIG-I and PKR have been shown to recognize free 5' triphosphate groups on cytoplasmic RNAs (23, 34, 37, 46), while RIG-I has been shown to recognize conserved RNA secondary structures (54) and PKR recognizes double stranded RNA (36). Given that a similar mutation at the P3 position of the nsP1/nsP2 cleavage domain of Sindbis virus has been shown to affect the kinetics of viral RNA synthesis, it was possible that the A532V mutant simply dysregulated viral RNA synthesis to produce more ligand and thereby more efficiently activated both RIG-I and PKR. Therefore, to assess the impact of the A532V mutation on viral RNA synthesis, BHK cells were infected with both the wild type RRV and mutant A532V viruses at an MOI of 10.0, which results in equivalent rates of infection by both viruses (data not shown) and total RNA were isolated at the indicated time-points. As shown in figure 3.5A, the RRV mutant displayed equivalent levels of the viral minus strand RNA at early times post-infection compared to the wild type virus. However, at later times post-infection, the A532V mutant produced more minus strand RNA than that of the wild type RRV. This is highlighted in figure 3.5B by densitometry quantification of the viral minus strand RNA bands that have been normalized to β -actin to ensure equivalent RNA loading. Furthermore, similar results were observed when analyzing full length genomic plus strand and the 26S subgenomic RNAs (figures 3.5C and 3.5D). Overall, these results suggest that the nsP1 mutation, like that observed for the Sindbis mutant, does dysregulate viral RNA

synthesis leading to increased production of viral RNAs by the mutant viruses over their wild type counterparts.

26S RNAs isolated from RRV and RRV A532V infected cells differentially activate the IFN- β promoter.

Disregulation of viral RNA synthesis by the A532V mutant resulting in increased production of viral RNA could provide more ligand for RIG-I and PKR, thereby resulting in excess type I IFN induction by the mutant virus. However, given that MDA-5 also contributes to the IFN response against the wild type virus, if increased levels of viral RNA were the sole reason that the mutant virus elicits a more potent IFN response, it would stand to reason that MDA5 would also contribute to the enhanced type I IFN response against the mutant virus. However, since only RIG-I and PKR were required for the enhanced IFN response by the viral mutant, this raised the possibility that in addition to driving increased viral RNA synthesis, the A532V mutation might also lead to modifications of the viral RNA that make it a better ligand specifically for RIG-I and/or MDA-5. Therefore, we sought to determine whether the RNAs from RRV and RRV A532V infected cells differ in their ability to activate the IFN- β promoter. To accomplish this, we utilized a luciferase assay in 293 cells that were transiently transfected with an IFN- β -luciferase reporter. For the RNA isolation, BHK-21 cells were either mock infected or infected with the RRV and RRV A532V mutant viruses at an MOI (10) and total RNAs were harvested 8 hours later. The 8-hour timepoint best represents the time of peak RNA synthesis by the RRV A532V mutant, although we have tested RNAs isolated at 4 and 6 hours post

infection (data not shown). Equal amounts of RNA were then titrated and transfected into the reporter cells. As shown in figure 3.6A, the RNAs from both the RRV and A532V mutant activated the reporter to significantly higher levels than the RNAs from mock-infected cells, which is consistent with previous reports that PRRs do not respond to self RNAs (23). Interestingly, the RNAs from the A532V infected cells are a better activator of the IFN- β -luciferase reporter than those of the wild type RRV and this induction was ablated if the RNA was previously treated with phosphatase (figure 3.6B), which suggests that free 5' triphosphates on the RNA were responsible for this IFN induction. This was also the case if the RNAs were introduced into cells overexpressing RIG-I, (figure 3.6C), suggesting that the RNA from the mutant virus infected cells was a more potent activator of RIG-I. Similar results are also observed at 6 hours post infection, however, at 4 hours post infection, the total RNAs from both wild type RRV and A532V infected cells were equivalent at activating the IFN- β reporter. This is consistent with our Northern Blot analysis (figure 3.5) as enhanced RNA synthesis by the mutant occurs later in infection.

While the above results suggest that RNA from cells infected with the mutant virus led to enhanced type I IFN induction compared to the wild type virus, given that higher levels of viral RNA were present in the preparations from the mutant virus-infected cells, these studies did not directly assess whether RNAs derived from the mutant virus might be better ligands for RIG-I and/or PKR-mediated type I IFN induction. Therefore, we initially tested whether the equivalent amounts of viral genome from the wild type or mutant viruses differed in their ability to elicit type I IFN

responses by extracting viral RNAs from RRV and A532V virion particles. Equal amounts of virion RNA were then titrated and transfected into the reporter cells. As demonstrated in figure 3.7, both RRV and A532V full length genomic RNAs activated the IFN- β -luciferase reporter equivalently, suggesting that the full length positive strand genomes of the wild type and mutant virus did not differ in their ability to induce type I IFN.

Though the results with full length genomic RNA demonstrated no difference in their capacity to activate the type I IFN system, these studies could not rule out a differential ability of the mutant virus 26S RNA or even minus strand RNA to elicit type I IFN induction. Therefore, we sought to directly evaluate the ability of the 26S RNAs from the two viruses to activate the type I IFN system by isolating the 26 RNA from wild type and mutant virus infected cells and testing these RNAs in the luciferase assay. To that end, we employed continuous sucrose gradient centrifugation to separate the full-length genomes from the shorter 26S RNAs. As shown in figures 3.8A and 3.8B, total RNAs were harvested from Mock, RRV, or A532V infected BHK-21 cells at 8 hours post-infection. The isolated RNAs were then fractionated through a 15-30% sucrose gradient as described in the materials and methods. An aliquot from each fraction was analyzed on a RNA gel (figure 3.8B). As indicated, the full-length genomes and 26S RNAs were observed in the RRV and A532V infected cells but not in the Mock infected cells. The full length RNAs for both viruses were present in fractions 6-8 while the 26S RNAs were present in fractions 12-14. Most importantly, fractions 12-14 did appear to include carryover of full-length genomes. Therefore, fractions 12-14 from each infected cell

preparation were pooled to concentrate the amount of 26S RNAs. The 26S RNAs were then treated with CIP enzyme or CIP buffer only and equal amounts were transfected into L929 cells for analysis of IRF-3 phosphorylation. As demonstrated in figure 3.8C, the isolated 26S RNAs from the A532V infected cells robustly induced IRF-3 phosphorylation compared to equivalent amounts of RNA from the wild type virus. Furthermore, IRF-3 activation was dependent upon CIP treatment, suggesting that free 5'-triphosphates on uncapped 26S RNA produced by the mutant virus is a better ligand for RIG-I and/or PKR, thereby resulting in enhanced activation of the type I IFN system. This hypothesis was further evaluated by testing the ability of these same RNAs to activate the IFN- β luciferase reporter. As shown in figure 3.8D, the A532V 26S RNA was a better inducer of RIG-I mediated induction of the IFN- β -luc reporter than the 26S RNAs of the wild type RRV.

To further demonstrate that the A532V mutation produces uncapped 26S RNAs within infected cells, we analyzed whether an antibody to the 5'-cap structure (anti-TMG from EMD) would immunoprecipitate the A532V 26S RNA. As demonstrated in figure 3.9, both the wild type RRV and A532V 26S RNAs are readily detectable before incubation with the anti-TMG coated beads. However, only the wild type RRV 26S RNA was detected in the anti-TMG bound fractions. Both mock and A532V 26S RNAs did not bind to the beads, supporting our results that the A532V 26S RNAs are uncapped. Also, to help rule out any non-specific RNA binding, a negative control was employed. The negative control consist of *in vitro* transcribed 26S RNAs that do not contain a 5'-cap structure. As seen in figure 3.9, the negative control did not bind to the beads, suggesting that antibody binding to

the 5'-cap structure is specific. We further demonstrated that the presence of uncapped 26S RNAs also leads to lower protein translation. We measured GFP expression levels using RRV replicon particles. The replicon particles encode a single GFP gene behind the 26S promoter (supplemental figure 3.1A). Therefore, L929 cells were infected with RRV replicon particles encoding either the wild type Alanine or Valine mutation at an MOI (5). At 12 hours post-infection, all cells were analyzed for GFP expression by Flow cytometry analysis. As demonstrated in the supplemental data (supplemental figures 3.1B and 3.1C), equal numbers of cells were infected by the wild type RRV and A532V replicon particles, however, the mean fluorescent intensity was lower in cells infected with the A532V replicon. Furthermore, similar to the results seen with RRV A532V viruses in our previous bioassays, the A532V replicon particles also induced more IFN production in comparison to the wild type (supplemental figure 3.1D). Altogether, our data provide strong evidence that the A532V mutant affects type I IFN induction through effects on the viral capping apparatus, thereby resulting in the production of more uncapped 26S RNA which serves as a better ligand for RIG-I and/or PKR.

DISCUSSION

Alphavirus interactions with the type I IFN system play a major role in regulating viral pathogenesis and understanding these interactions is essential for elucidating how these important pathogens evade the host response, establish infection, and cause disease. Recent studies by several groups have identified key roles for the cytoplasmic RNA sensors RIG-I, MDA-5, and PKR in alphavirus

recognition and initiation of the type I IFN response (3, 5, 16, 45), while additional studies investigating alphavirus evasion of the IFN system have begun to elucidate both nonspecific and specific mechanisms by which these viruses evade both IFN induction and signaling (4, 11, 18, 49, 58). Much of this latter work has focused on viral mutants that disrupt the virus's ability to shut off host macromolecular synthesis, and studies with these viruses have strongly suggested that non-specific antagonism of host cell RNA and protein synthesis plays a key role in limiting type I IFN induction by the virus (11, 13, 18). However, a growing body of evidence suggests that viral determinants that do not affect host cell macromolecular synthesis also regulate type I IFN induction and signaling (4, 6, 49), and relatively little is known about the mechanisms underlying these shutoff independent interactions between alphaviruses and the type I IFN system. The studies presented here investigated the mechanism(s) by which a determinant in the alphavirus nonstructural proteins modulates viral type I IFN induction, both at the level of understanding which host sensors contribute to viral recognition and in identifying the viral ligands that drive these processes. These studies confirm work by other groups demonstrating the three major cytoplasmic viral RNA sensors, RIG-I, MDA-5, and PKR contribute to the recognition of wild type alphaviruses, such as RRV, but that recognition of a mutant virus that induces very strong type I IFN responses is mediated by RIG-I and PKR, but not MDA-5 (figures 3.3 and 3.4). Additional studies strongly suggest that the viral mutation leads to the generation of uncapped viral subgenomic RNA (figure 3.9), thereby leading to the enhanced RIG-I/PKR dependent enhancement of type I IFN production. Not only do these studies further define the mechanisms by which

the host senses alphavirus infection, but they further illustrate how a virulence determinant in the alphavirus nonstructural proteins interacts with the host type I IFN system to limit viral recognition by the host.

The results presented here strongly suggest that the mutation within the nsP1/nsP2 cleavage domain disregulates viral RNA synthesis, resulting in the production of higher levels of all three viral RNA species (figure 3.5). Furthermore, the viral 26S RNA, but not the genomic RNA produced by the mutant virus appears to be better ligand for RIG-I and IRF-3 activation (figures 3.8B and 3.8C). Additionally, IRF-3 activation was dependent upon the presence of a 5'-triphosphate (figure 3.8C). Therefore, we have determined that the RRV A532V mutant, in addition to making more viral RNA species, also makes 26S RNAs that are capped less efficiently than the 26S RNA from the wild type virus (figure 3.9), making the mutant virus-derived 26S RNA a better ligand for RIG-I, and possibly PKR. This is consistent with the known roles of nsP1 and nsP2 in capping of the viral genomic and 26S RNAs through the nsP1 methyl and guanylttransferase activities and the 5' triphosphatase activities of nsP2 (1, 56). Though our results suggest that this effect on capping is specific for the viral 26S RNA, and previous studies have suggested that the 26S cap structure differs from the genomic RNA cap (8, 10), further analysis is needed to determine whether the nsP1 532 mutation specifically affects 26S capping and not the cap status of the genomic RNA.

It has been well established that both RIG-I and PKR can recognize free 5' triphosphates present on ssRNAs and dsRNAs. These are hallmark indicators of viral replicative intermediates as host mRNAs in the cytosol generally contain 7-

methyl-guanosine (7mG) cap or a 5'-monophosphate, and the presence of uncapped RNAs in the cytosol is considered to be a marker of non-self leading to RIG-I and PKR activation (23, 35, 37). However, it is possible that other modifications of the viral RNA also contribute to the excess type I IFN induction by the A532V mutant, the fact that phosphatase treatment ablates the ability of A532V-derived viral RNA to stimulate IRF3 activation strongly suggests that recognition of uncapped viral RNA is the major driver of type I IFN induction by the mutant virus. Though RIG-I and PKR interactions with free 5' triphosphate groups have been extensively studied at the biochemical level using cell free assays and at the cellular level by transfection of uncapped RNA ligands, to our knowledge, this is the first demonstration of a viral mutant where modulation of capping directly affects type I IFN induction. This further supports that idea that cap dependent viruses that replicate in the cytosol need to optimize their RNA capping activity as a means of avoiding the activation of RIG-I and/or PKR in the cell. In fact, a similar mutation at the P3 position of the nsP1/nsP2 cleavage site in Sindbis virus also results in modulation of type I IFN induction (6). In the case of this virus, a virulent Sindbis virus strain, AR86, appears to have acquired a gain of function mutation that promotes viral neurovirulence in adult mice. This virulence determinant, a Threonine in place of the consensus Sindbis virus Isoleucine at the P3 cleavage mutation increases virulence and decreases type I IFN induction. It remains to be determined whether this mutation also affects capping activity and studies are currently underway to address this possibility.

In summary, our findings demonstrate that the RRV viruses induce IFN activation through the RIG-I, MDA-5, and PKR RNA receptors via the IPS-1 signaling pathway. Furthermore, although MDA-5 does contribute to the IFN induction pool mediated by the wild type RRV virus, we demonstrate that the RRV A532V mutant is primarily driving excess IFN induction through the RIG-I and PKR RNA receptors. Additionally, we provide evidence that the RRV A532V mutant directly activates RIG-I through the production of an uncapped 26S RNA, thereby firmly establishing a mechanism in which the RRV A532V mutant drives excess IFN induction. Altogether, we propose a model in which a virulence determinant located within the conserved nsP1/2 cleavage site disregulates RNA synthesis, leading to the increased quantity of viral 26S RNAs that are not efficiently capped, and driving activation of RIG-I and/or PKR IFN responses. These studies further our current understanding of alphavirus pathogenesis and help define the mechanism(s) of IFN induction.

MATERIALS AND METHODS

Viruses, replicons, and cell culture: The wild type RRV (pRR64) and mutant RRV A532V (pRR64-A532V) viruses were generated as previously described (6, 33). A split-helper system was used to generate the RRV replicons (49). Briefly, all plasmids were linearized and used as templates for in vitro transcription of the full length genomic RNAs using mMessage Machine SP6 Kit (Ambion). Transcripts were electroporated into Baby Hamster Kidney cells (BHK-21) cells using a Bio-Rad electroporator. The supernatants were harvested 24 hours post electroporation and clarified through centrifugation for 20 minutes at 3,000RPM. The clarified supernatants were then concentrated through a 20% (w/v) sucrose/phosphate-buffered saline (PBS) cushion at 72,000 $\times g$ by ultracentrifugation (4 h). The pelleted viruses and replicons were resuspended in PBS and 0.1ml aliquots were stored at -80°C. All viruses were tittered by standard plaque assay performed on BHK-21 cells. The titers of the RRV replicons were determined by counting GFP positive cells in BHK-21 cells.

BHK-21 and L929 murine fibroblast cells were maintained in minimum essential medium (MEM) alpha medium (Gibco) supplemented with 10% Tryptose phosphate broth, 10% Donor Calf Serum (DCS), L-glutamine, and penicillin/streptomycin. Human embryonic kidney (HEK 293s) cells were maintained Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% Fetal Bovine Serum (FBS), L-glutamine, and penicillin/streptomycin. The IPS-1^{-/-}, RIG-I^{-/-}, MDA-5^{-/-} primary mouse embryonic fibroblast cells (and their wild type controls) were kindly provided

by Dr. M. Gale (University of Washington). MEFS were maintained in DMEM (Gibco) supplemented with 10% FBS, L-glutamine, penicillin/streptomycin, and non-essential amino acids. All other primary MEFS were generated from ~13-15 day old embryos and maintained in DMEM/F12 media containing 10% FBS, 10% tryptose phosphate broth, L-glutamine, and 50 µg/ml of gentamicin (Gibco). To generate the PKR and RNase L single deficient MEFS, Sv/129 wild type and PKR/RNase L doubly deficient (63) inbred mice were crossed to generate F1 heterozygous mice. The F1 generation was further crossed to generate F2 single deficient mice. All mice were bred in house in accordance with all University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee guidelines. The genotypes of all mice were confirmed by genotype protocols. The PKR primer sets were previously described in (63). The RNase L primers used were: 5'-GGAAGTCAGAGGACCGTTG-3' and 5'-GACGTCAATGAGTGTGACG-3'. The respective sizes are RNase L WT allele: ~319bp, RNase L KO allele: ~1.3kb, RNase L het: 0.3kb and 1.3kb.

Northern Blot analysis: BHK-21 cells seeded in 6 well dishes and either mock-infected or infected with RRV and A532V viruses at an MOI (5) in viral inoculum (1XPBS supplemented with 1%DCS, and Ca^{2+} - Mg^{2+}). After 1 hour incubation at 37°C, 2 ml of media was added directly to the inoculum. At 8 hours post-infection, the media was removed and cells were washed once with 1X Phosphate Buffer Saline (PBS-Gibco). The cells were then lysed with 1 ml of Trizol (Invitrogen) and total RNA was isolated using the PureLink RNA Mini Kit (Invitrogen) following the

manufacturer's protocol. Equal amounts of total RNA (as indicated) were denatured in glyoxal (BioRad) and dimethyl sulfoxide (DMSO-Sigma) for 1 hour at 50°C. The RNA samples were analyzed on a 0.08% Sodium phosphate agarose gel. The RNA samples were then transferred to a GeneScreen (Perkin Elmer) membrane by a capillary flow blotting system for a total of 48 hours. The RNAs were UV-crosslink to the membrane using a HL-2000 HybriLinker (UVP Laboratory Products) 1200 ($\times 100 \mu\text{J}/\text{cm}^2$) for 30 seconds. The membranes were incubated in Pre-hybridization buffer (50mM Tris, 3XSSC, 10XDenhardt's, 0.1%SDS, 2 $\mu\text{g}/\text{ml}$ of sonicated denatured salmon testes DNA-Sigma) that has been diluted (1:1) with Deionized Formamide at 55°C for 5 hours. Blots were then hybridized with 5×10^6 CPM of ^{32}P labeled riboprobes specific for the RRV plus or minus strand as described in (32) or specific for β -actin (murine pTRI- β -actin, Ambion) at 55°C overnight. Membranes were then washed three times with buffers (1XSSC with 0.1% SDS, 1XSSC, and 0.1XSSC) in consecutive order for 15 minutes each at 68°C. Membranes were then exposed to a phosphoimaging screen, scanned using a Storm Phosphoimager (GE Healthcare), and quantified using ImageQuant software (GE Healthcare).

Type I IFN bioassays: Type I IFN bioassays were performed as previously described. Briefly, L929 cell were seeded in 96 well plates. Sample supernatants, as well as a murine IFN- β (Chemicon) standard, were acidified to a pH<2 with 2N HCL for 24 hours. Then, supernatants and standards were neutralized to pH=7 with 2N NaOH and UV-treated for 10 minutes to inactivate any residual virus. The samples were then titrated into the L929 cells in serial 2-fold dilutions. After 24 hour

incubation, encephalomyocarditis virus (EMVC) was added to all cells at 4×10^6 PFU/ml. Cell viability was determined using 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay. The total amount of IFN in each well was determined by direct comparison to a known concentration of IFN standard that has been normalized to a reference by the National Institute of Health.

Quantitative real-time PCR analysis: MEFs were seeded in 24-well dishes and infected with RRV viruses at an MOI (5). After 6 hour post-infection, cells were washed once with 1X PBS, and lysed with 500ul of Trizol (Invitrogen). Total RNA was purified as previously described. An equal amount of RNA was reverse transcribed using random primers and superscript III RT (Invitrogen). The cDNAs were analyzed on a Prism 7000 machine (Applied Biosystems) using Taqman primer/probe sets for IFN- β and 18S rRNAs.

RNA isolations and sucrose gradient centrifugation: To isolate virion RNA, BHK-21 cells were seeded in 150mm dishes and infected with RRV and A532V viruses at an MOI (0.01). Supernatants at 24 hours post-infection were harvested, clarified, and concentrated as previously described. Concentrated virions were lysed and RNA extraction was performed using the MagMAX viral RNA isolation kit (Ambion). RNAs were then stored at -20°C for later use. To isolate RNAs from infected cells, BHK-21 cells were seeded in 6-well dishes and were mock infected or infected with RRV and A532V viruses at an MOI (10) for 1 hour. After the 1 hour incubation, 2ml of normal media or media supplemented with 1ug/ml of Actinomycin

D was added to the virus inoculum. At 8 hours post-infection, total RNAs from infected cells were harvested as previously described. All RNAs were eluted in nuclease free water, aliquoted, and stored at -20°C. The stored RNAs were then used as ligands in the luciferase assays and IRF-3 phosphorylation studies or further purified through 15-30% sucrose gradient centrifugation for isolation of the 26S RNAs. A 15% and 30% (w/v) sucrose buffer (140mM NaCl, 10mM Tris-HCl pH=7.4, 1mM EDTA) was prepared in nuclease free water. Then, a sucrose gradient maker was employed to make 10 ml gradients. Each gradient was overlaid with equal volumes of total RNAs from mock-infected or RRV and A532V infected cells. The sucrose gradients were then centrifuged at 23,000 RPM for 7 hours at 4°C using a Sorvall Surespin 630 rotor. Then, samples were fractionated and ethanol precipitated with 3M sodium acetate and 1mg/ml of yeast glycogen (Roche)). An aliquot of each fraction was electrophoresed in TBE gels and stained with Ethidium Bromide for RNA analysis. Fractions containing the 26S RNAs were pooled, aliquoted, and stored at -20°C.

Luciferase assays: Human RIG-I expression plasmids were constructed as previously described (61). Briefly, HEK-293 cells were treated with IFN- β (100 IU) for 24 hours. Then, total RNA was isolated using Ultraspec RNA Isolation (Biotecx) followed by AMV Reverse Transcriptase PCR (Promega) using RIG-I specific primers for first strand synthesis. An N-terminal FLAG tag was engineered on a forward PCR primer and FLAG-RIG-I was then assembled by overlap extension PCR cloned into pcDNA3.1+. All plasmids were confirmed by sequencing at the

University of North Carolina at Chapel Hill Genome Analysis Facility. For the Luciferase assay, HEK-293 cells were seeded in 48 well plates. After over night incubation, 200ng of FLAG-RIG-I or an empty plasmid (pcDNA3.1+) were co-transfected with 50ng of a plasmid encoding an IFN-beta promoter (kindly provided by Dr. J. Pagano, UNC-CH) driving the luciferase gene using Fugene 6 (Roche). Twenty-four hours post-transfection, HEK-293 cells were stimulated with 8ng of poly I:C (Invivogen) or with the indicated amounts of viral RNAs. All RNA ligands were transfected with Lipofectamine 2000 (Invitrogen) and cells were lysed with 1X Cell Culture Lysis Buffer (Promega) 7 hours later. Samples were then assayed and quantified for luciferase activity.

CIP Treatment and IRF-3 phosphorylation studies: Equal amounts of total RNA, 26S RNAs, or poly I:C (Invivogen) were incubated with 1-2ul of alkaline phosphatase (CIP, New England Biolabs) or with buffer only for 1 hour at 37°C. Then, RNAs were column purified using Invitrogen's PureLink RNA Mini kit and eluted in nuclease free water. L929 cells were seeded in 12 well plates. One microgram of either CIP or buffer treated RNAs were transfected into the L929 cells and at the indicated timepoints, total cells were washed 1X with PBS, and lysed in NP40 lysis buffer containing protease and phosphatase inhibitors. The amount of total protein in each sample was determined by Coomassie Plus (Bradford) Assay (Pierce). Equal amounts of total protein were resolved on a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) followed by a transfer onto a PVDF membrane (Bio-Rad). Anti-IRF-3 C-20 antibody (Santa Cruz) or anti- β -actin (Sigma) were used

as primary antibodies, followed by anti-goat antibody conjugated to a Horseradish peroxidase (HRP) secondary antibody. All membranes were then coated with ECL Plus (Amersham) and exposed to film.

RNA Immunoprecipitations: Equal amounts of sucrose purified, 26S RNAs were immunoprecipitated with anti-mouse 2,2,7-trimethylguanosine monoclonal antibodies (anti-TMG) bound to agarose beads (EMD K121). The *in vitro* transcribed 26S RNA control was generated from a linearized RRV Replicon helper plasmid using Ambion's MEGAscript (SP6) protocol. The following RNA IP assay was performed as previously described (17). Briefly, mock, RRV, or A532V 26S RNAs were purified from ribosomal RNAs using a MicroPoly (A) Purist Kit (Ambion). Then, the purified 26S RNAs were equilibrated in RNA binding buffer supplemented with RNasin Ribonuclease Inhibitor (Promega) and incubated with the anti-TMG beads at 4°C for 2 hours with continuous rocking. The anti-TMG beads were spun at 2,000 RPM for 2 minutes and washed three times to remove unbound RNAs. The RNAs were eluted from the beads by incubation in elution buffer for 10 min at 60°C, followed by a phenol chloroform extraction. To determine the presence of bound 26S RNAs, 2ul of recovered RNAs were used as templates for a reverse transcriptase-Polymerase Chain Reaction (RT-PCR) using oligo dT(20) primers and Superscript III RT (Invitrogen). Specific primers for the E1 gene were used in the subsequent PCR analysis to determine bound 26S RNAs. All PCR products were analyzed by gel electrophoresis.

Flow cytometry analysis: BHK-21 cells were seeded in 24 well plates. Cells were either mock-infected or infected with RRV-gfp and A532V-gfp viruses or the RRV and A532V Replicons at an MOI (5) in triplicate samples. At 12 hours post-infection, the media was removed and cells were washed once with 1X PBS. Cells were then incubated with Trypsin (Gibco) and fixed in 2% paraformaldehyde (PFA) overnight. GFP expressing cells were quantified using a CyAn Cytometer and Summit Software (Dako).

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FIGURE 3.1

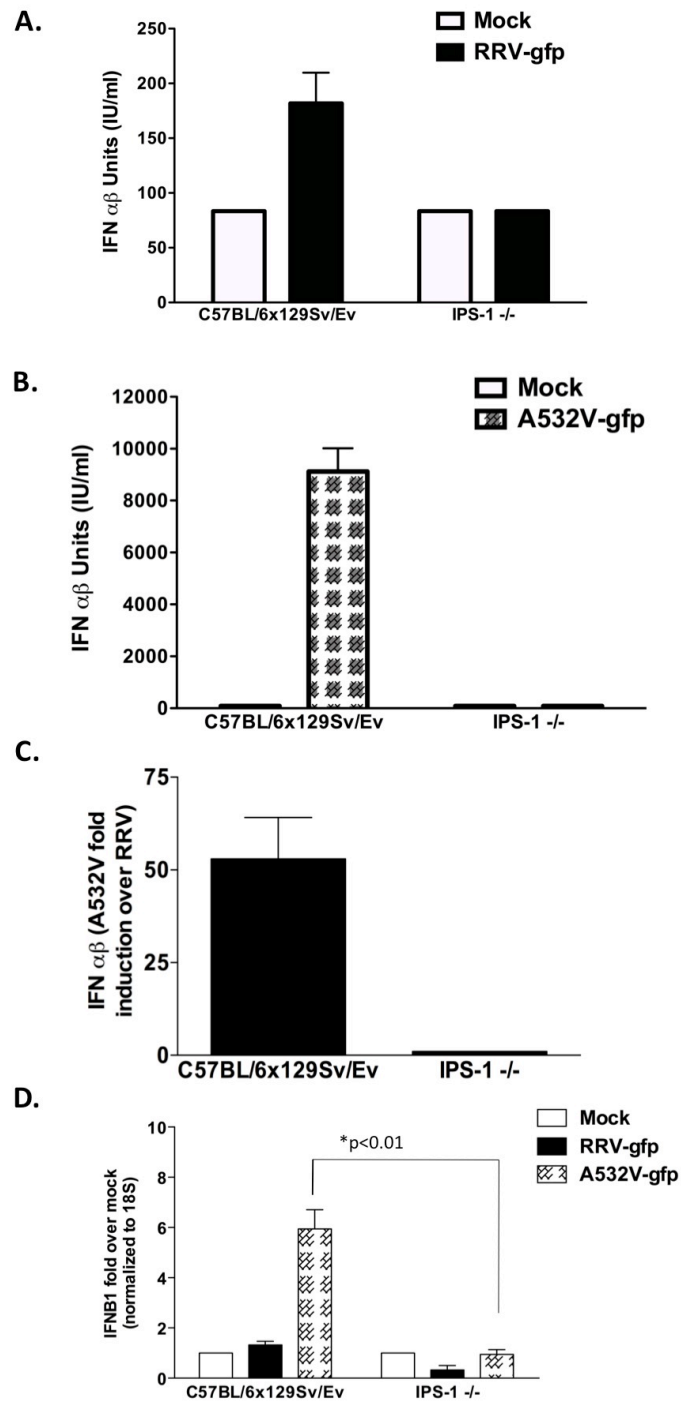


Figure 3.1. The IPS-1 molecule is critical for RRV A532V mediated IFN

induction. Wild type C57BL/6x129Sv/Ev and C57BL/6x129Sv/Ev IPS-1^{-/-} MEFS were either Mock infected or infected with RRV-gfp (A) and A532V-gfp (B) viruses at an MOI of 5. Supernatants harvested at 24 hours post-infection were analyzed for type I IFN using an IFN bioassay as described previously. C) The bioassay results in (A) and (B) are represented as the IFN fold induction of the RRV A532V mutant over that of wild type RRV. The limit of detection in each bioassay was ~83 IFN Units/ml. D) Total RNA was extracted from infected MEFS at 6 hour post-infection and analyzed by quantitative real-time PCR (Applied Biosystems) for IFN- β message. The data are represented as the fold induction over Mock infected cells and have been normalized to 18S rRNA. All samples were analyzed in triplicate and error bars represent the standard error of the mean. The p values were determined by ANOVA statistical analysis.

FIGURE 3.2

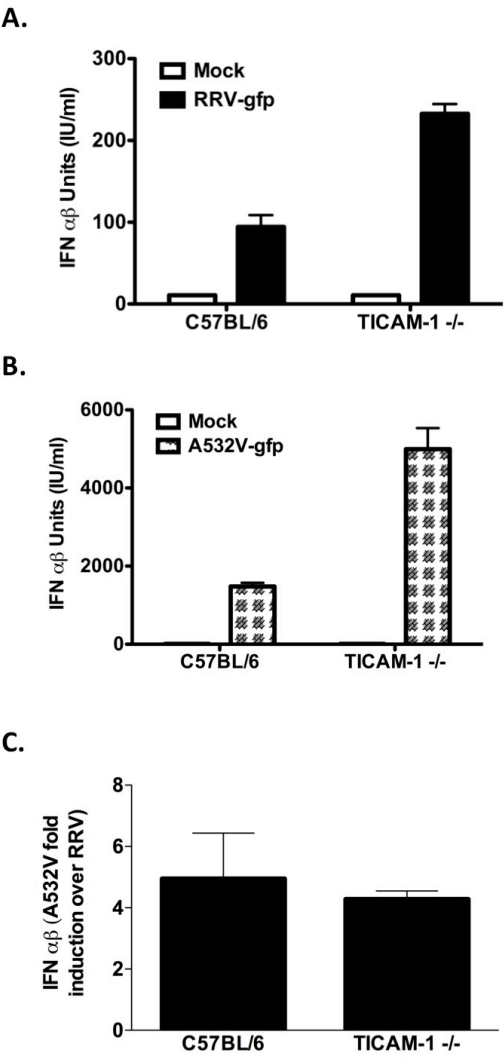


Figure 3.2. The RRV A532V mediated type I IFN induction is independent of

TICAM-1. C57BL/6 wild type and C57BL/6 TICAM-1^{-/-} MEFS were either Mock infected or infected with RRV-gfp (A) and A532V-gfp (B) viruses at an MOI of 5.

Supernatants at 24 hours post-infection were analyzed by an IFN bioassay. C) The bioassay results in (A) and (B) are represented as the IFN fold induction of the RRV A532V mutant over that of wild type RRV. The limit of detection was ~11 IFN

Units/ml. Samples were analyzed in triplicate and error bars represent the standard error of the mean.

FIGURE 3.3

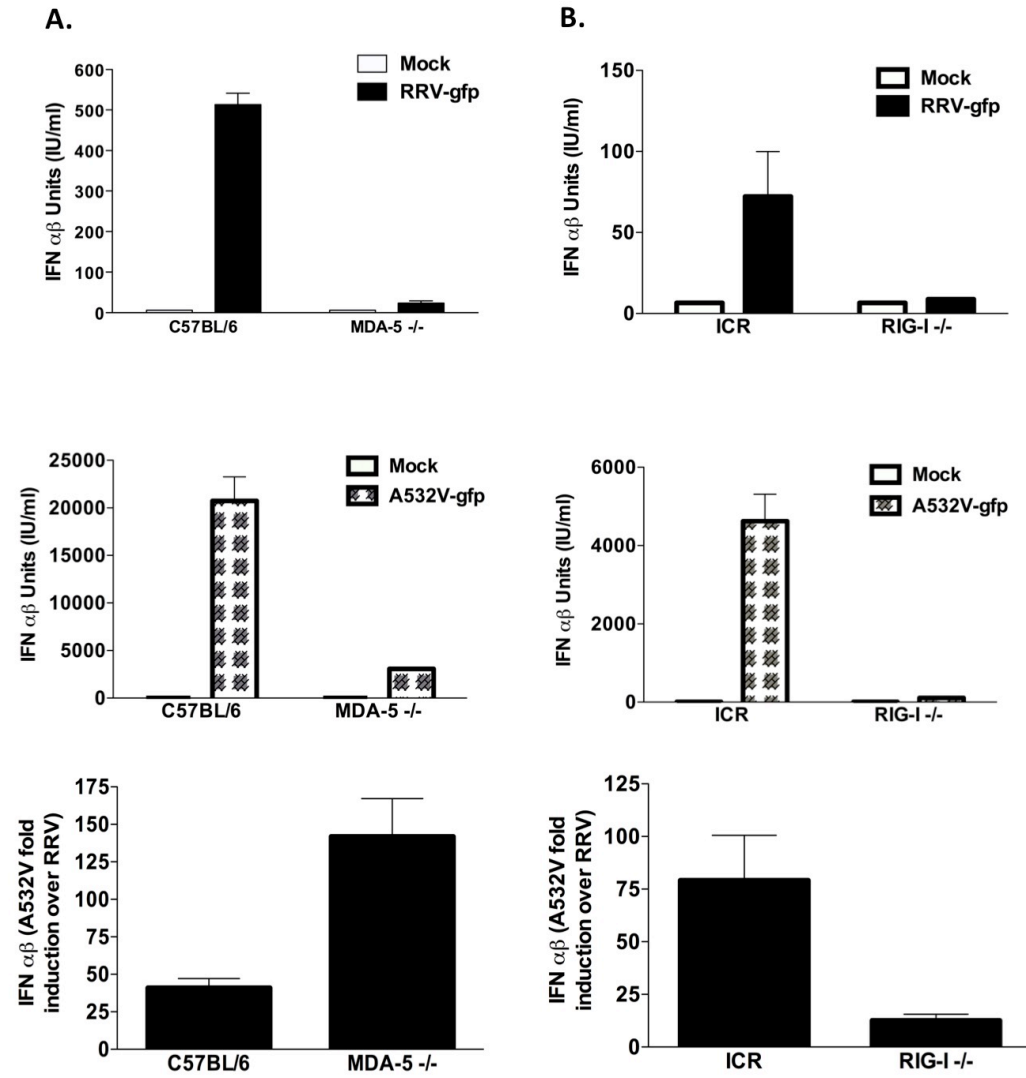


Figure 3.3. The RRV A532V mediated IFN induction is dependent upon RIG-I and not MDA-5. A) Wild type and MDA-5^{-/-} MEFS and B) Wild type and RIG-I^{-/-} MEFS were either Mock infected or infected with RRV-gfp and A532V-gfp viruses at an MOI of 5. Supernatants were harvested at 24 hours post-infection and analyzed for type I IFN using a bioassay. The upper panels depict the RRV-gfp results, while the middle panels depict the A532V-gfp results. The IFN fold induction of RRV A532V over that of wild type RRV in each corresponding MEF experiment is represented in the bottom panels. The limit of detection in the bioassay was 6 IU/ml. Samples were analyzed in triplicate and error bars represent the standard error of the mean.

FIGURE 3.4

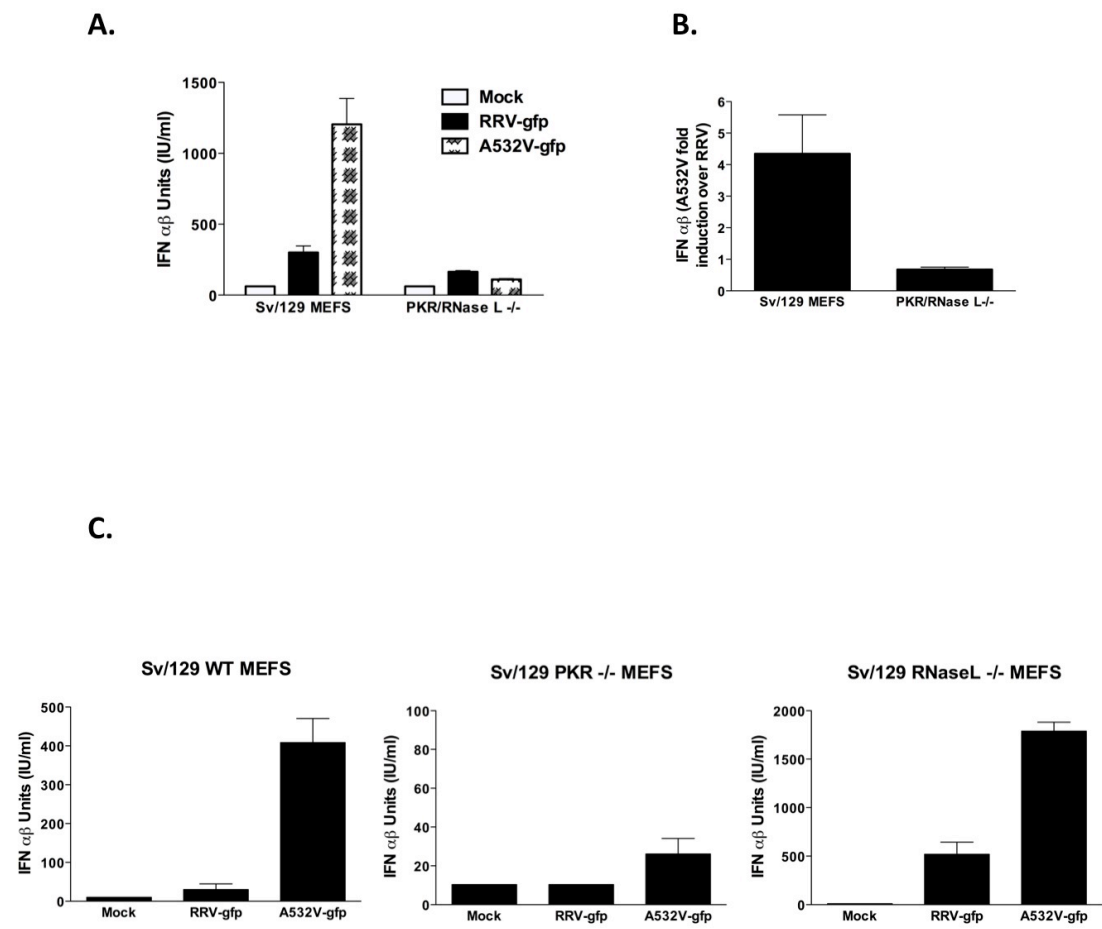


Figure 3.4. PKR contributes to the RRV A532V IFN induction. A) Sv129 wild type and Sv129 PKR and RNase L double deficient MEFS were either Mock infected or infected with RRV-gfp and A532V-gfp viruses at an MOI (5). Bioassays were performed on supernatants harvested at 24 hours post-infection and the limit of detection was 61 IFN Units/ml. B) The bioassay result in (A) is represented as the fold IFN induction of the A532V mutant over wild type RRV. C) Sv129 wild type, Sv129 PKR^{-/-}, and Sv129 RNase L^{-/-} single deficient MEFs were either Mock infected or infected with RRV-gfp and A532V-gfp viruses at an MOI of 5. Bioassays were performed on supernatants harvested at 24 hours post-infection and the limit of detection for all bioassays was 11 IFN Units/ml. All samples were analyzed in triplicate and error bars represent the standard error of the mean.

FIGURE 3.5

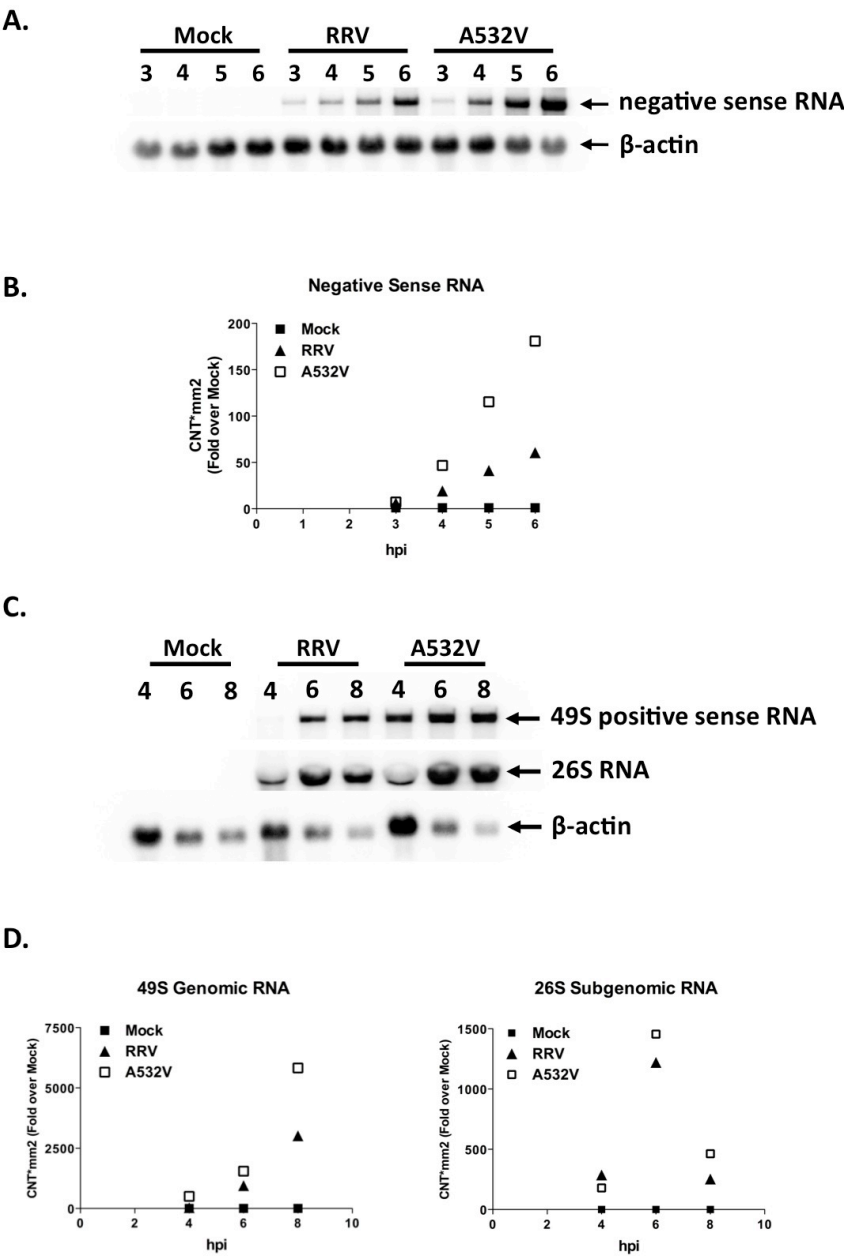


Figure 3.5. The RRV A532V mutant displays increased full length minus, plus

strand, and 26S RNA synthesis. BHK-21 cells were either Mock infected or infected with RRV and RRV A532V viruses at an MOI of 10. Total RNAs were isolated from infected cells at the indicated time points and analyzed by Northern Blot analyses. A) Five micrograms of the total RNAs were analyzed for viral minus strand and β -actin RNAs. C) Northern Blot analysis for full length plus strand and 26S. Equal amounts of RNA were analyzed between virus samples but varying amounts were used across time points. Five micrograms per sample was used for the 4 hour time point, two micrograms was used for 6 hour time point, and one microgram was used for the 8 hour time point. B and D) The levels of full length genomic (49S) and 26S subgenomic RNAs were determined by densitometry quantification and have been normalized to the amount of β -actin levels found within the same lane. Each data point is represented as fold over Mock. The data shown are representative of three independent experiments.

FIGURE 3.6

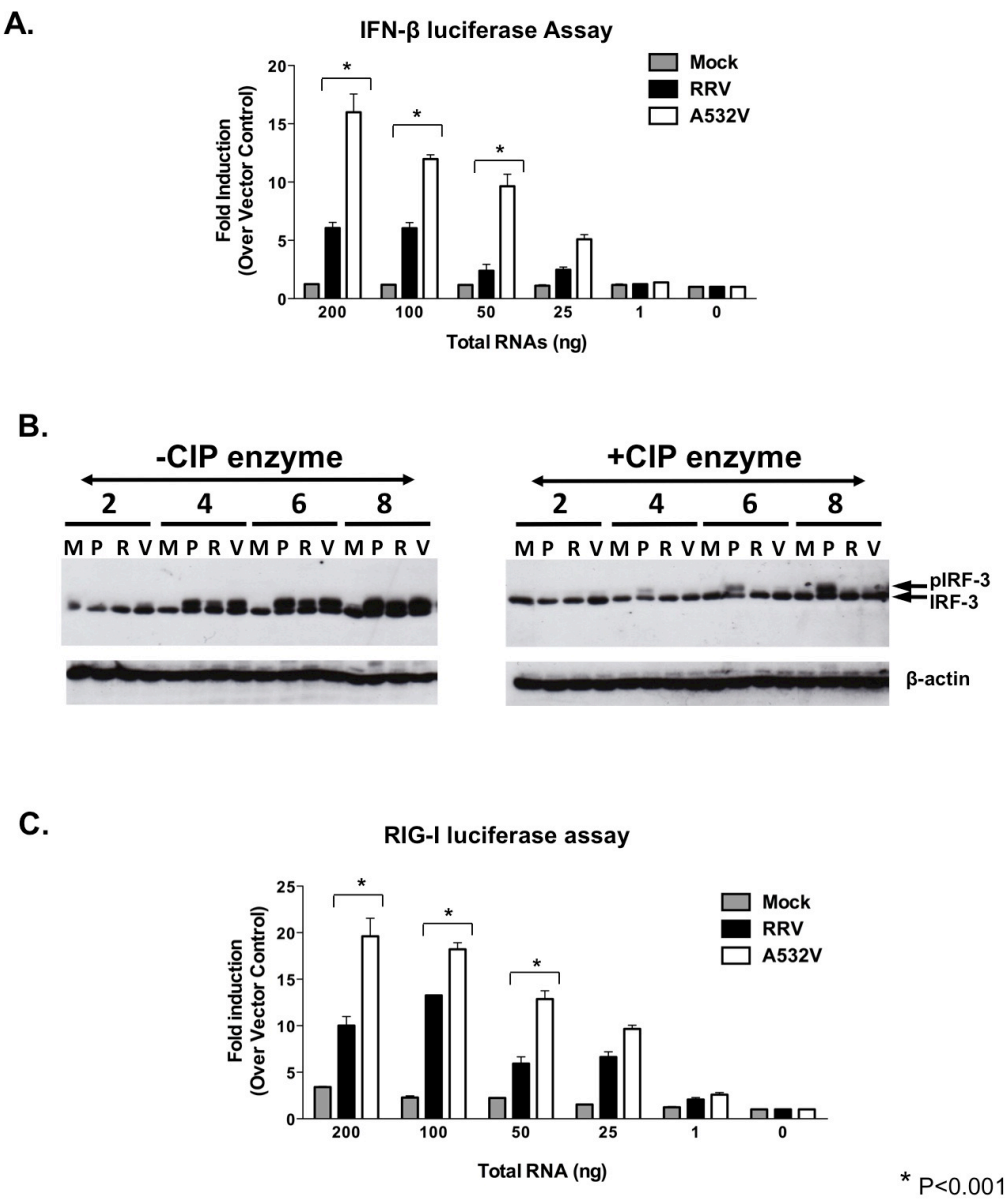


Figure 3.6. The RRV A532V viral RNAs more robustly activate IFN induction.

BHK-21 cells were either Mock infected or infected with RRV and A532V viruses at an MOI (10). At 8 hours post-infection, total RNAs were isolated following Invitrogen's PureLink RNA Mini Kit protocols and used in the following assays. A) IFN- β -luciferase assay. Briefly, 293 cells were transiently transfected with an IFN- β -luciferase reporter along with the PCDNA3.1+ plasmid using Eugene 6 (Roche). After 24 hours, the indicated RNAs were transfected into the 293 cells and assayed 7 hours later for luciferase activity. B) IRF-3 phosphorylation assay. Total RNAs were treated with or without CIP enzyme as described in the materials and methods. Then, equal amounts (1 microgram) were transfected into L929 cells. At the times indicated, total cell lysates were harvested and equal proteins were loading onto an SDS-PAGE gel for Western Blot analysis. Anti-IRF-3 C-20 (Santa Cruz) and anti- β -actin (Sigma) antibodies, followed by anti-goat-HRP (Sigma) secondary were used to detect IRF-3 and β -actin proteins. The arrows indicate phosphorylated IRF-3 (p-IRF-3), total IRF-3 (IRF-3), and β -actin bands. Samples are represented as M=Mock, P=poly I:C, R=RRV, and V=A532V. C) RIG-I luciferase assay. Equal amounts of total RNAs were titrated and then transfected into 293 cells transiently overexpressing a plasmid containing the human RIG-I gene or an empty plasmid (PCDNA3.1+) along with an IFN- β -luciferase reporter. At 7 hours post-transfection, cells were lysed and assayed for luciferase activity. All luciferase data were performed in triplicates and the error bars represent the standard error of the mean. The data are represented as the fold induction over PCDNA3.1+ control. The p values were determined by ANOVA analysis.

FIGURE 3.7

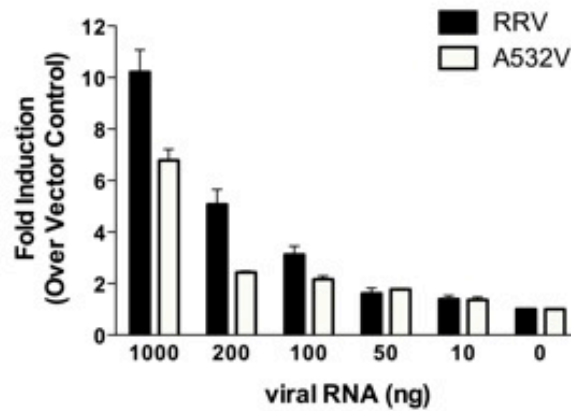


Figure 3.7. The RRV A532V full length viral RNAs do not differently activate IFN induction. Full length plus strand RNAs were isolated from RRV wild type and A532V mutant virion particles using Ambion's MagMAX Viral RNA isolation kit. After purification, equal amounts of RNA were titrated and transfected into the previously described RIG-I luciferase assay. All data were performed in triplicates and the error bars represent the standard error of the mean. The data are represented as the fold induction over PCDNA3.1+ control.

FIGURE 3.8

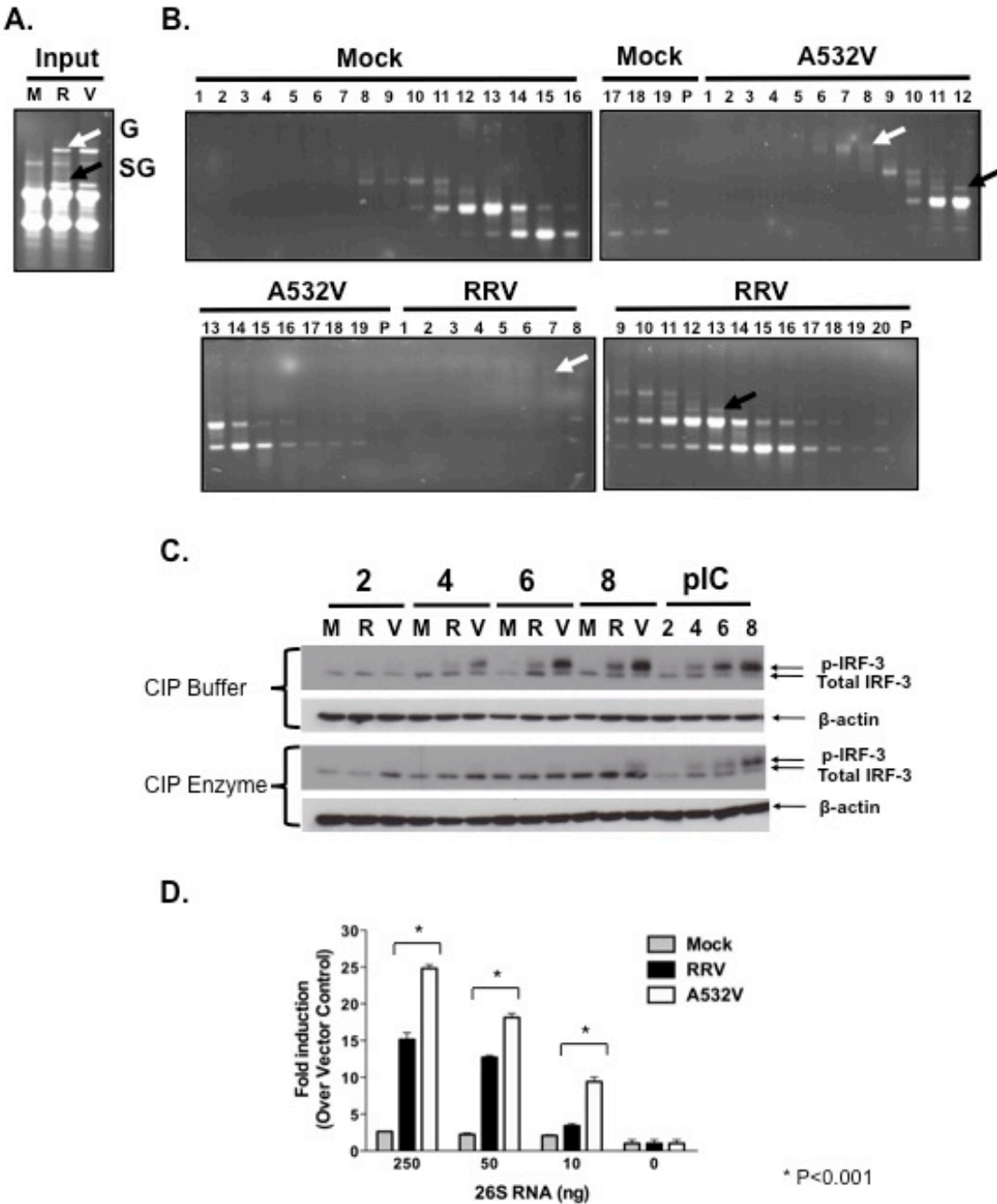


Figure 3.8. The A532V viral 26S RNA is a better activator IFN inducer. BHK cells were either Mock infected or infected with RRV and A532V viruses at an MOI of 10 in the presence or absence of 1ug/ml of Actinomycin D. Total RNAs were isolated at 8 hours post-infection, layered on top of a 15-30% sucrose gradient, and centrifuged as described in the materials and methods. After centrifugation, the gradients were fractionated in the order of bottom of the gradient (1) to the top (19 or 20). Any pellets formed were resuspended in buffer and indicated as (P). A) RNA gel analysis of isolated total RNAs before sucrose gradient centrifugation. B) RNA gel analysis of the sucrose gradient fractions. The full length genomic RNAs (G) are indicated by a white arrow and the 26S RNAs (SG) are indicated by a black arrow. Fractions containing the 26S RNAs from (12-14) from the Mock, RRV, and A532V sucrose gradients were pooled, concentrated, and subsequently used in the following experiments. C) Equal moles of 26S RNA were treated with CIP buffer or CIP enzyme and then transfected into L929 cells for IRF-3 phosphorylation analysis as previously described. Samples are indicated as M=Mock, R=RRV, and V=A532V. Data shown are a representative of two independent experiments. D) Equal moles of 26S RNAs were titrated and transfected into HEK 293 cell for RIG-I luciferase assay analyses. Each bar represents triplicate samples and the error bars represent the standard error of the mean. The p values were determined by ANOVA analysis.

FIGURE 3.9

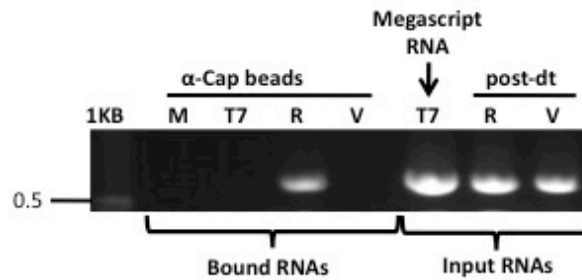
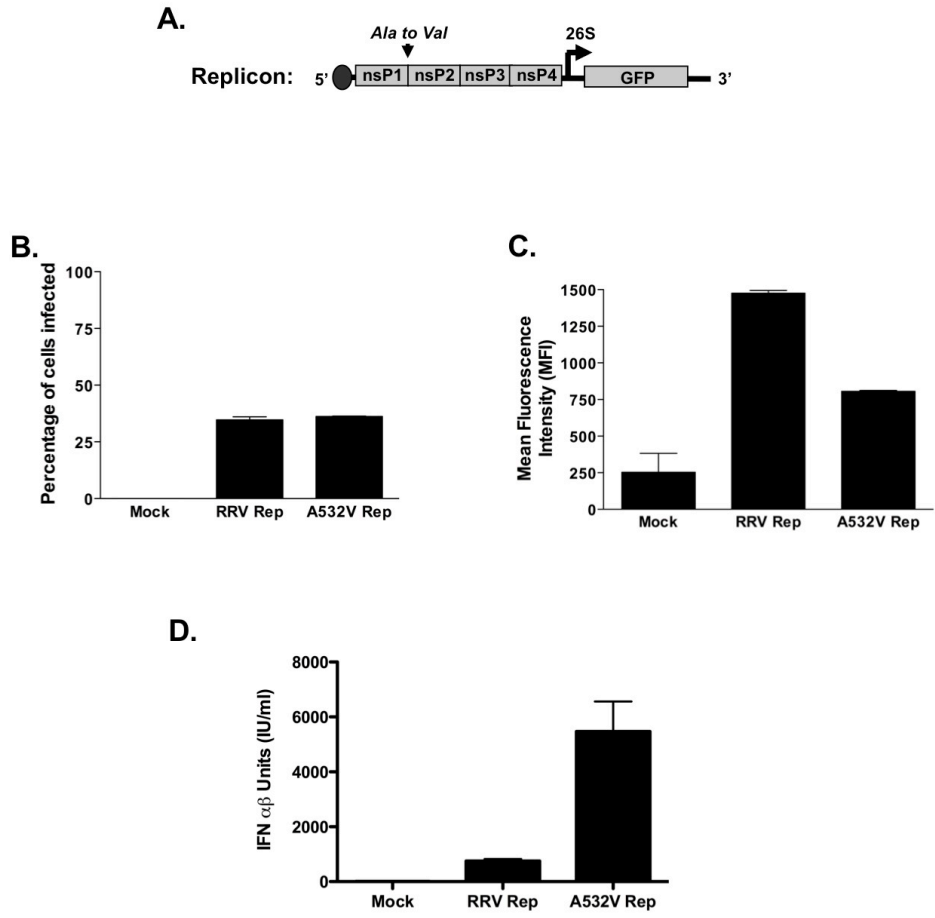


Figure 3.9. The RRV A532V virus makes uncapped 26S RNA.

Equal amounts of an *in vitro* transcribed 26S RNA or sucrose purified Mock, RRV, and A532V 26S RNA were immunoprecipitated (IP) with 2,2,7-trimethylguanosine monoclonal antibodies (EMD K121) as described in the materials and methods. 200ng of starting 26S RNAs (input) or 2ul of IP RNAs were then subjected to RT-PCR using an oligo dT (20) primer for CDNA synthesis. 5ul of each CDNA sample were then used for subsequent PCR amplification using virus specific primers for the E1 gene.

SUPPLEMENTAL FIGURE 3.1



Supplemental Figure 3.1. GFP expression from the A532V encoded 26S RNAs is lower than those of the wild type RRV. A) Schematic diagram of the RRV replicon genome encoding either the Alanine or Valine mutation followed by the GFP gene under a single 26S subgenomic promoter. B, C, and D) L929 cells were either Mock infected or infected with RRV and A532V Replicon particles at an MOI of 5. At 24 hours post-infection, cells were harvested and analyzed for GFP expression by FLOW cytometry analysis. B) Data depicts the percentage of cells infected and C) depicts the mean fluorescence intensity. D) Supernatants from replicon infected L929 cells were analyzed for IFN $\alpha\beta$ proteins using an L929 IFN bioassay. All samples were done in triplicate and error bars represent the standard error of the mean.

CHAPTER FOUR
DISCUSSION AND FUTURE DIRECTIONS

The type I IFN response plays a crucial role in controlling alphavirus infections. These processes are highlighted by studies using animal models in which mice that are deficient for one or more IFN molecules, displayed increased susceptibility, replication, and tropism upon various alphavirus infections (16, 17, 24, 25, 30). Interestingly, specific alphavirus interactions with the host IFN response are poorly understood. However, like many other viruses, it has been proposed that alphaviruses employ mechanisms to evade the host type I IFN. These proposals are based upon *in vivo* mouse studies demonstrating that virulent alphaviruses can persist and cause mortality in mice even in the presence of an intact IFN system (7, 13, 28, 30). Furthermore, these studies suggest the presence of genetic determinants within alphaviruses that modulate the host IFN responses, since attenuating mutations within virulent viruses made them more susceptible to IFN actions. Therefore, studies to identify the mechanism(s) of alphavirus IFN evasion, as well as the genetic determinants to modulate these processes, have been a high priority within the field. In this dissertation, we explore the role of a single determinant located within the conserved nsP1/nsP2 cleavage domain of two different, but closely related alphaviruses, to modulate host IFN induction as well as to identify the mechanism(s) underlying these effects.

Heise et al. (2000) previously identified a unique genetic determinant located in the nsP1 protein, position 538, of the adult mouse neurovirulent Sindbis AR86 virus (13). This determinant lies in the P3 position within the conserved nsP1/2 cleavage domain and was found to be essential for virus pathogenesis *in vivo*. A single amino acid mutation of the determinant, resulting in a Threonine to Isoleucine

change, attenuates this virus resulting in restricted spread within the brains of infected mice. The exact mechanism(s) by which this determinant regulates viral virulence, however, are not clearly defined. Interestingly, the Thr to Ile change within Sindbis AR86 affected both the kinetics of nonstructural polyprotein processing and regulated 26S RNA synthesis. Therefore, we hypothesized that an increase in RNA synthesis would lead to additional ligands that may more actively induce IFN. As type I IFN plays a critical role to control alphavirus infections, we questioned whether the attenuated Isoleucine in Sindbis AR86 (T538I), would result in robust IFN responses, possibly initiating its clearance by the host immune response. In accordance with this hypothesis, we found that the T538I mutant was a more robust IFN inducer in mice and in cell culture than the wild type Sindbis AR86 virus. As this determinant lies within the conserved nsP1/2 cleavage domain and was found to partially rescue virulence of other Sindbis viruses, this lead us to believe that the determinant would also modulate the IFN responses of other alphaviruses. We found that a similar change, Alanine to Valine, within Ross River Virus (RRV) also induces robust IFN responses *in vitro*. Although, we have yet to fully characterize the role of the Valine mutation on RRV pathogenesis *in vivo*, preliminary studies suggests that this mutant is also slightly attenuated (Cruz, C.C., Morrison, T.E., and Heise, M.T., unpublished data). Therefore, these studies highlight the role of a single determinant in modulating the host type I IFN response by two different alphaviruses, and suggest that at least in the case of Old World alphaviruses, this determinant is a major regulator of IFN responses and virulence. Therefore, it would be interesting to determine whether this determinant also affects

IFN induction by New World alphaviruses, such as Venezuelan Equine Encephalitis virus (VEEV). If a similar mutation in VEE exhibits a similar effect on IFN induction, it would establish that this determinant plays a general role in regulating IFN induction by alphaviruses. However, given that VEEV is known to interact with the host differently than that of the Old World alphaviruses (8), it is also likely that the nsP1 mutation may lead to different IFN results. This would suggest that the nsP1 determinant is unique to the Old World alphaviruses, and further studies to define this specificity would be needed.

Viruses employ many mechanisms to evade the host type I IFN response. A major mechanism that has been proposed for alphaviruses is through shutoff of host RNA and protein synthesis. Gorchakov et al. (2005) demonstrates that mutations within the nsP2 protein of a genetically related Sindbis virus leads to defective viruses incapable of inhibiting host cellular transcription and translation (10). Additionally, they found that their shutoff defective viruses also induced more type I IFN. Although the mechanism(s) of host shutoff is not clearly defined, it is likely due to the translocation of free nsP2 to the nucleus to mediate transcription shutoff (11). Therefore, though our nsP1 mutation do not coincide with the positions of their nsP2 mutations, it does lie within the nsP1/2 cleavage domain, potentially affecting either processing of the nonstructural polyprotein (nsP) or free nsP2 translocation to the nucleus. However, we have found that the nsP1 mutations in both the Sindbis AR86 (T538I) and RRV (A532V) did not adversely affect the host shutoff kinetics from that observed with the wild type viruses. Further, we found that the Sindbis T538I, which displays increased kinetics of nsP processing, was a better inhibitor of host RNA and

protein synthesis than the wild type AR86 virus. Additionally, we observed increased kinetics of IRF-3 phosphorylation upon infections with our nsP1 mutant viruses. IRF-3 phosphorylation occurs independently of *de novo* protein synthesis (22) and should not be affected by virus mediated host shutoff. Therefore, our studies suggest the mechanism(s) modulating the IFN responses by our mutants are independent of host shutoff processes. We, however, are not the first to report shutoff independent IFN modulation. Several studies have also reported similar findings using Semliki Forest Virus (SFV) and VEEV (4, 27). Although we agree that global inhibition of host macromolecular synthesis to prevent initiation of the type I IFN response, while simultaneously favoring virus replication is a potent means to antagonize the IFN response, complete host shutoff does not occur until later times in the virus replication cycle. Therefore, shutoff does not address potential early alphavirus interactions with the host type I IFN system. Therefore, it is likely, that alphaviruses employ many mechanism(s), both specific and non-specific, to efficiently antagonize the host response. We propose a biphasic model in which our nsP1 determinant acts early to specifically limit or evade the type I IFN response, until a second phase, mediated by host shutoff, takes over at later times.

There are several possibilities in which the nsP1 determinant could modulate the host type I IFN response. One mechanism is that the wild type viruses encode an IFN antagonist that is directly inhibiting IFN induction or molecules within the signaling pathway. This hypothesis correlates with previously identified roles for the nonstructural proteins of other alphaviruses to actively antagonize the IFN system (4, 27, 31). Alternatively, another mechanism is that the nsP1 determinant may

delay viral 26S RNA synthesis in an attempt to avoid recognition by the host's pattern recognition receptors until complete host shutoff has taken full effect. This suggests that the 26S RNA is a target for PRRs to induce IFN. Therefore, further experiments to test this theory are needed. Additionally, the nsP1 mutation may disrupt mechanisms to properly cap the viral RNAs, as nsP1 and nsP2 make up the viral capping apparatus (1, 29). As a result, uncapped RNAs are made, triggering IFN induction through the RIG-I and PKR pathways (14, 21). Therefore, identifying the PRR pathways that are involved in IFN induction by the nsP1 mutant will further our understanding of the nsP1 determinant to modulate the host IFN responses.

Recent evidence suggests that alphaviruses could directly inhibit type I IFN responses. In these studies, the authors demonstrate that the nonstructural proteins of Sindbis and VEEV can directly inhibit STAT-1 phosphorylation when stimulated with type I IFN (27, 31). Interestingly, Simmons et al. (manuscript in progress) also demonstrates that the T538I mutation disrupts wild type Sindbis AR86 from efficiently blocking IFN- β stimulated STAT-1 activation. Therefore, it is possible that the nsP1 determinant encodes an IFN antagonist that directly interferes with the IFN signaling pathway to limit IFN amplification and that insertion of Ile and Val into nsP1 disrupts this activity, thus, accounting for the huge IFN differences observed in our earlier studies. However, we found that infection with the nsP1 mutants of IFN- $\alpha\beta$ receptor deficient (or defective) cells, which cannot amplify IFN through the JAK-STAT pathway, still induced more IFN than the wild type viruses. Therefore, our nsP1 mutants were still capable of inducing IFN even in the absence of IFN amplification. Interestingly, preliminary experiments suggest that wild type RRV,

unlike AR86, does not actively inhibit STAT-1 phosphorylation (Simmons, J.S. unpublished data). Therefore, although, we cannot completely rule out a role for the nsP1 determinant in inhibition IFN signaling or that the determinant in Sindbis and RRV are acting through the same mechanisms, it is likely that our determinant is also modulating the IFN responses directly at the level of IFN induction.

In addition to the possibility that the nsP1 538 determinant is acting through JAK/STAT inhibition, it is also possible that our wild type viruses are actively suppressing initial IFN induction. Breakwell et al. (2007) demonstrates, using a SFV nsP2 mutant, that the nsPs of SFV are able to suppress IFN induction, through a shutoff independent mechanism (4). However, as they observe no difference in kinetics of IRF-3 activation between their viruses, they propose that antagonism occurs after IRF-3 translocation to the nucleus, which differs from our results. Therefore, it is likely that our nsP1 mutation is modulating IFN induction through a different mechanism than that observed with SFV and additional studies are needed to more fully address the possibility that either AR86 or RRV directly antagonize type I IFN induction.

Our data, thus far, suggests that the nsP1 determinant is modulating IFN directly at the level of IFN induction, possibly at steps prior to IRF-3 activation. This is consistent with our hypothesis that the nsP1 mutants are generating more/or better RNA ligands to induce IFN. As a first step in understanding how the nsP1 538 determinant affects type I IFN induction, we set out to define the host pathways that contribute to type I IFN responses against either the wild type or mutant viruses. Specifically, we wanted to identify the PRRs that would respond to the nsP1 mutant

in hopes that identifying a specific PRR pathway would shed light on the mechanisms of IFN modulation and possibly identifying potential viral RNA ligands. In the course of our studies, several different groups using Sindbis and chikungunya viruses reported that the IPS-1 pathway was important for alphavirus mediated IFN induction (5, 9, 26). More specifically, Burke et al. (2009) reports that MDA-5, but not RIG-I, contributes to IFN induction by Sindbis whereas Schilte et al. (2010) demonstrates that both RIG-I and MDA-5 responds to chikungunya virus (5, 26). Therefore, we evaluated the same PRR pathways to recognize the RRV A532V nsP1 mutant. Similar to their reports, we also found that the IPS-1 molecule was critical for RRV A532V to mediate IFN induction. Interestingly, while we demonstrate that both RIG-I and MDA-5 play a role in recognition of the wild type RRV, we found that RIG-I was required for the high level of IFN induction by the A532V mutant. This led us to hypothesize that the A532V mutation may be modifying or overproducing an RNA that is a RIG-I specific ligand. However, even in the absence of RIG-I, cells were still initiating low IFN responses to the RRV A532V mutant virus that were higher than the response mounted against the wild type virus. Although, this could be due to the actions of MDA-5, we could not rule out the possibility that another receptor was involved. Therefore we analyzed the role of PKR as PKR has been shown to recognize uncapped RNAs and several reports demonstrate that PKR contributes to the IFN induction mediated by both Sindbis and SFV (3, 5, 25). We found that like RIG-I, PKR also contributes to the IFN induction by the RRV A532V mutant. Additionally, we were able to rule out a role for both RNase L and TICAM-1 to mediate IFN to our nsP1 mutant as these molecules have

also been implicated to play a role in IFN induction (6, 18). Though we cannot rule out a role for other RNA receptors, such as TLRs, in recognition of the A532V mutant, our finding that IPS-1 is essential for A532V-mediated IFN induction strongly suggests that RIG-I and PKR are the major pattern recognition receptors responsible for IFN induction by the A532V mutant. Although, we have yet to determine if RIG-I and/or PKR also play a role to recognize the Sindbis T538I mutant, preliminary studies demonstrate that the mutant also drives IFN induction through the IPS-1 pathway (Cruz, C.C. and Heise, M.T., unpublished data).

Previous studies suggested that the Sindbis virus nsP1 T538I mutant results in early induction of viral 26S RNA, therefore, we assessed whether this determinant also led to excess RNA synthesis in the context of RRV. Therefore, to further test our hypothesis, we evaluated kinetics of viral RNA synthesis with the RRV A532V mutant virus. Similar to the T538I mutant, we also found a slight increase in overall 26S RNA synthesis. However, unlike Sindbis T538I, we also observed increased synthesis of full length minus and plus strand RNAs. Therefore, it is likely that the RRV A532V mutant simply disregulates overall viral RNA synthesis, providing the host cell with additional ligands to induce IFN. Although, additional analyses are needed to determine which specific RNA species is responsible for IFN induction and whether IFN induction is directly due to increased quantities of viral RNAs.

The fact that the nsP1 mutants display increased RNA synthesis, strongly supports our hypothesis that the nsP1 mutants are actively inducing type I IFN by overproducing potential ligands for RIG-I and/or PKR. However, these results do not rule out the possibility that the nsP1 mutants also lead to the production of altered

RNAs that are able to serve as better ligands for either RIG-I or PKR. Interestingly, it has been previously reported that free 5'-triphosphates present on uncapped RNAs can induce both RIG-I and PKR activation. 5'-triphosphates are indicators of viral replication as host mRNAs in the cytosol generally contain either a 5'-cap or a 5'-monophosphate (20). Therefore, host cellular RNAs are not recognized by RIG-I or PKR, allowing the RNA receptors to differentiate between self and non-self RNAs. Furthermore, viruses specifically remove the 5'-triphosphate on their RNAs (12) or attach a viral protein, such as VPg (32), or a 5'-cap (23) to the 5'-terminal end of its RNA in an attempt to disguise their RNAs from RIG-I detection, highlighting the fact that uncapped RNAs are potential viral ligands. Given that nsP1 and nsP2 are involved in capping of viral RNAs, it was possible that the mutant was not only disregulating viral RNA synthesis, but that one or more of the viral RNAs was also capped less efficiently, thereby making it a better ligand for RIG-I and/or PKR. Therefore, to directly test this possibility, we first determined whether the RNAs from wild type and RRV A532V infected cells would differentially activate IRF-3. We found that not only was the RNAs from A532V infected cells a better activator of IRF-3, but that activation was completely dependent upon the presence of a phosphate. Furthermore, we determined that the same RNAs also differentially activated RIG-I. Therefore, these studies support our hypothesis that the A532V mutant generates uncapped viral RNAs and that these RNAs are activating the RIG-I/PKR pathway.

The previous studies firmly demonstrate that RRV A532V uncapped viral RNAs are activating RIG-I. They have not, however, addressed the specific RNA

species that is mediating these responses. Therefore, we found that the A532V 26S RNA, rather than the full-length genome, was responsible for differential RIG-I activation. More importantly, IFN induction required a 5'-triphosphate and was a better RIG-I activator. Altogether, these data suggested that the RRV A532V specifically makes uncapped 26S RNAs. Given that the nsP1 and nsP2 proteins are involved in the capping of the viral RNAs, it is possible that the nsP1 mutation is disrupting the viral capping apparatus (1, 2, 19, 29). The viral nsP2 protein is a 5'-triphosphatase that removes a 5'-phosphate from the viral RNA as the nsP1 protein attaches a methylated cap. Although our mutation does not lie within the reported catalytic domains of both nsP1 and nsP2, we cannot rule out an effect to disrupt the capping process. Therefore, we assessed the presence of a 5'-cap structure between the wild type and A532V mutant 26S RNAs. In line with our earlier results, we found that the RRV wild type RNA contained a 5'-cap whereas the A532V mutant did not. These results were intriguing as we have previously observed delayed structural protein synthesis within the A532V infected cells and uncapped 26S RNAs would most likely explain these results. To further confirm our results, we utilized a replicon particle system containing either the Ala or Val to launch GFP from a single 26S promoter. Similar to our earlier results, we also observed lower GFP protein expression from A532V (Replicon) 26S RNAs, further emphasizing that the RRV A532V mutant makes uncapped 26S RNAs. Therefore, given our data, we demonstrate that in addition to making more viral RNA ligands, the A532V specifically makes uncapped RNAs to drive IFN induction through the RIG-I and/or PKR pathway. Although our data suggests that the disruption of the capping

apparatus is specific for the 26S RNA, since we do not observe differential RIG-I activation with the full-length genomes, we cannot completely rule out an effect to modify the cap structures on the viral genomes. Therefore, further analyses of the 5'-cap structure on the full-length genomes are needed.

In summary, this dissertation provides novel insight into how a single virulence determinant modulates the host type I IFN response. We found that mutation of the nsP1 determinant directly attenuates the virus, resulting in increased RNA synthesis, thus leading to its efficient recognition by the host RNA receptors to induce IFN induction. We further demonstrate that in addition to an increase in potential RNA ligands, the nsP1 mutants also disrupt the viral capping apparatus, generating uncapped RNAs that drive excess IFN induction through the RIG-I and PKR sensors. Furthermore, we propose that the nsP1 mutation specifically modifies the cap structures of the 26S RNAs, as the full length plus sense RNAs do not differ in their ability to activate IFN. However, it should be noted that we have yet to assess the capacity of the negative sense or the dsRNA replicative intermediates, to activate IFN. Although, given that the 26S RNAs are made in 5-molar excess to that of the full length RNAs (15), it is more likely that the 26S RNA is the target RIG-I/PKR ligand. This is not surprising, as our earlier studies with the Sindbis T538I mutant had indicated that the 26S RNA was a potential ligand. Therefore, it would be interesting to determine whether the 26S RNAs of the Sindbis mutant are also uncapped and if those RNAs lead to more robust IFN inducers and whether the same pattern recognition receptors are involved. These studies would further define the mechanism of IFN induction by the nsP1/nsP2 determinant as the Sindbis

viruses only differ in the quantity of 26S RNA, thus helping to rule out other RNA species. Finally, as the nsP1/nsP2 mutation attenuates the neurovirulent Sindbis virus *in vivo*, defining the specific IFN interactions will further our understanding of the pathogenesis of this virus.

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