MOLECULAR PATHOGENESIS OF THE
SINDBIS-GROUP VIRUS STRAIN AR86

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ABSTRACT

MEHUL S. SUTHAR: Molecular Pathogenesis of the Sindbis-group virus strain AR86
(Under the direction of Mark T. Heise, Ph.D.)

Sindbis virus infection of mice has provided valuable insight into the viral and host factors that contribute to virus-induced neurologic disease. In an effort to further define the viral genetic elements that contribute to adult mouse neurovirulence, the virulent Sindbis-group virus strain AR86 was compared to the closely related (22 single amino acid coding differences and the presence or absence of an 18 amino acid sequence in nsP3), but avirulent Girdwood strain. Detailed mapping studies identified three major determinants in the nonstructural region at nsP1 538 (Ile to Thr; avirulent to virulent), an 18 amino acid deletion in nsP3 (positions 386-403), and nsP3 537 (Opal to Cys; avirulent to virulent), as well as a single determinant in the structural genes at E2 243 (Leu to Ser; avirulent to virulent) that were essential for AR86 adult mouse neurovirulence. Of these four virulence determinants, we found that the virulence determinant at nsP1 538 plays a particularly important role. This determinant has been shown to regulate viral nonstructural processing, viral RNA synthesis, and play a major role in regulating adult mouse neurovirulence. Analysis of the effect of the nsP1 538 determinant on modulating the host antiviral response demonstrated that an attenuating mutation at nsP1 538 resulted in enhanced type I interferon induction. While most alphaviruses have been shown to inhibit type I interferon induction through virus-induced host cell shutoff, we demonstrated that the Sindbis-group virus strain AR86 inhibits type I interferon induction through an additional mechanism by inhibiting the activation of interferon regulatory factor 3 (IRF-3), a key mediator of type I interferon induction. In an

effort to extend these findings, we examined virus interactions with the type I interferon induction signaling pathway. We found that although AR86 genomic RNA is efficiently recognized by retinoic-acid inducible gene I (RIG-I), a host cytoplasmic sensor of viral dsRNA, AR86 virus actively antagonized IRF-3 activation induced from an exogenous source of dsRNA. Furthermore, the viral nonstructural proteins (nsPs), when expressed independently of virus infection, inhibited RIG-I and melanoma-differentiation-associated gene 5 (Mda5), a related host cytoplasmic sensor of viral dsRNA, signaling. Strikingly, co-expression of the viral nsPs proteins led to the loss of RIG-I, Mda5, and IRF-3 expression in a manner that was dependent on the nsP2 protease. These results demonstrate that the viral nonstructural proteins specifically antagonize the type I interferon induction signaling pathway and suggest that the virus may directly target components of the type I interferon induction signaling pathway. The results from these studies have provided significant insight into the viral and host factors that mediate virus-induced disease and have advanced our understanding of the mechanisms underlying alphavirus pathogenesis.

This thesis is dedicated to my wife, Sucheta,

Who has helped me maintain a positive attitude

and supported me throughout

And to my mother, father, and brothers

For their support and encouragement

And this would not be possible without Maharaj and Swami,

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LIST OF ABBREVIATIONS

BFC   Barmah Forest virus complex

CARD   Caspase recruitment domain

IFN   Interferon

IRF-3   Interferon Regulatory Factor-3

i.c.   Intracranial

kDa   Kilodalton

Mda5   Melanoma-differentiation-associated gene 5

NSV   Neuroadapted Sindbis virus

nsP   nonstructural protein

NF-κB   Nuclear factor kappa B

PAMP   Pathogen-associated molecular patterns

POGD   Pogosta virus disease

PRR   Pattern recognition receptors

RIG-I   Retinoic-acid inducible gene I

RRV   Ross River virus

RRVD   Ross River virus disease

SFV   Semliki Forest virus

SFC   Semliki Forest complex

SINV   Sindbis virus

SLC   Sindbis-like complex

SIRS   Systemic Inflammatory Response Syndrome

VEE   Venezuelan Equine Encephalitis virus

CHAPTER ONE

INTRODUCTION

OVERVIEW OF ALPHAVIRUSES

Genetics and Epidemiology of Alphaviruses

Alphaviruses are mosquito-borne viruses containing a positive-sense single-stranded RNA genome and are members of the *Togaviridae* family. Most alphaviruses are found within an enzootic cycle in nature, cycling between a mosquito vector and a principle vertebrate reservoir host, consisting of a small mammal or bird. Occasionally, alphaviruses are transmitted through the bite of a mosquito to large mammal hosts, such as horses, turkeys, and humans, resulting in an epizootic transmission cycle. Infection of large mammal hosts can be divided into two categories based on the outcome of disease (281). New World alphaviruses are generally associated with fever, moderate to severe encephalitis, and occasionally resulting in fatal disease (249). Old World alphaviruses are generally associated with fever, rash, and debilitating and often persistent polyarthritis (145, 249).

New World alphaviruses are primarily found in North and South America and compose of three main virus members, Venezuelan Equine Encephalitis (VEE), Eastern Equine Encephalitis (EEE), and Western Equine Encephalitis (WEE) viruses. VEE is found within an enzootic cycle between *Culex* species of mosquitoes and a small mammal, primarily the cotton rat, spiny rat, and opossums (44, 75, 76, 92, 93, 229, 279). One theory is that viral mutations within the enzootic transmission cycle can result in a crossover to the epizootic cycle resulting in a broader mosquito host range and infection of large mammal hosts, such as humans and horses (29, 30, 94, 280). Infection with enzootic strains of VEE in equines and humans are generally avirulent, with the exception of a few reported cases of human fatalities, however, epizootic strains are virulent and do cause significant disease

(189, 275). In particular, subtype IAB and IC viruses have been estimated to cause between 19-83% mortality in equines and less than 1% in humans, with 4-14% showing neurologic symptoms (26, 64, 189, 228, 275). Most recently, a major outbreak of VEE was reported in 1995 in Venezuela and Colombia, causing disease in nearly 75,000-100,000 individuals and a case fatality rate of 0.7% (4, 5, 284, 284).

EEE virus is maintained within an enzootic cycle by Ornitophillic species of mosquitoes and a narrow host range of Passerine birds (180). During the enzootic cycle, the virus becomes available to other mosquito species, otherwise known as the "bridge vector," that have broader feeding preferences and can result in transmitting the virus to hosts, such as horses and humans (109).   Although EEE virus has a low incidence rate within the Americas (8.2 cases per year within the United States), it is the most pathogenic of the encephalitic alphaviruses, causing between 35-70% mortality, with children and the elderly being most susceptible (1, 28, 36).

Similar to EEE virus, WEE virus is maintained by a bird/mosquito enzootic cycle and is a significant equine pathogen. WEE virus was first isolated from equines suffering encephalomyelitis during an outbreak in 1931 in the San Joaquin Valley (175). An estimated 6,000 equines showed signs of neurologic disease and approximately 3,000 equines succumbed to infection (175). WEE virus is thought to have evolved from a recombination event between a Sindbis-like virus and EEE virus that probably occurred between 1,300 and 1,900 years ago (103, 283). Finally, similar to VEE and EEE virus, WEE virus poses a significant threat to humans due to possibility of transmission via the aerosol route (206).

Old World alphaviruses can be found throughout most of the world, including Africa, Asia, Australia, and Europe. Similar to the New World alphaviruses, Old World

alphaviruses are maintained in nature either by a mosquito/bird or mosquito/large vertebrate host cycle. Old World alphaviruses can be divided up into three main groups based on their antigenic complexes, namely Semliki Forest complex (SFC), Bharma Forest complex (BFC), and Sindbis-like virus subcomplex (SLC) within the WEE virus complex (204).

The SFC consists of several viruses that are significant causes of human polyarthritis and rash. Ross River virus (RRV), which is endemic to Australia, spread to the island of Fiji causing a major outbreak in 1979 (6, 21, 66). On the island of Fiji alone, an estimated 500,000 individuals were infected and about 50,000 patients showed symptoms of severe pain in one or more joints, rash, and fever (6, 21, 66, 258). Chikungunya virus (CHIKV), another SFC virus, was first isolated in 1952-53 during an epidemic in Tanzania (162, 168, 210). Similar to RRV, CHIKV causes severe and debilitating polyarthritis, headache, fever, and rash within 2-4 days from initial infection (31, 32). Since the initial discovery of CHIKV, several small-scale outbreaks have been reported in India and Africa. However, in February 2006, a major outbreak of CHIKV was reported on the island of Reunion (54). In a matter of 6-9 months, the virus spread rapidly and reached epidemic proportions in East African countries bordering the Indian Ocean and reaching as far as India and Sri Lanka (2, 3, 235). In India alone, as of October 2006, nearly 1.25 million suspected cases have been reported, although this maybe underestimated due to likelihood of misdiagnosing patients with similar symptoms from dengue virus infection (3, 25, 205, 224).

The BFC consists of one member, Barmah Forest virus (BFV), and is the second most common mosquito-born disease in Australia. The incidence rate has been reported to be lower than that of RRV and appears to be geographically restricted along the Australian coastline (108). However, BFV has the potential to pose a serious public health risk due to

its similarities to RRV and its ability to replicate in greater than 73 species of mosquitoes (184).

The Sindbis-like subcomplex consists of the prototype strain of Sindbis virus (SINV) AR339 and several related strains. AR339 was isolated in Egypt in 1952 from a pool of *Culex* species of mosquitoes (256). Sindbis and Sindbis-like viruses are widespread and can be found throughout Africa, Europe, Asia and Australia (204). However, clinical disease associated with SINV infection is common in geographically restricted areas, such as South Africa, Egypt, and Northern Europe (145). The broad distribution of SINV is primarily due to bird migrations patterns, which serve as the major blood source for vector mosquitoes, and the availability of competent mosquito reservoir hosts (onithophilic mosquitoes of the genra *Culex, Culiseta*) (65, 163). Similar to EEEV, the epizootic cycle involves introduction of the virus to the *Aedes* sp. of mosquitoes to serve as the "bridge vector" (50).

It was not until 1963 in Johannesburg, South Africa, that the first SINV was isolated from a 45-year-old woman suffering from debilitating arthralgia in several joints, maculopapular rash on the trunk and limbs, and fever (166). Comparative studies of this new strain of SINV, named GirdwoodS.A., was found to be antigentically closely related to another SINV strain, AR86, which was isolated from a pool of *Culex* species of mosquitoes near the same area 9 years earlier (166, 285).

In 1982, Ockelbo virus was isolated in central Sweden from a pool of *Culiseta* mosquitoes and found to be the etiological agent for the Sindbis-like human symptoms afflicting the area for the past 15 years (187). The Ockelbo82 isolate cross-reacted with convalescent sera from patients with symptoms of Ockelbo disease and found to be closely related to SINV (187). Similar diseases were reported in eastern Finland and the Karelian

region of the former Soviet Union and were later termed "Pogosta disease" and "Karelian fever", respectively (145). Endemic outbreaks of Pogosta disease are typically found to occur at a frequency of every 7 years and between late July and early October (34, 140). While the exact reason for this cyclical pattern is not yet known, environmental effects and herd immunity in tetranoid birds are thought to play a contributing role (34). Similar frequencies are thought to occur with Ockelbo disease and Karelian fever (145). The last Pogosta disease outbreak occurred in the fall of 2002 in Finland and one study reported that approximately 11% of serum samples from a case study tested positive for antibodies against Pogosta virus, with the highest reported cases from western Finland (15%) to the lowest in northern Finland (6%) (146). Another study reported that nearly 50% of Pogosta virus infected patients with joint symptoms lasted for greater than 12 months (139). Currently there is no vaccine against SINV infections and the only therapy is supportive.

Genome Organization and Virus Life Cycle

Viral Particle

Alphaviruses are plus-strand RNA viruses with a genome of approximately 11.7 kilobases in length. The viral RNA consists of a methylated cap on the 5' end and a polyadenylated tail at the 3' end. The RNA is encapsidated with a single capsid protein arranged with icosahedral surface symmetry consisting of a triangulation number of 4 (T=4) (193). The virion is enclosed within a lipid-protein envelope consisting of a lipid-bilayer derived from the host cell plasma membrane and two envelope glycoproteins (E2 and E1) protruding from the surface. The E2-E1 glycoprotein's form 80 trimeric spikes that are involved in receptor interaction and fusion, respectively (reviewed in reference (249)).

Virus attachment and entry

Alphaviruses have a broad host range which include infecting vertebrate (mammals and birds) and invertebrate (mosquito vectors) hosts, as described in detail earlier. Within each of the hosts, the virus is able to infect a variety of cell types, including but not limited to dendritic cells, muscle cells, connective tissues, and neurons. Similarly, within cultured cells, most alphaviruses are able to infect a variety of cell types with fairly high efficiencies. This indicates that there maybe one or more receptors and co-receptors mediating alphavirus infectivity. Early studies from Wang et al. revealed a 67 kiloDalton (kDa) high-affinity laminin protein as a receptor for SINV entry in mammalian cells (276). This protein was found to be highly conserved in mammals and mosquitoes and expressed in a variety of cell types, which may partially explain the very broad host range of alphaviruses. Furthermore, Wang et al. also identified a distinct 63 kDa protein in chicken cells as a major receptor for SINV, suggesting differences in receptor usage between mammalian and avian host species (276). Ubol and Griffin identified two proteins (110 kDa and 74 kDa), primarily expressed on neural cells at birth, believed to serve as a receptor for SINV (270). However, expression of these proteins was found to be short-lived after birth. In 4-day old mice, expression decreased dramatically, which may explain the age-related susceptibility of SINV infection in mice (270). The identity of these putative attachment receptors has not yet been determined. Furthermore, recent work by La Linn et al. identified the collagen-binding α1β1 integrin as a receptor for RRV, however, its use as a receptor appears to be limited to BFV and RRV and complete inhibition of virus infection, as measured by antibody specific for the

α1β1 integrin, was not achieved (141). This suggests that α1β1 integrin is not the only receptor for RRV.

Identification of attachment receptors for alphaviruses has been complicated by the fact that most alphaviruses rapidly adapt in cell culture to interact with glycosaminoglycans, specifically heparan sulfate, and act as an attachment receptor. Acquisition of this adaptation has a tendency to occur after continuously passaging virus in cultured cells (135). While binding heparan sulfate increases the efficiency of infection in cultured cells, this adaptation has detrimental effects on the virulence of the virus (22, 134). Klimstra et al. has demonstrated that two C-type lectins, DC-SIGN (for DC-specific ICAM-3 grabbing non-integrin) and L-SIGN (for liver/lymph node-specific ICAM-3 grabbing non-integrin), expressed specifically on dendritic cells and on specialized liver and lymph node endothelial cells, respectively, can be used as co-receptors for SINV (133). Treating cells with inhibitors to DC-SIGN or L-SIGN, such as yeast mannan, EDTA, or antibody considerably reduced infectivity (133). C-type lectins contain a carbohydrate recognition domain that can bind carbohydrate structures in a Ca++ dependent manner (295). Based on this feature, Klimstra et al.. also discovered that viruses grown in mosquito cells, more efficiently bind to DC-SIGN or L-SIGN expressing cells as compared to virus grown in mammalian cells (133). This finding suggests that carbohydrate modifications on the viral glycoproteins alter the efficiency of virus to bind to DC-SIGN or L-SIGN. Furthermore, it has been demonstrated that mosquito-grown RRV virus, which has simple glycans on the glycoproteins, infect dendritic cells with higher efficiencies than mammalian-grown virus consisting of complex glycans (133, 237).

Alphaviruses are thought to deliver their genome RNA into the host cell cytoplasm of a cell through one of two pathways, direct fusion of the virion with the host cell plasma membrane or receptor-mediated endocytosis (38, 42, 55, 114, 131). This has been a subject of significant controversy in the literature. In the pathway involving direct fusion with the cell plasma membrane, it is believed that the first event triggering fusion is interaction of the E2 glycoprotein with a receptor at the cell surface (63). This interaction triggers a structural rearrangement of the heterodimeric E2-E1 glycoprotein spikes (176), resulting in fusion with the cell plasma membrane at neutral pH and the subsequent release of the virion genome RNA into the cytoplasm (194). These studies were completed using the SINV strain AR339 and several laboratory strain variants. Early studies demonstrated that fusion occurred independent of the endocytosis pathway by using lysosomotropic weak bases, such as chloroquine and ammonium chloride and a temperature-sensitive Chinese hamster ovary mutant cell line defective in its ability to acidify endosomes (38, 42, 52). However, a conflicting report from Glomb-Reinmund and Kielian demonstrated that SINV and SFV fusion occurs in the endosome in a pH-dependent manner (88). Studies by Flynn et al. demonstrated that the E2-E1 glycoprotein spikes undergo structural rearrangement at neutral pH (63), while Meyer et al. demonstrated that the structural rearrangements could be mimicked in vitro by treatment with low pH, brief exposure to heat, or treatment with a reducing agent (176). Furthermore, Abell and Brown demonstrated that thiol-disulfide exchange reactions were integral to the fusion event, which has been shown to disrupt the rigid E1 glycoprotein associations, a step which is suggested to directly follow the structural protein rearrangement (7).

In regards to the receptor-mediated endocytosis fusion pathway, studies were initially conducted using SFV and later studies directly compared SINV to SFV. Early studies, demonstrated that SFV enters the cell through a receptor-mediated endocytosis pathway (114, 167, 290). Using a liposome-based fusion assay to analyze alphavirus entry, studies showed that fusion was pH-dependent and required cholesterol and sphingolipids (130, 185, 289). Studies with SFV have shown that the virus is first internalized upon receptor-recognition at the cell surface, followed by uptake into a relatively neutral pH containing endocytic compartment, delivery to an early endosome with an acidic pH, transition to a late endosome with a lower pH, and fusion between the viral membrane and the lysosomal membrane, and finally delivery of the viral genome into the cytoplasm (114, 230). Similar to studies with SINV, conformational changes were also observed with SFV structural proteins upon low pH treatments, however unlike SINV, virions exposed to low pH were able to fuse directly to the plasma membrane, suggesting treatment with an acidic pH is all that is required to trigger the fusion event (33, 85, 272, 273, 290).

Interestingly, both competing groups agree that the structural rearrangement induced by exposure to low pH results in the formation of E1 homotrimeric proteins (194, 272). Isolation and crystallization of these trypsin-resistant E1 trimeric proteins revealed that five of these trimers assemble to form a fusion pore (85, 288). These fusion pores are believed to facilitate cell penetration (194, 288).

Viral RNA Synthesis

Upon successful entry and uncoating, the genomic RNA is immediately translated by the host cell translation machinery. The first of the viral proteins that are translated are the viral

nonstructural proteins, producing one of two polyproteins, nsP123 or nsP1234. The larger polyprotein is produced by read-through of an opal termination codon located between nsP3 and nsP4. While most alphaviruses encode for an opal termination codon proximal to the 3' end of the nsP3 gene, the SINV strain AR86 and the prototype SFV strain SFV4 carry a sense codon (241, 255). Translation readthrough of the opal termination codon occurs at a frequency of about 5-20%, thus limiting the quantities of the nsP4 protein relative to the other nonstructural proteins (158, 160).

The nonstructural coding region encodes for four nonstructural proteins. Nonstructural protein 1 (nsP1) is involved in capping newly synthesized genomic and subgenomic viral RNA's by functioning as a guanine-7-methyltransferase and guanylyltransferase (8). In addition, nsP1 has been shown to be tightly bound to host cell membranes and is palmitoylated (9, 142, 199). NsP2 is a multifunctional protein exhibiting papain-like thiol proteolytic activity (105), nucleoside triphosphatase activity (208, 271), and helicase activity (89). The nsP2 protease is responsible for cleaving the various nonstructural polyprotein precursors and, as a result, plays an important role in regulating viral RNA synthesis.

The nucleoside triphosphatase activity initiates the viral RNA capping process by cleaving the γ-β 5'triphosphate bond from newly made viral RNAs followed by the addition of a methylated cap by nsP1 (8, 271). NsP2 also is believed to play a major role in regulating virus-host interactions. NsP2 has been found to be important in regulating virus-induced host translation and transcription shutoff (77, 90). Approximately 50% of nsP2 is found within the nucleus of infected cells, however, its role in the nucleus is not well understood (90, 200, 209).

NsP3 is a phosphorylated protein that plays an essential role in viral minus-strand and subgenomic RNA synthesis, however, the functions of nsP3 are not well defined (148, 201, 277). The N-terminus of nsP3 is highly conserved amongst alphaviruses while the C-terminus varies in length and sequence (136, 249). Virus-host interaction studies have shown that nsP3 interacts with several host proteins, however, the importance of these interactions are not well understood (43, 72). In addition, studies have found that nsP3 aggregates to form dynamic spherule complexes within the cytoplasm (72).

The nsP4 protein is highly conserved amongst alphaviruses and functions as the viral RNA-dependent RNA polymerase and was found to possesses terminal adenylyltransferase activity (249, 261). NsP4 is also found to be tightly regulated within infected cells, in that excess nsP4 is highly unstable and shown to rapidly degrade by the N-end rule pathway (48).

Virus minus-strand RNA synthesis is initiated when nsP4 is cleaved from the nascent nonstructural polyprotein at the nsP3/nsP4 site by the nsP2 protease and associates with nsP123 to form the nsP123-nsP4 complex (150-152, 239, 277). This viral replication complex forms on the cytoplasmic side of endosomes and lysosomes and is capable of binding viral genome RNA and synthesizing 49S minus-strand RNA (70, 74, 149, 218). Subsequent cleavage at the nsP1/nsP2 site forms a short-lived nsP1-nsP23-nsP4 replication complex capable of minus-strand and plus-strand synthesis. The final cleavage event at the nsP2/nsP3 site forms the stable nsP1-nsP2-nsP3-nsP4 replication complex that is active in genomic plus-strand RNA synthesis and recognition of the internal 26S promoter in the minus-strand template to initiate 26S subgenomic RNA synthesis (239, 253). It is thought that cleavage of the nsP2/nsP3 serves to halt minus-strand RNA synthesis and amplify 26S subgenomic RNA synthesis. Minus-strand RNA synthesis occurs early during infection,

requires ongoing protein synthesis, and typically ceases between 3-6 hours post-infection while plus-strand RNA synthesis is ongoing for the remainder of the viral life cycle (152, 223).

Virus assembly and budding

The subgenomic RNA is translated to make the structural genes. The structural proteins are translated as a single polyprotein and the capsid protein, which is the first protein translated, is processed from the nascent polyprotein by the autoprotease activity of the capsid protein (11, 104, 173). Once the capsid protein is released, an exposed signal peptide in pE2 translocates the pE2-6k-E1 polyprotein into the lumen of the ER (24, 79, 80). Following insertion into the ER membrane, a host signal peptidase is believed to cleave pE2 from pE2-6K-E1 polyprotein (159). This cleavage event exposes a signal sequence on the N-terminus of 6K and leads to ER membrane translocation of the 6K-E1 polyprotein (174). While in the ER, the E1 protein is folded into its mature metastable conformation by changes in disulfide bonding (183). In addition, PE2 heterodimerizes with E1 to form the premature viral glycoprotein followed by formation of the glycoprotein spike, consisting of three sets of the PE2-E1 heterodimer (20). During transport through the secretory pathway to the plasma membrane, fatty acid modifications and oligosaccharide modifications are added to the glycoproteins (as reviewed in reference (249) and PE2 is processed by the host endoprotease furin to form the mature E2 glycoprotein (222, 296).

Budding is initiated by oligomerization of the capsid protein and encapsidation of the viral genome RNA. Selection of the viral genome RNA occurs by interaction of a packaging signal within nsP1 or nsP2, which varies depending on the strain of alphavirus, along with a

stretch of 68 amino acid residues in the capsid protein (71, 84, 286, 287). One-to-one interactions between the cytoplasmic tail of the E2 glycoprotein spike and the capsid protein initiates assembly of the capsid core (as reviewed in reference (81). Once fully assembled, the virion contains 240 copies of the capsid protein and 240 copies of the E2 and E1 glycoproteins in the envelope (193). The mature virion buds from the plasma membrane and into the extracellular space.

ANIMAL MODELS TO STUDY HUMAN-DISEASE ASSOCIATED WITH ALPHAVIRUS INFECTIONS

Virus-induced bone/joint disease

Old world alphaviruses are a general cause of infectious rheumatic complaints, including severe arthritis, arthralgia, and myalgia (98, 145). Ross River virus, Sindbis group viruses, Chikungunya virus, O'nyong-nyong virus, and Mayaro virus are recurrent problems in Australia, Southern Africa, Northern Europe Asia, and South America where they cause several hundred to several thousand reported cases of disease each year (2, 3, 145, 252, 257). Of note, Mayaro virus is the only arthralgia-inducing alphavirus located in the Americas (259). However, many of these viruses, including RRV, O'nyong-nyong, and CHIKV, are also capable of emerging in new locales and causing epidemics on a massive scale, as illustrated by an outbreak of RRV disease (RRVD) in the South Pacific in 1979-80 that affected 50-60,000 individuals just on the island of Fiji (6, 66, 106), an outbreak of O'nyong-nyong in the 1960's that caused upwards nearly two million cases of infectious arthritis/arthralgia (292), and currently an outbreak of CHIKV of epidemic proportions

affecting individuals residing in East African countries bordering the Indian Ocean, India, and Sri Lanka (2, 3, 54, 235).

Disease manifestations vary among arthritogenic alphaviruses. RRVD is characterized by rheumatic manifestations characterized by arthritis in several joints and constitutional effects, including fever, myalgia, and general fatigue (106). The rheumatic manifestations can range from mild pain in one or more joints to tenderness and swelling with restricted movement (106). Sindbis virus-induced disease can generally be characterized as arthralgias with pain in and around tendons and other tissues surrounding the joint. In particular, Pogosta disease (POGD), Ockelbo disease, and Karelian fever disease are characterized by fever, rash, arthralgia (139, 269).

Though alphavirus-induced arthridities are generally found to be acute and self-limiting (145, 252), the rheumatic symptoms can be severe and are often debilitating. In many reported cases of alphavirus-induced arthridities, a subset of patients complain of chronic pain and sometimes loss of function in one or more limbs (145). Approximately 50% of patients showing typical signs of RRVD experience additional symptoms of headache, lethargy, and maculopapular rashes (66, 106). Whether these symptoms are directly related to virus infection or other factors is not well understood.

Although alphavirus-induced arthridities can be extremely severe, the mechanisms underlying alphavirus-induced arthritis/arthralgia in humans are not well understood. Direct viral replication in and around affected joints and connective tissues are thought to contribute to disease. This is largely based on studies of individuals suffering from RRVD, where viral nucleic acid or antigen have been recovered from synovial aspirates (68, 243). In the case of patients suffering from POGD, skin biopsies and blood samples have tested positive for viral

nucleic acid and infectious virus from patients showing symptoms of POGD (140). It is believed that individuals suffering from chronic arthralgia, who previously tested positive for IgM or IgG antibodies to Pogosta virus (POGV), Ockelbo virus, or RRV is due to viral persistence in and around joints, connective tissues, or surrounding muscle (144, 186, 188, 243).

While attempts at recovering POGV from human synovial fluids have been unsuccessful, it is thought that virus persistence within the synovium contributes to prolonged joint symptoms often seen with a majority of individuals (146). This is supported by the finding that IgM specific antibodies can be detected several months to years after initial onset of symptoms (144). However, an IgM specific antibody response typically wanes 1-2 weeks after initial exposure to an antigen and correlates with an increase in an IgG specific antibody response. Therefore, it is unclear as to how a persistent POGV in a human can lead to a persistent IgM antibody response that can be detected several months after initial infection. Way et al. has shown that RRV is capable of persisting in macrophages, a predominate cell type present in synovium effusions recovered from RRVD patients (278). However, RRV has not been isolated from patients several months or years after the initial onset of RRVD and the mechanism of persistence is not well understood.

Although the immune response to alphavirus-induced arthralgia/arthritis in humans has also not been well characterized, studies with RRVD and POGD patients have provided limited, but significant, insight into the immune response to virus infection. Synovium effusions from RRVD patients were found to be mainly composed of macrophages, CD4+ T cells, B cells, and monocytes (68, 69, 110, 243). IFN-γ, mainly produced by natural killer (NK) cells and T cells, has also been shown to be present in synovial effusions, although its

role in recovery from RRVD is not well understood (213). Histological examination of maculopapular rashes from recovering RRVD patients were composed mainly of infiltrating CD8+ T cells, suggesting a role for lymphocytes in resolution of virus infection (67). In addition, examination of skin lesions from POGD patients revealed pronounced lymphohistocytic inflammatory infiltrates and lymphoblast-like cells (17).

In order to better understand the pathogenesis of arthritogenic alphaviruses, improved animal models would be useful for dissecting the pathogenesis of these diseases. To date, two mouse models have been reported for studying the pathogenesis of alphavirus-induced arthritis and myositis. Heise et al. found that the SINV strain AR86 replicates in bone and joint associated connective tissues of adult mice (111). However, no virus-induced disease was observed, thus limiting the potential of this model for studying the pathogenesis of SINV-induced arthritis/arthralgia. Morrison et al. recently reported a mouse model to study RRV-induced arthritis and myositis (182). In this study, RRV was found to replicate in bone, joint, and skeletal muscle tissues. Furthermore, macrophages, NK cells, CD4+ and CD8+ T cells were detected in skeletal muscle of RRV-infected mice, which is similar to synovial effusions and skin biopsies from patients suffering RRVD, suggesting that the mouse model reflects a valid system for studying RRV pathogenesis (243). However, for alphaviruses such as SINV, which cause arthralgias without overt inflammation, the RRV mouse model is likely to be of limited use in understanding the pathogenesis of virus-induced arthralgias. Therefore, improved animal models are needed if advances are to be made in studying the mechanisms underlying alphavirus-induced arthralgia.

Alphavirus-induced neurologic disease

Sindbis virus

While infection of mice with SINV causes arthralgia in humans, infection of mice provides an excellent model for studying virus-induced neurologic disease. The outcome of SINV infection in mice has been well characterized and found to correlate with the age and strain of the animal, virus dose, route of inoculation, and the virus strain (99, 100, 143, 267). Infection of neonatal mice with most SINV strains result in a lethal disease characterized by elevated levels of proinflammatory cytokines and high viral titers in the muscle, brain and serum, without causing overt encephalitis (134, 263, 264). Increasing the age of the animal or infecting with an attenuated mutant virus results in a shift from the systemic disease towards a central nervous system (CNS)-specific disease (262). However, infection in adult mice is limited in that most Sindbis-group viruses are avirulent in mice greater than 14 days of age, with the notable exceptions of the neuroadapted Sindbis virus (NSV) and the Sindbis-group virus strain AR86, which are lethal in adult mice (101, 214, 241).

Most laboratory variants of SINV have originated from the original SINV strain AR339, with the notable exception of AR86 and Girdwood. The AR339 strain of SINV was first isolated in 1952 from a pool of *Culex pipiens* and *C. univittatus* mosquitoes near Egypt, Cairo (aptly named after the Sindbis district from where it was isolated) (256). Although the exact passage history of the AR339 strain from when it was isolated is not known, it is believed to have been passaged 8 times in suckling mouse brains (personal communication R.E. Johnston) and further propagated by laboratories through either chicken embryo fibroblasts or baby hamster kidney cells, giving rise to several laboratory strain variants, such as HRsp (small plaque), HRlp (large plaque), SB, or SB-RL, or were additionally passed through neonatal and weanling mouse brains (14, 19, 101, 285). One such virus was NSV,

which was passed serially six times by intracranial inoculations of alternating passages between neonatal and weanling mouse brains (101). Klimstra et al. constructed an infectious cDNA clone for AR339 by assembling the consensus sequences of several laboratory variants and eliminating several predicted cell-culture adapted mutations. The predicted sequence is believed to be very close to the original AR339 isolate, prior to propagation in cell culture. The virus produced from the infectious clone is was called TR339 and is known as the prototypic SINV strain (135).

Two notable low cell culture passaged Sindbis-group virus isolates are AR86 and Girdwood. AR86 virus was isolated from a pool *Culex* mosquitoes near Johannesburg, South Africa in 1955 (285). Girdwood virus was recovered from a human patient near Johannesburg, South Africa suffering from febrile illness, rash, and arthritis and has the distinction of being one of the first Sindbis viruses to be isolated from a human case (166). It was immediately clear from antigenicity tests that Girdwood was closely related to AR86 and sequencing revealed that these two strains differed by only 22 single amino acid coding changes plus an 18 amino acid deletion in nsP3 (166, 241).

This next section on SINV pathogenesis describes three murine models to study SINV-induced neurologic disease. TRSB and TR339 virus infection of newborn mice have served as models to study virus-induced systemic inflammatory response syndrome (SIRS). SV (the strain of NSV prior to neuroadaptaion) and NSV infection of adult mice have served as models to study nonfatal and fatal virus-induced encephalitis, respectively. Finally, AR86 infection of adult mice has served as a model to study virus-induced neurologic disease.

TRSB/TR339

Early reports with a virulent strain of AR339 virus in newborn mice showed high levels of replication in the CNS, robust induction of type I interferon (IFN), and it was believed that the infected mice were succumbing to infection from a fatal acute encephalitis (207, 238). Two laboratory strain variants of AR339, Toto1101 or HRSP, were found to be attenuated in newborn mice and showed virus-specific IgG antibody production seven days after infection, which correlated with virus clearance (164, 203, 238). Studies with the TRSB strain, derived from an infectious cDNA clone for the SB strain, showed a lack of encephalitis and a more systemic inflammatory response (263). Several reports attributed these differences in virulence and immune response to mutations within the E2 and E1 glycoproteins (134, 263).

In support of this, Klimstra et al. demonstrated that virus passaged repeatedly in cell culture attenuated virulence because the virus readily adapted to using heperan sulfate as an attachment receptor (135). These adaptive mutations were linked to the E2 and E1 glycoproteins (135). Therefore, the differences in virulence between various the laboratory strains is most likely attributed to their passage history. Klimstra et al. also suggested that SINV strains that are more closely related to the consensus sequence of AR339 cause a lethal systemic inflammatory response, while attenuated laboratory strain variants cause an encephalitic disease in neonatal mice (134). Comparing infection of neonatal mice with TRSB and an attenuated mutant TRSB (TRSB-114) virus, which contains a cell-culture adapted mutation in the E2 glycoprotein at position 114, found that TRSB infected mice caused a lethal disease with no overt signs of virus-induced encephalitis, while the mutant TRSB-114 was attenuated in virulence and showed signs of encephalomyelitis (263). Further characterization of TRSB infected mice showed virus replication in several tissues, induction of proinflammatory cytokines, including type I IFN and tumor necrosis factor-α

(TNF-α), and induction of the stress hormones corticosterone and adrenalcorticotropin-releasing hormone (ACTH) (262).

Studies with TR339 showed similar virus localization and immune response in newborn mice, showing strong induction of proinflammatory cytokines, including type I IFN, TNF-α, IFN-γ, IL-12(p40), and IL-6, and induction of a stress response (134). Based on the similarities to LPS-treatment in newborn mice, it is believed the TR339 or TRSB infected mice are most likely dying from SIRS.

Virulence studies by Tucker et al., using two laboratory strains of SINV (TE and TES), found that an Arg to Gly (virulent) change at E2 position 172 was an important virulence determinant in newborn mice (265). The mechanism of this determinant is believed to correlate with kinetics of viral RNA synthesis, penetration and infection of neuronal cells, and viral growth in the CNS (265). Furthermore, sequence comparison between AR339 and NSV revealed that a His at position 55 in the E2 glycoprotein in combination with the Gly at position 172 in E2 are important virulence determinants in newborn and weanling mice (164, 267).

SV and NSV

The laboratory adapted SINV strain SV causes fatal disease in newborn mice and nonfatal encephalitis in adult mice (207). In contrast, NSV, causes acute fatal encephalitis in adult mice (101). These two SINV laboratory strains have served to study the molecular pathogenesis of virus-induced acute nonfatal and fatal encephalitis. Infection of adult mice with SV causes acute nonfatal asymptomatic encephalitis. After an i.c. inoculation, SV replicates primarily in neurons in the brain and the spinal cord. Virus replication peaks by

day 2 and detectable infectious virus is cleared by day 7. Perivascular cuffing in the CNS, representing infiltrating mononuclear inflammatory cells, are first observed between day 3 and 4, and peak by day 8 (169). The infiltrating inflammatory cells consisted mainly of CD4+ and CD8+ T cells, B cells, and macrophages/microglia (179, 212). SV infection also resulted in the induction of several proinflammatory cytokines, including IL-6, IL-1β, and TNF-α. Cytokine induction was found to correlate with a Th1 immune response, characterized by low levels of IL-4, high levels of IL-10 producing CD4+ T cells, and induction of IL-12, IL-2, and IFN-γ (212).

The appearance of virus-specific neutralizing antibodies correlated with clearance of infectious virus from the CNS (156). Although infection of adult *scid*/CB17 mice with SV exhibit nonfatal persistent infection, infectious virus was readily cleared by administering virus-specific monoclonal antibodies to the E2 glycoprotein. *In vitro* studies of viral persistence in primary neuronal cultures were shown to be cleared of virus upon treatment with neutralizing E2-specific monoclonal antibodies (156). In contrast, adoptive transfer of sensitized T cells into SV-infected scid/CD17 mice had no effect on persistence. Interestingly, SV-infected μMT mice, which lack functional B cells, showed clearance of infectious virus in a tissue-specific manner. While virus in the brain showed intermediate clearance in μMT and μMT mice depleted for CD8+ T cells, the spinal cord exhibited clearance similar to that seen in wild-type C57BL/6 mice. Because virus was cleared from the spinal cord in the absence of antibody and the fact that no μMT-infected mice showed clinical signs of infection, this suggested an additional mechanism of viral clearance from neurons in a noncytolytic manner. Using a double-promoter expressing virus, it was demonstrated that interferon-γ contributed to the noncytolytic clearance of virus from

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neurons (23).   Additional studies confirmed this observation in two different persistently infected neuronal cell lines (35).   The mechanism underlying IFN-γ mediated neuronal clearance is not well understood, however, it is believed to induce antiviral factors that alter the efficiency of virus production in infected neurons (35).

In contrast to SV-infected adult mice, NSV-infected mice displayed clinical signs that are characteristic of an acute encephalitis, such as ruffled fur, hunched posture, and hind limb paralysis. Mice typically succumb to NSV-induced disease between 6-11 days post-infection with an average mortality rate between 80-100%, depending on the strain of the mouse (101, 118). From an intranasal route of inoculation, NSV replicates in neurons within the olfactory region and spreads to the brain along neuronal tracts. By day 4, NSV antigen and viral RNA was shown to disseminate into large foci in the brain and virus appears to primarily localize to neurons (260). Following an i.c. inoculation, NSV infects neurons primarily in the hippocampal gyrus, the adjacent white matter, and the cerebral cortex (132). Regardless of the route of inoculation, NSV causes significant neuronal loss.

To understand the immune components involved in fatal NSV infection, Griffin et al. passively transferred immune sera, spleen cells, or lymph node cells (LNC) from mice surviving nonfatal NSV infection and found that only immune sera significantly protected mice from a lethal challenge of NSV (101). This study suggested that virus-specific IgG or IgM antibodies play an important role in protection against NSV infection, similar to the observations with persistent SV infection.   Infection of mice depleted of CD4+ T cells revealed reduced hippocampal damage and fewer infiltrating macrophages while mice depleted of CD8+ T cells showed more extensive hippocampal damage and greater infiltrating macrophages (132, 211). These results suggest that T cells may play an important

role in contributing to mortality following NSV infection. This is supported by work from Rowell et al.. that showed TCR-/- (α and β/δ) mice, CD4-/-, and CD8-/- mice displayed reduced virulence from NSV infection (211). In addition, nearly all of the IFN-γ knockout mice were protected from NSV infection. This study demonstrates a role for immune-mediated pathology to virus-induced fatal encephalitis. Furthermore, this result also demonstrates an alternative role for IFN-γ during fatal encephalitis, as compared to its role during nonfatal encephalitis.

To identify the genetic determinants of virulence within SV and NSV, initial studies using a panel of monoclonal antibodies to E1 and E2 showed that the E2 and E1 glycoproteins were altered during neuroadaptation with NSV and suggested this region of the genome was important for virulence in adult mice (247). Subsequent studies identified a His at position 55 in the E2 glycoprotein as major determinant of NSV adult mouse neurovirulence (164, 267). In addition to its importance as a virulence determinant in newborn mice, Gly at position 172 in the E2 glycoprotein appeared to play a minor role in adult mouse virulence (265). The mechanism underlying the His 55 determinant is not yet completely understood, however, E2 His 55 was shown to correlate with improved binding and entry into neuronal cells (266), increased levels of viral replication (51), and the ability to overcome the protective effect of bcl-2 overexpression in neurons (155, 157).

AR86

Previous studies from our laboratory with AR86 have demonstrated that a Thr at position 538 in nsP1, plays an important role in adult mouse neurovirulence. Replacing this Thr of AR86 with an Ile, found in most Sindbis-group viruses, attenuates virulence in adult mice, while

introduction of the Thr into a non-neurovirulent laboratory strain of SINV leads to an increase in neurovirulence (112). Introduction of the attenuating Ile at position 538 in nsP1 did not adversely affect viral growth, as viruses that contained this change replicated at least as well as wild-type AR86 virus both in cell culture and in the brains of infected animals (112). Furthermore, the presence of an Ile at nsP1 538 accelerated processing of the nonstructural polyprotein precursor nsP123 and showed earlier induction of viral 26S RNA synthesis (113).

In an effort to further define the viral determinants that contribute to adult mouse neurovirulence, mapping studies were conducted between the neurovirulent Sindbis-group virus strain AR86 and the closely related but avirulent Girdwood strain. This study identified four major genetic determinants of adult mouse neurovirulence within the SV AR86 (254). These determinants localized to the nonstructural coding region, at nsP1 position 538 (Ile=non-neurovirulent, Thr=neurovirulent), at nsP3 positions 386-403 (18 amino acid deletion= neurovirulent), and at nsP3 position 537 (Opal termination codon=non-neurovirulent, Cys=neurovirulent), as well as one change in the structural coding region at E2 position 243 (Leu=non-neurovirulent, Ser=neurovirulent). Although the attenuating mutations result in a loss of virulence, *in vitro* replication kinetics were not adversely affected and the attenuated mutant AR86 viruses were able to establish infection in the brain and spinal cord as efficiently as the virulent wild-type AR86 virus (254).

While the attenuated and virulent viruses appear to establish infection within the CNS with similar efficiency, at late times post-infection the wild-type AR86 virus continues to replicate throughout the brain while the attenuated mutant S363 virus, containing the four attenuating determinants in the AR86 background, was cleared (254). This suggests that the

mutant S363 virus may be controlled more effectively by the host immune response than the wild-type AR86 virus. This is supported by the observations that the mutant S363 virus induces higher levels of inflammation within the brain than wild-type AR86 virus (Suthar M.S. and Heise M.T., unpublished data). Furthermore, while the mutant S363 virus shows no mortality in wild-type C57BL/6 mice, this virus causes 100% mortality in RAG-/- mice, suggesting that the adaptive immune response is required to protect against the mutant S363 virus-induced disease (Suthar M.S. and Heise M.T., unpublished data). This is further supported by preliminary studies where the mutant S363 virus induced higher levels of CD8+ T cells and macrophages at late times post-infection as compared to the wild-type AR86 virus (Suthar M.S. and Heise M.T., unpublished data). These results were further corroborated by the finding that introduction of the attenuating Ile at nsP1 538, led to more type I IFN induction both *in vivo* and *in vitro* as compared to wild-type AR86 virus.

Venezuelan Equine Encephalitis virus

VEE virus infection in equines and humans can be grouped based on their virulence, with the avirulent viruses generally causing no overt signs of infection and virulent viruses causing fairly high mortality in equines (20-80%) and febrile illness associated with neurologic disease in humans. The avirulent viruses are generally associated with the enzootic subtypes IE, II, III, IV, while the virulent viruses are generally associated with the epizootic subtypes IAB and IC (as reviewed in reference (282)). Experimental infection with VEE virus in the laboratory has been shown to cause fatal disease in several mammalian species (37, 236, 274). Early studies in guinea pigs revealed that the epizootic strains (with the exception of ID) caused fatal disease while enzootic strains were avirulent (120, 227). VEE viral

pathogenesis has been studied extensively in the mouse model using the subtype IAB Trinidad donkey strain (TRD). This strain was isolated in 1943 from the brain of a donkey and was passaged once in a guinea pig brain followed by 13 passages in embryonated chicken eggs (178). Infection of mice with the TRD virus results in a fatal disease, regardless of the age, strain, or route of inoculation, although mice infected from the intranasal or intracranial routes appear to more rapidly succumb to virus-induced disease (40, 137, 138, 248). Infection of adult mice with TRD virus causes encephalomyelitis, similar to infections seen in humans and equines (137).

Following peripheral inoculation of VEE virus in adult mice, disease progression is characterized in two phases, a lymphoid phase and a CNS phase (39, 45, 96). Immediately after a footpad inoculation, VEE virus migrates to and replicates in the draining popliteal lymph node. Studies by Macdonald et al. have demonstrated using viral replicons that following a subcutaneous inoculation in the footpad, Langerhan cells, which reside in the skin, are readily infected (165). Extensive replication in the lymph node likely seeds a high-titered viremia by 18 hours post-infection, followed by virus infection in several vascularized organs, including spleen, thymus, heart, lung, kidney, liver, and adrenal glands (39). Detectable VEE virus in the serum begins to wane upon production of VEE-specific IgM responses by 72 hours post-infection (39). The CNS phase is characterized by virus entry into the CNS and extensive replication in neurons and astrocytes (232). A robust immune response within the CNS leads to high levels of inflammation in the brain and immune-mediated pathology (39, 231). Virus infection of the peripheral nervous system is believed to be seeded from virus that is circulating in the blood or through infection of olfactory or dental neuronal tissues (45, 96). Removal of the olfactory bulb or neuroepithelium by

surgery or chemical disruption, did not prevent VEE virus from entering the CNS from an intranasal route of inoculation, suggesting other peripheral nervous system pathways by which the virus can gain entry into the CNS (40).

It has become apparent through several studies that both viral and host factors contribute to VEE virus-induced encephalitis. Infection of mice with an attenuated VEE virus, such as TC-83, leads to a protective immune response (41, 245). In contrast, infection with virulent VEE virus leads to a fatal and cytotoxic immune response in the CNS. Greider et al. identified three attenuating mutations within the E2 and E1 glycoproteins which were defective in spreading to the draining lymph node (DLN), producing a high titer viremia, or efficiently invading the CNS (96). These virulence determinants localized to E2 Lys at position 76, E2 Lys at position 209, E2 Asn at position 239, and E1 Thr at position 272 (96).

The host innate and adaptive immune responses have been shown to be important in controlling VEE virus infection. Cytokine expression analysis within the DLN upon a footpad inoculation with wild-type VEE virus (V3000) showed elevated levels of IFN-γ, IL-6, IL-12, IL-10, and TNF-α (95). Several lines of evidence have demonstrated the importance of type I IFN in protecting mice from virulent VEE virus infection and disease. Infection of IRF-1-/- mice, IRF2-/- mice, IFN-α/βR$^{-/-}$ mice with virulent VEE virus exhibited rapid kinetics of death and enhanced viral tropism (97, 233, 291). However, infection of mice treated with pegylated IFN-α prior to infection with VEE virus showed a reduction in virulence (161).

The host adaptive immune response has been shown to be important in protection from lethal VEE virus infection. Vaccination of mice with attenuated VEE mutant viruses (V3032 and TC-83) have shown near complete protection against a lethal challenge with

wild-type VEE virus, further demonstrating a protective immune response induced by attenuated mutants of VEE virus (46, 107, 121). In these studies, protection from the lethal challenge administered s.c. or by aerosol was due to a combination of virus-neutralizing IgG antibodies in the serum and mucosal IgA responses.

Semliki Forest virus

SFV has been shown to experimentally infect several different mammalian species, including rabbits, guinea pigs, hamsters, mice, and rats (27, 57). Infection of mice with SFV has been well characterized and shown to result in one of three disease outcomes—acute encephalitis, demyelinating meningoencephalo-myelitis, or persistent infection of the central nervous system. SFV-induced disease in the murine model depends on the strain of the virus and age and strain of the mouse. All known strains of SFV cause a lethal disease in neonatal and suckling mice (15, 62). The age-dependent susceptibility is observed in mice greater than 14 days of age, in which most SFV strains, including A7, and A7(74), and are avirulent (15, 62, 195). However, the prototype SFV strain SFV4 (derived from an infectious clone) and L10 strain are virulent in adult mice (18, 83, 87).

The SFV AR2066 strain was isolated from a pool of *Aedes argenteopunctatus* mosquitoes in Namancurra, Mozambique and was subsequently passaged seven times intracerebrally in suckling mice giving rise to the A7 strain (170). The A7 strain was further selected from plaques in monolayers of primary chicken embryo cells resulting in the A7(74) strain (27, 251). SFV was originally isolated in 1942 from a pool of mosquitoes in Uganda and was passaged intracerebrally eight times in adult mice followed by two passages through the brains of suckling mice to give rise to the L10 strain (102). The prototype SFV SFV4

strain was derived from an unknown number of passages in BHK cells of the L10 strain, resulting in decreased virulence in adult mice as compared to the original L10 strain (87, 181).

Following peripheral inoculation in adult mice, SFV replicates in smooth, skeletal, and cardiac muscles (57). A transient high titer viremia is seeded by 24 hours post-infection and subsequently leads to neuroinvasion (59). The viremia is controlled and eventually cleared by 4 days post-infection by a combination of virus-specific IgM and IgG antibody responses (59). Interestingly, the virulent (SFV4 and L10) and avirulent (A7 and A7(74)) strains are able to efficiently enter the CNS, suggesting attenuation of the avirulent strains occurs once the virus enters the CNS (18, 191, 197, 198). The mechanism of neuroinvasion by SFV has been well characterized. Early studies by Pathak et al. suggested virus enters the brain from the blood by passing across cerebral endothelial cells (197). Fazakerley et al. confirmed this finding in neonatal and adult mice by observing scattered perivascular foci of viral RNA around cerebral capillaries early during infection (59). Furthermore, SFV may enter the CNS by infecting endothelial cells causing increased permeability of the blood-brain barrier (244). Following intranasal inoculation, SFV is believed to enter through the olfactory neuronal pathway in an age-dependent manner (125, 191).

Several lines of evidence demonstrate that the age-dependent virulence of the A7 or A7(74) strains correlate with the maturity of CNS cells. In neonatal mice, A7(74) is able to enter, producing the perivascular foci, and spread from these foci unhindered throughout the brain (59, 190). However, in adult mice, A7 or A7(74) viruses replicate slower and reach lower peak titers than the virulent SFV4 or L10 viruses (59, 190, 191). *In situ* hybridization, immunocytochemistry, and electron microscopy of A7(74)-infected adult mouse brains show

restricted patterns of virus localization, mostly appearing as small perivascular foci (18, 59, 191, 195, 198). The restricted spread within the CNS has been shown to be independent of a maturing adaptive immune response, in that, *nu/nu* adult mice, which lack T cells, and SCID adult mice, which lack T and B cells, displayed similar patterns of virus localization as compared to wild-type mice (12, 59). Similar results have been reported in mice lacking a functional type I interferon pathway (191). These studies suggest that the defect in viral spread in adult mice is due to alteration in virus infection and/or replication in maturing neurons, however, the mechanism restricting A7 or A7(74) viral replication in the CNS is not well understood.

Viral and cellular factors may contribute to the observed restriction in viral replication, including altered expression of a developmentally regulated neuronal receptor or a developmentally regulated change in neurons that influences the outcome of a productive viral infection. As mentioned earlier, Ubol et al. identified a putative neuronal receptor for the closely related SINV and showed that expression of the receptor diminished with the age of the mouse (270). However, the receptor for SFV has not been identified and have thus limited the studies on this front. On the other hand, pretreatment of adult mice with aurothiolates, which induce smooth endoplasmic membrane production in neurons, followed by infection with the avirulent A7(74) virus caused increased replication, virulence, and displayed similar virus dissemination within the CNS as compared to the virulent L10 virus (171, 172, 226).

Furthermore, electron microscopic studies of CNS cells from adult mice infected with A7(74) indicated a defect in virion maturation. Within these CNS cells, large viral core aggregates, later identified as viral capsid protein, form in the cytoplasm (59, 196). In

contrast to the adult mice, neonatal mice infected with A7(74) show normal virion formation in CNS cells (59).

Studies by Scallan et al. implicated a role for the antiapoptotic factor bcl-2 in limiting viral replication and delaying virus-induced apoptosis (225). However, studies by Murphy et al. showed that SFV-infection induced apoptosis in three cell lines over-expressing bcl-2 with comparable kinetics to cells normally expressing bcl-2 (91). Furthermore, it was shown that SFV overcomes bcl-2 over-expression by proteolytic cleavage and inactivation of bcl-2 (91). The viral function which mediates this activity has not been identified. In addition, the biological significance of this finding is controversial in that staining for bcl-2 in the CNS of adult rats revealed a lack of bcl-2 expression in neurons, the main culprit in restricting viral spread of the avirulent SFV strains in the CNS (219).

Infection of mice with avirulent strains of SFV causes focal demyelinating meningoencephalomyelitis and represents a viral model to study multiple sclerosis (MS) in humans (16, 251). The demyelinating lesions occur in focal areas in the white matter of the CNS and are believed to be caused by the host immune response and direct infection of oligodendrocytes (83, 251). In support of this, infection of SCID mice, *nu/nu* mice, or immunosuppressive therapy, including treatment with cyclophosphamide, γ-irradiation, and cycloleucine, resulted in viral persistence in the CNS without overt signs of cytopathic effect or demyelination (13, 61, 82, 119). Reconstitution of *nu/nu* mice with spleens from *nu/+* littermates or recovery of immunosuppressed mice abrogated viral persistence and resulted in demyelination (13, 58, 82). Furthermore, adoptive transfer studies into immunosuppressed mice, infection of nude mice (T-cell deficiency), and administration of anti-CD4 or anti-CD8 antibodies in wild-type mice demonstrated the demyelination was due to a pathogenic

immune response and, for the most part, mediated by CD8+ T cells (60, 83, 250). Studies by Keogh et al. demonstrated that Th1 CD4+ T cells play an important role in controlling SFV infection in a manner that does not contribute to virus-induced neuropathology (128). Infection of mice deficient for IL-12, a Th1 promoting cytokine, with SFV A7 showed no effect on demyelination, increase in neuronal necrosis, higher virus brain titers, and reduced IFN-γ production as compared to wild-type mice (128). In contrast, infection of mice deficient for IL-4, a Th2 promoting cytokine, showed reduced demyelination, lower virus brain titers, less severe neuronal necrosis, and higher levels of IFN-γ production as compared to wild-type mice (128). In addition, infection of mice genetically deficient in expression of IFN-γ indicated that IFN-γ plays an important role in mediating neuronal necrosis and little, if any, role in mediating virus-induced demyelination (129).

Based on sequences of the virulent and avirulent SFV strains and the availability of a full-length infectious clone (SFV4), several studies were initiated to identify the genetic determinants of SFV virulence. Early studies identified two determinants of adult mouse virulence within the E2 glycoprotein consisting of a Val to Ile at position 37 and Asn to Ser at position 212 (221). Tuittila et al. identified the nsP3 gene as the major virulence factor in SFV4 (268). Within the nsP3 gene, the opal termination codon to an arginine codon at position 469 was found to be a major virulence determinant. Furthermore, several amino acid changes within nsP3, namely Val to Ile at position 11, Ala to Glu at position 48, Gly to Ala at position 70, Leu to Phe at position 201, Asp to Asn at position 249, Thr to Ala at position 435, and Phe to Leu at position 442 were important virulence determinants (268). Introduction of the Arg codon at position 469 and specific combinations of two of the

remaining seven amino acids in nsP3 into the avirulent A7(74) strain fully conferred adult mouse virulence (268).

VIRUS MODULATION OF THE HOST TYPE I INTERFERON INDUCTION

Type I interferon signaling pathway

The innate and adaptive arms of the immune system play critical roles in controlling virus replication, viral clearance, and establishing long-term protection from re-infection. Although the adaptive immune response is critical, this response is ultimately determined by the innate immune response. Type I IFN is the first line of defense for a host cell infected with a virus and is responsible for deterring virus replication and spread within the host.

Interferons were first discovered by Isaacs and Lindenmann in a study that identified a host factor that was induced upon treatment of cells with heat-inactivated influenza A virus (117). This factor was shown to interfere with establishing influenza A virus infection in cells, and thus this interference factor was aptly termed "interferons." An immense amount of research has been conducted on characterizing interferons since this initial observation. Interferons can be grouped into three subsets, type I, II, and III (as reviewed in reference (202)). Type I IFNss are comprised of seven classes, IFN-α, IFN-β, IFN-δ, IFN-ε, IFN-κ, IFN-τ, and IFN-ω. Type I IFNs can be induced and secreted by most cell types. Type II IFN is comprised mainly of IFN-γ and is mainly secreted by T cells and NK cells. Type III IFNs are a new set of IFN genes that include IFN-λ1, IFN-λ2, and IFN-λ3 (otherwise known as IL-29, IL-28A, and IL-28B, respectively). Not much is known about the cell types that primarily produce Type III IFN, however, comparisons have been made to IFN-α/β, and have

been shown to be induced to comparable levels and kinetics as that of IFN-α/β (192). The remainder of this review will focus on virus-host interactions with type I IFN, mainly IFN-α/β.

In humans and mice, there are 13 and 14 subclasses of IFN-α, respectively, and only one class of IFN-β (202). In a virally-infected cell, IFN-β is the first type I IFN to be induced. Secreted IFN-β binds to its receptor on uninfected and infected cells, leading to activation of signaling pathways that result in the synthesis of IFN-α and interferon-stimulated genes (ISG). Type I IFNs can be induced in response to RNA virus infection through two distinct pathways, recognition of viral RNA by toll-like receptors (TLRs) or cytosolic receptors (177).

TLRs are a type of pattern recognition receptors (PRRs) and recognize conserved pathogen specific molecules that are often referred to as pathogen-associated molecular patterns (PAMPs). To date, 13 different TLRs have been identified in humans and mice (10). Of these 13, TLR3, TLR7, and TLR8 are responsible for sensing viral RNA and inducing a proinflammatory cytokine response, particularly type I IFN (10). TLR3 is expressed in myeloid dendritic cells (mDCs), NK cells, neuronal cells, and astrocytes, and is thought to recognize double-stranded RNA (dsRNA) within the endosomal compartment (47, 234). In contrast, TLR7 is expressed in mDCs and plasmacytoid DCs (pDCs), B cells, and monocytes and TLR8 is expressed in NK cells, T cells, B cells, and monocytes (10). Both TLR7 and TLR8 recognize single-stranded RNA molecules present in the endosomal compartment (49, 242). While TLR7 and TLR8 immune effects require the adaptor protein MyD88 for signaling, TLR3 signals through Toll/IL-1 receptor (TIR)-domain-containing adaptor inducing IFN-β (TRIF) in a MyD88-independent manner (10). MyD88-mediated signaling

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activates IRF-7 and NF-κB leading to the induction of IFN-α and ISGs. In contrast, TLR3

signals through TRIF to activate IRF-3 and NF-κB leading to the induction of IFN-β.

While TLR signaling depends on the presence of ssRNA or dsRNA in the endosomal

compartment, dsRNA in the cytoplasm is recognized by a newly identified set of RNA

helicases retinoic acid inducible gene-I (RIG-I) and melanoma differentiation antigen 5

(Mda5) (126, 294). These sensory molecules are found in nearly every cell type analyzed,

except for pDCs (127). RIG-I and Mda5 contain two caspase recruitment domains

(CARDS), helicase domain, and a repressor domain (RD) (217, 294). The repressor domain

has only been identified in RIG-I and has not yet been identified in Mda5. RIG-I is normally

found in an inactive conformation where the RD is interacting with the CARD domains.

Upon binding the RNA substrate, a conformational change occurs that releases the RD

domain and exposes the CARD domain and leads to in RIG-I multimerizing (217). This

complex then signals through the CARD domains to the mitochondrial associated protein

IPS-1, otherwise known as MAVS, Cardif, and VISA (122). This interaction recruits the

"noncononical" kinases IκB kinase-ε (IKKε) and TANK-binding kinase-1 (TBK1). These

kinases phosphorylate IRF-3 at serine residues located between 386 and 403. This, in turn,

leads to the formation of IRF-3 homodimers and translocation into the nucleus.

IPS-1 is also activates the NF-κB signaling pathway through interactions with Fas-

associated death-domain protein (FADD) and receptor-interacting protein (RIP-1) or by

direct recruitment of the inhibitory kappa B kinases (IKK), IKKα and IKKβ (122, 293). IPS-

1 association with FADD and/or RIP-1 is thought activate IKK and cause the

phosphorylation of the NF-κB inhibitor protein, Iκβα, leading to its ubiquitination,

degradation, and release of free NF-κB, which subsequently translocates to the nucleus.

Activation of these signaling pathways results in the formation of the enhancesome consisting of IRF-3, NF-κB, CBP/p300, and ATF-2/c-Jun. The enchancesome binds to the promoter region of the IFN-β gene and activates its transcription (115).

In addition to the recognition of viral dsRNA by TLR3 and RIG-I/Mda5, two other well characterized dsRNA-dependent interferon-inducible pathways are the 2-5A oligoadenylates synthetase/RNaseL and the protein kinase R (PKR) pathways. In response to IFN, host cells synthesize proteins encoded by ISGs. One such pathway is the 2-5A system which is composed of two types of enzymes: 2-5A oligoadenylates synthetases (OAS) and RNaseL. After activation by dsRNA, 2-5A OAS catalyzes ATP to produce 2'5'-linked oligoadenylates (2-5A) that can range from dimers to 30mers (as reviewed in reference (124)). The 2-5A substrates bind to and activate RNaseL, a potent endoribonuclease which specifically cleaves cellular and viral single-stranded RNA substrates and effectively limiting virus replication, inhibiting cell proliferation, and inducing apoptosis.

PKR was one of the first identified dsRNA-dependent interferon-inducible enzymes (as reviewed in reference (147)). PKR is normally found within the cytoplasm of most cells in an inactive state. Upon binding dsRNA substrates PKR homodimerizes and undergoes autophosphorylation. Activated PKR phosphorylates the α subunit of eIF2 thereby inhibiting of host protein synthesis. The effects of PKR can also lead to induction of apoptosis or promote cell survival and this is mediated by association with TRAF and activated IKK (86).

As described in detail below, type I IFN induction and the antiviral response induced through type I IFN signaling play important roles in controlling virus replication in the host. This next section describes alphavirus interactions with the host type I IFN response with

focus on three main topics: Host factors limiting alphavirus replication, viral evasion of type I IFN, and alphavirus modulation of type I IFN.

Host factors limiting alphavirus replication

The direct role of the host type I IFN response to alphavirus infection has been directly analyzed using mice genetically deficient in type I IFN-α/β receptors (IFN-α/βR$^{-/-}$). These mice are unresponsive to type I IFN and exhibit increased susceptibility to a number of viruses, including alphaviruses ((97, 116, 215, 220, 240). While infection of adult wild-type 129Sv/Ev mice with TR339 causes no morbidity or mortality, infection of adult IFN-α/βR$^{-/-}$ mice causes 100% mortality with rapid kinetics of death (average survival time is between 2-3 days postinfection) (215). Infection of the knockout mice exhibited increased viral replication and increased tropism for tissues that normally do not harbor high levels of replication, including the stomach, pancreas, intestines, and heart. Furthermore, DCs derived from IFN-α/βR$^{-/-}$ mice exhibit increased susceptibility to alphavirus infection (215, 237).

Interferon-stimulated genes have also been shown to play an important role in controlling alphavirus infection. Microarray analysis of SINV-infected brain tissue showed that several host antiviral response genes were upregulated, including IRF-1, IRF-7, Stat1, and ISG15 (123). Interestingly in this study, PKR and the coupled 2-5A synthetase/RNaseL genes were not found to be upregulated. In support of this finding, Ryman et al. demonstrated that PKR/RNase/Mx-1 did not play a major role in controlling virus replication, in that mice deficient in PKR, RNaseL, and Mx-1 (triply-deficient [TD]) did not exhibit any increased tissue tropism, virus dissemination or virulence (216). This suggested

that an alternative pathway dependent on the IFN-α/β receptor was important in controlling virus replication.

In a study to identify host genes responsible for conferring age-dependent resistance to SINV infection, ISG12 was identified and shown to extend average survival time in neonatal mice infected with SINV (143). The mechanism by which ISG12 functions to delay death in neonatal mice is not currently understood, however, ISG12 expression was found to be expressed at higher levels in CNS tissues of infected weanling mice as compared to neonatal mice.

ISG-15 was recently reported to protect mice from infection with a virulent SINV (153, 154). Using a recombinant SINV expressing ISG15 from a second subgenomic promoter, IFN-α/βR^{-/-} mice, were protected from a lethal dose while wild-type virus caused greater than 90% mortality (153, 154). Furthermore, infection with the recombinant SINV expressing ISG15 reduced virus replication in several tissues in infected IFN-α/βR^{-/-} mice. Infection of ISG15^{-/-} mice with wild-type SINV showed increased virulence, while infection of these mice with the recombinant SINV expressing ISG15 exhibited reduced virulence (154). While the mechanism of the antiviral activity of ISG15 during SINV infection is not understood, it is thought to function as a cytokine or increase stability of host proteins through direct conjugation.

Alphavirus evasion of type I interferon

 Successful transmission of alphaviruses from the mosquito vector to the human host is believed to be a complex process involving delivery of virus to the host, establishing virus infection, and dissemination of virus. Upon delivery of the virus from the mosquito vector, it

is believed that alphaviruses likely interact with mDCs in the skin. Shabman et al. discovered that infection of mDCs with virus derived from mosquito cells showed considerably reduced type I IFN induction compared to infections with virus derived from mammalian cells (237). Furthermore, passage of the mosquito grown virus over mammalian cells followed by infection of mDCs restored the type I IFN induction phenotype, strongly suggesting nongenetic viral factors as playing an important role in modulating type I IFN induction.

Alphavirus modulation of type I interferon

Most alphaviruses induce shutoff of host macromolecular synthesis in mammalian cells (249). This shutoff mechanism is believed to be employed by alphaviruses to suppress the host antiviral response. Infection of vertebrate cells has been shown to induce shutoff at the level of host transcription and translation and are thought to be two independent events (90). Two viral proteins, nsP2 and capsid (C), have been shown to important regulators of host cell macromolecular synthesis.

Mutational analysis within the carboxy- terminus of nsP2 revealed its functions in virus-induced host cell translation and transcription shutoff (90). Specifically, an amino acid change from a Pro to Leu at position 726 in nsP2 significantly reduced the efficiency of virus-induced host translation shutoff and correlated with an increase in type I IFN induction and upregulation of ISGs mRNA production (73). In addition, ablating the nsP2/3 cleavage site altered kinetics of virus-induced host transcription shutoff without affecting host translation shutoff and resulted in the induction of type I IFN (90), similar to that seen with the mutation at nsP2 position 726. This result suggested that mature nsP2 was required for

the shutoff of host cell transcription. In support of this, Garmashov et al. demonstrated that expression of nsP2 alone inhibited RNA polymerase I and RNA polymerase II-dependent transcription and this was independent of the protease and helicase functions of nsP2 (77). Furthermore, overexpression of nsP2 caused cytotoxic effects in host cells and this was believed to be mediated by nsP2-inhibition of host cell transcription (77).

The role of the viral C protein in shutoff of host cell macromolecular synthesis has been a source of controversy. Early studies with SFV suggested a role for C protein in host shutoff by two key observations: i) increased expression of C protein during infection inversely correlated with shutoff of host protein synthesis (53) and ii) PKR phosphorylation during infection correlated with expression of the C protein (56). It was this later study by Favre et al. that demonstrated that expression of C protein at high concentrations induced phosphorylation of PKR, phosphorylation of the α subunit of eIF2, and in turn lead to shutoff of host protein synthesis (56). Using a cell-free translation system, it was demonstrated that purified C protein added to cytoplasmic extracts triggered phosphorylation of PKR and eIF2α. However, C protein did not co-immunoprecipitate with PKR, suggesting that C protein maybe involved in interactions with host cell proteins that can influence the activation of PKR.

Recent studies by Garmashova et al. reported that the C protein from New World alphaviruses, such as VEEV and EEEV, plays an important role in host transcription shutoff, whereas Old World alphaviruses, such as SINV and SFV, host transcription shutoff depends on the nsP2 protein and not the C protein (78). These studies also indicated the N-terminal portion of the C protein from VEEV or EEEV was important and independent of the RNA binding domain or protease function. Most of the C protein is found within the cytoplasm of

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infected cells, therefore, these studies imply that C protein may block transcription through effects on nuclear export of host mRNA and rRNA or degradation of host mRNA and rRNA in the cytoplasm.

Infection of IFN-α/βR$^{-/-}$ mice with several VEE strains found that virulence was associated with the determinants within the 5'UTR and glycoproteins (246). Interestingly, epizootic VEE strains were found to be more IFN resistant than enzootic VEE strains. Studies by White et al. identified a single change at nucleotide 3 (G to A) within the 5'UTR that modulated sensitivity to IFN-α/β (291). While infection of wild-type mice with the attenuating mutation at nt 3 showed no mortality, infection of IFN-α/βR$^{-/-}$ mice exhibited 100% mortality and mice died with similar kinetics as those infected with wild-type VEE. While both viruses exhibited strong induction of type I IFN, the virus containing the attenuating mutation showed enhanced sensitivity to type I IFN. While the mechanism of this virulence determinant is not understood, it is believed that it maybe affecting viral RNA synthesis, virus-induced host shutoff, or efficiency of viral nonstructural protein synthesis.

DISSERTATION OBJECTIVES

SINV infection of mice has provided valuable insight into the viral and host factors that contribute to virus-induced neurologic disease. Specifically, the Sindbis-group virus strain AR86 infection in mice provides an excellent viral pathogenesis model. Infection of adult mice with AR86 virus causes a lethal disease characterized by a severe neurologic disease (112, 241, 254), uncontrolled virus replication and spread within the CNS (112, 254), and an apparent lack on inflammation in the CNS (Suthar, M.S. and Heise, M.T., unpublished). Furthermore, AR86 virus can be manipulated at the molecular level with the

use of the full-length infectious clone and a well-established alphavirus-reverse genetic system (242).

As mentioned earlier, AR86 virus was found to be closely related to the Sindbis-group virus strain Girdwood (166). In contrast to AR86 virus, Girdwood virus is avirulent in adult mice however, and perhaps most importantly, Girdwood virus establishes infection in the CNS as well as that of AR86 virus. These two viruses differ by only 22 amino acid coding changes plus an 18 amino acid deletion in nsP3 between positions 386-403 found in AR86 virus.

The viral and host factors involved in AR86 virus pathogenesis are not well understood. Previous studies by Heise et. al identified a unique amino acid at nsP1 position 538 as playing an important role in adult mouse neurovirulence (112). Introduction of an attenuating Ile, which is found in most SINV, at this position reduced AR86 virulence, however, did not completely attenuate AR86 virulence (112). This strongly suggested the presence of additional viral factors that contribute to AR86 adult mouse neurovirulence. This provides a unique opportunity to use the Girdwood virus in order to identify the additional AR86 virulence determinants followed by studies aimed at uncovering the mechanism of these virulence determinants, with specific focus on characterizing viral interactions with the host immune response. Therefore, the objectives of this dissertation are as follows:

1) To identify the major determinants of AR86 adult mouse neurovirulence.

2) To determine if the AR86 virulence determinant at nsP1 position 538 plays a role in modulating the host innate immune response, specifically type I IFN.

3) To determine if the AR86 virus nonstructural proteins are interacting with the type I IFN induction signaling pathway.

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CHAPTER TWO

AN ARTHRALGIA ASSOCIATED SINDBIS VIRUS EXHIBITS ENHANCED REPLICATION IN MURINE JOINT AND MUSCLE TISSUE

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ABSTRACT

Sindbis viruses, like many alphaviruses, cause rash, fever, and arthralgia in humans, however, the pathogenesis of alphavirus-induced arthritis/arthralgia is poorly understood. Therefore, a molecularly cloned Sindbis-group virus, Girdwood, that is associated with infectious arthralgia humans, was assessed for its ability to replicate in joint associated tissues and cause disease in a mouse model. In 14 day-old mice, Girdwood exhibited tropism for periosteum, joint associated connective tissue, and tendons within the hind limbs. Furthermore, comparison of the Girdwood clone to TR339, a molecularly cloned Sindbis virus that represents an early, non-cell culture adapted strain of the original AR339 strain of Sindbis, showed that Girdwood virus caused more severe disease than TR339 following peripheral inoculation of 14 day-old mice. Although both viruses exhibited similar levels of replication within the serum and CNS, the arthralgia associated Girdwood virus exhibited significantly higher viral titers in bone and joint associated tissue and skeletal muscle as compared to TR339. Therefore, this virus represents a useful tool for dissecting the viral molecular determinants that promote replication within joints and associated connective tissues.

KEYWORDS

Sindbis virus
Alphavirus
Girdwood
Virus-induced arthralgia
TR339

INTRODUCTION

Old world alphaviruses are a common cause of infectious rheumatic complaints, including severe arthritis, arthralgia, and myalgia (Griffin, 2001; Laine et al., 2004). Viruses such as Ross River virus (RRV), Sindbis viruses (SINV), and Chikungunya are recurrent problems in Australia, Southern Africa, Europe and Asia, where they cause several hundred to several thousand cases of disease each year (2006a; 2006b; Enserink, 2006; Laine et al., 2004; Suhrbier and La Linn, 2004). However, many of these viruses, including RRV, O'nyong-nyong, and Chikungunya virus, are also capable of emerging in new locales and causing epidemics on a massive scale, as illustrated by an outbreak of RRV disease in the South Pacific in 1979-80 that affected 50-60,000 individuals just on the island of Fiji (Aaskov et al., 1981; Fraser, 1986; Harley et al., 2001), an outbreak of O'nyong-nyong in the 1960's that caused upwards of nearly two million cases of infectious arthritis/arthralgia (Williams et al., 1965), and currently an epidemic of Chikungunya virus affecting individuals residing in East African countries bordering the Indian Ocean, India, and Sri Lanka (2006a; 2006b; Enserink, 2006; Seneviratne and Perera, 2006).

Though alphavirus-induced arthridities are generally acute and self-limited (Laine et al., 2004; Suhrbier and La Linn, 2004), the rheumatic symptoms can be severe and are often debilitating. Furthermore, with many alphavirus-induced arthridities, a subset of patients complain of chronic pain and loss of function (Laine et al., 2004), though whether this is directly related to persistent virus infection or other factors is not well understood. Disease manifestations also vary between alphavirus infections, with viruses such as RRV are generally associated with frank arthritis involving virus-induced inflammation (Fraser et al., 1983; Hazelton et al., 1985; Soden et al., 2000), while SINV are generally associated with

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arthralgia characterized by pain within tendons and other tissues surrounding the joint (Kurkela et al., 2005; Turunen et al., 1998).

Alphavirus-induced arthridities can be extremely severe, however, the mechanisms underlying alphavirus-induced arthritis and arthralgia in humans are not well understood. Direct viral replication and inflammation within and around affected joints and connective tissues are thought to contribute to disease. RRV nucleic acid or antigen has been recovered from synovial aspirates of individuals suffering from RRV-induced infectious polyarthritis (Fraser et al., 1981; Soden et al., 2000). Characterization of the inflammatory response within synovium effusions revealed that it mainly consists of macrophages, CD4+ T cells, B cells, and monocytes (Fraser et al., 1981; Fraser et al., 1983; Hazelton et al., 1985; Soden et al., 2000). For Sindbis-group viruses, skin biopsies and blood samples have tested positive for viral nucleic acid and infectious virus has been recovered from patients showing symptoms of Pogosta disease, however, no virus has been recovered from joint-associated tissues (Kurkela et al., 2004).

In order to better understand the pathogenesis of arthritogenic alphaviruses, improved animal models would be useful for dissecting the pathogenesis of these diseases. Currently, two mouse models have been described for studying the pathogenesis of alphavirus-induced arthritis and myositis. Morrison et al. has recently characterized RRV infection in an adult mouse model to study virus-induced arthralgia/arthritis and myositis (Morrison et al., 2006). In this model, RRV shows tropism for bone, joint, and skeletal muscle from a peripheral route of inoculation. Furthermore, infiltrating inflammatory macrophages, NK cells, CD4+ and CD8+ T cells were present in skeletal muscle of RRV-infected mice, which is similar to synovial effusions and skin biopsies from patients suffering from RRV-induced polyarthritis

(Soden et al., 2000), suggesting that the mouse model reflects a valid system for studying RRV pathogenesis. However, for alphaviruses such as SINV, which cause arthralgias without overt inflammation, the RRV model is likely to be of limited use in understanding the pathogenesis of virus-induced arthralgia. One study has shown that the Sindbis-group virus strain AR86 replicates within joint associated tissues of adult mice (Heise et al., 2000a). However, no virus-induced disease was observed, thus limiting the potential of this model for studying the pathogenesis of SINV-induced arthralgia. Therefore, improved animal models are needed if advances are to be made in studying the mechanisms underlying alphavirus-induced arthritis/arthralgia.

In this study, we report the characterization of a molecular clone for the Sindbis-group virus, Girdwood, a virus originally isolated from a human suffering from virus-induced arthralgia (Malherbe et al., 1963). Characterization of the cloned virus (G100) demonstrated similar *in vitro* and *in vivo* growth and virulence characteristics as compared to the natural Girdwood isolate. Studies to evaluate Girdwood replication in 14 day-old mice, an age where Girdwood virus (G100) causes hind limb dysfunction without a lethal outcome, showed high levels of replication in periosteum, tendon, and joint-associated connective tissues. Furthermore, direct comparison between the arthralgia associated Girdwood virus and the prototypic laboratory strain of SINV (TR339), which has not been associated with arthralgia, showed striking differences in both viral replication and disease induction. While both G100 and TR339 showed similar levels of replication in neural tissues, G100 exhibited significantly higher titers in several peripheral tissues, including ankle joints and skeletal muscle. In addition, G100 consistently caused more severe disease signs (hind limb dysfunction and weakness) than TR339, which suggest that the molecularly cloned G100

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virus may prove to be a useful reagent for studying the pathogenesis of alphavirus-induced arthritis/arthralgia and for identifying viral genetic determinants that promote viral replication in joint and skeletal muscle tissues.

RESULTS

Characterization of the full-length infectious clone of the arthralgia associated Sindbis-group virus Girdwood. The Sindbis-group virus, Girdwood, was isolated from a human suffering from arthralgia (Malherbe et al., 1963) and is closely related to the arthralgia associated Northern European isolates of Sindbis virus, such as Ockelbo, Karelian Fever, and Pogosta (Laine et al., 2004; Shirako et al., 1991). Our group has previously described the use of the full-length infectious clone of Girdwood in a study to map virulence determinants within the adult mouse virulent Sindbis-group virus strain AR86 (Suthar et al., 2005). However, given that Girdwood virus caused arthralgia in a human (Malherbe et al., 1963), we sought to investigate whether this virus might also be a useful reagent for studying the pathogenesis of alphavirus-induced arthralgia. As a first step in this process, a detailed analysis was performed to ensure that the cloned virus was phenotypically identical to the disease associated natural isolate. This was particularly important given that the infectious clone derived virus contained the 5' and 3' UTRs from the closely related Sindbis-group virus, AR86, which resulted in a single nucleotide change at position 16 (from a T to C) in the 5' UTR and a single nucleotide change at position 11558 (from a T to a C) in the 3' UTR (Suthar et al., 2005). Both G100 and the natural isolate exhibited a large plaque size phenotype, in which the two viruses were indistinguishable (data not shown). The specific infectivity (i.e. PFU per cpm as measured on BHK-21 cells) of the Girdwood clone and natural isolate were also compared (Fig. 1). The clone derived virus and the natural isolate had comparable particle to PFU ratios. Both viruses exhibited similar specific infectivities to TR339, a non-cell culture SINV that represents the prototypic AR339 strain of Sindbis, and all three were less than TRSB, a well characterized SINV that contains a cell culture

adaptation mutation in E2 (Arg at position 1) resulting in enhanced BHK-21 cell infectivity (Klimstra et al., 1998).

The growth characteristics of the cloned virus was compared to the natural isolate by performing single- and multi-step growth curves in BHK-21 cells. In the single-step growth assay, in which cells were infected at an MOI of 5.0, the infectious clone derived virus and the natural isolate displayed similar growth kinetics (Fig. 2A). Furthermore, both viruses exhibited similar growth kinetics in a multi-step growth curve in which BHK-21 cells were infected at an MOI of 0.01 (Fig. 2B). By 36 hours post-infection, both viruses reached comparable peak viral titers of approximately 10^6 -10^7 PFU/ml.

Previous studies using the Girdwood infectious clone demonstrated that this virus was non-neurovirulent in mice greater than 4 weeks of age. To further characterize the virulence of the Girdwood clone, suckling and adult mice were infected and the virulence was compared to that of the natural isolate (Table 1). Overall, the infectious clone showed similar virulence properties to that of the natural isolate in 5, 8, 14, and 42 day-old mice. While infection of five-day-old mice exhibited 100% mortality, infection of 14-day-old mice with the clone G100 virus exhibited no mortality, however, mice exhibited moderate to severe morbidity, characterized by ruffled fur, hunched posture, and moderate to severe hind limb dysfunction (Fig. 3B). In mice greater than 2 weeks of age, no mortality or morbidity was observed with either virus, confirming previously published studies (Suthar et al., 2005).

The results from these studies demonstrate that the Girdwood clone exhibits similar *in vitro* and virulence properties as that of the natural isolate. Furthermore, infection of 14 day-old mice may serve as a non-lethal model to study virus-induced disease.

Girdwood virus exhibits tropism for bone and joint associated tissues in 14-day-old mice. Peripheral inoculation of adult mice with AR86, which is closely related to Girdwood, demonstrated virus tropism and replication in bone and joint associated connective tissues (Heise et al., 2000a). However, one limitation of these studies was that AR86 infection was restricted to older animals (greater than 6 weeks of age) mainly due to the fact that AR86 is neuroinvasive and lethal in younger mice (Suthar, M.S. and Heise, M.T., data not shown). Since SINV replication in joint associated tissues within the mouse becomes more restricted with age (Heise M.T., unpublished), we examined whether Girdwood would exhibit tropism for bone and joint associated tissue in younger animals. In this study, 14-day-old mice, in which Girdwood showed moderate to severe hind limb dysfunction and no mortality, were infected with a modified Girdwood virus expressing green fluorescent protein (GFP) from a second subgenomic promoter. GFP-positive cells were observed in the hind limbs at 24, 48, and 72 hours post-infection, however the most intense signal was observed at 48 hours post-infection. At 48 hours post-infection, virus-specific signal within the ankle joint and foot were observed within periosteum, joint associated connective tissues, and tendons (Fig. 4). These results, which were also confirmed by *in situ* hybridization (data not shown), demonstrate that Girdwood exhibits tropism for bone and joint-associated tissues in 14 day-old mice.

Girdwood virus replication in 14-day-old mice. Based on the finding that Girdwood virus showed tropism for joint associated tissues, we compared Girdwood replication in 14 day-old mice to the molecularly cloned SINV, TR339, a non-cell culture adapted representative of the prototypic AR339 strain of SINV that is largely avirulent in mice greater than 11 days of age and which has not been associated with arthralgia (Klimstra et al., 1998; Ryman et al.,

2007). Girdwood infection consistently caused more severe disease (DS= 3.0 on day 7), and exhibited greater than 30% weight differences by day 9 p.i. as compared to TR339 infected mice (DS=1.0 on day 7) (Fig. 3).   The Girdwood virus-induced disease was striking in that unlike the severe debilitating disease induced by the closely related, but adult mouse neurovirulent AR86 virus (Heise et al., 2000b), the hind limb disease induced by Girdwood infection was relatively moderate and mice eventually recovered (data not shown).

Additional studies were performed to evaluate the sites and magnitude of Girdwood and TR339 replication in the infected mice. Overall levels of virus replication were evaluated in ankle joint, muscle, spleen, serum, and CNS tissues of infected mice. Consistent with previous studies with the Sindbis-group virus strain AR86, both G100 and TR339 exhibited replication in joint associated tissues (Fig. 5). Though both viruses showed equivalent levels of replication in the CNS, the arthralgia associated G100 virus had significantly higher titers in the ankle, skeletal muscle, and spleen (Fig. 5). This suggests that determinants within the G100 virus promote enhanced growth in peripheral tissues, and further studies are required to identify these determinants and assess their role in virus-induced disease.

DISCUSSION

Alphaviruses are a common cause of infectious arthridities worldwide, with several viruses, such as Chikungunya and RRV associated with large outbreaks of severe arthritis/arthralgia (Aaskov et al., 1981; Enserink, 2006). Although alphaviruses are a significant cause of human disease, relatively little is known about the pathogenesis of alphavirus-induced rheumatic diseases. We and others have described a mouse model of RRV-induced arthritis/myositis, however, unlike RRV, which causes inflammatory arthritis in humans, SINV, such as Ockelbo and Girdwood are associated with arthralgia (Harley et al., 2001; Laine et al., 2004; Morrison et al., 2006). It is therefore likely that the pathogenesis of arthritis associated viruses, such as RRV will differ from arthralgia associated viruses such as the SINV. With this in mind, we tested whether a molecularly cloned Sindbis virus that was found to cause arthralgia in a human would also replicate in bone and joint associated tissues and cause disease in a mouse model.

It is thought that direct virus replication within the affected joints contributes to the development of alphavirus-induced arthritis/arthralgia in humans. This is largely based on studies with RRV, where viral RNA and viral antigen positive cells have been isolated from the knee joint and synovium effusions of afflicted individuals (Fraser et al., 1981; Soden et al., 2000). In support of this, RRV has been shown to replicate to high levels in synovial tissues of infected mice and in hind limb skeletal muscle (Morrison et al., 2006), while the data presented here also demonstrates that joint associated tissues and skeletal muscle of mice are major sites of replication for the arthralgia associated Girdwood virus. Perhaps most importantly, these results also demonstrate that the arthralgia associated Girdwood virus replicates to higher levels in joint and muscle tissues, than the prototypic TR339 strain of

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SINV, which has not been linked to arthralgia in humans. This result is particularly striking in that both viruses replicated to equivalent levels in the CNS, which suggests that Girdwood virus may either exhibit an enhanced tropism for extraneural tissues, or an enhanced ability to replicate in cells within these tissues. Either of these possibilities may have an important impact on our understanding of the pathogenesis of alphavirus-induced arthralgia, since this would suggest that specific viral determinants contribute to the ability of arthralgia associated viruses to target to and/or replicate within joints associated or other connective tissues. This would in turn raise the possibility of mapping the determinants within the cloned Girdwood virus that contribute to the enhanced replication within muscle and joint tissues. The availability of molecularly cloned versions of Girdwood and TR339, which can be readily used to create a set of chimeras to map the determinants of bone/joint tropism, provides a set of tools that could be used significantly enhance our understanding of the viral and host factors that contribute the development of arthralgia following alphavirus infection.

The mechanisms underlying alphavirus-induced arthralgia are poorly understood, since the joint pain induced by SINV lacks the inflammatory infiltrates that presumably mediate the arthritic symptoms caused by viruses such as RRV (Harley et al., 2001; Rulli et al., 2005). Infection of 14 day-old mice with Girdwood did result in a transient hind limb dysfunction which is similar to that seen with RRV-infected mice (data not shown; (Morrison et al., 2006). Though we cannot rule out a role for neurologic effects, several lines of evidence suggest that this disease is due to direct viral replication within muscle and joint associated tissues within the limb, rather than virus-induced nerve damage within the CNS. This includes the transient nature of the disease, with infected mice recovering full use of their hind limbs after the resolution of the viral infection. Though it is possible for mice to

regain partial hind limb function following virus-induced damage of the CNS (Suthar et al., 2005), it is unlikely that the affected animals would regain full function as rapidly as the those in our study if the hind limb dysfunction were simply due to nerve damage. While Girdwood caused greater disease than TR339, both viruses replicated to similar levels in the CNS, but differed in their ability to replicate in joint and muscle tissue. Furthermore, hematoxylin and eosin stained brain and spinal cord sections revealed no overt neuronal damage during the course of infection (data not shown). This result supports the hypothesis that direct viral replication by the arthralgia associated Girdwood virus is responsible for the hind limb dysfunction, though additional studies are needed to address this issue in greater detail. However, even if the hind limb disease proves to be due to virus-induced damage to the CNS, this mouse model should still be useful for identifying viral genetic factors that promote replication within joint associated tissues.

In summary, we have characterized a molecular clone of the arthralgia associated Girdwood virus for its ability to replicate in bone and joint associated tissues and cause disease in a mouse model. Not only did Girdwood virus cause more severe disease than the non-arthralgia associated TR339 virus, but showed enhanced ability to replicate in bone and joint associated tissues and skeletal muscle. This enhanced ability to replicate in bone and joint associated tissues and skeletal muscle may promote the induction of arthralgia by viruses such as Girdwood, while this difference in replication between TR339 provides an opportunity to identify viral determinants that promote replication in extraneural tissues, which has not previously been reported for alphaviruses.

MATERIALS AND METHODS

Viruses and virus stocks. Plasmids containing the viral cDNA are designated with the prefix "p" while the infectious virus derived from the cDNA clone does not contain the "p" designation; i.e., G100 represents virus derived from the plasmid pG100. TRSB and TR339 virus stocks were generously provided to us by the laboratories of Robert E. Johnston and William Klimstra.

Virus stocks were made as described previously (Suthar et al., 2005). Briefly, viral cDNA plasmids were linearized with *PmeI* and used as templates for the synthesis of full-length transcripts using SP6-specific mMessage Machine *in vitro* transcription kits (Ambion). Transcripts were electroporated into BHK-21 cells grown in α-minimal essential medium (α-MEM: 10% fetal calf serum [Gibco], 10% tryptose phosphate broth [Sigma], and 0.29 mg of L-glutamine per ml [Gibco]). Supernatants were harvested 24 to 27 hours after electroporation and subjected to centrifugation at 3000 RPM (Sorvall rotor RTH-250) for 20 minutes at 4°C and frozen in 1-ml aliquots. Virus stocks were titrated on BHK-21 cells as previously described (Simpson et al., 1996).

BHK-21 cells (ATTC CCL-10) were maintained at 37°C in α-MEM (Gibco) supplemented with 10% donor calf serum, 10% tryptose phosphate broth, and 1% L-glutamine, for a maximum of 10 passages. Single-step and multi-step *in vitro* growth curves were performed as previously described. Briefly, BHK-21 cells were plated at 10^5 cells/well in 24-well plates (Sarstedt) for 18 h at 37°C. Medium was removed, and wells were infected with virus in triplicate at a multiplicity of infection (MOI) of 5.0 or 0.01. Cells were incubated at 37°C for 1 h. Wells were then washed three times with 1 ml of PBS supplemented with 1% DCS and Ca^{2+}-Mg^{2+}. One milliliter of growth medium was then

added to each well, and cells were incubated at 37°C. Samples of supernatant were removed at various time points, with an equal volume of fresh medium added to maintain a constant volume within each well. Samples were frozen at −80°C until analysis by plaque assay.

Construction of the full-length Girdwood clone. The Girdwood infectious clone, pG100, was generated by RT-PCR amplification of the Girdwood genome from low cell culture passaged virus stocks. The Girdwood genome was amplified as overlapping one to three kB fragments that spanned the genome, which were then introduced into the AR86 pS300 infectious clone plasmid (Suthar et al., 2005) using restriction sites that are conserved between Girdwood and AR86. The 5' and 3' untranslated regions (UTR) of this clone were maintained on the AR86 background (single nucleotide changes at positions 16 (T to C) and 11558 (T to C)). The resulting clone, pG100, was sequenced to ensure that the cloned sequence matched the previously published Girdwood sequence (NCBI Accession No. U38304) (Simpson et al., 1996). Several single amino acid differences were found in the Girdwood clone that differed from the published sequence (Simpson et al., 1996). Several of these changes were present on multiple clones and changed a codon that was unique to Girdwood back to the SINV consensus sequence and therefore likely represent differences between our Girdwood isolate and the published sequence. These changes are located at nt 1721, 2200, 2407, 2496, 2498, 4588, 4780, 5524, 5526, 5527, 5704, 5705, 5824, 5826, 5843, 6328, 6329, 6714 and were maintained within the clone. In addition to these changes, PCR artifacts were found in the completed clone and nucleotide changes that resulted in coding changes were corrected either by recloning the fragment or by PCR mediated mutagenesis. There were also three non-coding nucleotide changes present at nucleotide position 536 (T to an A), 8639 (C to T), and 8985 (A to G). These were not corrected and were instead used as

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marker mutations to differentiate the cloned virus from the Girdwood natural isolate. Other than the consensus sequence changes discussed above, the virus produced from pG100 has an amino acid sequence identical to that reported for Girdwood (Simpson et al., 1996).

Specific Infectivity Assay. Virus specific infectivity assays were performed as described by (Ryman et al., 2004) Briefly, radiolabeled virus was prepared by infection of a BHK cell monolayer with an MOI of 2-3 followed by incubation with 10 µCi/ml [^{35}S]-methionine (Amersham). Virus was purified on two subsequent discontinuous 20%/60% sucrose gradients and concentrated by pelleting through 20% sucrose. The BHK cell-specific infectivity (measured as PFU per count per minute [cpm]) was calculated for each virus preparation by plaque assay on BHK cells.

Animal Studies. Specific-pathogen-free timed pregnant CD-1 mice (13-15 days gestation) or six-week old female CD-1 mice were obtained from Charles River Breeding Laboratories (Raleigh, NC). Mice that were born within a 12-hour period were pooled and divided randomly as 10-12 pups per litter. Animal housing and care were in accordance with all University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee guidelines. Virulence studies were performed in 5, 8, or 14-day-old CD-1 mice by s.c inoculation in the ventral thorax with 10^3 PFU of virus diluted with diluent containing phosphate-buffered saline (PBS [pH 7.4]), supplemented with 1% donor calf serum (DCS) (Gibco). Mock infected mice received diluent alone. Mice were monitored daily for mortality and/or morbidity for a period of 21-days postinfection. Six-week-old mice were anesthetized with either isoflourane (Halocarbon Laboratories) or ketamine supplemented with xylazine (Barber Med.) prior to intracranial (i.c.) inoculation with a standard dose of 10^3 PFU of virus in diluent. Mock infected mice received diluent alone. Mice were monitored

daily for body weight and scored for disease signs as follows: 1= Ruffled fur and/or hunched body posture, 2= Mild hind-limb dysfunction, 3= Prominent hind-limb dysfunction, 4= Severe hind-limb dysfunction, 5= Paralysis of hind-limbs, and 6= Terminal morbidity. As required by the UNC-CH animal protocol, infected mice were euthanized during the experiment either when mice dropped to 30% of initial body weight or when mice exhibited severe disease signs. For *in vivo* growth studies, 14-day-old mice were inoculated as described and sacrificed by exsanguination followed by perfusion with 10 ml of PBS. Tissues from these mice were harvested at 18, 36, 48, 60, 72, and 96 hours post infection and viral loads per gram or ml of tissue were determined in the the hind limb ankles, quadricep muscle, spleen, brain, and serum by plaque assay on BHK-21 cells. Statistical analysis were performed using Students two-tailed t-test and differences were considered significant when $p<0.05$ (Instat 3.0). Alternatively following exsanguination, mice were perfused with 4% paraformaldehyde and hind limbs were removed. The hind limbs were decalcified for approximately two week in 4% paraformaldehyde and 8% EDTA. Frozen sections were then prepared from the decalcified hind limbs.

ACKNOWLEDGMENTS

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Figure 2-1: Relative specific infectivity of the Girdwood clone (G100), Girdwood natural isolate and TRSB.
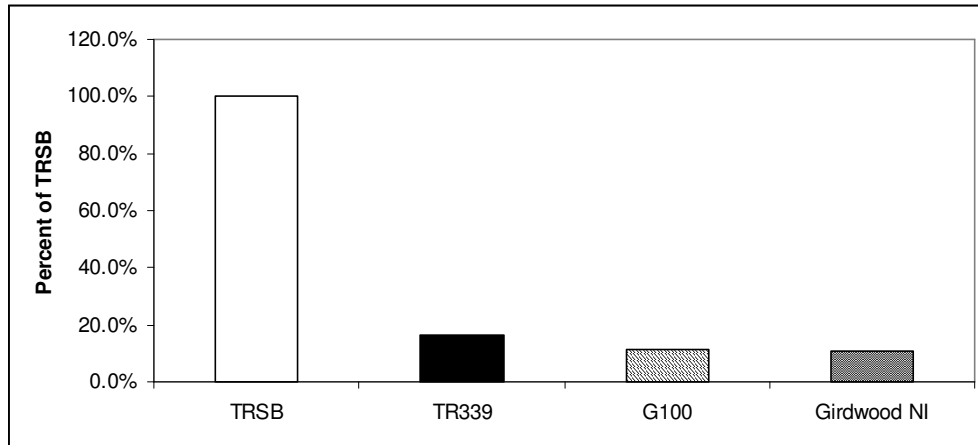
Figure 2-1. Relative specific infectivity of the Girdwood clone (G100), Girdwood natural isolate and TRSB. Radiolabeled virus was prepared, diluted, and assayed for plaque formation on BHK-21 cell monolayers. The specific infectivity (measured as PFU/cpm) was calculated for each of the viruses and the values were normalized to TRSB (100%). The data shown are from one of three representative experiments.

Figure 2-2: The Girdwood clone displays similar in vitro growth kinetics as that of the natural isolate.
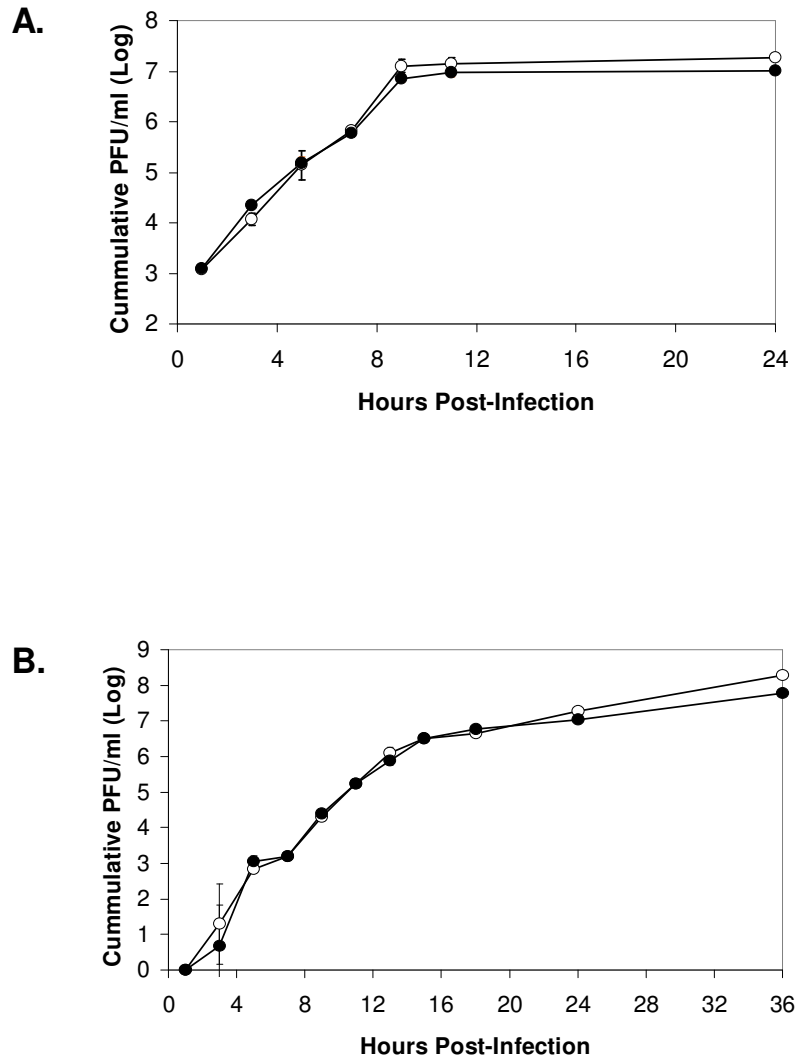
Figure 2-2. The Girdwood clone displays similar *in vitro* growth kinetics as that of the natural isolate. Single-step (A) and multi-step (B) *in vitro* growth assays were performed in which BHK-21 cells were infected with the Girdwood clone (G100; solid circles) or the natural isolate (open circles) at an MOI of 5.0 or 0.01, respectively. Samples of supernatant were removed at the indicated time points and evaluated for virus yield by plaque assay. Each point represents the average titer of three independent samples with the standard deviation. The data shown are from one of three representative experiments.

Table 2-1: Girdwood virulence in mice.

Age (days)	Route	Girdwood Natural Isolate			Girdwood Clone (G100)		
		% Morbidity (DS)	% Mortality	AST (SD)	% Morbidity (DS)	% Mortality	AST (SD)
5	s.c	ND	100%	4.4 (1.4)	ND	100%	4.2 (1.2)
8	s.c	ND	45.5%	10.8 (3.1)	ND	69.6%	8.3 (1.7)
14	s.c	100%	0%	N/A	100%	0%	N/A
42	i.c	0% (0)	0%	N/A	0% (0)	0%	N/A

ND=Not Determined
DS= Clinical score as measured on peak day of disease

Table 1
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Figure 2-3: Girdwood infected mice show more severe disease signs than TR339.

A.

B.
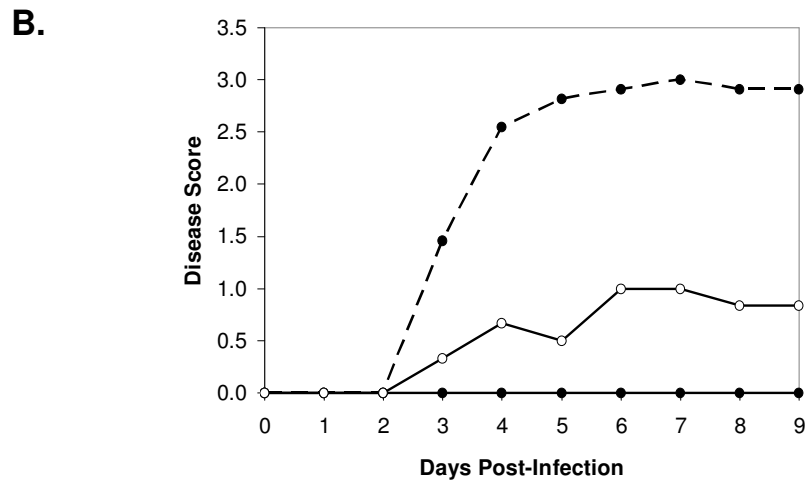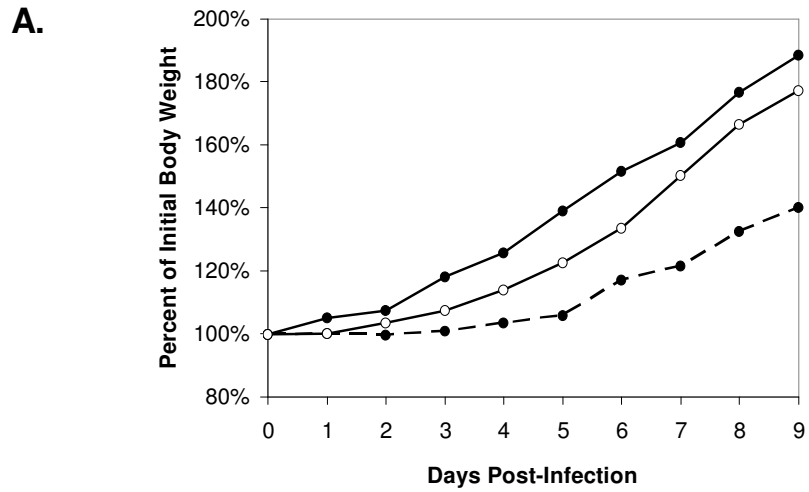
Figure 2-3. Girdwood infected mice show more severe disease signs than TR339. Groups of fourteen day-old CD-1 mice were infected s.c. in the ventral thorax with mock or 10^3 PFU of Mock (closed circle; solid line), G100 (open circle; solid line), or TR339 (closed circle; dashed line). Mice were monitored daily for (A) weight and (B) disease score. Each data point represent an average for weight or disease score of infected mice at each timepoint (Mock=6, G100=11, TR339=10). The data shown are from one of four representative experiments.

Figure 2-4: Girdwood exhibits tropism for bone and joint-associated connective tissues.
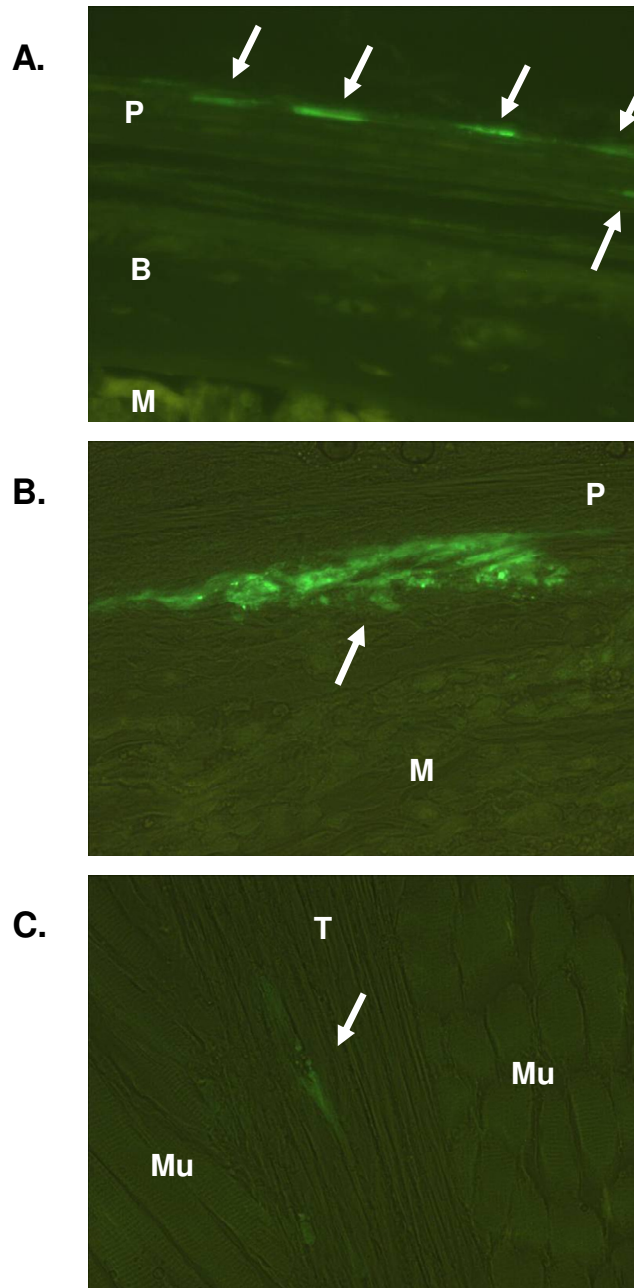
Figure 2-4. Girdwood exhibits tropism for bone and joint-associated connective tissues.
Fourteen day-old CD-1 mice were infected s.c. in the ventral thorax with 10^3 PFU of G100-egfp and ankle joint and foot bones were evaluated for virus-specific signal at 48 hours post-infection. (M= marrow, P= periosteum, T=tendon, B=Bone, Mu=Muscle). Panels A, B, and C represent different locations within the foot and ankle joints of positive virus signal.

Figure 2-5: Girdwood exhibits enhanced replication in peripheral tissues as compared to TR339.

Figure 2-5
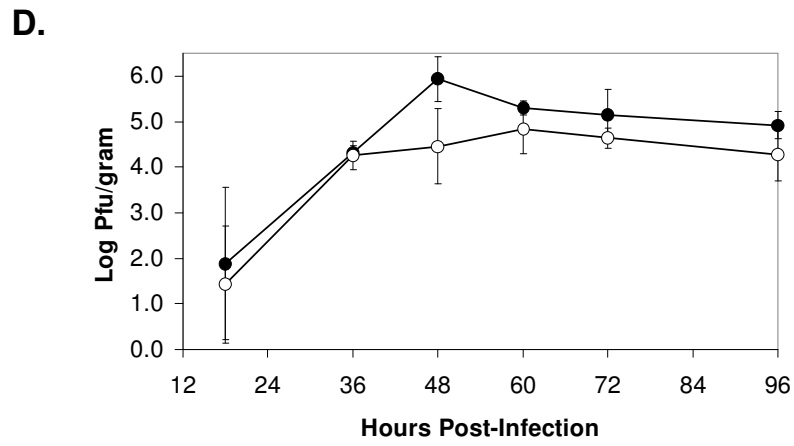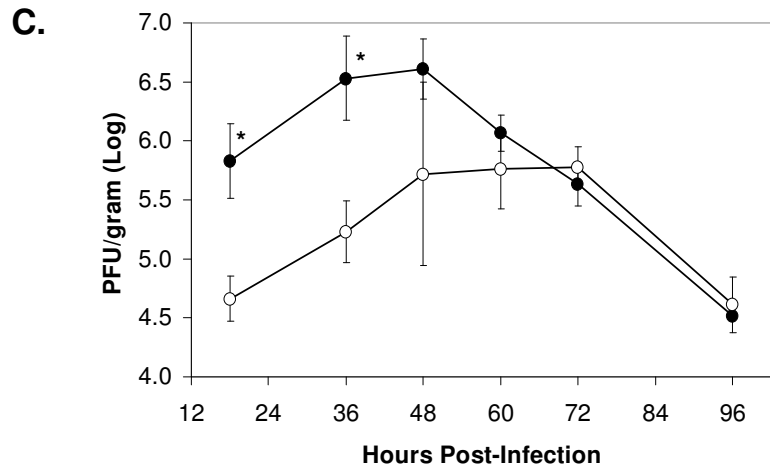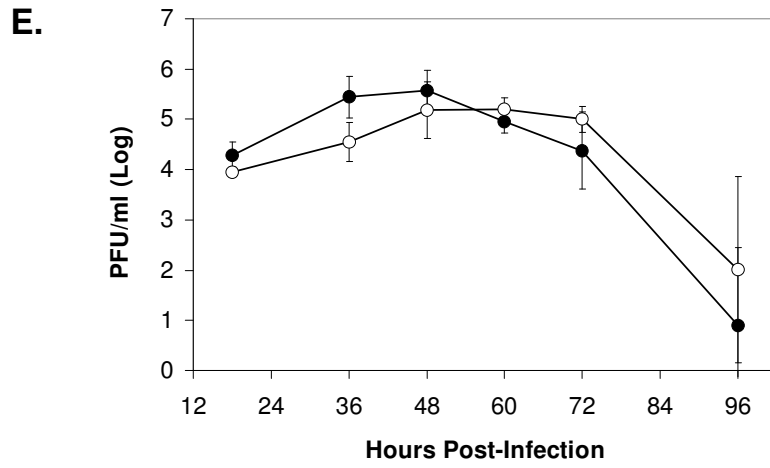
C.

D.

Figure 2-5

E.

Figure 2-5. Girdwood exhibits enhanced replication in peripheral tissues as compared to TR339. Fourteen day-old CD-1 mice were infected s.c. in the ventral thorax with 10^3 PFU of G100 (solid circle) or TR339 (open circle). Mice were sacrificed by exsanguination at 18, 36, 48, 60, 72, and 96 hours post infection and perfused with PBS (pH7.4). The hind limb (A) ankles, (B) quadricep muscle, (C) spleen, and (D) brain were removed, weighed, and titered by plaque assay on BHK-21 cells. Serum (E) was collected and evaluated for viral load. Each point represents the average titer from three mice per virus per time point along with the standard deviation (* $p<0.05$.). The data shown represents one of three experiments.

CHAPTER THREE

IDENTIFICATION OF ADULT MOUSE NEUROVIRULENCE DETERMINANTS OF THE SINDBIS VIRUS STRAIN AR86

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ABSTRACT

Sindbis virus (SINV) infection of mice has provided valuable insight into viral and host factors that contribute to virus-induced neurologic disease. In an effort to further define the viral genetic elements that contribute to adult mouse neurovirulence, the neurovirulent Sindbis-group virus strain AR86 was compared to the closely related (22 single amino acid coding changes and the presence or absence of an 18 amino acid sequence in nsP3 (positions 386-403)), but avirulent Girdwood strain. Initial studies using chimeric viruses demonstrated that genetic elements within the nonstructural and structural coding regions contributed to AR86 neurovirulence. Detailed mapping studies identified three major determinants in the nonstructural region at nsP1 538 (Ile to Thr; avirulent to virulent), an 18 amino acid deletion in nsP3 (positions 386-403), and nsP3 537 (Opal to Cys; avirulent to virulent), as well as a single determinant in the structural genes at E2 243 (Leu to Ser; avirulent to virulent) that were essential for AR86 adult mouse neurovirulence. Replacing these codons in AR86 with those found in Girdwood resulted in attenuation of AR86, while the four corresponding AR86 changes in the Girdwood genetic background increased virulence to the level of wild-type AR86. The attenuating mutations did not adversely effect viral replication *in vitro* and the attenuated viruses established infection in the brain and spinal cord as efficiently as the virulent viruses. However, the virus containing the four virulence determinants grew to higher levels in the spinal cord at late times post-infection, suggesting that the virus containing the four attenuating determinants either fails to spread or are cleared more efficiently than the virus with the virulence determinants.

INTRODUCTION

Infection of mice with Sindbis-group viruses provides an excellent model for studying virus-induced neurological disease. The outcome of SINV infection in the mouse model has been found to correlate with the age and strain of the animal, virus dose, route of inoculation, and the virus strain (5, 6, 13, 31). Infection of neonatal mice with SINV results in a lethal disease characterized by elevated levels of proinflammatory cytokines and high viral titers in the muscle, brain and serum, in the absence of overt encephalitis (11, 26, 27). Increasing the age of the animal or infecting with an attenuated mutant virus results in a shift from the systemic disease towards a neurologic disease, however, most Sindbis-group viruses are avirulent in mice greater than 14 days of age (28).

Previous neurovirulence studies with Sindbis-group viruses have identified virulence determinants in both the E2 glycoprotein gene (29, 30) and the 5' untranslated region (3, 12). Studies with the neuroadapted Sindbis virus (NSV) identified a His at amino acid position 55 in the E2 glycoprotein that plays a major role in adult mouse neurovirulence (8, 30). The mechanism underlying this change is not yet completely understood; however, E2 His 55 was shown to correlate with improved binding and entry into neuronal cells (30), increased levels of viral replication (2), and the ability to overcome the protective effect of bcl-2 overexpression in neurons (15, 16). Additionally, a single substitution of a G at position 8 in the 5' noncoding region of NSV was responsible for conferring neurovirulence in adult rats (12).

In addition to demonstrating the role of the structural genes in adult mouse neurovirulence, studies with the Sindbis-group virus S.A.AR86 (AR86), and Semliki Forest Virus (SFV) indicate that the viral nonstructural genes contribute to adult mouse

neurovirulence (9, 32, 33). Studies with AR86 have shown that the presence of a Thr at position 538 in nsP1 plays an important role in contributing to adult mouse neurovirulence. Replacing this Thr of AR86 with an Ile, found in most Sindbis-group viruses, attenuates the virus, while introduction of the Thr into a non-neurovirulent laboratory strain of SINV lead to an increase in neurovirulence (9). The introduction of the attenuating Ile at position 538 in nsP1 did not affect viral growth, as viruses that contained this change replicated as well as wild-type AR86 both in cell culture and in the brains of infected animals. Furthermore, the presence of an Ile at position 538 accelerated processing of the nonstructural protein precursor (P123) into the mature nonstructural proteins, leading to earlier induction of viral 26S RNA synthesis during infection (10). In the case of SFV, mapping studies performed using virulent and avirulent strains demonstrated the importance of the nsP3 gene, including replacement of the opal termination codon (located within nsP3) with a sense codon, in reconstituting adult mouse neurovirulence (32, 33).

In this study, we utilized a newly generated clone of the Sindbis-group virus Girdwood to map determinants of adult mouse neurovirulence within AR86. While AR86 causes a lethal disease in adult mice, Girdwood is avirulent, even when administered intracranially. However, these viruses differ by only 22 single amino acid coding changes, as well as the presence of an 18 amino acid sequence in nsP3 that are present in Girdwood, but are deleted from AR86. Detailed mapping studies localized determinants to both the nonstructural and structural genes. Furthermore, the virus containing the attenuating mutations did not adversely effect *in vitro* viral replication and the attenuated viruses established infection in the brain and spinal cord as efficiently as the virulent viruses. However, at late times post-infection, the virus containing the virulence determinants grew to

higher levels in the spinal cord, suggesting the virus containing the attenuating determinants either fail to spread or are cleared more efficiently than the virus with the virulence determinants.

MATERIALS AND METHODS

Virus stocks and cell culture. Plasmids containing the viral cDNA are designated with the prefix "p" while the infectious virus derived from the cDNA clone does not contain the "p" designation; i.e., S300 represents virus derived from the plasmid pS300. Clone pS300 was derived from AR86 RNA, and S300 is comparable to AR86 virus both in its growth and virulence properties (23). Clone pS300 is identical to clone pS55 (23) except for a *Pme*I restriction site engineered at the 3' end of the viral 3'UTR to facilitate generating chimeras with the Girdwood clone. Virus derived from the Girdwood cDNA clone also exhibits *in vitro* and *in vivo* phenotypes similar to the Girdwood natural isolate (M. Suthar, manuscript in preparation).

Virus stocks were made as described previously (9). Briefly, viral cDNA plasmids were linearized with *PmeI* and used as templates for the synthesis of full-length transcripts by using SP6-specific mMessage Machine *in vitro* transcription kits (Ambion). Transcripts were electroporated into BHK-21 cells grown in α-minimal essential medium (α-MEM: 10% fetal calf serum [Gibco], 10% tryptose phosphate broth [Sigma], and 0.29 mg of L-glutamine per ml [Gibco]). Supernatants were harvested 24 to 27 hours after electroporation and subjected to centrifugation at 3000 RPM (Sorvall rotor RTH-250) for 20 minutes at 4°C and frozen in 1-ml aliquots. Virus stocks were titrated on BHK-21 cells as previously described (23).

BHK-21 cells were maintained at 37°C in α-MEM, for a maximum of 10 passages Single-step *in vitro* growth curves were performed as previously described (9). Briefly, BHK-21 cells were plated at 10^5 cells/well in 24-well plates (Sarstedt) for 18 h at 37°C. Medium was removed, and wells were infected with virus in triplicate at a multiplicity of

infection (MOI) of 5.0. Cells were incubated at 37°C for 1 h. Wells were then washed three times with 1 ml of room temperature PBS supplemented with 1% DCS and Ca^{2+}-Mg^{2+}. One milliliter of growth medium was then added to each well, and cells were incubated at 37°C. Samples of supernatant were removed at various time points, with an equal volume of fresh medium added to maintain a constant volume within each well. Samples were frozen at − 80°C until analysis by plaque assay.

Construction of full-length cDNAs and chimeric viruses. Standard recombinant DNA techniques were used to construct cDNA clones as previously described in (21). The chimeric cDNA clone pS350, encoding the nsP1, nsP2, and nsP3 genes of Girdwood in the AR86 genome, was constructed by replacing the *Mfe*I (nt 43) to *Bst*BI (nt 6411) fragment of pS300 with that of pG100 (Fig. 1). The chimeric cDNA clone pG106, encoding the structural genes of Girdwood in the AR86 genome, was constructed by exchanging the *Mfe*I (nt 43) to *Bst*BI (nt 6411) fragment of pG100 with that of pS300 (Fig. 1).

Clone pS341 (changes of Ile to Val at position 648 and Leu to Glu at position 651 in nsP2), clone pS342 (Gly to Glu change at nsP3 position 344), and clone pS344 (Cys to Opal termination codon change at nsP3 position 537) were created by PCR-mediated site-directed mutagenesis on the wild-type pS300 background (Fig. 2). Clone pS343, containing the Girdwood 18 amino acid region in nsP3 between positions 386-403, was constructed by replacing the *Sfi*I (nt 5122) to *Aat*II (nt 5285) fragment of pS300 with that of pG100. However, introduction of this region also included a single amino acid change of a Glu at nsP3 position 344, which was subsequently corrected to a Gly by primer-directed mutagenesis.

For the following panel of nonstructural chimeric cDNA clones, AR86 genomic fragments or point mutations were introduced into the chimeric virus pS350 (Fig. 3). Clone pS351 was derived by replacing the thymidine with cytidine at nucleotide position 1672 using PCR megaprimer mutagenesis (22), which replaced an Ile with a Thr codon at nsP1 position 538. Clone pS352 was made by replacing the adenine with a thymidine at nucleotide position 5793 to replace the opal termination codon at nsP3 position 537 with a Cys codon. Clone pS353 was constructed by deleting the sequences between nucleotide positions 5257-5311, corresponding to amino acid positions 386 to 403 of nsP3 in Girdwood, by primer directed mutagenesis. Clones pS354, pS355, pS356, and pS364, which contain different combinations of the changes at nsP1 position 538, nsP3 positions 386-403, and nsP3 position 537, were constructed by exchanging restriction fragments from clones pS351, pS352, pS353.

For the panel of structural gene chimeric viruses listed in Fig. 4, AR86 genomic fragments or point mutations were introduced into the chimeric virus pG106. The *Stu*I-*BssH*II fragment (nt 8110 to 9766) including most of the E2 coding region from clone pS300 was introduced into clone pG106 to create the chimeric clone pG107, and the *BssH*II-*Pme*I fragment (nt 9766 to 11343), encompassing most of the E1 coding region of clone pS300, was introduced into clone pG106 to create chimeric clone pG108. Clone pG117 was constructed by replacing the thymidine at position 9319 of pG106 with cytidine by PCR megaprimer mutagenesis, resulting in a coding change from a Ser to Leu at E2 position 243. Chimeric clone pG163 was constructed by primer-directed mutagenesis of pG100 as follows: substitution of thymidine for cytidine at nucleotide 1672 (Ile to Thr at nsP1 538), deletion of nucleotides between 5257-5311 (18 amino acid deletion from nsP3 386-403), substitution of

adenine for thymidine at nucleotide 5765 (Opal to Cys at nsP3 537), and thymidine for cytidine at nucleotide 9319 (Leu to Ser at E2 243). The reciprocal set of changes were made in pS300 to create clone pS363. Introduction of the various mutations were confirmed by sequencing at the University of North Carolina at Chapel Hill Genome Analysis Facility with a model 373A DNA sequencing apparatus (Applied Biosystems). Chimeric virus stocks derived from full-length cDNAs were made as described above.

Animal studies. Specific-pathogen-free female CD-1 mice were obtained from Charles River Breeding Laboratories (Raleigh, NC). Animal housing and care were in accordance with all University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee guidelines. Six-week-old mice were anesthetized with either isoflourane (Halocarbon Laboratories) or 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. Mice were monitored daily for body weight and scored for clinical signs as follows: 1= Ruffled fur and/or hunched body posture, 2= Mild hind-limb dysfunction, 3= Prominent hind-limb dysfunction, 4= Severe hind-limb dysfunction, 5= Paralysis of hind-limbs, and 6= Terminal morbidity. The average clinical score was calculated on day 5. This day was chosen because by day 6 infected mice reproducibly showed severe disease progression and occasionally were euthanized, with death being recorded for the following day. As required by the UNC-CH animal protocol, infected mice were euthanized during the experiment either when mice dropped below 30% of initial body weight or when mice exhibited severe disease signs. Statistical analysis for mortality was performed using Fisher's exact chi-squared test and differences were

considered significant when p<0.05 (Instat 3.0). For the *in vivo* growth studies, mice were inoculated as described and sacrificed by exsanguination followed by perfusion with 10 ml of PBS at 6, 12, 24, 48, 72, 96, and 120 hours postinfection. The whole brain was removed, weighed, and viral load assessed by plaque assay on BHK-21 cells (9, 23). The spinal cord was removed, divided into thoracic and lumbar regions, weighed, and viral loads evaluated by plaque assay on BHK-21 cells.

RESULTS

Neurovirulence determinants are localized within both the nonstructural and structural genes of AR86. SINV adult mouse neurovirulence determinants have previously been found in the viral 5' UTR and the E2 glycoprotein (12, 29). In addition, previous studies with AR86 identified a single coding change at nsP1 position 538 that was essential for adult mouse neurovirulence (9). In an effort to identify additional virulence determinants within AR86, comparisons were made between AR86 and Girdwood virus, a Sindbis-group virus that is closely related to AR86, but non-neurovirulent in adult mice. To confirm neurovirulence phenotypes, mice were inoculated intracranially (i.c.) with 10^3 PFU of either S300 (AR86) or G100 (Girdwood) and monitored for virus-induced morbidity and mortality. Mice were observed daily and scored for clinical signs such as weight loss, ruffled fur, hunched body posture, hind-limb dysfunction (mild, moderate, and severe), paralysis of hind-limbs, and terminal morbidity. The average clinical score for each group was calculated from observations made on day 5. Consistent with previous reports, S300 caused 100% morbidity and 100% mortality in 4 week-old mice, while G100 caused no detectable morbidity or mortality in the infected mice (Fig. 1A). G100 and S300 differ at 22 single

amino acid positions and by the presence (G100) or absence (S300) of an 18 amino acid sequence in nsP3 (Table 1). Given that the coding differences between S300 and G100 were distributed throughout the genome, our initial strategy involved defining whether the viral nonstructural or structural genes contained the major determinants of AR86 virulence. Therefore, two chimeras containing either the AR86 nonstructural coding region with the G100 structural genes (G106) or the G100 nonstructural genes with the AR86 structural genes (S350) were constructed and evaluated for virulence following i.c. infection of four week-old CD-1 mice. G106 caused 91.7% mortality and 100% morbidity, while S350 caused neither morbidity or mortality (Fig. 1A), though this virus grew as well as either wild-type parent S300 or G100, both *in vitro* and *in vivo* (data not shown). Furthermore, *in vitro* transcripts of pS300, pG100, pS350 and pG106 exhibited similar specific infectivities (data not shown). These results strongly suggested that the major determinants of AR86 neurovirulence were located in the nonstructural coding region. However, when older mice (6 week-old) were inoculated i.c. with G106, only 26.1% mortality was observed, in comparison to 95.5% mortality with S300 (Fig. 1B). Therefore, it was likely that determinants in both the nonstructural and structural genes were required for the full neurovirulence of AR86.

Since AR86 virulence determinants appeared to be located in both the structural and nonstructural coding regions, mapping of these virulence determinants were done separately, with the initial focus on the nonstructural region. While it was possible that noncoding differences were at least partly responsible for the virulence differences between AR86 and Girdwood, the initial analysis focused on the coding differences, which were located in nsP1, nsP2, and nsP3, but not nsP4 (Table 1). Previous analysis indicated that eight of the coding

differences, as well as the nsP3 deletion, were unique to AR86 when compared the known coding sequences of five non-virulent SINV (23). Furthermore, in previous studies, the unique Thr at nsP1 538 of AR86 was shown to be essential for adult mouse neurovirulence, as viruses with Ile at this position no longer caused lethal disease in adult mice (9). This suggested that the other coding changes that were unique to AR86 might be virulence determinants. Therefore each of the AR86 specific coding changes, with the exception of two changes at codons 442 and 446 in nsP3, were changed in the S300 background to the codon found at the corresponding position in Girdwood (clones pS341 and pS342). The role of the 18 amino acid deletion was also assessed by reintroducing the 18 amino acid sequence found at nsP3 positions 386 to 403 of Girdwood and the other non-neurovirulent SINV, back into the S300 clone (clone pS343), and an opal codon was introduced at nsP3 position 537 (pS344). The specific changes introduced into each clone was confirmed by sequencing. Viruses derived from these clones were evaluated for a loss of virulence following i.c. inoculation of 10^3 PFU into 6 week-old CD-1 mice. In the process of performing this analysis it became apparent that the reported Arg codon at position 256 of nsP2 in the AR86 infectious clone (23) was actually an Ala codon, which is present in all of the other SINV genomes. Therefore, this position was dropped from our analysis. Three changes at positions 648 and 651 of nsP2 (clone pS341) and at position 344 of nsP3 (clone pS342) did not appear to be major contributors to the adult mouse neurovirulence phenotype of AR86, since changing these codons to the corresponding codon found in Girdwood did not affect virulence in S300 (Fig. 2). However, reintroduction of the 18 amino acid deletion in nsP3 (clone pS343), or changing the unique Cys codon at nsP3 position 537 back to the opal termination codon (clone pS344) found in most non-neurovirulent SINV altered S300

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virulence (Fig. 2). In addition, both of these viruses exhibited equivalent growth kinetics to the wild-type S300 virus *in vitro*, suggesting that the decreased virulence was not simply due to a decrease in virus growth (data not shown). These results suggested that in addition to nsP1 position 538 (9), additional AR86 virulence determinants included the 18 amino acid deletion in nsP3 and the Cys codon at nsP3 537, while the unique codons at nsP2 648 and 651 or nsP3 344 did not appear to contribute to adult mouse virulence. The role of nsP3 442 and 446 was not addressed in this analysis, but the contribution of these determinants to virulence was evaluated in later studies (see below).

Introduction of AR86 determinants into the non-virulent Girdwood background results in a gain of virulence. Though mutation of nsP3 537 (Cys to Opal) or the reintroduction of the 18 amino acid deletion into nsP3 on the S300 background resulted in a loss of virulence, it was possible that rather than being true virulence determinants, these changes simply decreased virus fitness and thereby caused a subtle decrease in virus replication which affected virulence. Therefore, a more rigorous test of whether these or other determinants within the nonstructural region were virulence determinants was performed by introducing changes into the non-neurovirulent Girdwood background (clone pG100) and assaying for a gain of virulence. Since the AR86 nonstructural genes did not confer wild-type levels of virulence in the absence of the AR86 structural genes, all nonstructural mapping studies were performed using the clone pS350, which contained the Girdwood nonstructural region and the AR86 structural genes. Therefore, if all of the nonstructural determinants of virulence were introduced into the pS350 clone, the resulting virus should exhibit a level of neurovirulence comparable to AR86. As an initial step in this process, the three loci that were implicated in the S300 analysis (Fig. 2 and (9)) were analyzed in the S350 background

by introducing the codon found in the virulent AR86 virus back into the corresponding position in the Girdwood nonstructural region and looking for a gain of virulence (Fig. 3).

Introduction of a Thr codon in place of the Ile at nsP1 538 in the non-neurovirulent SINV TR339 was previously shown to increase virulence in 18 to 21 day old mice (9), which strongly suggests that this determinant plays a major role in adult mouse neurovirulence. When a Thr codon was placed at nsP1 538 of the ps350 clone, the resulting virus (S351) caused 100% morbidity, with a day 5 average clinical score of 2.2 and 16.7% mortality (Fig. 3), compared to 27.3% morbidity (day 5 average clinical score of 0.4) and 0% mortality with the S350 virus. A virus (S352) where the consensus opal termination codon at nsP3 position 537 was replaced with the AR86 derived Cys codon caused 66.7% morbidity (day 5 average clinical score of 1.3), but no mortality (Fig. 3). Likewise, deleting the 18 amino acids from position 386 to 403 in nsP3 of clone s350 (virus designated as S353) gave 78.6% morbidity (day 5 average clinical score of 1.2), but no mortality (Fig. 3). Therefore, all three changes independently increased S350 virulence as determined by an increase in virus-induced morbidity, however none of the individual determinants were able to raise S350 virulence to the level observed with AR86 (clone S300). Therefore, the individual coding changes were evaluated in combination. Introduction of both the 18 amino acid deletion and the Cys codon at nsP3 position 537 of clone ps350 resulted in a virus (S364) that caused 91.7% morbidity (day 5 average clinical score of 1.5) and 6.7% mortality (Fig. 3). However, when the Thr at nsP1 position 538 was introduced into clone pS350 in combination with either the 18 amino acid deletion (clone ps355) or the Cys codon at nsP3 position 537 (clone ps354), the resulting viruses were significantly more virulent than the parental S350 virus (27.3% morbidity, 0% mortality) (Fig. 3). The S355 virus (Thr at nsP1 538, 18 amino acid deletion at nsP3 386-

403) caused 100% morbidity and 65.4% mortality in 6 week-old mice, while the S354 virus (Thr at nsP1 538, Cys at nsP3 537) caused 100% morbidity and 76.9% mortality (Fig. 3). Finally, when all three changes were introduced into the same virus (clone pS356) the resulting virus caused 100% morbidity and 100% mortality in 6 week-old mice following i.c. inoculation. Therefore, the Thr at nsP1 position 538, the 18 amino acid deletion from nsP3 386 to 403, and the opal to Cys change at nsP3 527 are the major determinants of AR86 neurovirulence within the AR86 nonstructural protein coding region. Minor roles for other coding changes, such as nsP3 positions 442 and 446, or noncoding changes cannot be ruled out, however they were not necessary for the full adult mouse neurovirulence phenotype in the Girdwood background.

Serine at Position 243 in the E2 glycoprotein is a major determinant of AR86 adult mouse neurovirulence. Both the AR86 nonstructural coding region determinants and the AR86 structural genes were required for complete neurovirulence (Fig 1). Therefore, studies were initiated to identify the structural gene determinants that contributed to adult mouse virulence. Since complete virulence required the AR86 nonstructural coding region, all structural gene mapping studies were conducted using the G106 clone, which contains the AR86 nonstructural coding region and the Girdwood structural genes, and causes 87.0% morbidity (day 5 average clinical score of 2.0) and 26.1% mortality. Gene segments or individual coding changes from the structural genes of wild-type AR86 (pS300) were introduced back into pG106 and the resulting viruses evaluated for increased virulence following i.c. inoculation of adult mice. The AR86 and Girdwood structural genes contained 5 coding differences, 1 in the E2 glycoprotein, 1 in the 6k protein, and 3 in the E1 glycoprotein. The relative contribution of the change in E2 versus the changes in the 6k/E1

protein coding regions were assessed using two chimeras where the E2 coding region or the 6k/E1 coding region of AR86 was introduced into the G106 background. When these viruses were evaluated for virulence, the virus with the AR86 E2 gene (G107) caused 100% morbidity and 82.4% mortality (Fig. 4), while the virus with the AR86 6k/E1 coding region caused 100% morbidity (day 5 average clinical score of 2.2) and 33.3% mortality (Fig. 4). While these results suggest that minor determinants of AR86 neurovirulence reside within the 6k/E1 coding region, it is clear that a major determinant of adult mouse neurovirulence was located in the E2 glycoprotein. Since AR86 and Girdwood differ only at position 243 of E2, it was likely that this was the major determinant of adult mouse neurovirulence within the AR86 structural proteins. This was directly tested by introducing the Ser codon at E2 position 243 into the G106 clone to create the virus G117. While G106 caused 87.0% morbidity (day 5 average clinical score of 2.0) and 26.1% mortality, clone G117 caused 100% morbidity (day 5 average clinical score of 2.0) and 92.6% mortality, suggesting that the Ser at E2 position 243 was a major determinant of adult mouse neurovirulence (Fig. 4). Although the average peak clinical scores for G106 and G117 are the same on day 5, G117-infected mice progress to show more severe disease and eventually die with an extended AST than G106.

Determinants at nsP1 538, nsP3 386-430, nsP3 537, and E2 243 are able to confer an adult mouse neurovirulence phenotype in the Girdwood genetic background. In order to determine whether the three nonstructural determinants and the Ser codon at E2 position 243 were the major determinants of adult mouse neurovirulence in the AR86 virus, the four determinants were introduced into the G100 background. While G100 caused no morbidity or mortality in six week-old mice, the virus G163, which was identical to G100 except for the

presence of an Ile to Thr change at nsP1 538, an 18 amino acid deletion from nsP3 positions 386 to 403, an opal to Cys change at nsP3 537, and Leu to Ser change at E2 243, caused 100% morbidity (day 5 average clinical score 3.0) and 84.6% mortality (Fig. 5). For comparison, the same four changes were introduced into the S300 background, however, in this case the changes were Thr to Ile at nsP1 538, reconstitution of nsP3 codons 386 to 403, Cys to opal at nsP3 537, and Ser to Leu at E2 243. This virus, which was designated S363, caused 71.4% morbidity (day 5 average clinical score of 0.9) and 0% mortality, in contrast to the parental S300 virus, which caused 100% morbidity (day 5 average clinical score of 3.1) and 95.5% mortality (Fig. 5). These results show that introduction of the four AR86 determinants back into the non-virulent Girdwood background resulted in a virus that exhibited neurovirulence that was comparable to AR86, demonstrating that these four changes play a major role in the adult mouse neurovirulence phenotype of AR86. However, minor contribution from other coding or non-coding differences between Girdwood and AR86 cannot be ruled out.

***In vitro* and *in vivo* growth analysis of the attenuated and virulent viruses.** The differences in virulence between the attenuated viruses (G100 or S363 chimera) and the virulent viruses (S300 or G163 chimera) could simply be explained by replication efficiency or the ability to successfully establish infection within neural tissues. Therefore, a single-step *in vitro* growth assay was performed for each virus in BHK-21 cells. As shown in Figure 6A, the attenuated viruses grew at least as well as the virulent viruses at all time points tested. Viral growth in brain and spinal cords of infected mice was evaluated next. Though G100 and S363 are significantly attenuated with regard to causing disease, both of these viruses grew at least as well as the virulent S300 and G163 viruses in the brains of

infected mice at all times post infection (Figure 6B). Furthermore, when viral growth in the spinal cord was evaluated, viral titers were equivalent at 48 hours post infection, suggesting that both viruses were able to establish infection within the spinal cord. However, at 96 hours post infection, which is a time point when mice are starting to develop virus-induced disease signs, the virulent S300 virus exhibited significantly higher titers ($p < 0.05$) in the spinal cord than the attenuated S363 virus (Figure 6 C and D). This suggests that the virus containing the four attenuating determinants are either defective in their ability to spread within the spinal cord or cleared more efficiently than the virus containing the four virulence determinants. Additional studies to evaluate the extent of viral replication within these tissues will be required to address this question.

DISCUSSION

The identification of the molecular determinants of alphavirus virulence represents an important step in understanding the pathogenesis of alphavirus-induced neurologic disease. The majority of studies looking at alphavirus neurovirulence determinants have identified the structural gene E2 and the 5' UTR as major determinants of virulence in both neonatal and adult mice (3, 19, 29). However, recent work with the Sindbis-group virus AR86 and Semliki forest virus (SFV) has demonstrated a role for the nonstructural genes in mediating alphavirus neurovirulence (9, 32, 33). In this report, we describe the identification of four coding changes at nsP1 position 538, nsP3 position 537, an 18 amino acid deletion from nsP3 386 to 403, and at E2 position 243 as major determinants of AR86 neurovirulence. The fact that these four changes conferred adult mouse neurovirulence on a normally non-neurovirulent SINV argues that these changes represent the major determinants of AR86

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neurovirulence, although contributions from additional AR86 determinants cannot be ruled out. It is also important to note that the neurovirulence determinants did not appear to simply affect viral replication *in vitro* or in neural tissue at early times post-infection (Figure 6). However, studies in the spinal cord suggest that the attenuated viruses are either defective in spreading at late times in the infection, or may be cleared more efficiently than the virulent viruses (Figure 6). Additional work is required to assess these possibilities and also determine whether the differences between the virulent and attenuated virus are spinal cord specific or simply a lack of sensitivity in detecting differences in the brain by plaque assay.

It is striking that all four of the identified AR86 neurovirulence determinants are, to our knowledge, unique to AR86, and not present in the published sequences of non-adult mouse neurovirulent SINV (23). Furthermore, these determinants do not appear to be shared with other adult mouse neurovirulent SINV, such as the NSV strain (3, 19, 31). This suggests that AR86 and NSV have evolved different strategies for causing neurologic disease in adult mice. For instance, while the nonstructural gene determinants of AR86 are essential for neurovirulence in adult mice, the E2 243 change is only essential in mice greater than 4 weeks of age (Figure 1). In contrast, the structural genes of the NSV strain of Sindbis are the major determinants of adult mouse neurovirulence, with a His at E2 position 55 being particularly important (31). There are several potential explanations for the differences in virulence determinants between AR86 and NSV, including passage histories and strain variation in the original viral isolates from which these neurovirulent viruses were derived. NSV was originally derived from the AR339 strain of Sindbis (7), while AR86 and Girdwood, which are closely related to each other, were isolated in South Africa. In fact, Girdwood is one of the few SINV isolated from an infected human (20). Therefore, it is

possible that genetic differences between this strain and the South African Sindbis-group virus strains such as AR86 and Girdwood biased these viruses toward different types of virulence determinants. NSV and AR86 were also placed under different types of selective pressure during their early passage. NSV was derived from a virus that was passaged an unknown number of times in cell culture, and then selected through six rounds of passage in neonatal and weanling mice (7). In contrast, AR86 was isolated from a mosquito pool, and subjected to 45 to 60 alternating rounds of intracranial passage in neonatal and weanling mice (34), with very limited exposure to cell culture prior to the generation of the infectious clone (23). It is likely that these vastly different passage histories are in part responsible for the differences in virulence determinants between AR86 and NSV.

While all four changes are essential for the complete mouse neurovirulence phenotype of AR86, it appears that the Thr at nsP1 538 plays a particularly important role. Previous studies have shown that changing the Thr at nsP1 538 in AR86 to the consensus Ile found in non-neurovirulent viruses results in a complete loss of virus-induced mortality, though this virus was still capable of causing disease (9). Likewise, introduction of a Thr codon at nsP1 position 538 into S350 (which contains the AR86 structural genes in the Girdwood virus background), creating S351, did not result in a complete restoration of neurovirulence, but, a partial gain of virulence was observed (100% morbidity, 16.7% mortality, see Fig. 3) As for the mechanism underlying the role for nsP1 538 in virulence, we have previously reported that substitution of the wild-type Thr with an Ile residue accelerates processing of the P123 polyprotein precursor into the mature nsP1, nsP2, and nsP3 proteins (10). This coincided with a more rapid induction of 26S RNA synthesis contributing to earlier expression from the 26S promoter in infected cells, but did not

measurably affect the levels of viral minus- or plus-strand RNA synthesis (10). The role of altered nonstructural polyprotein processing and/or accelerated induction of 26S RNA synthesis in adult mouse neurovirulence has yet to be determined. They may be acting through one or more mechanisms, including induction of immune mediators by the earlier 26S RNA expression, enhanced cytopathic effect due to high level expression of the viral structural genes, or differential effects on host macromolecular synthesis due to differences in the ratios of the nonstructural polyprotein precursor versus mature nonstructural proteins.

The 18 amino acid deletion between residues 386-403 in nsP3 is located within the C-terminal region, which is highly variable between alphaviruses (as reviewed in reference (25)). The function for the nsP3 protein has yet to be defined. However, it has been shown to be a phosphoprotein that is required for the synthesis of both viral minus-strand and subgenomic RNA (14, 17). With this in mind, it is interesting to note that the 18 amino acid deletion results in the removal of 7 Ser residues, which may affect the overall phosphorylation of nsP3. Mutational analysis within the nonconserved C-terminal region of SINV nsP3 altered levels of viral minus-strand RNA synthesis, along with levels of nsP3 phosphorylation (14). Therefore, it will be important to assess the effect of the 18 amino acid deletion on viral minus-strand synthesis. Recently, it was reported that mutations within the nsP3 gene of SFV, including a seven amino acid deletion within the C-terminal region, fully restored neurovirulence in adult mice (32), which suggests that nsP3 may contribute to the virulence of multiple alphaviruses. However, more analysis is required to determine the exact role for the AR86 nsP3 in adult mouse neurovirulence.

While most alphaviruses encode an opal termination codon proximal to the 3' end of the nsP3 gene, the Sindbis-group virus strain AR86 (23) and the SFV strain SFV4 carry a

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sense codon, which has been shown to be an important contributor to neurovirulence in adult mice (23, 32, 33). Tuittila et al. performed a detailed mapping study within the replicase genes using virulent and avirulent SFV strains and found that an Arg (virulent SFV4 strain) at nsP3 position 469, in place of the opal termination codon, was an important contributor to the adult mouse neurovirulence phenotype (32). The mechanism of this change in pathogenesis is not well understood. Translational readthrough of the opal termination codon occurs at a frequency of about 5-10% (as reviewed in reference (4)), leading to limiting quantities of the nsP4 protein relative to the other nonstructural proteins. Interestingly, the nsP4 protein was found to be tightly regulated within infected cells, in that excess nsP4 was shown to be rapidly degraded by the N-end rule pathway (1). It is also worth noting that a different nsP3 C-terminus is produced in viruses encoding either an opal termination codon or a sense codon. In the presence of an opal termination codon, the predominate nsP3 C-terminus is produced by the translational stop codon, while in the presence of a sense codon, an extra seven amino acids are added to the C-terminus of nsP3. It is well known that the opal termination codon regulates both nonstructural polyprotein processing and viral RNA synthesis. Li et al. replaced the opal termination codon of SINV with different sense codons and found increased levels of the nsP3/4 polyprotein precursor and in reduced levels of mature nsP3 early during infection (18). This study also demonstrated that replacing the opal termination codon with a sense codon lead to reduced levels of both 49S genomic and 26S subgenomic viral RNA synthesis early during infection (18). Based on these studies, it will be important to determine whether the Cys codon in AR86 affects neurovirulence through alterations of nonstructural polyprotein processing and/or viral RNA synthesis, which may

exert subtle effects on viral replication or affect an as yet to be defined interaction with the host.

The neurovirulence determinant within the E2 glycoprotein at position 243, where AR86 encodes a unique serine residue, most likely affects early viral interactions with neurons or other cell types in the infected animal. This change is near a region of E2 that is associated with receptor attachment, which raises the strong possibility that the Ser residue might affect virus/receptor interactions either through direct receptor interactions or by changing the conformation of the E2 glycoprotein (24). This is supported by findings within the E2 glycoprotein of SINV virus, in that a Gly residue at position 172 enhanced viral binding to neuronal cells (29). Therefore, additional studies to evaluate the effect of Ser versus Leu at E2 position 243 on virus binding or infection of neurons may provide useful information on the role of this determinant in regulating viral infection of neurons or other cell types. However, the lack of a clearly defined neuronal receptor for alphaviruses currently prevents a direct analysis of this determinant's role in virus/receptor interactions.

In conclusion, we have identified major determinants of SINV adult mouse neurovirulence using two closely related neurovirulent and non-neurovirulent Sindbis-group viruses. These determinants are nsP1 position 538, a deletion in nsP3 between 386-403, nsP3 position 537, and E2 position 243. Further studies are underway to determine whether these virulence determinants act by affecting viral RNA synthesis, cell tropism, or through some as yet undefined interaction with the infected host.

ACKNOWLEDGMENTS

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Table 3-1. Amino acid sequence comparison between AR86 and Girdwood.

Gene	Amino Acid Position	AR86	Girdwood
nsP1	469	Met	Ile
	538	Thr	Ile
nsP2	30	Ile	Thr
	187	Thr	Ala
	218	Glu	Gln
	295	His	Tyr
	485	Ala	Arg
	634	Glu	Lys
	648	Ile	Val
	651	Lys	Glu
nsP3	344	Gly	Glu
	346	Val	Ala
	386-403*	Deletion	
	386	Tyr	Asp
	442	Asp	Gly
	446	Ile	Met
	537*	Cys	Opal
E2	243*	Ser	Leu
6k	30	Val	Ile
E1	61	Arg	Lys
	112	Val	Ala
	169	Leu	Ser

Footnote: a) AR86 amino acid numbering

b) nsP3 18 amino acid deletion found in AR86

c) First amino acid after 18 amino acid deletion

Figure 3-1: Both the nonstructural and structural genes of AR86 contain neurovirulence determinants.

A.

| nsP1 | nsP2 | nsP3 | nsP4 | C | E2 | E1 |

Name	Plasmid Construct	n	Morbidity (CS)	Mortality	AST (SD)
pS300		7	100% (2.3)	100%	6.0 (1.4)
pG100		7	0% (0)	0%	N/A
pS350		12	0% (0)	0%	N/A
pG106		12	100% (2.8)	91.7%	7.1 (0.8)

B.

| nsP1 | nsP2 | nsP3 | nsP4 | C | E2 | E1 |

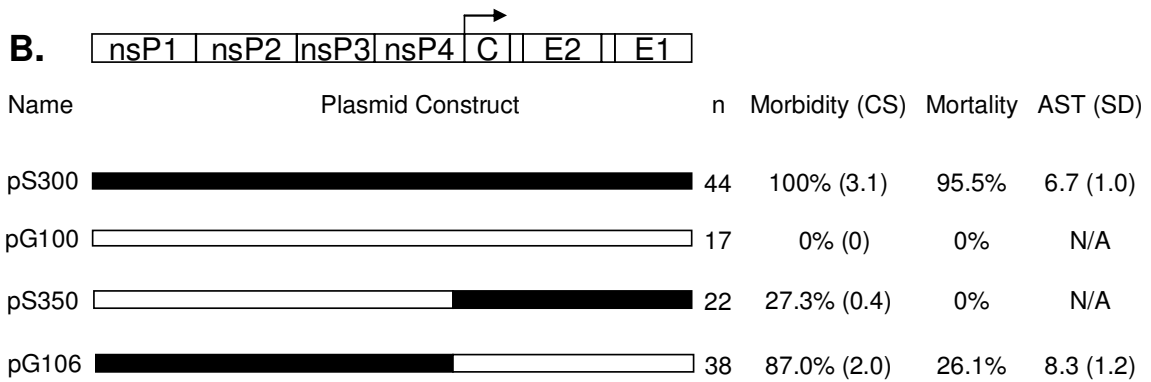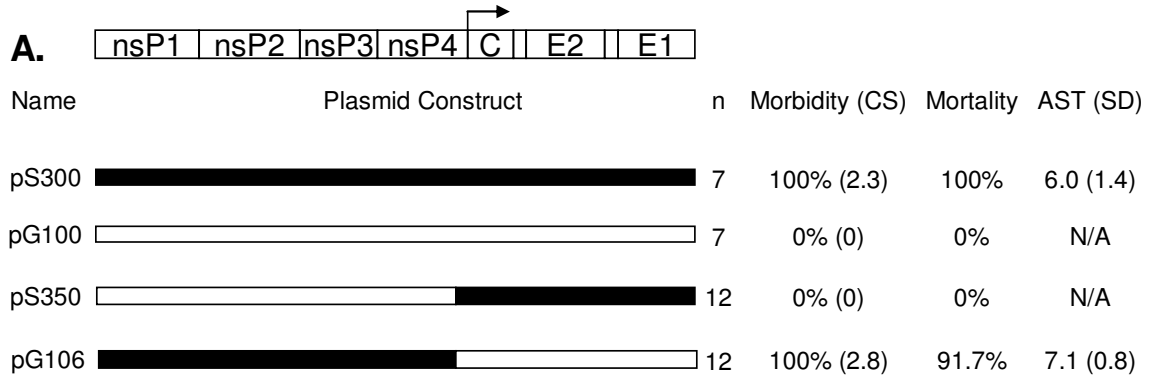| Name	Plasmid Construct	n	Morbidity (CS)	Mortality	AST (SD)
pS300		44	100% (3.1)	95.5%	6.7 (1.0)
pG100		17	0% (0)	0%	N/A
pS350		22	27.3% (0.4)	0%	N/A
pG106		38	87.0% (2.0)	26.1%	8.3 (1.2)

Figure 3-1. **Both the nonstructural and structural genes of AR86 contain neurovirulence determinants.** A diagram of the *Alphavirus* genome organization is located at the top. On the left are the names of the cDNAs encoding full-length virus, beginning with the parental strains, pS300 (WT AR86) and pG100 (WT Girdwood) followed by the chimeric viruses. The cDNA constructs are diagrammed to show the sequences derived from pS300 (shaded box) and pG100 (opened box). The chimeric clone pS350 contains AR86 nt sequences between 1 to 6411 and the chimeric pG106 clone contains AR86 nt sequences from 6411 to 11343. Groups (n= number of mice per group) of either (A) four week-old or (B) six week-old female CD-1 mice inoculated intracranially (i.c.) with 10^3 PFU of each virus and observed daily for clinical signs. Virulence was assessed by morbidity (average clinical score (CS) on day 5), mortality, and average survival time (AST, days ± standard deviation [SD]).

Figure 3-2: Mutational analysis of neurovirulence determinants within the nonstructural genes of AR86.

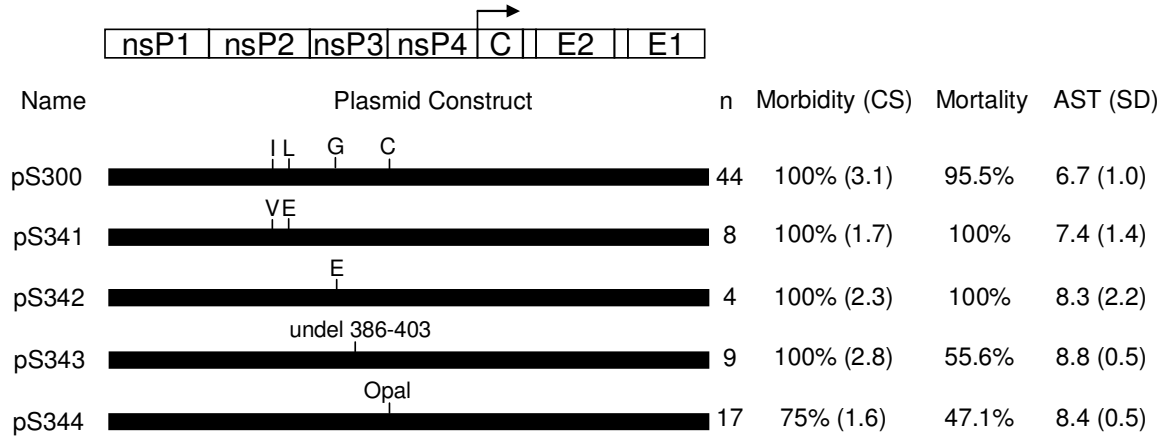| Name	Plasmid Construct	n	Morbidity (CS)	Mortality	AST (SD)
pS300		44	100% (3.1)	95.5%	6.7 (1.0)
pS341		8	100% (1.7)	100%	7.4 (1.4)
pS342		4	100% (2.3)	100%	8.3 (2.2)
pS343		9	100% (2.8)	55.6%	8.8 (0.5)
pS344		17	75% (1.6)	47.1%	8.4 (0.5)

Figure 3-2. Mutational analysis of neurovirulence determinants within the nonstructural genes of AR86. The cDNA constructs are diagrammed to show sequences derived from AR86 (pS300) along with amino acids located in nsP2 at positions 648 and 651, and nsP3 position 344, 386-403, and 537. Groups (n) of six week-old female CD-1 mice were inoculated i.c. with 10^3 PFU of either wild-type AR86 (S300) or each mutant virus and observed daily for clinical signs. Virulence was assessed by morbidity (average clinical score (CS) on day 5), mortality, and AST (days ± standard deviation [SD]). The mortalities for the mutant viruses S343 and S344 were statistically significant when compared to S300 ($p<0.05$).

Figure 3-3: AR86 neurovirulence determinants within the nonstructural genes.
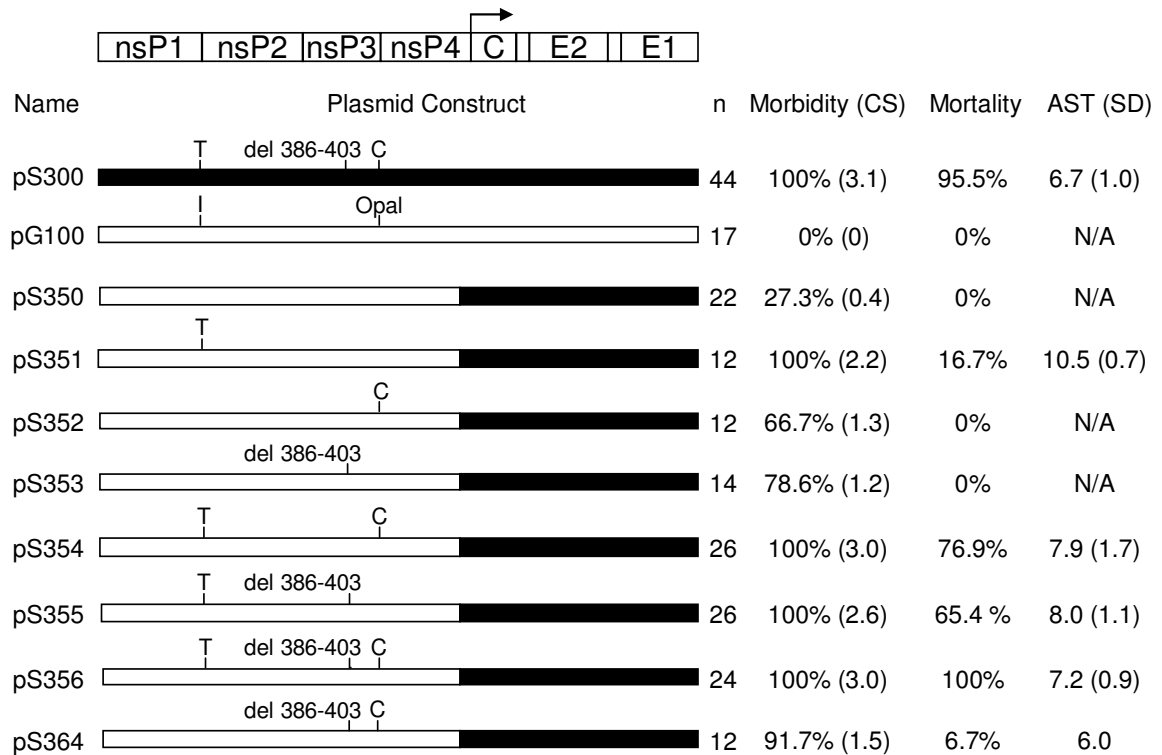
Figure 3-3. AR86 neurovirulence determinants within the nonstructural genes. The cDNA constructs are diagrammed to show the sequences derived from pS300 (shaded box) and pG100 (opened box) along with amino acids located in nsP1 at position 538 (Thr= pS300 and Ile= pG100), nsP3 between positions 386-403 (del 386-403= pS300) and nsP3 at position 537 (Cys= pS300 and Opal= pG100). All of the chimeric clones contain AR86 nt sequences between 6411 and 11343. Groups (n) of six week-old female CD-1 mice were inoculated i.c. with 10^3 PFU of each virus and observed daily for clinical signs. Virulence was measured by morbidity (average clinical score (CS) on day 5), mortality, and AST (days ± standard deviation [SD]). The mortalities for the chimeric viruses S354, S355, and S356 were statistically significant when compared to the parental chimeric virus S350 ($p<0.05$).

Figure 3-4: AR86 neurovirulence determinants within the structural genes.
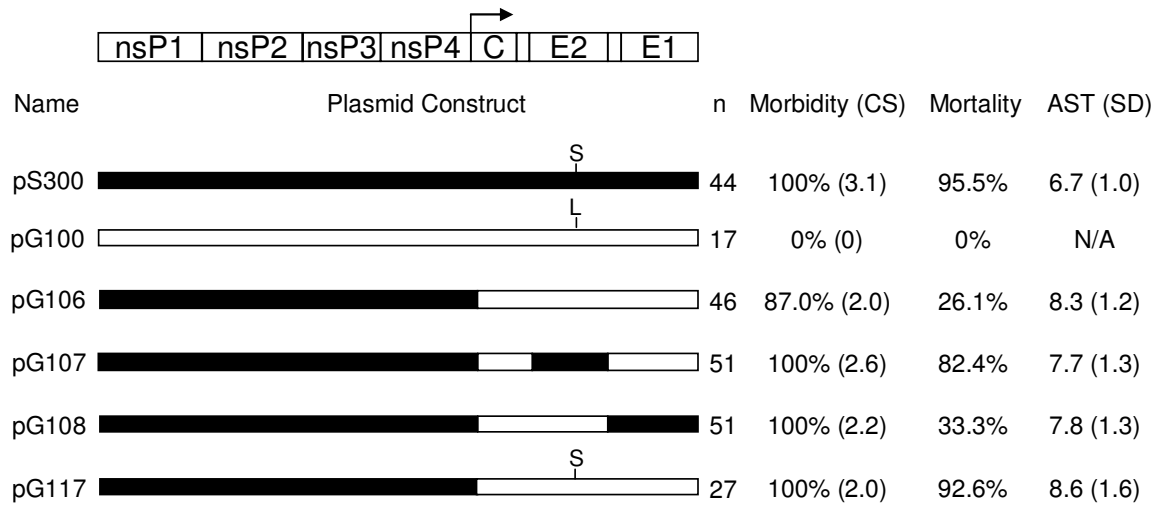
Figure 3-4. AR86 neurovirulence determinants within the structural genes. The cDNA constructs are diagrammed to show the sequences derived from pS300 (shaded box) and pG100 (opened box) along with the amino acid located in E2 at position 243 (Ser= pS300 and Leu = pG100). All of the chimeric clones contain AR86 nt sequences between 1 and 6411. Groups (n) of six week-old female CD-1 mice were inoculated i.c. with 10^3 PFU of virus and observed daily for clinical signs. Virulence was assessed by morbidity (average clinical score (CS) on day 5), mortality, and AST (days ± standard deviation [SD]). The mortalities for the chimeric viruses G107 and G117 were statistically significant when compared to the parental chimeric virus G106 (p<0.05).

Figure 3-5: Identification of the major determinants of neurovirulence within AR86.

| nsP1 | nsP2 | nsP3 | nsP4 | C | | E2 | | E1 |

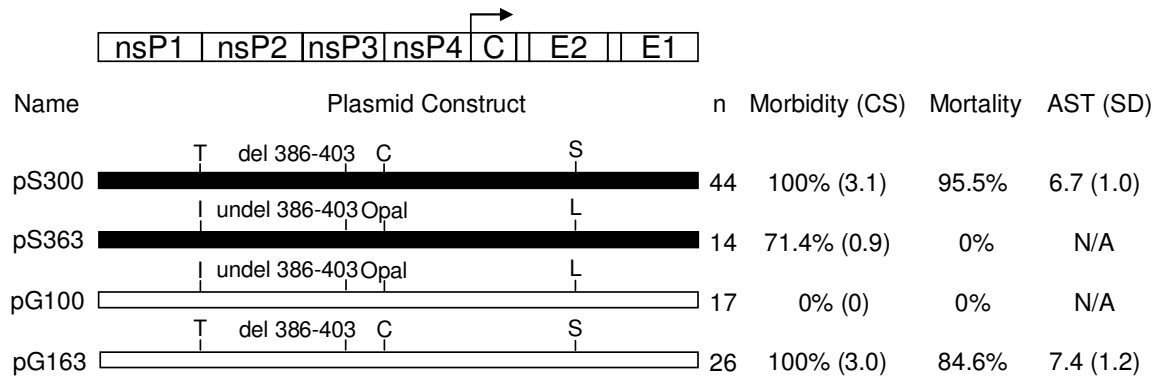| Name	Plasmid Construct	n	Morbidity (CS)	Mortality	AST (SD)
	T　　del 386-403　C　　　　　　S				
pS300		44	100% (3.1)	95.5%	6.7 (1.0)
	I　undel 386-403 Opal　　　　L				
pS363		14	71.4% (0.9)	0%	N/A
	I　undel 386-403 Opal　　　　L				
pG100		17	0% (0)	0%	N/A
	T　　del 386-403　C　　　　　　S				
pG163		26	100% (3.0)	84.6%	7.4 (1.2)

Figure 3-5. Identification of the major determinants of neurovirulence within AR86.

The cDNA constructs are diagrammed to show the sequences derived from pS300 (shaded box) and pG100 (opened box) along with amino acids located in nsP1 at position 538 (Thr= pS300 and Ile= pG100), nsP3 between positions 386-403 (del 386-403= pS300), nsP3 at position 537 (Cys= pS300 and Opal= pG100), and E2 at position 243 (Ser= pS300 and Leu = pG100). Groups (n) of six week-old female CD-1 mice were inoculated i.c. with 10^3 PFU of virus and observed daily for clinical signs. Virulence was measured by morbidity (average clinical score (CS) on day 5), mortality, and AST (days ± standard deviation [SD]).

Figure 3-6: In vitro and in vivo growth analysis of virulent and attenuated viruses.
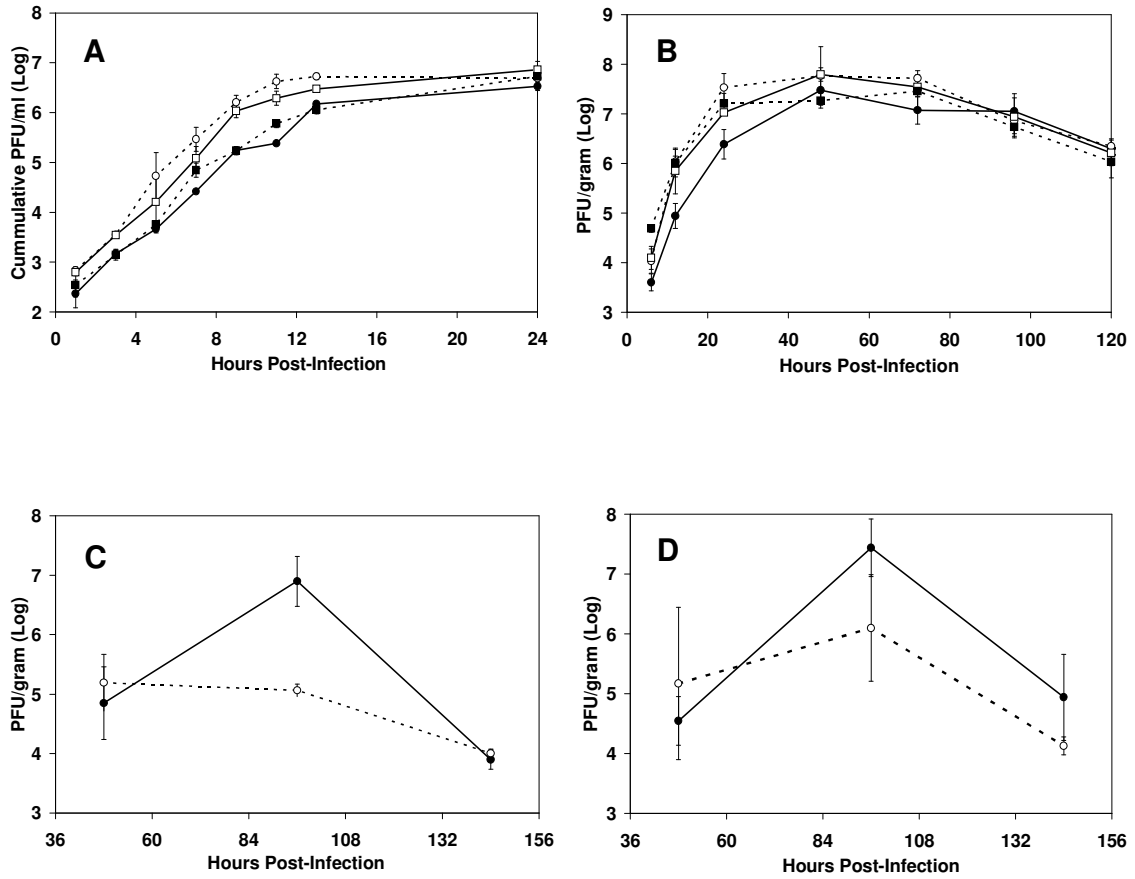
Figure 3-6. *In vitro* and *in vivo* growth analysis of virulent and attenuated viruses. A. Single-step *in vitro* growth curve was performed on BHK-21 cells infected with S300 (solid line, closed circle), S363 (broken line, open circle), G100 (sold line, open square), or G163 (broken line, closed square) at an MOI of 5.0. Shown is a representative experiment where each point represents the average of three independent samples plus or minus the standard deviation. B. Six-week-old female CD-1 mice were infected i.c. with 10^3 PFU of S300 (solid line, closed circle), S363 (broken line, open circle), G100 (sold line, open square), and G163 (broken line, closed square). Mice were sacrificed by exsanguination at 6 12, 24, 48, 72, 96, and 120 hours post infection and perfused with PBS (pH7.4). The brain was harvested and evaluated for viral load by plaque assay on BHK-21 cells. The data shown represents one of three experiments for the brain. C and D. Six week old CD-1 mice were infected with S300 (solid line, closed circle) or S363 (broken line, open circle) as in Figure 6B. Mice were sacrificed at 48, 96, or 144 hours post-infection, perfused with PBS, and viral titers in the thoracic or lumbar spinal cord determined by plaque assay. C. Viral titers in the thoracic spinal cord. n= 3 mice per time point and data shown represents one of two identical experiments. D. Viral loads in the lumbar spinal cord. Data was pooled from two experiments and n = 6 mice per time point. Differences in viral loads in the thoracic and lumbar spinal cord at 96 hours post-infection are statistically significant (p<0.05) as measured by two-tailed student's t test.

CHAPTER FOUR

A VIRULENCE DETERMINANT AT NSP1 538 MODULATES TYPE I INTERFERON INDUCTION BY THE SINDBIS-GROUP VIRUS STRAIN AR86

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ABSTRACT

A virulence determinant at nsP1 538 of the adult mouse virulent strain of Sindbis-group virus AR86, regulates viral nonstructural polyprotein processing, viral RNA synthesis, and adult mouse neurovirulence. An attenuating mutation at nsP1 538 resulted in enhanced type I interferon (IFN) induction *in vivo* and *in vitro*. Further characterization of this determinant demonstrated that its effects on type I IFN induction were independent of effects on virus-mediated host cell transcription and translation. Analysis of Interferon Regulator Factor-3 (IRF-3), a key mediator of type I IFN induction, revealed that in the presence of the attenuating mutation at nsP1 538, more rapid IRF-3 phosphorylation and dimerization was observed as compared to wild-type virus. The results from this study suggest that the nsP1 538 locus modulates viral type I IFN induction and exerts a major influence on viral virulence.

INTRODUCTION

Alphaviruses are mosquito borne viruses that cause a range of human diseases, including encephalitis and severe arthritis/arthralgia. Though Sindbis viruses (SINV) do not generally cause severe disease in humans, experimental SINV infection models in rodents have been used to identify viral determinants that contribute to virulence, and identify host factors that control infection and mediate viral clearance (11, 20, 41, 42, 49). Such virulence determinants are distributed throughout the genome and their activities demonstrate an important role for the innate and adaptive immune response in controlling viral replication and virus-induced disease. However, specific interactions between alphavirus virulence determinants and the host immune system have not been extensively studied.

The alphavirus nonstructural proteins constitute the viral RNA replicase and are translated as a polyprotein that is postranslationally processed to produce the mature nonstructural proteins, nsP1, nsP2, nsP3, and nsP4 (reviewed in reference (47)). nsP1 exhibits methyltransferase and guanyltransferase activity and is involved in viral RNA capping (2, 33). nsP2 is a multifunctional protein exhibiting papain-like thiol proteolytic activity (19), nucleoside triphosphatase activity (39, 55), and helicase activity (15). NsP3 is a phosphorylated protein that plays an essential role in viral minus-strand and subgenomic RNA synthesis; however, the functions of nsP3 are not well defined (24, 37, 56). NsP4 functions as the viral RNA-dependent RNA polymerase and was recently found to possess terminal adenylyltransferase activity (47, 51). Processing of the nonstructural polyprotein regulates viral RNA synthesis. The nsP123 polyprotein, in combination with nsP4, mediates viral minus strand synthesis (25, 26, 45), while processing of the nsP123 and nsP23

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polyproteins into the mature nonstructural proteins leads to a shutoff of minus strand RNA synthesis and induction of viral positive strand genomic and 26S RNA synthesis (45, 48).

Control of alphavirus infection *in vivo* is mediated by components of both the innate and adaptive immune system (17, 18, 41, 52). Type I IFN plays an essential role in the early control of alphavirus infection, with mice deficient in the type I IFN receptors being highly susceptible to normally nonpathogenic strains of SINV (41) or Venezuelan equine encephalitis virus (VEE) (57). Other components of the adaptive immune response, including antibody, T cells, and IFN-γ contribute to the control and clearance of non-pathogenic SINV strains (5, 6, 29), while T cells may contribute to virus-induced immune pathology with at least some neurovirulent SINV strains (23, 40).

Most SINV strains are nonpathogenic in adult mice; however, adult mouse virulent viruses, such as NSV and AR86 have been used to identify viral determinants that contribute to adult mouse virulence and/or neuroinvasion (10, 49, 54). The major determinant of adult mouse virulence for the NSV strain is a determinant at E2 position 55, where a histidine codon is associated with increased virulence in adult mice (54). Though the exact role of this determinant in promoting virus-induced disease is undefined, this determinant appears to enhance viral infection of neurons (9, 53), overcome the protective effect of bcl-2 overexpression in neurons (27, 28), and modulate the adaptive immune response in promoting virus-induced disease (23, 40). Virulence studies with the AR86 strain of Sindbis, which causes 90-100% mortality in mice greater than 6 weeks of age when delivered by the intracranial route (46), identified four major genetic determinants of adult mouse neurovirulence which localized to a Thr at nsP1 538, an 18 amino acid deletion from amino acids 386 to 403 in nsP3, Cys at nsP3 position 537, and Ser at E2 position 243. Though all

four of these determinants are required for full adult mouse virulence, one determinant at nsP1 538 appeared to play a major role in promoting AR86 virulence (20, 49). In comparison to an attenuating Ile codon at this position, the presence of the wild-type Thr codon exhibited delayed processing of the viral nonstructural polyprotein and delayed RNA synthesis from the 26S subgenomic promoter (21), however the actual mechanism(s) by which this determinant affects viral virulence is not currently understood.

Though the viral nonstructural proteins play an essential role in viral replication, their interactions with the host are less well defined. Shutoff of host cell RNA and protein synthesis is mediated by the viral nonstructural proteins (16), with nsP2 believed to play a major role in this process through an undefined mechanism (12, 13, 16). Host shutoff is thought to enhance viral replication by limiting competition for cellular translational machinery, while also limiting the ability of the infected cell to mount an antiviral response, including the production of type I IFN (11, 35). However, the mechanisms by which the viral nonstructural proteins modify the host cell and promote viral virulence are still largely undetermined.

As described above, previous work from our laboratory has defined three determinants of adult mouse virulence within the nonstructural proteins of the AR86 strain of Sindbis (49), and one of these determinants, at nsP1 position 538, affects the kinetics of viral nonstructural protein processing and viral subgenomic RNA synthesis (21). Based on importance of the virulence determinant at nsP1 position 538 and the role of type I IFN in controlling virus infection, we hypothesized that this virulence determinant modulates type I IFN induction. In this report, we demonstrate that a virus with an attenuating mutation at nsP1 538 (Thr to Ile) is a potent inducer of type I IFN *in vivo* and *in vitro*. Furthermore, this

determinant did not affect the kinetics of viral host cell shutoff, but did affect the kinetics of IRF3 activation in the infected cell. These results demonstrate that an alphavirus virulence determinant modulates type I IFN induction and suggests potential mechanisms for effects on virulence.

MATERIALS AND METHODS

Viruses and cells. Plasmids containing the viral cDNA are designated with the prefix "p" while the infectious virus derived from the cDNA clone does not contain the "p" designation; i.e., S300 represents virus derived from the plasmid pS300. Virus stocks were made as described previously (49). Briefly, viral cDNA plasmids were linearized with *PmeI* and used as templates for the synthesis of full-length transcripts by using SP6-specific mMessage Machine *in vitro* transcription kits (Ambion). Transcripts were electroporated into BHK-21 cells grown in α-minimal essential medium (α-MEM: 10% fetal calf serum [Gibco], 10% tryptose phosphate broth [Sigma], and 0.29 mg of L-glutamine per ml [Gibco]). Supernatants were harvested 24 to 27 hours after electroporation and subjected to centrifugation at 3000 RPM (Sorvall rotor RTH-250) for 20 minutes at 4°C and frozen in 1-ml aliquots. Virus stocks were titered on BHK-21 cells as previously described (46).

BHK-21 and L929 cells were maintained at 37°C in α-MEM, for a maximum of 10 passages. A549 cells (ATCC) are human lung carcinoma cells and were grown in Dulbecco's modified medium (DMEM) containing 10% fetal calf serum [Gibco], 0.29 mg of L-glutamine per ml [Gibco], and penicillin/streptomycin. HEC-1B cells (ATCC) are human endometrial carcinoma cells and were used in this study to analyze IRF-3, since these cells are nonresponsive to IFN (8, 50). HEC-1B cells were grown in MEM containing 10% fetal bovine serum, 0.29 mg of L-glutamine per ml [Gibco], and penicillin/streptomycin. Neuro2A cells (ATCC) are mouse neuroblastoma cells and were grown in MEM containing nonessential amino acids and 10% FBS.

Examination of total RNA synthesis. To examine host transcription, Neuro2A cells were mock infected (M), infected with S300, or S340 at an MOI of 10.0. At 2, 5, 8, and 16 hours

post-infection, media was removed and replaced with complete α-MEM medium containing

[^{3}H]-uridine at 20 μCi/ml. After 3 hours incubation at 37°C, cells were washed 1X in PBS

and cells lysed with Trizol (Invitrogen) and RNA was isolated. RNAs were denatured in

glyoxal and dimethyl sulfoxide for one hour at 50°C and analyzed on a 0.8% agarose 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 ddH$_2$O to

precipitate the PPO, dried, and exposed to film (Kodak Biomax MR). For quanitition, 18S

rRNA and 28S rRNA fragment and the fragment corresponding to host cellular mRNA, as

previously described (16), were excised from the dried gel and cpm's were measured by

liquid scintillation.

Examination of protein synthesis. To monitor protein synthesis with S300, S340- or mock-

infected cells, at various time-points post-infection, Neuro2A cell monolayers were washed

with PBS and starved of methionine and cysteine by providing Minimum Essential Medium

lacking methionine and cysteine (Sigma). After 1 h incubation at 37°C, the medium was

replaced with starvation medium supplemented with 33 μCi/mL 35S-methionine/35S-

cysteine (Pro-mix, Amersham Pharmacia). Immediately following incubation for 1 h at

37°C, monolayers were harvested in NP-40 lysis buffer as described above. Equal volumes

of lysate from each time point were separated on 10% SDS-PAGE gel. The gels were fixed

in a solution of 10% acetic acid and 40% methanol, dried, exposed to a Phosphorimaging

screen, scanned using a Storm Phosphorimager (GE Healthcare) and analyzed using

ImageQuant software (GE Healthcare). Residual host cell protein synthesis was evaluated by

measuring the amount of radioactivity detected in the protein band corresponding to actin

and normalized to the amount of radioactivity detected in the same protein band in mock infected cells.

Animal studies. Specific pathogen-free C57BL/6 mice were obtained from The Jackson Laboratory and bred in-house. Specific pathogen-free six-week old female CD-1 mice were obtained from Charles River Breeding Laboratories (Raleigh, NC). Animal housing and care were in accordance with all University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee guidelines. Six- to eight-week-old mice 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 was frozen at -80°C until analyzed for type I IFN by bioassay (see below).

Type I IFN bioassay. Type I IFN α/β were measured in cell culture supernatants and in sera using a biological assay as previously described. Briefly, for a mouse IFN bioassay, L929 cells were seeded at 3 x 10^4 cells/well in a 96 well plate one day prior to the addition of IFN standards or experimental samples. Human IFN bioassay was performed in a similar manner except A549 cells were used. Cell culture supernatant or mouse sera (diluted 1:10 in L929 media) were centrifuged at 60,000 RPM for one hour at 4°C followed by acidification to a pH of 2.0 using hydrochloric acid (HCl). Twenty-four hours later, supernatants and sera were neutralized with sodium hydroxide, after which cell culture supernatants were treated with UV light for 20 minutes to eliminate any residual virus. Duplicate supernatants and sera samples were then applied to the 96-well plates in two-fold dilutions along with an NIH mouse- or human-specific IFN α/β standard. The following day, EMCV challenge virus was

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added to the cells in 50 ul/well at an MOI of 5.0. Twenty-four hours later, 50% CPE was measured by blind scoring the wells and IFN in the supernatant or sera was calculated based on the respective NIH IFN standard. Statistical analysis were performed using Students two-tailed t-test and differences were considered significant when $p<0.05$ (Instat 3.0).

Native IRF-3 PAGE. HEC-1B cells were plated at 5×10^5 cells/well and infected with mock (diluent), S300, or S340 at an MOI of 1.0 for 1 hour at 37°C. After 1 hour of infection, HEC-1B media was added and cells were incubated at 37°C for the remainder of the infection. At various times post-infection, cells were washed one time with PBS (phosphate buffered saline) and cell pellets were frozen at -80°C. Whole cell extracts were prepared by mixing cells with NP-40 lysis buffer containing protease and phosphatase ihhibitors (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 10% glycerol, 30 mM NaF, 1.0 mM Na_3VO_4, 40 mM β-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, and 5 µg/ml of each leupeptin, pepstatin, and aprotinin) and centrifuged at 13,000 RPM at 4°C for 10 minutes. Equal cell lysates (10 ul) were mixed on ice with a 2X loading buffer (125 mM Tris-Cl, pH 6.8, 30% glycerol, 0.002% bromphenol blue). A 7.5% native PAGE was pre-run at 4°C for 30 minutes at 45 mA in gel running buffer (25 mM Tris-Cl, pH 8.4, 192 mM glycine, in the presence of or absence of 0.2% deoxycholate in the cathode and anode buffer, respectively). Samples were loaded on the gel and run at 25 mA for 60 min at 4°C followed by transfer to polyvinylidene difluoride (PVDF) membranes. After an hour of transfer, membranes were blocked for 1 hour at room temperature with PBS containing 0.1% Tween-20 (Sigma) (wash buffer) and 5% dry milk (blocking buffer). The membrane was incubated in primary antibody overnight at 4°C with rabbit anti-IRF3 FL-425 (Santa Cruz, sc-9082) at 1:500. After three washes, secondary

antibody was incubated for 1 hour at room temperature with anti-rabbit conjugated HRP at 1:10,000 (Sigma) in blocking buffer. The membrane was washed three times followed by detection of proteins was using ECL chemiluminescence (Amersham Biosciences).

SDS-PAGE and Western Blot Protein extracts were resolved on 10% SDS-PAGE followed by transfer to a PVDF membrane. After an hour of transfer, membranes were blocked for 1 hour at room temperature with blocking buffer. Membranes were incubated in primary antibody (rabbit anti-IRF-3 FL-425, and mouse anti-alpha-tubulin [Sigma]) diluted in blocking buffer overnight at 4°C. For detection of phosphorylated IRF-3, rabbit anti-IRF-396 (anti-His5033; kindly provided by Dr. J. Hiscott) was diluted in wash buffer containing 5% bovine serum albumin and incubated overnight at 4°C. Membranes were washed, incubated in the appropriate anti-rabbit or anti-mouse secondary antibodies, and proteins were detected as described earlier.

RESULTS

The attenuated mutant S340 virus induces more type I IFN in cell culture. The determinant at nsP1 538 has previously been characterized and profoundly affects adult mouse neurovirulence (20). Introduction of an attenuating Ile codon (virus designated S340) in place of the virulence-associated Thr codon at nsP1 538 significantly reduced virulence. The attenuating Ile codon also accelerates processing of the nonstructural polyprotein precursor P1234, resulting in rapid induction of viral RNA synthesis from the viral subgenomic 26S promoter (21). Since altered viral RNA synthesis may affect type I IFN induction and type I IFN plays a major role in controlling alphavirus infection, type I IFN induction by the mutant S340 virus was evaluated in cell culture. L929 cells were mock infected or infected with S300 (wild-type) or S340 (mutant) at an MOI of 5.0. At 18 hours post-infection, the supernatant was removed and assayed for type I IFN. As shown in Figure 1, the mutant S340 virus induced significantly more type I IFN than the wild-type S300 virus. While the mutant S340 virus exhibited IFN induction greater than 250 IU/ml, the wild-type S300 virus showed levels comparable to mock-infected cells. Similar differences in IFN induction were observed in other cell types, including A549 (Fig. 1B), HeLa, and Neuro2A cells (data not shown). These results demonstrated that the presence of an attenuating mutation at nsP1 position 538 increases the ability of the virus to induce type I IFN.

The attenuated mutant S340 virus induces a strong IFN response *in vivo*. To determine whether the differential type I IFN induction between S300 or the mutant S340 virus also occurs *in vivo*, type I IFN levels were measured in the serum of infected adult mice. Six-week old CD-1 (Fig. 2A) or C57BL/6 (Fig. 2B) were mock infected or infected with S300 or

S340 by the intracranial route (i.c.) and bled between 9 and 18 hours post-infection. Serum type I IFN responses were measured using an IFN bioassay on L929 cells. As shown in figure 2, serum from mice infected with the mutant S340 virus exhibited robust type I IFN levels compared to mice infected with the wild-type S300 virus. In CD-1 mice, the mutant virus displayed higher levels of type I IFN between 12 and 18 hours post-infection as compared to the wild-type S300 virus. Similarly, in the C57BL/6 mice, the mutant virus induced greater than 16,000 IU/ml IFN by 12 hours post-infection as compared to the wild-type virus which induced levels comparable to mock infected mice. Furthermore, wild-type S300 did not induce type I IFN 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 IFN. Interestingly, similar IFN differences were found in TLR3-/-, Myd88-/-, and TD-/- (PKR, RNaseL, MxA) between the wild-type and mutant viruses (data not shown), which indicates that the source of the IFN in the serum is most likely independent of the TLR3, Myd88, or PKR pathways. These results demonstrate that the mutant S340 virus induces more type I IFN in cultured cells and *in vivo* than the wild-type S300 virus, suggesting that this determinant plays a major role in modulating viral type I IFN induction.

The mutant and wild-type S300 virus show similar kinetics of virus-induced host shutoff. One potential explanation for the differences in IFN induction between the wild-type and mutant viruses is that the mutant S340 virus may be impaired in its ability to shutoff host macromolecular synthesis, since mutations within nsP2 of a genetically related SINV, that impaired virus-induced host transcription and/or translation shutoff, led to increased type I IFN induction (16). Therefore, virus-induced host shutoff was examined between the wild-type S300 and mutant S340 viruses. To examine kinetics of host transcription shutoff,

Neuro2A cells, a mouse neuroblastoma cell line, were mock infected or infected with S300 or S340 at an MOI of 10.0. At 2, 5, 8, or 16 hours postinfection, cells were incubated with media containing [³H]-uridine for 3 hours, RNA was isolated, and analyzed by agarose gel electrophoresis (Fig. 3). Up to 8 hours post-infection, both viruses show similar kinetics of host transcription shutoff, as observed by the loss of host mRNA, 28S rRNA, and 18S rRNA. Interestingly, by 16 hours postinfection, wild-type S300 virus exhibited less efficient shutoff of host mRNA, 28S rRNA and 18S rRNA as compared to the mutant S340 virus. This result strongly suggests that the enhanced type I IFN induction by the S340 virus is not due to a defect in host transcription shutoff.

To examine kinetics of host translation shutoff, Neuro2A cells were mock infected or infected with S300 or S340 at an MOI of 10.0. At various times post-infection, cells were labeled with ³⁵S-methionine/cysteiene for one hour and lysates collected and analyzed by SDS-PAGE (Fig. 4A). By as early as 4 hours post-infection, both viruses exhibit greater than 50% shutoff of host protein synthesis and by 10 hours post-infection greater than 80% of host protein synthesis shutoff is observed. Most importantly, when comparing the 42 kDa band, which corresponds to β-actin, the mutant S340 virus displayed similar kinetics of host protein shutoff as compared to wild-type S300 virus (Figure 4B). Similar observations were also made in BHK-21 cells (data not shown). These results demonstrate that the IFN induction by the mutant S340 virus is not simply due to defects in host transcription or translation shutoff and suggest that the determinant at nsP1 538 modulates host type I IFN induction by another mechanism.

The mutant S340 virus induces early IRF-3 activation. Although the results of Gorchakov et al. (16) suggest that virus-induced host shutoff plays an important role in

antagonizing type I IFN induction, the observation that the attenuated mutant S340 virus did not show defects in host cell shutoff suggests that the determinant at nsP1 position 538 modulates type I IFN induction through a different mechanism. IRF-3 activation is an essential step in type I IFN induction and is marked by phosphorylation of six serine residues grouped into two clusters (S385/386 and S396/398/402/405) (36, 44), which leads to dimerization, nuclear translocation, association with CBP/p300 and binding to the IFN-beta promoter (31, 43, 58). Importantly, it has been reported by Preston et al. (38) that IRF-3 is capable of becoming activated independent of *de novo* host translation or transcription. Therefore, IRF-3 dimerization and phosphorylation were examined in HEC-1B cells. HEC-1B cells were mock infected or infected with S300 or S340 at an MOI of 1.0. Other cultures were infected with Sendai virus (SeV) to serve as the positive control for IRF-3 activation. At various times post-infection, lysates were collected and analyzed for IRF-3 dimerization by native PAGE and phosphorylation of IRF-3 by using a phospho-specific antibody for IRF-3 (serine at position 396; Fig. 5). By 6 hours postinfection, IRF-3 dimerization was apparent with the mutant S340 virus but not the wild-type S300 virus. By 8 hours post-infection, the wild-type S300 virus showed slight activation of IRF-3; however, IRF-3 phosphorylation and dimerization levels were still lower than the mutant S340 virus. These results suggest that the mutant S340 virus more rapidly induces IRF-3 activation than the wild-type S300 virus, and moreover that the determinant at nsP1 538 plays a major role in modulating type I IFN induction by AR86.

DISCUSSION

Type I IFN 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 induction of a type I IFN response. These mechanisms range from nonspecific effects, such as rapid shutoff of host cell macromolecular synthesis (1, 16), masking viral RNA from host cell sensory proteins (7, 34), or specific inhibition of host cell dsRNA sensors or signaling molecules that link these sensor molecules to transcription factors that regulate type I IFN transcription (3, 4, 30, 32). Studies with SINV have demonstrated that host cell shutoff plays a major role in regulating viral type I IFN induction (16). However, complete host cell shutoff does not occur until late in infection, which raises the question of whether alphaviruses utilize other mechanisms to suppress or avoid type I IFN responses prior to host cell shutoff. In this report, we present evidence of a viral virulence determinant at nsP1 538 in the neurovirulent strain of SINV that modulates type I IFN induction without affecting host cell macromolecular synthesis.

While wild-type S300 virus exhibited reduced IFN induction *in vitro* and *in vivo*, the mutant S340 virus containing an Ile at nsP1 position 538 induced robust levels of type I IFN. Given that type I IFN plays a central role in regulating alphavirus-induced disease (41, 57), it is likely that the determinant at nsP1 538 affects viral virulence through effects on type I IFN induction. Accordingly, the mutant S340 virus, which unlike wild-type S300 exhibited no mortality in WT 129Sv/Ev mice, shows 100% mortality in IFNα/βR-/- mice, which cannot respond to type I IFN (data not shown). Interestingly, IFN induction by the mutant S340 virus appeared to be independent of the Toll-like pathways, as TLR3-/- or Myd88-/- mice

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infected with the mutant S340 virus induced comparable levels of type I IFN to that of wild-type C57BL/6 mice (data not shown).

Using a genetically related SINV, Gorchakov et al. demonstrated that viruses with mutations resulting in defects in host translation or transcription shutoff induced more type I IFN (16). Therefore, virus-induced host transcription and translation shutoff between wild-type S300 and the mutant S340 virus were evaluated. While both viruses exhibited similar overall kinetics of host transcription shutoff, the wild-type S300 virus exhibited defects in shutoff of cellular RNA polymerase I-dependent transcription at 16 hours postinfection (Fig. 3). Recently, mature nsP2 has been shown to be critical in the inhibition of RNA polymerase I- and II-dependent transcription (12, 13). Thus, the differences in rRNA inhibition at late times postinfection between the wild-type and mutant AR86 viruses could possibly be due to differences in processing of the nonstructural polyprotein, where wild-type S300 has a more stable polyprotein than the mutant S340 virus (21). Furthermore, our studies also demonstrated that the mutant S340 virus exhibited similar kinetics of host translation shutoff as compared to wild-type S300. These results suggest that altered host cell shutoff is not the sole explanation for enhanced IFN induction by the mutant S340 virus. This is further supported by the finding that the mutant S340 virus led to more rapid induction of IRF-3 dimerization and phosphorylation, a step which is independent of *de novo* host protein synthesis and will not be affected by virus-induced host shutoff (38).

Therefore, the determinant at nsP1 538 affects type I IFN induction independently of virus-induced host shutoff, suggesting three possible mechanisms by which the determinant at nsP1 538 modulates type I IFN induction. First, the wild-type S300 virus may downregulate or delay 26S promoter induction until virus-induced host shutoff is efficiently

able to antagonize type I IFN induction, and this effect is disrupted by the determinant at nsP1 538. This would suggest that the subgenomic RNA may be a potential target for sensing by the host cell, a question which requires further investigation. Second, Gitlin et al. has reported that RIG-I plays an important role in responding to SINV infection (14). RIG-I interacts with 5'triphosphates on uncapped RNAs, and given that nsP1 and nsP2 proteins are involved in capping viral RNAs, it is possible that the determinant at nsP1 538 is altering the efficiency of capping viral genomic or subgenomic RNA (2, 22, 33, 39, 55). Third, wild-type S300 may actively suppress early type I IFN induction, and this effect is disrupted by the attenuating Ile at nsP1 538. This scenario raises the possibility that alphaviruses employ a multi-step approach to blocking type I IFN induction, whereby a specific inhibitory effect is active early in infection, while a generalized host shutoff would block IFN synthesis late in infection.

Additional studies are underway to distinguish between these possibilities, and determine the mechanism by which AR86 virus mediates this effect. In summary, we have demonstrated that wild-type S300 virus was a poor inducer of type I IFN, while the mutant S340 virus containing an attenuating Ile codon at nsP1 position 538 induces a robust type I IFN response *in vitro* and *in vivo*. The altered type I IFN induction was independent of virus-induced host translation or transcription shutoff, suggesting that the determinant at nsP1 538 acts through a novel mechanism to modulate the host type I IFN response.

ACKNOWLEDGEMENTS

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Figure 4-1: The mutant S340 virus induces more type I IFN in cell culture.
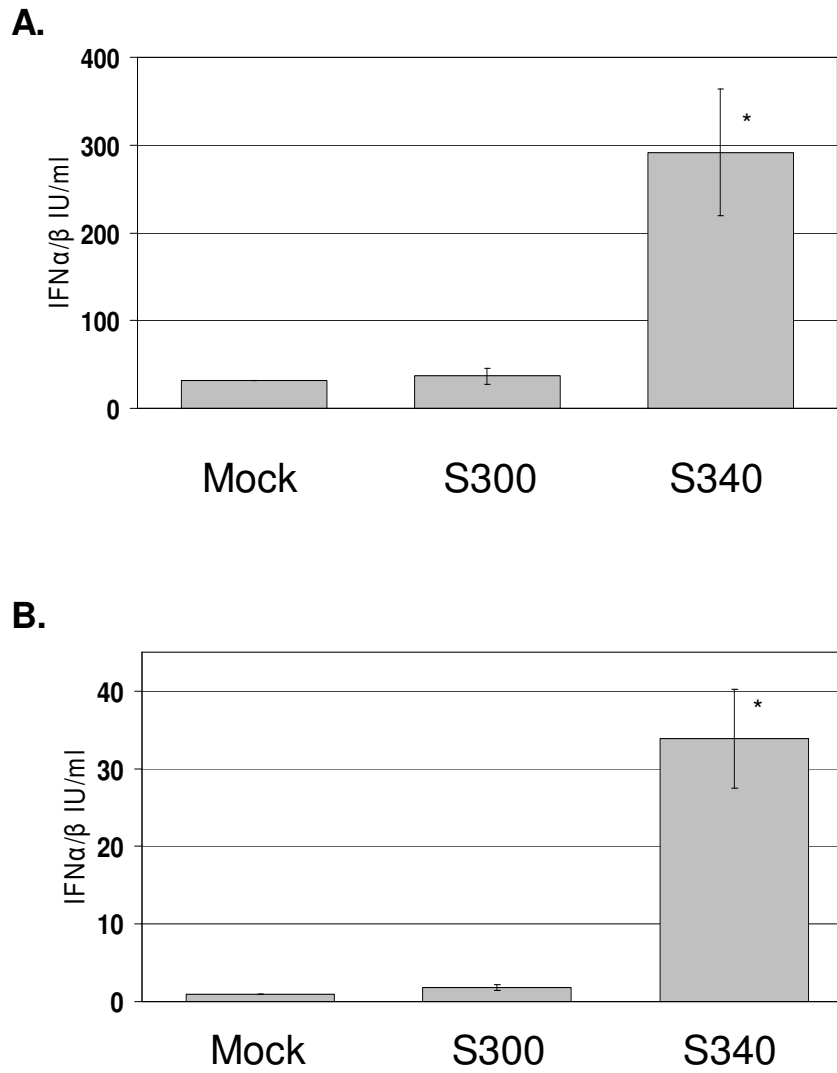

A.

B.

Figure 4-1. The mutant S340 virus induces more type I IFN in cell culture. (A) L929 or (B) A549 cells were infected with mock, wild-type AR86 (S300) or mutant AR86 (S340; Ile at nsP1 position 538) at an MOI of 5.0 and supernatants were harvested at 18 hours post-infection. Supernatents were subjected to an IFN bioassay on L929 or A549 cells, as described in the Materials and Methods. Each bar represents the average of triplicate samples and standard deviation was calculated (*p<0.05).

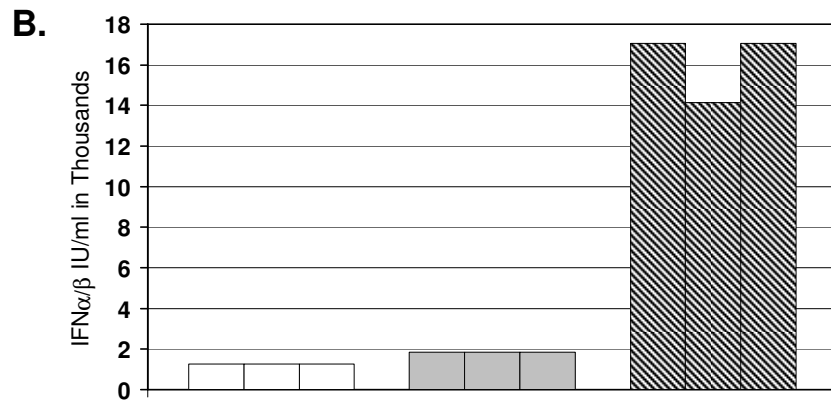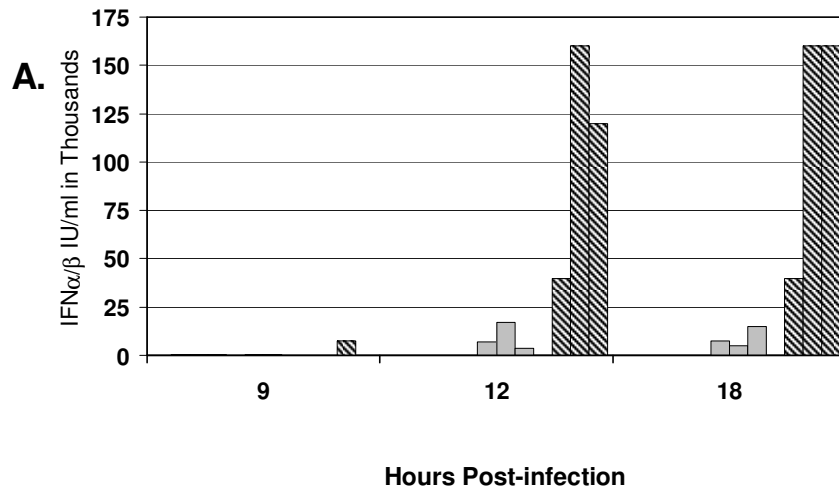**Figure 4-2: The mutant S340 virus induces a robust type I IFN response *in vivo*.**

Figure 4-2. The mutant S340 virus induces a robust type I IFN response *in vivo*. Groups of six-week old (n=3) (A) CD-1 or (B) C57BL/6 mice were infected with PBS alone, S300 (wild-type AR86) or S340 (mutant AR86; Ile at nsP1 position 538) at $1x10^3$ PFU via the intracranial (i.c.) route. Serum was harvested by tail vein bleeding at (A) 9, 12, and 18 hours post-infection or (B) 12 hours post-infection and was subjected to a L929 mouse IFN bioassay, as described in the Materials and Methods. Each bar represents an individual animal (Mock- white, S300- grey, S340- hatched). The data shown are representative of three independent experiments.

Figure 4-3: The mutant S340 virus exhibits similar kinetics of host cell transcription shutoff as compared to wild-type S300 virus.
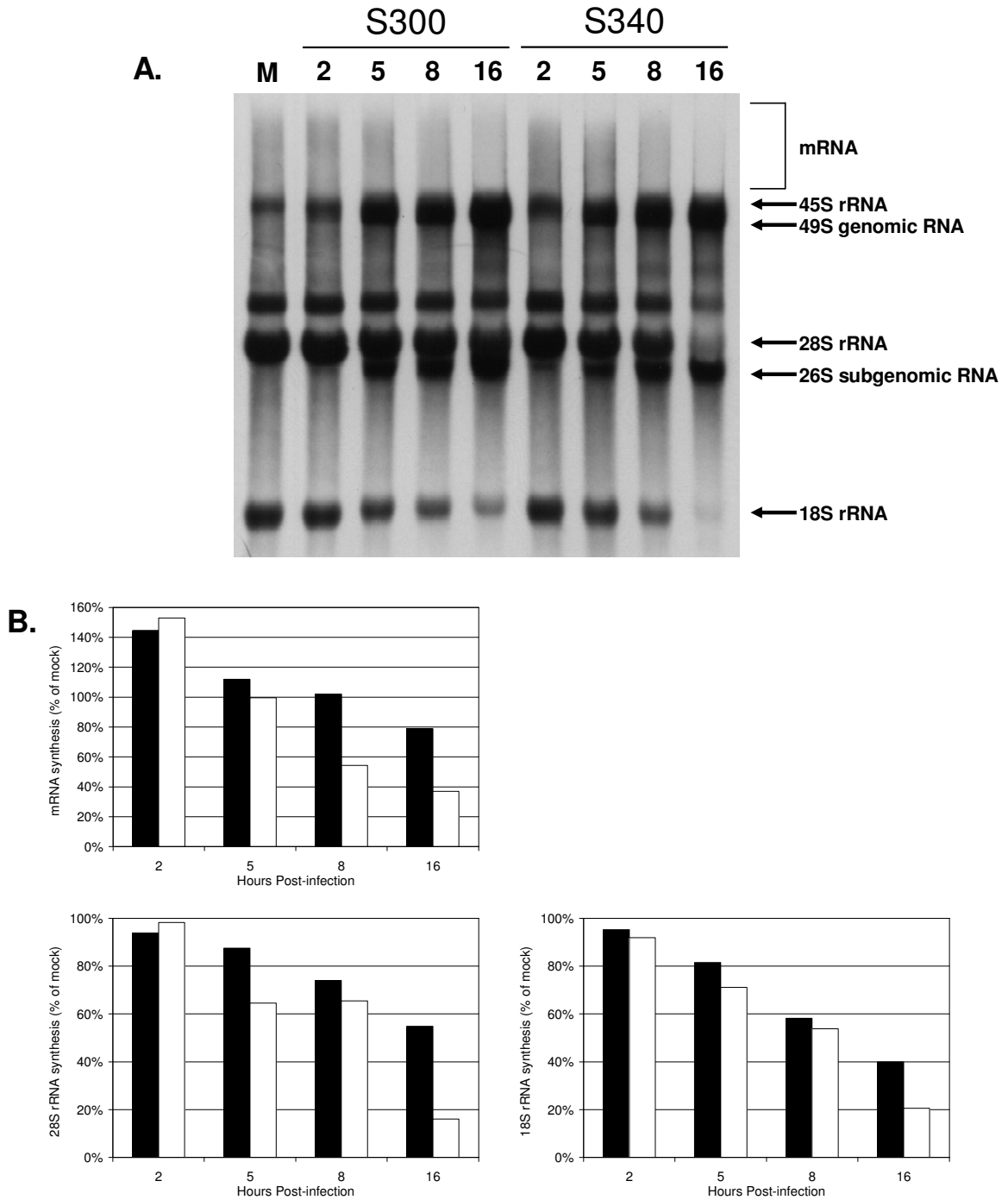
Figure 4-3. The mutant S340 virus exhibits similar kinetics of host cell transcription shutoff as compared to wild-type S300 virus. (A) Neuro-2A cells were mock infected (M) or infected with S300 (wild-type AR86) or S340 (mutant AR86) at an MOI of 10.0. At the timepoints indicated, media was removed and replaced with complete α-MEM medium containing [3H]-uridine at 20 μCi/ml. After 3 hours incubation at 37°C, cells were washed 1X in PBS, RNAs were isolated using Trizol and RNAs were analyzed by agarose gel electrophoresis as described in the Materials and Methods. (B) Residual host cell transcription was measured by excising fragments from the dried gel followed by liquid scintillation counting. The data are reported as counts per minute normalized to the amount of radioactivity detected in the same band in mock infected cells. The data shown are representative of two independent experiments.

Figure 4-4: The mutant S340 virus shuts off host cell translation with similar kinetics as the wild-type S300 virus.

Figure 4-4. The mutant S340 virus shuts off host cell translation with similar kinetics as the wild-type S300 virus. (A) Neuro-2A cells were mock infected (M) or infected with S300 (wild-type AR86) or S340 (mutant AR86) at an MOI of 10.0. Cells were labeled with ^{35}S Met/Cys for 1 hour at the time points indicated and cell lysates were analyzed by PAGE. (B) Residual host cell protein synthesis 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 (S300- solid line; S340- dashed line). The data shown are representative of three independent experiments.

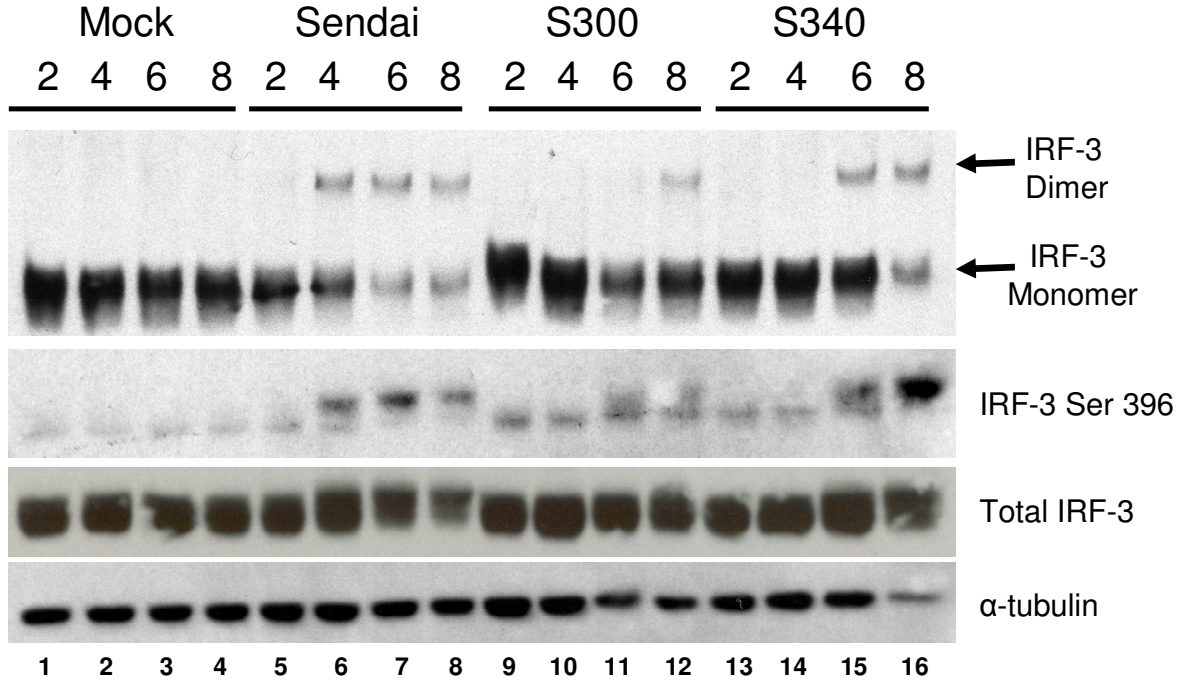**Figure 4-5: The mutant AR86 virus induces earlier IRF-3 activation.**

Figure 4-5. The mutant AR86 virus induces earlier IRF-3 activation. HEC-1B cells were infected with mock (1-4), Sendai virus (5-8), S300 (wild-type AR86; 9-12) or S340 (mutant AR86; 13-16) at an MOI of 1.0 and at the indicated timepoints, cells were washed one time with PBS and whole cell extracts were prepared by mixing cells with NP-40 lysis buffer containing protease and phosphatase inhibitors. Native PAGE (upper panel) and SDS-PAGE western blots (lower panels) were performed as described in the Materials and Methods. Blots shown are representative of three independent experiments.

CHAPTER FIVE

THE SINDBIS VIRUS NONSTRUCTURAL PROTEINS ANTAGONIZE TYPE I INTERFERON INDUCTION BY INHIBITING RIG-I AND IRF-3 ACTIVITY

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ABSTRACT

Many viruses have evolved strategies to interfere with type I interferon induction and promote viral virulence. Alphaviruses have been shown to inhibit type I IFN induction by virus-mediated shutoff of host cell macromolecular synthesis. However, whether alphaviruses possess specific mechanisms to inhibit host type I IFN induction signaling is not known. We have recently shown that the adult mouse neurovirulent Sindbis-group virus AR86 inhibits type I IFN induction by inhibiting Interferon Regulatory Factor-3 (IRF-3) activation early during infection. In this study, we extend these findings and show that while AR86 genomic RNA is efficiently recognized by RIG-I, AR86 virus actively antagonizes IRF-3 activation early during infection. Furthermore, the viral nonstructural proteins (nsPs) inhibited RIG-I and Mda5 signaling when expressed independently of virus infection. Strikingly, co-expression of the viral nsPs proteins led to the loss of RIG-I, Mda5, and IRF-3 expression in a manner that was dependent on the nsP2 protease. These results demonstrate that the viral nonstructural proteins specifically antagonize the type I IFN induction signaling pathway and suggest that the virus may directly target components of the type I IFN induction signaling pathway.

INTRODUCTION

Alphaviruses are mosquito borne viruses that are a significant human pathogen, causing disease ranging from encephalitis to arthritis. Sindbis viruses (SINV) are one of the best model systems for studying alphavirus interactions with the host. SINV have been well characterized at the molecular level and cause diseases in mice ranging from a shock-like inflammatory response syndrome (SIRS) in young mice to lethal encephalitis in adult mice (15, 16, 30). SINV pathogenesis models have been used to define specific viral and host factors that regulate virus-induced disease. One of the most important components of the innate antiviral response is the type I IFN system. Deficiency in IFN signaling significantly increases sensitivity to SINV infection (24, 25). However, relatively little is known about how these viruses interact with the host cell to initiate and/or avoid type I IFN response. Virus-induced shutoff of host cell macromolecular synthesis has been shown to inhibit type I IFN induction signaling, however, whether these viruses specifically antagonize type I IFN induction has not been evaluated.

Type I IFN induction is initiated upon recognition of viral products by host cell pattern recognition receptors (PRRs), specifically Toll-like receptors (TLRs) and recently identified retinoic acid inducible gene-I (RIG-I) and melanoma differentiation antigen 5 (Mda5) (21). While TLR7 and TLR3 recognize single-stranded (ssRNA) and double-stranded RNA (dsRNA) within the endosomal compartment, respectively, dsRNA in the cytoplasm is recognized by RIG-I and Mda5 (13, 34). Upon binding dsRNA substrates, TLR3 and RIG-I/Mda5 recruit their respective adaptor proteins, Toll/IL-1 receptor (TIR)-domain containing adaptor inducing IFN-β (TRIF) and IPS-1/MAVS/Cardif/VISA via the caspase recruitment domain leading to activation of IFN regulator factor-3 (IRF-3), Nuclear

factor kappa B (NF-κB), ATF2/c-Jun and formation of the enhancesome complex that binds to the promoter region of the IFN-β gene and activates transcription (21).

Alphaviruses contain a single-stranded positive-sense RNA genome of approximately 11.5 kb in length that encodes for the nonstructural proteins in the 5' two-thirds of the genome and the structural proteins in the remaining one-third. The nonstructural proteins are translated as a polyprotein that are postranslationally processed to produce the mature nonstructural proteins, nsP1, nsP2, nsP3, and nsP4. While the nsP polyprotein precursors and mature nsPs are mainly involved in viral RNA synthesis, they have also been shown to modulate the host antiviral response through specific virus-host interactions. The nsP2 protein has been well-studied and shown play an important role in antagonizing the host antiviral response through shutoff of host macromolecular synthesis. Introducing mutations in the carboxy-terminus of nsP2, which render it defective in host translation or transcription shutoff, correlated with increased type I IFN induction (6, 9). Furthermore, mature nsP2 has been implicated in causing cytopathic effect in cells, most likely attributed to its ability to shutoff host RNA polymerase I and RNA polymerase II transcription (7).

Recent studies with the adult mouse neurovirulent AR86 virus has demonstrated that a virulence determinant at nsP1 position 538 modulates type I IFN induction. Introduction of an Ile for the virulence-associated Thr codon at this position attenuated virulence and resulted in induction of a type I IFN response (10, Suthar, M.S. and Heise, M.T., unpublished). In addition to virus-induced shutoff of host macromolecular synthesis, AR86 virus inhibits type I IFN induction through an additional mechanism by inhibiting IRF-3 activation early during infection. This study further demonstrates the importance of the viral nsPs in modulating the host antiviral response through specific virus-host interactions.

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Therefore, we hypothesized that the AR86 viral nsPs specifically antagonize components of the type I IFN signaling pathway upstream of IRF-3.

In this study, we demonstrate that RIG-I efficiently recognizes AR86 virus genomic RNA. Although the viral RNA is capable of being recognized, AR86 was found to actively antagonize IRF-3 activation in infected cells. Since the viral nonstructural proteins are known to play a major role in regulating viral type I IFN induction, we evaluated whether the AR86 nonstructural proteins were antagonizing the RIG-I/Mda5-mediated signaling pathway. These studies demonstrate that the AR86 nonstructural proteins interfered with RIG-I and Mda5 mediated IFN-β induction through a mechanism that requires a functional nsP2 protease. We also found that RIG-I, Mda5, and IRF-3 expression were rapidly lost in the presence of the viral nonstructural proteins. This work demonstrates a novel role for the alphavirus nonstructural proteins in blocking IFN induction and suggests that the viral nsP2 protease may directly target components of the type I IFN induction signaling pathway.

MATERIALS AND METHODS

Viruses and cell lines. Virus stocks were made as described previously (30). Briefly, viral cDNA plasmids were linearized with PmeI and used as templates for the synthesis of full-length transcripts by using SP6-specific mMessage Machine in vitro transcription kits (Ambion). Transcripts were electroporated into BHK-21 cells grown in α-minimal essential medium (α-MEM: 10% fetal calf serum [Invitrogen], 10% tryptose phosphate broth [Sigma], and 0.29 mg of L-glutamine per ml [Invitrogen]). Supernatants were harvested 24 hours after electroporation and subjected to centrifugation at 3000 RPM (Sorvall rotor RTH-250) for 20 minutes at 4°C and frozen in 1-ml aliquots. Virus stocks were titrated on BHK-21 cells as previously described (28).

BHK-21 cells were maintained in α-MEM, for a maximum of 10 passages. Human embryonic kidney (HEK) 293 cells (ATCC) were maintained in Dulbecco's MEM containing 10% fetal bovine serum [Invitrogen], 0.29 mg of L-glutamine per ml [Gibco], and 1x penicillin/streptomycin [Invitrogen]. HEC-1B cells (ATCC) are human endometrial carcinoma cells and were used in this study to analyze IRF-3, since these cells are nonresponsive to IFN (5, 31). HEC-1B cells were grown in MEM containing 10% fetal bovine serum, 0.29 mg of L-glutamine per ml [Gibco], and penicillin/streptomycin.

Plasmid Constructs. Standard recombinant DNA techniques were used to construct cDNA clones. To construct the AR86 nsP1234 construct, replicon plasmid pRep89-egfp was digested with MfeI and NotI, which encompasses nsP1234, and cloned into pcDNA3.1+ (Invitrogen) digested with EcoRI and NotI. To construct nsP123, PCR-mediated site-directed mutagenesis of the nsP1234 construct was modified to create the authentic cleaved

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end of nsP3. To construct the protease defective nsP expression plasmids, a mutation of a Cys codon at nsP2 481 to a Gly codon was engineered by site-directed mutagenesis (clones are designated as P2C481G). Flag-tagged nsP plasmids were constructed by inserting a FLAG-tag amino acid sequence (DYKDADDDK) upstream of nsP1. The reporter construct p56-luc was kindly provided by Dr. G.Sen. The reporter plasmid 3X-KB-luc and pcDNA-FLAG-p65 expression plasmids were a kind gift from Dr. A.Baldwin. The reporter plasmid pIFN-β-Luc and pcDNA-FLAG-IRF-3 expression plasmids were a kind gift from Dr. J.Pagano. pcDNA3.1+-egfp was purchased from Invitrogen. RIG-I, and Mda5 expression plasmids were constructed as described by (1, 34). Briefly, HEK293 cells were treated with IFN-β for 24 hours, RNA was isolated and RT-PCR was performed using oligo dT primers (Invitrogen) or Mda5 specific primers for first strand synthesis following Promega's AMV-RT (HC) protocol. FLAG-RIG-I and FLAG-Mda5 were assembled by overlap extension PCR and cloned into pcDNA3.1+. FLAG-constituitively active RIG-I expression plasmid was constructed by PCR amplification of the CARD domains of RIG-I and cloned into pcDNA3.1+. All plasmids were confirmed by sequencing at the University of North Carolina at Chapel Hill Genome Analysis Facility with a model 373A DNA sequencing apparatus (Applied Biosystems).

Native IRF-3 PAGE. HEC-1B cells were plated at 5 x 10^5 cells/well and infected with mock (diluent), S300, or S340 at an MOI of 1.0 for 1 hour at 37°C. After 1 hour of infection, the inoculum was removed and HEC-1B media was added to the cells and were incubated at 37°C for the remainder of the infection. At 1, 2, and 4 hours post-infection, cells were mock transfected or transfected with 100 poly-(I:C) using Lipofectamine 2000 (Invitrogen) and harvested 2 hours post-transfection. Cells were washed one time with PBS

(phosphate buffered saline) and cell pellets were frozen at -80°C. Whole cell extracts were prepared by mixing cells with RIPA lysis buffer containing protease inhibitors (5 µg/ml of each leupeptin, pepstatin, and aprotinin) and centrifuged at 13,000 RPM at 4°C for 10 minutes. Equal cell lysates (10 ul) were mixed on ice with a 2X loading buffer (125 mM Tris-Cl, pH 6.8, 30% glycerol, 0.002% bromphenol blue). A 7.5% native PAGE was pre-run at 4°C for 30 minutes at 45 mA in gel running buffer (25 mM Tris-Cl, pH 8.4, 192 mM glycine, in the presence of or absence of 0.2% deoxycholate in the cathode and anode buffer, respectively). Samples were loaded on the gel and run at 25 mA for 60 min at 4°C followed by transfer to polyvinylidene difluoride (PVDF) membranes. After an hour of transfer, membranes were blocked for 1 hour at room temperature with PBS containing 0.1% Tween-20 (Sigma) (wash buffer) and 5% dry milk (blocking buffer). The membrane was incubated in primary antibody overnight at 4°C with rabbit anti-IRF3 FL-425 (Santa Cruz, sc-9082) at 1:500. After three washes, secondary antibody was incubated for 1 hour at room temperature with anti-rabbit conjugated HRP at 1:10,000 (Sigma) in blocking buffer. The membrane was washed three times followed by detection of proteins was using ECL chemiluminescence (Amersham Biosciences).

SDS-PAGE and Western Blot. Western blot analysis was performed as previously described (22). Samples were quantitated using Bradford assay and equal protein was loaded onto SDS-PAGE gel followed by transfer to polyvinylidene difluoride (PVDF) membranes. After an hour of transfer, membranes were blocked for 1 hour at room temperature with PBS containing 0.1% Tween-20 (Sigma) (wash buffer) and 5% dry milk (blocking buffer). Blots were stained with rabbit anti-RIG-I (dilution 1:15,000), mouse anti-Flag (1:1000), rabbit anti-gfp (1:500), rabbit anti-nsP1 (1:10,000) (generously provided by Dr.C.Rice) , and rabbit anti-

IRF3-FL425 (1:500) (Santa Cruz Biotechnology; sc-9082) in blocking buffer. After three washes, secondary antibody was incubated for 1 hour at room temperature with anti-rabbit conjugated HRP at 1:6,000 (Amersham Biosciences) or anti-mouse conjugated HRP at 1:2500 (Amersham Biosciences) in blocking buffer. The membrane was washed three times followed by detection of proteins using ECL chemiluminescence (Amersham Biosciences).

RNA transcription and virion RNA isolation. RNA transcriptions were performed as previously described ((30). Briefly, viral cDNA plasmids were linearized with PmeI and used as templates for the synthesis of full-length transcripts by using SP6-specific mMessage Machine *in vitro* transcription kits (Ambion). RNAs were isolated as described in the manufacturer's protocol. Virion RNA was extracted from 1×10^7 PFU of purified virus using the MagMax Viral RNA Isolation Kit (Ambion) and RNAs were resuspended in 50 ul of nuclease-free water.

Luciferase Assay. HEK293 cells (6×10^4 cells per well in 48-well plates) were transfected in triplicate with the indicated expression vectors using Fugene6 [Roche] at 3 ul per ug of plasmid DNA. Where indicated, in vitro transcript RNA, virion RNA, or Poly-(I:C) were subsequently transfected using Lipofectamine 2000 [Invitrogen] and cells were lysed with 1X Cell Culture Lysis Reagent (Promega) and assayed for luciferase activity. Where indicated, relative luciferase activity was normalized to total protein and multiplied by a factor of 10 and reported as normalized relative luciferase units.

RESULTS

AR86 viral RNA is more efficiently recognized by RIG-I than Mda5. Recent work by several groups have shown that RIG-I and Mda5 vary in their specificity for recognizing RNA viruses. While RIG-I has been shown to be critical for recognizing Influenza virus, Sendai virus, Vesicular Stomatitis virus, Hepatitis C virus, and Japanese encephalitis virus, Mda5, on the other hand, has been shown to be critical for recognizing Encephalomyocarditis virus (8, 14). In addition, Mda5 was reportedly not critical for responding to SINV infection (8).

To further evaluate the specificities of RIG-I and Mda5 for recognizing SINV RNA, HEK293 cells were co-transfected with an IFN-β promoter reporter plasmid expressing the luciferase gene along with either RIG-I or Mda5 expression plasmids. Twenty-four hours post-transfection, cells were subsequently transfected with various RNA inducers, including carrier RNA, poly-(I:C), AR86 virion-derived RNA, and *in vitro* transcribed full-length wild-type AR86 viral RNA, followed by measuring luciferase activity seven hours post-stimulation (Fig. 1). RIG-I and Mda5 efficiently responded to poly-(I:C), however, RIG-I was more efficient than Mda5 in responding to RNA extracted from virions or full-length virus RNA derived from *in vitro* transcription.

Due to the complexities of transfecting replication competent viral RNA into cells, a mutation within nsP2 (Cys to Gly at position 481), which has been shown to ablate nsP2 protease function and viral RNA synthesis (29), was engineered into the full-length infectious clone of AR86. *In vitro* transcripts from this clone were transfected into cells under the same conditions and similar specificities were observed as that of *in vitro*

transcripts derived from that of the wild-type AR86 infectious clone. The results from this study demonstrate that RIG-I, and partially Mda5, are capable of responding to SINV RNA.

AR86 virus infection inhibits dsRNA-induced IRF-3 activation. Previous studies from our laboratory have shown AR86 virus inhibits activation of IRF-3 early during infection, suggesting that AR86 virus is either actively suppressing type I IFN induction or evading type I IFN induction. Therefore, we determined whether AR86 can block activation of IRF-3 induced from an exogenous source of dsRNA. HEC-1B cells were infected with AR86 at an MOI of 1.0 and poly-(I:C) was transfected into cells at 1, 2, and 4 hours post-infection and cells were harvested 2 hours post-transfection (equivalent to 3, 4, and 6 hours post-infection, respectively). As shown in Figure 2, early during infection, AR86 virus inhibited IRF-3 dimerization upon treatment with poly-(I:C) in contrast to mock infected cells, which exhibited normal IRF-3 dimerization (compare lanes 2 and 4). This result suggests that AR86 virus is able to actively antagonize the type I IFN induction signaling pathway.

AR86 nsPs inhibit RIG-I and Mda5 mediated IFN-β promoter activity. Though RIG-I clearly recognizes AR86 genomic RNA (Fig. 1), the fact that AR86 infected cells were refractory to poly-(I:C)-induced IRF-3 activation suggests that AR86 is interfering with type I IFN induction. Since the viral nonstructural proteins have previously been implicated in modulating the type I IFN response, we directly evaluated whether the AR86 nsPs blocked RIG-I/Mda5-mediated type I IFN induction. To evaluate this, HEK293 cells were transfected with the IFN-β-luc reporter plasmid, RIG-I or Mda5 expression plasmids, and a nsP1234 expression plasmid. Cells were subsequently transfected with poly-(I:C) and luciferase activity was measured (Fig. 3). In the context of vector alone, poly-(I:C) exhibited strong induction of RIG-I or Mda5-mediated IFN-β promoter activity. In contrast,

expression of nsP1234 efficiently inhibited RIG-I- and Mda5-mediated IFN-β promoter activity.

The AR86 viral nonstructural proteins are translated as a single polyprotein (nsP1234), however, nsP4 is rapidly cleaved off so that the major polyprotein precursor at early times post-infection is nsP123. Therefore, we next determined whether nsP123 is able to inhibit RIG-I or Mda5 mediated IFN-β promoter activity (Fig. 3). Similar to the findings with nsP1234, nsP123 was found to efficiently inhibit RIG-I and Mda5-mediated IFN-β promoter activity. These results demonstrate that the AR86 nonstructural proteins, independent of nsP4, are able to inhibit the IFN-β induction signaling pathway.

AR86 nsPs inhibit RIG-I-mediated IFN-β signaling. We next determined if the viral nsPs were antagonizing the ability of RIG-I to recognize dsRNA through its DExD/H box helicase domain or antagonizing downstream signaling through its N-terminal CARD domains. Ectopic expression of the RIG-I N-terminal CARD domains has been shown to induce IFN-β signaling independent of a dsRNA substrate (19, 33, 34). Therefore, HEK293 cells were co-transfected with the IFN-β-luc reporter plasmid, constituitively active RIG-I expression plasmid, and nsP1234 (Fig. 4). In the presence of an empty plasmid transfection control, constituitively active RIG-I exhibited robust IFN-β promoter activity, while expression of wild-type AR86 nsPs completely abrogated IFN-β promoter activity. This result suggests that the AR86 viral nonstructural proteins are either directly antagonizing RIG-I signaling or downstream RIG-I-mediated signaling.

Viral nsP2 protease function is required for antagonizing RIG-I. Recent reports have implicated viral proteases as playing an important role in modulating type I IFN induction. The SINV nsP2 protease reportedly plays an important role in the induction of cell death (7).

In addition, the Hepatitis C virus NS3/4A protease has been shown to antagonize the IFN-β signaling pathway through cleavage of the TLR3-adaptor protein TRIF and the RIG-I/Mda5 adaptor protein IPS-1 (18, 20).

Based on these findings, we next determined whether the nsP2 protease functions in antagonizing the RIG-I- or Mda5-activated IFN-β signaling pathway. A mutation within the nsP2 protease active site at position 481 from a Cys to Gly (P2C481G) was introduced into the nsP123 expression plasmid to ablate proteolytic processing of the nonstructural polyprotein (29). HEK293 cells were co-transfected with the IFN-β-luc reporter plasmid, RIG-I or Mda5 expression plasmids, and nsP123 P2C481G expression plasmid. Cells were subsequently transfected with poly-(I:C) and luciferase activity was measured. In contrast to the wild-type AR86 nsPs (Fig. 5), the viral nonstructural proteins containing a defective nsP2 protease were unable to downregulate RIG-I or Mda5 mediated IFN-β promoter activity (Fig. 5A and 5B, respectively). Moreover, expression of the nsP123 P2C481G had no effect on constituitively active RIG-I mediated IFN-β promoter activity (Fig. 5C). This result suggests that the AR86 nonstructural proteins antagonize type I IFN induction through a novel mechanism that is dependent on the viral nsP2 protease.

AR86 nsPs inhibit RIG-I/IRF-3 specific pathway, but not p65. Upon binding dsRNA substrate, the RIG-I CARD domains interact with the CARD domains of the mitochondrial-associated adaptor protein IPS-1/MAVS/Cardif/VISA and leads to the activation of IRF-3 and NF-κB, two components of the enhancesome complex required for IFN-β transcription (12). We next determined whether the AR86 viral nonstructural proteins are antagonizing RIG-I signaling to IPS-1/MAVS/Cardif/VISA or antagonizing signaling downstream of IPS-1/MAVS/Cardif/VISA. To evaluate this, HEK293 cells were co-transfected with either an

IFN-β-luc reporter plasmid, an IRF-3 specific reporter plasmid (p56-luc) or an NF-κB specific reporter plasmid (3X-kB-luc), along with a MAVS expression plasmid, and empty vector, AR86 nsP1234 or nsP1234 P2C481G expression plasmids. As shown in Figure 6, expression of the wild-type AR86 nsPs efficiently inhibited MAVS-mediated IFN-β promoter activity and this was dependent on the nsP2 protease. Moreover, the AR86 nsPs exhibited strong inhibition of MAVS-mediated IRF-3 promoter activity (2.7-fold induction) as compared to empty vector control (35-fold induction) or nsP1234 P2C481G (114-fold induction).   Furthermore, the wild-type AR86 nsPs inhibited MAVS-mediated NF-κB promoter activity (5-fold induction) as compared to empty vector control (21-fold induction) or nsP1234 P2C481G (62-fold induction).   This result suggests that the AR86 nsPs are antagonizing MAVS-mediated signaling to IRF-3 and NF-κB in an nsP2-protease dependent manner.

While the AR86 nsPs inhibited MAVS-mediated signaling, it appeared as though the AR86 nsPs were exerting greater inhibition on IRF-3 promoter activity as compared to NF-κB promoter activity. To further evaluate virus interactions with the type I IFN induction signaling pathway, we next directly evaluated the effect the AR86 nsPs have on IRF-3 and NF-κB activity. To evaluate this, HEK293 cells were co-transfected with either an IRF-3 specific reporter plasmid (p56-luc) or an NF-κB specific reporter plasmid (3X-κB-luc), along with IRF-3 or p65 expression plasmids, and empty vector, AR86 nsP1234 or nsP1234 P2C481G expression plasmids. Expression of the wild-type AR86 nsPs efficiently inhibited IRF-3-mediated IRF-3 promoter activity (1-fold induction) as compared to empty vector control (9.4 fold induction) and nsP1234 P2C481G (11.6 fold induction).   In contrast, expression of the wild-type AR86 nsPs showed no inhibition of p65-mediated NF-κB

promoter activity, demonstrating the AR86 nsPs have no effect NF-κB activity. These results demonstrate that the AR86 nsPs are antagonizing IRF-3 activity and confirm our earlier findings that AR86 virus actively suppresses IRF-3 activation in infected cells (Fig. 2).

AR86 nsPs leads to loss of RIG-I, Mda5, and IRF3 expression. Due to the fact that expression of the wild-type AR86 nsPs led to strong inhibition of RIG-I, Mda5, and IRF-3 activities and this was dependent on a functional nsP2 protease, we next determined whether the viral nonstructural proteins were affecting expression of the various components of the type I IFN induction signaling pathway. To evaluate this, HEK293 cells were co-transfected with either empty vector, wild-type nsP123, or nsP123 P2C481G along with RIG-I, Mda5, constituitively active RIG-I, MAVS, IRF-3, and p65. Twenty-four hours post-transfection, cell lysates were collected and analyzed for protein expression (Fig. 7) In the presence of wild-type nsP123, RIG-I, Mda5, constituitively active RIG-I, and IRF-3 exhibit nearly complete loss of expression. However, co-expression of wild-type nsP123 with MAVS or the p65 subunit of NF-κB exhibited slightly reduced or normal expression levels, respectively. Furthermore, normal expression levels were observed when the wild-type nsP123 were co-expressed with egfp (data not shown). Consistent with its lack of inhibiting reporter activity, nsP123 P2C481G expression did not effect the expression of any of the signaling proteins. This result demonstrates that the viral nonstructural proteins are specifically targeting IFN-β signaling proteins and this is dependent on a functional nsP2 protease.

DISCUSSION

The innate and adaptive arms of the immune system play critical roles in controlling early virus replication, viral clearance, and establishing long-term protection from re-infection (11, 27). Type I IFN is the first line of defense during virus infection and is responsible for deterring virus replication and spread within the host (17, 23, 32). Many viruses have evolved strategies to interfere with type I IFN induction to promote viral virulence. Our laboratory has previously demonstrated that a single virulence determinant at nsP1 538 modulates type I IFN induction by altering IRF-3 activation. In the presence of a virulence-associated Thr at nsP1 538, wild-type AR86 exhibited reduced type I IFN induction and delayed IRF-3 activation early during infection. This indicated that the AR86 nonstructural proteins were interacting with components of the type I IFN signaling pathway and blocking IRF-3 activation early during infection. In this report, we provide evidence that AR86 genomic RNA is capable of being recognized by RIG-I and Mda5, but that this effect is blocked by the viral nonstructural proteins ability to antagonize RIG-I, Mda5, and IRF-3 expression, through a viral nsP2 protease-dependent mechanism.

Studying host protein interactions with the alphavirus nonstructural proteins can be difficult, in part, due to their specific role in viral RNA synthesis. To circumvent this issue, the viral nonstructural proteins were expressed independent of the context of virus infection using a CMV-promoter based overexpression plasmid. Although this has been shown to be a viable alternative to studying virus-host interactions (7, 22), several concerns can be raised by overexpression of the viral nonstructural proteins, including nsP2-induced cell death and host transcription/translation shutoff. However, in our studies, we failed to observe cell death while expressing the viral nsPs up to 72 hours post-transfection (data not shown).

Furthermore, several lines of evidence argue against the issue that overexpression of the viral nsPs are inducing host cell shutoff. First, co-expression of the viral nsPs with the p65 subunit of NF-κB or egfp (data not shown) did not alter protein or RNA expression levels (Fig. 5 and data not shown, respectively). Secondly, p65-medated 3X-κB promoter activity was unaltered in the presence of the viral nsPs, which suggests that the viral nsPs specifically target steps in the IFN induction pathway rather than inducing a generalized host cell shutoff.

Perhaps, one of the most intriguing observations was that while wild-type AR86 nsPs efficiently induced the loss of RIG-I, Mda5, and IRF-3 expression, the nsPs containing a defective nsP2 protease were unable to block RIG-I, Mda5, or IRF-3 activity or reduce protein stability. This strongly suggests a novel role for the viral protease in antagonizing type I IFN induction and raises two possibilities by which the protease is affecting protein expression. First, the viral nsP2 is targeting and cleaving RIG-I and Mda5. One such examples is the finding that the Hepatitis C virus NS3/4A protease cleaves the mitochondrial-associated adaptor protein IPS-1 and the TLR-adaptor protein TRIF to antagonize type I IFN induction (18, 20). Examination of the amino acid sequences for RIG-I and Mda5 revealed nsP1/2 and nsP2/3 virus-like cleavage sites within the proteins, respectively. This suggests that AR86, which has been shown to have a stable nsP123 early during infection, is able to efficiently target host proteins containing nsP1/2 virus-like cleavage sites, such as RIG-I. As the nsP123 precursor polyprotein is processed into nsP23, this polyprotein would be able to efficiently target host proteins that contain nsP2/3 virus-like cleavage sites, such as Mda5. However, further studies are required to determine if these host proteins are truly targets of the viral nsP2 protease. These finding would demonstrate an important role for the early nonstructural polyproteins and viral protease in antagonizing the

host type I IFN response. This raises the possibility that viruses, not only alphaviruses but any virus containing a protease, would be able to target host proteins for cleavage.

Another possibility for the observed loss of RIG-I, Mda5, and IRF-3 expression is that the viral nsPs could be targeting these proteins for degradation. Recently, it has been reported that the Classic swine fever virus N(pro) protein and Rotavirus nonstructural protein NSP1 target endogenous IRF-3 for degradation in a proteasome-dependent manner (3, 4). It is well known that activated IRF-3 is efficiently targeted to the host proteasome through the function of peptidylprolyl isomerase Pin1 (26), however, the mechanism of degrading endogenous IRF-3 is not known. Mda5 has recently been shown to be cleaved during poliovirus infection and this appeared to be mediated by the induction of apoptosis during virus infection (2). These findings imply that the host cell has efficient mechanism to downregulate expression of components of the type I IFN signaling pathway. The ability of a virus to target these negative regulating pathways would be a mechanism to completely abrogate the ability of the host cell to respond to virus infection. Therefore, it will be interesting to determine if the viral nsPs interact with and target these components for degradation or induce a negative regulator within the host cell that efficiently targets these proteins for degradation.

In summary, we have demonstrated that AR86 viral RNA is recognized by RIG-I and Mda5 and that the viral nonstructural proteins antagonize type I IFN induction by reducing the stability of RIG-I, Mda5 and IRF-3. Perhaps most importantly, we demonstrated an important role for the viral nsP2 protease in antagonizing type I IFN induction signaling. Further studies are underway to investigate the mechanism by which the AR86 nonstructural proteins mediate these effects.

ACKNOWLEDGEMENTS

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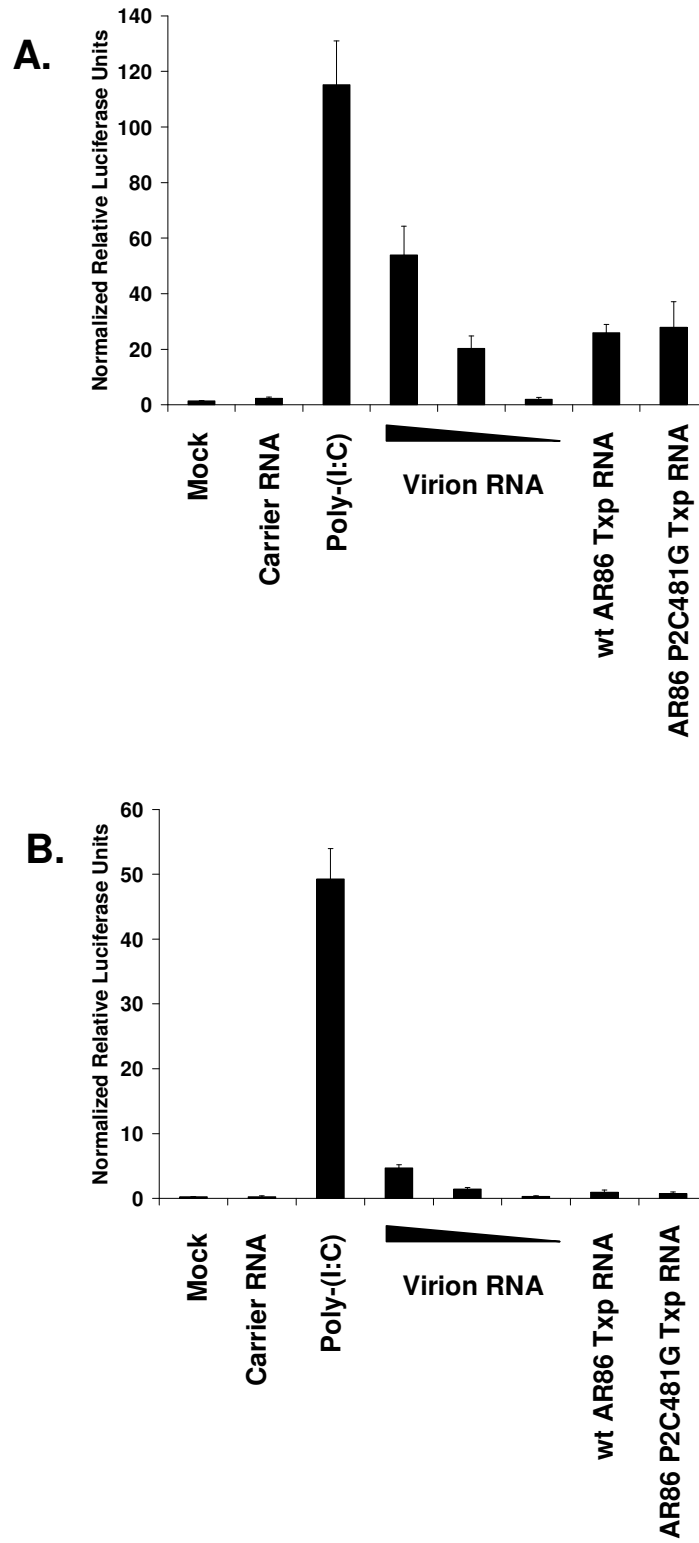**Figure 5-1: RIG-I is more efficient in responding to AR86 viral RNA than Mda5.**

Figure 5-1. RIG-I is more efficient in responding to AR86 viral RNA than Mda5. HEK293 cells were co-transfected with the IFN-β-luc reporter plasmid (50 ng), (A) RIG-I (50 ng) or (B) Mda5 (50 ng) expression plasmids. After 24 hours, cells were transfected with carrier RNA, 8 ng of poly-(I:C), AR86 virion RNA (undiluted, 1:10, 1:100), 1 ug wild-type txp RNA, or 1 ug P2C481G AR86 transcript (Txp) RNA and luciferase activity was measured after seven hours post-stimulation. Each bar represents an average of triplicate samples normalized to total protein and standard deviation was calculated. The data shown are representative of three independent experiments.

Figure 5-2: AR86 virus blocks poly-(I:C)-mediated IRF-3 activation.
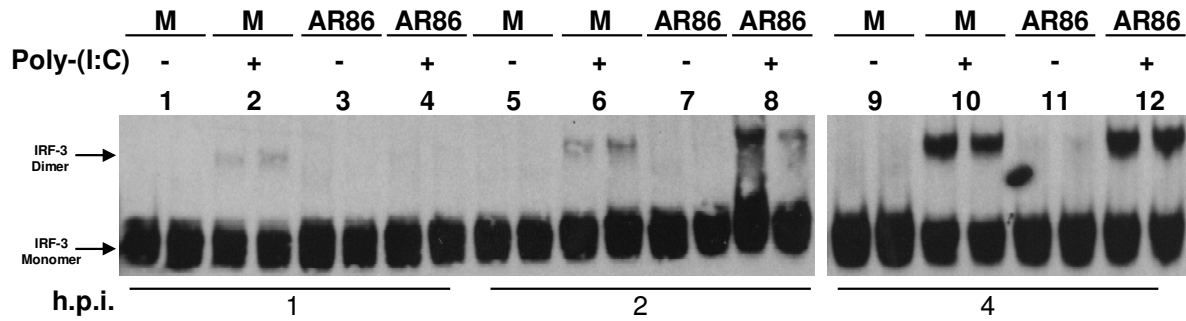
Figure 5-2. AR86 virus blocks poly-(I:C)-mediated IRF-3 activation. HEC-1B cells were mock infected or infected with wild-type AR86 at an MOI of 1.0 in duplicate followed by mock transfections or transfections with 100 ng poly-(I:C) at one (lanes 1-4), two (lanes 5-8), or four (lanes 9-12) hours post-infection. Two hours post-transfection, cells were harvested and lysates were analyzed for IRF-3 dimerization by Native PAGE.

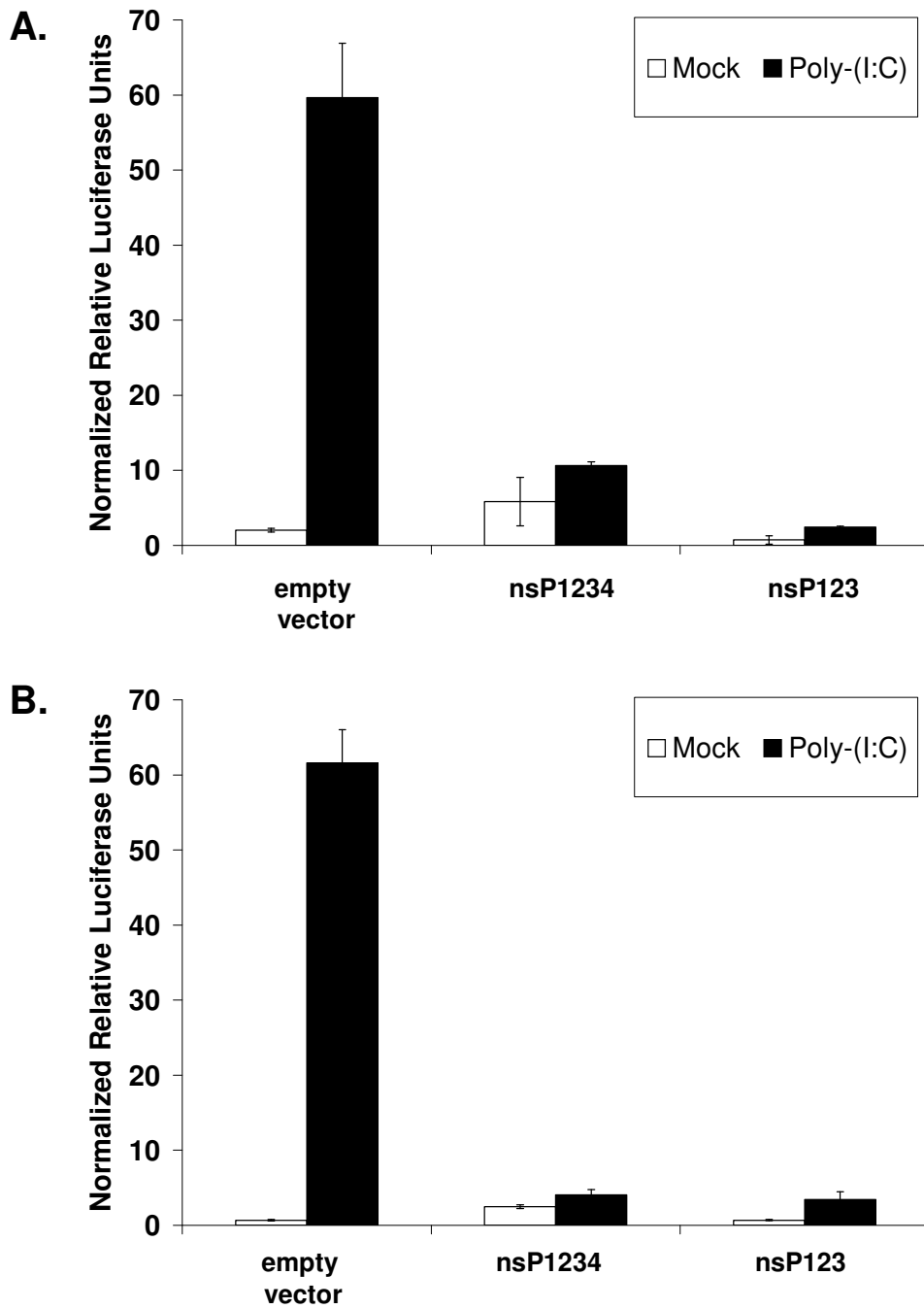**Figure 5-3: The AR86 nsPs inhibit RIG-I and Mda5 mediated IFN-β promoter activity.**

Figure 5-3. The AR86 nsPs inhibit RIG-I and Mda5 mediated IFN-β promoter activity. HEK293 cells were co-transfected with the IFN-β-luc reporter plasmid (50 ng), empty vector, nsP1234 (200 ng) or nsP123 (200 ng) expression plasmids, and (A) RIG-I (50 ng) or (B) Mda5 (50 ng) expression plasmids. After twenty-four hours of incubation, cells were subsequently mock transfected or transfected with 8 ng of poly-(I:C) and luciferase activity was measured seven hours after post-stimulation. Each bar represents an average of triplicate samples normalized to total protein and standard deviation was calculated. The data shown are representative of three independent experiments.

Figure 5-4: The AR86 nsPs inhibit constituitively active RIG-I mediated IFN-β promoter activity.
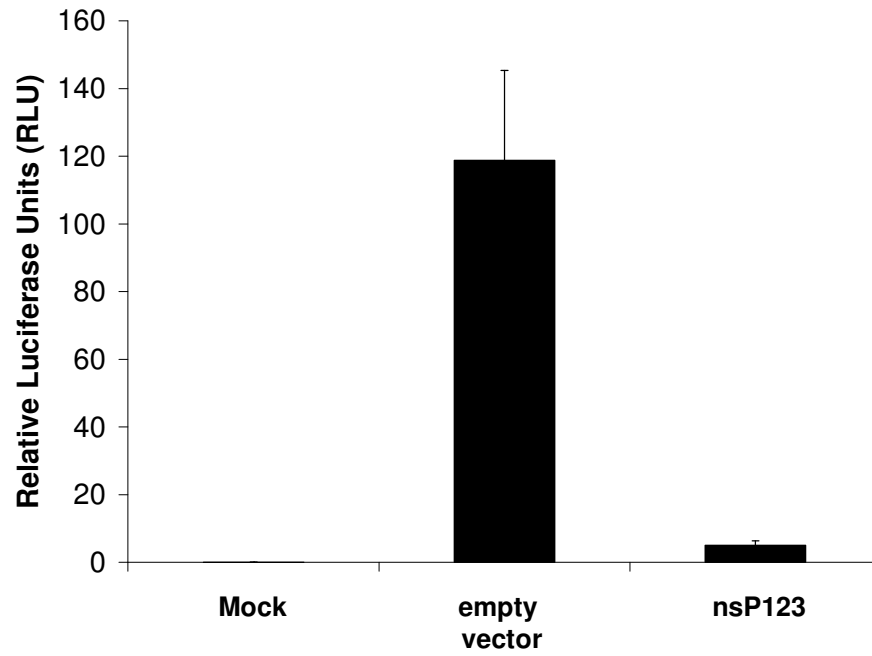
Figure 5-4. The AR86 nsPs inhibit constituitively active RIG-I mediated IFN-β promoter activity. HEK293 cells were co-transfected with the IFN-β-luc reporter plasmid (50 ng), constituitively active RIG-I (100 ng) or empty vector control (100 ng), and empty vector or nsP123 (200 ng) expression plasmids. Twenty-four hours post-transfection luciferase activity was measured. Each bar represents an average of triplicate samples and standard deviation was calculated. The data shown are representative of three independent experiments.

Figure 5-5: The protease function of nsP2 is critical in antagonizing RIG-I and Mda5 mediated IFN-β promoter activity.
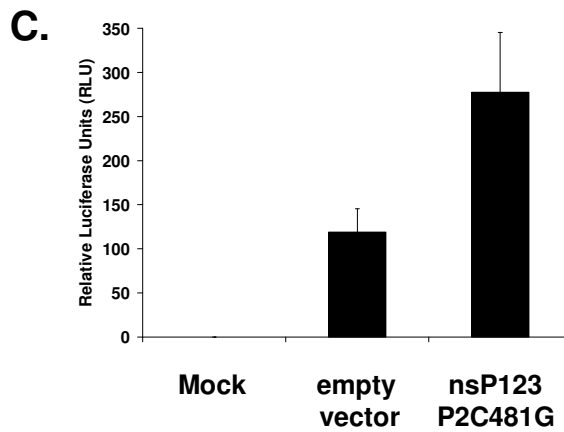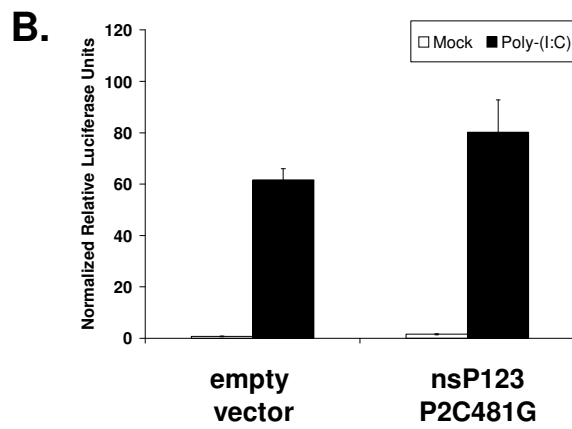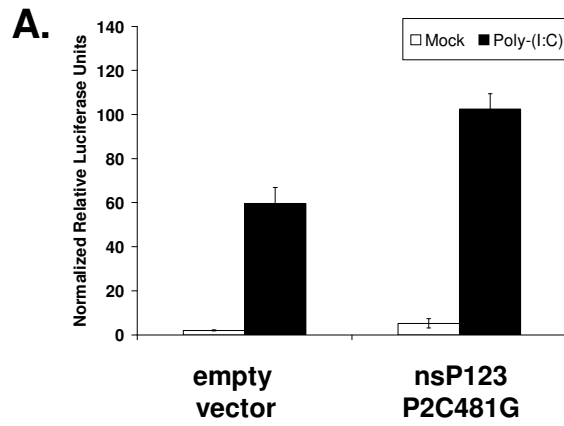
Figure 5-5. The protease function of nsP2 is critical in antagonizing RIG-I and Mda5 mediated IFN-β promoter activity. HEK293 cells were co-transfected with the IFN-β-luc reporter plasmid (50 ng), empty vector, or nsP123 P2C481G (200 ng) expression plasmids, and (A) RIG-I (50 ng) or (B) Mda5 (50 ng) expression plasmids. After twenty-four hours of incubation, cells were subsequently mock transfected or transfected with 8 ng of poly-(I:C) and luciferase activity was measured seven hours after post-stimulation. (C) HEK293 cells were co-transfected with the IFN-β-luc reporter plasmid (50 ng), constituitively active RIG-I (100 ng) or empty vector control (100 ng), and empty vector or nsP123 P2C481G (200 ng) expression plasmids. Twenty-four hours post-transfection luciferase activity was measured. Each bar represents an average of triplicate samples normalized to total protein and standard deviation was calculated. The data shown are representative of three independent experiments.

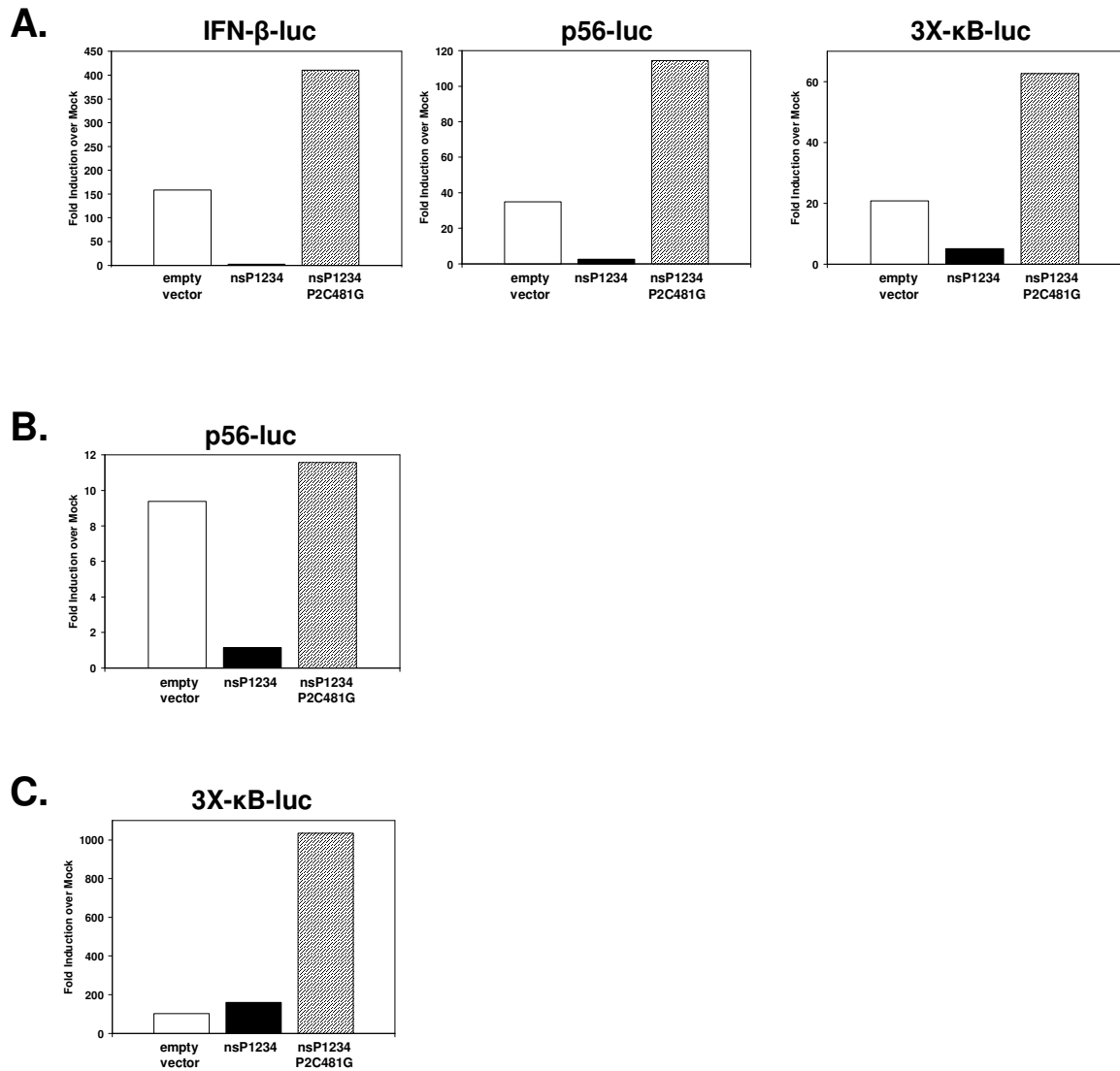**Figure 5-6: AR86 nsPs inhibit MAVS/IRF-3 signaling pathway.**

Figure 5-6. AR86 nsPs inhibit MAVS/IRF-3 signaling pathway. (A) HEK293 cells were co-transfected with either IFN-β-luc reporter plasmid (50 ng), p56-luc reporter or 3X-κB reporter plasmids (50 ng), empty vector, nsP1234, or nsP1234 P2C481G, and empty vector or MAVS expression plasmid (100 ng). (B) Transfections were performed in a similar manner except IRF-3 was used as the inducer and p56-luc promoter activity was measured. (C) Transfections were performed in a similar manner except p65 was used as the inducer and 3X-κB-luc promoter activity was measured.   Twenty-four hours post-transfection luciferase activity was measured.   Each bar represents an average of triplicate samples normalized to cells induced with an empty vector. The data shown are representative of three independent experiments.

Figure 5-7: Expression of AR86 nsPs induces the loss of RIG-I, Mda5, and IRF-3 expression.
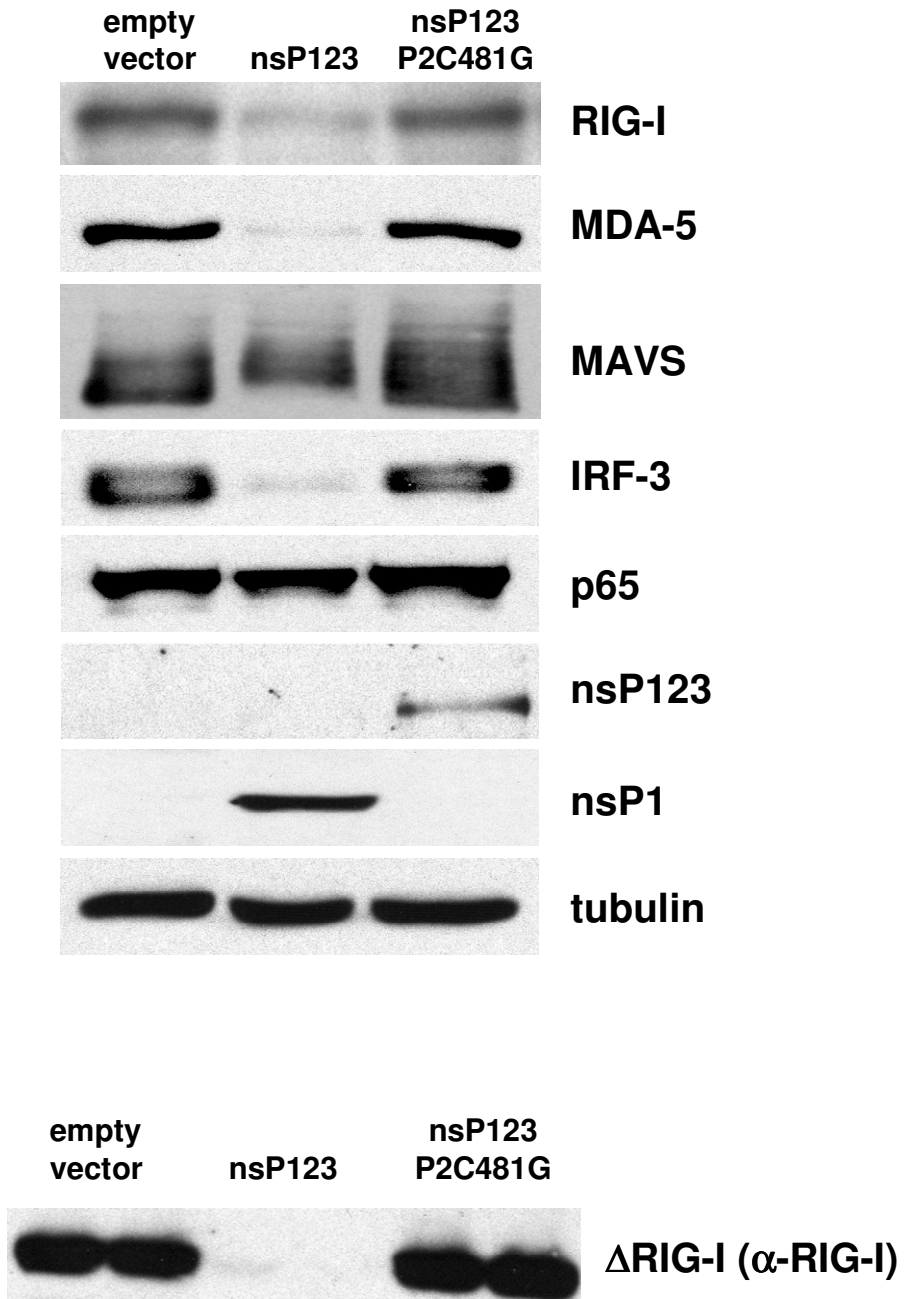
Figure 5-7. Expression of AR86 nsPs induces the loss of RIG-I, Mda5, and IRF-3 expression. HEK293 cells were co-transfected with empty vector, nsP123, nsP123 P2C481G (1 ug) and either RIG-I, Mda5, constituitively active RIG-I, MAVS, IRF-3, or p65 (500 ng). Cells were lysed after twenty-four hours and protein expression was evaluated by western blot as described in the Materials and Methods. The data shown are representative of three independent experiments.

CHAPTER SIX

DISCUSSION

Girdwood virus-induced arthralgia in mice

We hypothesized that the Sindbis-group virus strain Girdwood, that is associated with infectious arthralgia in humans, would be useful for studying the pathogenesis of bone and joint disease in a mouse model. While arthritogenic alphaviruses are a significant cause of human disease, the mechanisms underlying alphavirus-induced arthritis and arthralgia in humans are not well understood. In order to better understand the pathogenesis of arthritogenic alphaviruses, improved animals models are needed to dissect the pathogenesis of these diseases. Currently, two mouse models have been described for studying the pathogenesis of alphavirus-induced arthritis and myositis. One study has shown that the Sindbis-group virus strain AR86 replicates in joint associated tissues of adult mice (20). However, no virus-induced disease was observed, which limits the potential of this model for studying the pathogenesis of SINV-induced arthralgia. Furthermore, Morrison et al. has described a mouse model of RRV-induced arthritis/myositis; however, unlike RRV, which causes inflammatory arthritis in humans, SINV, such as Ockelbo, Pogosta, and Girdwood cause arthralgias without overt inflammation (19, 26, 38). It is therefore likely that the pathogenesis of arthritis associated viruses will differ from arthralgia-associated viruses.

It is thought that direct virus replication within the affected joints contributes to the development of alphavirus-induced arthritis/arthralgia in humans. This is largely based on studies with RRV, where viral RNA and viral antigen-positive cells have been isolated from the knee joint and synovium effusions of afflicted individuals (13, 46). RRV has been shown to replicate to high levels in synovial tissues of infected mice and in hind limb skeletal muscle (38), In support of this, our studies demonstrated that joint-associated tissues and skeletal muscle of mice were major sites of replication for the arthralgia-associated Sindbis-

group virus strain Girdwood. Perhaps most importantly, we found that the arthralgia-associated Girdwood virus replicates to higher titers in joint and muscle tissues than the prototypic TR339 strain of SINV, which has not been linked to arthralgia in humans. This result is particularly striking in that both viruses replicated to equivalent titers in the CNS, which suggests that the arthralgia-associated Girdwood virus may either exhibit an enhanced tropism for extraneural tissues, or an enhanced ability to replicate in cells within these tissues. Either of these possibilities may have an important impact on our understanding of the pathogenesis of alphavirus-induced arthritis/arthralgia, since this would suggest that specific viral determinants contribute to the ability of arthralgia-associated viruses to target to and/or replicate within joints or associated connective tissues. This in turn raises the possibility of mapping genetic determinants within the cloned Girdwood virus that contribute to the enhanced replication within muscle and joint tissues. The availability of molecular clones of Girdwood and TR339 viruses, which can be readily used to create a set of chimeras to map the determinants of bone/joint tropism, provides a set of tools that could be used to significantly enhance our understanding of the viral and host factors that contribute the development of arthritis/arthralgia following alphavirus infection.

The mechanisms underlying alphavirus-induced arthralgia are poorly understood, since the joint pain induced by SINV lack the inflammation that presumably mediates the arthritic symptoms caused by viruses such as RRV (19, 42). Infection of 14 day-old mice with Girdwood virus did result in a transient hind limb dysfunction which is similar to that seen with RRV-infected mice (data not shown; (38), respectively). Though we cannot rule out a role for neurologic effects, several lines of evidence from this study suggest that this disease is due to direct viral replication within muscle and joint-associated tissues within the

limb, rather than virus-induced nerve damage within the CNS. This includes the observed transient nature of the disease, with infected mice recovering full use of their hind limbs after the resolution of the viral infection. Though it is possible for mice to regain partial hind limb function following virus-induced damage of the CNS (48), it is unlikely that the affected animals would regain full function as rapidly as the those in our study if the hind limb dysfunction were due to nerve damage. Furthermore, while Girdwood virus replicated to higher titers than TR339 virus in joint and muscle tissues and caused more severe disease, both viruses replicated to similar titers in the CNS. Hematoxylin and eosin stained brain and spinal cord sections revealed no overt neuronal damage during the course of infection (data not shown). This result supports the hypothesis that direct viral replication by the arthralgia-associated Girdwood virus is responsible for the hind limb dysfunction, though additional studies are needed to address this issue in greater detail. However, even if the hind limb disease proves to be due to virus-induced damage to the CNS, this mouse model should still be useful for identifying viral genetic factors that promote replication within joint-associated tissues.

The genetic determinants of the adult mouse neurovirulent AR86 virus

SINV infection of mice has provided valuable insight into the viral and host factors that contribute to virus-induced neurologic disease. The identification of molecular determinants of alphavirus virulence represent an important step in understanding the pathogenesis of alphavirus-induced neurologic disease. While the Sindbis-group virus strain AR86 causes a lethal disease in adult mice, the closely related Girdwood strain is avirulent (21, 44, 48). These two strains differ by only 22 amino acid coding changes plus an 18

amino acid deletion within nsP3 (44). We hypothesized that the avirulent Girdwood virus will aid in the identification of genetic determinants of adult mouse neurovirulence within the AR86 virus. Initial studies using chimeric viruses demonstrated that genetic elements within the nonstructural and structural regions contribute to AR86 neurovirulence. Detailed mapping studies identified four major genetic determinants of adult mouse virulence that localized to nsP1 position 538 (Ile to Thr; avirulent to virulent), an 18 amino acid deletion from nsP3 386 to 403, nsP3 position 537 (Opal to Cys; avirulent to virulent), and E2 position 243 (Leu to Ser; avirulent to virulent). The fact that these four changes conferred adult mouse neurovirulence on a normally non-neurovirulent SINV (Girdwood) clearly argued that these changes represent the major determinants of AR86 neurovirulence, although contributions from additional AR86 determinants cannot be ruled out.

These findings are unique in that all four of the identified AR86 neurovirulence determinants are not present in published sequences of any other adult mouse neurovirulent SINV, such as neuroadapted Sindbis virus (NSV). This suggests that AR86 and NSV have evolved different mechanisms for causing neurologic disease in adult mice. Previous neurovirulence studies with NSV have shown the importance for regions within the E2 glycoprotein and the 5' untranslated region (UTR) in contributing to virus-induced neurologic disease. Within the E2 glycoprotein, a His at position 55 in the E2 glycoprotein was found to play a major role in adult mouse neurovirulence (18, 50). The mechanism underlying this change is not yet completely understood; however, E2 His 55 was shown to correlate with improved binding and entry into neuronal cells (50), increased levels of viral replication (12), and the ability to overcome the protective effect of bcl-2 overexpression in

neurons (28, 29). Additionally, a single substitution of a G at position 8 in the 5' noncoding region of NSV was responsible for conferring neurovirulence in adult rats (25).

While all four changes are essential for the complete mouse neurovirulence phenotype of AR86, it appears that the Thr at nsP1 538 plays a particularly important role. Previous studies with this determinants have shown that changing the Thr at nsP1 538 in AR86 to the consensus Ile found in non-neurovirulent viruses resulted in a complete loss of virus-induced mortality, though this virus was still capable of causing disease, albeit less severe (21).

One of the unique findings in our mapping studies was that the other two nonstructural virulence determinants (18 amino acid deletion and nsP3 537) showed very little effect on adult mouse neurovirulence when introduced, either individually or in combination, into an attenuated chimeric virus. However, combining the Thr at nsP1 538 with either of the two nsP3 virulence determinants resulted in a synergistic effect on adult mouse neurovirulence. This indicates that the nsP1 538 determinant is interacting with the 18 amino acid deletion and nsP3 537 determinants. As for the mechanism underlying the role for nsP1 538 in virulence, we have previously reported that substitution of the wild-type Thr with an Ile residue accelerates processing of the P123 polyprotein precursor into the mature nsP1, nsP2, and nsP3 proteins (22). This coincided with a more rapid induction of 26S RNA synthesis contributing to earlier expression from the 26S promoter in infected cells, but did not measurably affect the levels of viral minus- or plus-strand RNA synthesis (22). Based on the difference in nsP polyprotein processing, this raises the possibility that the two nsP3 virulence determinants enhance adult mouse neurovirulence only when there are more stable nonstructural polyprotein precursors present during infection. In addition to

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the modulating nsP polyprotein processing and viral RNA synthesis, this determinant was found to modulate type I IFN induction. Details of this finding are discussed in more detail in the next section.

The 18 amino acid deletion between residues 386-403 in nsP3 is located within the highly variable C-terminal region of the protein (as reviewed in reference (47)). One of the intriguing features of this 18 amino acid region is that Girdwood virus, which was originally isolated in South Africa (34), has the same set of amino acid residues within this region as that of other SINV strains, including the infectious clone for the original AR339 strain (TRSB), which was isolated near Egypt, Cairo (35, 54). This indicates that this 18 amino acid domain is highly conserved amongst SINV. While the function for the nsP3 protein has yet to be defined, it has been shown to be a phosphoprotein that is required for the synthesis of both viral minus-strand and subgenomic RNA (27, 30). It is interesting to note that the 18 amino acid deletion within AR86 results in the removal of 7 Ser residue and 1 Thr residue, which may affect the overall phosphorylation of nsP3. Mutational analysis within the C-terminal region of nsP3, which included this 18 amino acid domain, altered levels of viral minus-strand RNA synthesis and levels of nsP3 phosphorylation (27). It will be intriguing to determine if the Ser/Thr residues are important or whether the specific amino acid sequence within the 18 amino acid region is important. Due to the fact that nsP3 can tolerate insertions of green fluorescent protein (GFP), red fluorescent protein (RFP), or luciferase protein within the C-terminal region, this suggests that the specific amino acid sequence within the 18 amino acid region may not be as important as the Ser/Thr residues (8, 10, 14).

In addition to its role in viral RNA synthesis, nsP3 has been shown to interact with several host proteins during the course of virus infection. Cristea et al. has demonstrated that

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early during infection nsP3 transiently interacts with Ras-GTPase activating protein (G3BP), a nuclear transport factor, while late in infection, nsP3 interacts with 14-3-3, a cellular protein which interacts with phosphorylated proteins and regulates various host cell processes (10). In addition, Frolova et al. demonstrated that nsP3 forms higher ordered structures during infection and similarly found that nsP3 interacts with 14-3-3 late in infection (14). While the importance of these nsP3-host protein interactions are not yet defined, it does demonstrate that nsP3 is not exclusively involved in viral RNA synthesis. It will be interesting to determine the effect this 18 amino acid region plays in altering the efficiency of these interactions.

While most alphaviruses encode an opal termination codon proximal to the 3' end of the nsP3 gene, the Sindbis-group virus strain AR86 (44) and the Semliki Forest virus (SFV) strain SFV4 carry a cysteine and arginine sense codon, respectively (44, 51, 52). Tuittila and colleagues performed a detailed mapping study within the replicase genes using virulent and avirulent SFV strains and found that an Arg (virulent SFV4 strain) in place of the opal termination codon at nsP3 position 469 was an important contributor to adult mouse neurovirulence (51).

While the mechanism of this determinant in viral pathogenesis is not well understood, studies have characterized the role of the opal termination codon in regulating nonstructural polyprotein processing and viral RNA synthesis. Translational read-through of the opal termination codon occurs at a frequency of about 5-10% (as reviewed in reference (17)), leading to limiting quantities of nsP4 relative to the other nonstructural proteins. Li et al. replaced the opal termination codon of SINV with different sense codons and found increased levels of the nsP3/4 polyprotein precursor and reduced levels of mature nsP3 early

during infection (31). A different C-terminus of nsP3 is produced in viruses encoding either

an opal termination codon or a sense codon. In the presence of an opal termination codon,

the predominate nsP3 protein form consists of a C-terminus produced by translational stop at

nsP3 537. In contrast, in the presence of a sense codon, the predominate nsP3 protein is

formed by cleavage at the nsP3/nsP4 site, which results in the addition of an extra seven

amino acids to the C-terminus of nsP3 (47). Furthermore, replacing the opal termination

codon with a sense codon reduced levels of both 49S genomic and 26S subgenomic viral

RNA synthesis early during infection (31). Based on these findings, it will be important to

determine whether the Cys sense codon in AR86 affects neurovirulence through alterations

of nonstructural polyprotein processing and/or viral RNA synthesis.   Additionally, it will be

interesting to study the relevance of the nsP34 precursor polyprotein in viral RNA synthesis

or modulating virus-host interactions.

The neurovirulence determinant within the E2 glycoprotein at position 243, in which

AR86 encodes a unique serine residue, is likely affecting viral interactions with neurons or

other cell types in the infected animal. This hypothesis is based on the fact that E2 is

involved in host cell receptor interactions and this particular change is near a region of the E2

glycoprotein that is thought to be involved in direct contact with host receptors (39, 45). This

raises the strong possibility that the Ser residue might affect virus/receptor interactions by

either altering the efficiency of receptor interaction or by changing the conformation of the

E2 glycoprotein. This is supported by findings within the E2 glycoprotein of other SINV, in

which a Gly residue at position 172 enhanced viral binding to neuronal cells or a His residue

at position 55 improved binding, entry, and replication in neuronal cells (12, 49, 50).

Preliminary evidence from our laboratory showed no difference in the infectivity of neuronal

cells. However, this determinant did appear to alter the infectivity of astrocytes, in which the attenuating Leu exhibited higher infectivity than the virulent Ser residue (Suthar, M.S. and Heise, M.T., unpublished data). This preliminary finding, if true, would have some important implications on viral pathogenesis. Astrocytes are the major glial cell within the CNS and are critical in the formation and maintenance of the blood-brain barrier, antigen presentation to CD4+ T cells through expression of major histocompatibility complex II (MHC class II), and induction of proinflammatory cytokines and chemokines such as type I IFN, IL-6, IP-10, IL-1, RANTES, and TNF-α (11). This would imply that infection of this cell type may result in the induction of a strong proinflammatory response which would ultimately restrict virus replication and spread within the CNS. It will be interesting to determine if this virulence determinant also shows differences in infectivity of microglial cells, which are the resident macrophages within the CNS. Therefore, additional studies to evaluate the effect of the Ser versus Leu at E2 position 243 on virus binding or infection of neurons, astrocytes, or microglial cells may provide useful information on the role of this determinant in regulating viral infection. However, the lack of a clearly defined receptor for alphaviruses currently hinders a direct analysis of this determinant's role in virus/receptor interactions.

AR86 virus antagonism of type I IFN induction

Type I IFN is an essential component of the host response to viral infection because it directly activates antiviral effects 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 induction of a type I IFN response. These mechanisms

range from nonspecific effects, such as shutoff of host cell macromolecular synthesis (1, 16), masking viral RNA from host cell sensory proteins (9, 37), or specific inhibition of host cell dsRNA sensors or signaling molecules that link these sensor molecules to transcription factors that regulate type I IFN transcription (3, 6, 32, 33).

Although studies with SINV and other alphaviruses have demonstrated that host cell shutoff plays a major role in regulating viral type I IFN induction (16), we found that in our studies with AR86, the virulence determinant at nsP1 538 modulates type I IFN induction through an additional mechanism by altering the activation of Interferon Regulatory Factor-3 (IRF-3). We found that while wild-type AR86 virus exhibits reduced IFN induction *in vitro* or *in vivo*, a mutant AR86 virus containing the attenuating Ile at nsP1 position 538, induced robust levels of type I IFN. While these two viruses did not differ in their kinetics of host translation of transcription shutoff, the mutant AR86 virus exhibited more rapid induction of IRF-3 dimerization and phosphorylation, a step which is independent of *de novo* host protein synthesis and is not affected by virus-induced host shutoff. To further support this finding, it will be important to evaluate type I IFN induction and virulence in IRF3-/- mice.

Since the determinant at nsP1 538 affects type I IFN induction in addition to effects on host cell macromolecular synthesis, this raises three possible mechanisms by which the determinant at nsP1 538 modulates type I IFN induction. One possibility is that wild-type S300 virus intentionally delays RNA synthesis from the 26S promoter until late in infection and this effect is disrupted by the determinant at nsP1 538. This would suggest that the subgenomic RNA may be a potential target for sensing by the host cell. A recent report by Gitlin et al., suggested that RIG-I plays an important role in responding to SINV infection (15). Secondly, based on the recent finding that RIG-I has been shown to interact with

5'triphosphates on uncapped RNAs and given that virus nsP1 and nsP2 proteins are involved capping viral RNAs, it is possible that the determinant at nsP1 538 is altering the efficiency of capping viral genomic or subgenomic RNA (2, 23, 36, 41, 53). Thirdly, wild-type AR86 nonstructural proteins may actively suppress early type I IFN induction through direct interaction with host type I IFN signaling component(s), and this effect is disrupted by the attenuating Ile at nsP1 538. This scenario raises the possibility that alphaviruses employ a multi-step approach to blocking type I IFN induction, where a specific inhibitory effect is active early in infection, while a generalized host shutoff would block IFN synthesis late in infection.

Given the role of the SINV nonstructural proteins in modulating type I IFN induction, we hypothesized that the AR86 nonstructural proteins were actively suppressing the type I IFN response. Towards this end, we investigated the role of the two host cell cytoplasmic sensors of dsRNA, RIG-I and Mda5, during SINV infection. We found that AR86 genomic RNA is capable of being recognized by RIG-I and Mda5. In future studies, it will be interesting to determine the region of the viral genome RIG-I is recognizing and whether the subgenomic RNA is more efficiently recognized by RIG-I than full-length genomic RNA.

Although the AR86 genomic RNA is capable of being recognized by RIG-I, type I IFN induction is blocked by the viral nonstructural proteins. We found that RIG-I, Mda5, and IRF-3 expression were rapidly lost in the presence of the viral nonstructural proteins in a viral nsP2 protease-dependent manner. This result suggests a novel role for the viral nsP2 protease in antagonizing type I IFN induction and raises two possibilities by which the protease is effecting protein expression. The first is the idea that the viral nsP2 protease is targeting and cleaving RIG-I and Mda5. Examination of the amino acid sequences for RIG-I

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and Mda5 revealed nsP1/2 and nsP2/3 virus-like cleavage sites within the proteins, respectively. The cleavage preferences for nsP2 depend on its context with the other nonstructural proteins (as reviewed in reference (47)). Briefly, cleavage at the nsP1/2 site occurs only by the nsP123 polyprotein and inhibiting the nsP1/2 cleavage site blocked cleavage at nsP2/3 by the nsP123 polyprotein. Furthermore, the nsP23 polyprotein is responsible for cleaving the nsP2/3 site. The relevance of the cleavage site preferences on type I IFN induction is demonstrated by the finding that a Thr at nsP1 538 exhibits delayed nsP processing early during infection. This would imply that wild-type AR86, which has been shown to have a stable nsP123 early during infection, is able to efficiently target host proteins that contain nsP1/2 virus-like cleavage sites, such as RIG-I. As the nsP123 precursor polyprotein is processed into nsP23, this polyprotein would be able to efficiently target host proteins that contain nsP2/3 virus-like cleavage sites, such as Mda5.

In addition to RIG-I and Mda5, we have identified other host cell proteins that regulate type I IFN induction that contain virus-like cleavage sites, including the mitochondrial associated adaptor protein IPS-1, containing an nsP2/3 like site, and histone deacetylase 6 (HDAC6), containing an nsP1/2 like site. The adaptor protein IPS-1, also known as MAVS, Cardif, and VISA, interacts with activated RIG-I and Mda5, via their respective CARD domains, to activate IFN-β gene transcription (24). While HDAC-1 and HDAC-8 were found to function as repressors of viral type I IFN infection, HDAC6 was found to function as a co-activator of IRF-3 dependent transcription (40). While targeted cleavage of these proteins would be an efficient means of antagonizing the host cell antiviral response, further studies are required to determine if these host proteins are targets of the viral nsP2 protease. These studies would include identification of the potential cleaved target

protein fragments when co-expressed with the viral nsPs or during virus infection. Moreover, this raises the possibility that targeting host proteins by viral proteases may be a general viral immune evasion strategy. One such example is the observation that the Hepatitis C virus NS3/4A protease cleaves the mitochondrial-associated adaptor protein IPS-1 and the TLR-adaptor protein TRIF to antagonize type I IFN induction (32, 33).

Another possibility for the observed loss of RIG-I, Mda5, and IRF-3 expression is that the viral nsPs could be targeting these proteins for degradation. Recently, it has been reported that the Classic swine fever virus N(pro) protein and Rotavirus nonstructural protein NSP1 target endogenous IRF-3 for degradation in a proteasome-dependent manner (5, 7). It is well known that activated IRF-3 is efficiently targeted to the host proteasome through the function of peptidylprolyl isomerase Pin-1 (43), however, the mechanism of degrading endogenous IRF-3 is not known. Mda5 has recently been shown to be cleaved during poliovirus infection and this appeared to be mediated by the induction of apoptosis during virus infection (4). These findings imply that the host cell has efficient mechanism to downregulate expression of components of the type I IFN signaling pathway. The ability of a virus to target these negative regulatory pathways would be a mechanism to completely abrogate the ability of the host cell to respond to virus infection. Therefore, it will be interesting to determine if the viral nsPs interact with and target these components for degradation or induce a negative regulator within the host cell that efficiently targets these proteins for degradation.

These studies have provided significant insight into the viral and host factors that mediate alphavirus-induced disease and the mechanisms underlying alphavirus pathogenesis.

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SINV infection of mice is one of the best characterized model systems for studying virus-induced diseases. These studies have shown the spectrum of diseases SINV can cause in mice, ranging from virus-induced arthralgia to virus-induced neurologic disease. Identifying the viral determinants responsible for modulating virus-induced neurologic disease provides the fundamental basis for future studies characterizing the mechanism of these determinants. Undoubtedly, future studies with the Sindbis-group virus strains Girdwood and AR86 will unveil novel mechanisms through which these viruses modulate the host antiviral response.

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