

ABSTRACT

MAGLIOCCA, JOSEPH WILLIAM. Alphavirus Entry, Assembly, and Egress. (Under the direction of Dennis T. Brown).

Arboviruses in the alphavirus genus can cause a variety of serious infections in humans and domesticated animals. Sindbis virus (SV) serves as the prototypical model used in studies of viruses in this genus, and a detailed understanding of the biological processes of its lifecycle is essential for the SV's use as a model organism. Sindbis virus was long thought to enter its host cells by a fusion mechanism similar to the entry mechanism of influenza viruses, but data suggesting this model are mostly of questionable relevance. Newer data have suggested that SV forms a pore through the host cell membrane, and the viral RNA passes through this pore to enter its host. The mechanism driving this pore formation is still unknown. In chapter 2, vesicles derived from baby hamster kidney (BHK) cells were exposed to a high titer, wild-type strain of SV (SVHR). This infection was fixed and examined via electron microscopy. This "pore structure" forms between the virus and vesicles, but viral RNA does not pass through it, suggesting that a process in the host cell is required to drive the entry of alphavirus RNA into its host, but not the formation of the pore structure itself.

In chapter 3, a panel of SV strains with mutations at 2 separate sites in the SV glycoproteins was examined in BHK cells. These mutants were suspected to have deficiencies in their replication cycles. At position E1_H230, a deletion (Δ H230) and a substitution (H230A) mutant were studied, and similar mutants at position E2_G309 (G209A, G209D, and Δ G209) were also examined. Both mutations at position E1_H230 were lethal to the production of progeny virus, only unassembled viral proteins were produced, but repeated passaging of the H230A mutant led to a second site mutant (V231I)

that was able to partially rescue the virus. Neither of the E2_G209 substitution mutants had observable differences from wild type strains, but the deletion mutant (Δ G209) showed very low titer when grown at 37°C and no titer when grown at 28°C. No completed virus particles were seen when this virus was grown at 37 °C, but at 28°C, partially budded virus particles as well as chains and clumps of virus particles were observed in the host cell. S-35 labeling of the Δ G209 mutant grown at 28 °C confirmed that viral protein synthesis occurred as it does in wild type strains, so protein synthesis is not the cause of the partial-budding phenotype. Together, these data lead us to conclude that E1_H230 is necessary for complete SV assembly, and an amino acid is required at position 209 in E2 for complete assembly and budding, but multiple amino acids can serve this role. The data presented in the two chapters provides new insights into the SV lifecycle, specifically what occurs during the cycle and what is required for it to proceed properly.

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Alphavirus Entry, Assembly, and Egress

by
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DEDICATION

This work is dedicated to my mother, Cynthia Branager Magliocca -- Despite all of the hardships in her life, she was able to give me all the love and support I could have asked for, to my father John Anthony Magliocca -- Even though he passed away when I was 13, he was able to instill in me the values that guide my life and decision making, and to my great-grandfather Anthony Alphonse Schwenkler-He rose from a small coal mining town to graduate college and become a chemistry instructor. Even though I am just one of the dozens of his great-grandchildren whom he never met, his commitment to science education was passed down to me and many of my cousins.

BIOGRAPHY

I was born September 28, 1986 in Silver Spring, Maryland to John and Cynthia Magliocca. I was first exposed to science at an early age when my mother read me children's books about topics such as the human body, animals, the planets, and other topics at night before bed. She could tell I had an innate desire to learn and to talk about what I had learned with others.

When I was 2, my brother, Thomas Magliocca was born. He suffered from chronic ear infections for the first few years of his life and had difficulty learning to speak properly. This language impairment along with diagnoses of autism and ADHD meant that my brother had several learning disabilities and would require special education. My mother tried to teach him as she taught me, and while he made significant progress, it was clear that teaching him would be difficult.

In 1994, we moved to the Raleigh, North Carolina area, where my mother's parents and sisters lived and where my parents had met. It was a different environment, but I eventually adjusted. My love of learning continued, and I became better able to understand my brother's struggles. I grew to love science and history especially, and while the former was out of my parents areas of expertise, they tried to support my interest in these fields. With their support, I won second place in the 1999 North Carolina state Geography Bee and fourth in the 2001 Bee.

Unfortunately in 1997, tragedy struck my family. In April, my maternal grandfather was diagnosed with terminal bladder cancer and in August, my father was diagnosed with lung cancer. The timing of these two diagnoses, and especially the shock of my 37-year-old,

nonsmoking father's disease impacted us greatly. My grandfather died shortly after my father's diagnosis, but we hoped for the best for my father, even though his prognosis was grim. Through a combination of chemotherapy, radiation and surgery, he was able to live nearly three more years, but on May 11, 2000 he lost his battle. He and my mother tried to hide his suffering from my brother and me, but we could still tell what was going on.

After his death, my mother returned to NCSU to finish her master's degree in English and she got a job as a technical writer at the State Employee's credit union to support us. This job meant she had less time to work with my brother, so throughout high school, I began to teach him math and science. He was difficult to teach at first, but when I realized that his main issue with these subjects was that he did not understand the meaning of many words, I tried to show him ways to learn things that did not require as many words and to clearly explain the meanings of words in terms he could understand. This experience helped him make significant progress and helped me realize that I loved teaching and that I wanted teaching to be at least a part of my career.

The social aspect of high school was difficult for me, but it was here that I decided that I wanted to study chemistry or biology in college, with help from teachers in these fields including Ruth Wormald, Gail Barkes, Rebecca Townshend, and Beverly Graziani. I participated in the state Science Olympiad competition at NCSU in 2003 and 2004, by winning in a human anatomy/physiology competition, I won a small scholarship to NCSU. This scholarship, along with the school's proximity to my home and my mother's status as an alumna led me to choose this school for my post-secondary education.

In 2005, I graduated high school and became an undergraduate at NCSU majoring in chemistry. During the next four years I found that I especially loved organic chemistry. When I took BCH 451 as an elective in my senior year, I realized that this was the field I wanted pursue in graduate school. After talking to my advisor Dr. Phil Brown, I decided that the best option would be to add biochemistry as a second major. The requirements for a chemistry degree were similar to those of biochemistry, which meant I only needed to take one more year to get this second degree. I attended two NCSU graduations, Spring 2009 for chemistry and Spring 2010 for biochemistry.

Through my undergraduate years, I honed my teaching skills working at NCSU's undergraduate tutorial center, where successful undergraduate students can tutor other undergrads in certain courses. I also got my first taste of real research working in the lab of Dr. Seth Kullman in NCSU's toxicology department. Here I was able to learn many basic techniques for research in the biological sciences.

During my second senior year I applied to several biochemistry graduate programs, but after taking biochemistry courses from Dr. Stu Maxwell, Dr. Trino Ascencio-Ibáñez, and Dr. Carla Mattos, I decided to continue at NCSU for graduate school. After rotations in the labs of Dr. Bob Rose and Dr. John Cavanagh, my interest in virology led me to join the lab of Dr. Dennis Brown and Dr. Raquel Hernández in the spring of 2011. Since then I have worked on several projects with Sindbis virus. After graduation, I hope to find a career in academia, teaching biochemistry or a related field, but I will also look for jobs in research.

ACKNOWLEDGMENTS

In our lab, first and foremost I must thank Drs. Dennis Brown and Raquel Hernandez for giving me the opportunity to work and study in their lab and for advising me in the direction of my research and teaching me so much about virology in general. I also must thank lab managers Amanda Piper and Mariana Ribeiro for teaching me many of the techniques that I had to learn for my projects and for putting up with the many mistakes I made while learning them! I am also indebted to our postdocs Dr. Ricardo Vancini, and Dr. Denitria “Alex” Breuer for their help in teaching me several techniques, especially Ricardo’s electron microscopy prowess, without which my research would have been impossible. I must also thank our other graduate students, Sabrina Hunt, Krysty Yamamoto, and Ryan Schuchman as well as undergraduates Sofonias Mehari, Bliss Green, David Cookmeyer, Mark Messana, Spencer Moore, Mariene Ribeiro, and Kate Emmerich and the Arbovax employees, especially Emerson Huitt, who kept our lab running during their times working in it.

Outside of our lab, I must thank my committee members Drs. Cynthia Hemenway, Bob Rose, and Frank Scholle for their advice over the years as well as Drs. Stu Maxwell, Trino Ascencio-Ibáñez, and Dave Presutti for your guidance when I was a T.A., and the people in the front office Curt Moore, Nancy House, Pam Francis, Angie Barefoot, and Tiayonna Liska for keeping our department running and Dr. Paul Wollenzien, Richele Thompson, and Logan Draughn for help with equipment and materials that we have needed over the years.

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CHAPTER 1

Sindbis Virus and Its Importance

Section 1: Arboviruses, Alphaviruses, and their Structure and Function

Arthropod-born (arbo-) viruses are a major worldwide health concern. Each year hundreds of millions of people are infected by this type of virus, which includes flaviviruses such as yellow fever virus, dengue virus, west Nile virus, and the emerging Zika virus, as well as togaviruses in the alphavirus genus and other viral groups (Kuno and Chang 2005). These animal viruses are defined by their mode of transmission--they are usually passed between vertebrate hosts by arthropod vectors, such as mosquitos of the genus *aedes* (Kraemer, Sinka et al. 2015). This transmission occurs when infectious virus is present in the blood of an infected vertebrate host at a high enough titer to infect the mosquito's cells when it feeds on the vertebrate's blood. The virus then replicates in the infected mosquito's cells and is shed into its saliva (Sim, Jupatanakul et al. 2014). When the infected mosquito bites an uninfected vertebrate, the virus particles in the saliva will enter the blood of this vertebrate and will infect and replicate in the new vertebrate host's cells again to a high enough titer for the virus to infect a new mosquito vector that bites it. This lifecycle requires the virus to have the ability to replicate in both vertebrate and insect cells. These 2 cell types differ significantly in temperature, composition, and other conditions, so these viruses have had to evolve the ability to carry out biochemical processes that can occur in these very different environments (Brown and Hernandez 2012).

Sindbis Virus (SV) is an enveloped, positive-sense RNA arbovirus in the family togaviridae. This viral family contains a number of animal pathogens in the genus alphavirus, which includes 3 equine encephalitis virus species (Eastern, Western, and Venezuelan equine encephalitis viruses-EEEV, WEEV, and VEEV respectively), Ross River virus (RRV), chikungunya virus (CHIKV), and Semliki Forest virus (SFV), in addition to SV (Leung, Ng et al. 2011). As arboviruses, most of these viruses primarily infect small mammals and birds in an enzootic manner mediated by hematophagous insect vectors, but sometimes they can cause disease in humans and domesticated animals, with CHIKV in particular being a serious and growing human health concern (Brown and Hernandez 2012). There are currently no approved vaccines or antiviral treatments for diseases caused by these viruses although research and development into these methods of management is ongoing (Lundstrom 2014).

Decades of study of SV and its properties have led to the development of a successful set of laboratory protocols for the growth and maintenance of this virus in a research setting (Hernandez, Sinodis et al. 2010). The ability to use SV in this manner, along with its relation to more serious pathogens make it an ideal model organism for the development of vaccines, treatments, and other responses to alphavirus-caused disease (Brown and Hernandez 2012). However, the development of these methods of control requires extensive knowledge of the replication cycle of this virus. Past research has led to the development of models of the process of this cycle, but many details remain unconfirmed and controversial.

The Sindbis virion is about 70 nm in diameter and consists of a nucleocapsid core complex containing positive sense RNA and 240 copies of the viral capsid protein (Leung,

Ng et al. 2011, Brown and Hernandez 2012). This core is surrounded by a host cell membrane-derived envelope and a glycoprotein shell consisting of 80 trimers of heterodimers of two glycoproteins, known as E1 and E2 (Brown and Hernandez 2012). The glycoproteins each have a transmembrane domain that crosses the envelope, and this domain in E2 binds to the capsid protein, anchoring the glycoprotein spike to the nucleocapsid (Figure 1.1)

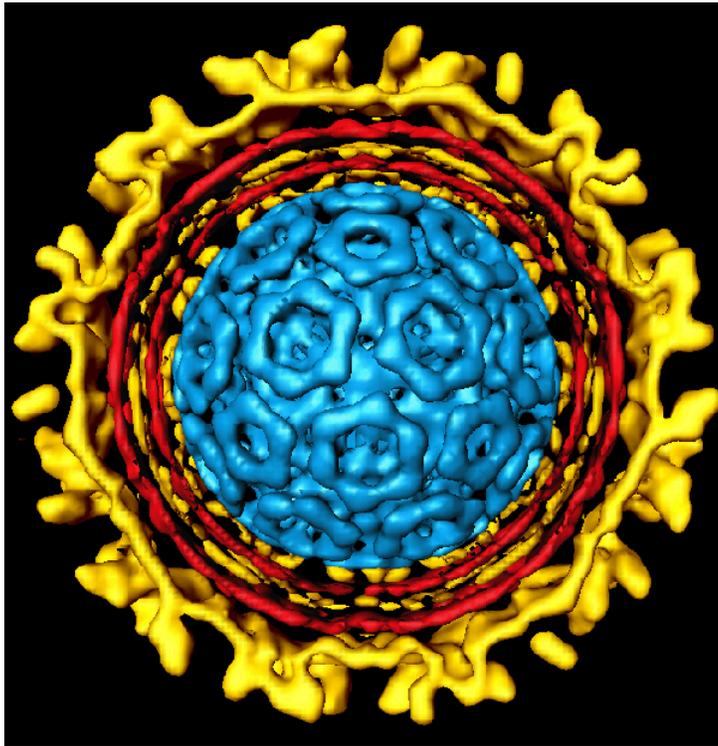


Figure 1.1: Model of Sinbis virion structure. Blue represents the nucleocapsid complex; red represents the host cell membrane-derived envelope; and yellow represents the trimers of E1 and E2 glycoprotein heterodimers.

As an alphavirus, SV has a genome consisting of a single strand of positive sense RNA. This strand contains the genes for nine proteins: four are nonstructural and are only synthesized in the host cell and five are structural and are used to form the progeny virus

(Paessler and Weaver 2009). Each of these groups of proteins is translated as a polyprotein that is processed post-translation by viral and cellular proteins. The four nonstructural proteins (nsp1-4) are located near the 5' terminus of the viral RNA and serve as an RNA-dependent RNA polymerase (RdRp) and associated accessory proteins for RNA transcription and replication. The five structural proteins are encoded in genes located toward the 3' terminus of the RNA genome. These consist of the capsid protein (C), the two glycoproteins E1 & E2, and two small proteins, E3 & 6K, which are not included in the completed progeny virion (Paessler and Weaver 2009, Sherman and Weaver 2010). (Figure 1.2)

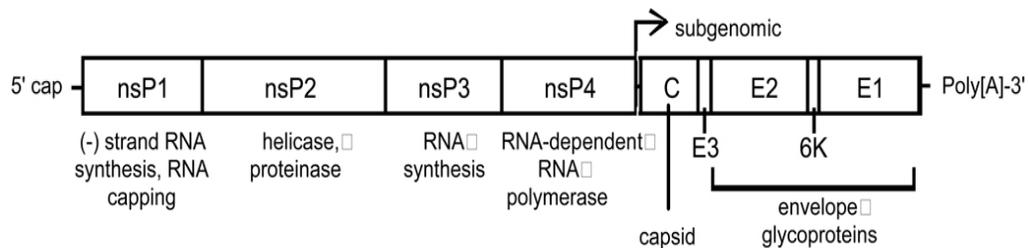


Figure 1.2: Map of the Alphavirus Genome. Names of genes and proteins. With permission from *Vaccine*.

Section 2: Sindbis Virus Entry

Like all viruses, The SV genome must gain entry into a host cell in order to replicate. The most popular model for SV entry is derived from entry models of other enveloped viruses such as that of influenza (White, Kielian et al. 1983, Samji 2009). This model assumes that when a Sindbis virion encounters a potential host cell, its glycoproteins bind to a receptor on the host cell surface. Protein conformational changes induced by this binding cause the host cell to engulf the infecting virion in an endosome, which is then pulled into the cell. Once the endosome is inside of the host cell, its pH is lowered by ATPases in the lipid

membrane. At a critical pH, conformational changes in the virus's and host's proteins allow the virus to escape the vesicle and the viral RNA to escape the nucleocapsid so that it can be translated (Brown and Hernandez 2012).

However, there are several problems with this model. Most direct evidence for this model comes from experiments that are potentially irrelevant. Direct fusion between SV and living cells can be induced, but only if the pH is lowered to 5.8 and then increased back to neutral (Paredes, Ferreira et al. 2004, Brown and Hernandez 2012). There is no evidence that this increase can occur *in vivo*. SV can fuse with artificial liposomes without this pH increase, but these liposomes have some key differences from the living cells SV must infect, making any conclusions drawn from these experiments questionable. One example of this can be seen in the observation that SV-liposome fusion can only occur if the liposomes contain cholesterol, which is not found in large quantities in the mosquito cells that must be infected by SV when the virus goes through its lifecycle in nature (Hafer, Whittlesey et al. 2009).

Data suggesting an alternate model of entry for this virus have been found over the past few decades. In 1978, a study showed that cells infected with SV have a much lower sensitivity to antiviral antibodies than cells infected with a virus known to enter via a fusion pathway. The authors concluded that this is evidence against this type of entry mechanism for SV (Fan and Sefton 1978). This conclusion was later supported by a 2004 study of SV structure at neutral pH, acidic pH, and returned to neutral pH from acidic pH. The observation that the virion does not disassemble at the lowered pH suggests that fusion is unlikely, because according to the fusion model, disassembly would have to occur at this pH

in order to release viral RNA into the host cell (Paredes, Ferreira et al. 2004). Also, the investigators observed via electron microscopy of SV-infected Baby Hamster Kidney (BHK) cells no fusion in any stage between the virions and cells, but rather virus particles attached to the host cells by stalks containing a pore through which the viral RNA appears to pass through. They also observed SV particles near the host cell with much lower electron density than most SV particles. They hypothesized that these were “empty” SV particles that had already deposited their RNA into the host cell (Paredes, Ferreira et al. 2004).

These observations led the investigators to develop a new potential model for SV entry in which viral binding to host cell receptors induces conformation changes that form this pore structure in the host membrane. Next in this hypothetical pathway, viral RNA enters the host through this pore, and finally the remaining “shell” of the virus is released from the host. Later studies by the same group provided further evidence for this entry model when they showed that the ratio of “empty” to “full” SV particles increases during infection in a time- and temperature-dependent manner (Vancini, Wang et al. 2013). Using these data as a rate of infection in an Arrhenius plot suggested that SV RNA is not “injected” into host cells as occurs in some bacriophages (Hu, Margolin et al. 2013). Instead the viral RNA is either pulled into the host by something in the cell, or viral RNA simply diffuses into the host through the pore (Vancini, Hernandez et al. 2015).

Section 3: Sindbis Virus Replication and Assembly

Models for the biological processes of SV-infected cells post infection are less controversial. According to current models, immediately after SV RNA enters the host cell, the nonstructural proteins are translated as a polyprotein from the viral RNA (Jose, Snyder et

al. 2009). These proteins serve as an RdRp and associated accessory proteins that transcribe minus-sense RNA from the initial positive-sense genome before they fully mature (Jose, Snyder et al. 2009). Later, these four proteins cleave into separate proteins. At this time, they transcribe new full length positive sense RNA for the genomes of the progeny virus as well as shorter positive-sense subgenomic RNA, both from the previously synthesized negative-sense RNA (Jose, Snyder et al. 2009). Next, the subgenomic RNA is translated to synthesize the structural proteins for the progeny virus.

Like the nonstructural proteins, the SV structural proteins are also synthesized as a single polyprotein. This contains the capsid protein at the N-terminus, followed by PE2, 6K and E1 before the C-terminus. After being synthesized, autoproteolysis removes the capsid protein, which then forms the nucleocapsid complex with the newly formed full-length, positive-sense RNA for the progeny virus. The remaining polyprotein enters the endoplasmic reticulum (ER) membrane. Here signal peptidase cleaves out the 6K protein, and the resulting PE2 and E1 glycoproteins form heterodimers and then trimerize, forming the precursors to the glycoprotein spikes (West, Hernandez et al. 2006). Next, these glycoprotein trimers of heterodimers travel to the Golgi apparatus, at which point furin protease cleaves off the E3 protein, leaving mature E2 complexed with E1. These glycoproteins are exported to the cell membrane. Here the newly formed nucleocapsid complex joins with the glycoprotein spikes and is enveloped and released from the host cell as a progeny virion (West, Hernandez et al. 2006, Whitehurst, Willis et al. 2006).

Section 4: Summary of Research Chapters

The data presented in the preceding chapters give new insight into SV entry, assembly, and egress. In Chapter 2, new evidence for the pore structure mechanism of entry is presented. In this study, vesicles derived from BHK cell membranes were isolated and then infected with heat resistant Sindbis virus (SVHR), the SV strain with the highest stability and titer. The vesicles were examined with electron microscopy after 10 minutes of infection and were seen to have virus particles attached to the vesicles via the pore structure seen in complete cells in the previous study.

In Chapter 3, five mutant strains of SV were developed and studied. These mutant strains have deletion or substitution mutations at one of two amino acid residues in the SV glycoproteins, namely the histidine at position 230 (H230) in E1 and the glycine at position 209 (G209) in E2. Both H230 mutants showed lethal assembly defects, but they could be partially rescued by a second substitution mutation found via serial infection. The G209 substitution mutants showed no defects in titer, but the deletion mutant showed a newly observed phenotype. SV particles began to bud from the host cell, but budding arrests, leaving bulging virions and clumps and chains of virions on the surface of the host cell. This observation suggests a role for E2 late in the budding of progeny virus.

CHAPTER 2

Sindbis Virus Binds to BHK Membrane-Derived Vesicles by Forming a Pore Structure

Section 1: Summary

Despite decades of investigation, a consensus model for the entry of Sindbis virus (SV) into host cells has not been developed. For years it has been thought that this virus's envelope fuses with the host cell's membrane like other enveloped viruses, but observations in the last decade have called this model into question. Electron microscopy images of SV infection of Baby Hamster Kidney (BHK) cells at neutral pH have shown a pore structure forming between the virus and cell with viral RNA leaving the virus through this pore. The mechanism for the formation of this pore structure and how RNA travels through it is unknown. In the present study, we isolated vesicles derived from BHK cell membranes and exposed them to SV at neutral pH. By examining this interaction with electron microscopy (EM), we show that this pore structure forms between the heat resistant Sinbis virus strain (SVHR) and BHK membrane-derived vesicles

Section 2: Introduction

Sindbis Virus (SV) is an arthropod-borne (arbo-) virus in the alphavirus family. It is an enveloped virus and contains positive sense RNA and serves as an excellent model organism for studies of other alphaviruses such as chikungunya, as well as flaviviruses like dengue, yellow fever, and west Nile viruses (Brown and Hernandez 2012). The SV replication cycle has been studied for decades, but a consensus model for viral entry into host cells has not been developed, and current models for this process remain unconfirmed and controversial. The most popular model for SV entry suggests that the viral envelope fuses with the host cell membrane in a pH-dependent process similar to that of influenza virus entry (White, Kielian et al. 1983, Brown and Hernandez 2012). According to this model,

when a Sindbis virion encounters a potential host cell, its glycoproteins first bind to a receptor protein. The virion is then pulled into an endosome vesicle at the membrane. Next, the pH of the forming endosome is lowered by ATPases in the vesicle's membrane. Once the pH reaches a specific level, the virion is pulled into the cell by conformational changes induced by the pH change, and the nucleic acid is released from the nucleocapsid due to conformational changes (Brown and Hernandez 2012).

Much of the direct evidence for this entry model comes from experiments with SV and other alphaviruses that have questionable biological relevance. Experiments performed to confirm the fusion model have successfully induced fusion between SV virions and living cells or artificial liposomes, but under conditions unlikely to be found *in vivo* (Paredes, Ferreira et al. 2004, Brown and Hernandez 2012). Experimental data has found that to induce fusion between SV and living cells, the pH must be lowered to 5.8 and then returned to neutral pH, but the fusion model does not account for a pH increase (Paredes, Ferreira et al. 2004, Brown and Hernandez 2012). Also, while the fusion of SV to artificial liposomes can be accomplished without raising the pH, the fusion with liposomes requires the liposomes to contain cholesterol, which is not present in large quantities in mosquito cells, and these cells must be infected by SV to propagate the virus in nature (Hafer, Whittlesey et al. 2009).

Other data offer evidence that this model is incorrect. As early as 1978, a study found that SV-infected cells have much lower sensitivity to anti-viral antibodies than a virus known to enter host cells by this model. These investigators concluded that SV likely does not enter host cells by fusion (Fan and Sefton 1978). This conclusion was later supported by a 2004

study of the structure of SV at pH 7.2, then lowered to 5.3, and returned to 7.2 using cryo-electron microscopy. This study found that while the virion structure changed at the lower pH, it did not fully disassemble, which would be required for the fusion model to be correct. Also, electron micrographs of SV/BHK cell complexes showed no fusion, but SV virions appeared to be attached to the host cell by a “pore structure” between the virion and cell through which the viral nucleic acid enters the cell [Figure 2.1]. Also, some empty virions (identified by their lower electron density) were seen on the cell membrane. These data helped to develop a new model of entry for SV. It was hypothesized that after receptor binding, conformation changes in the virion form this pore to allow entry of the viral nucleic acid (Paredes, Ferreira et al. 2004). A later investigation studied the rate of SV RNA entry into BHK cells by measuring the full virion/empty virion ratio at different time points. This study found that the nucleic acid enters the cell at the membrane without fusion in a temperature dependent manner (Vancini, Wang et al. 2013). Fitting these data into an Arrhenius plot suggests that the RNA is not “injected” into the cell like some bacteriophages (Hu, Margolin et al. 2013), that is the virus particle does not force the RNA in; either it is pulled in by some structure in the cell or diffuses into the cell through the pore (Vancini, Hernandez et al. 2015).

The mechanism and energy driving this “pore-structure” model of SV entry is still unknown. To further investigate the interaction of SV with host cell membranes, in the present study, the interaction of SV with BHK membrane-derived vesicles was examined. These vesicles have none of the cell’s internal structures, but any membrane proteins from the BHK cells should remain. The heat resistant Sindbis virus strain (SVHR) was used in

this study because its stability and high titer should guarantee optimum infection efficiency (Renz and Brown 1976).

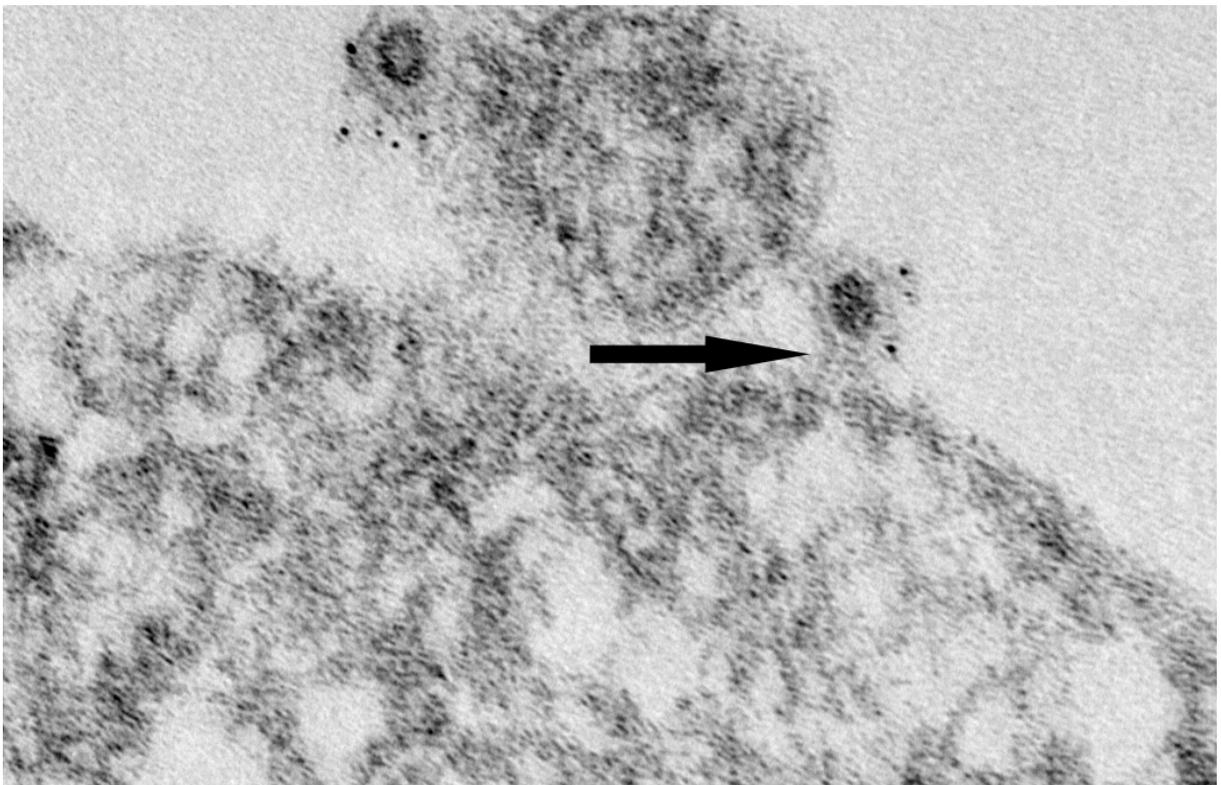


Figure 2.1: SV particle bound to BHK cell. Here a Sindbis virus virion is attached to a BHK cell via the “stalk-pore” structure. This structure is noted by the arrow.

Section 3: Results

After the BHK vesicles were isolated from the sucrose gradient, and their presence was confirmed under a light microscope [Figure 2.2], they were exposed to tartrate gradient-purified SVHR for 10 minutes at 25°C and neutral pH and then fixed for electron microscopy. The image obtained shows SVHR virions gathered around a vesicle [Figure 2.3] and between the vesicle and at least 2 virions, the “stalk” of the pore structure seen previously with whole cells (Paredes, Ferreira et al. 2004, Vancini, Wang et al. 2013) is clearly visible [Figure 2.2, insets 1 and 2]. Several other virions appear to be attached similarly, but the pore-stalk structure is less clearly defined due to the location of the virion relative to the EM section. From these observations we can conclude that SV can bind to vesicles in the same manner as to cells and that no internal cell structure or active cellular process is required for its formation. Also, all the visible virions--both attached and unattached to the vesicle--have about the same electron density. Because the previous studies showed an electron density reduction in virions that were attached to cells and low electron density “empty” particles floating away from the cell (Paredes, Ferreira et al. 2004, Vancini, Wang et al. 2013, Vancini, Hernandez et al. 2015), the similar electron density throughout suggests that viral RNA is not entering the vesicle. This is clearly visible in the virions in Figure 2.4.

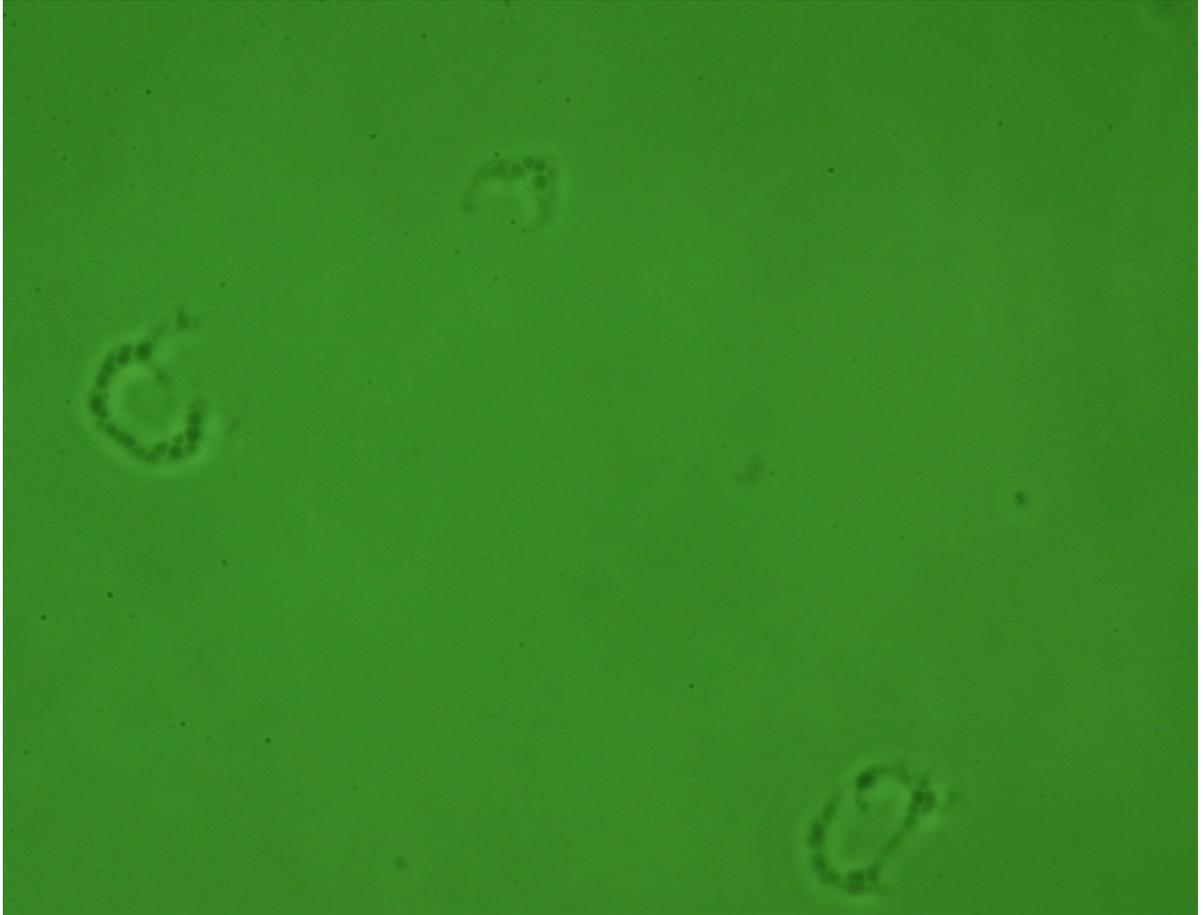


Figure 2.2: Purified BHK Membrane Vesicles. Under light microscope with 360 x magnification. Notice the variety of shapes and sizes of the vesicles.

Figure 2.3 (pages 18-19): Tartrate Gradient-purified SVHR and Sucrose Gradient-purified BHK Membrane Vesicle. The circled virions as well as some others appear to be attached to the membrane vesicle through the previously observed pore structure. See insets 1 and 2. Arrows indicate locations of insets.

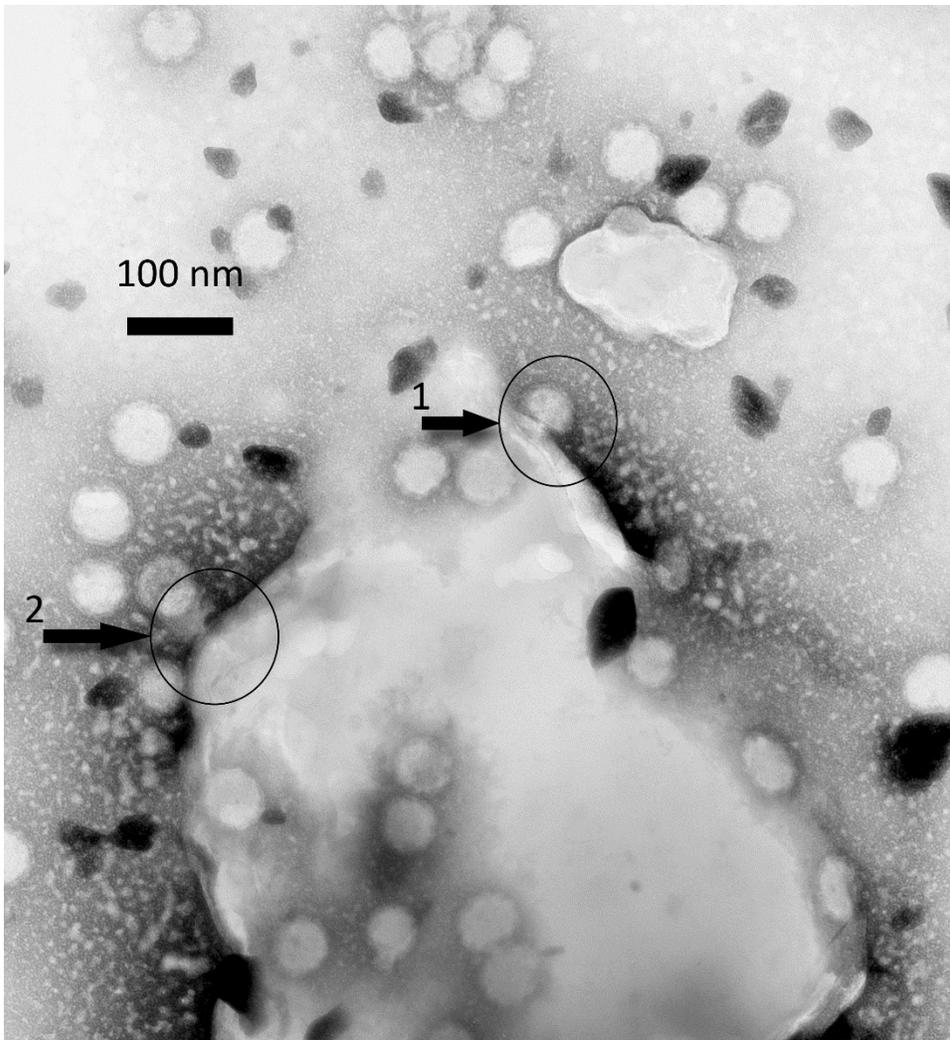


Figure 2.3 inset 1

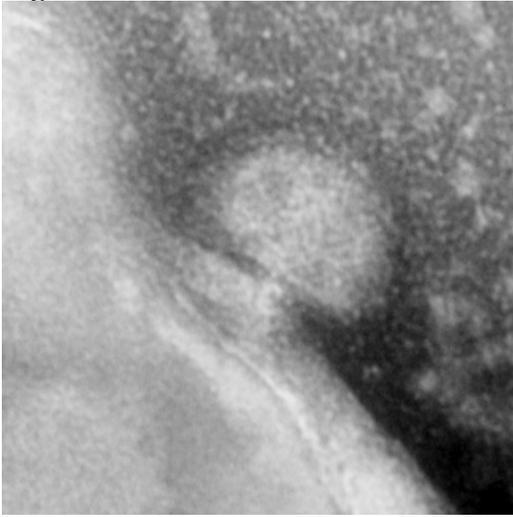
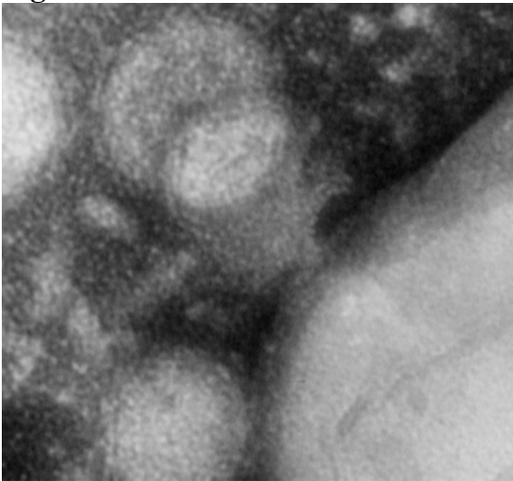


Figure 2.3 inset 2



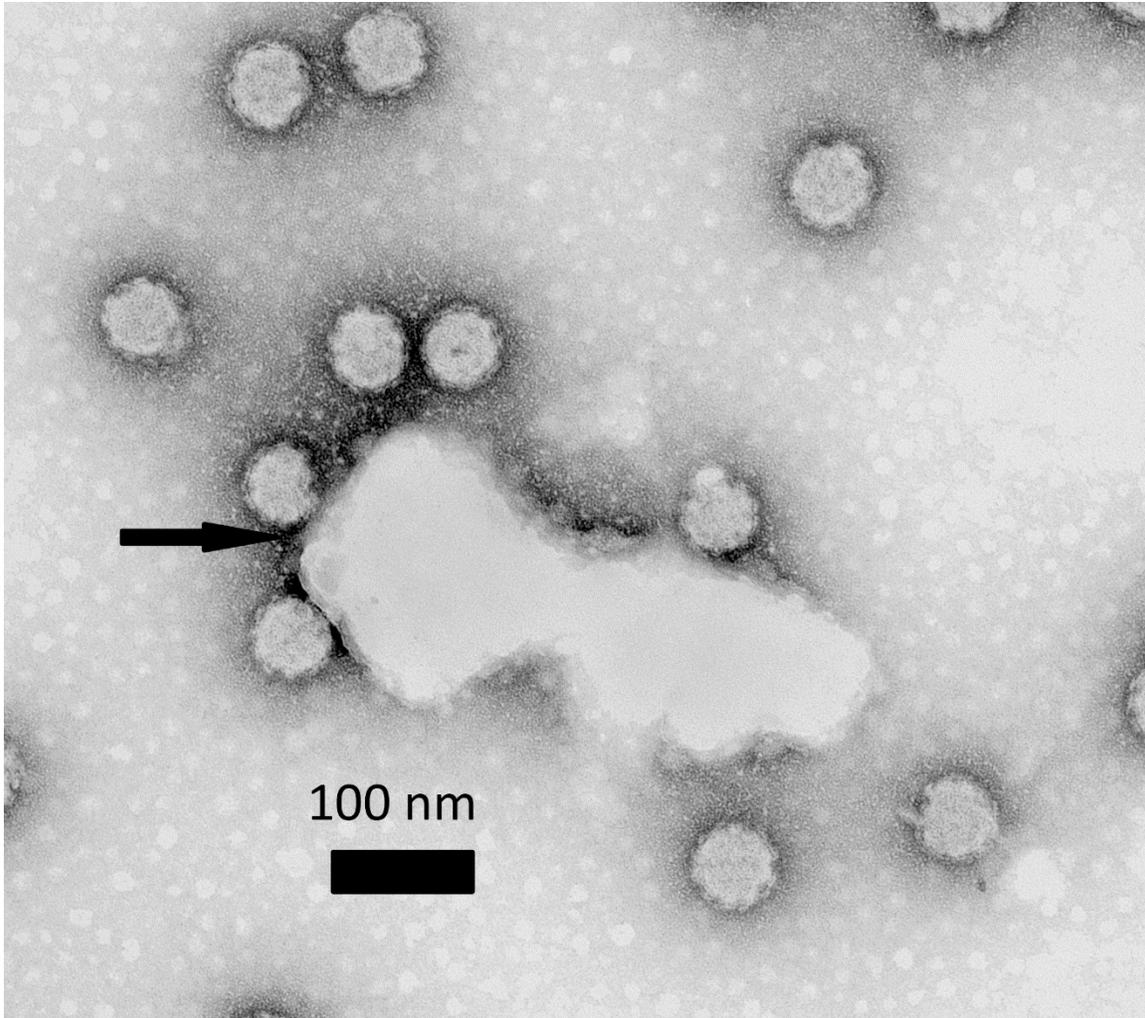


Figure 2.4: Another Purified Vesicle with Attached SVHR-Electron density view. Again virion appears to be attached to BHK membrane vesicle by a pore structure. Arrow points to possible “stalk-pore” structure. Notice that all virions, attached and unattached, appear to have the same electron density. This suggests that viral RNA has not left the virion and entered the cell.

Section 4: Discussion

From these observations, we acquire more evidence for the pore formation model of SV entry. Also, because we can see this pore structure forming between SVHR particles and BHK membrane-derived vesicles, we can conclude that the formation of this pore is not driven by an active process of the cell because membrane vesicles have little to no metabolism or means of maintaining homeostasis other than membrane proteins. The lack of “empty” SV virions suggests that RNA is not entering the vesicles. This is evidence that a cell structure is required to pull the viral RNA into its host. This hypothesis suggests SV genome entry may occur similarly to a hypothetical model of phage DNA entry into its hosts in which bacterial transcription factors are thought to pull the viral DNA into bacteria as they begin transcription (Molineux and Panja 2013).

To enhance these conclusions, this experiment can be repeated using other alphaviruses to determine how widespread this method of entry into host cells is. Because SV is an arbovirus, its replication cycle takes place in two very different groups of animals in nature and therefore, any SV observations should be examined in both mammalian and mosquito cells to prove their biological relevance. The next step in studying SV entry by pore should be to prove that SV nucleic acid does not enter the vesicles. If this can be established, cells with knocked out candidates for pulling SV RNA into cells could be developed and used to confirm this hypothetical aspect of this “pore” entry model.

CHAPTER 3

Single Site Glycoprotein Mutants Inhibit a Late Event in Sindbis

Virus Assembly

Section 1: Summary

A panel of Sindbis virus mutants that were suspected to have deficiencies in one or more aspects of their replication cycles was examined in Baby Hamster Kidney (BHK) cells. These included an amino acid deletion (Δ H230) and substitution (H230A) in the Sindbis glycoprotein E1_H230 and similar mutants in E2_G209 (G209A, G209D, and Δ G209). Neither H230 mutation produced measurable titer, but repeated passaging of the H230A in BHK cells produced a second site compensatory mutant (V231I) that partially rescued both H230 mutants. Electron micrograph (EM) images of these mutants showed assembled viral nucleocapsids but no completed, mature virions. Neither E2_G209 substitution mutant had any effect on virus production; however, the deletion mutant (Δ G209) showed a very low titer when grown at 37°C and no titer when grown at 28°C. When the deletion mutant grown at 28°C was examined by EM, partially budded virions were observed at the cell surface. S-35 labeling of this mutant confirmed the presence of mutant virus protein in the transfected BHK cell lysate. We conclude that H230 is essential for the assembly of complete infectious Sindbis virions and that the presence of an amino acid at E2 position 209 is required for complete budding of Sindbis virus particles although several different amino acids can be at this location without affecting titer.

Section 2: Introduction

Sindbis virus (SV) is an enveloped, positive sense RNA alphavirus that, while not a significant human pathogen, is a useful model for studying other members of the alphavirus genus, which includes, for example, chikungunya, Eastern Equine Encephalitis and Ross River Virus (Brown and Hernandez 2012). SV's relation to these important human and

animal pathogens, along with its ability to grow well in a laboratory setting, make it an excellent model organism for investigations into details of the lifecycles of these viruses, which can lead to strategies for controlling these virus-caused diseases. To be used effectively in this manner, the details of the SV lifecycle must be thoroughly understood so that knowledge acquired with SV can be applied to other arboviruses (Brown and Hernandez 2012).

The current model for SV assembly in mammalian cells proceeds as follows: After virus entry and uncoating, the four nonstructural proteins (nsp1-4) are translated as a polyprotein from the positive-sense RNA genome (Jose, Snyder et al. 2009). These consist of an RNA-dependent RNA polymerase (RdRp) and accessory proteins, which immediately transcribe minus-sense RNA from the initial positive-sense template (Jose, Snyder et al. 2009). Later on as the nonstructural proteins cleave fully, they synthesize full length positive sense RNA for the progeny virus and a subgenomic RNA molecule that is translated to synthesize the structural proteins for the progeny virus (Jose, Snyder et al. 2009). SV structural proteins are then translated as a single polyprotein consisting of N-terminus, capsid protein, PE2, 6K, E1, C-terminus. The capsid protein is then cleaved from the polyprotein by autoproteolysis. The remaining polyprotein is translocated into the membrane of the endoplasmic reticulum (ER), where signal peptidase removes the 6K protein, and the PE2 and E1 glycoproteins heterodimerize and then trimerize (West, Hernandez et al. 2006). The PE2 /E1 trimers of heterodimers enter the Golgi apparatus where furin protease cleaves PE2 into E2 and E3. The trimers of heterodimers are exported to the cell plasma membrane. While this occurs, the capsid protein packages the positive-sense RNA, and together they

travel to the plasma membrane as the nucleocapsid complex. Here, envelopment takes place, and the mature virions are released from the cell (West, Hernandez et al. 2006, Whitehurst, Willis et al. 2006).

The E1/E2 heterodimers have critical roles in the process of entry into host cells as well as in assembly. As glycoproteins, they have a role in recognition of host receptors, and they also likely facilitate the entry of the viral RNA into the host cell. Deficiencies in assembly of some SV and other alphaviruses with mutations in E1 and E2 confirm that they also have roles in assembly of virus particles (Hahn, Rice et al. 1989). Characterization of mutants with these deficiencies can help us understand what domains of the proteins are essential for its various functions.

The identification of the amino acid residues of alphavirus E1 and E2 proteins most essential for proper function can only be definitively accomplished by making substitution or deletion mutants, but finding conserved amino acid residues in sequences of various alphaviruses can help identify likely candidates. A previous series of studies identified the importance of a single conserved histidine residue in the E1 protein of the alphavirus Semliki Forest virus (SFV) (Chanel-Vos and Kielian 2004, Chanel-Vos and Kielian 2006). The investigators found that when this histidine was substituted with alanine, SFV particles were either not fully assembled when grown at 37°C or were assembled but were noninfectious when grown at 28°C (Chanel-Vos and Kielian 2004). They later identified several second-site revertant mutations that rescued the H230A mutant, but found no revertants to the wild type sequence (Chanel-Vos and Kielian 2006).

The histidine at position 230 in E1 is conserved in all alphavirus E1 proteins (Chanel-Vos and Kielian 2004, Roussel, Lescar et al. 2006). This residue is part of a short conserved sequence. It is preceded by isoleucine or valine and followed by valine [Table 3.1A]. Highly conserved residues in related proteins are often the most essential for proper protein structure and function, and therefore mutations of these residues have the most significant consequences (Engelman and Craigie 1992). Conserved histidine residues specifically are of additional interest due to histidine's relatively unique properties among amino acids and its many methods of interacting with other amino acid residues and other molecules (Liao, Du et al. 2013). These trends caused us to conclude that this location merited study in SV.

Another single amino acid location that is of interest in alphaviruses is at position 209 in E2. The residue at this location can vary significantly without effecting viral production in SV, but it is glycine in most alphavirus strains [Table 3.1B] [Protein BLAST sequence comparison] (Johnson, Zaretskaya et al. 2008). The identity of this specific residue has been shown to affect interaction with antibodies and virus neutralization (Stanley, Cooper et al. 1985, Lustig, Jackson et al. 1988, Mendoza, Stanley et al. 1988). These observations suggested that this domain merits more detailed examination by testing mutants at this site.

To examine the role of these sites in SV assembly, single site deletion and substitution mutants were made using site-directed mutagenesis in a plasmid containing the full length SV genome [Table 3.2]. After isolating mutant plasmids, positive-sense RNA was made and transfected into BHK cells. The plasmid containing the mutant SV genome also contained the gene for Venus yellow fluorescence protein, which served as a reporter. Twenty-four hours after transfection, cells that have taken up RNA from this plasmid appear

yellow-green when exposed to UV light. Studies of these mutants revealed several surprising properties with respect to assembly and release from host cell.

Table 3.1: Comparison of Relevant Glycoprotein Sequences in Various Alphavirus Species. Abbreviations are as follows: SV=Sindbis virus, Chik=Chikungunya virus, SFV=Semliki Forest virus, VEE=Venezuelan equine encephalitis virus, WEE=Western equine encephalitis virus, RRV=Ross River virus. Bold represents consensus sequence with amino acid of interest. All sequences were found in the NCBI BLAST database.

Virus	Relevant E2 Sequence (positions 199-219)	Relevant E1 Sequence (positions 220-240)
SV	YECKCGDYKT G TVSTRTEITG	RLLKPSAKN VH VPYTQASSGF
Chik	YKCNCGGSNE G LTTTDDKVINN	VLQRPAVGT VH VPYSQAPSGF
SFV	YNCTCGTGN V GTTNSDMTINT	KLARPSGM VH VPYTQTPSGF
VEE	VECECGGK I SETINKTKQFS	VLQRPKAGAI H VPYTCAPSGF
WEE	YECKCGDYST G IVSTRTKMNG	RLLKPSVKNI H VPYTQAVSGY
RRV	YNCTCGRDN V GTTSTDKTINT	KLSRPSGV VH VPYTPPSGF

Table 3.2: Relevant Sequences of RNA and Proteins of each Mutant. Bold represents the codon/amino acid residue of interest. Italicized represents the codon/amino acid residue changed in the revertant mutants.

Location	SV Strain	Relevant RNA sequence (5'→3')	Relevant Amino Acid Sequence (N-term→C-term)
E1 mutants	Wild Type-E1 H230	AACGUG CA UGUCCCG	Asn-Val- His -Val-Pro
	E1H230A	AACGUG GCG GUCCCG	Asn-Val- Ala -Val-Pro
	E1ΔH230	AACGUGGUCCCG	Asn-Val-Val-Pro
revertants	E1H230AV 231I	AACGUG GCGA UCCCG	Asn-Val- Ala - <i>Ile</i> -Pro
	E1ΔH230V 231I	AACGUGA U CCCG	Asn-Val- <i>Ile</i> -Pro
E2 mutants	Wild Type-E2 G209	AAGACC GA ACCGUU	Lys-Thr-Gly-Thr-Val
	E2G209A	AAGACC GCA ACCGUU	Lys-Thr-Ala-Thr-Val
	E2G209D	AAGACC GAC ACCGUU	Lys-Thr-Asp-Thr-Val
	E2ΔG209	AAGACCACCGUU	Lys-Thr-Thr-Val

Table 3.3: A: Titers of all mutant, wild type, and revertant SV strains transfected and infected in BHK at 37°C. B:Titers of all mutants and wild type transfected and infected in U4.4 at 28°C. C:Titers of mutants and wild type transfected and infected in BHK at 28°C. All titers in Plaque Forming Units (Pfu)/mL.

A.

SV Strain	BHK Transfection (37°C)	1st Infection	2nd Infection	3rd Infection	4th Infection	5th Infection
H230A	0	1.50E+04	1.62E+06	1.40E+07	1.00E+06	1.50E+07
ΔH230	0	0	0			
G209A	3.50E+07	1.25E+09				
G209D	9.50E+06	2.05E+09				
ΔG209	0	5.00E+00	0	0		
Y420	4.00E+08					
revertants						
H230AV2						
31I	3.50E+01	7.50E+04				
ΔH230V2						
31I	3.60E+02	3.55E+06				

B.

SV Strain	U4.4 Transfection (28°C)	1st Infection
H230A	0	0.00E+00
ΔH230	0	0.00E+00
G209A	6.00E+03	2.00E+03
G209D	3.00E+04	1.00E+04
ΔG209	1.00E+01	1.50E+01
Y420	1.10E+07	

C.

SV Strain	BHK Transfection (28° C)	1st infection
Y420	3.50E+07	
ΔG209	0.00E+00	0
H230A	0.00E+00	
ΔH230	0.00E+00	

Section 3: Results

Section 3.1-Summary of mutants made: Deletion and substitution mutants were made for each location of interest. The deletion mutants (Δ H230 and Δ G209) should show the effects of the absence of that amino acid residue at that location, but the deletion may have additional effects on the protein structure and function unrelated to the deletion itself. Substitution mutants (to alanine) will eliminate the risk of this variable; these mutants (H230A and G209A) should only show the effects of eliminating the side chain. However, because glycine has no side chain and is even smaller than alanine, the G209A mutant would not be very different from the wild type. Therefore, a second substitution mutant, G209D was made at that site. Aspartate is significantly larger than alanine, and while it may cause other unintended effects, studying both of these substitution mutants should allow for relevant conclusions to be made. Table 3.2 shows details of the mutant sequences.

Section 3.2-E1_H230 Mutants-BHK Transfection and Infection: First, the RNA of the H230A and Δ H230 mutants was transfected into BHK cells. The success of these transfections was confirmed after 24 hours by observation of the presence of green cells under UV light [Figure 3.1 A-B]. Media harvested from cells transfected by both mutants were titrated by plaque assay; however, no virus titer was observed. Next, the media from cells infected by both mutants was used to infect new monolayers of BHK cells. After 24 hours, some green cells were observed in the H230A mutant infection, but none were seen in the Δ H230 infection. The plaque assay still showed no titer for the Δ H230 mutant, but the H230A mutant showed a titer of 1.5×10^4 pfu/mL [Table 3.3A]. We concluded that the Δ H230 RNA did not produce infectious virus particles, but we were still unsure whether the

H230A RNA produced infectious particles or whether a compensatory mutation had occurred that restored infectivity.

To search for such a compensating mutation, the virus was passaged four more times in BHK cells with virus production measured after each infection. The titer increased to 1.62×10^6 pfu/mL after the second passage and to 1.4×10^7 after the third. The titer stayed within one order of magnitude for the next two passages [Table 3.3A]. This suggested to us that a stable compensatory mutation had occurred in the H230A viral stock, but it was unable to fully restore titer to that of the wild-type (WT) Y420 strain.

Section 3.3-E1_H230A Compensatory Mutant Identification: To confirm that this was a compensatory mutant, RNA was extracted from virus isolated from the media from the fifth passage of the H230A mutant, and RT-PCR was used to make cDNA for the SV E1 gene. Sequencing the cDNA showed that the substitution at position 230 was still present, but the next codon in the sequence showed one guanine substituted with adenine. This substitution changes the coded amino acid from valine to isoleucine. No other substitutions were seen.

In order to determine whether or not this substitution truly was a compensatory mutant, the V231I substitution was added to the H230A and Δ H230 plasmids by site-directed mutagenesis. RNA from these new plasmids was next transfected into BHK cells. After 24 hours, green cells were visible, and the virus media was harvested and titrated by plaque assay. The transfections' titers were 3.5×10^1 and 3.6×10^2 pfu/mL for the mutants H230AV231I and Δ H230V231I respectively. Because viral titers of transfections are often dependent on the efficiency of the transfection, the harvested virus was used to reinfect

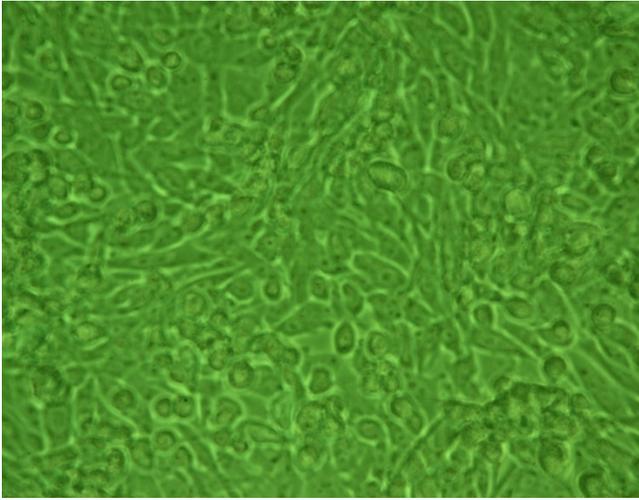
uninfected BHK cells. After 24 hours, the media of these infections was harvested and titrated. The measured titers for the infection were 7.5×10^4 pfu/mL and 3.55×10^6 pfu/mL for H230AV231I and Δ H230V231I [Table 3.3C]. From these data we can conclude that the V231I substitution is a second site revertant that can partially rescue both the H230 deletion and substitution.

Section 3.4-E2_G209 Mutants-BHK Transfection and Infection: RNA transcribed from the two substitution mutants (G209A and G209D) and the one deletion mutant (Δ G209) was transfected into BHK cells. As with the H230 mutants, green cells were observed 24 hours after transfection [Figure 3.1C], and the media was harvested and titrated by plaque assay. No titer was observed in the media from the Δ G209 transfection, but the G209A and G209D mutants showed titers of 3.5×10^7 and 9.5×10^6 pfu/mL respectively. BHK cells were infected with this media, and the media was harvested 24 hours later and titrated. This time both of the two substitution mutants showed titer of about 10^9 pfu/mL, which is about equal to the wild type titer [Table 3.2A]. These data led us to conclude that the specific amino acid residue at this position has little effect on titer, which is consistent with previous data showing that having either glycine or arginine at this location does not affect viral titer (Lustig, Jackson et al. 1988). On the other hand, the Δ G209 mutant showed a very low titer (5×10^0 pfu/mL) [Figure 3.1D], but a second and third passage with this media again showed no titer [Table 3.3A]. The presence of green cells in the plaques themselves ensured that these plaques were the result of infection and not simply errors in preparation of the assay [Figure 3.2A]. This led us to conclude that the Δ G209 mutant was

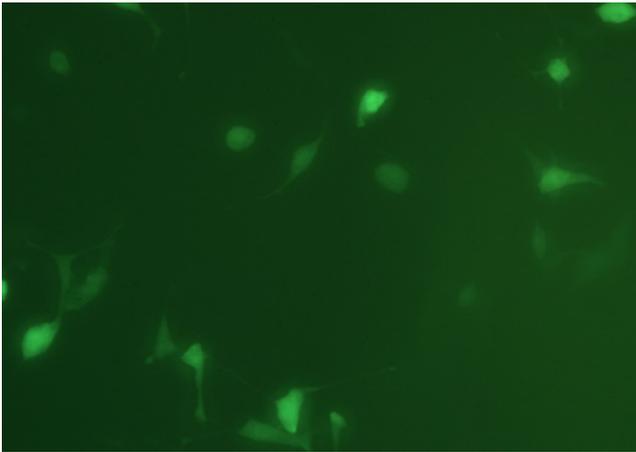
somewhat stable and very slightly infectious in BHK cells, but it produced only a very small number of infectious virus particles.

Figure 3.1 (pages 36-37): Images of transfected/infected cells expressing Venus YFP under UV light. **A.** H230A transfection in BHK with visible light. **B.** H230A transfection in BHK with UV. **C.** G209D infection in BHK with UV. **D.** Δ G209 infection in BHK with UV. **E.** Δ G209 infection in BHK with UV. All images were taken approximately 24 hours post transfection/infection under 160X magnification. All BHK images under visible light appeared similarly to image A. The visibility of the Venus YFP in the transfection images means the green cells have taken up and are translating the viral RNA. The visibility of venus YFP in the infection images means that these cells were infected by viruses containing the venus YFP gene. Note that the G209D infection has wild type titer and that the Δ G209 infections have very low titers.

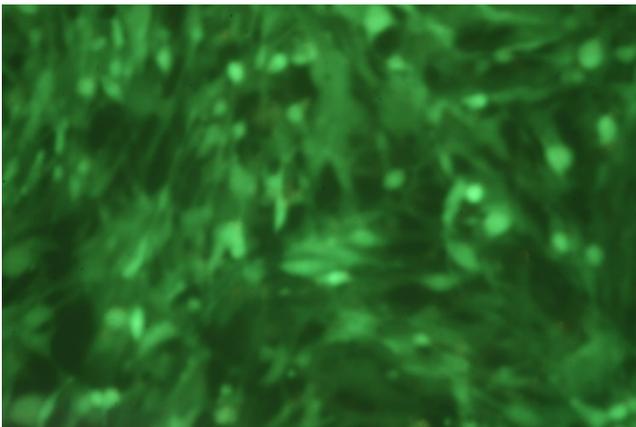
A.



B.



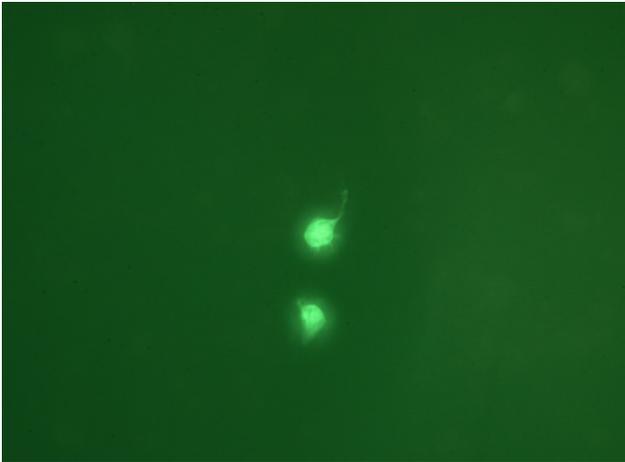
C.



D.



E.



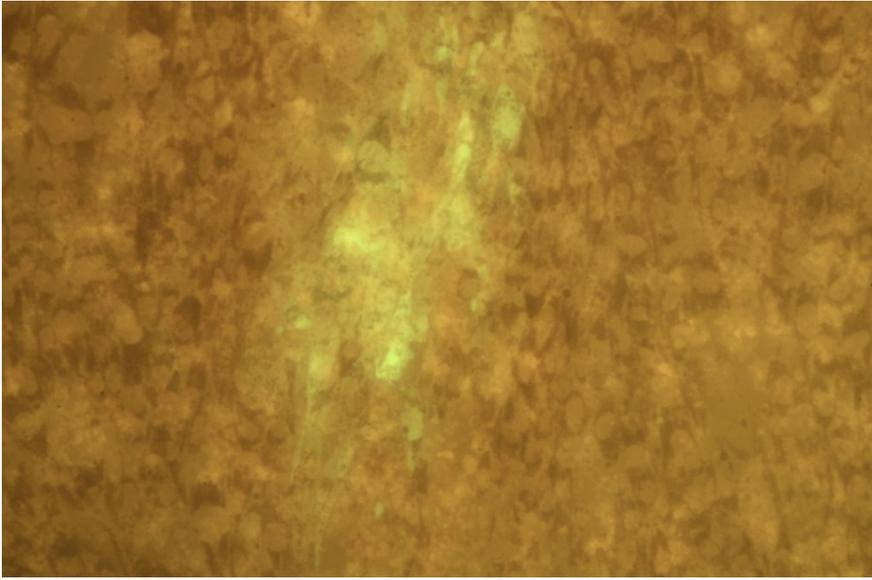
Section 3.5-E1_H230 mutants and E2_G209 mutants-U4.4 Transfection and

Infection: To study any effects these mutations have on host range, we repeated the above experiments using mosquito-derived U4.4 cells. U4.4 is an *Aedes albopictus* cell line that has been used to test host range properties of SV mutants (Whitehurst, Willis et al. 2006). Twenty-four hours after RNA of each of the H230 mutants was transfected into U4.4 cells, some cells appeared green. Titration of the media from these transfections showed no titer from either mutant. This media was used to reinfect new U4.4 cells. No green cells were observed, and titration of this media harvested after 24 hours again showed no titer [Table 3.3B]. Because the appearance of green cells means that the transfection was successful, we conclude that these mutant strains do not produce virus particles in U4.4 cells.

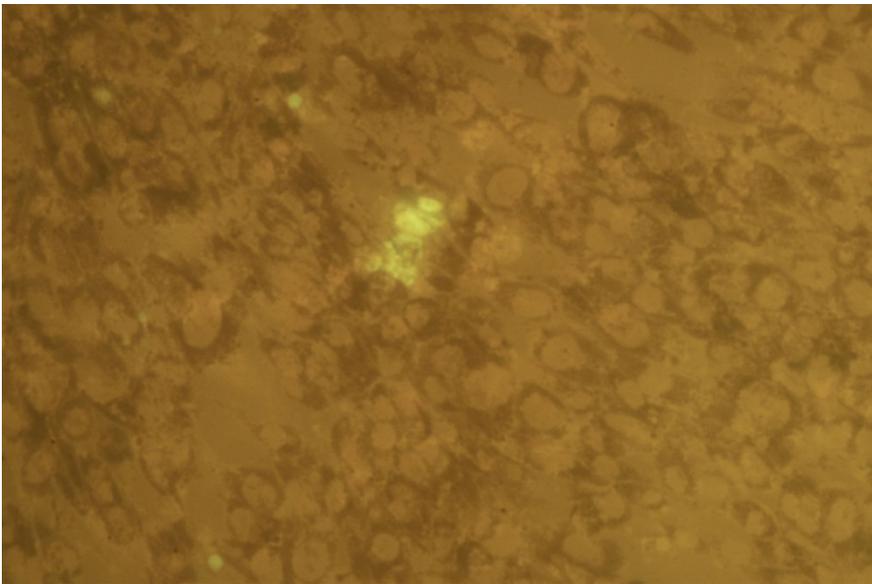
When the G209 substitution mutants' RNA was transfected into U4.4 cells and titrated, the measured titers were 6×10^3 and 3×10^4 pfu/mL for G209A and G209D respectively. After infecting new U4.4 cells and harvesting and titrating the media, we found that the titers were still in these orders of magnitude. This may signify some host range effects of these substitution mutants. When the Δ G209 mutant was transfected [Figure 3.1E], the measured titer was 1×10^1 pfu/mL, which is extremely low, just as it was in BHK cells. An infection with this mutant showed a similar titer, 1.5×10^1 pfu/mL [Table 3.3B]. Again, the presence of green cells in the plaque showed that the plaque was legitimate [Figure 3.2B]. This result suggests that there is some stability in the Δ G209 mutant and is very slightly infectious, and that there is little host range difference in these properties.

Figure 3.2 (page 40): Images of Δ G209 plaques in BHK cells under UV light. A. Plaque from virus originally grown in BHK cells. **B.** Plaque from virus originally grown in U4.4 cells. Both images were taken under 240X magnification approximately 48 hours after infection and 4 hours after overlay with 1% agar and 3% neutral red stain. The cells expressing the fluorescence protein have been infected by mutant virus. Notice plaque from BHK-grown virus is larger.

A.



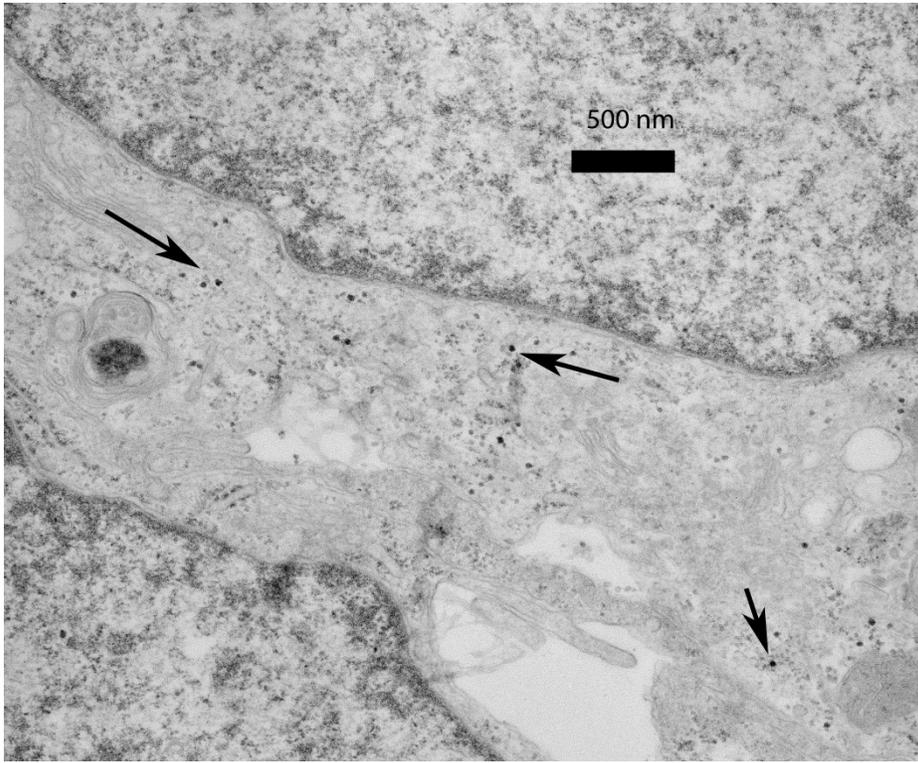
B.



Section 3.6: E1_H230 mutants and E2_ΔG209-Electron Microscopy: To further examine the deficiencies in these mutants, electron microscopy was used to view cells after transfection with these mutants' RNA. Images of cells infected with all three of these mutants showed assembled nucleocapsids in the cytoplasm, but no completed virus particles [Figures 3.3 and 3.4]. These images (along with the presence of green cells after transfection or infection) suggest that the low to nonexistent titers of these mutants are the result of viral assembly defects; that is, that viral structural proteins are synthesized from these mutants' RNA, but the proteins are unable to assemble mature particles. The second site revertant of the H230A mutant and the nonzero titer of the ΔG209 mutant could be explained by the presence of a very small number of functional virus particles produced that might not be visible in the EM images or possibly from a mutation that occurred during the *in vitro* transcription. Also the nucleocapsids in the cells are not associated with the ER or other membranes. This characteristic has been seen in other SV mutants with reduced titers, notably in a 1975 paper that compared an SV mutant with this property to another whose capsid proteins were membrane-associated (Brown and Smith 1975). This contrasts with the previously studied SFV H230A mutant that also showed assembly defects but showed nucleocapsids attached to the cell membrane (Chanel-Vos and Kielian 2004).

Figure 3.3 (pages 43-44): EM of BHK cells transfected with H230 Mutants. A. H230A transfection. Bar represents 500 nm. Arrows point to free capsid proteins in cytoplasm. **B.** Δ H230 transfection. Bar represents 200 nm. Arrows point to free capsid proteins in cytoplasm. The presence of free capsid protein in the cytoplasm along with the absence of completed virus particles means viral proteins are being expressed, but full assembly is not occurring.

A.



B.

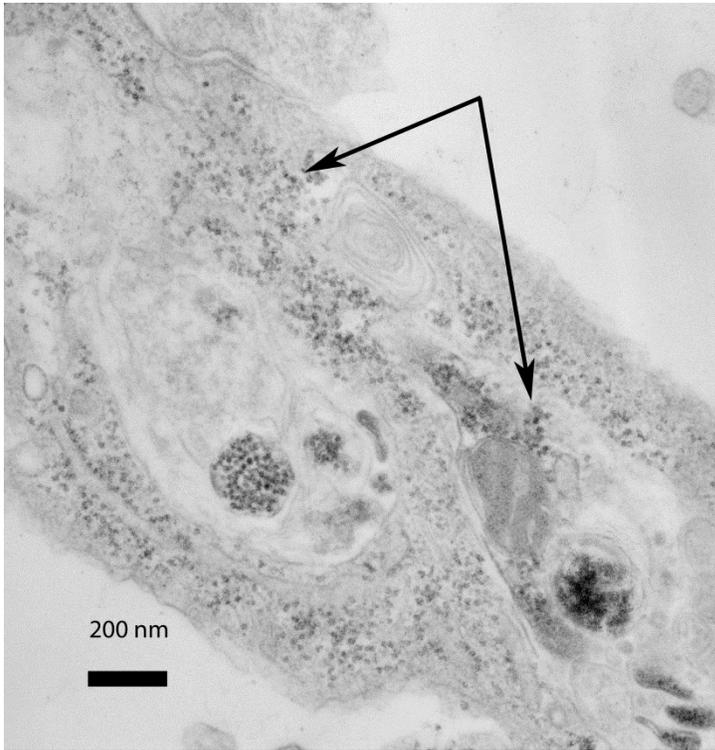




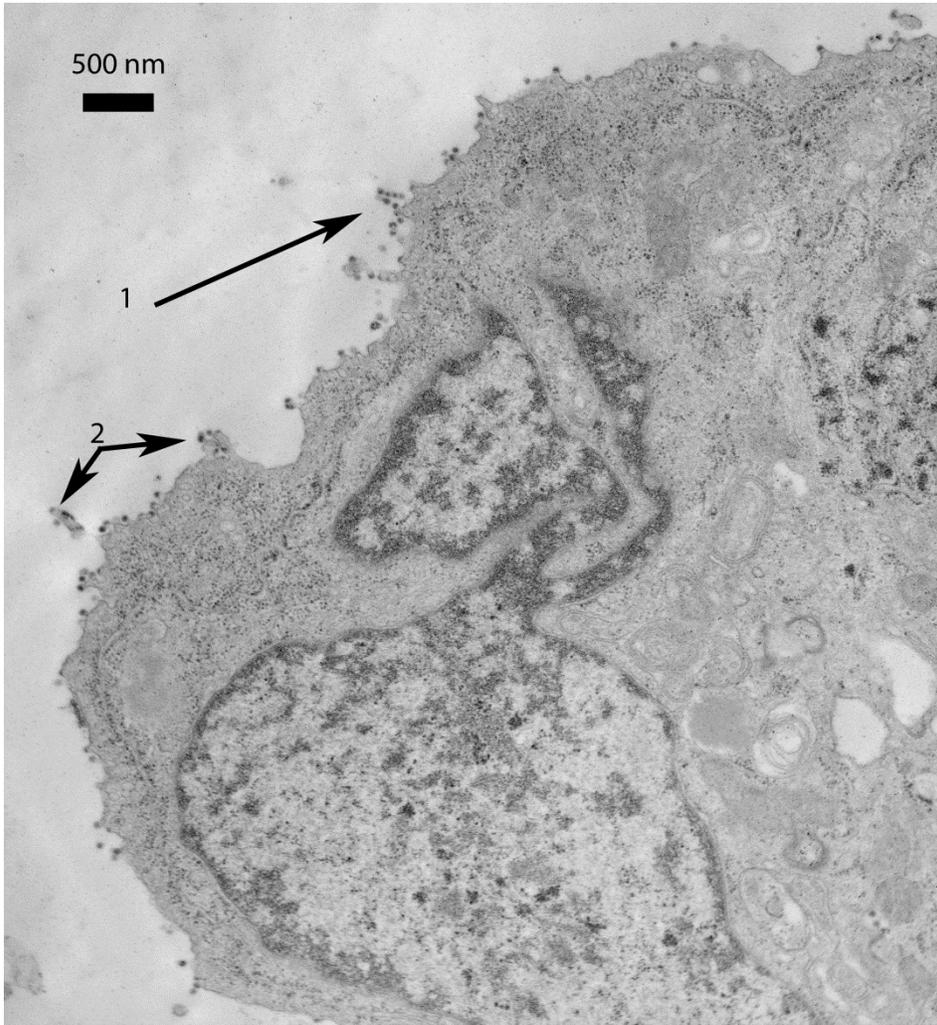
Figure 3.4: EM of BHK cells transfected with Δ G209 Mutant grown at 37 °C. Bar represents 200 nm. Arrow points to free capsid protein in cytoplasm.

Section 3.7-H230 mutants and Δ G209 at 28°C in BHK: Because the SFV study showed significantly different properties of the H230A mutant when it was grown in BHK at 28°C (Chanel-Vos and Kielian 2004), we repeated the transfection of the assembly mutants but grew the transfected cells overnight at 28°C. None of these transfections showed any titer, but the EM images of Δ G209 produced a surprising result. A large number of partially budded particles were seen at the surface of the cell [Figure 3.5A-B]. Also, some clumps and chains of virus particles were seen protruding from the cell. To the best of our knowledge, this phenotype has not been reported for any other alphavirus mutant. The wild type Y420 strain that was transfected as a positive control showed the normal budding phenotype as expected [Figure 3.5C]. Also, unlike in the SFV study (Chanel-Vos and Kielian 2004), no

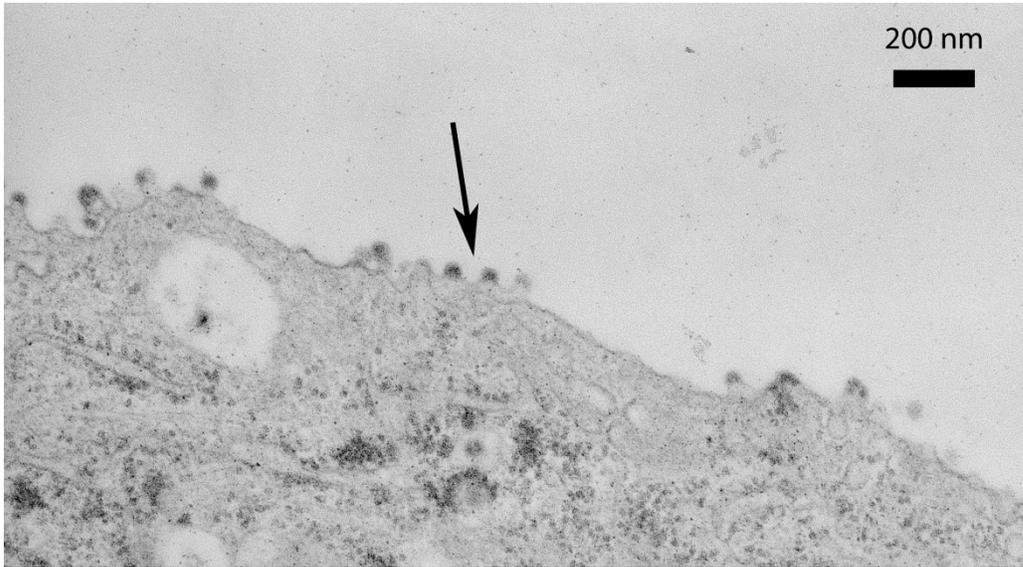
complete SV particles were seen in BHK cells transfected with the H230 mutants when grown at 28°C.

Figure 3.5 (pages 48-49): EM of BHK cells transfected with Δ G209 mutant and wild type grown at 28 °C. A. Δ G209 transfection. Bar represents 500 nm. Notice groups of partially budded virions on cell surface. Arrow 1 points to chains of virions coming off the membrane. Arrow 2 points to possible clumps of virions that detached from the cell. **B. Δ G209 transfection.** Bar represents 200 nm. Arrow points to higher magnification of arrested budding. **C. Y420 transfection.** Bar represents 200 nm. Arrow 1 points to virions in various stages of budding with multiple completed virions free from the host cell. Arrow 2 points membrane-associated capsid proteins.

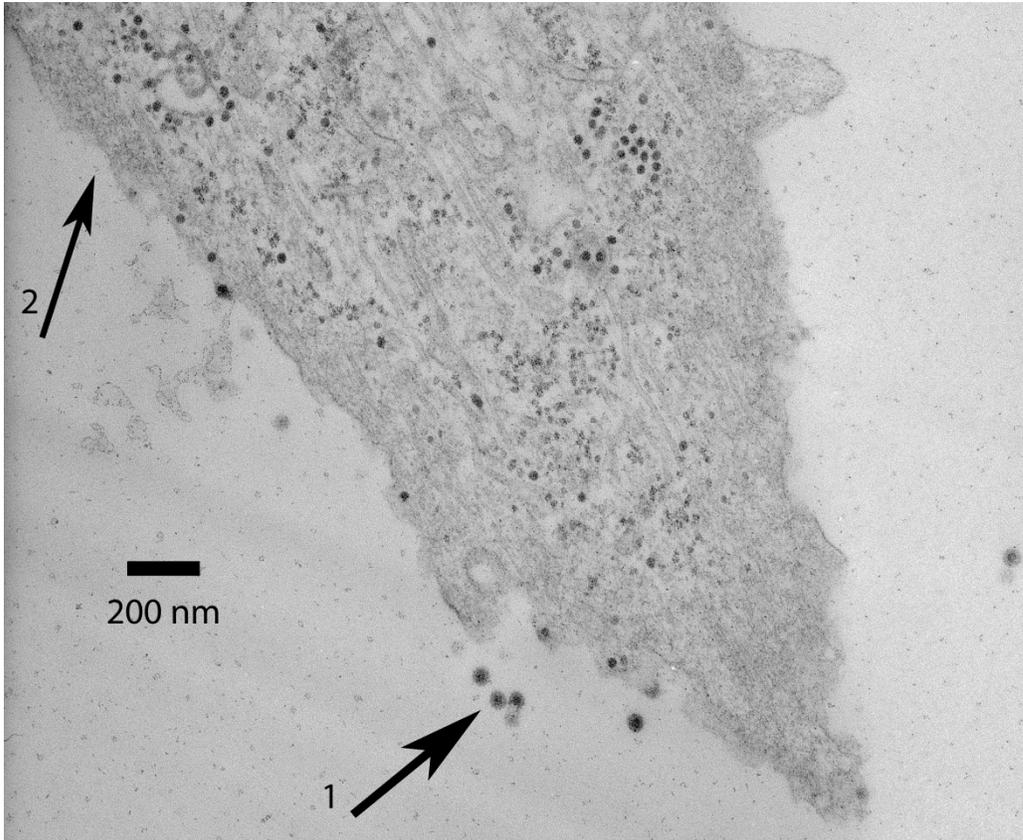
A.



B.



C.



Section 3.8: Δ G209 S35 metabolic labeling: To see if this arrested budding phenotype was caused by a difference in gene expression, we used S35-labeled methionine and cysteine to examine the proteins produced in cells transfected with Y420 and Δ G209 and grown at 28°C. Twenty-four hours after transfection (and 18 hours after labeling), when green cells were visible, the media was harvested, and virus particles in the media were purified by tartrate gradient ultracentrifugation. The transfected cells themselves were lysed, homogenized, and bound to polyclonal anti-Sindbis virus antibodies bound to sepharose beads to collect SV proteins. These purified virus proteins were run on an SDS-PAGE gel that was used to expose x-ray film to show only the labeled proteins. Radioactive Y420 viral proteins were seen in the media, but no Δ G209 proteins. This was consistent with the partial budding phenotype. The purified virus lysate showed many proteins, but the same proteins, including SV E1/E2 dimers, were seen in both the mutant and control [Figure 3.6]. The SV E1/E2 proteins form the darkest band in the lysate lanes. Therefore, we can conclude that the Δ G209 deletion does not affect protein synthesis and that the partial budding phenotype results from assembly defects.

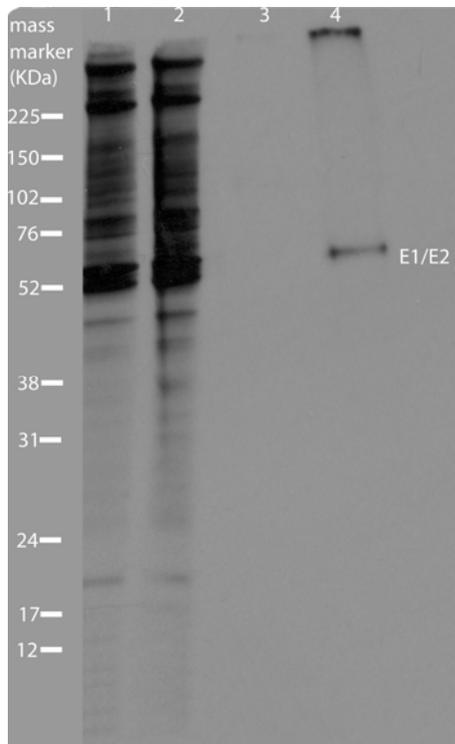


Figure 3.6: Image of x-ray film exposed to polyacrylamide gel with labeled mutant and Y420 run. Lane 1: Lysate of cells transfected with Δ G209 grown at 28° C. **Lane 2:** Lysate of cells transfected with Y420 grown at 28° C. **Lane 3:** Tartrate gradient purified media from cells transfected with Δ G209 grown at 28° C. **Lane 4:** Tartrate gradient purified media from cells transfected with Y420 grown at 28° C. Notice the presence of same proteins in mutant and wild type cell lysate in lanes 1 and 2 and the presence of E1/E2 protein complexes in the lysate and WT media. The presence of E1/E2 proteins in only the media of the wild type (lane 4) is consistent with the EM observations that Δ G209 virus particles are not released from the cell membrane at 28°C.

Section 4: Discussion

The single conserved histidine at position 230 in the alphavirus E1 protein clearly plays an essential role in SV assembly because removing this residue either via substitution or deletion is detrimental to the production of completed virus particles. The removal of this histidine may still allow for a few infectious particles to be produced, hence the presence of the revertant, but this cannot be confirmed because the mutation could also have occurred

due to an error during sp6 transcription. Histidine's unique properties (its aromaticity, ionizability, ability to bind to metal cations etc.) allow it to play many roles in protein interactions (Liao, Du et al. 2013), but we show that the titer of these virus mutants can be partially rescued by the replacement of the next valine with the very similar amino acid isoleucine. Isoleucine shares few of histidine's properties, so this suggests that the importance of histidine specifically at this location is limited; however, histidine may still have some importance due to the fact that WT titer was not fully restored by this mutant. Histidine's limited importance was also suggested by the previously reported data that show that many different second-site revertants can rescue the H230A mutant in SFV (Chanel-Vos and Kielian 2006). Further experiments such as replacing histidine with a panel of different amino acids could provide confirmation of this hypothesis and could lead to more information regarding the biochemical cause of this phenotype.

The role of the glycine at position 209 in SV E2 is less clear. Again, the importance of glycine specifically at this location appears to be limited, due to our observations and those of previous studies (Lustig, Jackson et al. 1988, Mendoza, Stanley et al. 1988) that allow for a variety of amino acid residues there, but the residue at this position clearly plays an essential role in assembly because deleting it produces few infectious particles (none visible in EM at 37°C, but a small number of plaques). The G209 deletion mutant grown in BHK cells at 28°C showed very unusual properties. The EM images show partially budded particles and chains and clumps of virus particles; a phenotype that has never been seen before in alphaviruses. However, a similar phenotype has been observed in mutants of HIV (Gottlinger, Dorfman et al. 1991, Huang, Orenstein et al. 1995) and in HIV-infected cells

with a host protein silenced (Garrus, von Schwedler et al. 2001). In the HIV mutant, it was hypothesized that the presence of HIV surface proteins in the cell membrane is sufficient to cause bud-like structures on the cell membrane (Garrus, von Schwedler et al. 2001). This is less likely to be the cause of this arrested budding phenotype in the SV mutant because a previous study of an SV assembly mutant that had E1/E2 dimers present in the host cell membrane did not show partial budding (Li, Liao et al. 1999). Likewise, the previously cited 1975 SV study reported experiments that suggested the presence of SV proteins in the cell membranes, but no partial budding was observed (Brown and Smith 1975).

Previous freeze-etching studies of SV budding led to a stepwise model of budding (Brown, Waite et al. 1972) in which SV nucleocapsid approaches the host cell membrane and binds to it. Next, the membrane containing the nucleocapsid bulges from the cell. Then the membrane under the nucleocapsid pinches off to form a stalk under the emerging virion. Finally the membrane seals the stalk structure releasing the virus particle. We observe that when grown at 28°C the G209 deletion mutant appears to reach the stalk stage in budding and then is arrested at this step. S-35 labeling tells us that arrested budding is not due to a change in protein synthesis, so this suggests an additional property of the glycoproteins in SV assembly. The E2 glycoprotein may be involved in unknown protein-protein interactions that drive the separation of the virion from the membrane. At 28°C, this interaction in the Δ G209 mutant may be altered to the point where the separation of the membrane step cannot be completed. Future studies involving other substitutions at this location, other temperatures, and other alphaviruses can determine more details about this property.

CHAPTER 4

Materials and Methods

Section 1: Chapter 2

Section 1.1-Growth and Propagation of BHK cells and SVHR: BHK cells were grown and maintained as described previously (Hernandez, Sinodis et al. 2010). SVHR was grown and propagated as described in the same source (Hernandez, Sinodis et al. 2010).

Section 1.2-Tartrate Gradient Purification of SVHR: Ten flasks of BHK cells at approximately 90% confluency were infected with 500 μ L SVHR in TM Diluent (Hernandez, Sinodis et al. 2010) with a titer of approximately 1×10^9 pfu/mL for 1 hour at room temperature with rocking. 5 mL BHK media was then added, and the infected cells were allowed to grow at 37°C overnight. The next day, the media from these flasks was harvested as before (Hernandez, Sinodis et al. 2010), and then run on a 15%-35% tartrate step gradient overnight as before (Nelson, Hernandez et al. 2005). The next morning the virus band was pulled and run on a continuous 15%-35% tartrate gradient for 3.5 hours at 26,000 rpm. The light blue virus band pulled at the end of this run was used for these experiments.

Section 1.3-Preparation of BHK Cell Membrane-Derived Vesicles: The BHK membrane-derived vesicles were isolated in a manner derived from a previous procedure (Smith and Brown 1977). One flask of BHK cells at approximately 95% confluency was washed with 5 mL PBS-D buffer [30mM KCl, 15.2 mM KH₂PO₄, 80 mM Na₂HPO₄, 140 mM NaCl]. This buffer was removed and then 5 mL lifting buffer [10 mM HEPES-KOH pH 7.2, 15mM KCl, 2mM EDTA, 10 mM MOPS] was added to remove the cells from the flask. The lifting buffer containing the BHK cells was poured off from the flask and spun down for five minutes at mid speed in a bench-top centrifuge. The lifting buffer supernatant was

poured off and the BHK cell pellet was resuspended in 5 mL hypotonic homogenization buffer [.001 M tris pH 7.0, 5% sucrose, .001 M magnesium acetate] to swell cells. The homogenization buffer/BHK cell suspension was transferred to a 15 mL dounce homogenizer. The pestle was slowly brought down 10 times. Next the homogenized cell suspension was spun down in a Sorvall centrifuge for 5 minutes at 2000 rpm to remove nuclei and cell debris. The supernatant from this mixture was then run through 7 mL of a 20% sucrose in PBS-D gradient onto 6 mL of a 60% sucrose in PBS-D cushion overnight at 20000 rpm. This centrifugation was performed at room temperature to prevent excessive viscosity of the sucrose solutions.

The next day the membrane vesicles were recovered from the 20%/60% interface. Sucrose was added to the recovered membrane vesicles to make the solution 70% sucrose (measured by refractometer). The vesicles were run up through a 3 mL 60% sucrose gradient onto 9 mL 30% sucrose cushion at 20,000 rpm overnight at room temperature. The next day the membrane vesicles were recovered from the 30%/60% interface and used for the experiments.

Section 1.4-Infection of Vesicles with SVHR: 500 μ L of the recovered BHK membrane-derived vesicle suspension was mixed with 500 μ L tartrate gradient-purified SVHR. This mixture was incubated at room temperature for 10 minutes before it was fixed for electron microscopy.

Section 1.5-Immuno-EM: Equivalent monolayers of BHK cells were grown in 6-well culture plates at 37°C. Purified Sindbis virus was attached to cell monolayers at 4°C at neutral pH for 30 minutes while rocking, cells were washed with cold buffer to remove

unattached virus and then the virus-cell complex was immediately fixed with 3% paraformaldehyde for electron microscopy for 30 minutes. The virus-cell complexes were subsequently labeled with immuno-gold using an anti-Sindbis serum and gold (6nm) conjugated anti-rabbit IgG antibody, before proceeding to electron microscopy processing.

Section 1.6-Thin-sections: BHK infected cell monolayers were fixed with 3% paraformaldehyde in 0.1 M cacodylic acid buffer, pH 7.4 (Ladd Research Industries, Williston, VT). The cells were washed three times with 0.1 M cacodylic acid buffer and stained by 1% osmium tetroxide in cacodylic buffer for 1 hour. Cells were then washed as before and embedded in 2% agarose. The agarose containing the cell sample was then pre-stained with 2% uranyl acetate (Polaron Instruments Inc, Hatfield, PA) overnight at 4°C. The samples were washed and carried through ethanol dehydration. Infiltration was done using SPURR compound (LADD Research Industries). Blocks were then sectioned on a Leica UCT6 (Leica Microsystems, Inc. Deerfield, IL). Ultrathin sections were stained with 4% uranyl acetate in distilled water for 60 minutes and in Reynolds lead citrate, pH 12 (Mallinkrodt Baker Inc. Paris, KY) for 4 minutes. The samples were examined at 80kV in a JEOL 1210 transmission electron microscope.

Section 1.7-Negative Contrast Staining: Purified virus-vesicle complexes were attached to carbon-coated copper grids for 2 minutes, washed 3 times with distilled water and briefly stained with 2% uranyl acetate before viewing on a JEOL 1210 transmission electron microscope at 80kV.

Section 2: Chapter 3

Section 2.1-Site-Directed Mutagenesis: The plasmid containing the template for all mutants (pTE3'2J) was described previously (Vancini, Wang et al. 2013). Site-directed mutagenesis was performed using the following pairs of DNA primers (from 5' to 3'):

E1_H230A-f: CAAGAACGTGGCGGTCCCGTACACG and E1_H230A-r:

CGTGTACGGGACCGCCACGTTCTTG, melting point (MP)-65.4°C; E1_ΔH230-f:

CAAGAACGTGGTCCCGTACACG and E1_ΔH230-r:

CGTGTACGGGACCGCCACGTTCTTG, MP-60.1 °C; E2_G209A-f:

CGACTACAAGACCGCAACCGTTTCG and E2_G209A-r:

CGAAACGGTTGCGGTCTTG TAGTCG, MP-61.8 °C ; E2_G209D-f:

CGACTACAAGACCGACACCGTTTCG and E2_G209D-r:

CGAAACGGTGTCGGTCTTG TAGTCG, MP-61.2 °C; E2_ΔG209-f:

CGGCGACTACAAGACCGTTTCG and E2_ΔG209-r:

CGAAACGGTGGTCTTG TAGTCGCCG MP-63.4 °C.

For compensatory mutant site-directed mutagenesis, the primer pairs were as follows:

E1_H230AV231I-f: GTGGCGATCCCGTACACGCAGG and E1_H230AV231I-r:

CCTGCGTGTACGGGATCGCCAC, MP-64.6 °C; E1_ΔH230V231I-f:

CGTGATCCCGTACACGCAGGCC and E1_ΔH230V231I-r:

GGCCTGCGTGTACGGGATCACG, MP-64.6 °C.

Mutagenesis began with PCR using *PfuUltra* High Fidelity DNA polymerase. The PCR protocol began with the manufacturer's instruction manual but through

experimentation, the following protocol was developed. The PCR mixture for all reactions consisted of 100 ng template DNA, 1.5X concentrated PfuUltra Buffer, 250 ng of each primer, and 800 μ mol each dNTPs mixed all diluted to 25 μ L with dH₂O with 2.5 U PfuUltra enzyme added. The thermocycler program consisted of 2 minutes of melting at 95°C followed by 30 cycles of 30 seconds melting at 95°C, 30 seconds annealing at 5 degrees less than the primer melting temperature and 29 minutes elongation (2 minutes per kilobase in template) at 68°C. Following the reactions, 1 μ L DpnI was added to each mixture and allowed to digest the template at 37°C for 2 hours.

Section 2.2-Large DNA Prep and Transcription: Following template digestion, 2 μ L of each mutant plasmid PCR product was transformed into DH5 α one-shot cells by heat shocking at 42°C for 40 seconds. Following 1 hour recovery by shaking at 37°C with 750 μ L SOC added, 350 μ L of this mixture was plated on LB-Agar plates containing 85 μ g/mL carbenicillin (carb.) and grown overnight at 37°C. The next day, individual colonies were grown into 10 mL overnight cultures with LB containing carb. at 37°C. This large culture was used to inoculate 1 liter LB with carb. This culture was allowed to grow to an optical density at 600 nm of about .6. At this point 4 mL of 34 mg/mL chloramphenicol in ethanol was added to amplify the plasmids. The next day alkaline lysis and CsCl/Ethidium bromide (EtBr) gradient centrifugation were performed in a manner similar to that of a standard protocol (Heilig, Elbing et al. 2001), to harvest, clean and recover the plasmid.

After ultracentrifugation, the EtBr was removed from the plasmid solution by adding 5 mL of a solution of NaCl saturated in isopropanol. This mixture was vortexed for 30 seconds, and the organic layer containing EtBr was aspirated off. This was repeated until no

pink/orange color was seen in the organic layer. After this, the aqueous layer was diluted with an equal volume dH₂O, and 6 volumes cold 100% ethanol was added to precipitate out the plasmid. The mixture was stored at -20 °C overnight.

The precipitated DNA was recovered by centrifugation at 10,000 rpm for 30 minutes and then washed with 70% ethanol. The pellet was resuspended in 1 mL TE buffer. The DNA was cleaned up by treatment with 15 µL RNase Cocktail overnight at room temperature. Next enough proteinase K was added to make the concentration 50µg/mL along with 1/10 volume 10x proteinase K buffer (.1 molar Tris pH 7.8, .05 molar EDTA, 5% SDS). This was incubated for 2 hours at 37°C.

Once plasmids containing each mutant were obtained, they were purified by phenol extraction, resuspended in 400 µL TE buffer and linearized by XhoI treatment. 50 µL of each plasmid was mixed with 7.5 µL NEB buffer 2, 1 µL BSA, 2 µL XhoI stock, and 14.5 µL dH₂O. The mixtures were digested at 37°C overnight. The linearized plasmids were again phenol extracted and resuspended in depcH₂O.

Next RNA transcripts were made from each plasmid. Approximately 290 ng of each linearized plasmid was added to 2 µL 10x RNA polymerase buffer, 2 µL NTP mixture with 5' cap analogue (10mM ATP, CTP, UTP; 5mM GTP; 20 mM 5' cap analogue), .5 µL DTT, 1 µL RNaseI, enough depc H₂O to raise the final volume to 20 µL, and 2 µL Sp6 RNA polymerase. This mixture was reacted at 40°C for 1 hour. The success of each transcription reaction was evaluated by running 2 µL reaction products on a 1% agarose gel. To complete preparation for transfection, the template was digested by adding 2 µL DNase I and incubating at 37°C for one hour.

Section 2.3-BHK and U4.4 Transfection & Infection and Titration by plaque

assay: BHK and U4.4 cells were obtained, grown, and maintained as described previously (Hernandez, Sinodis et al. 2010). The transfection process was performed by electroporation as described previously (Hernandez, Sinodis et al. 2010), but the cells were pulsed twice with the voltage reduced to .75 KV for BHK and 1 KV for U4.4.

All BHK and U4.4 infections were performed as described previously (Hernandez, Sinodis et al. 2010).

All plaque assays were performed in BHK cells as described previously (Hernandez, Sinodis et al. 2010).

For the BHK transfections at 28°C, the transfections were performed as above, but after transfection, the cells were allowed to recover at 37°C for 1 hour and then grown overnight at 28°C.

Twenty-four hours after all transfections, their success was evaluated by observation of green cells under UV light.

Section 2.4-RNA Extraction/RT-PCR & Sequencing: To determine the presence of any compensatory mutants or revertants in the serially passaged E1H230A virus, viral RNA was extracted, and cDNA was made via RT-PCR. 5 mL of virus-containing media was centrifuged for 1 hour at 50000 rpm in a Beckman Sw55Ti rotor. The supernatant was poured off and the pellet was resuspended in 100 µL 2x concentrated viral lysis buffer (100mM Tris-Cl pH 7.0, 20 mM EDTA, 1% SDS) and 100 µL TE. This was incubated for 20 minutes at 37°C with occasional vortexing. The contents were transferred to an RNase-free 1.5 mL screw cap microfuge tube and mixed with 250 µL Phenol at 60°C. This was

vortexed for 30 seconds and centrifuged for 2.5 minutes at 15000 rpm in a tabletop centrifuge. The aqueous layer was transferred to a new tube, and the remaining organic layer was back extracted with 100 μ L TE buffer. The extracted aqueous layer was mixed with 350 μ L fresh phenol and the previous steps were repeated. The aqueous layer from the second extraction was mixed with 500 μ L 1:1 phenol: chloroform/isoamyl alcohol, and the extraction was repeated. Next the aqueous layer was added to 500 μ L chloroform and was vortexed and centrifuged 1 last time. This final aqueous layer was mixed with 1/10 volume 3M sodium acetate pH 5.0 and 3 volumes ice cold 100% ethanol, and the RNA was precipitated out overnight at -80°C.

The next day the RNA was recovered, washed with 70% ethanol and resuspended in 20 μ L depc H₂O. 10 μ L of this sample was added to 4 μ L MgCl₂, 2 μ L 10x PCR buffer, 1 μ L RNase I, 2 μ L dNTP mixture, and 1 μ L sin E111138R primer (5'-GTGCTAAAGTGTACTGTCACC-3'). This mixture was heated to 99°C for five minutes, allowed to cool, and 1 μ L Mulv reverse transcriptase was added. This mixture was reacted at 42°C for 35 minutes followed by 5 minutes enzyme deactivation at 99°C.

The products of this RT reactions were added to 8 μ L MgCl₂, 10 μ L Taq buffer, 60 μ L depc H₂O, 2 μ L dNTP mixture, 1 μ L sin E111138R primer, 1 μ L sin E10708F primer (5'-CCAGCACAGACATTAGGCTAC-3'), and 1 μ L amplitaq polymerase. This mixture was reacted in a thermocycler with the following program: 30 cycles of 1 minute at 95 °C, 2 minutes at 60°C, and 2 minutes at 72°C after which the reaction finished with 8 minutes at 72°C. After the success of the RT-PCR reactions was evaluated by running 5 μ L on a 4% agarose gel, the products were sequenced by Eurofins.

Section 2.5-Electron Microscopy: All microscopy was performed as described previously (Vancini, Wang et al. 2013).

Section 2.6-Metabolic Labeling: The S-35 methionine and cysteine labeling of the E2ΔG209 was performed mostly as described previously (West, Hernandez et al. 2006), but cells were grown at 37°C for one hour and at 28°C for 5.5 hours before the labeling process began.

CHAPTER 5

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