ABSTRACT

WEST, JOHN ALLEN. Investigation of the Role of the E2 Endodomain in Sindbis Virus Assembly. (Under the direction of Dr. Dennis T. Brown)

Sindbis virus (SV) is the prototype member of the alphavirus genus belonging to the family Togaviridae. SV is a mosquito-borne virus that can be transmitted to both humans and other animals via mosquito bloodmeal. Structurally, SV is composed of three proteins; two envelope glycoproteins (E1 and E2) and capsid protein (C). The outer shell (envelope) of the virus particle is composed of E1 and E2 proteins and is stabilized by E1-E1 protein interactions. Between the outer shell and the inner shell (nucleocapsid), which is composed of capsid protein and the viral RNA, is a host derived membrane bilayer. Connecting the outer shell to the nucleocapsid core is a critical step in virus maturation without which a mature virus particle could not be formed. The proteins involved in this interaction are E2 (specifically a 33 amino acid cytoplasmic domain) and the capsid protein. The research presented here provides new information on this interaction and sheds light on the role of two specific regions within the E2 endodomain. We have determined that two residues within the conserved TPY domain (aa 398-400) in the E2 endodomain are dispensable for virus assembly, however they are critical for the production of infectious virus. Additionally, a domain encompassing amino acids 409-417 in the E2 endodomain has been determined to be critical for both virus assembly and function. These observations identify two specific regions in the E2 endodomain that play different roles in virus assembly. The conserved TPY domain is critical for infectivity, while the domain from amino acids 409-417 is essential for virus assembly. The identification of amino acids 409-417 as a critical domain.
for nucleocapsid binding is an important step in understanding how these viruses assemble.

In the future this domain could be a target for vaccine development.
Investigation of the Role of the E2 Endodomain in Sindbis Virus Assembly

By

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Approved by:

_________________________ _________________________
Co-chair of Advisory Committee    Chair of Advisory Committee
DEDICATION

First, I would like to dedicate this to my fiancée Shelly. Thank you for being there every time that I got stressed out or thought that I might never get through everything. You are my light at the end of the tunnel and you never gave up on me and always supported me no matter what. I love you and thank you very much.

I would also like to dedicate this to my family; Mom, Dad, and my sister. Thanks, Mom and Dad, for giving me the tools to succeed in life and supporting me when I needed it. Thank you also for believing in me those times that I did not believe in myself. It has been a rough few years and not everything has gone as planned but we are all still here and I appreciate everything that both of you have done for me.

To my sister, I would like to say that if I can get through this then you can also accomplish whatever you want to do in life. I also want to tell you that knowing that you are as proud of me as I am of you means a lot to me. I hope that you and Seth and me and Shelly grow to be great friends and even better family.

Thank you and I love all of you.
BIOGRAPHY

I grew up in Raleigh, NC and have lived here my entire life. I grew up as an N.C. State fan and knew that I always wanted to go to college there. My Dad worked at State for over twenty years and my Mom graduated from State in 1971. So it was destined for me to attend NCSU. Some of my hobbies include playing golf, reading (mysteries and suspense novels), and also watching movies. I am currently engaged and hopefully will be getting married next fall.

I have always had an interest in science and have wanted to do research since I got my first lab experience. Virus research appealed to me most because of the real life application of the research, possible vaccine development is my long term goal for whatever virus I am working on. I hope, at some point, that the knowledge I have gained in Dr. Brown’s lab as a virologist will give me the opportunity to contribute to the creation of a vaccine.

My experience at N.C. State has provided me with a wealth of knowledge that I will take with me in the future. I will remain in the area for the time being to begin my post-graduate career, but at some point I would like to move and experience another part of the country.
ACKNOWLEDGMENTS

I would like to acknowledge Dr. Dennis Brown, my dissertation advisor, first for accepting me into his lab and showing a belief in me that I could accomplish what I set out to accomplish. I would also like to thank Dr. Brown for his trust in my decision making as I progressed through my career and began to propose some of my own ideas and take control of my project. In addition I would like to thank Dr. Raquel Hernandez for allowing me to grow as a student and being there anytime that I had a question. Finally I would like to thank the members of my committee who forced me to start thinking further ahead with my experiments while always keeping the big picture in mind.
## TABLE OF CONTENTS

List of Tables ........................................................................................................................................ vii

List of Figures ......................................................................................................................................... viii

Chapter 1: Literature Review .........................................................................................................................1

1.1 Sindbis virus lifecycle and genome organization ..............................................................................1

1.2 Structural Properties of Sindbis virus ..............................................................................................3

1.3 Previous Research on E2 Endodomain ..............................................................................................6

Chapter 2: The Role of a Conserved Tripeptide in the Endodomain of Sindbis Virus Glycoprotein E2 in Virus Assembly and Function ..........................................................13

2.1 Abstract ........................................................................................................................................14

2.2 Introduction ..................................................................................................................................14

2.3 Methods ......................................................................................................................................20

2.4 Results .........................................................................................................................................24

2.5 Discussion ....................................................................................................................................34

2.6 References ..................................................................................................................................37

Chapter 3: Mutations in the Endodomain of Sindbis Virus Glycoprotein E2 define domains critical for virus assembly ..........................................................................................40

3.1 Abstract ......................................................................................................................................41

3.2 Introduction ..................................................................................................................................42

3.3 Materials and Methods ..................................................................................................................46

3.4 Results .........................................................................................................................................53

3.5 Discussion ....................................................................................................................................73

3.6 References ..................................................................................................................................77

v
LIST OF TABLES

Chapter 1
Table 1.1 A brief summary of previous investigations on the role of the E2 endodomain in virus assembly .................................................................9

Chapter 2
Table 2.1 A summary of previous investigations on the role of the E2 endodomain in virus assembly .................................................................19
Table 2.2 Low pH mediated fusion of cells by mutant and wild type Sindbis virus ........................................................................................................33

Chapter 3
Table 3.1 Protection of Capsid residue Y180 upon association with peptides corresponding to fragments of the E2 Endodomain ........................56
LIST OF FIGURES

Chapter 1

Figure 1.1 Genome organization of Sindbis virus ..............................................................2

Figure 1.2 Cross-section of Sindbis virus from a cryo-electron microscopy reconstruction. In yellow is the envelope composed of E1 and E2. In red is the host-derived lipid bilayer, and in blue is the nucleocapsid core. (from Paredes et al., 1993) ................................................................................5

Chapter 2

Figure 2.1 Functional map of the E2 endodomain. T398 and Y400 are putative phosphorylation sites .........................................................................17

Figure 2.2 Production of infectious virus by cells transfected with RNA of the double mutant E2 T398A/Y400N or wild type (WT). Virus titers were performed on BHK-21 cells as described in and Methods ................................................................................................26

Figure 2.3 Production of virus of wild type virus density by E2 T398A/Y400N. Labeled wild type and mutant virus were purified and analyzed as described in Methods. The refractive index for gradients containing the wild type or E2 T398A/Y400N was measured using a refractometer and the distribution of radio labeled protein was determined by scintillation spectrometry ........................28

Figure 2.4 Relative infectivity (particle:pfu) of virus produced by mutant E2 398A/Y400N. Cells were transfected with RNA of the double mutant or wild type (WT) as described in Methods. Particle: pfu ratios of E2 T398A/Y400N compared to wild type virus were determined on BHK-21 cells as described in Methods .................................29

Figure 2.5 Electron microscopy of virus produced by mutant E2 T398A/Y400N. Negative staining of virus preparations was carried out as described in methods. Density gradient purified wild type and mutant virus preparations were examined though only the mutant is shown ........................................................................................................30
Chapter 3

Figure 3.1 Functional map of the Sindbis Virus E2 Endodomain, including a proposed capsid interacting domain (Based on data presented in the text) ....44

Figure 3.2 Iodination of Capsid residue Y180 as an indicator of E2 protein binding. Capsid protein was iodinated in the presence (A) or absence (B) of a peptide representing the 33 amino acid E2 endodomain. The reaction was quenched and then treated + or – with V8 protease to release a peptide containing capsid Y180 ...................................................55

Figure 3.3 Production of virus after transfection with virus RNA containing substitution mutations in the putative capsid binding domain. Titers were determined on BHK cells as described in Materials and Methods .....................................................................................59

Figure 3.4 Production of infectious virus from BHK cells transfected with virus RNA containing deletions in the putative capsid binding domain. Titers were determined on BHK cells as described in Materials and Methods .....................................................................................61

Figure 3.5 Proteins released into the media of BHK cells transfected with E2 Endodomain deletion mutants. Equal volumes of media surrounding equal numbers of transfected cells was applied directly to PAGE. Arrows indicate positions of virus structural proteins E1, E2, Capsid and E3 .....................................................................................64

Figure 3.6 Electron micrographs of BHK cells transfected with E2 endodomain deletion mutants. A, wild type; B, delta406-407; C, delta409-411; and D, delta414-417. E is an enlargement of an area in D. Arrows indicate nucleocapsids. Magnification bars =200nm ........67

Figure 3.7 Growth of E2 Endodomain deletion mutants in Insect cells. Aedes albopictus (clone U4.4) cells were transfected with wild type or mutant contain virus RNA as described in methods ........69

Figure 3.8 Electron micrographs of Aedes albopictus cells transfected with E2 endodomain deletion mutants. A, wild type (arrows indicate virions); B & C, delta409-411. Magnification bars =100nm ............................72
Chapter 1: Literature Review

1.1 Lifecycle and Genome Organization of Sindbis Virus

As the prototype of the alphavirus genus, Sindbis virus (SV) has been well studied in the field of virology. It was first isolated in 1952 in the Sindbis health district (hence its name) outside of Cairo, Egypt (Hurlbut, 1953; Taylor and Hurlbut, 1953; Taylor et al., 1955). Sindbis was later classified as a member of the family Togaviridae. Sindbis is a positive sense RNA virus, meaning that upon introduction of the viral genome into the target cell the RNA can immediately be translated by the host cell machinery, and virus protein production begins. The viral genome is organized as shown in (Figure 1.1) and is approximately 11,700 kb in length (Strauss and Strauss, 1994). Upon infection the first proteins to be translated from the viral RNA are the nonstructural proteins, nsp1, nsp2, nsp3, and nsp4. The non-structural proteins function in a variety of processes, but their main function is to facilitate the replication of the (+) strand RNA to (-) strand. The negative sense strand then serves as the template for the synthesis of the (+) strand which ultimately is incorporated into the mature virus particle and used in subsequent infections (Strauss and Strauss, 1994).

A second critical function of the negative strand RNA is its role in production of a subgenomic (26S) RNA that serves as the template for the production of the virus structural proteins. A mature virus particle contains three structural proteins, Capsid (C), Envelope glycoprotein 1 (E1), and Envelope glycoprotein 2 (E2). These proteins are the product of translation of the 26S subgenomic RNA by host cell machinery. The structural proteins are translated in the order NH4-C-PE2-6K-E1-COOH. Capsid protein
Figure 1.1 Genome organization of Sindbis virus.
contains an intrinsic autoprotease activity which cleaves the protein from the developing polyprotein. As the polyprotein chain develops it is translocated into the membrane of the Endoplasmic Reticulum (ER). This 100kD protein is inserted in the order NH4-PE2-6K-E1-COOH. In the ER the polyprotein is processed by the cellular enzyme signal peptidase at two sites; one located between PE2 and 6K and the other located between 6K and E1 (Liljestrom and Garoff, 1991). Processing at these sites by signal peptidase results in the production of mature E1 protein and the precursor to E2 protein (PE2). Signal peptidase cleavage also results in the release of the 6K protein from the polyprotein. Before export to the Trans-Golgi network, E1 and PE2 form heterodimers and subsequently trimerize (Carleton et al., 1997, Mulvey & Brown, 1994, Mulvey & Brown, 1996). These trimers are exported to the TGN where further processing and modification of the proteins takes place. Once at the TGN PE2 is processed by furin protease (Nelson et al., 2005) to produce E2 and E3 proteins. The E3 protein (15kD) is released into the media and is not present in mature virus particles (Mayne et al., 1985). Additional modifications such as glycosylation and fatty acid acylation are also made to the glycoproteins in the TGN (Strauss and Strauss, 1994). There are two glycosylation sites on each of the structural proteins E1 (aa 139 and 245) and E2 (aa 196 and 318) (Hseih et al., 1983; and Mayne et al., 1985). Fully processed E1-E2 complexes are transported to the plasma membrane where they participate in budding of new viruses.

1.2 Structural Properties of Sindbis Virus

There are 240 copies of each of the three structural proteins (C, E1, and E2) in a mature virion. The outer shell, or envelope, of the particle is made up of E1 and E2
glycoproteins, has T=4 icosahedral symmetry and is 70 nm in diameter (Paredes et al., 1993) (Figure 1.2). The inner shell (nucleocapsid/core) also has T=4 symmetry and is connected to the outer shell via specific protein-protein interactions between E2 and the capsid protein (Choi et al., 1991). Between the envelope and the nucleocapsid lies a host derived lipid bilayer, which can differ in composition based on the cell that has been infected (vertebrate or invertebrate). Mosquitoes are the natural host for alphaviruses; however alphaviruses can also infect humans and other animals. The membranes of mammals and insects differ in composition in that insect membranes do not contain cholesterol, whereas mammals contain a significant amount of cholesterol. It has not been determined what effect (if any) this difference in membrane composition has on virus assembly. Within the membrane bilayer both E1 and E2 are embedded, however only E2 has a cytoplasmic domain (33 amino acids in length) that extends beyond the membrane; it is predicted that only two amino acids in E1 extend into the cytoplasm.

A significant portion of the E2 tail (approximately 13 amino acids) is buried in the membrane when the polyprotein is initially inserted in ER. Exposure of the cytoplasmic domain of E2 is essential for the full maturation of the virus particle, however the point in the secretory pathway at which exposure takes place is unknown. It is also not known by what mechanism exposure of the endodomain occurs. The endodomain is believed to play a role in the extraction of the tail from the membrane, contain the signal sequence for the insertion of 6K protein in the ER membrane, and contain the signal peptidase cleavage site (Liljestrom and Garoff, 1991). Still, the primary function of this domain is to bind to the nucleocapsid during virus budding.
Figure 1.2 Cross-section of Sindbis virus from a cryo-electron microscopy reconstruction. In yellow is the envelope composed of E1 and E2. In red is the host-derived lipid bilayer, and in blue is the nucleocapsid core. (Paredes et al., 1993).
1.3 Previous Research on the E2 Endodomain

Originally it was not known that E2 contained a domain which extended through the membrane of the virus particle. Early work by several labs simply identified E2 as the protein that interacted with the nucleocapsid to connect the outer and inner shells of the virus. Brown and Smith (1975) identified E2 as the protein responsible for interacting with the nucleocapsid using temperature sensitive mutants of Sindbis virus. More specifically, evidence from both Garoff and Simons (1974) and Ziemiecki and Garoff (1978) suggested that this interaction occurred between a cytoplasmic domain of either E1/E2 with the nucleocapsid in SFV. In 1986, Roman and Garoff showed that truncations in the cytoplasmic domain in Semliki Forest virus (SFV) E2 protein did not affect the transport of the E1-E2 protein complex. Further support for the proposed E2 tail-nucleocapsid interaction came when Metsikko and Garoff (1990) identified a specific interaction between the E2 cytoplasmic domain and the nucleocapsid in SFV. In this study, truncated peptides corresponding to the endodomain of E2 prevented binding of nucleocapsids and they also showed that oligomerization of full length peptides was the preferred orientation for binding to the nucleocapsids.

More recent studies have further characterized the interaction of the E2 tail and the nucleocapsid. Liu and Brown showed that the E2 endodomain, a large part of which is initially buried in the membrane bilayer, is extracted from the membrane after export from the ER but prior to arrival at the cell surface (1993). In 1994, Lee and Brown showed that mutation of two amino acids in the capsid protein resulted in the formation of aberrant particles and a loss of infectivity. This suggested a specific interaction between the exposed E2 endodomain and the nucleocapsid at the cell surface. Support for a specific interaction
was provided through studies using a chimeric virus of Ross River and Sindbis virus. Lopez and colleagues (1994), using a chimeric virus containing the E2 of SV and the capsid protein of RR, showed that substitution of SV amino acids in the tail for RR amino acids adapted the endodomain and allowed binding to the RR nucleocapsid and subsequent particle formation.

A breakthrough in understanding this critical protein-protein interaction occurred when a crystal structure of the carboxy terminal portion of the capsid protein was resolved (Choi et al., 1991; Lee et al., 1996; Choi et al., 1996). The crystal structure revealed a hydrophobic cleft on the surface of the capsid protein extending from approximately amino acid 170 to 250 that was proposed as the binding site for the E2 endodomain (Lee et al., 1996). It was also proposed that within the hydrophobic cleft an aromatic interaction between Y400 in the E2 tail and two amino acids in the capsid protein (Y180 and W247) stabilized the protein-protein interaction (Lee et al., 1994a; Lee et al., 1994b; Lee et al., 1996; and Skoking et al., 1996). A model, which fit the E2 tail into the cleft based on the first ten amino acids of the tail (from the amino terminus), juxtaposed the Y400 (SV) in close proximity to capsid residues Y180 and W247.

The tyrosine residue at position 400 (SV), implicated in the aromatic interaction is part of a three amino acid sequence, 398-TPY-400, that is conserved throughout the alphaviruses (Strauss and Strauss, 1994). Mutational analysis of this particular residue (Y400) by multiple groups has revealed that it is critical for virus maturation. Liu et al, showed that mutation of Y400 in combination with mutation of the T398 caused a loss of infectious virus production (1996). This double mutation also prevented phosphorylation of these residues which had been proposed to be required for virus maturation, along with dephosphorylation (Liu & Brown, 1993). Additional single point mutations of this residue
have also indicated that this residue is critical for virus maturation (Lee et al., 1996; Gaedigk-Nitschko and Schlesinger, 1991).

It has been suggested by (Owen and Kuhn, 1997) that amino acids 400 (Tyr) and 402 (Leu) occupy the cleft in the capsid protein and are responsible for the binding of E2 to the nucleocapsid. As mentioned previously, position Y400 is part of the ‘TPY’ domain which is conserved throughout the alphaviruses; however position L402 is not a completely conserved residue. Mutation of position 402 does not result in a loss of infectious virus production in all cases, indicating that this residue is not required for virus maturation. The proposed interaction of residues 400 and 402 is based on a fit of the first ten amino acids of the endodomain into the electron density map of the capsid protein (from the crystal structure) (Choi et al., 1991). Because the fit of the tail in the hydrophobic cleft is based on the first ten amino acids of the E2 endodomain only, it is possible that the remaining 23 amino acids not included in this model do make contact with the capsid protein, thereby altering the proposed interaction.

Along with the TPY domain there are several other residues along the E2 tail that are highly conserved within the alphavirus genus. There have been extensive efforts to determine which of these residues are responsible for the interaction between the E2 tail and the nucleocapsid protein (Table 1.1). There are specific point mutations which result in the total loss of infectious virus production, however, multiple substitutions at the same position later revealed that some mutations can be tolerated, and result in significant levels of infectious virus production. Mutagenesis has been performed on amino acids
Table 1.1 A brief summary of previous investigations on the role of the E2 endodomain in virus assembly.

<table>
<thead>
<tr>
<th>Position</th>
<th>Mutation</th>
<th>Effect</th>
<th>Compensatory</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>K391</td>
<td>Deletion of K391</td>
<td>No virus produced in BHK</td>
<td>none</td>
<td>Hernandez et al., 2000</td>
</tr>
<tr>
<td>T398Y400</td>
<td>T398A/Y400N</td>
<td>Originally no virus produced, blocked in phosphorylation, no transport defects, no synthesis defects</td>
<td>Revertants observed at 400 but not 398, also had deletion of 402-406, substitution of L402 with V</td>
<td>Liu et al., 1996 (Vir) 222; 236-246</td>
</tr>
<tr>
<td>F399</td>
<td>P399G</td>
<td>Released lower than normal levels of virus, produced multi-core particles, more heat stable than wt</td>
<td>None reported</td>
<td>Ivanova and Schlesinger (IV) 1993, 2546-2551</td>
</tr>
<tr>
<td>A401</td>
<td>A401H</td>
<td>Released lower levels of particles, multi-core particles, more heat stable</td>
<td>None reported</td>
<td>Same as above</td>
</tr>
<tr>
<td>P401</td>
<td>P401G</td>
<td>Released lower levels of particles, multi-core particles, more heat stable</td>
<td>SIH2N in capsid (no real effect alone) T398M in E2 tail (not studied)</td>
<td>Same as above</td>
</tr>
<tr>
<td>Y409</td>
<td>Y409F</td>
<td>Reduced particle formation 3-4 logs</td>
<td>None reported</td>
<td>Gudosh-Nitschko and Schlesinger (Yi) 1993, 183; 206-214</td>
</tr>
<tr>
<td>C416</td>
<td>C416A</td>
<td>Produced virus slower than wt</td>
<td>None reported</td>
<td>Ivanova and Schlesinger (IV) 1993, 2546-2551</td>
</tr>
<tr>
<td>C417</td>
<td>C417A</td>
<td>Highly defective in virus release</td>
<td>T256M in capsid (alone no effect) SA11.1 in E2 tail (not studied)</td>
<td>Ivanova and Schlesinger (IV) 1993, 2546-2551</td>
</tr>
<tr>
<td>S420</td>
<td>S420C</td>
<td>Affected Fv2-6k cleavage</td>
<td>None reported</td>
<td>Gudosh-Nitschko and Schlesinger (IV) 1993, 183; 206-214</td>
</tr>
</tbody>
</table>
throughout the tail (both amino terminal and carboxy terminal) and has revealed that no specific domain or residue is responsible for nucleocapsid binding. Up to this point single point mutations, whether they are located in the amino terminal half or the carboxy terminal half of the endodomain, have not been efficient enough in disrupting SV assembly to provide the information needed to elucidate the binding interactions between E2 and the nucleocapsid.

Little has been done to study the effects of truncations or deletions of specific domains/amino acids within the E2 tail. Three studies in particular have focused on truncations of the E2 tail and their effects on virus maturation. Roman and Garoff (1986) showed that truncations of the tail did not affect the transport of the E1-E2 protein complex to the plasma membrane in mammalian cells (in SFV). In a controversial study, Kail and colleagues used anti-idiotype antibody studies and synthetic peptides corresponding to the E2 tail of SFV to identify the C-terminal eight amino acids as being crucial for binding to the nucleocapsid and initiation of virus budding (1991).

More recently, a single deletion of the membrane proximal K391 in SV was analyzed for its effect on the assembly of the virus particle (2000). This single deletion in the E2 endodomain resulted in a complete loss of infectious virus production from mammalian cells; however this deletion caused no defect in virus production from insect cells. This indicated that in mammalian cells the length of the E2 tail is more critical to virus assembly than in insect cells. It is possible that the thickness of the host derived membrane bilayer is partially responsible for the toleration of this deletion in insect cells.

As mentioned previously, insect cells lack cholesterol which in turn results in thinner membranes. These thinner membranes may be able to tolerate shorter membrane spanning
domains created by deletions within a specific region of the E2 tail. Up to this point no studies have been done using deletions in the membrane spanning domain of the E2 tail. However, a single deletion at position K391, which is upstream of the transmembrane (TM) domain in the E2 tail, has been analyzed in both vertebrate and invertebrate cell lines (Hernandez et al., 2000). This single deletion prevented virus assembly in mammalian cells but did not affect virus assembly in insect cells. This indicated that the length of the E2 tail may be more critical in mammalian cells than in insect cells, possibly due to the thickness of the membrane. Also, deletions upstream of the membrane spanning domain within the E2 tail could cause incorrect extraction of the tail and consequently have a deleterious impact on virus assembly.

There is evidence suggesting that the length of membrane spanning domains may dramatically affect the assembly of Sindbis virus and that shortening of a TM domain can be compensated for by growth in insect cells (Hernandez et al., 2003). Overall, little is known about the requirements for the interaction of the E2 endodomain despite numerous studies. Recently, a new model for the interaction of the E2 endodomain with the nucleocapsid has been proposed that disagrees with the current model. Using both NMR spectroscopy and artificial phospholipid bilayers studded with truncated E2 proteins, it was concluded that the binding site for the E2 endodomain with the virus capsid lies within the N-terminal 80 amino acids of the capsid protein, not in the hydrophobic cleft observed in the crystal structure (Wilkinson et al., 2005). At this point there is no other data to support this new proposed binding site. The goal of my research is to more specifically determine what amino acids in the E2 endodomain are necessary for nucleocapsid binding and subsequent virus budding. We will employ both substitutions and a battery of deletion mutants to better characterize the
interaction between the E2 and nucleocapsid proteins. These mutations were examined in mammalian cells to determine what is required for binding but to also investigate the role of the host derived membrane in virus assembly. In addition, the mutants examined in the studies discussed below, are targeted to specific regions of the E2 endodomain that contain highly conserved amino acids and were identified by our own experiments as being critical for the interaction of the E2 endodomain with the preformed nucleocapsid. Specifically, my research focuses on the TPY domain and a domain within the E2 tail identified by our own peptide mapping studies. My research shows that both of these domains play critical roles in the assembly of Sindbis virus and the function of the mature particle (infectivity).
Chapter 2:

The role of a conserved tripeptide in the endodomain of Sindbis virus glycoprotein E2 in virus assembly and function

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2.1 Abstract

Envelopment of Sindbis virus (SV) at the plasma membrane begins with the interaction of the E2 glycoprotein endodomain with a hydrophobic cleft in the surface of the pre-assembled nucleocapsid. The driving force for this budding event is thought to reside in this virus type specific association at the surface of the cell. The specific amino acids involved in this interaction have not been identified; however it has been proposed that a conserved motif (TPY) at aa 398-400 in the E2 tail plays a critical role in this interaction. We have examined this interaction with virus containing mutations at two positions in this conserved domain, T398A and Y400N. The viruses produced have very low infectivity (as determined by particle to PFU ratios) however there appears to be no defect in assembly, as the virus has wild type density and electron microscopy shows assembled particles with no obvious aberrant structural changes. The loss of infectivity in the double mutant is accompanied by the loss of the ability to fuse cells after brief exposure to acid pH. These data support the idea that these residues are vital for production of infectious/functional virus; however they are dispensable for assembly. These results combined with other published observations expand our understanding of the interaction of the E2 endodomain with the capsid protein.

2.2 Introduction

Sindbis virus, (SV), the prototype of the alphaviruses, is a popular model system in which to study the assembly of a particular class of membrane containing viruses, those that have icosahedral morphology (Strauss & Strauss, 1994). Sindbis virus is a well-ordered, structurally complex virion composed of three structural proteins; capsid (C), and envelope
proteins E1 and E2. There are 240 copies of each protein in a 1:1:1 stoichiometric arrangement in a mature virus particle. A mature Sindbis virion is organized as nested T=4 icosahedrons with a host-derived lipid membrane bilayer sandwiched between the outer shell and the inner shell (nucleocapsid) (Paredes et al., 1993). E1 and E2 are organized as trimers of heterodimers and form 80 trimeric spikes on the surface of the virus. The integrity of the outer shell is maintained through lateral E1- E1 interactions on the virus surface (Anthony & Brown, 1991, Pletnev et al., 2001). The inner shell (nucleocapsid), also organized as a T=4 icosahedron (Coombs & Brown, 1987, Paredes et al., 1993, Paredes et al., 1992), is an aggregate of the 49S viral RNA and capsid protein. The E2 protein contains a membrane spanning domain and a cytoplasmic domain (endodomain/tail) (Hernandez et al., 2003, Liu & Brown, 1993b). The endodomain is a 33 amino acid domain that specifically interacts with the capsid protein during assembly (Cheng et al., 1995, Lee & Brown, 1994, Lee et al., 1996, Lopez et al., 1994, Owen & Kuhn, 1997, Wilkinson et al., 2005). This interaction connects the outer shell to the inner shell, an interaction that persists in the mature virus particle.

The assembly of the mature virion is a complex process involving multiple specific protein-protein interactions. Initially the virus structural proteins are translated from a 26S subgenomic RNA (NH2-C-pE2-6K-E1-COOH) (Lilijestrom & Garoff, 1991). During translation the C protein is autoproteolytically cleaved from the developing polyprotein chain. The C protein then assembles in the cytoplasm with the 49S viral RNA to form the nucleocapsid (Ferreira et al., 2003). The remaining proteins are integrated into the membrane of the endoplasmic reticulum (ER). In the ER the polyprotein is processed by signalase at specific sites to produce pE2 and E1 and release the intervening 6K protein.
(Lilijestrom & Garoff, 1991). The role of 6K in virus maturation is unresolved. PE2 and E1 form trimers of heterodimers (Carleton et al., 1997, Mulvey & Brown, 1994, Mulvey & Brown, 1996), which are exported to the trans-golgi network (TGN). In the TGN PE2 is processed by furin protease to E2 and E3 (Nelson et al., 2005). At this point E3 protein is released into the surrounding media. The E2-E1 heterotrimeric complex is then exported to the plasma membrane, where the process of virus envelopment takes place (Brown et al., 1972).

It is at the plasma membrane where the virus assembles into its mature functional form. It is in the initiation of the process of envelopment that one of the critical events in virus assembly takes place, the association of the E2 endodomain with the preformed nucleocapsid (Ferreira et al., 2003, Owen & Kuhn, 1996, Owen & Kuhn, 1997). It has also been suggested that a preformed nucleocapsid is not necessary for assembly of alphaviruses (Forsell et al., 1996), however preformed nucleocapsids attached to membranes are the preferred association as shown by Ferreira et al. (2003). This association is a highly specific interaction between the 33 amino acid endodomain (E2 aa 391-aa 423) of E2 and the protein sequence in the hydrophobic cleft of the assembled nucleocapsid (C aa 175 -aa 250) (Lee & Brown, 1994, Lee et al., 1996). The E2 tail is a multifunctional protein domain (Figure 2.1); however its primary function is to serve as the site of attachment for the capsid protein. The COOH terminal portion of the E2 endodomain is initially buried in the membrane of the ER (Liu & Brown, 1993b). It is not known at what point in the secretory pathway this domain becomes exposed to the cytoplasm; however exposure occurs after export from the ER and prior to arrival at the cell surface. We have suggested that a transient phosphorylation of either the T398 or
Figure 2.1 Functional map of the E2 endodomain. T398 and Y400 are putative phosphorylation sites.
Y400 may play a role in tail extraction (Liu & Brown, 1993a). Once at the cell surface the interaction of the E2 endodomain with the nucleocapsid core is the first step in virus budding. Previous studies have provided insight into which amino acids play a critical role in this interaction (Gaedigk-Nitschko & Schlesinger, 1991, Lopez et al., 1994, Owen & Kuhn, 1996, Owen & Kuhn, 1997, Ryan et al., 1998, Weiss et al., 1994) (Table 2.1). The crystal structure of the C protein indicates that there is an aromatic interaction that could take place between two residues in the capsid Y180, W247 and one residue in the E2 tail, Y400, which is conserved throughout the alphavirus family (Lee et al., 1996, Skoging et al., 1996). Experiments from our lab support the conclusion that Y400 plays a critical role in this association (Liu et al., 1996). We have investigated the importance of Y400 in combination with another completely conserved residue, T398 in virus assembly and function. This mutant was previously characterized in our lab as a tool to study the role of phosphorylation at T398 and Y400 in virus production (Liu et al., 1996). We chose to revisit the properties of this mutant after the observation by Hernandez (2000) which showed that shifting the location of the Y400 in the endodomain caused a complete loss of virus production.
Table 2.1 A summary of previous investigations on the role of the E2 endodomain in virus assembly

<table>
<thead>
<tr>
<th>Position</th>
<th>Mutation</th>
<th>Effect</th>
<th>Compensatory</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>K391</td>
<td>Deletion of K391</td>
<td>No virus produced in BHK</td>
<td>None</td>
<td>Hernandez et al., 2010</td>
</tr>
<tr>
<td>T398/Y400</td>
<td>T398A/Y400N</td>
<td>Orally no virus produced, blocked in phosphorylation, no transport defects, no synthesis defects</td>
<td>Revertants observed at 400 but not 398, also had deletion of 400–400, substitution of L102 with V</td>
<td>Liu et al., 1996 (Vir) 222; 236-246</td>
</tr>
<tr>
<td>P399</td>
<td>P399G</td>
<td>Reduced lower than normal levels of virus, produced multi-cored particles, more heat stable</td>
<td>None reported</td>
<td>Ivanova and Schlesinger (IV) 1993, 2546-2551</td>
</tr>
<tr>
<td>A401</td>
<td>A401H, A401K</td>
<td>Released lower levels of particles, multi-cored particles, more heat stable</td>
<td>None reported</td>
<td>Same as above</td>
</tr>
<tr>
<td>P404</td>
<td>P404G</td>
<td>Released lower levels of particles, multi-cored particles, more heat stable</td>
<td>S122N in capsid (no real effect alone) T398M in E2 tail (not studied)</td>
<td>Same as above</td>
</tr>
<tr>
<td>Y400</td>
<td>Y400F</td>
<td>Reduced particle formation 3–4 logs</td>
<td>None reported</td>
<td>Gaedigk-Nitschko and Schlesinger (Vir) 1991 183: 206-214</td>
</tr>
<tr>
<td>E402</td>
<td>V, G, T, G, N, D, and R</td>
<td>V: Medium plaques and 2 log decrease C and T: Small plaques 4 log decrease G, N, and D: Very small plaques 5 log decrease R: Dead No processing, transport, or translocation defects</td>
<td>None reported</td>
<td>Gwos and Bokas (VGC) 1997 236: 105-196</td>
</tr>
<tr>
<td>C416</td>
<td>C416A</td>
<td>Produced virus slower than wt Produced multi-cored particles and site of palmitoylation</td>
<td>None reported</td>
<td>Ivanova and Schlesinger (IV) 1993, 2546-2551</td>
</tr>
<tr>
<td>C417</td>
<td>C417A</td>
<td>Highly defective in virus release Produced multi-cored particles Site of palmitoylation Defective in processing FV26K protein</td>
<td>T256M in capsid (alone no affect) S411L in E2 tail (not studied)</td>
<td>Ivanova and Schlesinger (IV) 1993, 2546-2551</td>
</tr>
<tr>
<td>S420</td>
<td>S420C</td>
<td>Affected FV2-6K cleavage</td>
<td>None reported</td>
<td>Gaedigk-Nitschko and Schlesinger (Vir) 1991 183: 206-214</td>
</tr>
</tbody>
</table>
2.3 Methods

Cells, viruses, and media

Baby hamster kidney (BHK-21) cells were maintained in eagle’s minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 5% tryptose phosphate broth, and 2mM glutamine as described previously (Renz & Brown, 1976). The cells were maintained at 37°C under 5% CO₂. The wild-type Sindbis virus used in these studies was produced from the SV clone Toto 1101 (Rice et al., 1987, Strauss et al., 1984). This clone contains a tyrosine at position 420 in the E2 protein that is an S in Toto 1101. This substitution has no observable phenotype (Liu & Brown, 1993b). In this investigation we employ a double mutant described in 1996 (Liu et al., 1996).

In vitro transcription, RNA transfection, and Plaque assay

Full-length mutant and wild-type cDNA’s were linearized using the enzyme Xho1, treated with proteinase K, phenol extracted and ethanol precipitated. The templates were transcribed as described previously (Hernandez et al., 2003, Rice et al., 1987). The infectious RNAs were transfected by electroporation as described by Liljestrom and Garoff (Liljestrom & Garoff, 1991). Cells were pelleted and washed in RNase free phosphate buffered saline (pH 7.0). Washed cells were resuspended to a concentration of 1x 10⁷ cells/ml. RNA transcripts (20 µl) were added to 400 µl of BHK cells. The electroporation conditions were 1.5 kV, 25 µF, and ∞ resistance. Cells were pulsed once, allowed to sit for ten minutes, and then transferred into 10 ml of MEM medium (no gentamicin) in 25cm² tissue culture flasks. Virus was harvested at 24 hrs post-transfection and flash frozen in
liquid nitrogen in 1 ml aliquots to be stored at -80°C. To determine the titer of each virus plaque assays were carried out as described previously (Renz and Brown 1976).

Mutagenesis and RT-PCR

Mutagenesis was done as described previously (Liu et al., 1996). Briefly, using the megaprimer method of PCR mutagenesis the mutations were inserted at the correct positions (T398 and Y400) using AmpliTaq polymerase and specific PCR conditions. To analyze the mutant by RT-PCR virus from transfections was first pelleted at 50,000 rpm for one hour in a Beckmann SW55Ti rotor. The pellet was then incubated in 100µl of Tris-EDTA and 100µl of 2X lysis buffer (100mM Tris-Cl [pH 7.0], 20mM EDTA, 1% SDS) for twenty minutes at 37°C (vortexing every 5 minutes). The lysed virus was then extracted sequentially with phenol (twice), phenol/chloroform (once), and chloroform (once). Extracted RNA was then precipitated in RNase-free ethanol overnight at -80°C. The RNA was then pelleted and resuspended in 10µl of DEPC (di ethyl pyrocarbonate) - treated water. RNA was transcribed using MuLV RT under the following conditions. The reaction contained 10X PCR buffer (10 mM Tris-Cl (pH 8.3), 50 mM KCl, and 5 mM MgCl₂), 20 U of SupeRNasin™ (Ambion, Austin TX), 200 µM each deoxynucleoside triphosphates, 1.0 µM reverse primer, and 50 U of MuLV RT in a final volume of 20 µl. Reverse transcription was performed at 42°C for 20 min and 99°C for 5 min. After the transcription reaction, the volume of the reaction mixture was increased to 100 µl and the concentrations of the deoxynucleoside triphosphates and MgCl₂ were adjusted for the increased volume. Forward primer was added to a final concentration of 2.0 µM, and an additional 1.0 µM reverse primer was added. Taq DNA
polymerase was added to a final concentration of 2.5 U/100-µl reaction mixture. The primers used to sequence through the E2 endodomain and capsid protein were the following; (capsid) 5’- ggggtcgtctaattgtcttc -3’, and (endodomain) 5’- caaggtgcaactgc -3’.

**Metabolic labeling of infected cells**

Subconfluent monolayers of BHK cells were first treated with 5ml of media containing 4µg of actinomycin-D (Calbiochem, San Diego, CA) for one hour. Cells were then infected with both wild type and T398A/Y400N at a multiplicity of infection (MOI) of one. Virus was diluted in 1X PBS-D containing 3% FBS. Infection was carried out at room temperature for one hour. After one hour the media was removed and 5 mls of fresh media was added to the cells, the cells were then incubated for 5 hours at 37°C. Following the five-hour incubation the cells were then starved of cysteine and methionine for one hour. Finally the monolayers were labeled overnight with a [35S] methionine-cysteine protein labeling mixture at a concentration of 50 µCi/ml.

**Purification and particle/PFU ratio determination of T398A/Y400**

Transfections were carried out as described above. The supernatants from transfected monolayers were harvested at 20-24 hrs post-infection and layered over a sedimentation gradient of 15% tartrate (11mls) over a 35% cushion of tartrate (6 mls) (in 1X PBS-D). The labeled virus was centrifuged overnight at 24,000 rpm in a Beckmann SW28 rotor. A band was observed for both the wild-type and mutant, the refractive index was taken for each of the samples. These bands were analyzed by plaque assay to determine the titer of the purified virus. Purified virus protein concentrations were determined by Micro BCA analysis
(Pierce, Rockford, IL). The same fractions were titered on BHK-21 cells as described above. The number of particles in a preparation of wild-type virus was determined using electron microscopy by the agar filtration protocol created by Kellenberger and Bitterli (1976) and the particle count was correlated to the protein concentration as determined by BCA (Hernandez et al., 2003). These calculations were used to determine the particle/PFU ratio for the mutant and wild type. Virus collected from sedimentation gradients was also layered over a tartrate density gradient (15%-30%) and centrifuged for two hours at 26,000 rpm in a Beckmann SW28 rotor. Virus bands were collected and analyzed by electron microscopy for aberrant particle formation.

**Negative staining**

Virus collected from potassium tartrate density gradients was attached to carbon-coated grids, washed 3 times with sterile H2O and negatively stained with 1% uranyl acetate. The grids were viewed under a JEOL 100S transmission electron microscope.

**Low pH mediated virus-cell fusion**

Fusion experiments were carried out as described previously (Edwards & Brown, 1986, Edwards et al., 1983). BHK-21 cells were split into 12 well plates to perform the low pH mediated fusion from without. Plates were preincubated on an ice/water bath for 15-20 minutes in order to reach 4°C. Either T398A/Y400N or wild type virus were attached to cell monolayers for fifteen minutes at 4°C. After fifteen minutes the inoculum was removed the wells were washed with 1X PBS-D and the treated with fusion media (pH 5.3) for five minutes at room temperature. The fusion media was washed off and the cells were then
treated with fusion media (pH 7.4) for five minutes at room temperature. The pH 7.4 fusion was then washed off and growth media (1X MEM) was added and the plates were incubated for one hour at 37°C. After one hour the cell monolayers were analyzed for fusion and photographed.

2.4 Results

Construction of Sindbis virus E2 endodomain mutants

X-ray crystallographic analysis of expressed capsid protein has revealed a hydrophobic cleft in the surface of the capsid protein (Choi et al., 1991). The cleft is bordered by two aromatic residues, one on either side, Y180 and W247 (Lee et al., 1996). Experiments in which capsid - Y180 was converted to S identified Y180 as critical for attachment of the capsid to the E2 tail and for function of the mature virion (Lee & Brown, 1994, Lee et al., 1994). These observations led to the hypothesis that these residues participate in an aromatic ring interaction with Y400 of the Sindbis virus E2 tail (Skoging et al., 1996). Y400 is part of a sequence of three amino acids which are conserved among all the Alphaviruses. We have previously shown that Y400 but not T398 are required for virus production (Liu & Brown, 1993a). To further examine the role of these amino acids in the interaction of the endodomain with the capsid protein a mutant was constructed at E2 Y400 that also contained a point mutation at position T398. This double mutant was constructed to examine; 1) the requirement for the aromatic association of E2 Y400 with C Y180 and W247, 2) the role phosphorylation of E2 T398 or E2 Y400 plays in virus production. The double mutant T398A/Y400N prevents phosphorylation at either position and precludes any aromatic interaction. In a previous study, a point mutation at E2 Y400F caused a decrease in

**Growth of the mutant T398A/Y400N in BHK cells**

To determine the phenotype of the double mutant (T398A/Y400N) viral RNA was synthesized by in vitro transcription and the infectious RNA was introduced into BHK-21 cells via electroporation as described in Methods. Cells were transfected with either wild type or mutant RNA and the culture media was harvested 18-24 hrs post transfection when cytopathic effect was evident. Media was flash frozen in liquid nitrogen and stored at -80°C prior to analysis. Plaque assay was used to assay the media for the presence of infectious virus as described in Methods. Growth of the mutant virus is shown in figure 2.2. Compared to the wild-type parental virus the double mutant showed a significant reduction (3-4 orders of magnitude) in infectious virus production in BHK cells. A low but significant amount of infectious virus was consistently produced from this double mutant indicating that the proposed aromatic interaction between the Y400 and the capsid residues Y180 and W247 is not absolutely required for the production of infectious virus. Analysis by RT-PCR through the E2 endodomain has shown that virus produced from T398A/Y400N retained the mutations at both positions. In addition, these data also indicate that while phosphorylation of the E2 tail occurs it is not absolutely required for production of infectious virus as previously thought (Liu & Brown, 1993a), as both potential phosphorylation sites (T398, Y400) are lost in the double mutant.
Figure 2.2 Production of infectious virus by cells transfected with RNA of the double mutant E2 T398A/Y400N or wild type (WT). Virus titers were performed on BHK-21 cells as described in Methods.
Infectivity and particle assembly of mutant E2 T398A/Y400N

The mutant E2 T398A/Y400N produces infectious virus particles; however, the result of the insertion of a mutation in a virus structural protein may be loss of infectivity but not necessarily loss of particle formation. To determine if the affect of the mutation T398A/Y400N was to produce non-infectious virus or if particle assembly itself was inhibited we determined the relative infectivity (particle: pfu ratio) of the double mutant. Virus was purified by density gradient centrifugation as described in Methods. The density of the collected virus bands is shown in figure 2.3 and the virus produced by the mutant was found to band at the same density as wild type virus. BCA analysis of the purified virus proteins indicated that the T398A/Y400N produced a slightly lesser amount of virus protein compared to that produced by the wild type virus (figure 2.3). Purified virus collected from the gradient was also analyzed by plaque assay. The particle: pfu ratio was determined using the obtained protein concentration and the titer of the purified virus (figure 2.4). The virus produced by the double mutant was found to be significantly less infectious than that produced by the wild-type virus. Particle to PFU ratios for wild type virus was 6.25:1 while the double mutant was found to be $4.8 \times 10^4$:1. These data support the hypothesis that the mutations present in T398A/Y400N do not prevent virus assembly but are important for virus infectivity. In order to determine if the protein produced by T398A/Y400N observed in the BCA assay is actually incorporated into typical virus particles; twice purified T398A/Y400N mutant virus was examined by negative stain electron microscopy (figure 2.5). Virus particles did not appear to be empty nor did they appear to have any aberrant structural properties compared to wild type virus preparations.
Figure 2.3 Production of virus of wild type virus density by E2 T398A/Y400N. Labeled wild type and mutant virus were purified and analyzed as described in Methods. The refractive index for gradients containing the wild type or E2 T398A/Y400N was measured using a refractometer and the distribution of radio labeled protein was determined by scintillation spectrometry.
Figure 2.4 Relative infectivity (particle: pfu) of virus produced by mutant E2 T398A/Y400N. Cells were transfected with RNA of the double mutant or wild type (WT) as described in Methods. Particle: pfu ratios of E2 T398A/Y400N compared to wild type virus were determined on BHK-21 cells as described in Methods.
Figure 2.5 Electron microscopy of virus produced by mutant E2 T398A/Y400N. Negative staining of virus preparations was carried out as described in methods. Density gradient purified wild type and mutant virus preparations were examined though only the mutant is shown.
Compensating mutations in the capsid protein

RNA viruses are known for their propensity for acquiring mutations (Drake & Holland, 1999). It is a common occurrence in RNA viruses that the effects of a mutation made in one residue or protein domain can be offset by insertion of a compensating mutation at another position in the genome. These compensating mutations are frequently found in the protein with which the mutated protein interacts during virus assembly (Lopez et al., 1994). Compensating mutations provide important information on protein-protein interactions essential for virus assembly and function. It has been hypothesized that SV E2 protein interacts through its endodomain with a hydrophobic cleft in the surface of the capsid protein (Lee & Brown, 1994, Lee et al., 1996). In order to determine if the observed virus production in T398A/Y400N is due to a compensating mutation in or around the cleft of the capsid protein, RT-PCR was performed on RNA extracted from virus. RT-PCR was limited to the region of the capsid believed to interact with E2, the hydrophobic cleft (aa 160-264). As described in Methods virus harvested from a transfection was pelleted at 50,000 rpm and subjected to multiple extractions to remove virus proteins. Virus RNA was then reverse transcribed and the resulting DNA was amplified by PCR. The DNA was sequenced and the resulting sequence compared to wild type through the capsid protein. This examination of the hydrophobic cleft region between residues 160-264 revealed no mutations in this region of the capsid protein. This was an unexpected result because this mutant has such a deleterious effect on the production of infectious virus, however repeated analyses of virus produced by this mutant were consistent; the mutations were maintained in the tail, and compensating mutations were not found in the capsid or in other regions of the E2 tail. This
suggests that the virus can tolerate the T398A and Y400N mutations during the assembly process but not during the infection cycle.

**Fusion of cells by mutant E2 T398A/Y400N**

The glycoproteins of Sindbis virus have the ability to produce cell-cell fusion after brief transient exposure to acid pH (Edwards & Brown, 1986, Edwards et al., 1983, Mann et al., 1983, Paredes et al., 2004). It is possible that the mutations produced in the endodomain of E2, which reduce virus infectivity, also reduce the ability to mediate cell fusion.

To examine the ability of the double mutant to produce cell fusion, mutant or wild type virus were attached to BHK 21 cells in different amounts to determine if ability to fuse cells correlated with the number of total particles or the number of infectious particles absorbed (Table 2.2). The low titers obtained for the mutant virus restricted the amount of virus that could be employed in this assay. Fifteen minutes at 4°C was allowed for absorption of virus and then the monolayers were washed into fusion media (pH 5.3) at room temperature for 5 min. after which the low pH media was replaced with media at pH 7.2 for one hour. At the end of the incubation the cells were examined in the phase contrast microscope and the % of cells fused was determined (Table 2.2). The amount of infectious virus employed in this experiment was low, limited by the concentration of the double mutant. The amount of fusion obtained appeared to correlate to the amount of infectious virus. 10 times the number of mutant virus particles as wild type particles resulted in equivalent multiplicities of infection and produced the same
Table 2.2 Low pH mediated fusion of cells by mutant and wild type Sindbis virus*

<table>
<thead>
<tr>
<th>Virus</th>
<th>MOI</th>
<th>Particles/Cell</th>
<th>Percent Cells Fused</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.3</td>
<td>14</td>
<td>5%</td>
</tr>
<tr>
<td>E2 T398A/Y400N</td>
<td>0.3</td>
<td>210</td>
<td>5%</td>
</tr>
<tr>
<td>Wild type</td>
<td>17</td>
<td>765</td>
<td>95%</td>
</tr>
<tr>
<td>E2 T398A/Y400N</td>
<td>0.18</td>
<td>126</td>
<td>1%</td>
</tr>
</tbody>
</table>

*Cells were exposed to virus and fused as described in Methods
amount of fusion (Figure 2.2 top). Increasing the number of infectious virus increased the percentage of cells fused. This result indicated that the defect in the double mutant which reduced virus infectivity also reduced its ability to fuse cells after brief exposure to acid pH.

2.5 Discussion

Previous studies have suggested that the E2 endodomain specifically interacts with a hydrophobic pocket in the surface of the SV capsid protein (Lee & Brown, 1994, Lee et al., 1996, Owen & Kuhn, 1997, Wilkinson et al., 2005). This interaction is critical for the assembly of mature virus particles and represents the initial stage of the budding process by which mature SV particles exit an infected cell. The endodomain of E2 is a 33 amino acid domain containing the sequence KARRECLTPYALAPNAVIPTSLALLCCVRSANA (391-423). The domain itself has multiple functions, the primary function being attachment to the capsid to initiate the budding process. Secondary functions of this domain include producing the signal sequence for insertion of the 6K protein into the membrane of the ER prior to processing of the polyprotein (Liljestrom & Garoff, 1991). The 33 amino acid endodomain also contains the signalase cleavage site for separation of PE2 from 6K protein (Liljestrom & Garoff, 1991).

One region of the E2 tail that has been well studied and proposed to be involved in the binding to the capsid protein is the ‘TPY’ domain, amino acids 398-400 (Skoging et al., 1996). This domain is conserved throughout the alphavirus family and has been proposed to be involved in several different ways with the binding of capsid protein. It has been suggested that Y400 is involved in an aromatic interaction along with two residues in the capsid protein, Y180 and W247 (Skoging et al., 1996). It was proposed that this interaction
stabilized the protein-protein interaction and allowed for the budding process to continue to completion (formation of mature viruses). The ‘TPY’ domain was also proposed to be a site of phosphorylation during assembly and that phosphorylation and dephosphorylation were both required in order to form mature virus particles (Liu & Brown, 1993a). The data presented here suggest that the TPY sequence is not essential for the formation of virus particles but is essential for virus function. The ability of the double mutant to assemble virions suggests that aromatic interactions and phosphorylation events are not essential for this process. A revertant of the double mutant restored the Y at position 400 but left the A at position 398 (Liu et al., 1996) suggesting that the component essential for infectivity is the Y at position 400. In a previous study we found that this double mutant did not produce detectable amounts of virus particles (Liu & Brown, 1993a). In this study the RNA produced from the original clone transfected into BHK cells does produce virus particles. The reason for this difference is not known but is likely related to changes in the BHK cell in the intervening 12 years which also includes a geographical change in the location of the laboratory. We have shown that a change in the host cell can alter the phenotype of a particular mutation (Hernandez et al., 2005, Hernandez et al., 2000, Hernandez et al., 2003, Nelson et al., 2005). The mutant is still severely inhibited in its ability to produce virions.

The mutations we have produced in the E2 endodomain insert residues at positions 398 and 400 that are less bulky than those in the wild-type sequence. The change in atomic density probably affects the specific nature of this critical association resulting in the reduced infectivity of the virus produced. Previous studies have demonstrated that there are other regions of the endodomain involved in this interaction (Lee et al., 1996, Lopez et al., 1994, Owen & Kuhn, 1997, Wilkinson et al., 2005) and these interactions may be more critical to
the process of assembly than the TPY domain. Because of the changes in the T398A/Y400N mutant the capsid protein may be less tightly bound to the endodomain and this association may be critical for release of the RNA.

The data presented above suggest that alterations at position Y400 results in a production of virus particles which have low infectivity. It is noteworthy that other research has demonstrated that the position of the conserved Y in the TPY sequence also plays a critical role in the assembly process (Hernandez et al., 2000). That research has shown that a distance of 9 to 10 amino acids from the inner surface of the membrane to the conserved Y is required for the production of virus particles. A single deletion at the membrane interface resulted in the inability to assemble virus and blocked the attachment of capsids to the endodomain (Hernandez et al., 2000). Other insertions or deletions in the region between the membrane and Y400 resulted in the loss of virus production and the production of structurally aberrant virions (Hernandez et al., 2005). The number of amino acids in the endodomain between the membrane and Y400 seems to be more critical for virus assembly while Y at 400 seems to be critical for the infectivity of assembled particles.

Acknowledgements

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2.6 References


Chapter 3:

Mutations in the Endodomain of Sindbis Virus Glycoprotein E2 define domains critical for virus assembly

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submitted to Journal of Virology
3.1 Abstract

Envelopment of Sindbis virus at the plasma membrane is a multi-step process in which an initial step is the association of the E2 protein via a cytoplasmic endodomain with the preassembled nucleocapsid. Sindbis virus is vectored in nature by blood sucking insects and grows efficiently in a number of avian and mammalian vertebrate hosts. Assembly of Sindbis virus, therefore, must occur in two very different host cell environments. Mammalian cells contain cholesterol which insect membranes lack. This difference in membrane composition may be critical in determining what requirements are placed on the E2 tail for virus assembly. To examine the effect of membrane composition on the assembly of Sindbis virus we have produced substitutions and deletions in a region of the E2 tail that is initially integrated into the Endoplasmic Reticulum (E2 aa 408-415). The effects of these mutations on virus assembly and function were determined in both vertebrate and invertebrate cells. Amino acid substitutions reduced infectious virus production in a position dependant fashion but were not efficient in disrupting assembly in mammalian cells. Deletions in the E2 endodomain resulted in failure to assemble virions in mammalian cells. Examination of BHK cells transfected with these mutants, by electron microscopy, revealed assembly of nucleocapsids which failed to attach to membranes. Introduction of these deletion mutants into insect cells resulted in the assembly of virus like particles but no assayable infectivity. These data suggest a fundamental difference between Sindbis virus assembly in mammalian cells as compared to insect cells.
3.2 Introduction

Sindbis virus (SV) is the prototype of the alphaviruses, a family of viruses vectored in nature by mosquitoes and transferred via blood meal to humans and other vertebrates including birds and mammals (48). SV is an icosahedral virus with T=4 architecture composed of three structural proteins, capsid (C), envelope glycoprotein 1 (E1), and envelope glycoprotein 2 (E2) (37). There are 240 copies of each of the structural proteins in a mature virus particle in a 1:1:1 stoichiometric arrangement. The outer envelope of the virus is composed of E1 and E2, which form 80 heterotrimeric spikes on the surface of the virus. Sandwiched between this outer shell and the inner shell or core (an aggregate of capsid protein and the virus RNA) is a host derived lipid bilayer. The membrane is traversed by both of the outer envelope proteins E1 and E2. Two amino acids exit the membrane and are exposed on the cytoplasmic side of E1 whereas E2 has a cytoplasmic domain that is 33 amino acids in length. The integrity of an intact virion is maintained by two distinct interactions between the structural proteins. Lateral E1-E1 protein interactions stabilize the outer shell (1, 39) and an association involving the cytoplasmic domain of E2 and capsid connects the inner and outer shells, holding the particle together (6, 17, 19, 24, 35, 50).

Assembly of the virus particle involves multiple, specific protein-protein interactions. The structural proteins are first translated from 26S subgenomic RNA in the sequence NH2-C-PE2-6K-E1-COOH (20). Capsid is released from the polypeptide via an auto-proteolytic activity and subsequently associates with the 49S viral RNA to form an assembled nucleocapsid (10). After capsid release from the polypeptide chain the remaining protein is inserted into the endoplasmic reticulum (ER) where cleavage by signal peptidase releases the 6K protein (20). Prior to export to the Golgi apparatus E1 and PE2 form heterodimers
followed by trimerization of these heterodimers (5, 31, 32). E1 and E2 heterotrimers are exported to the Golgi where PE2 is processed by furin protease to form E2 releasing the E3 protein (34, 48). E3 is not present in a mature virus particle and is released into the surrounding media. The E1-E2 protein complex is transported to the plasma membrane where the process of virus envelopment takes place (4). At an unknown point in the secretory pathway the endodomain of E2 is pulled through the membrane and exposed to the cytoplasm for nucleocapsid binding (23).

The differences in the chemical and physical properties of membranes between insect and mammalian cells suggest that interactions of virus proteins with these membranes may differ. Insect cells (the natural host for alphaviruses) do not contain significant amounts of cholesterol (less than 1%) in their membranes (7, 8, 42) whereas mammalian cells have cholesterol as a significant contributor to their structure and function (2). We have previously shown that the difference in the composition of mammalian and insect membranes places different requirements on the transmembrane domains of the virus glycoproteins for proper virus assembly (13-15).

The association of the E2 endodomain with the preformed nucleocapsid is one of the critical events in the envelopment process (10, 17-19, 35, 36). This 33 amino acid endodomain (aa391-423) is a multifunctional domain which plays a critical role in the integration and processing of the structural proteins in the ER, however its primary function in assembly is binding to the nucleocapsid (figure 3.1). X-ray crystallographic analysis of the capsid protein revealed a hydrophobic cleft in the protein extending from
Figure 3.1 Functional map of the Sindbis Virus E2 Endodomain, including a proposed capsid interacting domain (Based on data presented in the text).
amino acid 170-250 (19, 46). It has been proposed that this is the site of binding for the E2 endodomain during the process of envelopment.

Many studies have been directed at determining the nature of the E2-capsid interaction and which residues from each protein are involved in binding (11, 24, 35, 36, 44, 49). None of these studies, however, have determined with certainty which residues in either the E2 tail or the capsid cleft are responsible for the interaction of these two proteins. A large portion of the carboxyl terminus of the E2 endodomain is initially buried in the membrane of the ER and is exposed to the cytoplasm after export from the ER but before arrival at the cell surface (23). Previous studies have suggested a model for a conformational change in the capsid protein which accompanies binding of the E2 tail (19). Iodination of nucleocapsids isolated from virus particles identified one exposed tyrosine at on the surface of the nucleocapsid at position 180 (9). This tyrosine was not found to be exposed in the crystal structure of the capsid protein (19). These data were used to develop a model which proposed that in the mature virus particle, the nucleocapsid was bound to the E2 tail and the tyrosine at 180 was internalized into the hydrophobic cleft (19). In the absence of the E2 tail, tyrosine 180 becomes exposed on the surface of the capsid protein (9). We have used this model to confirm that a conformational change occurs in the capsid protein in response to association with the E2 endodomain. We have used the ability to induce this conformational change in the capsid protein to identify the region of E2 that would be responsible for associating with the capsid cleft. This experiment identified E2 amino acids 408-415 as the most critical region of the E2 endodomain involved in nucleocapsid binding. This region of the E2 endodomain was then targeted for mutational analysis. Deletions and substitutions
were made in this region and then analyzed in both mammalian cells and insect cells for their effect on virus assembly and infectivity.

### 3.3 Materials and Methods

**Cells, viruses, and media**

Baby hamster kidney (BHK-21) cells were maintained in Eagle’s minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 5% tryptose phosphate broth, and 2mM glutamine as described previously (40). The cells were maintained at 37°C under 5% CO₂. The U4.4 cells used were cloned from cells provided by Sonya Buckley (Yale Arbovirus Research Unit, New Haven, Conn.). This cell line is maintained in Mitsuhashi and Maramorosch (M&M) medium(29). U4.4 cells are grown at 28°C under 5% CO₂. The wild-type Sindbis virus cDNA used in these studies was the SV Toto 1101 (41, 47).

**Iodination of capsid protein**

Purified capsid protein from amino acids 106-264 expressed in E. coli (a generous gift from Richard Kuhn, Purdue University) was used as the capsid source. Peptides tested included the full length 33 amino acid endodomain, 391-KARRECLTPYALAPNAVPISTSLALLCCVRSANA-423 and truncated peptides from this sequence, 408-IPTSALALLCCVRSANA-423, 416-CCVRSANA-423, 400-YAL-402, 399-PYALAP-404, 391-KARRECLTPYAL-402, and a control peptide of an unrelated sequence of moderate hydrophobicity NH₂-ILEPVHGV-COOH. These peptides were synthesized and purified at California Institute of Technology and were provided by Dr. James Strauss. All
peptides were resuspended in 100% DMSO at a concentration of 1 mg/ml as this was the condition required to dissolve the full length 33mer. The 3mer and 6mer were added in a 1000 fold molar excess to the capsid protein. All other peptides were added at a 10 fold molar excess. Capsid protein 106-264 (10µg) was used in all binding reactions. Capsid was bound to peptides by incubation in TEN (50 mM Tris pH 7.6, 50 mM NaCl, and 1 mM EDTA) buffer. All capsid-peptide binding reactions were adjusted to 40% DMSO which was the final concentration of DMSO present in the binding reaction with the full length 33mer. The capsid-peptide binding reaction was allowed to proceed slowly at 4°C overnight. Capsid–peptide complexes were removed from unbound peptide in centricon Amicon 10 spin centricron (Millipore, Bellerica, MA) by washing 4X each with 2 ml TEN buffer. As a control for the possibility that the denatured capsid protein could expose Y 180 to iodination, C was denatured in 8M urea. In this reaction 10µg of C in TEN was added to 8M urea and incubated at 37°C for 5 hr. The denatured C was washed 3 X with 2ml TEN in an Amicon 10 centricon and brought up to 40µl with TEN prior to iodination. The iodination reaction was essentially as described by Coombs (9). In brief, to 40µl of capsid-peptide complex was added to 40mM glucose, 200 µCi [125I] (1227Ci/mM, Perkin Elmer, Irvine), 0.02 U glucose oxidase (diluted in PBS, 0.9 mM CaCl2, 1.4 mM KH2PO4, 0.5 mM MgCl2 6H2O, 136 mM NaCl, 7.9mM) (Worthington biochemicals, Lakewood, NJ), and 0.01 U lactoperoxidase (diluted in PBS), (Worthington biochemicals, Lakewood, NJ) and brought up to a total volumn of 63µl in TEN buffer. Iodination was terminated by the addition of 25µl of stop buffer consisting of 10 mg/ml tyrosine, 10% glycerol and 0.1% xylene cyanol incubated at room temperature for 30min. Unincorporated label and any labeled free peptide was removed from the protein-peptide complexes by passing the protein through a 5 ml
Sephadex G-25 (Sigma, St. Louis, MO) column. Column matrix was swollen in PBS with 0.5% BSA and washed with 10 column volumes of PBS. A V-8 (endoproteinase-Glu-C) digest was made of the Capsid-peptide complexes by adding V-8 (dissolved in H2O) to a final concentration of 40µg/ml. The digest was allowed to proceed for 3 hr. at 35°C. Peptides generated from the V-8 digest were run on 30 cm Tricine slab gels (25) at 30 mA for 19 hrs.

**Site-directed Mutagenesis of Toto 1101 and RT-PCR**

Using standard megaprimer site-directed mutagenesis protocols (45) and *Pfu* DNA polymerase, substitution and deletion mutants were generated within the E2 endodomain of Sindbis virus. Substitutions were made at the following positions in the E2 endodomain; 408I, 410T, 411S, and 413A. Positions 408, 410, and 411 were each mutated to alanine (I408A, T410A, and S411A) while the alanine at position 413 was mutated to glycine (A413G). Deletions were made at positions E2 406-407, 409-411, and 414-417. Primer pairs used in creating the deletions were as follows; (406-407) (sense) 5’-GCCCTGGCCCCAACATCCCAAACCTTCGCTGGC-3’ (antisense) 5’-GCCAGCGAAGTTGGGATGTTTGGGGCCAGGGC-3’, (409-411) (sense) 5’-CCCAAACGCCGTAATCCTGGCAGCTTGTGC-3’ and (antisense) 5’-GCACAAGAGTTGGGGATTACGGCGTTTGGG-3’, (414-417) (sense) 5’-CCCAAACGCCGTAATCCTGGCAGCTTGTGC-3’ and (antisense) 5’-GCACAAGAGTTGGGGATTACGGCGTTTGGG-3’. PCR products from the mutagenized plasmid were placed into the wild-type vector using unique *BssH II* and *BsiW I* sites. After
confirmation of the correct sequence throughout the insert, infectious RNA was transcribed in vitro using SP6 polymerase and introduced into cells by electroporation as described below.

To analyze the mutant viruses produced from transfection for the retention of the original mutations and for the presence of pseudorevertants RT-PCR was used. Virus from transfections was pelleted at 50,000 rpm for one hour. The pellet was then incubated in 100µl of Tris-EDTA (10 mM Tris pH 8.0, 1 mM EDTA) and 100µl of 2X lysis buffer (100 mM Tris-Cl (pH 7.0), 20 mM EDTA, 1% SDS) for twenty minutes at 37°C (vortexing every 5 minutes). The lysed virus was then extracted sequentially with phenol (twice), phenol/chloroform (once), and chloroform (once). Extracted RNA was precipitated in RNase-free ethanol overnight at -80°C. RNA was pelleted and resuspended in 10µl of DEPC (diethyl pyrocarbonate) treated water. RNA was transcribed using MuLV reverse transcriptase (RT) (Applied Biosystems, Foster City CA) under the following conditions. The reaction contained 10X PCR buffer (10 mM Tris-Cl (pH 8.3), 50 mM KCl, and 5 mM MgCl₂), 20 U of SuperRNasin™ (Ambion, Austin, TX), 200 µM each deoxynucleoside triphosphates (dNTPs) (Roche Diagnostics, Indianapolis, IN), 1.0 µM reverse primer, and 50 U of MuLV RT in a final volume of 20 µl. Reverse transcription was performed at 42°C for 20 min and 99°C for 5 min. After transcription, the volume of the reaction mixture was increased to 100 µl and the concentrations of dNTPs and MgCl₂ were adjusted for the increased volume. Sense primer was added to a final concentration of 2.0 µM, and an additional 1.0 µM antisense primer was added. Taq DNA polymerase was added to a final concentration of 2.5 U/100-µl reaction mixture.
In vitro transcription, RNA transfection, and plaque assay

Full-length mutant and wild-type cDNA’s were linearized using the enzyme Xho1, treated with proteinase K, phenol extracted and ethanol precipitated. The templates were transcribed as described previously (15, 41). The infectious RNAs were transfected by electroporation essentially as described by Liljestrom and Garoff (21). Briefly, BHK cells were trypsinized, pelleted and washed in RNase free phosphate buffered saline (pH 7.0). Washed cells were resuspended to a concentration of 1x 10^7 cells/ml. RNA transcripts (20 µl) were added to 400 µl of BHK cells. The electroporation conditions were 1.5 kV, 25 µF, and ∞ resistance. Cells were pulsed once, allowed to sit for ten minutes, and then transferred into 10 ml of MEM medium (no gentamicin) in 25cm² tissue culture flasks. Virus was harvested at 24 hrs post-transfection and flash frozen in liquid nitrogen in 1 ml aliquots and stored at -80°C. To determine the titer of each virus plaque assays were carried out as described previously (40). All virus titrations were done on BHK-21 cells.

Transfection of U4.4 (Aedes Albopictus) cells

RNA transcripts of each of the mutants were prepared as described above. Cells were pelleted and washed 3X in RNase free HBS buffer (20mM Hepes, 137mM NaCl, 5mM KCl, 0.7mM Na₂HPO₄, and 6mM D-Glucose). Washed cells were resuspended to a concentration of 5 x 10⁷ cells/ml. RNA transcripts (20µl) were added to 400 µl of cells. The electroporation conditions were 1.5kV, 25µF, and ∞ resistance. The cells were pulsed once and allowed to sit at room temperature for ten minutes. After ten minutes the mixture was transferred to a 25cm² vented flask and M&M growth media was added in a drop wise
fashion. Transfections were incubated at 28°C under 5% CO₂. Virus was harvested at approximately 48 hrs post-transfection.

**Immune Precipitation of Cells transfected with E2 Endodomain deletion mutants**

Anti-whole virus antibody was produced in rabbits and Sindbis virus specific IgG was purified using a Hi Trap, protein-A column (Amersham Pharmacia Biotech, Piscataway, NJ). Metabolically labeled transfections of BHK-21 cells were carried out as described above. At 6.5-hrs post-transfection, 5 ml of fresh media containing 4 μg of actinomycin-D/ml (Act-D) (Calbiochem, San Diego, CA.) was added to 25-cm² flasks of cells (≈5 × 10⁶ cells) and incubated at 37 °C for 1 hr. The flasks were then washed with 5 ml of room temperature PBS-D and placed into 5 ml of starvation medium (MEM deficient in methionine and cysteine, supplemented with 2 mM glutamine, 3% FBS) and returned to 37°C for 1 hr. The transfected cells were then labeled with 50 μCi of [³⁵S] methionine-cysteine (Met/Cys)/ml in 5 ml of starvation medium and incubated at 37°C overnight or until advanced cytopathic effect (CPE) was visible. The labeled virus (media) was removed and stored 4°C. The cells were washed once with cold 1X-PBS-D. Labeled cell associated proteins were processed for immune precipitation as described previously(34). Two μl of anti-whole virus antibody was added to the cell supernatants and rocked at 4°C. To the cell-antibody suspension 200 μl of Protein-A beads was added and allowed to rock overnight at 4°C. Polyacrylamide gel electrophoresis (PAGE) analysis of the supernatant from the precipitation and the labeled media was done to confirm the efficiency of the antibody to remove viral proteins.
Transmission electron microscopy

Samples were analyzed by TEM as described previously (38). BHK-21 and Aedes albopictus cell monolayers were transfected with RNA transcribed from either wild type or each of the E2 endodomain deletion mutants (Δ406-407, Δ409-411, and Δ414-417) as described above. Approximately 18-20 hrs post-transfection for BHK cells and 48 hrs. for mosquito cells, the media was harvested and the cell monolayers were scraped from the flasks and pelleted by low-speed centrifugation. Cell pellets were washed twice with PBS-D and fixed with 3% glutaraldehyde (Ladd Research Industries, Williston, VT) in 0.1M cacodylic acid buffer (pH 7.4) (Ladd Research Industries, Williston VT). After the cells were washed three times with 0.1 M cacodylic acid, the cells were stained with 2% osmium tetroxide in cacodylic buffer for 1 hr. Cells were then washed as before and embedded in 2% agarose. The agarose containing the cell sample was then prestained with 1% 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 trimmed on an LKB NOVA Ultrotome (Leica Microsystems, Inc. Deerfield, IL). Ultra-thin sections were then obtained and were stained with 5% uranyl acetate in distilled water for 60 min and in Reynolds lead citrate (pH 12) (Mallinkrodt Baker Inc., Paris, KY.) for 4 min. The samples were examined at 80 kV in a JEOL JEM 100S transmission electron microscope.
3.4 Results

Protection of capsid protein residue Y180 by E2 endodomain peptides

Sindbis virus capsid protein contains four tyrosine residues at positions 162, 180, 189, and 198. One property of tyrosine is its ability to be iodinated by the enzyme lactoperoxidase. In 1987, Coombs determined that only one of these four tyrosine residues (Y180) is accessible to lactoperoxidase iodination in the intact nucleocapsid and is thus exposed on the surface of the protein in the intact core structure (9). The structure of the capsid protein determined by X-ray crystallography suggested that the tyrosine at position 180 could exist in two conformations (19). In the absence of association with the E2 endodomain, Y180 is exposed on the surface of the protein but in association with the E2 endodomain Y180 is buried in the protein complex. These observations allowed us to develop an assay for the interaction of the E2 endodomain with the capsid protein based on the ability of amino acid sequence derived from the endodomain to protect the tyrosine at position 180 from iodination as described in Methods. Protection studies were performed using peptides of differing lengths, corresponding to selected regions of the E2 endodomain, and purified capsid protein. E2 peptide and capsid protein were mixed, and lactoperoxidase and [125I] were added as described in Methods. The reaction was quenched, incubated with V-8 protease and then analyzed by Tricine gel electrophoresis as described previously (25). We have previously shown that this treatment releases a peptide containing capsid protein Y180, in the peptide 177-AFTYTSE-183 (9) from purified nucleocapsids. The in vitro experiment revealed that in the presence of a peptide corresponding to the full-length E2 endodomain, capsid protein residue Y180 could not be labeled by lactoperoxidase
(protected), while in the absence of E2 peptide it was exposed to labeling by $[^{125}\text{I}]$ (exposed) as shown in figure 3.2. Table 3.1 shows the results obtained when this experiment was conducted with a number of peptides representing fragments of the E2 endodomain. This experiment revealed that the conformational change in capsid protein resulting in the protection of capsid Y180 could be obtained with the full length endodomain. Progressive truncations of the endodomain revealed that the minimum domain needed for protection of Y180 was a peptide containing amino acids 408-415, in the carboxyl portion of the E2 tail. Surprisingly, peptides containing the conserved TPY (aa398-400) sequence, in the absence of the carboxyl portion of the E2 endodomain, did not produce the conformational change resulting in the protection of Y180. It has been proposed that the Y400 in the TPY sequence interacted with Y180 and W247 in the capsid and that this interaction was critical for virus assembly and virus infectivity (46).
Figure 3.2 Iodination of Capsid residue Y180 as an indicator of E2 protein binding. Capsid protein was iodinated in the presence (A) or absence (B) of a peptide representing the 33 amino acid E2 endodomain. The reaction was quenched and then treated + or – with V8 protease to release a peptide containing capsid Y180.
Table 3.1 Protection of Capsid residue Y180 upon association with peptides corresponding to fragments of the E2 Endodomain

<table>
<thead>
<tr>
<th>E2 Endodomain Peptides</th>
<th>Protection of Capsid Y180</th>
</tr>
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<tbody>
<tr>
<td>391- KARRECLTPYALAPNAV IPTSLALLCCVRSANA -6K</td>
<td>YES</td>
</tr>
<tr>
<td>ILEPVHG (control)</td>
<td>NO</td>
</tr>
<tr>
<td>391- KARRECLTPYAL</td>
<td>NO</td>
</tr>
<tr>
<td>YAL</td>
<td>NO</td>
</tr>
<tr>
<td>PYALAP</td>
<td>NO</td>
</tr>
<tr>
<td>CCVRSANA -6K</td>
<td>NO</td>
</tr>
</tbody>
</table>
**Construction of E2 endodomain mutants**

The peptide protection experiments presented above revealed that the amino acid sequence from 408-415 in the E2 tail was required for the interaction with capsid protein in the *in vitro* assay. To elucidate the role that this sequence plays in virus assembly and function, mutations were created in this region. Both substitution and deletion mutants were produced as described above. Amino acid substitutions were made at four positions within the targeted domain; I408A, T410A, S411A and A413G. These positions were selected because they had the highest degree of conservation among the alphaviruses in the targeted region of E2, aa 408-415. Deletion mutants were made removing residues delta406-407 (AV), delta409-411 (PTS), and delta414-417 (LLCC). We hypothesized that deletions in the E2 tail would further aid in defining the critical E2-capsid interactions which take place during virus assembly. The mutants produced cover the entire region of the E2 endodomain identified in the peptide protection experiments (described above) as a domain which recognizes the capsid protein. As the COOH terminus of the E2 endodomain is initially integrated into the ER membrane, deletion and substitution mutants in this region were also expected to elucidate the role these amino acids play in this initial event in membrane protein assembly.

**Growth of E2 endodomain substitution mutants in BHK cells**

To determine the effect of amino acid substitutions in the E2 endodomain on virus assembly and function, viral RNA was produced from constructs containing the mutations by *in vitro* transcription and transfected into BHK cells as described in Methods. Wild type SV produced an average of 2x10⁹ pfu/ml of virus from BHK cells and the mutants A413G and
S411A produced no change in virus production compared to wild type (figure 3.3). However, the mutants T410A and I408A showed a reduction in the amount of infectious virus produced. I408A produced $1 \times 10^6$ pfu/ml and T410A produced $1 \times 10^8$ pfu/ml (figure 3.3). With the exception of the mutant A413G all of these substitutions introduce changes in the atomic density of the E2 endodomain. The alanine residues that were substituted into the E2 tail are less bulky than the wild type amino acids that are present; isoleucine (408), threonine (410), and serine (411). The observation that these changes, with the exception of mutant I408A, produce little reduction in infectious virus production and no alteration in protein production (data not shown) suggest that the precise amino acid sequence of this portion of the E2 endodomain is not critical in assembly of the virion nor does it play a significant role in virus function. It does indicate that as substitutions are made closer to the membrane bilayer, there is a more deleterious effect on the production of infectious virus. In addition it suggests that position 408 is more critical for infectious virus production than the positions downstream (410, 411, and 413) that were also analyzed. From these data and data published by others (11, 16, 35, 43) it seems that many single amino acid substitutions do not modify the endodomain structure to significantly disrupt assembly.
Figure 3.3 Production of virus after transfection with virus RNA containing substitution mutations in the putative capsid binding domain. Titers were determined on BHK cells as described in Materials and Methods.
Growth of E2 endodomain deletion mutants in mammalian cells

We have previously demonstrated that the deletion of a single amino acid at the point where the E2 endodomain emerges from the membrane bilayer (E2 K391) blocked virus production from mammalian cells (14). We concluded that this deletion reduced a critical distance between the membrane bilayer and a sequence in the endodomain which interacted with the capsid protein. Deletions in other regions of the E2 endodomain have not been examined for their effect on the assembly and growth of Sindbis virus.

To determine the effect of deletions in the C-terminal portion of the E2 endodomain (identified by the C-Y180 protection assay) on virus assembly and infectivity, viral RNA from endodomain deletion mutants delta406-407, delta409-411, and delta414-417 was transcribed and transfected into mammalian cells (figure 3.4). Virus production from wild type and mutant constructs was assayed as described. Mammalian cells transfected with wild type viral RNA produce a titer of $2 \times 10^9$ pfu/ml, however all three deletion mutants showed a reduction in infectious virus production. The mutant delta406-407 produced an average of $4 \times 10^7$ pfu/ml of virus in BHK cells. This reduction in infectious virus production indicates that positions 406 (alanine) and 407 (valine), are important for virus assembly and function. These deletions are immediately upstream of the I408A substitution which was also found necessary for virus production. The deletion mutations delta409-411 (deletion of PTS) and delta414-417 (deletion of LLCC) both completely blocked the production of infectious virus from mammalian cells (figure 3.4).
Figure 3.4 Production of infectious virus from BHK cells transfected with virus RNA containing deletions in the putative capsid binding domain. Titers were determined on BHK cells as described in Materials and Methods.
This result indicates that these motifs are absolutely required for assembly and production of infectious Sindbis virus in BHK cells. It is noteworthy that P409 and 415-417 (LCC) are conserved among the alphaviruses. Substitutions at P409 and CC 416 and 417 severely limit virus production (11). These data in combination with the observation that virus production was only moderately affected when single amino acids in this region were substituted in this study indicates that the length of the carboxyl end of the E2 endodomain as well as the strictly conserved sequence is a critical factor in virus assembly in mammalian cells.

**Protein synthesis in BHK cells transfected with E2 endodomain deletion mutants**

We have established that no infectious or non infectious virus is produced from two of the deletion mutants (delta409-411 and delta414-417) and that the deletion mutant delta406-407 produces significantly less infectious virus from vertebrate cells compared to wild type (shown in figure 3.3). In order to determine if defective protein processing is the reason for the complete loss of virus production from BHK cells, cells were transfected with each of the deletion mutants and analyzed for virus protein production, processing and release.

Sindbis virus membrane proteins are integrated into the membranes on the endoplasmic reticulum where they follow a complex pathway of folding and oligomerization to produce a heterotrimer which is exported from the ER to the plasma membrane (1, 5, 31-33). The ER controls for the production of correctly folded and assembled proteins and only those proteins deemed correctly assembled are shipped to the cell surface. En route to the cell surface the PE2 precursor is proteolytically processed to E2 by Furin, a resident protease in the Trans Golgi Network. The product of this proteolytic event is the membrane associated
E2 and the 16kD E3 protein which is secreted. The production of E3 by a virus or mutant virus indicates that the proteins have passed the quality control of the ER and have been exported to the Golgi apparatus. We examined the proteins secreted from cells transfected with wild type Sindbis and the deletion mutants to assay the production and export of proteins from the ER.

Virus proteins released into the surrounding medium were analyzed by PAGE using equal volumes of cell supernatant as described in Materials and Methods and shown in figure 3.5. BHK cells transfected with both the wild type and the E2 deletion mutant delta406-407 produced a normal complement of virus proteins. Equal volumes of supernatant also revealed equal amounts of the E3 glycoprotein produced by wild type and the double deletion. BHK cells transfected with both delta409-411 and delta414-417 also produced amounts of E3 equal to the wild type infection; however, neither of these mutants released significant amounts of the virus structural proteins E1 or E2 into the media. The mutant delta409-411 did release capsid protein into the media but there was little indication of the presence of E1 or E2, whereas delta414-417 showed little to no release of any structural proteins including capsid (figure 3.5). This result is supported by electron microscopy described below.
Figure 3.5 Proteins released into the media of BHK cells transfected with E2 Endodomain deletion mutants. Equal volumes of media surrounding equal numbers of transfected cells was applied directly to PAGE. Arrows indicate positions of virus structural proteins E1, E2, Capsid and E3.
These results suggest that these deletions in the COOH terminus of the E2 endodomain (delta406-407, delta409-411 and delta414-417) do not affect the insertion of the polyprotein or the processing of the structural proteins. To determine if mature, functional E1 protein was present at the cell surface, BHK cells transfected with deletion mutants delta409-411 and delta414-417 were subjected to analysis by fusion from within. Cell-cell fusion from within (27) was observed for both large deletion mutants which indicated that functional E1 protein was present at the cell surface (data not shown) in a conformation allowing for low pH induced fusion. This further suggests that there are no processing or transport defects in mammalian cells transfected with these deletions (delta409-411 and delta414-417). Collectively these data suggest that the defect in the E2 deletion mutants, specifically delta409-411 and delta414-417, is most likely in the ability of E2 to bind the nucleocapsid.

**Electron microscopy of BHK cells transfected with E2 endodomain deletion mutants**

To confirm that the defect in the E2 deletion mutants was in the ability to bind nucleocapsids to modified cell membranes, we analyzed transfected cells by electron microscopy. BHK cells transfected with wild type SV showed nucleocapsids attached to membranes, nucleocapsids at the plasma membrane and virus particles budding from the plasma membrane as is typical in a mammalian cell infection (figure 3.6A). Budding viruses were also observed in cells transfected with the E2 deletion mutant delta406-407 as expected based on the amount of infectious virus produced. However, a significant number of nucleocapsids were seen free in the cytoplasm with this double deletion mutant (figure 3.6B). This result suggests that the decrease in the amount of infectious virus production from this mutant is due, in part, to a reduced ability of the E2 endodomain to bind nucleocapsids.
resulting in an accumulation of non membrane associated capsids. The electron micrographs of cells transfected with deletion mutants delta409-411 (3.6C) and delta414-417 (3.6D & E) support the conclusions derived from PAGE analysis of proteins secreted from the infected cells. No virus particles were observed budding from the plasma membrane and none of the assembled nucleocapsids were attached to internal membranes, and no nucleocapsids were associated with the plasma membrane. All of the nucleocapsids produced by the triple and quadruple deletions were found free in the cytoplasm. These observations support the conclusion presented above that the defects in these deletion mutants are in the ability of the E2 endodomain to bind the preassembled nucleocapsid.
Figure 3.6 Electron micrographs of BHK cells transfected with E2 endodomain deletion mutants. A, wild type; B, delta406-407; C, delta409-411; and D, delta414-417. E is an enlargement of an area in D. Arrows indicate nucleocapsids. Magnification bars =200nm.
**Electron microscopy of insect cells transfected with E2 endodomain deletion mutants**

Sindbis virus is an Arbovirus and as such must be capable of assembling virus in cells of both vertebrate and invertebrate origins (3). We have previously shown that deletions in the transmembrane domain of the E2 glycoprotein could restrict the ability of Sindbis to grow to the invertebrate host (14, 15). We suggested that this was a result of the different chemical and physical properties of the cell membranes of the two hosts. These observations suggested that the deletions which prevented the formation of virus particles in mammalian cells may be tolerated to some degree in the invertebrate cells as the targeted domain is also, initially, a transmembrane domain (23). Insect U4.4 cells transfected with wild type virus produced $5 \times 10^9$ pfu/ml. The phenotype of the E2 endodomain deletion mutants in insect cells mimicked the phenotype observed in mammalian cells (figure 3.7). The delta406-407 mutant produced an average of $1 \times 10^8$ pfu/ml of virus and both the delta409-411 and delta414-417 mutants produced no infectious virus from U4.4 (*Aedes albopictus*) cells. These data are similar to the results obtained in BHK cells (figure 3.4) and confirm that these domains are vital for the production of infectious virus in insect cells as well as mammalian cells.
Figure 3.7 Growth of E2 Endodomain deletion mutants in Insect cells. *Aedes albopictus* (clone U4.4) cells were transfected with wild type or mutant contain virus RNA as described in methods.
To determine if the failure to produce infectious virus in insect cells was also the result of a failure to assemble virus particles as in BHK cells, U4.4 cells transfected with these deletion mutants were analyzed by electron microscopy. We have previously shown that Sindbis maturation in insect cells takes place in internal vesicles rather than at the plasma membrane (3, 12, 28) with some cells displaying large numbers of matured virus particles. The virus contained in the vesicles is released into the media by a process resembling exocytosis in which the vesicle membrane fuses with the cell membrane releasing the contents into the surrounding medium (3, 12, 28). In agreement with the data showing production of infectious virus cells infected with wild type Sindbis or the double deletion (406-407) mutant virus particle were seen within vesicles as is typical of infection of insect cells (figure 3.8A only wild type shown).

Transfections of insect cells with the larger deletions (409-411 and 414-417) produced a remarkable result. Although no infectivity could be recovered from these transfections, large numbers of virus like particles were seen within vesicles for both of the deletion mutants (figure 3.8 B & C only 409-411 shown). Insect cells transfected with wild type virus or the double mutant contained relatively low amounts of cell associated virus whereas cells infected with the triple and quadruple deletions contained very large numbers of cell associated virus like particles (figure 3.8 B&C). This result may suggest that release of the virus like particles is impaired in the triple and quadruple deletion mutant infected cells. We have been unable to recover any virus infectivity (determined by assay on BHK cells) and attempts to release infectious virus from these cells by disrupting the cells also failed. For this reason we refer to these structures as virus like particles. These results suggest that the virus produced in the triple and quadruple mutant transfected cells is non
infectious for BHK cells. The virus like particles seen in the insect cells transfected with the triple and quadruple deletion mutants was not seen in the wild type, double deletion or mock transfected cells. Some of these particles are not typical in morphology although they are consistent in size at 670 to 700 angstroms in cross sections as is wild type Sindbis (figure 3.8 B&C). Some of the particles appear to lack the electron dense centers typical of thin sectioned Sindbis virus. More of these virus like particles were seen in the triple deletion than in the quadruple deletion transfected cells suggesting that assembly of the virus like particles may have been more impaired by the larger deletion. These virus like particles were found to be very fragile as attempts to purify them from cell homogenates by gradient centrifugation was not successful.
Figure 3.8 Electron micrographs of *Aedes albopictus* cells transfected with E2 endodomain deletion mutants. A, wild type (arrows indicate virions); B & C, delta409-411. Magnification bars =100nm
3.5 Discussion

Previous studies have indicated that the E2 endodomain specifically interacts with a hydrophobic cleft in the surface of the Sindbis virus nucleocapsid (6, 17, 19, 24, 35, 50). This interaction is one of the initial steps in the process of virus envelopment at the plasma membrane and is critical in the formation of a mature, infectious virus particle. Attachment of the E2 endodomain to the nucleocapsid is a primary function of this multifunctional domain. Imbedded within the E2 endodomain is the signal sequence for insertion of the 6K protein into the ER membrane (21). The C-terminal end of the E2 endodomain also contains the signalase recognition motif necessary for removal of 6K protein from the polyprotein.

The binding of the E2 endodomain to the preformed nucleocapsid appears to be a two step event (22). The first step involves binding of the C-terminal portion of the endodomain to the nucleocapsid followed by a reorientation of the E2 endodomain within the capsid cleft in which E2 Y400 is positioned in close proximity to C Y180 and W247(19). The mutants analyzed in this paper were targeted to a specific region of the E2 tail identified by in vitro peptide binding experiments, specifically the domain encompassing residues 408-415 within the E2 tail. This is also the region predicted to be involved in the initial step of capsid binding.

Substitutions of single amino acid residues in the E2 endodomain (I408A, T410A, S411A, and A413G) had little effect on the ability of Sindbis virus to assemble and produce infectious particles. Two of the substitutions, at positions 408 and 410, had the greatest effect, a reduction of two to three orders of magnitude and one order of magnitude respectively in virus production. However substitutions at positions 411 and 413 had no effect on infectious virus production. Additionally, none of these substitution mutants were
defective in virus protein production or processing. Despite the fact that three of the four substitutions introduced significant changes in the atomic density of the E2 endodomain little change in virus production was noted indicating that the sequence in this region of the E2 endodomain is not a critical factor in the interaction with the nucleocapsid.

If sequence is not a determining factor in the binding of the E2 endodomain the length of the tail could be a determining factor in binding. The deletion mutants in this study shorten the length of the E2 tail by two, three, and four amino acids respectively ($\Delta_{406-407}$, $\Delta_{409-411}$, and $\Delta_{414-417}$). It was our hypothesis that these deletions in the E2 endodomain would interfere with the initial step in E2-nucleocapsid binding and that these deletions might also aid in identifying the region of the endodomain which interacted with ER membrane during the initial stages of polyprotein translocation. The phenotypes of these deletion mutations might also aid in elucidating the role of the composition of the host derived lipid bilayer in the integration of the polyprotein into the host membrane and how this domain participates in the assembly of infectious virions.

The deletions described herein are located in a region of E2 that is predicted to initially be buried in the membrane of the ER when the polyprotein is inserted (23). It is not known at what point in the secretory pathway this domain is extracted from the membrane however extraction occurs after export from the ER but prior to arrival at the plasma membrane (23). Extraction of this domain and the proteolytic processing of PE2 produce a mature E2 protein whose primary function is to associate with the preassembled nucleocapsid. We have observed that the larger deletions of three and four amino acids expressed in mutants delta409-411 and delta414-417 respectively, are lethal mutations. The larger deletion mutants prevent infectious virus production in both mammalian and insect
cells, while the smaller deletion, delta406-407, produces infectious virus from both cell types. Electron microscopy has revealed that these mutants do assemble nucleocapsids in mammalian cells, however they are not capable of binding membranes and the assembled nucleocapsids are found free in the cytoplasm. It is possible that the defect in capsid binding seen with these deletion mutants is failure to attach nucleocapsids to the truncated endodomain or a failure to extract the E2 tail from the membrane and expose it to the cytoplasm. This hypothesis will be tested using a clone of Sindbis virus that allows the extraction of the tail to be monitored (23).

In mammalian cells we observed that nucleocapsids are assembled inside of cells and that the E3 protein is released into the media in equivalent amounts for each of the deletion mutants. This indicates that deletions made in the C-terminal portion of the E2 endodomain (specifically between amino acids 406 and 417) have little effect on the insertion, processing, transport of the structural proteins of Sindbis virus in mammalian cells. Collectively, the evidence indicates that amino acids 406-417 in the E2 endodomain are a critical specifically for nucleocapsid envelopment in BHK cells.

The deletion mutants described above were found to display a phenotype similar to that seen in mammalian cells for the production of infectious virus when transfected into insect cells. Significant infectious virus production was observed for the double deletion but in the case of the larger deletions no infectious virus was produced. However, electron microscopy revealed that assembled virus particles, or virus like particles were present within vesicles of insect cells infected with each of the deletion mutants. This indicates that the larger of these deletion mutants can assemble virus like particles in the insect cells, a circumstance not seen in mammalian cells. It is possible that the difference in membrane
composition between insect and mammalian cells is responsible for this contrastive result. Mosquito membranes do not contain cholesterol which, among other effects, makes them significantly thinner than cholesterol rich mammalian cell membranes (2, 8, 26, 30). This feature may allow a shortened membrane spanning domain to be successfully inserted into the membranes of these cells but not the membranes of mammalian cells. Although virus like particles can be identified in insect cells infected with the larger deletions, no infectivity can be recovered from these cells indicating that the assembly process is defective in some important and unknown aspect. Collectively these observations support the notion that there may be a more fundamental difference in the assembly of Sindbis virus in insect cells compared to the assembly of Sindbis virus in mammalian cells than previously thought.

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3.6 References


Chapter 4: Conclusions and Future Directions

Understanding the interaction between the E2 protein and the nucleocapsid core of Sindbis virus is critical for understanding the process of virus assembly. The association between the endodomain of E2 and the nucleocapsid core is one of the initial events in virus envelopment and is the driving force in budding of mature particles. In the studies discussed above, we have used both deletion and substitution mutations targeted to specific domains of the E2 endodomain to investigate the association between this domain and the Sindbis virus nucleocapsid.

The E2 endodomain serves several purposes in the maturation process of SV. It contains the signal sequence for the insertion of the 6K protein during translocation. It also contains the signal peptidase recognition sequence used by signal peptidase to release the 6K protein from the precursor polyprotein. However, the primary function of the endodomain is to bind to the nucleocapsid during assembly and drive the process of envelopment. The specific association between the endodomain and the capsid protein has been well studied, but presently the requirements for binding have not been identified. It has been proposed that the conserved ‘TPY’ domain is critical in this interaction. We have employed a double mutation in this domain to further define its role in capsid binding. In addition, previous research has also involved analysis of substitutions throughout the remainder of the E2 endodomain and their affect on virus assembly. There have also been several studies analyzing mutations within the capsid protein that have implicated a specific region of the capsid protein in the binding process. Unfortunately, none of these studies have identified any particular domain within the E2 tail as being primarily responsible for the association with the capsid protein.
The ‘TPY’ domain is the only conserved three amino acid domain within the E2 endodomain of alphaviruses, and therefore has been proposed to play an important role in the interaction of the endodomain with the nucleocapsid. We examined the role of this conserved domain using a double mutant T398A/Y400N. This mutation both prevents phosphorylation and removes the aromatic Tyrosine residue from the domain. Both of these functions have been proposed to be important in the E2-nucleocapsid interaction (Liu & Brown, 1993; Lee et al., 1996). The results of our studies indicate that this domain plays a critical role in virus maturation. Our studies show that this domain is not critical for virus assembly, but absolutely required for virus infectivity. This mutant produced assembled particles that sediment to wild type density, produced an equivalent amount of protein as wild type virus, and assembled particles containing no obvious aberrant structural changes.

These results suggest that phosphorylation is not required for the assembly of Sindbis virus; however it could play a role in determining the infectivity of an assembled particle. Also, the aromatic association that has been proposed between the Y400 in the E2 endodomain and the Y180 and W247 in the capsid protein is not required for assembly of Sindbis virus. However, this interaction may also play a role in determining the infectivity of the mature particle. Overall, these results show that the conserved ‘TPY’ domain is critical for the function of the assembled particle.

In the future substitution or deletion of the entire ‘TPY’ domain may further elucidate the role of this domain in SV assembly and function. Additionally, replacement of the Y400 with a different aromatic amino acid may aid in determining if E2-nucleocapsid binding is affected by the presence of an aromatic residue at that position. This would lend credence to the proposal that the E2 endodomain interaction with the nucleocapsid cleft is partially
stabilized by the strength of this bond. Lastly, similar investigations of the role of this conserved domain should be done using related viruses such as Semliki Forest virus and Ross River virus.

In addition to examining the role of the ‘TPY’ domain further studies were conducted using both substitutions and deletions within a specific domain in the carboxy terminal half of the E2 endodomain. An examination of previously published data up to this point reveals no trend in the defects seen in assembly when substitutions have been made in the E2 endodomain. Several mutations in the amino terminus have proven to be detrimental to virus assembly, as well as a few of the mutations in the carboxy terminal half of the tail. On the other hand, substitutions have been made in the tail that have little or no effect on the assembly of Sindbis virus. As a whole, single substitutions in the E2 endodomain have not proven to be efficient enough in disrupting virus assembly to determine, with any specificity, the nature of the interaction between the endodomain and the capsid protein.

Prior to selecting sites within the endodomain for substitution or deletion, we first attempted to determine if any particular region of the E2 tail was more critical for inducing the conformational change necessary for capsid binding. Peptide protection experiments revealed that a domain within the E2 tail extending from aa 408-415 was critical for inducing the conformational change needed to protect capsid residue Y180. Based on these data, we created a battery of substitution mutations within this domain to test the hypothesis that this domain contains the amino acids critical for binding the E2 endodomain to the nucleocapsid during assembly. Substitutions were made at positions 408 (I to A), 410 (T to A), 411 (S to A), and 413 (A to G). These positions were chosen because they are the most conserved residues contained in the target domain.
Our experiments using these mutants confirm that, in general, single substitutions in the E2 endodomain do not efficiently disrupt the assembly of Sindbis virus. Despite the fact that the substitutions inserted in these experiments were targeted to a domain identified as critical for inducing the conformational change necessary to bind the capsid protein, these single substitutions still provided little insight into the specificity of the E2-nucleocapsid association. Only one of the mutants analyzed in this study resulted in a significant loss of infectious virus production. Substitution of position I408A resulted in a decrease of three orders of magnitude in infectious virus production from mammalian cells compared to wild type virus production. The remaining mutants at positions T410A, S411A, A413G had little or no effect on infectious virus production from mammalian cells. One trend does emerge from these substitutions indicating that mutations inserted closer to the membrane bilayer have a more detrimental affect on virus assembly and production. Still, these mutations revealed little about the specificity of the interaction between the E2 endodomain and the nucleocapsid.

Based on these results and previous data reported from substitutions in the E2 endodomain, we decided to take a new approach to elucidate the nature of the interaction between the E2 tail and capsid. A limited number of studies have been performed using deletions in the E2 endodomain to study virus assembly and the interaction of E2 with the nucleocapsid. As discussed previously, early studies truncated large portions of the E2 tail and determined that large deletions caused no defects in the transport of E2 to the plasma membrane and in addition that the E2 tail was responsible for binding the capsid. However, not until recently were smaller deletions in the tail analyzed for their affect on virus assembly and function. A single deletion at the membrane interface in SV (deltaK391) resulted in a
complete loss of infectious virus production from mammalian cells but showed no decrease in virus production from insect cells (Hernandez et al., 2000). This suggested that deletions in the E2 tail could be tolerated more easily in invertebrate cells than in vertebrate cells, and that deletions within the endodomain may reveal much more about the specificity of the interaction between the E2 tail and the nucleocapsid.

We targeted deletions to a domain identified by peptide protection assays as being critical for the protection of capsid residue Y180 located in the proposed hydrophobic cleft in the surface of the capsid protein. The domain identified in these experiments extended from amino acid 408-IPTSLALL-415. Interestingly, these residues are incorporated into the membrane of the ER upon translocation of the 100kD polyprotein. The fact that the domain targeted for deletion is predicted to be in a transmembrane domain allowed us to observe the effect of each deletion on virus assembly and also any effect on translocation of the polyprotein.

Our results indicated that each of the mutations caused defects in virus assembly and therefore virus function. The most severe defects were caused by the larger deletions (delta409-411 and delta414-417). Both of these mutations resulted in the complete loss of infectious virus production from mammalian cells, while the smallest deletion (delta406-407) resulted in a decrease of only one order of magnitude. Further examination by immune precipitation revealed that these deletions did not alter protein production, protein processing, or transport virus proteins to the plasma membrane. This was confirmed by the detection of E3 protein, one of the products of Furin protease cleavage of the PE2 protein in the TGN, in the media of transfected cells. The E3 was detected in equivalent amounts for all of the mutants as compared to wild type, indicating that equivalent amounts of protein were being
exported from the ER for each mutant and that these proteins were being properly processed. This also indicated that the proteins had bypassed all of the quality control mechanisms of the cell, and were recognized as properly processed and properly folded proteins. Fusion of cells transfected with these mutants also revealed mature E1 protein present at the plasma membrane, indicating that virus proteins produced by these deletion mutants were fully processed and transported to the plasma membrane.

Since there was no defect in protein trafficking of these mutants we examined cells transfected with these mutants by electron microscopy. Each of the mutants showed the same phenotype, free nucleocapsids in the cytoplasm with no evidence of nucleocapsids binding to membranes inside the cell. Combined with the data that showed no defect in protein production, processing, or transport had occurred in these mutants the EM data suggested that the defect caused by these deletions in the E2 endodomain was a failure to bind to the nucleocapsid. The above data indicates that the domain encompassing amino acids 406-417 in the E2 endodomain is critical for both virus assembly and infectious virus production in mammalian cells.

These deletion mutants were also analyzed in insect cells (invertebrate) and revealed remarkable new evidence. As shown, the ability of these mutants to produce infectious virus from insect cells was similar when compared to the production of infectious virus from mammalian cells. The double deletion produced a significant amount of infectious virus while the larger deletions produced no infectious virus. However, analysis by EM of insect cells transfected with the mutant RNA’s revealed that in insect cells virus like particles are assembled. A large number of virus like particles were observed in cells transfected with the triple deletion whereas cells transfected with the quadruple deletion did not show as many
assembled virus like particles. This suggests that the insect cells maybe more efficient assembling the smaller deletions but that assembly of the largest deletion still occurs. In addition this suggests that the assembly process in insect cells differs dramatically from that in mammalian cells. It is possible that this could be attributed to the difference in chemical composition between insect and mammalian membranes. Insect membranes do not contain cholesterol making them thinner, this could account for the tolerance of deletions made in a predicted membrane spanning domain, as analyzed in the studies discussed above.

Taken together, the results of these studies along with previous work examining deletions in the E2 endodomain, it is clear that deletions as opposed to substitutions will provide much more useful data on both the assembly and function of SV, specifically information on the interaction of E2 protein with the nucleocapsid. Several directions can be taken with these specific deletion mutants (delta406-407, delta409-411, and delta414-417) to clarify how these mutations in the E2 endodomain affect the assembly and function of the virus.

The most important question that arises from the results of this study regards whether or not these mutations interfere with the extraction of the tail, or whether the defect is in the ability to bind capsid protein. It would be advantageous to insert these deletions into SV containing a tyrosine residue at position 420 in the E2 tail as opposed to the wild type serine located at that position. Previous studies have shown that this tyrosine can be iodinated to determine if the E2 tail has been successfully extracted. Analysis of these deletions in this background (Y420) would reveal whether these mutations prevent E2 tail extraction, or if these deletions are responsible for binding to the capsid. Given the data already shown for
these deletions, evidence of tail extraction would suggest that the defect lies in the ability to bind the capsid protein.
Chapter 5: References


