Abstract

Whitehurst, Christopher Benton. Structure and Assembly of the Sindbis Virus E1 and E2 Transmembrane Proteins. (Under the direction of Dr. Dennis T. Brown.)

Sindbis virus is composed of two nested T=4 icosahedral protein shells containing 240 copies each of three structural proteins: E1, E2, and Capsid in a 1:1:1 stoichiometric ratio. E2 is a 423 amino acid glycoprotein with a membrane spanning domain 26 amino acids in length. A previous study had determined that deletions in the transmembrane domain could affect virus assembly and infectivity (Hernandez et al, 2003 J.Virol **77**(23)**,** 12710-9). Unexpectedly, a single deletion mutant (from 26 to 25 amino acids) resulted in a 1000-fold decrease in infectious virus production while another deletion of eight amino acids had no affect on virus production. To further investigate the importance of these mutants, other single deletion mutants and another eight amino acid deletion mutant were constructed. We found that deletions located closer to the cytoplasmic (inner) leaflet of the membrane bilayer had a more detrimental effect on virus assembly and infectivity than those located closer to the luminal (outer) leaflet of the membrane bilayer. We also found that selective pressure can restore single amino acid deletions in the transmembrane domain but not necessarily to the wild type sequence. These results suggest the position of the deletion and the length of the C terminal region of the E2 transmembrane domain are vital for normal virus production. Deletion mutants resulting in decreased infectivity, produce particles that appear to be processed and transported correctly suggesting a role involved in virus entry.

A complex network of disulfide bonds in the E1 and E2 glycoproteins is developed through a series of intermediates as virus maturation occurs. E1 and E2 cysteine residues were labeled with iodoacetamide in the native virus particle and analyzed by mass spectrometry. This analysis identified cysteines of E1 and E2 that were found to be free in the native virus particle as well as those that were either solvent inaccessible or blocked by their involvement in disulfide bonds. Native virus labeled with iodoacetamide yielded a four log decease in viral infectivity. This suggests either the bound iodoacetamide alone may be involved in the loss of infectivity by destabilizing the virus particle or a rearrangement of disulfide bonds, which is required for infectivity, is blocked by the presence of iodoacetamide. In addition, cysteines that were determined to be free in the E1 glycoprotein were mutated to serine. Mutation of these cysteines greatly decreased the amount of infectious virus production suggesting a disulfide bond rearrangement may occur as virus maturation proceeds.

Structure and Assembly of the Sindbis Virus E1 and E2 Transmembrane Proteins

by

Christopher Benton Whitehurst

A dissertation submitted to the Graduate Faculty of

North Carolina State University

In partial fulfillment of the

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Doctor of Philosophy

Biochemistry

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2006

Approved by:

Chair of Advisory Committee

Dedication

To my wife, Jane, my two boys, Watson and Henry, and to any future offspring I may spawn. To my parents, Howard and Madeline, my bother, Vincent, his wife, Ann, their son, Wren, and their soon to be #2, and my sister, Annette.

To my father and mother-in-law, Charlie and Jane Sutherland, and to their other children, Taylor, James, and Morgan.

To the rest of those who have supported me.

Biography

I was born Christopher Benton Whitehurst in Laurinburg, NC on November 29,1973 to Howard Hugh and Madeline Condino Whitehurst. I have one older brother, Vincent, and a younger sister, Annette. In 1981, we moved to Abbeville, South Carolina. I attended Abbeville High School and graduated in 1992. I received my BS in Biochemistry from Clemson University. I met my wife, Jane, there. We broke up. Got back together. Broke up. I moved to Raleigh. She moved to Milwaukee. I worked at Surgicot and Bayer. Work sucked. I decided to go to graduate school. I moved to Milwaukee, WI and moved in with Jane (but only because I got a good deal on the rent) and attended the University of Wisconsin - Milwaukee. Got married to Jane in 2000 (got a better deal on the rent). I received an MS in Chemistry in 2001. I came to NC State University to pursue a PhD in Biochemistry. Watson was born in 2002, and Henry was born in 2004. My wife was the mother of both. I will finish this summer, 2006, with a PhD in Biochemistry. Next I will go to the University of North Carolina for a post doc.

I cut and pasted my CV below if you want to see the educational stuff. Don’t call me.

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Characterization of the Sindbis Virus E2 Transmembrane Domain. Whitehurst, C., Willis, J., Sinodis, C., Hernandez, R. and Brown, D. American Society for Virology Conference. 2005. Pennsylvania State University, University Park, PA

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Poxviral VP11: A Big Role for a Small Protein. Reddy, M., Afshan, G., Ohigashi, Y., Du, Y., and Whitehurst, C. American Society for Virology Conference. 2000. Colorado State University, Fort Collins, CO

Publications:

Location of free and disulfide bound cysteines in the Sindbis virus E1 and E2 glycoproteins. Christopher B. Whitehurst, Erik J. Soderblom, Michael B. Goshe, and Dennis T. Brown. *In preparation.*

Single and Multiple Deletions in the Transmembrane Domain of the Sindbis Virus E2 Glycoprotein Identify a Region Critical for Normal Virus Growth. Christopher B. Whitehurst, John H. Willis, Christine N. Sinodis, Raquel Hernandez, and Dennis T. Brown. Virology. 2006 Mar 30;347(1):199-207.

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Childhood enemy: Donnie Sutton.

College friends: Geoff Chambers, Brian Morris, Peter Kahle, Aaron Kimmerling, Kirsten G. Pierson, Jason Hemphill, Patrick Hughes, and Nancy Neal.

College enemy: Elvino Menoldovo (I hope I screwed up the spelling of your name).

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Thanks.

Chris

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Chapter 1

Introduction to Sindbis Virus

Sindbis virus was first isolated in August 1952 from a pool of mosquitoes in the Sindbis health district, which is located about 30 km from Cairo, Egypt (Hurlbut, 1953; Taylor and Hurlbut, 1953). It is a member of the alphavirus genus within the togavirus family. The Alphaviruses currently have 26 members (Calisher et al., 1988) and of the genomes sequenced they share about 45% sequence identity in the structural proteins and about 60% identity in the nonstructural proteins (Strauss and Strauss, 1994). Alphaviruses are arthropod borne with mosquitoes being the usual vector. They have a very wide geographical distribution and have been isolated from every continent except Antarctica (Strauss and Strauss, 1994). Alphaviruses are a potential threat to humans as Eastern Equine Encephalitis Virus and Western Equine Encephalitis virus both cause fatal encephalitis in both North and South America (Griffin, 1986; Peters, 1990). Sindbis virus, the prototype alphavirus, is usually considered to be avirulent in humans.

Sindbis virus is a positive sense RNA virus with a genome of approximately 11.7 kB (49S)(Strauss and Strauss, 1994), containing both a 5' cap and a 3' poly A tail (Strauss, 1986)(Figure 1.1). The genome is divided into 2 regions consisting of nonstructural and structural genes. Initially only the first 7600 bases are translated from the genomic RNA directly into a polyprotein, which is then cleaved into the four nonstructural proteins: nsp1, nsp2, nsp3, and nsp4 (Voyles, 1993). These genes include a protease and at least one RNA dependent RNA polymerase. Expression of the 3' end of the Sindbis viral genome requires two RNA synthesis steps. First a 49S minus strand RNA, complementary to the genome, is synthesized and serves

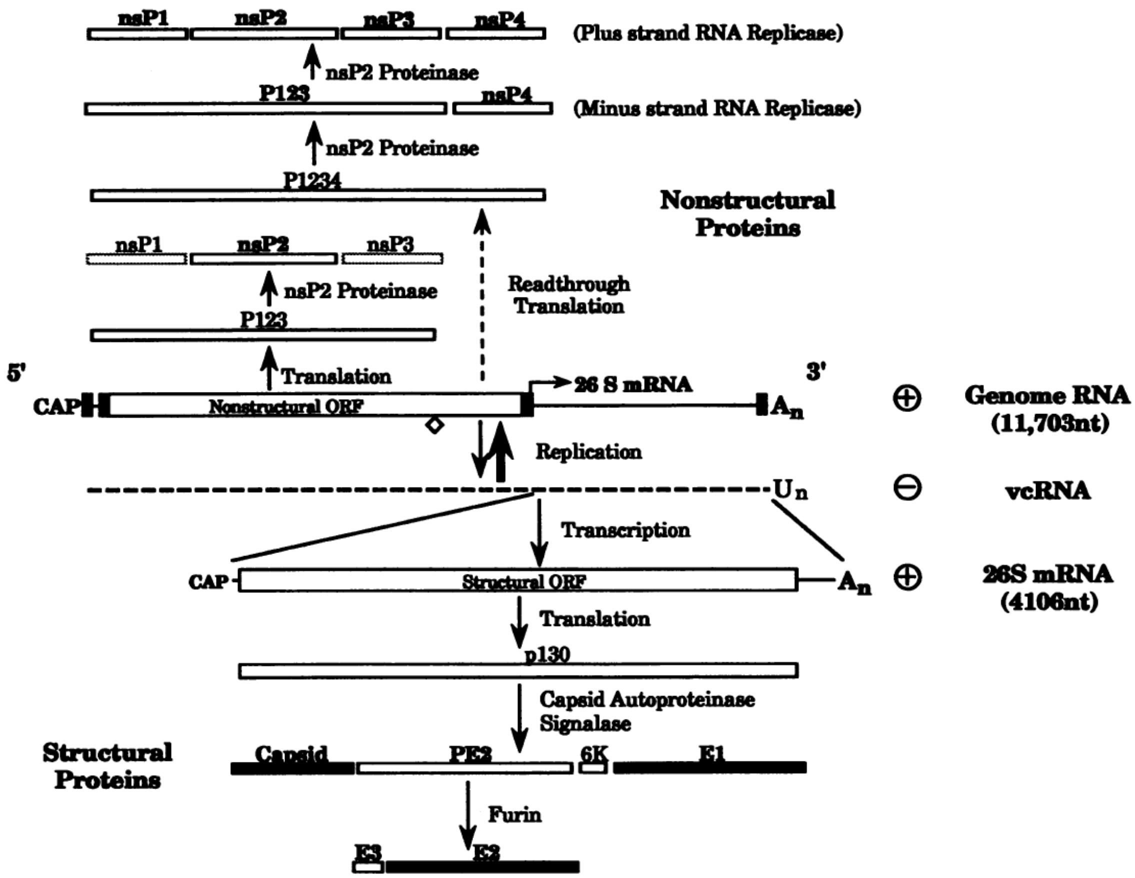

Figure 1.1: Genome organization of Sindbis virus. The 49S genomic RNA is illustrated in the center. The 26S subgenomic mRNA is expanded to show the structural open reading frame and its gene products. vcRNA is the minus-strand complement of the genomic RNA.

as the template for a new shorter 26S positive strand molecule, which is equivalent to the 3' end of the genome. The 26S positive strand RNA is then capped and tailed and is translated into a polyprotein. A series of cleavages of the translated polyprotein results in the formation of the structural proteins: Capsid, E3, E2, 6K and E1.

The mature virus particle contains three of the structural proteins (E1, E2, and capsid) in a 1:1:1 stoichiometric ratio. The Sindbis virus particle is 70 nm in diameter, contains a cell membrane derived bilayer, and serves as an excellent model for structural studies. Sindbis virus is organized into two geometrically identical T=4 icosahedral shells containing 80 equivalent units composed up of 240 copies of each structural protein (E1, E2, and capsid)(Paredes et al., 1993)(Figure 1.2). The outer protein shell is composed solely of the E1: E2 dimers which are then organized into trimers (Anthony and Brown, 1991; Carleton et al., 1997; Pletnev et al., 2001). Each one of these trimers represents an equivalent unit on the surface of the viral particle. The inner protein shell, which is surrounded by the outer protein shell, is composed entirely of the capsid protein. The 11.7 kB genomic RNA is packaged within the inner shell. A host derived membrane bilayer is positioned between the inner and outer shell of the Sindbis virus particle and is penetrated by the transmembrane domain anchors of the E1 and E2 proteins (Lilijestrom and Garoff, 1991; Rice et al., 1982; Strauss, Lenches, and Strauss, 2002). The endodomain of the E2 protein interacts with the capsid protein and serves to link the two shells together.

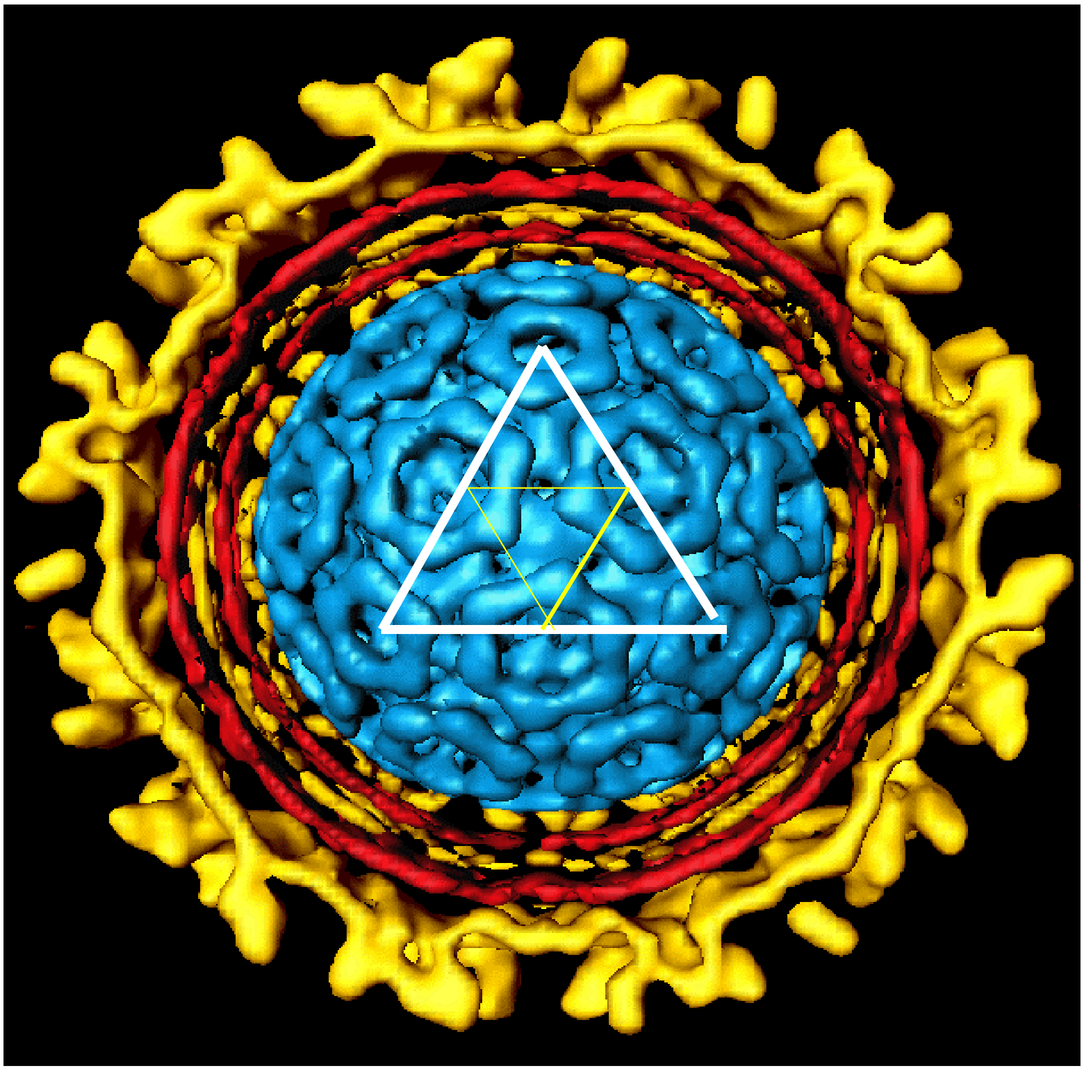

Figure 1.2: Cross-section of Sindbis virus from a cryo-electron microscopy reconstruction. In yellow is the envelope composed of E1 and E2. In red is the host-derived lipid bilayer, and in blue is the nucleocapsid core. The large white triangle connects three five fold axises and can be subdivided into 4 equivalent units indicating T= 4 icosahedral symmetry. (Paredes et al., 1993).

The 26S mRNA produced by Sindbis virus encodes a polyprotein that is post-translationally cleaved into Capsid, E1, E2, E3, and 6K. Auto-protease activity removes the capsid protein after its translation on the cytoplasmic ribosome and exposes a transmembrane signal sequence that is necessary for the insertion of the polyprotein into the membrane bilayer. The transmembrane polyprotein contains six membrane spanning domains (Figure 1.3) (Liljestrom and Garoff, 1991) and is cleaved into its structural components by signalase (PE2, 6K, and E1) (Liljestrom and Garoff, 1991). 6K, which is only found at low levels in the mature virus particle (Gaedigk-Nitschko and Schlesinger, 1990), is necessary for correct integration of E1 into the membrane and plays a role in the efficient budding of viral particles (Liljestrom and Garoff, 1991). E1, which contains a functional domain involved in virus entry, is then folded into a stable high energy configuration as it is assembled into heterodimers with PE2 (Carleton et al., 1997; Mulvey and Brown, 1994; Mulvey and Brown, 1995; Mulvey and Brown, 1996) . E1/PE2 is then transported from the ER into the trans-golgi where PE2 is processed to E2 and E3 by furin (Moehring et al., 1993). E3 is released and is not found in the mature virus particle. The E1/E2 heterotrimer is then recruited to the cell surface. As transport of the E1/E2 heterotrimer to the plasma membrane occurs, a second membrane-spanning domain (Domain 3 of Figure 1.3) of E2 is retracted from the membrane and results in a 33 amino acid endodomain being exposed to the cytoplasm. This 33 amino acid endodomain interacts with a hydrophobic cleft in the capsid protein and allows the E1-E2 heterotrimers to assemble into the outer protein shell at the cell surface

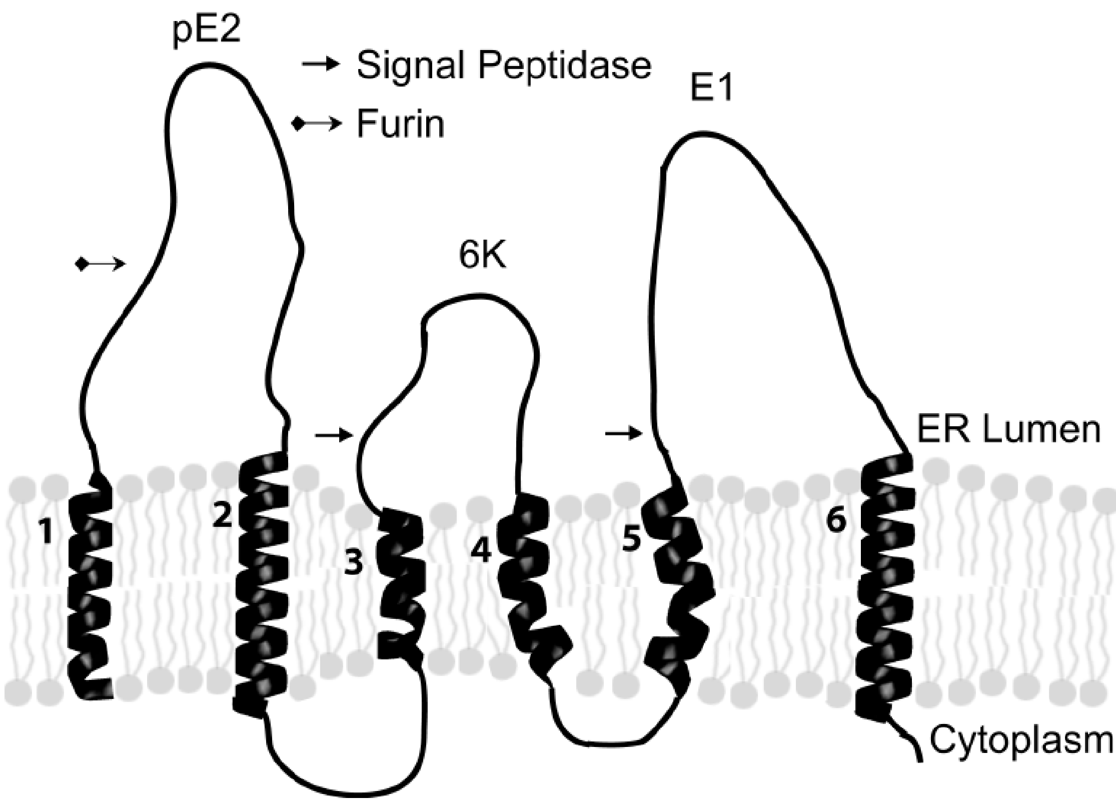

Figure 1.3: A schematic representation of the organization of the Sindbis virus structural proteins in the membrane of the Endoplasmic Reticulum. The integrated polyprotein has six transmembrane domains labeled 1 to 6. The 6K protein is eliminated from the developing spike complex after processing by signal peptidase. The N-terminal region of PE2 is removed in the Golgi by Furin protease. Of the remaining three domains that are incorporated into virus, domain 3 is withdrawn from the membrane and attaches to the nucleocapsid, and domains 2 and 6 are membrane anchors.

(Lee and Brown, 1994; Lee et al., 1996; Oates et al., 1992; Owen and Kuhn, 1996; Owen and Kuhn, 1997). The association of the E2 endodomain with the capsid protein gives stability to the structure of the virus (Lee, Ricker, and Brown, 1994) and plays a critical role in the formation of the outer protein shell around the preformed inner protein shell as the process of envelopment takes place (Ferreira et al., 2003)(Figure 1.4). The mature virus particle is then released.

This thesis focuses on two aspects of the E1 and E2 glycoprotein structure. The first deals with investigating the role of the Sindbis virus E2 transmembrane domain. Previously, sequential deletions were made in the 26 amino acid E2 transmembrane domain to investigate the interaction of the membrane-spanning domain with the membrane bilayer (Hernandez et al., 2003). Deletion of a single amino acid in the transmembrane domain resulted in a four-log loss of infectious virus production (Hernandez et al., 2003). This was a surprising find since 25 amino acids is long enough to span the membrane bilayer. Previous results also showed that deleting eight amino acids from the N terminal half of the transmembrane region had no effect on normal virus growth. To further investigate these results a series of single amino acid deletions were made within the E2 transmembrane domain along with another mutant where eight amino acids were deleted from the C terminal half of the transmembrane domain. The results revealed that deletions located closer to the cytoplasmic face of the transmembrane domain resulted in a greater loss of infectivity than deletion located closer to the lumenal side of the membrane (Whitehurst et al., 2006). These and other results suggest that the location of the deletion on the helix vertically within the transmembrane domain is important for

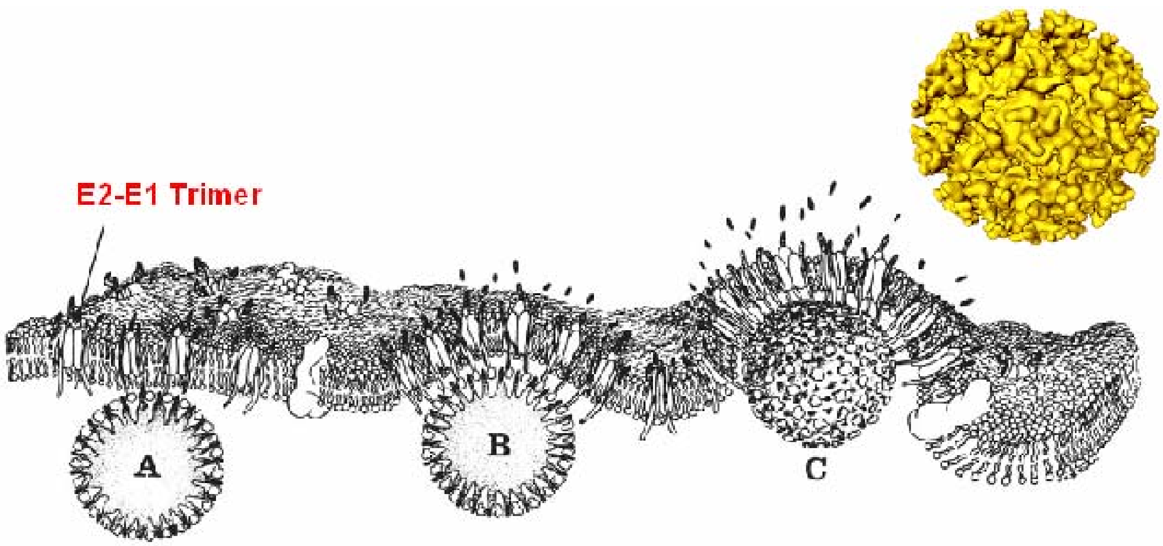

Figure 1.4: Viral release from the cell. Representation of (A) nucleocapsid binding to E2-E1 trimers, (B) budding through a host membrane bilayer, and (C) release of the viral particle.

normal virus growth. This may be explained by allowing for more degrees of freedom so that the E2 endodomain may interact properly with the capsid protein (Whitehurst et al., 2006). There is also evidence to suggest that the E1 and E2 transmembrane domains may interact with each other (Strauss, Lenches, and Strauss, 2002; Whitehurst et al., 2006).

The second part of the thesis focuses on identifying free and disulfide bound cysteines in the E1 and E2 proteins by using mass spectrometry as a tool. Little is known about the E2 disulfide network. Gidwitz et al. (Gidwitz et al., 1988) reported that all cysteines located in the E2 protein were either involved in disulfide bonds or were inaccessible when treated with 14C iodoacetamide. Much more is known for the E1 protein. The Semliki Forest virus E1 crystal structure was solved and the disulfide bonds present were indicated (Roussel et al., 2006).

In the second half of this thesis the location of free and disulfide bound cysteines in the Sindbis virus E1 and E2 proteins were identified. Native virus particles were incubated in the presence of iodoacetamide to identify the location of free cysteines. A series of cysteine to serine mutations were also constructed for the cysteines that were found to be free in the E1 native virus particle. These all yielded a decrease in infectious virus production. This data taken along with other data suggests that a disulfide bond rearrangement may occur as virus maturation proceeds.

CHAPTER 2

Single and multiple deletions in the transmembrane domain of the Sindbis virus E2 glycoprotein identify a region critical for normal virus growth

Christopher B. Whitehurst, John H. Willis, Christine N. Sinodis, Raquel Hernandez, and Dennis T. Brown

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Abstract

Sindbis virus is composed of two nested T = 4 icosahedral protein shells containing 240 copies each of three structural proteins: E1, E2, and Capsid in a 1:1:1 stoichiometric ratio. E2 is a 423 amino acid glycoprotein with a membrane spanning domain 26 amino acids in length and a 33 amino acid cytoplasmic endodomain. The interaction of the endodomain with the nucleocapsid is an essential step in virus maturation and directs the formation of the outer protein shell as envelopment occurs. A previous study had determined that deletions in the transmembrane domain could affect virus assembly and infectivity (Hernandez et al., 2003. J. Virol. 77 (23), 12710–12719). Unexpectedly, a single deletion mutant (from 26 to 25 amino acids) resulted in a 1000-fold decrease in infectious virus production while another deletion of eight amino acids had no affect on infectious virus production. To further investigate the importance of these mutants, other single deletion mutants and another eight amino acid deletion mutant were constructed. We found that deletions located closer to the cytoplasmic (inner) leaflet of the membrane bilayer had a more detrimental effect on virus assembly and infectivity than those located closer to the luminal (outer) leaflet of the membrane bilayer. We also found that selective pressure can restore single amino acid deletions in the transmembrane domain but not necessarily to the wild type sequence. The partial restoration of an eight amino acid deletion (from 18 to 22 amino acids) also partially restored infectious virus production. The amount of infectious virus produced by this revertant was equivalent to that produced for the four amino acid deletion produced by site directed mutagenesis. These results suggest that the position of the deletion

and the length of the C terminal region of the E2 transmembrane domain is vital for normal virus production. Deletion mutants resulting in decreased infectivity produce particles that appear to be processed and transported correctly suggesting a role involved in virus entry.

Introduction

Sindbis virus (SV) is an Alphavirus, which is propagated in nature via a complicated life cycle involving insect vectors and mammalian hosts. Mature SV contains 240 copies each of three structural proteins E1, E2, and Capsid (C) in a 1:1:1 stoichiometric ratio. These proteins are organized into two geometrically identical T=4 icosahedral shells (Paredes et al., 1993). The outer protein shell is composed of the E1 and E2 glycoproteins organized in trimers of heterodimers (Anthony and Brown, 1991; Carleton et al., 1997; Pletnev et al., 2001). This outer shell surrounds the inner shell, composed of protein C, which is assembled around the viral RNA. A host-derived membrane bilayer is positioned between the two shells and is penetrated by the transmembrane (TM) domain anchors of E1 and E2 (Lilijestrom and Garoff, 1991; Rice et al., 1982; Strauss, Lenches, and Strauss, 2002).

SV structural proteins are produced from a subgenomic mRNA which encodes a polypeptide that is posttranslationally cleaved into capsid, E1, E2, E3, and 6K. The capsid protein is removed by auto-proteolysis after translation on cytoplasmic ribosomes. Removal of capsid exposes a signal sequence at the amino terminus of the developing polypeptide. This sequence directs the translational complex to the endoplasmic reticulum where the envelope glycoproteins are synthesized. Synthesis on the ER results in the incorporation of the protein as a multipass membrane protein with six membrane-spanning domains (Lilijestrom and Garoff, 1991) (see Figure. 2.1). The polyprotein is processed into PE2, 6K, and E1 by signal peptidase (Lilijestrom and Garoff, 1991). Transmembrane domain five of the 6K protein plays a role in the correct integration of E1 into the membrane (Lilijestrom and Garoff, 1991). The 6K protein, which contains transmembrane domains four and five, is released by signal peptidase and is only found at very low levels in mature virus (Gaedigk-Nitschko and Schlesinger, 1990). Prior to export to the cell surface, the E1 glycoprotein is folded into a stable compact high-energy conformation as it is assembled into heterotrimers with PE2 (Carleton et al., 1997; Mulvey and Brown, 1994; Mulvey and Brown, 1995; Mulvey and Brown, 1996). En route to the cell surface, PE2 is processed to E2 and E3 by the furin protease, which resides in the trans Golgi network, (Moehring et al., 1993) and E1 is converted from a stable to a metastable configuration. E3, which contains transmembrane domain one, is not present in the mature virus. During transport of the trimeric complexes to the plasma membrane, the second membrane-spanning region of E2 (see Figure 2.1, domain 3) is retracted from the membrane and the 33 amino acid endodomain is exposed to the cytoplasm. The E2 endodomain specifically interacts with a hydrophobic cleft in the capsid protein (Lee and Brown, 1994; Lee et al., 1996; Oates et al., 1992; Owen and Kuhn, 1996; Owen and Kuhn, 1997). The interaction between E2 and C gives stability to the structure of the virus (Lee, Ricker, and Brown, 1994) and plays a critical role in the formation of the outer virus protein shell

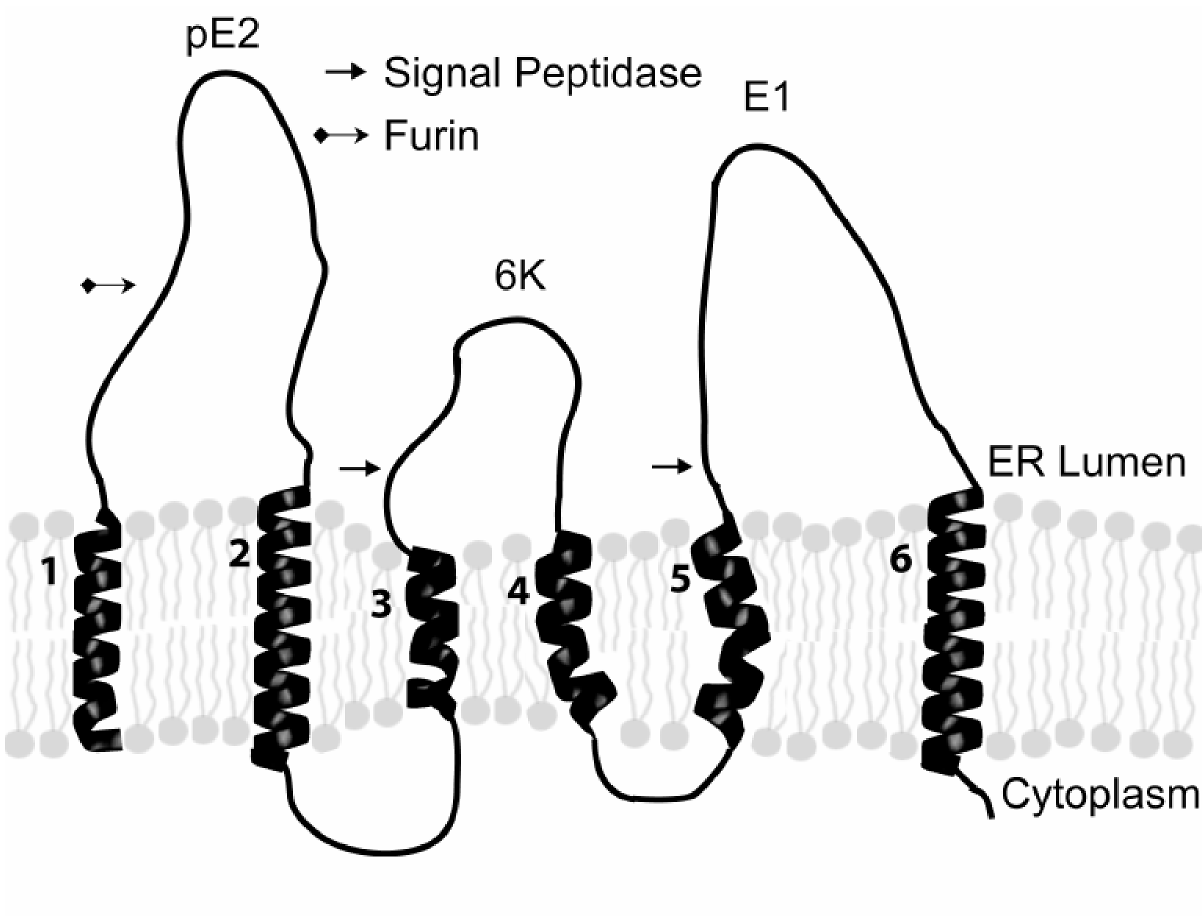


Figure 2.1: A schematic representation of the organization of the Sindbis virus structural proteins in the membrane of the Endoplasmic Reticulum. The integrated polyprotein has six transmembrane domains labeled 1 to 6. The 6K protein is eliminated from the developing spike complex after processing by signal peptidase. The N-terminal region of PE2 is removed in the Golgi by Furin protease. Of the remaining three domains that are incorporated into virus, domain 3 is withdrawn from the membrane and attaches to the nucleocapsid, and domains 2 and 6 are membrane anchors.

around the preformed inner protein shell as the process of envelopment takes place (Ferreira et al., 2003). At the plasma membrane the E1-E2 heterotrimers are recruited into the outer protein shell by association of the E2 endodomain with the icosahedral nucleocapsid (Owen and Kuhn, 1997).

In mature SV only transmembrane regions two (E2) and six (E1) remain membrane associated (see Figure 2.1). The amino acid sequences of the transmembrane regions are not conserved among the alphaviruses, however the hydrophobic nature and approximate length (26-28 amino acids) are conserved. Previously, sequential deletions were made in the E2 transmembrane domain to investigate the interaction of the 26 amino acid membrane-spanning domain with the membrane bilayer. Deletion of a single methionine at position 379 (TM25) resulted in a 3 to 4 log loss in production of infectious virus (Hernandez et al., 2003). Many different factors could contribute to the observed phenotype of the TM25 mutant. i) The observed phenotype could be a result of a single specific amino acid deletion at position 379. ii) The angular location of the deletion on the face of the transmembrane helix may account for the loss of infectivity (ie. if the deletion is made 90 degrees, 180 degrees or another angle from a set point of reference). iii) The location of the deletion vertically (ie closer to the luminal side or closer to the cytoplasmic side of the membrane) may contribute to the observed phenotype. iv) A specific interaction between the E1 and E2 transmembrane regions may be necessary for normal virus infectivity and this interaction may be impaired by the mutation. Strauss and coworkers have demonstrated an interaction between E1 and E2 transmembrane domains using a chimeric virus composed of Ross River E1 and Sindbis virus E2 (Strauss, Lenches, and Strauss, 2002).

To further characterize the E2 membrane-spanning domain and investigate the importance of the factors presented above, additional single deletion mutants and an eight amino acid deletion mutant were constructed in the E2 transmembrane domain. Mutant sites were chosen to encompass all faces of the helix and to traverse the helix from N terminal to C terminal within the E2 transmembrane domain. It was found that deletions located closer to the cytoplasmic face of the membrane region resulted in a greater loss of infectivity than deletions located closer to the lumenal side of the membrane. Also deletions located on one face of the helix and close to the cytoplasmic interface resulted in reduced infectious virus production. The results presented herein suggest i) the observed phenotype of the TM25 mutants is not due to a single specific amino acid deletion, ii) the location of the deletion vertically is important for normal virus growth, iii) the angular location of the deletion on the face of the helix does not affect normal virus growth and iv) an interaction may be occurring between the E1 and E2 transmembrane domains in the cytoplasmic half of the transmembrane domain. In addition, other factors may influence the observed phenotype. The membrane composition differs from the inner leaflet to the outer leaflet of the membrane (Stoffel, Anderson, and Stahl, 1975). Also, deletions within the transmembrane domain may have a log range effect on the interaction between the E2 endodomain and the capsid protein.

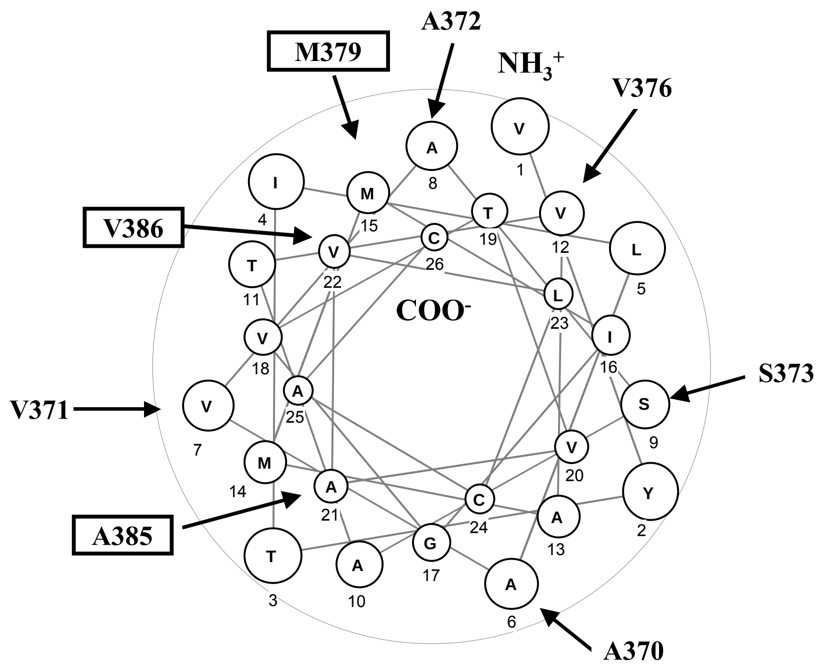
Results

Virus Production by Sindbis virus E2 single amino acid (TM25) mutants

Transmembrane domain two (see Figure 2.1) was chosen for mutagenesis because of E2's interaction with both the inner and outer icosahedral protein shells (Hernandez et al., 2003). Previously, a series of sequential deletions in the E2 transmembrane domain were produced (Hernandez et al., 2003). The goal of these experiments was to elucidate the requirements placed on the length of the transmembrane domain for integration into cell membranes and for assembly of infectious virus. In that study, a single deletion of a methionine at position 379 yielded a decrease in infectious virus production of four orders of magnitude (Hernandez et al., 2003). The relative infectivity of this mutant measured as the particle/pfu ratio was approximately 10^5 suggesting that virus assembly occurred, but that the resulting virus population was largely noninfectious. To further determine the effect of single deletion mutations in the transmembrane domain on virus assembly and infectivity, a series of single amino acid deletions were produced in the E2 transmembrane domain (Figure 2.2). Deletions were placed at positions ranging from the luminal to the cytoplasmic side of the membrane bilayers. In addition, the deletions also covered all faces of the transmembrane helix (Figure 2.2).

Virus production by these mutants in BHK cells is shown in Figure 2.3A. The data show that the wild-type virus, SVHR, yields a titer of approximately 10^9 pfu/ml. The single amino acid deletion mutants ΔM379, ΔA385 and ΔV386 all gave reduced titers of approximately 10^6 pfu/ml. Mutants ΔA370, ΔV371, ΔA372, ΔS373, and

A

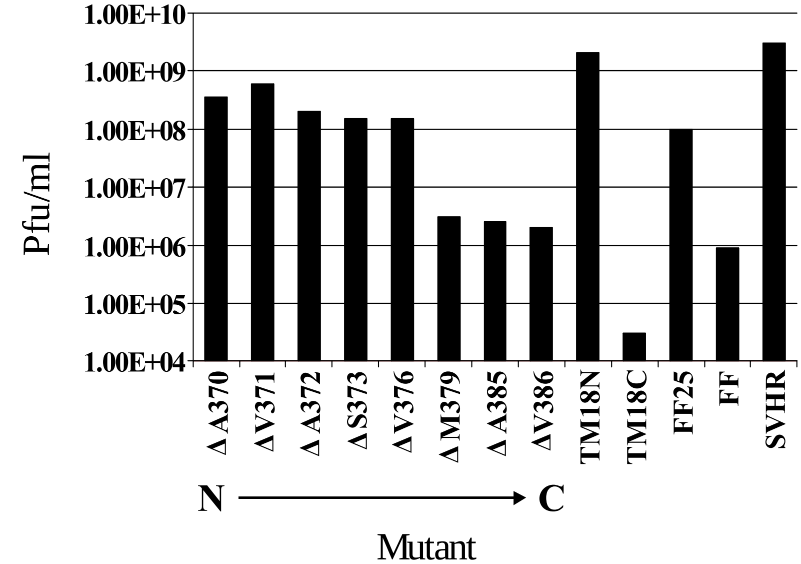


B

wt	VYTILAVASATVAMMIGVTVAVLCAC	26AA
ΔA370	VYTIL-VASATVAMMIGVTVAVLCAC	25AA
ΔV371	VYTILA-ASATVAMMIGVTVAVLCAC	25AA
ΔA372	VYTILAV-SATVAMMIGVTVAVLCAC	25AA
ΔS373	VYTILAVA-ATVAMMIGVTVAVLCAC	25AA
ΔV376	VYTILAVASAT-AMMIGVTVAVLCAC	25AA
ΔM379	VYTILAVASATVAM-IGVTVAVLCAC	25AA
ΔA385	VYTILAVASATVAMMIGVTV-VLCAC	25AA
ΔV386	VYTILAVASATVAMMIGVTVA-LCAC	25AA
TM18N	VYTILAV--------IGVTVAVLCAC	18AA
TM18C	VYTILAVASATVAM--------LCAC	18AA
FF	VYTILAVASATVAMMIGVTVAVLCACF	27AA
FF25	VYTILAVASATVAM-IGVTVAVLCACF	26AA

Figure 2.2: Representations of the E2 transmembrane. (A) A helical wheel representation of the E2 transmembrane domain from the N-terminal region to the C-terminal region. Locations of single amino acid deletion mutations are noted with arrows. Mutations cover all faces of the helix and span the entire membrane domain. Boxed labels indicate mutants, which produced10^6 pfu/ml, and others represent virus, which produced 10^8 pfu/ml. (B) Sequence of the deletions in the E2 transmembrane domain (transmembrane domain #2 from Fig. 1). (-) denotes location of the deletion. The resulting number of amino acids is listed to the right.

A



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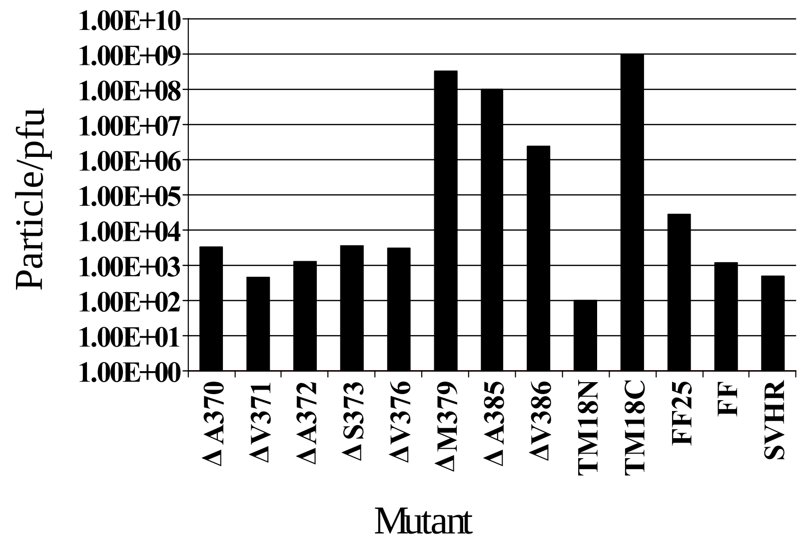

Figure 2.3: Infectious virus production and relative infectivity of the TM25, TM18, FF, and FF25 mutants. (A) Production of infectious virus by the TM25, TM18, FF, and FF25 mutants from transfection. The arrow at the bottom highlights the relative location of the TM25 deletions from N terminal to C terminal. SVHR represents wt Sindbis Virus Heat Resistant strain. (B) Relative infectivity of the TM25, TM18, FF and FF25 mutants as determined by particle-to-pfu assay. Virus utilized in this experiment was released from the cell into the media and purified (see Methods). Mutant virus particles banded to wt density. Those mutants producing higher titers had lower particle-to-pfu ratios indicating more of the particles produced from these mutants were infectious, with the exception of the FF mutant. Except for FF, the mutants produce similar amounts of particles.

ΔV376 produced relatively higher titers of approximately 10^8 pfu/ml but still an order of magnitude below wild type. Single deletion mutants located in the N terminal region of the E2 transmembrane domain produced two logs more infectious virus than those located in the C terminal region of the transmembrane domain. The difference in infectious virus production from mutants in the N terminal and the C terminal TM region did not represent a gradual decline but a two-log decrease that occurred between position 376 and 379. This suggests that single deletion mutations located closer to the N terminal portion of the transmembrane domain have a less detrimental effect than those located closer to the C terminal domain. These domains are separated by as few as three amino acids.

Affect of transmembrane domain length on virus infectivity

We have described a mutant, FF391/392 (FF), which inserts an extra amino acid into the E2 glycoprotein at the interface of the cytoplasmic side of the membrane bilayers (Hernandez et al., 2005). This insertion results in a 3000-fold loss of infectious virus production in BHK cells. This mutant was utilized as the parent into which the deletion at M379 was placed to determine if infectious virus production could be rescued by restoring the wild-type number of amino acids to the carboxyl terminal end of E2. These two mutants (FF and ΔM379) have one extra or one fewer amino acid respectively in the C terminal domain of E2 and both yield reduced production of infectious virus. The resulting mutant was designated TM25(ΔM379)391/392, (abbreviated to FF25) and results in the restoration of a wt number of amino acids in the COOH region of E2 . The parent FF mutant produced a titer of approximately 10^6 pfu/ml, whereas the FF25 mutant produced a titer of approximately 10^8 pfu/ml (Figure 2.3A). When these two mutations (FF and ΔM379) are combined the wt number of amino acids is restored and the level of infectious virus production is rescued. A critical number of amino acids may be required in the C terminal region of E2 for interaction with E1 or with the capsid protein.

Virus production by Sindbis virus containing large E2 TM deletions

We have previously shown that a deletion of eight amino acids (TM18) in the N terminal region of the E2 transmembrane domain results in normal virus production (Hernandez et al., 2003). This very puzzling observation raised a number of questions regarding the role of the TM domain in virus assembly and infectivity. For example; why does Sindbis virus have 26 amino acids in the TM domain if only 18 will suffice for normal virus production? The data presented above show that single deletions in the E2 TM domain have a significantly less deleterious effect if they are located in the region of the TM domain where the original TM 18 deletion was produced (amino acids 372-379). To further investigate the finding that mutations located closer to the C terminal end of the transmembrane had a greater effect on virus production than those located closer to the N terminal end two TM18 mutants were studied (Figure 2.2), the original eight amino acid N terminal deletion of residues 372-379 (TM18N) and a C terminal deletion of residues 379-386 (TM18C). Three of the single deletion mutants yielding a titer of 10^8 pfu/ml (ΔA372, ΔS373, and ΔV376) were located in the N terminal sequence included in the TM18N deletion and the single deletion mutants yielding a titer of 10^6 pfu/ml (ΔM379, ΔA385, and ΔV386) were all located in the C terminal sequence included in TM18C deletion. TM18N produced similar levels of infectious virus as wild type (10^9 pfu/ml) (Figure 2.3A). Whereas, TM18C produced greatly reduced levels of infectious virus (10^4 pfu/ml). Similar findings were found when TM18N and TM18C were transfected into an insect cell line (Aedes albopictus clone U4.4). In insect cells TM18C and TM18N yielded a titer of 7.3×10^3 and 7.0×10^9 pfu/ml respectively. Taken together these data demonstrates the N terminal region of the E2 transmembrane domain is not as essential for normal virus growth in BHK-21 or U4.4 cells as is the C terminal region.

Relative infectivity of the E2 TM deletion mutants

Equal volumes of virus released from the cell by the various TM mutants were purified by equilibrium density gradient centrifugation on linear potassium tartrate gradients as described in Materials and Methods. All of the TM mutants were found to produce virus that banded at a mean density of 1.20 gm/cm^3 indicating that they are similar in composition to wild type virus in that they contain a normal complement of protein, lipid, and RNA.

The relative infectivity (particle-to-PFU ratio) of the TM deletion mutants was determined as described in Materials and Methods. Particle-to-pfu it is a measure of total viral particles to infectious particles which were released into the cell supernatant. For example, if a mutant has a particle/pfu of 10, then 1 of 10 viral particles released from the cell are infectious. The results are shown in Figure 2.3B. The profile of particle-to-PFU ratio for the TM25 mutants is approximately the inverse of the virus production profile shown in Figure 2.3A. All of the TM25 mutants produce similar amounts of virus particles (within 1 log), but the relative infectivity of these viruses differ greatly. Deletion mutants ∆A370, ∆V371, ∆A372, ∆S373, and ∆V376 yield lower particle/pfu ratios and thus produce virus with a relatively high infectivity. Deletion mutants ∆M379, ∆A385, and ∆V386 all give higher particle/pfu ratios and thus have a relatively lower infectivity.

The FF and FF25 mutants produced particle/pfu ratios of 1.2 X 10^3 and 2.8 X 10^4 respectively. In comparison to ∆M379, ∆A385, and ∆V386 vastly more of the particles produced by FF and FF25 are infectious. This result suggests the loss of infectious virus production for FF may be due to the inability of this mutant to correctly assemble virus particles (Hernandez et al., 2005).

Deletion mutations TM18C and TM18N have particle/pfu ratios of 9.6 X 10^8 to 1 and 4.5 X 10^1 to 1 respectively (Figure 2.3B). This demonstrates a large number of particles are produced for TM18C however only a very small amount of the particles assembled are infectious in comparison to TM18N.

SDS-PAGE analysis of purified virus particles produced the same protein banding pattern and ratio of incorporated proteins as wt (Table 2.1). These data indicate that the high particle to PFU ratios obtained for the ∆M379, ∆A385, and ∆V386 mutants are not the result of a failure to incorporate normal amounts of protein into the virus particles. These data could not be obtained for the TM18C mutant due to the very low yield of virus produced. Electron microscopy of negatively stained preparations of the ∆M379, ∆A385, ∆V386 and TM18C mutants revealed virus that was morphologically similar to wt (data not shown). The

Table 2.1: Relative density of viral proteins produced from selected mutants. Purified virus released from cells was analyzed by SDS-PAGE and visualized by autoradiography. The density of the E1 band for each lane was set to an arbitrary unit of one. The E2 and capsid band densities were reported as a fraction of the E1 value.

Mutant	E1	E2	Capsid
ΔM379	1.00	1.04	0.92
ΔA385	1.00	0.95	0.93
ΔV386	1.00	0.95	0.93
ΔV376	1.00	0.97	1.05
SVHR	1.00	1.05	0.95

particle/pfu data supports these conclusions in that the density of the mutant virus particles is similar to wt density suggesting a normal complement of protein, lipid, and RNA. If the altered particle to PFU ratio was due to failure to incorporate structural proteins in normal ratios the density of the virus produced would be expected to be different and the particle to PFU ratio artificially low.

Compensatory mutations for TM mutants

Deletion mutants yielding low amounts of infectious virus (ΔM379, ΔA385, ΔV386, and TM18C) were subjected to serial passage in an attempt to produce mutations, which may rescue virus infectivity (compensatory mutations). Other mutants yielding higher infectious virus production were not subjected to serial passage as their titers were near wild type levels and the lack of selective pressure might result in the production of amino acid changes not related to the original defect. After three passages for ΔM379, ΔA385, and ΔV386 and five passages for TM18C an increase in infectious virus production was observed (Figure 2.4). After passage the single deletion mutants produced infectious virus at the same level as wt and the TM18C mutant infectious virus production was increased by approximately two orders of magnitude. RT/PCR followed by sequencing of both the E1 and E2 transmembrane domains of the revertants yielded no differences in the E1 transmembrane domain or the E2 endodomain (33 amino acid tail which interacts with capsid) but mutations compensating for the loss of virus growth (compensatory mutations) were found for all of these mutants in the E2 transmembrane domain (Figure 2.5). ΔM379 converted an alanine to a valine at position 372 and a serine

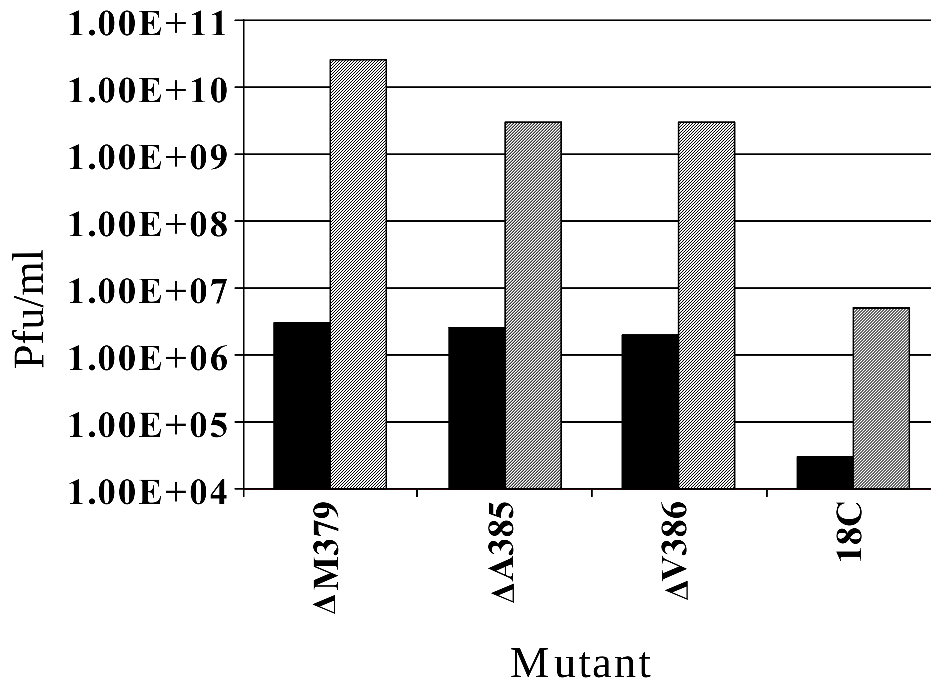

Figure 2.4: Production of infectious virus by the TM25 and TM18 mutants before and after serial passage. Virus was passaged between three and five times for the mutants that produced lower titers (ΔM379, ΔA385, ΔV386 and TM18C). Infectious virus growth was rescued after serial passage. Solid bars represent virus growth before passage. Light bars represent virus growth after passage.

wt	VYTILAVASATVAMMIGVTVAVLCAC	26AA
ΔM379	VYTILAVASATVAM-IGVTVAVLCAC	25AA
CMΔM379	VYTILAV**VS**SATVAMIGVTVAVLCAC	26AA
ΔA385	VYTILAVASATVAMMIGVTV-VLCAC	25AA
CMΔA385	VYTILAVASATVAMMIGVTV**V**VLCAC	26AA
ΔV386	VYTILAVASATVAMMIGVTVA-LCAC	25AA
CMΔV386#1	VYTI**H**AVASATVAMMIGVTVA**V**LCAC	26AA
CMΔV386#2	VYTILAVASATVAMMIGVTVA**V**LCAC	26AA
CMΔV386#3	VYTILAVASATVAMMIGVTVA**V**LCAC	26AA
wt	VYTILAVASATVAMMIGVTVAVLCAC	26AA
TM18C	VYTILAVASATVAM--------LCAC	18AA
CM18C	VYTILAVASATVAM----LCAC**ACAC**	22AA

Figure 2.5: Compensatory mutations (CM) found in the E2 transmembrane domain. After serial passage and an increase in infectious virus production, virus was isolated, purified, and RT/PCR followed by sequencing was performed. Mutants compensating for the increase in virus growth are underlined and highlighted in bold lettering. All mutants were examined in triplicate and all three trials resulted in identical sequences except for ΔV386. The resulting number of amino acids in the TM domain is noted on the right. No compensatory mutations were found in the E1 transmembrane domain or in the E2 tail domain that interacts with capsid.

was inserted adjacent to it. This compensatory mutation restored the wild type length (26 amino acids) to the transmembrane domain but did not restore M at 379. The ∆A385 compensatory mutation inserted a valine at the position of the alanine deletion, which also resulted in a wt length transmembrane domain. The ∆V386 compensatory mutation reverted back to the original wt sequence and length, with the exception of a leucine being converted to a histidine for ∆V386#1 (Figure 2.5). These revertants suggest that wt length is important for normal virus growth in the C terminal half of the transmembrane domain.

After five passages the TM18C mutant demonstrated a 100-fold increase in infectious virus production. The increase in virus production was accompanied by a four amino acid insertion, ACAC in the C terminal domain of the E2 transmembrane. The ACAC that was inserted contains the same nucleotide sequence as a repeat of the wt AC sequence preceding it (Figure 2.5). The TM18C compensatory mutation results in an increase from 18 to 22 amino acids spanning the transmembrane domain. When this compensatory mutation is compared to a previously described TM22 mutation (Hernandez et al., 2003) it was found that it produces approximately the same amount of infectious virus (10^6 pfu/ml) and the location of the remaining four amino acid deletion is in approximately the same location (Figure 2.6). This stresses the importance of the size and location of the deletion in the E2 transmembrane domain.   No compensating mutations were found in the E2 endodomain or the transmembrane domain of the E1 glycoprotein for any of the mutants. This interesting result suggests that selective pressure favors restoring the

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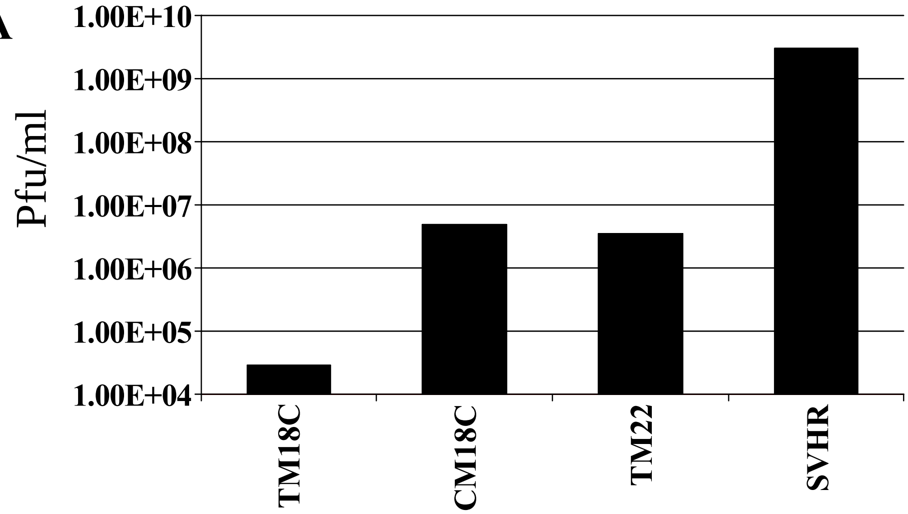

B

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wt      VYTILAVASATVAMMIGVTVAVLCAC 26AA
18C     VYTILAVASATVAM--------LCAC 18AA
CM18C   VYTILAVASATVAM----LCACACAC 22AA
TM22    VYTILAVASATVA----VTVAVLCAC 22AA
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Figure 2.6: Comparison of the TM18C, CM18C, and TM22 growth and sequence (see text for description of these mutants). (A) Infectious virus production of TM18C, CM18C, and TM22 from transfection. (B) Sequence comparison of TM18C, CM18C, and TM22. CM18C results in 22 amino acids and a similar titer to a previously described TM22 mutant. The location of the deletion is in approximately the same place suggesting the length and location of the deletion are important for normal virus production.

deletion rather than altering a protein domain with which the mutated sequence is presumed to interact.

Discussion

The data presented herein demonstrate that a single deletion in the E2 transmembrane domain results in a significant loss of ability to produce infectious virus. It also shows that the effect of this deletion varies with its position in the TM sequence. The production of infectious virus is low for deletion mutations ΔM379, ΔA385, and ΔV386, located near the carboxyl terminus of the TM domain, while ΔA370, ΔV371, ΔA372, ΔS373, and ΔV376, located near the amino terminal end produce significantly more infectious virus. We also found that the inhibitory effect of a single insertion (FF) at the membrane interface is rescued when a compensating single amino acid deletion is placed in the middle of the transmembrane domain restoring the wild type number of amino acids in the E2 C terminal region (FF25). The relative importance of the two halves of the transmembrane domain was illustrated by the observation that the N terminal region of the E2 transmembrane domain could be deleted (TM18N) and the resulting mutant virus maintained wt growth whereas, deletion of the COOH terminal region (TM18C) resulted in a 10,000 fold decrease in infectious virus production.

Viral protein processing and transport appears normal as evidenced by SDS-PAGE analysis of purified virus released from cells. In addition, we have previously shown that protein processing and export from the ER by mutants containing deletions in the transmembrane domain is not affected until the deletion is larger than 12 amino acids (Hernandez et al., 2003). The deletion mutants produced all band at wild type virus density indicating that they are similar in composition to wild type virus in that they contain a normal complement of protein, lipid and RNA. SDS-PAGE of proteins in purified virus produced by the mutants indicated that they contained the same relative amounts of E1, E2 and capsid (Table 2.1). The process of assembly of these viruses is dependent upon a strict interaction of the three structural proteins in a 1:1:1 stoicheometric relationship. These data rule out the possibility that the reduced infectivity of the mutant viruses is the result of failure to assemble the normal amounts (240 copies of each) of virus proteins. Such an event would have, also, altered the buoyant density of the virus produced. Therefore, for all of the mutations produced, we have proposed a set of possible explanations to address the hypotheses stated in the introduction. These hypotheses were initially posed to account for the observed loss of infectious virus production produced by the original TM25 mutant (Hernandez et al., 2003):

Hypothesis i) The observed phenotype of the original TM25 deletion (Hernandez et al., 2003)could be a result of a single specific amino acid deletion at position 379 of E2. We found that the observed phenotype is not the result of the specific deletion of a methionine located at amino acid 379. ∆A385 and ∆V386 give similar reduced titers of approximately 10^6 pfu/ml. In addition the compensatory mutations identified after serial passage of ∆M379 resulted in a restoration of titer without replacing M379. These data suggest that deletion of a specific amino acid is not responsible for the TM 25 phenotype.

Hypothesis ii) The angular location of the deletion on the face of the helix may account for the loss of infectivity (ie. if the deletion is made 90 degrees, 180 degrees or another angle from a set point of reference). Figure 2.2 reveals that the single amino acid deletions at positions ∆M379, ∆A385 and ∆V386 are located on the same side of the hydrophobic helix. These three deletions resulted in reduced virus growth, however ∆V371 is located on the same face of the hydrophobic helix and produces 100 times more infectious virus. This result suggests that the angular location on the face of the helix does not account for the loss of infectivity.

Hypothesis iii) The location of the deletion vertically (ie closer to the lumenal side or closer to the cytoplasmic side of the membrane) may contribute to the observed phenotype. We found that single deletion mutants located closer to the lumenal face of the transmembrane had a less detrimental effect on virus infectivity than those located closer to the cytoplasmic face (Figure 2.2 and 2.3). This relationship was also true for the eight amino acid deletion (TM18) mutants where the TM18C mutant grew to a titer of 10^4 and the TM18N mutant grew to a titer of 10^9 pfu/ml (Figures 2.2 and 2.3). These data suggest that the position of the deletion along the length of the hydrophobic helix plays a determinative role in the effect on virus assembly and infectivity. Interaction between E1 and E2 occurs above the luminal face of the transmembrane region (Anthony and Brown, 1991). This could serve to anchor the proteins in a fixed position in the developing protein shell and all single deletions made in the transmembrane region may result in a one hundred degree rotation distal to the mutation (toward the COOH end). However, only single deletions in the C terminal half of the transmembrane domain result in reduced virus growth. This phenomenon may be explained by positioning the mutant E2 endodomain in an orientation unfavorable for correct interaction with the capsid protein. Deletions closer to the N terminus may allow for more degrees of freedom for E2 to interact properly with the capsid protein. Mutations located closer to the luminal face may have more flexibility to restore the wild type exit point of the protein from the cytoplasmic face of the transmembrane region.

The single deletion in the FF insertion mutant (FF25) mutant restores normal growth to the FF mutant. The FF25 mutant also restores the wild type number of amino acids in the C terminal region of E2. This result suggests the titer was restored because the length of the C terminal domain is critical for normal virus growth whereas the length in the N terminal domain is not critical. This is supported by the TM18N and TM18C comparison.

Hypothesis iv) An interaction between the E1 and E2 transmembrane regions may be necessary for normal virus infectivity. This interaction may stabilize the E2 protein in a configuration where the E2 tail can most efficiently bind with the capsid protein. Figure 2.2 shows that deletion mutations ∆M379, ∆A385, and ∆V386 are all located on one face of the helix and are all also located in the C terminal region of the E2 transmembrane domain. ∆M379, ∆A385 and ∆V386 all give reduced titers (10^6) and therefore may be involved in interactions with the transmembrane region of E1 near the cytoplasmic interface of the transmembrane domain. Strauss et al demonstrated an interaction between E1 and E2 transmembrane domains using a chimeric virus composed of Ross Rover E1 and Sindbis virus E2 (Strauss, Lenches, and Strauss, 2002). They identified a compensatory mutation in the center of the transmembrane domain of E2 (I380). In addition structural evidence from Mancini et al (Mancini et al., 2000) shows E1 and E2 protein densities in the transmembrane domain have the greatest degree of separation at the N terminal region and a minimal amount of separation halfway through the transmembrane domain. Zhang et al also suggest that the E1 and E2 proteins are separated at the N terminal region of the transmembrane domain but come together in a coil-coil structure toward the C terminal region of the transmembrane domain (Zhang et al., 2002). These results suggest the E1 and E2 transmembrane domains interact at a point halfway across the membrane bilayer and the association continues through the C terminal region of the transmembrane domain. This data may also explain why eight amino acids may be removed from the N terminal region of the E2 transmembrane domain with no loss of infectivity while deletion of eight amino acids in the C terminal domain results in a five log decrease of infectious virus production. These E1-E2 associations described in the electron cryomicrographs of Mancini et al (Mancini et al., 2000) may be necessary for virus stability and infectivity.

Only the FF insertion mutant seems to have an affect on virus particle assembly. The other mutants described in this study result in the production of approximately the same number of particles although the percentage of virus that is infectious vary greatly. The deletion mutants described in this study are not inhibited in their ability to transport membrane proteins to the cell surface as significant numbers of virus particles are produced. It has been demonstrated that association with PE2 is necessary for the transport of E1 to the cell surface (Carleton et al., 1997). However, the E2 transmembrane domain deletions described here do not prevent this function. Mutations located closest to the cytoplasmic interface of the E2 transmembrane interface produce the lowest amount of infectious virus but have the highest particle/pfu ratio. This along with SDS-PAGE analysis and negative stains of purified virus particles indicates proteins are being processed correctly, assembled into particles, and released, but the infectivity of these mutants is reduced by some other mechanism – likely by inhibiting cell entry.

After several passages the production of infectious virus by ΔM379, ΔA385, and ΔV386 was restored to wt levels and a 100-fold increase in infectious virus production was observed for the eight amino acid deletion (TM18C) mutant. Compensating mutations were found in the E2 transmembrane domain for all of these mutants (Figure 2.5). For all of the single deletion mutants a restoration to wt length in the TM domain accompanied restoration of wild type phenotype. Wt length in the TM domain may be necessary in the C terminal region of the transmembrane domain to produce a functional interaction with E1 or the capsid protein. The presence of a specific number of amino acids in this region may be necessary for correct protein-protein associations.

In addition to wt length, wt sequence is also restored for all the single deletion compensatory mutations to the C terminal half of the E2 transmembrane domain, except for ΔA385 where an A or V substitution occurred (Figure 2.5). This C terminal sequence may be necessary for proper interaction with E1 in the transmembrane domain. This is supported by the fact that TM18N grows to wt levels but TM18C only grows to a titer of 10^4pfu/ml. Both of these mutants result in

18 amino acids in the transmembrane domain suggesting the sequence may be important for normal infectious virus production.

Compensatory mutations in TM18C resulted in a two-log increase in infectious virus production and the insertion of the amino acid sequence ACAC in the C terminal region of the transmembrane domain. The resulting 22 amino acid transmembrane domain deletion matched that of the original TM22 deletion mutant, produced by deletion cloning, (Figure 2.6) and produced the same amount of infectious virus. This demonstrates that the location of the deletion is important for normal virus production. The insertion of the sequence ACAC in the C terminal region may indicate that it is necessary for normal virus growth. However, when the C at position 388 was converted to a serine from a wt parent no loss in infectious virus production was observed (data not shown). Schlesinger and others mutated both of these cysteines to alanines and found that there was no significant loss of virus infectivity (Ryan, Ivanova, and Schlesinger, 1998) suggesting the cysteines are not vital for normal virus growth. Collectively these results argue that transmembrane domains may serve functions other than to simply anchor the protein.

Data presented above suggest that deletion mutations in the TM domain of the E2 glycoprotein may inhibit events required for cell penetration since mutant viral proteins are processed correctly, assembled into particles, and released from the cell. We are presently examining the ability of the mutants to undergo the structural changes identified in the penetration process by Parades et al (Paredes et al., 2004).

Materials and methods

Cell culture and virus titration

Growth of baby hamster kidney cells (BHK-21) were described previously (Renz and Brown, 1976). BHK cells were grown and maintained in minimal essential medium containing Earl's salts (Invitrogen, Carlsbad Calif.) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 5% tryptose phosphate broth, and 2 mM glutamine as described previously (Renz and Brown, 1976). The virus strain chosen for mutagenesis was Toto1101 (Rice et al., 1987) or Y420 which contains a substitution in E2 at position 420 changing a serine to a tyrosine. Y420, and has been described previously (Liu and Brown, 1993). Titration of virus produced was performed using BHK-21 cell monolayers (Renz and Brown, 1976). Virus was titered in triplicate.

TM mutants and reverse transcription (RT)-PCR analysis of mutant viruses

The TM mutants were all made by QuikChange site-directed mutagenesis (Stratagene, La Jolla, Calif.) as described previously (Hernandez et al., 2003). Mutant viruses were named for the specific amino acid deleted from the amino terminus of the E2 protein. The PCR conditions were 95°C for 1 min, 55°C for 2 min, and 68°C for 18 min. This cycle was repeated 15 times. After the desired mutations were made and confirmed by sequencing, they were subcloned into the Toto 1101 or Y420 vector using the *Bcl*I and *BssH*II (New England Biolabs, Beverly, Mass.)

unique sites and linearized with Xho1. Infectious RNA was transcribed in vitro using SP6 RNA polymerase (New England Biolabs). To confirm that the desired deletions remained in the virus grown in cell culture, the RNA was extracted from the virus, reverse transcribed, and amplified by PCR (RT-PCR) (Hernandez et al., 2003).

In vitro transcription and RNA transfection

The mutant and wild-type cDNA constructs were prepared for transcription and transcribed in vitro as described previously (Hernandez et al., 2000) and (Hernandez et al., 2003). The RNA transcripts were electroporated into BHK or U4.4 cells. The electroporation was performed as described by Lilijestrom and Garoff (Lilijestrom and Garoff, 1991). Virus was harvested 24 h posttransfection.

Virus growth, purification, quantitation and particle/PFU determination

Virus growth in U4.4 and BHK cells and subsequent purification and quantitation were described previously (Hernandez et al., 2003). The highest multiplicity of infection possible for each of the mutant viruses was used for infection. An infection of the Toto1101 parent virus using a corresponding multiplicity of infection was also done (multiplicity of infection range was from 0.006 to 0.1). Virus was harvested from the cell supernatant after 24 h and was spun to equilibrium on 30 to 45% sucrose gradients in PBS-D buffer at 24,000 rpm in a Beckman SW-28 rotor overnight. 0.5 mL fractions were collected and titered (Hernandez et al., 2003). The amount of viral protein was determined by using the Micro BCA protein assay reagent kit (Pierce, Rockford, Ill.). ^{35}S labeled virus proteins were loaded on a

10.8% poly-acrylamide gel to verify normal protein processing and to analyze the stoichiometric ratio of structural proteins produced. The resulting gels were exposed to X-ray film to produce exposures which did not, in any part of the gel, exceed the recording capacity (saturation density) of the film. The resulting protein profiles were scanned with an optical densitometer to obtain optical densities. For each mutant the optical density of the protein E1 was arbitrarily set as 1 and other protein optical densities were presented relative to E1 in Table 2.1.

The number of particles in a preparation of wild-type virus (SVHR) was determined under the electron microscope by the agar filtration protocol described by Kellenberger and Bitterli (Kellenberger and Bitterli, 1976), and the particle count was correlated to the amount of protein as obtained by BCA. The total particle count was divided by the amount of infectious virus particles produced to determine the total number of particles per infectious particle (particle/pfu).

Serial passage of transmembrane mutants

Mutants yielding low infectious virus production were chosen for serial passage. Transfection of BHK cells with mutagenized RNA was performed as referenced above. Virus was harvested and titered on BHK-21 cells (Renz and Brown, 1976). BHK monolayers were subsequently infected with harvested virus at an MOI of 1 or lower depending on the amount of infectious virus produced. Each passage was harvested after cytopathic effect was observed (24-48h) and titered on BHK-21 cells. Plaques were picked, placed in 1 ml PBS-D containing 3% FBS and stored at 4°C O/N. Plaques were flash frozen in N_2 and placed at –80°C until use.

Plaques were then used for a final round of infection. After 24h virus was harvested, extracted, and RT/PCR was performed as described earlier. Resulting PCR products were purified on a 4% agarose gel and were sent directly to be sequenced or subcloned using the pGem T-Vector System 1 (Promega, Madison, WI).

Chapter 3

Identification of free and disulfide bound cysteines in the Sindbis virus E1 and E2 glycoproteins

Abstract

Sindbis virus is a single stranded positive sense RNA virus. It is composed of 240 copies of three structural proteins, E1, E2, and capsid. These proteins are arranged in a 1:1:1 molar ratio and form a mature virus particle composed of two nested T=4 icosahedral shells. A complex network of disulfide bonds in the E1 and E2 glycoproteins is developed through a series of structural intermediates as virus maturation occurs. E1 and E2 cysteine residues were labeled with iodoacetamide in the native virus particle and analyzed by mass spectrometry. This analysis identified cysteines of E1 and E2 which were found to be free in the native virus particle, as well as those that were either solvent inaccessible or blocked by their involvement in disulfide bonds. Native virus particles alkylated with iodoacetamide yielded a four-log decrease in viral infectivity. This suggests that either the bound iodoacetamide alone may be involved in the loss of infectivity by destabilizing the virus particle or that a rearrangement of disulfide bonds, which is required for infectivity, is blocked by the presence of iodoacetamide. To examine if a structural effect due to the alkylation of cysteine, or if a possible disulfide rearrangement was the cause of the loss in virus infectivity, cysteines that were determined to be free in the E1 glycoprotein were mutated to serines. Mutation of these cysteines yielded a drastic effect on infectious virus production. These data suggest that a disulfide bond rearrangement may occur as virus maturation proceeds.

Introduction

Sindbis virus (SV) is positive sense, single stranded RNA virus of the Alphavirus genus in the *Togaviridae* family and is propagated in nature by both insect and mammalian hosts. The mature SV particle contains three structural proteins E1, E2, and capsid (C) in a 1:1:1 stoichiometric ratio. The SV particle is approximately 70 nm in diameter and serves as an excellent model for structural studies. Each virus particle is composed of 240 copies of each of these proteins producing 80 equivalent units which are organized into two geometrically identical T=4 icosahedral shells (Paredes et al., 1993). An outer protein shell, composed of the E1 and E2 glycoproteins, is organized into trimers of E1/E2 heterodimers (Anthony and Brown, 1991; Carleton et al., 1997; Pletnev et al., 2001). The outer protein shell surrounds an inner shell, which is composed entirely of the capsid protein. The 11.7 kb genomic RNA is packed inside the inner capsid shell. Positioned between the two protein shells is a host-derived membrane bilayer that is penetrated by the transmembrane (TM) domain anchors of E1 and E2 (Lilijestrom and Garoff, 1991; Rice et al., 1982; Strauss, Lenches, and Strauss, 2002). The endodomain of E2 interacts with the capsid protein linking the two shells together.

SV produces a subgenomic mRNA that encodes a polyprotein which is post-translationally cleaved into capsid, E1, E2, E3, and 6K. The capsid protein is processed by an auto-proteolitic activity after translation on cytoplasmic ribosomes. Removal of the capsid protein exposes the polyprotein signal sequences which directs insertion of the multipass polyprotein which contains six membrane spanning domains (Lilijestrom and Garoff, 1991). Signalase cleaves the polyprotein into the structural components: PE2, 6K, and E1 (Lilijestrom and Garoff, 1991). The 6K protein plays a role in the correct integration of E1 into the membrane and is necessary for efficient particle budding (Lilijestrom and Garoff, 1991). 6K is found only at very low levels in mature virus (Gaedigk-Nitschko and Schlesinger, 1990). The E1 glycoprotein, containing a functional domain necessary for viral entry, is folded into a stable compact high-energy conformation as it is assembled into heterotrimers with the precursor to E2, PE2 (Carleton et al., 1997; Mulvey and Brown, 1994; Mulvey and Brown, 1995; Mulvey and Brown, 1996). PE2 is transported to the cell surface through the trans Golgi network where it is processed to E2 and E3 by furin (Moehring et al., 1993). Evidence suggests that E2 is responsible for receptor recognition. E3 is not present in the mature virus. During transport of the trimeric complexes to the plasma membrane, a second membrane-spanning region of E2 is retracted from the membrane and a 33 amino acid endodomain is exposed to the cytoplasm. At the plasma membrane the E1-E2 heteotrimers assemble into the outer protein shell by association of the E2 endodomain with a hydrophobic cleft in the capsid protein (Lee and Brown, 1994; Lee et al., 1996; Oates et al., 1992; Owen and Kuhn, 1996; Owen and Kuhn, 1997) This association of the E2 endodomain with the capsid protein gives stability to the structure of the virus (Lee, Ricker, and Brown, 1994) and plays a critical role in the formation of the viral outer protein shell around the preformed inner protein shell as the process of envelopment takes place (Ferreira et al., 2003).

The Sindbis virus E1 protein contains 439 amino acids with an ectodomain consisting of the first 408 residues. E2 contains 423 amino acids and has an endodomain consisting of the first 362 residues. Each of these membrane proteins contains 17 cysteine residues (capsid does not contain any cysteines), which are completely conserved across the Alphavirus genus with the exception of E1 C430 and E2 C388 and C390. These non-conserved cysteines are located in the transmembrane domains. Residues C396, C416 and C417 of the E2 protein are located in the 33 amino acid endodomain. Of the remaining cysteine residues, the possibility of eight disulfide bonds in the E1 protein and six in the E2 protein exists.

Little is known about the E2 disulfide network. Gidwitz et al suggested that all cysteines located in the E2 protein were either involved in disulfide bonds or were not accessible when treated with ^{14}[C] iodoacetamide at a 1.1 molar excess of iodoacetamide to total cysteines (Gidwitz et al., 1988). The Semliki Forest Virus, also a member of the Alphavirus genus, E1 crystal structure has been solved at three angstrom resolution (Roussel et al., 2006). Herein the authors report disulfide bonds located at residues 49-144, 62-94, 63-96, 259-271, 301-376, 328-370, 301-376, and 306-380.

The E1 protein proceeds through a complicated set of interactions as folding occurs in the ER. Once translated, E1 quickly adopts an E1 alpha configuration, proceeds through a series of alternately folded disulfide bond intermediates (beta and gamma) and ultimately adopts a high energy E1 epsilon form (Carleton and Brown, 1996). E1 epsilon associates with PE2 in a heterotrimeric spike and leaves the ER for the trans golgi network (TGN). The presence of the E1 intermediates was determined through an analysis of virus proteins extracted from Brefeldin-A treated BHK cells (Carleton and Brown, 1996) which blocked export of E1 from the ER. It was concluded from these studies that the E1 protein acquires a stable conformation through the sequential formation of disulfide intermediates. It is imperative to emphasize that attempts to extract the E1 protein from mature virus particles with detergent resulted in the generation of a ladder that may represent all the disulfide intermediates (Mulvey and Brown, 1994). The propensity of E1 to adopt alternate conformations makes isolation away from the mature virus in its native form very difficult. The crystal structure presented by Roussel et al is of the E1 protein which was extracted by detergent based methods (Roussel et al., 2006) and may not represent the native form.

In this report, we identify the location of free and disulfide bound cysteines in the Sindbis virus E1 and E2 proteins by incubation of native virus particles in the presence of iodoacetamide. Iodoacetamide alkylates free sulfhydryl groups and, in this case, revealed the location of free cysteines in the E1 and E2 proteins of the native virus particle. While it was found that virus particles alkylated with iodoacetamide resulted in a four-log decrease in virus infectivity, this result was not completely unexpected. Previous models of particle assembly and the infection of host cells predicts the involvement of disulfide intermediates (Carleton and Brown, 1996; Mulvey and Brown, 1994; Mulvey and Brown, 1996; Paredes et al., 2004). To examine the role of specific disulfide intermediates, cysteines found to be free in the native E1 protein were mutated to serine. Each of these substitution mutations resulted in at least a three-log decrease in infectious virus production. These data taken together with identification of the cysteines suggests that free cysteines are present in the infectious virus particle and that disulfide bond rearrangements may occur as virus maturation proceeds.

Results

Location of the free cysteines in the Sindbis Virus E1 and E2 proteins

Mature virus particles were collected from the media of infected BHK cells and purified over potassium tartrate gradients. Purified virus was then dialyzed against PBS-D and alkylated by the addition of 500 fold molar excess iodoacetamide to total cysteine. After SDS PAGE analysis to separate the viral proteins and remove excess iodoacetamide, virus particles were reduced with DTT and treated with 20 mM iodomethane (see Materials and Methods). Using this labeling technique all cysteines containing a free sulfhydryl group (not involved in a disulfide bond) and accessible to solvent would be labeled with iodoacetamide. All other cysteines would be labeled with iodomethane. After labeling samples were examined by LC/MS/MS (ESI). Figure 3.1, Figure 3.2, and Table 3.1 represent a compilation of the data collected for the Sindbis virus E1 protein. This approach labeled 14 of the 17 cysteines of E1. E1 cysteines 49, 114, 259, 271, and 430 were found to be labeled by both iodoacetamide and iodomethane. This suggests that these cysteines contained free sulfyhydryl groups but were only partially accessible to iodoacetamide. The remainder of the population would be labeled by iodomethane after the virus had been reduced. E1 cysteines 62, 63, 68, 78, 301, 306, 328, 370, 376, and 380 were only labeled by iodomethane suggesting that these residues were either solvent inaccessible or their sulfyhydryl groups were

E1 structural protein - (14 of 17 identified)
YEHATTVPNVPQIPYKALVERAGYAPLNLEITVMSSEV
LPSTNQEYITCKFTTVVPSPKIKCCGSLECQPAAHADY
TCKVFGGVYPFMWGGAQCFCDSENSQMSEAYVELSA
DCASDHAQAIKVHTAAMKVGLRIVYGNTTSFLDVYVN
GVTPGTSKDLKVIAGPISASFTPFDHKVVIHRGLVYNY
DFPEYGAMKPGAFGDIQATSLTSKDLIASTDIRLLKPSA
KNVHVPYTQASSGFEMWKNNSGRPLQETAPFGCKIAV
NPLRAVDCSYGNIPISIDIPNAAFIRTSDAPLVSTVKCEV
SECTYSADFGGMATLQYVSDREGQCPVHSHSSTATLQ
ESTVHVLEKGAVTVHFSTASPQANFIVSLCGKKTTCNA
ECKPPADHIVSTPHKNDQEFQAAISKTSWSWLFALFGG
ASSLLIIGLMIFACSMMLTSTRR

Blue = iodomethane
Red = iodoacetamide
Green = Both

Figure 3.1: Representation of labeled cysteine residues in the amino acid sequence of the Sindbis virus E1 protein as determined by mass spectrometry. Cysteines highlighted in blue were alkylated by iodomethane. Cysteines highlighted in red were alkylated by iodoacetamide. Cysteines shown in green represent residues that were alkylated by both reagents.

E2 structural protein - (5 of 17 identified)
SVIDDFTLTSPYLGTCSYCHHTVPCFSPVKIEQVWDEA
DDNTIRIQTSAQFGYDQSGAASANKYRYMSLKQDHTV
KEGTMDDIKISTSGPCRRLSYKGYFLLAK**C**PPGDSVTV
SIVSSNSATSCTLARKIKPKFVGREKYDLPPVHGKKIP**C**
TVYDRLKETTAGYITMHRPRPHAYTSYLEESSGKVYA
KPPSGKNITYE**C**KCGDYKTGTVSTRTEITG**C**TAIKQCV
AYKSDQTKWVFNSPDLIRHDDHTAQGKLHLPFKLIPST
CMVPVAHAPNVIHGFKHISLQLDTDHLTLLTTRRLGAN
PEPTTEWIVGKTVRNFTVDRDGLEYIWGNHEPVRVYA
QESAPGDPHGWPHEIVQHYYHRHPVYTILAVASATVA
MMIGVTVAVLCACKARRE**C**LTPYALAPNAVIPTSLALL
CCVRSANA

Blue = iodomethane
Red = iodoacetamide
Green = Both

Figure 3.2: Representation of labeled cysteine residues in the amino acid sequence of the Sindbis virus E2 protein as determined by mass spectrometry. Cysteines highlighted in blue were alkylated by iodomethane. Cysteines highlighted in red were alkylated by iodoacetamide. Cysteines shown in green represent residues that were alkylated by both reagents.

Table 3.1: Free and bound cysteines of the Sindbis virus E1 and E2 proteins. Residues listed in the "Inaccessible or Bound" column represent cysteines that were only alkylated by iodomethane. Residues listed in the "Free" column were detected alkylated with iodoacetamide. "% Free" is the calculated amount of cysteine labeled with iodoacetamide when both labels were detected.

	Inaccessible or Bound	Free	% Free
E1	62, 63, 68, 78, 301, 306, 328, 370, 376, 380	49, 114, 259, 271, 430	114 = 90% 259 = 17% 271 = 73% 430 = 100%
E2	201, 411	105, 152, 220	105 = 100% 152 = 0.5% 220 = 1.5%

Reported E1 disulfide bonds 49-114, 62-94, 63-96, 259-271, 301-376, 328-370, 301-376, and 306-380 (Roussel et al., 2006) blocked due to the presence of disulfide bonds. E1 C430 was only detected in the form labeled by iodoacetamide suggesting it was accessible to solvent and was not involved in a disulfide bond. In Table 3.1 a column titled “% free” represents a measure of the percent of the sample alkylated by iodoacetamide (the population that was accessible and had free sulfhydryl groups) when both labels were detected. Precursor ions and extracted ion chromatograms were utilized to obtain this data and offer only an approximation of the true value. The percentage of cysteines alkylated by iodoacetamide for E1 cysteines 114, 259, 271, and 430 was 90%, 17%, 73%, and 100% respectively. The remaining percentage was found alkylated by iodomethane. These values indicate that some cysteines in the native virus particle are more accessible to iodoacetamide while some are less accessible and are buried deeper in the virus particle.

Five of the 17 cysteines were labeled in the E2 protein. C105, C152, and C220 were all labeled, at least partially, with the iodoacetamide reagent demonstrating that a population of these cysteines have free sulfhydryl groups and are accessible to solvent and therefore cannot be involved in disulfide bonds in the native particle. E2 C201 and C411 were only detected alkylated by iodomethane and may be involved in disulfide bonds. Analysis of precursor ions and extracted ion chromatograms revealed that only a small population of E2 152 and 220 were found to have free sulfhydryl groups (0.5% and 1.5% respectively). E2 C105 was only detected in the “free” state.

As a control, RNase B was examined under the same cysteine concentration and same conditions as the purified virus samples. All eight cysteines of RNase B are known to be involved in disulfide bonds. Five of the eight cysteines were labeled and detected by mass spectrometry (Figure 3.3). All cysteines detected were only alkylated by iodomethane indicating that they were not accessible to solvent or that they were blocked by the presence of disulfide bonds. This validates the labeling strategy since no cysteines of RNase B were found to have free sulfhydryl groups. This is in agreement with the published disulfide network present in RNase B (Shin et al., 2003).

Effect of iodoacetamide labeling on virus infectivity

Purified virus was incubated at 100 or 500 fold excess iodoacetamide to cysteine at 37 degrees C and pH 8.0 for 2 hours and was titered on BHK cells (Table 3.2). Purified virus treated with 100 fold excess of iodoacetamide resulted in a two log decrease in virus infectivity while treatment of virus with 500 fold excess iodoacetamide resulted in a three and a half log decrease of infectious virus when compared to wild type purified virus. This data reveals that iodoacetamide is alkylating free cysteine residues in the native virus particle and that the presence of the alkyl group has a detrimental effect on virus infectivity. The presence of the alkyl group alone may destabilize the virus structure or possibly the cysteines that are found to be free in the native virus particle may be involved in disulfide bonds during an early step in virus infection. It should also be noted that approximately the same amount of infectious virus was found to remain infectious when purified virus was stored at either pH 7.4 or 8.0 (data not shown). Iodoacetamide alkylation

RNase B - (5 of 8 identified)
KETAAAKFERQHMDSSTSAASSSNYCNQMMKSRNLT
KDR**C**KPVNTFVHESLADVQAV**C**SQKNVACKNGQTN**C**
YQSYSTMSITD**C**RETGSSKYPN**C**AYKTTQANKHIIVAC
EGNPYVPVHFDASV

Blue = iodomethane
Red = iodoacetamide
Green = Both

Figure 3.3: Representation of labeled cysteine residues in the amino acid sequence of RNase B as determined by mass spectrometry. Cysteines highlighted in blue were alkylated by iodomethane. Cysteines highlighted in red were alkylated by iodoacetamide. Cysteines shown in green represent residues that were alkylated by both reagents.

Table 3.2: Infectivity of purified virus alkylated with iodoacetamide as determined by plaque assay on BHK cell monolayers. Purified virus was incubated at 100 or 500 fold excess iodoacetamide to cysteine at 37 degrees C and pH 8.0 for 2 hours and was titered on BHK cells.

Virus	Titer
Purified virus	2.0×10^{10} pfu/ml
Purified Virus 100x	1.5×10^{8} pfu/ml
Purified virus 500x	9.0×10^{6} pfu/ml

of cysteine is most efficient at pH 8.0 and since no differences in infectious viral titer were observed at pH 8.0, this pH was adopted for experimentation.

Conformational changes induced with low pH do not alter the location of free cysteines

It has been well established that exposure of Sindbis virus to acid pH (5.3) and subsequent return to neutral pH (pH 7.4) induces cell to cell fusion from without (FFWO) of cell monolayers resulting in the formation of synctia (Paredes et al., 2004). This pH induced conformational change may correlate to a structural change which occurs on the virus surface during entry possibly induced by interactions with the host receptor (Paredes et al., 2004). Electron cryomicroscopy analysis has revealed that as the pH is reduced from 7.4 to 5.3 a new spike structure emerges from the E1 protein, which normally lays flat along the surface of the virus, within five-fold axis (Paredes et al., 2004). This reconfiguration of E1 may be necessary for delivery of the viral genome into the host cell (Paredes et al., 2004). Once the virus is returned to neutral pH the virus structure partially reverts to a more native structure where this new spike structure is withdrawn back into the virus surface. Since this low pH mediated fusion event may mimic entry, the location of the free cysteines was investigated to see if disulfide rearrangement occurred after induction of these conformational changes. The intermediate low pH form (5.3) could not be alkylated due to the kinetics of labeling with the iodoacetamide reagent. However the viral structure after exposure to low pH (5.3) and return to neutral pH was examined by alklylation with iodoacetamide.

The location of the free cysteines after the pH shift described above is illustrated in Figure 3.4, Figure 3.5, and Table 3.3. Six of the 17 cysteines of E1 were identified. The reduction in the amount of coverage for the pH shift experiments compared to the non-shifted sample were probably due to the sample concentration. When the pH was shifted to 5.3 the virus began to precipitate above certain concentrations. Therefore the concentration was adjusted to an amount where no precipitation occurred (see Methods). Both C259 and C271 of E1 were found to be alkylated by iodoacetamide suggesting that they are not involved in disulfide bonds and are accessible to solvent. Cysteines at positions 62, 301, 306, and 328 were only alkylated with iodomethane reagent suggesting that these residues are not available to reagent and that they may be involved in disulfide bonds. The % free (% labeled with iodoacetamide reagent) for C259 was 29% and 100% for C271. This shows a general increase of accessibility to reagent for the pH-shifted sample compared to the sample maintained at pH 8.0. However, no cysteines that were found to have free sulfyhydryl groups at pH 8.0 were found to be inaccessible or blocked after the pH shift or vice versa. No detectable rearrangement of disulfide bonds was observed after low pH exposure.

Three of the 17 cysteines of E2 were alkylated after the pH shift (7.4- 5.3-8.0). E2 C220 was found to have a free sulfhydryl group while C152 and C411 were only found alkylated with the iodomethane reagent indicating that they were inaccessible or in disulfide bonds after the pH shift. After the pH shift 6% of the E2 C220 was found to be alkylated by iodoacetamide while 94% was found to be alkylated by iodomethane indicating that six percent of the detected C220 were found to have a

E1 structural protein - (6 of 17 identified)
YEHATTVPNVPQIPYKALVERAGYAPLNLEITVMSSEV
LPSTNQEYITCKFTTVVPSPKIK**C**CGSLECQPAAHADYT
CKVFGGVYPFMWGGAQCFCDSENSQMSEAYVELSAD
CASDHAQAIKVHTAAMKVGLRIVYGNTTSFLDVYVNG
VTPGTSKDLKVIAGPISASFTPFDHKVVIHRGLVYNYDF
PEYGAMKPGAFGDIQATSLTSKDLIASTDIRLLKPSAKN
VHVPYTQASSGFEMWKNNSGRPLQETAPFG**C**KIAVNP
LRAVD**C**SYGNIPISIDIPNAAFIRTSDAPLVSTVK**C**EVSE
CTYSADFGGMATLQYVSDREGQ**C**PVHSHSSTATLQES
TVHVLEKGAVTVHFSTASPQANFIVSLCGKKTTCNAEC
KPPADHIVSTPHKNDQEFQAAISKTSWSWLFALFGGAS
SLLIIGLMIFACSMMLTSTRR

Blue = iodomethane
Red = iodoacetamide
Green = Both

Figure 3.4: Representation of labeled cysteine residues in the amino acid sequence of the Sindbis virus E1 protein as determined by mass spectrometry after exposure to low pH. Cysteines highlighted in blue were alkylated by iodomethane. Cysteines highlighted in red were alkylated by iodoacetamide. Cysteines shown in green represent residues that were alkylated by both reagents.

E2 structural protein - (3 of 17 identified)
SVIDDFTLTSPYLGTCSYCHHTVPCFSPVKIEQVWDEA
DDNTIRIQTSAQFGYDQSGAASANKYRYMSLKQDHTV
KEGTMDDIKISTSGPCRRLSYKGYFLLAKCPPGDSVTV
SIVSSNSATSCTLARKIKPKFVGREKYDLPPVHGKKIP**C**
TVYDRLKETTAGYITMHRPRPHAYTSYLEESSGKVYA
KPPSGKNITYECKCGDYKTGTVSTRTEITG**C**TAIKQCV
AYKSDQTKWVFNSPDLIRHDDHTAQGKLHLPFKLIPST
CMVPVAHAPNVIHGFKHISLQLDTDHLTLLTTRRLGAN
PEPTTEWIVGKTVRNFTVDRDGLEYIWGNHEPVRVYA
QESAPGDPHGWPHEIVQHYYHRHPVYTILAVASATVA
MMIGVTVAVLCACKARRE**C**LTPYALAPNAVIPTSLALL
CCVRSANA

Blue = iodomethane
Red = iodoacetamide
Green = Both

Figure 3.5: Representation of labeled cysteine residues in the amino acid sequence of the Sindbis virus E2 protein as determined by mass spectrometry after exposure to low pH. Cysteines highlighted in blue were alkylated by iodomethane. Cysteines highlighted in red were alkylated by iodoacetamide. Cysteines shown in green represent residues that were alkylated by both reagents.

Table 3.3: Free and bound cysteines of the Sindbis virus E1 and E2 proteins after exposure to low pH. Residues listed in the "Inaccessible or Bound" column represent cysteines that were only alkylated by iodomethane. Residues listed in the "Free" column were detected alkylated with iodoacetamide. "% Free" is the calculated amount of cysteine labeled with iodoacetamide when both labels were detected.

	Inaccessible or Bound (pH 7.4-5.3-8.0)	Free (pH 7.4-5.3-8.0)	% Free
E1	62, 301, 306, 328	259, 271	259 = 29% 271 = 100%
E2	152, 411	220	220 = 6%

free and accessible sulfhydryl group after the pH shift. There is an increase from 1.5% to 6% for the amount of free C220 when the pH is shifted from 7.4 to 5.3 to 8.0 compared to when it is held constant at pH 8.0. The low pH shift likely induces a conformation where C220 of E2 is more accessible to solvent. Once again no detectable rearrangement of disulfide bonds was observed after low pH exposure.

The effect of low pH exposure on virus infectivity was also examined. Virus was maintained at pH 8.0 or was reduced to pH 5.3 for 10 minutes at 37 degrees C and then returned to pH 8.0. Virus held constant at pH 8.0 had a titer of 3.0×10^{10} pfu/ml while virus exposed to low pH resulted in a three-log decrease in virus infectivity to 2.0×10^{7} pfu/ml. This is in agreement with previously published literature (Edwards, Mann, and Brown, 1983).

Effects of E1 cysteine mutations on infectious virus production

In order to examine the effects of the E1 cysteines that were found to be free, cysteine to serine substitution mutants were constructed for C49, C259, and C271. These three residues were all found to have free sulfhydryl groups according to the mass spectrometry analysis. Interestingly all of these mutants exhibited a large decrease in infectious virus production (Figure 3.6). No viable virus was detected for the C49S mutant while the C259S and C271S each yielded infectious virus production of 2.5×10^{3} pfu/ml and 3.5×10^{5} pfu/ml respectively. Wild type virus yielded a titer of 5.0×10^{8} pfu/ml. These data indicate that while these residues do not seem to be involved in disulfide bonds they are critical for normal virus production. These residues may be free in the native virus and after the low pH shift

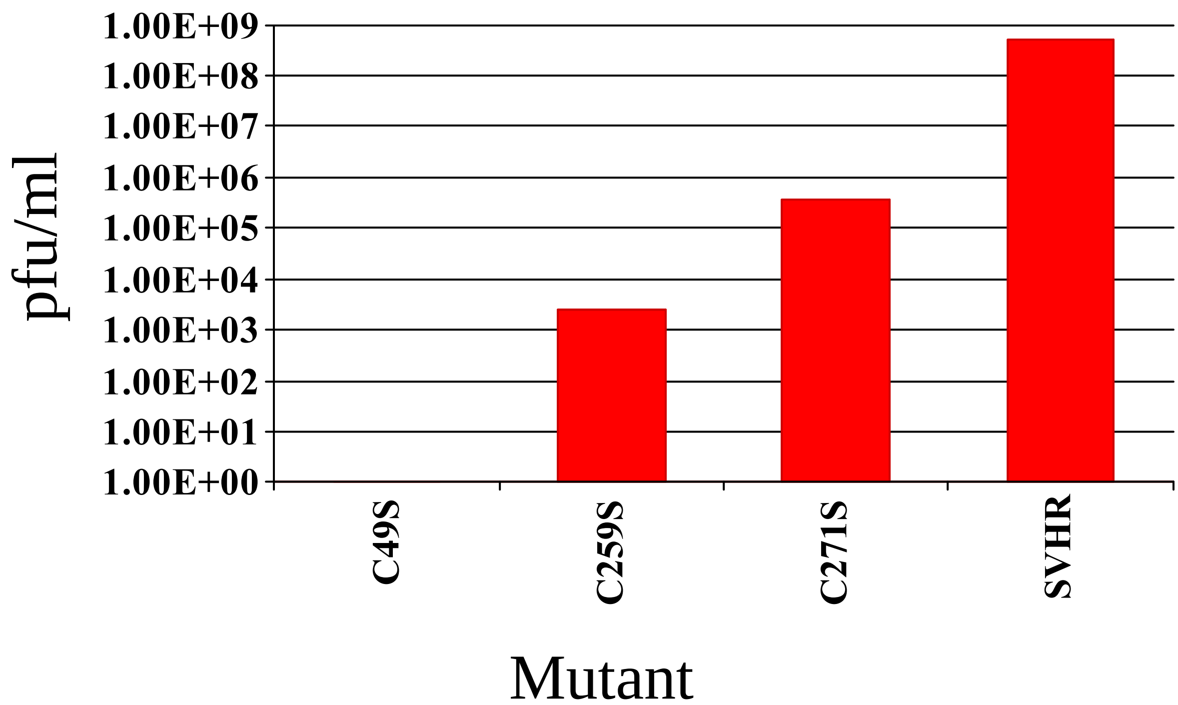

Figure 3.6: Infectious virus production of E1 cysteine mutants. BHK grown virus was harvested after 24h and titered on BHK cells.

(this could possibly be the result of the production of disulfide intermediates which form at low pH but return to the original configuration after adjusting the pH to 8.0) but may yet interact in a disulfide bond as virus maturation proceeds as evidenced by the different disulfide forms produced by E1 during folding in the ER (Carleton and Brown, 1996).

Discussion

Disulfide bonds are essential to Sindbis virus assembly and infectivity. Sindbis virus E1 and E2 proteins contain 17 cysteines apiece. In order to gain insight into the nature of the disulfide network a double labeling strategy, in combination with mass spectrometry, was employed that would allow for the identification of free cysteines present in the mature virus particle.

Native virus particles were collected from the supernatant of BHK cells infected with Sindbis virus. Particles were purified over tartrate gradients, dialyzed against pH 8.0 PBS-D and subsequently alkylated by incubation with 500 fold molar excess of iodoacetamide to total cysteine present. After separating the proteins thorough SDS PAGE, disulfide bonds of extracted proteins were reduced with DTT and the free sulfhydryl groups were then alkylated with iodomethane. Utilizing this technique all free sulfhydryl groups present in the mature virus particle should be labeled by iodoacetamide since only the native virus particle was exposed to iodoacetamide. Excess iodoacetamide was removed during the electrophoresis step and therefore there is no competition with iodomethane and subsequent false labeling of a cysteine after reduction with DTT. The efficiency of this technique was verified by the use of RNase B as a control (Figure 3.3). RNase B contains eight cysteines that are known to be involved in four disulfide bonds. No cysteines of RNase B were found to be alkylated with the iodoacetamide reagent demonstrating there were no free sulfhydryl groups available to iodoacetamide. After reduction of RNase B with DTT, the presence of cysteines labeled by iodomethane were detected. All cysteines detected were labeled by iodomethane demonstrating complete labeling under the conditions utilized.

Labeling of virus particles at pH 8.0 revealed E1 cysteines 49, 114, 259, and 271 were alkylated by both iodoacetamide and iodomethane reagents (Figure 3.1). The presence of the two labels is detected by the difference in mass of the corresponding label. Labeling with iodoacetamide results in a net increase of 57.02 Da per cysteine residue while labeling with iodomethane results in a net increase of 14.02 Da per cysteine residue. Final alkylation with iodomethane is a necessary step in order to prevent disulfide rearrangement from occurring before analysis by mass spectrometry. Since E1 residues 49, 114, 259, and 271 were labeled, at least in part, by iodoacetamide these cysteines must have free sulfhydryl groups and therefore cannot be involved in disulfide bonds. The finding that all of these residues were also partially detected with iodomethane alkylation may be due to the nature of folding of the mature virus particle. Certain cysteines may have limited access to reagent and therefore would not be completely alkylated by the iodoacetamide reagent in the native state. However, upon reduction of the proteins with DTT they become readily available and are alkylated by the iodomethane reagent. The percentage of cysteine labeled by iodoacetamide compared to the percentage of cysteine labeled by iodomethane is illustrated in Table 3.1. This data represents only an approximation based on extracted ion chromatograms and precursor ions. E1 114 and 271 were mostly alkylated by the iodoacetamide reagent (90% and 73% free) suggesting these cysteines were more accessible to the iodoacetamide reagent that cysteine 259 (17% free). E1 C259 may be buried deeper within the virus particle than C114 and C259. C430, which lies in the transmembrane domain, was easily accessible to reagent as 100% of the cysteines detected were labeled by iodoacetamide. E1 cysteines 62, 63, 68, 78, 301, 306, 328, 370, 376, and 380 were only detected labeled by iodomethane. This suggests that these cysteines are involved in a disulfide bond or are inaccessible to reagent in the native virus particle.

Much of this data is in agreement with the reported disulfide bonds of Roussel et al based on the crystal structure of the Semliki Forest Virus E1 protein (Roussel et al., 2006). Roussel reports that all E1 cysteines with the exception of 430 are involved in disulfide bonds (Roussel et al., 2006). Here we report that cysteines 49, 114, 259, 271, and 430 are found to have free sulfhydryl groups and are not involved in disulfide bonds. Roussel reports that E1 cysteines 49 and 114 and cysteines 259 and 271 are linked together in disulfide bonds. One reason for the discrepancy may be the utilization of detergent based extraction methods employed during the preparation of the Semliki Forest Virus E1 protein for crystallography. It was previously shown that Sindbis virus E1 adopts many disulfide states upon extraction from the native virus particle with detergent (Mulvey and Brown, 1994). The crystal structure of E1 from Semliki Forest Virus may be of one of these non-native states.

Five of the 17 cysteines of E2 were detected with mass spectrometry (Figure 3.2). Of these, E2 cysteines 105, 152, and 220 were partially labeled by the iodoacetamide reagent indicating that they contain free sulfhydryl groups and are not involved in disulfide bonds. E2 cysteines 201 and 220 were only detected labeled by the iodomethane reagent and therefore are either involved in disulfide bonds or are inaccessible to the reagent. E1 C152 and C220 were found to be labeled by iodoacetamide approximately 0.5% and 1.5% of the time. The vast majority of these cysteines were labeled by the iodomethane reagent after the virus was reduced by DTT. This suggests that these cysteines are deeply buried within the native virus particle and largely unavailable to the iodoacetamide reagent. In any case, a portion of E2 C152 and C220 were labeled by iodoacetamide suggesting that they are not involved in disulfide bonds.

The effect of labeling native virus particles by iodoacetamide was examined by plaque assay. Purified virus was incubated with either 100 or 500 fold molar excess iodoacetamide to total cysteine for two hours at 37 degrees Celsius and was titered on BHK cells (Table 3.2). The loss of infectivity was dependent on the concentration of iodoacetamide present. A two-log decrease in viral infectivity was observed when virus was treated with 100 fold excess iodoacetamide and a 3.5 log decrease was observed when virus was treated with 500 fold excess iodoacetamide. The presence of iodoacetamide alone accounts for this decrease in viral infectivity indicating iodoacetamide is alkylating free sulfhydryl groups. The alkyl group itself may be responsible for the loss of virus infectivity by destabilizing the structure of the native viral particle. Alternatively early steps in virus infectivity may be inhibited by the presence of the alkyl group on the free cysteines. Carleton et al demonstrated that Sindbis virus E1 protein adopts several different disulfide structures as virus maturation progresses composed of alpha, beta, and gamma forms (Carleton and Brown, 1996). Possibly a cysteine which is not involved in a disulfide bond in the native state is later involved in a disulfide bond during the viral life cycle. This later step would then be inhibited by the presence of the alkyl group added during the iodoacetamide reaction. These results would suggest that a disulfide bond rearrangement occurs as virus matures. This is substantiated by the loss in viral infectivity with increasing concentrations of iodoacetamide. For instance if a cysteine were found to be free and not involved in a disulfide bond throughout the virus life cycle such a large decrease in virus infectivity may not be expected.

Fusion of a cell monolayer may be achieved by adding virus particles to the cells, subjecting the cells to low pH (5.3) and then returning them to neutral pH (7.4) (Paredes et al., 2004) This pH shift results in a conformational change on the surface of the virus that may correlated to structural changes which occur during virus entry (Paredes et al., 2004). To investigate the effects this conformational change may have on the disulfide network, iodoacetamide and iodomethane labeling of free cysteines was utilized after the pH shift. Due to the inefficiency of labeling at low pH, the virus particle was labeled after the virus had been subjected to pH 5.3 for 10 minutes and then returned to pH 8.0.

Six of the 17 E1 cysteines were identified by mass spectrometry after the pH shift (Figure 3.4 and Table 3.3). E1 cysteines 259 and 271 were found to be labeled by both iodoacetamide and iodomethane. Since only the native virus particle was exposed to iodoacetamide C259 and C271 must have free sulfhydryl groups and therefore cannot be involved in disulfide bonds. 29% of the detected C259 and 100% of the detected C271 was found to be labeled with iodoacetamide after the pH shift. When compared to the percentage of C259 and C271 labeled with iodoacetamide from the samples that were maintained at pH there is a general increase in solvent accessibility. The percentage of C259 labeled with iodoacetamide at pH 8.0 was 17% and rose to 29% after the pH shift. The percentage of C271 labeled with iodoacetamide at pH 8.0 was 73% and rose to 100% after the pH shift. These findings indicate a structure that is more accessible to solvent after the pH shift. E1 cysteines at positions 62, 301, 306, and 328 were only found to be alkylated by the iodomethane reagent suggesting that these residues are either buried in the particle where they are not accessible to reagent or they are involved in disulfide bonds. It should also be noted that the same cysteines found to be inaccessible or in disulfide bonds at pH 8.0 were the same ones that were detected after the pH shift. The same is true for those cysteines that were found to have free sulfhydryl groups regardless of the pH. This correlating data implies that a disulfide bond rearrangement does not exist, based on the cysteines detected, between the native state and the state occupied after the pH shift to 5.3 and back to pH 8.0. If this pH shift does correlate to a change in conformation necessary for viral entry it may proceed without a disulfide bond rearrangement.

However the presence of free cysteines at pH 5.3 could not be determined allowing for the possibility of disulfide bond rearrangement at low pH followed by a subsequent rearrangement back to a more native state upon return to pH 8.0.

Only three cysteines of E2 were detected after the pH shift. E2 C220 was labeled partially by iodoacetamide indicating that it contains a free sulfhydryl group and is not involved in a disulfide bond. Six percent of C220 were found to be labeled with iodoacetamide. Before the pH shift only 1.5% of C220 was detected labeled with iodoacetamide indicating once again that after the pH shift the virus particle adopts a structure that is more accessible to reagent. E2 cysteines 152 and 411 were only alkylated by the iodomethane reagent indicating that they were inaccessible to the reagent or that they were involved in disulfide bonds. E2 cysteines 152 and 411 were found to be inaccessible or involved in disulfide bonds before and after the pH shift. C220 was found to have a free sulfhydryl group before and after the pH shift. This once again illustrates that no disulfide bond rearrangement occurred for the cysteines detected after the virus was treated with low pH and then returned to neutral pH and may indicate that disulfide bond rearrangement is not necessary for viral entry.

Labeling of cysteines by iodoacetamide in the Sindbis virus E1 and E2 proteins also indicates regions of the native virus particle that are accessible to reagent. Sindbis virus E1 (49, 114, 259, 271, and 430) and E2 (105,152, and 220) cysteine residues were found to be accessible to iodoacetamide at neutral pH. E1 (259 and 271) and E2 (220) cysteine residues were found to be accessible at pH

5.3. These data along with others help provide insight into the surface accessible regions of the virus particle under different conditions (Table 3.4).

Since several cysteines of E1 were found to not be involved in disulfide bonds, three mutants were constructed to evaluate the effect of E1 cysteines C49, 259, and C271 on infectious virus production. In each case cysteine was converted to serine. Interestingly all of these mutants had a great effect on the amount of infectious virus particles produced (Figure 3.6) as may be expected since all of these cysteines are completely conserved across the Alphavirus genus. No viable virus was detected for the C49S mutant even though it was found to have a free sulfhydryl group in the native particle. Similar results were found for the C259S and C271S mutants where C259S yielded a six-log decrease in infectious virus production and C271S yielded a three-log decrease in infectious virus production. All three of these residues were found to have free sulfhydryl groups and not be involved in disulfide bonds but all yielded a large decrease in infectious virus production. This finding reinforces the idea that disulfide bond rearrangement occurs as virus maturation proceeds. If these cysteines remain free during the complete Sindbis virus life cycle such a large decrease in infectious virus production would not be expected. These residues may contain free sulfhydryl groups in the native state but may then be involved in a rearrangement during virus entry or at some later point as virus maturation progresses.

The data presented herein suggest that Sindbis virus E1 cysteines 49, 114, 259, 271, and 430 and E2 cysteines 105, 152, and 220 are not involved in disulfide

Table 3.4: Accessible regions of the Sindbis virus E1 and E2 proteins. This table reflects a brief summary of the literature in reference to accessible regions located on the Sindbis virus E1 and E2 proteins as determined by multiple methods (see Reference column). All experiments were carried out at neutral pH unless stated otherwise.

E1	E2	Assay	Reference
K16	K70, K76, K97, K131, K149, K202, K235	Accessible to biotin	(Sharp et al., 2006)
1-21, 161-176, 212-220	31-84, 134-148, 158-186, 231-260, 299-314, 324-337	Accessible to protease at neutral pH	(Phinney, Blackburn, and Brown, 2000)
273-287, 145-158, 86-91, 1-29, 110-119, 121-128, 162-170, 177-200, 206-217	99-102, 262-309, 42-66, 168-179, 237-257, 330-338	Accessible to protease at pH 4.5	(Phinney, Blackburn, and Brown, 2000)
Y1, Y15, Y24	Y155, Y165, Y188, Y140, Y328, Y339, Y53	Accessible to Iodination	(Phinney, Blackburn, and Brown, 2000)
	181-216	Interacts with neutralizing antibody	(Strauss et al., 1991)
	173-220	Epitope recognized by antibody	(Wang and Strauss, 1991)
130, 133, 392, 393		Accessible to furin	(Nelson et al., 2005)
C49, C114, C259, C271, C430	C105, C152, C220	Accessible to iodoacetamide at neutral pH	Current text
C259, C271	C220	Accessible to iodoacetamide at pH 5.3	Current text

bonds. In addition, native virus particles labeled by iodoacetamide had a four-log decrease in virus infectivity as well as E1 mutants C49S, C259S, and C271S yielded a large decrease in infectious virus production. Taken together this data suggests that cysteines found free in the native virus particle may undergo a necessary rearrangement where they are later involved in a disulfide bond during virus maturation.

Materials and Methods

Cell maintenance, virus growth, purification and labeling

BHK-21 cells were grown and maintained in supplemented minimum essential media as described previously (Renz and Brown, 1976; Whitehurst et al., 2006). The Sindbis virus heat resistant strain was used to infect BHK cells at an MOI of 10 (Hernandez et al., 2003). Virus was harvested from the cell supernatant at 24 hpi and was purified over a 15%/35% step tartrate gradients by centrifugation at 24K rpm at 4 degrees C in a Beckman SW28 rotor o/n. The visible band of virus was extracted with a needle, diluted two fold and placed over a continuous 15-35% tartrate gradient. This gradient was centrifuged in a Beckman SW28 rotor at 26K for 5 h at 4 degrees C. The virus band was collected with a needle and dialyzed against PBS-D pH 8.0 o/n. The amount of viral protein was quantitated by using the Micro BCA protein assay kit (Pierce).

Purified virus along with cysteine equimolar amounts of RNase B (Sigma), to serve as an control, was either maintained at pH 8.0 or were adjusted to pH 5.3, placed at 37 degrees C for 10 min and then returned to pH 8.0. Samples that were reduced to pH 5.3 were diluted to approximately 50 μg/ml due to precipitation at the normal working concentration of approximately 200 μg/ml. At this time all samples were incubated with 500 fold molar excess iodoacetamide (Sigma) to total cysteine and were placed at 37 degrees C for 2 h. A portion of iodoacetamide labeled virus was removed for titration (Renz and Brown, 1976) and the corresponding result was compared to a non-labeled control treated in the same fashion.

SDS-PAGE and preparation of samples for LC/MS/MS

Samples were boiled in 5X SDS sample buffer devoid of any reductants and were run on a 10.8% polyacrylamide gel. The gel was fixed 2x with 50% methanol and 10% acetic acid for 15 min. at RT. The gel was stained O/N with Ruby Red (Invitrogen) and washed 2x for 30 min. with 10% methanol and 7% acetic acid. Bands were then visualized with UV and cut out and place in 1.5 ml eppendorff tubes.

Gel slices were washed 2x with pH 8.0 50 mM ammonium bicarbonate for 10 min and destained 3x for 20 min. with 90 min with 50% acetonitrile, 50% 50 mM ammonium bicarbonate. Samples were dehydrated with 95% acetonitrile and placed in a speed vacuum until dry. Samples were rehydrated in 50 mM ammonium bicarbonate (pH 8.0) containing 5 mM DTT and were placed at 37 degrees C for 1 h. Samples were placed in a speed vacuum until dry and they were incubated with 20 mM iodomethane (Sigma) in 50 mM ammonium bicarbonate at 37 degrees C for 1h. Gel slices were washed 3x for 10 min with 50 mM ammonium bicarbonate (pH 8.0) and dehydrated with 95% acetonitrile for 10 min. After drying in a speed vacuum the samples were rehydrated with 50 mM ammonium bicarbonate and the gel slices were crushed into pieces. Samples were then dried in the speed vacuum and were resuspended in 50 mM ammonium bicarbonate containing either trypsin or chymotrypsin in a 1:5 enzyme to protein ratio. Samples were then placed at 37 (trypsin) or 25 degrees (chymotrypsin) C O/N. Peptides were then extracted by sonication 3x for 20 min. with 60% acetonitrile, 35% 50 mM ammonium bicarbonate, and 5% formic acid. Samples were dried, resuspended in 50 mM ammonium bicarbonate with 5% acetonitrile and 0.1% formic acid.

Microcapillary reversed-phase LC/MS/MS analysis of SCXC fractioned peptides

Peptides were separated by microcapillary reversed-phase liquid chromatography–tandem mass spectrometry (µrpLC/MS/MS) using an Agilent 1100 Series capillary LC system (Agilent Technologies, Inc., Palo Alto, CA) coupled to an LCQ Deca ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) using an in-house manufactured electrospray interface operating in the positive ion mode at 2.2 kV. A reversed-phase capillary column was manufactured in-house by slurry packing 5 µm 300 Å Jupiter C_{18} stationary phase (Phenomenex, Torrence, CA) into a 60 cm × 360 µm o.d. × 150 µm i.d., capillary (Polymicro Technologies Inc., Phoenix, AZ) incorporating a 2 µm retaining mesh in a HPLC stainless steel union (Valco Instruments Co., Houston, TX) containing a flame-pulled capillary tip. The mobile phases consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. Peptide fractions were solubilized with 20 µl of 5% B/95% A. After a volume of 8 μl was loaded onto the reversed-phase column, a gradient program held the flow rate of 1.5 μl min^{-1} at 5% B for 20 min, then initiated a linear gradient to 95% B over 90 min. After washing the column with 95% B for 20 min the column was equilibrated with 5% B for 60 min prior to the next injection. The LCQ Deca was operated in the data-dependent MS/MS mode in which the four most intense ions detected in the precursor MS scan were selected for collision-induced dissociation (CID). A two-minute dynamic *m*/*z* exclusion list for selected precursor ions was utilized to increase the detection of lower abundant peptides during gradient elution. Data were acquired in the *m*/*z* range 400–2000 using a normalized collision energy setting of 45%.

Peptide identification

Peptides were identified by searching the MS/MS spectra against a Sindbis virus structural protein database using TurboSEQUEST (Bioworks 3.1, Thermo Finnigan, San Jose, CA). In this study, the SEQUEST scoring criteria used for a positive peptide identification were tryptic and chymotryptic peptides displaying a cross-correlation score (Xcorr) of greater than 2.0, 1.5, and 3.3, for +1, +2, and +3 charged precursor ions, respectively, and a delta-correlation score (ΔC_n) of greater than 0.1 as previously described (Peng et al., 2003). The Xcorr of a peptide is based on the "fit" of the MS/MS data to the theoretical distribution of ions produced for the peptide, and the ΔC_n is the "difference" between the top two Xcorrs for a given MS/MS spectrum.

Differential searches were conducted to identify the presence of alkylation of cysteines by iodoacetamide and iodomethane. Cysteines modified by iodoacetamide will have a net mass increase of 57.02 Da and cysteines modified by iodomethane will have a net mass increase of 14.02 Da. Percentage “free” was calculated by taking the area of peaks corresponding to peptides labeled with iodoacetamide in comparison to peptide labeled with iodomethane based on precursor ions and extracted ion chromatograms.

Mutants, transfection, and virus titration

Mutants were all constructed in the virus strain Toto 1101 (Rice et al., 1987) using the QuikChange mutagenesis protocol (Stratagene) as described (Hernandez et al., 2003). Mutants were named for the specific amino acid mutated from the amino acid terminus of the E1 protein. In each case a cysteine was mutated to a serine. The PCR primers utilized were:

C49S forward 5’ GAGTACATTACATCTAAATTCACCACTG 3’,

C49S reverse 5’ CAGTGGTGAATTTAGATGTAATGTACTC 3’,

C259S forward 5’ CCGCACCTTTCGGATCCAAGATTGCAG 3’,

C259S reverse 5’ CTGCAATCTTGGATCCGAAAGGTGCGG 3’,

C271S forward 5’ CCGAGCGGTGGACAGTTCATACGGG 3’, and

C271S reverse 5’ CCCGTATGAACTGTCCACCGCTCGG 3’. The PCR conditions were 94 degrees C for 2 min, then 95 C for 12 s, 50 C for 30 s, and 68 C for 30 min for 30 cycles. After the first 10 cycles the 30 min extension time was increased by 10 s/cycle. A final extension at 72 C for 30 min was performed followed by 4 C.

Mutations were confirmed by sequencing and after large scale growth and purification were linearized with Xho1 (New England Biolabs).

Mutant cDNA constructs were prepared for transcription and transcribed by Sp6 reverse transcriptase (New England Biolabs) as described previously (Hernandez et al., 2000; Hernandez et al., 2003). Electroporation was performed on BHK cells as described by Lilijestrom and Garoff (Liljestrom and Garoff, 1991). Virus was harvested after 24 hours and titered on BHK cells (Renz and Brown, 1976).

Chapter 4

Conclusions and Future Directions

Investigating the role of the E2 transmembrane domain

Understanding the role of the E2 transmembrane domain is an important step to understanding the structure of Sindbis virus and how its structure relates to infectivity. It was recently shown (Hernandez et al., 2003) that deletions in the E2 transmembrane domain resulted in an array of interesting data across different cell types (mammalian and insect). Surprisingly, it was found that a single deletion in the E2 transmembrane resulted in a large loss of infectious virus production while a larger deletion of eight amino acids could be tolerated. This suggested that the E2 transmembrane domain consisted of a larger function than simply anchoring the protein into the membrane. To investigate the role of the E2 transmembrane further, a series of single amino acid deletions were constructed in the transmembrane domain. These deletions were carefully placed where they represented all faces of the helix and also covered sites ranging from the N terminal to the C terminal end of the transmembrane domain (Whitehurst et al., 2006). In addition an eight amino acid deletion was constructed where the C terminal half of the transmembrane domain was deleted.

It was found that the single deletion mutants located in the C terminal half of the transmembrane domain resulted in a three log decrease in infectious virus production whereas, the single deletion mutants located in the N terminal half of the transmembrane domain resulted in only a one log decrease in infectious virus production. This data suggested that deletions located in the N terminal region of the transmembrane could be tolerated where deletions located to the C terminal end of the transmembrane domain had a much more detrimental effect on normal virus growth. This was also found to be the case with larger deletions. An eight amino acid deletion was tolerated when it was deleted form the N terminal half of the transmembrane domain but resulted in a six-log decrease in infectious virus production when eight amino acids were removed from the C terminal end. It was also shown that all of these single deletion mutants still released similar amounts of intact virus particles from the cell and appeared to be transported and processed correctly. This suggests mutants which result in decreased infectious virus growth may be defective at some step of viral entry.

Mutants that resulted in reduced normal virus growth were passage several times and eventually the amount of infectious virus production increased to wt levels (for the single amino acid deletion mutants) or was partially restored (for the eight amino acid deletion). RT/ PCR analysis revealed that selective pressure restored wt length, but not necessarily sequence, to the transmembrane domain for the single amino acid deletions and restored wt titer. The eight amino acid deletion was partially restored to wt length and an increase was observed in infectious virus production. These results indicated that wt length is necessary for normal virus growth in the C terminal region of the transmembrane domain.

The N terminal region of the transmembrane tolerates deletion better than the C terminal region of the transmembrane domain. These results may be explained by positioning the E2 tail in an unfavorable orientation. The E2 tail must interact with the capsid protein and deletions located closer to the N terminus of the transmembrane domain may allow for more degrees of freedom. It is also possible that the E1 and E2 transmembrane interact. Strauss et al (Strauss, Lenches, and

Strauss, 2002) have shown that by replacing the Sindbis virus E1 protein with the Ross River virus E1 protein, that selective pressure induces changes in the E2 transmembrane domain. The data in chapter two is in agreement with this data. If these two domain interact toward the C terminal half of the transmembrane domain it may be expected that deletion of the residues located in this area would have a greater affect on normal virus growth than those located outside of the region (toward the N terminal domain).

Since all deletion mutants produced similar amounts of particles that banded to wild type density on tartrate gradients, seemed to be processed and transported correctly, and produced stoichiometrically equivalent amounts of structural proteins as wild type, it was reasoned that the mutants resulting in reduced infectious virus growth may be defective at one stage of viral entry. This issue could be addressed with future experimentation. To this end, single deletion mutants could be examined by investigating induced cell fusion of BHK cells where either a single deletion mutant or SVHR is applied to the cells. It could be determined if the amount of cell fusion correlates to the total amount of viral particles present or to the amount of infectious particles present. If some of the mutants do not yield the same amount of fusion as the wild type while maintaining the same total amount of viral particles it may suggest that these mutants are defective at some stage of attachment and virus penetration. At this time one could follow up with EM work to determine if the mutant virus is visibly packed at the cell surface, indicating a defectivity in viral penetration or if it is able to penetrate the cell in wild type fashion. If no virus is observed, it may be defective in receptor recognition and attachment.

Location of the free and disulfide bound cysteines of the Sindbis virus structural proteins

E1 and E2 of Sindbis virus have 17 cysteines each. Capsid has none. With exception of the transmembrane domains every one of the cysteines is conserved. Very little is known about the disulfide network for the E2 protein. Gidwitz et al (Gidwitz et al., 1988) suggest that all E2 cysteines are either involved in disulfide bonds or are not available to solvent when labeling free sulfhydryl group with radioactive iodoacetamide (iodoacetamide alkylates free sulfhydryl groups) at a 1.1 molar excess. Much more is known for the E1 protein. Roussel et al (Roussel et al., 2006) published the crystal structure of the Semliki forest virus of E1 and reported the location of the disulfide bonds. During preparation of the E1 protein they removed it from the membrane with detergent. Removal of E1 with detergent has been shown to produce a set of disulfide bonds (Mulvey and Brown, 1994) suggesting the structure they crystallized may not be in the native form.

A strategy was devised to label the free cysteine residues (those not involved in disulfide bonds) in the native virus particle and then to detect them with mass spectrometry. Briefly, native virus particles were incubated with iodoacetamide, which allows for free and accessible cysteines to be alkylated. The proteins were then separated on SDS-PAGE gels and were subsequently reduced and incubated with iodomethane. The iodomethane will then alkylate the newly reduced cysteines. This will prevent disulfide shuffling from occurring before data collection. Using this method cysteines found free in the native virus particle will be alkylated by iodoacetamide and cysteines that may have been blocked by disulfide bounds or were inaccessible in the native virus particle will be labeled by alkylation with iodomethane. The difference in the mass of alkylation allows for detection by mass spectrometry.

Our findings revealed the location of several free cysteines for both the E1 and E2 proteins. There were some differences between the location of free cysteines found by us and Roussel et al (Roussel et al., 2006). Once again this may be due to crystal studies involving a non-native virus particle. In addition our findings revealed that after brief exposure to low pH that no differences were detected in the disulfide network. Since structural changes induced by exposure to low pH may represent structural changes on the surface of the virus during entry our findings suggest that disulfide rearrangement may not occur during entry.

Mature virus particles alkylated by iodoacetamide revealed a decrease in virus infectivity. This suggest that the labeling of free cysteines either reduces viral infectivity by destabilizing the virus particle by the presence of the label or that the labeled cysteines are normally involved in a disulfide bond later in the virus life cycle. Several of the cysteines that were found to be free in the native virus particle were converted to serine to investigate their role on infectious virus production and possible involvement in the disulfide network. All the mutants had a drastic effect on infectious virus production. If these cysteines remain in a free state during virus infection, such a large decrease in infectious virus production would not be expected. This data suggests that a disulfide bond rearrangement may occur during virus maturation.

To investigate the effects of each cysteine on infectious virus production all of the E1 cysteines are in the process of being converted to serine. These mutants will also serve as a tool to aid in the determination of the disulfide bonds in the native virus particle and their corresponding role in viral infectivity. Other aspects such as assembly, processing, and transport can be addressed with these mutants. In addition other methods of mass spectrometry are being explored to elucidate the structure of the disulfide network.

Other structural aspects of the mature virus particle are being examined using chemical cross-linkers and mass spectrometry. The mature virus particle has been cross-linked with commercial cross-linkers, BSS and DS3, and non-commercial cross-linkers being developed in the Goshe lab. All of these are off to a promising start as shifts are evident by SDS-PAGE analysis. Hopefully these will provide information on regions of contact between the E1 and E2 protein and allow us to further understand the structure of the Sindbis virus particle.

Chapter 5

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