Investigating the effects of pH on alphaviral E3-E2 glycoprotein association, organization, and cellular tropism

Jason Michael Sequra
Purdue University

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By  Jason M. Segura

Entitled
INVESTIGATING THE EFFECTS OF PH ON ALPHAVIRAL E3-E2 GLYCOPROTEIN ASSOCIATION, ORGANIZATION, AND CELLULAR TROPISM

For the degree of  Doctor of Philosophy

Is approved by the final examining committee:

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Approved by Major Professor(s):  David A. Sanders

Approved by:  Stephen Konieczny  8/25/2016

Head of the Departmental Graduate Program  Date
INVESTIGATING THE EFFECTS OF PH ON ALPHAVIRAL E3-E2 GLYCOPROTEIN ASSOCIATION, ORGANIZATION, AND CELLULAR TROPISM

A Dissertation
Submitted to the Faculty
of
Purdue University
by
Jason Michael Segura

In Partial Fulfillment of the
Requirements for the Degree
of
Doctor of Philosophy

December 2016
Purdue University
West Lafayette, Indiana
To Mom & Dad
ACKNOWLEDGMENTS

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<tr>
<td>AcMNPV</td>
<td><em>Autographa californica</em> Multiple Nucleopolyhedrovirus</td>
</tr>
<tr>
<td>AMV</td>
<td>Avian Myeloblastosis Virus</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulfate</td>
</tr>
<tr>
<td>BHK</td>
<td>Baby Hamster Kidney Fibroblast Cell</td>
</tr>
<tr>
<td>bMON</td>
<td>Bacmid AcMNPV Genome with Occlusion Negative Phenotype</td>
</tr>
<tr>
<td>CHIKV</td>
<td>Chikungunya Virus</td>
</tr>
<tr>
<td>CHO 22</td>
<td>Chinese Hamster Ovary Epithelial Cell with Heparan Sulfate</td>
</tr>
<tr>
<td>CHO 18.4</td>
<td>Chinese Hamster Ovary Epithelial Cell without Heparan Sulfate</td>
</tr>
<tr>
<td>DENV</td>
<td>Dengue Virus</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modification of Eagle’s Medium</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<tr>
<td>EEEV</td>
<td>Eastern Equine Encephalitis Virus</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein (<em>Aequorea Victoria</em>)</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan Sulfate</td>
</tr>
<tr>
<td>kD</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>LacZ</td>
<td>Lac Operon Gene Z (Encodes β-galactosidase)</td>
</tr>
<tr>
<td>LTR</td>
<td>Long Terminal Repeat</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of Infection</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>MW024</td>
<td>Recombinant Bacmid Strain Lacking gp64 Envelope Sequence</td>
</tr>
<tr>
<td>Abbreviation</td>
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<tr>
<td>MW033</td>
<td>Recombinant Bacmid Strain Rescued by gp64 Envelope Sequence</td>
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<tr>
<td>NRAMP</td>
<td>Natural Resistance-Associated Macrophage Protein</td>
</tr>
<tr>
<td>pAb</td>
<td>Polyclonal Antibody</td>
</tr>
<tr>
<td>pE2</td>
<td>Alphaviral E3-E2 Polyprotein Complex</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline Solution</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per Minute</td>
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<tr>
<td>RRV</td>
<td>Ross River Virus</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium-Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>Sf9</td>
<td><em>Spodoptera frugiperda</em> (Pupal Ovarian Cell - Fall Armyworm)</td>
</tr>
<tr>
<td>SF900III</td>
<td><em>Spodoptera frugiperda</em> Media (3rd Formulation)</td>
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<tr>
<td>SFV</td>
<td>Semliki Forest Virus</td>
</tr>
<tr>
<td>SIND</td>
<td>Sindbis Virus</td>
</tr>
<tr>
<td>ss(−)DNA</td>
<td>Single Stranded Negative-Sense DNA</td>
</tr>
<tr>
<td>ss(+)RNA</td>
<td>Single Stranded Plus-Sense RNA</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TU/mL</td>
<td>Transducing Unit per Milliliter</td>
</tr>
<tr>
<td>VEEV</td>
<td>Venezuelan Equine Encephalitis Virus</td>
</tr>
<tr>
<td>VLP</td>
<td>Virus-Like Paricle</td>
</tr>
<tr>
<td>VSV</td>
<td>Vesicular Stomatitis Virus</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
</tr>
<tr>
<td>φNXgp</td>
<td>PhoenixGP Expression System (HEK293T Cell) with Gag/Pol</td>
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ABSTRACT

Segura, Jason M. PhD., Purdue University, December 2016. Investigating the Effects of pH On Alphaviral E3-E2 Glycoprotein Association, Organization, and Cellular Tropism. Major Professor: David A. Sanders.

In alphaviruses the role of E3 is required in protecting the fusion peptide region of E1 during intracellular transport. Throughout viral processing, the association of E2 and E3 is required for the successful trafficking and incorporation of E1 into the mature virion. This E3-E2 association has been observed to extend to mature virions in the solved structure for the envelope of Semliki Forest virus (SFV) and supported by the solved structure for the entire Venezuelan equine encephalitis virion (VEEV) with exclusive contacts being made between E3-E2. Immunization with monoclonal antibodies against VEEV E3 provided protection for mice challenged by lethal doses of VEEV and suggests potential new targets for antibody neutralization, but it is currently unclear if E3 is retained on mature VEEV virus. Using non-replicating expression systems that avoid virus-culturing artifacts, we discovered that Moloney murine leukemia virus or baculovirus pseudotyped with the alphavirus envelope spike complex of VEEV demonstrates a pH-dependent retention of E3 on mature virus for both mammalian cells and insect cells through indirect-immunofluorescence assays and neutralization studies using polyclonal antibodies against VEEV E3.
In studies investigating the impacts of retaining the E3 glycoprotein on mature virus outside of a host cell, we found this retention of E3 decreases receptor-mediated entry of cell targets that can be rescued on cells containing heparan sulfate suggesting viruses containing E3 on mature envelopes can utilize the E3 protein as an attachment factor. We utilized a method for downregulating the cell-surface expression of the natural resistance-associated macrophage protein (NRAMP2), a definitive receptor for the prototypic alphavirus Sindbis (SIND), on cellular targets. We observed a significant decrease in entry of VEEV versus control Ross River (RRV) virus that does not utilize NRAMP2. This inhibition can be rescued with binding to heparan sulfate by VEEV retaining E3. Together, these data suggest that the E3 glycoprotein protects the fusion region of E1 on budded, mature virus dependent on the pH of the extracellular environment. This association potentially serves a role as an attachment factor on virus and presents new binding sites for protein interactions and potential inhibition.
CHAPTER 1. ALPHAVIRUS INTRODUCTION

1.1. Introduction

Alphaviruses are enveloped positive-sense RNA viruses that belong to the family *Togaviridae* and are also known as arboviruses for their modes of transmission (Strauss 1994; Sourisseau 2007; Cavrini 2009). Alphaviruses contain over 25 individual species of varying sequence similarity including the well-studied, prototypic members Semliki Forest (SFV) and Sindbis (SIND) as well as Ross River (RRV), Chikungunya (CHIKV), and Venezuelan Equine Encephalitis (VEEV). Further designation places these species into geographically classified clades of Old World or New World arboviruses characterized by a considerable range of illnesses including long lasting symptoms of arthralgia or debilitating encephalitis, respectively (Jose 2009).

Repeated outbreaks of these alphavirus species in countries within Asia, Africa, South America, and emergence in Europe and North America demonstrate not only epidemic occurrences, but also an increasing viral capacity to extend outside endemic territories (Chevillon 2007). Overall, these pathogens impact the health and economic stability within affected regions due to the prolonged symptoms experienced by those infected, and considering the global
distribution of the various species, alphaviruses pose a significant, ongoing health risk (Figure 1). Furthermore, the classification of VEEV as a bioterrorism agent and the absence of any alphaviral vaccine underscore the importance of understanding the entry and replication mechanisms of these viruses.

Figure 1. Global Distribution of Selected Alphavirus Species

Geographical distribution of alphavirus members is further designated to Old World vs. New World clades. New World viruses within the Americas demonstrate pathologies of encephalitis whereas Old World viruses cause arthralgia and myalgia. Map shows major endemic locations of recorded outbreaks of both clades according to the World Health Organization.
1.2. **Viral Genome & Structure**

Alphaviruses possess a single-stranded, positive sense RNA (+ssRNA) genome of approximately 11.8kb (Figure 2). The genomic RNA is characterized by a capped 5' terminus and contains the nonstructural protein-coding genes for nsP1 – 4 in the 5' proximal region. The 3' end of the genomic RNA is polyadenylated and is transcribed via a minus strand intermediate to yield a 26S subgenomic RNA responsible for 5 structural components of the virion as the capsid, pE2 (precursor containing E2 and E3), 6K, and E1 proteins (Solignat 2009).
**Figure 2. Genomic Organization of Alphaviruses**

The genomic organization of alphaviruses is shown. The typical genome consists of a ~11.5kb single-strand +RNA with the nonstructural, replication genes located at the 5' end and the structural genes located at the 3' end (Top). Organization of the polyprotein sequence encoding the structural proteins for capsid, E3, E2, 6K, and E1 (Middle). In the pE2 complex, containing E3 at the 5' end and the A-C domains of E2 at the 3' end, the late-stage furin cleavage between E3 and E2 is shown with a red arrow (Bottom). Figures adapted and modified from Weaver et al. (2004); Voss et al. (2010).

The structures of four alphaviruses, RRV, SFV, SIND, and VEEV, have been solved (Parades 1993, 1998; Cheng 1995; Mancini 2000; Mukhopadhyay 2006; Zhang 2011). The virions are approximately 70 nm in size, are icosahedral with a T=4 symmetry and consist of a nucleocapsid core surrounded by a lipid bilayer derived from host cell membrane. The core is made up of 240 copies of a
30 kDa capsid protein that associates to form pentamers and hexamers (Parades 1992). The viral envelope contains 80 glycoprotein spikes that mediate entry into the cell (Kielian 2000). The spike is comprised of a trimer of heterodimers of the entry proteins E1 and E2. The E1 and E2 glycoproteins each have a transmembrane region and interact to create a rigid structure across the membrane that exhibits the T=4 icosahedral symmetry common to alphaviruses (Von Bornsdoff 1975; Vogel 1986; Fuller 1987; Parades 1993). E1 lies at the base of the spike forming a lattice on the virus surface (Lescar 2001). In contrast, E2 extends upward from the lattice framework, appearing leaf-like (Zhang 2002). The fusion peptide, required for membrane fusion and entry, is found in E1, whereas E2 contains the receptor binding site responsible for recognizing both mammalian and insect host cell receptors and a C-terminal region that interacts with the nucleocapsid core through its transmembrane domain (Smith 1995; Mukhopadhyay 2006).

The E3 glycoprotein is found in all species of the alphavirus genera with varying degrees of sequence similarity, however the exact function of E3 is not fully understood. There is evidence that the presence of E3 is required for the successful maturation of the virus envelope proteins throughout the secretory pathway (Lobigs 1990; Parrott 2009; Uchime 2013). Replacement of E3 with a signal sequence causes a loss of E1 at the site of assembly and structural studies demonstrate a potential interaction between E1 and E3 (Lobigs 1990, Wu 2008). This interaction might not only be important for targeting E1 to the membrane, but it may also provide a stabilizing influence within the varying pH
ranges found in the Golgi itself, preventing premature exposure of the fusion loop. This is supported by evidence indicating that in VEEV and other alphaviruses, cleavage mutants were incapable of initiating entry into susceptible cells (Wahlberg 1989, 1992; Lobigs 1990, 1990). Observations with cleavage mutants presenting E3 on mature SIND virus have been shown to bind heparan sulfate moieties on the cell as well as offer new antigenic determinants for neutralization in VEEV (Klimstra 1999; Parker 2010, Sjöberg 2011). The possibility of an E2-E3 association on mature virus is supported by the solved structures of SFV and recently for VEEV at 4.4Å resolution suggesting that the retention of E3 on the viral envelope is an electrostatic association in a low-pH environment (Sjöberg 2011; Zhang 2011).

The increasing evidence for E3 presented on the virus surface adds another variable to the conformational changes the viral glycoproteins must undergo to mediate receptor binding and membrane fusion and presents an opportunity to investigate another potential mechanism for neutralizing these viruses. E3 consists of two β-strands and three α-helices linked in the case of CHIKV by three disulfide bonds. In pE2, E3 stabilizes the domain B in E2 so that a groove is created between domain B and domain A in which the fusion loop of E1 is hidden. Disruption of this groove occurs when E3 is cleaved from E2 allowing the fusion loop of E1 to become exposed (Voss 2010). The exact fate of E3 after cleavage is unknown, however E3 is reported to remain associated with SFV on the mature virus surface (Garoff 1974), but to be absent from other
alphaviruses (Simizu 1984). Further investigations into the role of E3 are hampered by the lack of reagents exclusive to recognizing the E3 glycoprotein.

1.3. Virus Infectious Life Cycle

Reflecting their global presence, these viruses have a broad host range; additionally, the receptors are not conserved across the various species of alphaviruses (Helenius 1980; Wang 1992; Byrnes 1998; Klimstra 1998; Heil 2001; Rose 2011). After binding a receptor, clathrin-mediated endocytosis brings the virus particle into the cell target (Strauss 1994; Bernard 2010). The low pH environment within the endosome is required to cause the conformational changes that destabilize the interactions between E1 and E2 (Strauss 1994; Kolokoltsov 2005; Colpitts 2007). This facilitates disassociation of E2 from E1 and permits the conformational recruitment of E1 monomers into trimers and position the fusion epitope of each E1 protein in these trimers to fuse with the endosomal membrane as supported by the pre-fusion and post-fusion structure determinations for CHIKV at pH 7 and SIND at pH 5.6, respectively (Li 2010; Voss 2010). The structural proteins of VEEV are targeted for translation from the subgenomic RNA as a single polyprotein C-pE2-6K-E1 on the membrane of the endoplasmic reticulum (Figure 3). The capsid protein (C) is translated first and released by auto-proteolysis leaving a signal sequence at the N-terminus of pE2 that targets the polypeptide for translocation into the endoplasmic reticulum (Aliperti 1978; Hahn 1990; Strauss 1994).
The infectious pathway of alphaviruses is summarized above. Early steps of entry include receptor-mediated binding and clathrin-mediated endocytosis by the E2 glycoprotein leading to a low pH-dependent fusion event by the E1 glycoprotein. Viral replication proceeds following the disassembly of the nucleocapsid core as the genomic strand is translated to yield the replication proteins nsP1-4, which facilitate template strand replication among other duties. As a temporal strategy, structural proteins are translated through a negative strand intermediate as a single polyprotein. Final maturation steps and assembly produce virus that exits the cell through budding. Figure taken from Kuhn et al. (2016).
Within the lumen of the ER, the proteins are separated by the action of signal peptidases to form pE2, 6K and E1, and post-translational modifications, such as glycosylation, occur (Garoff 1974; Raju 1991). The entry proteins pE2 and E1 associate as heterodimers following a translational strategy that retains pE2 in the ER since it is translated before E1 (Aliperti 1978; Melancon 1987; Hahn 1990; Kielian 1990; Andersson 2003; Sanz 2002). As the pE2-E1 heterodimer reaches the trans-golgi, pE2 is cleaved by a furin protease into E3 and E2. It is assumed that E3 remains associated with the E2-E1 complex until the complex leaves the Golgi towards the final assembly of the structural proteins with capsid protein near the cellular membrane. This association is believed to be due to the increasingly acidic cellular compartments through which processed viral glycoproteins transit before assembling at the cell membrane (Strauss 1994; Paroutis 2004; Uchime 2013; Fields 2015). Mutants with deletions in key tyrosine residues at the interface of interaction between E2 and E3 or complete replacement of E3 with a signal-peptide sequence prevents successful transport of E1 to the cell surface to form mature virions suggesting a critical role of E3 intracellularly in protecting the fusion IJ loop within E1 while associated with E2 before budding from producer cells (Lobigs 1990; Uchime 2013).

1.4. Glycoprotein Transport & Arrangement

The structural components of alphavirus proteins have specific roles where E2 mediates receptor recognition whereas E1 facilitates membrane class
II fusion (Strauss 1994; Mukhopadhyay 2006). The E3 glycoprotein, while a part of pE2, is believed to occlude the fusion peptide region of E1 thus preventing premature fusion by E1 during transport within the secretory pathway of the infected cell. The location of E3 has been mapped to the β-ribbon region between the A and B domains of E2 in cryo-EM studies (Parades 1998; Wu 2008; Li 2010). This proximity to E2 as a part of pE2 is believed to allow E3 to form several disulfide bonds with E2 during transport to maintain the stability of E2 and allow the successful heterodimerization of pE2-E1 (Parrott 2009; Li 2010). Variable cleavage of E3 from pE2 by a furin-like protease in the late secretory pathway is observed before the virus exits, but the retention of E3 has been observed structurally for SFV and VEEV. The absence of structural data showing E3 on other alphavirus members suggests that not all alphavirus members undergo the same cleavage efficiency or possess different requirements for pH protection (Lobigs 1990; Jose 2009; Zhang 2011).

Solved structures of SFV E1 in pre-fusion and post-fusion states along with recent whole-virion cryo-EM structures for SIND, CHIKV and VEEV at high resolution show that the envelope glycoproteins amongst the various species of alphaviruses share a common architecture despite differences in the sequence encoding them (Lescar 2001; Kielian 2006; Roussel 2006; Li 2010; Voss 2010; Zhang 2011) (Figure 4).
Figure 4. Alphavirus Capsid with Structural Proteins on Mature Particles

A) 3D reconstruction of mature VEEV virus particle showing E1 (green) and E2 (blue) arranged in a T=4 icosahedral symmetry. B) Asymmetrical alphavirus spike contains E2 (cyan), E1 (violet), and E3 (orange) associated in the mature VEEV virus particle within the cell-derived lipid bilayer (yellow). Membrane spanning contacts are also shown between E2 and E1 to the capsid (blue) surrounding the RNA genome (green). C) The pE2-E1 heterodimer spike of VEEV showing the structural arrangement of the E3 (gold), E2 (blue), and E1 (silver) glycoproteins. The association between E3 and E2 as pE2 is highlighted in the box and magnified to show important contacts between residues supporting prior work of an exclusive association between the E3 and E2 glycoproteins. Figures adapted from Voss et al. (2010); Zhang et al. (2011); Fields et al. (2015).

Using SFV as an archetype, the standing model for alphaviral fusion has been characterized with cryo-EM reconstructions and occurs within the maturing endosome through a low pH event (typically 5.5-5.9) that triggers irreversible conformational changes in the E2-E1 glycoprotein dimer promoting dissociation.
The dynamic rearrangement of E1-E2 dimers into E1 homotrimers during the fusion process exposes the hydrophobic fusion peptide region on E1 for insertion into a host cell endosomal membrane. This allows fusion to occur between the host cell and viral membranes and permits the delivery of the viral genomic material into the cytoplasm of the target cell (Hammar 2003; Zaitseva 2005; Liao 2005; Mukhopadhyay 2006; Sanchez-San Martin 2009; Li 2010; Wu 2007) (Figure 5).
Figure 5. Class II Fusion of Virus-Host Cell Envelopes

Current model for Class II fusion between two membranes observed in Alphaviruses and Flaviviruses. A) For Alphaviruses, E2-E1 envelope proteins present on the surface of the virus (clear membrane) are arranged as trimers of heterodimers. E2 is colored a light green while E1 is colored according to its individual domains where blue is DIII, red is DI, and yellow is DII. B) Upon a low pH event the envelope proteins dissociate and E1 undergoes conformational changes which extend the monomer towards the target membrane. C) E1 monomers associate into homotrimers while their distal loops containing the fusion peptide region insert into the target membrane. D) E1 monomers further undergo conformational changes where DIII folds over the hinge region present in DI to overlap DII. E) Hemifusion from these conformational changes occurs as the bridging of these two membranes is carried out through the “hairpin” force of the E1 homotrimers. F) Complete pore formation in which E1 homotrimers have successfully bridged the viral and target membranes for lipid mixing. The formed pore now allows the nucleocapsid to enter the target cell cytoplasm. Figure adapted from Martin et al. (2009).

1.5. Alphaviral Pseudotypes & Design

Vectorology is a branch of virology which involves the manipulation and construction of viral vectors to deliver payloads to specific cell targets. In
principle, this involves utilizing the core of a retrovirus, engineered with an application in mind, coupled with the envelope of another virus to add specificity. The range in tropism of retroviral vectors has grown over the years with the successful creation of vectors expressing envelope glycoproteins with ecotropic, amphotropic, or pantropic capabilities. (Wool-Lewis 1998; Palu 2000; Srinivasakumar 2002; Sinn 2003; Simmons 2004; Hafer 2009; Rausalu 2009; Barrett 2013).

The immunogenicity and cytotoxicity of viral vectors are important factors to consider when designing and applying pseudotype vectors to cell targets within a host. These effects are influenced by the expression and association of protein subunits from different viral sources within a host and the host’s immunologic response (Kahl 2005; Lundstrom 2005). The application for virus pseudotypes has traditionally been focused with gene therapy in mind, where the application of viral vectors must be managed against host immune defenses to avoid complications. However, considerable work has shown that pseudotype vectors can be utilized to illicit an immune response with the purpose of vaccination. The application of viral vectors with this goal depends on whether or not vectors are engineered to allow for replication. Whereas live-attenuated virus is optimal for establishing immunologic response and prolonged protection, pseudotype virus and virus-like particles (VLPs) are ill-suited as long-term solutions with varying levels of success (Akahata 2010; Kramer 2013). However, replication-incompetent recombinant particles can serve a specialized role in investigations of protein interactions specifically because they cannot replicate.
This lack of replication eliminates the portion of the infectious pathway involved in exit and allows only for the mechanisms of entry to be studied (Sinn 2003; Simmons 2004; Cho 2008).

The usage of retrovirus-based packaging vectors in the creation of pseudotype virus is not a new concept and has been successful for decades, building off the research on murine retroviruses from the late-1960’s and into the early-1970’s. The initial work incorporating the envelope proteins of Vesicular stomatitis virus (VSV) with Moloney murine leukemia virus (MuLV) and avian myeloblastosis virus (AMV) capsid cores had introduced the potential for developing chimeras expressing proteins from separate viruses by altering the tropism of these viral cores due to the molecular attributes from pseudotyped envelope proteins (Zavada 1972; Love 1974). The choice of using MuLV is supported by its ability to incorporate a wide range of heterologous protein complexes into its capsid, allowing for the potential to create an assortment of high-titer, retroviral-based pseudotypes (Burns 1993). This success with initial pseudotypes branched into the usage of lentivirus subtypes and further expanded both the vector systems available and envelope protein sources to include filovirus and flavivirus (Wool-Lewis 1998; Bruett 2001; Sinn 2003; Hu 2007). The increasing library of envelope protein candidates from various viral families grew to include alphaviruses and allowed for the investigation of both entry mechanisms and the design of therapeutic delivery systems (Sharkey 2001).
The usage of replication-incompetent, envelope-deficient pseudotyped virus allows for multiple directions of research from a single approach simply by altering relatively similar retroviral packaging systems. Through the use of pseudotype vectors, progress has been made in detecting neutralizing antibodies to VEEV envelope proteins and in developing gene therapy vectors based on the tropism of pseudotyped Ebola envelope proteins (Sinn 2003; Kolokoltsov 2006). Most importantly, the approaches of pseudotype creation have shown that the processing of chosen envelope proteins and the entry pathways used by them follow the same criteria seen in the wild-type virus from which they are chosen. This allows researchers to investigate the properties of the protein interactions with a cell target and characterize the molecular attributes of specific envelope proteins (Sharkey 2001; Hu 2007).

The creation of pseudotype virus generally involves the production of a packaging core capsid that is retroviral in origin from a stably-transfected producer cell line that has been engineered to be replication-incompetent (Palu 2000). Advances in mammalian packaging systems expressing envelope-deficient, replication-incompetent retrovirus for high-titer chimera production allow for the substitution of envelope protein candidates to study particular viral protein interactions on cell targets (Pear 1993; Ory 1996; Swift 2001). These packaging cells, termed Phoenix System (NXgp), are modified human embryonic kidney 293 cells and stably express the sequences encoding the core retroviral capsid (gag) and polymerase (pol) proteins of MuLV under the control of the Rous sarcoma virus (RSV) promoter. Stable transfection of these
packaging cells with a transducible lacZ gene or fluorescent reporter allows for assaying successful entry following the transient transfection of a helper plasmid containing the sequence that encodes the envelope protein of a chosen virus including alphaviruses under the control of the cytomegalovirus promoter (CMV) for high-level mammalian expression (Figure 6).

Figure 6. Mammalian Expression of Pseudotype Virus Using the Phoenix System

PhoenixGP cells are altered human kidney 293T cells engineered to express the gag and pol genes from Moloney murine leukemia virus lacking the LTR regions and ψ signal sequence. These cells produce replication-incompetent, envelope-deficient MuLV particles. Stable transfection with MFG.nls.LacZ, a recombinant retroviral genome containing the gene that encodes β-galactosidase flanked by LTR regions and possessing a ψ signal sequence for incorporation, allows for the transduction of a reporter gene into target cells that can be assayed with the addition of X-gal. The transient expression of a helper plasmid containing the chosen envelope protein (e.g. VEEV) produces retroviral pseudotypes with alphaviral envelope proteins that dictate the tropism of the pseudotyped viruses based on the interactions of the viral envelope proteins and receptors/attachment factors on target cells.
The creation of pseudotyped virus from insect cells follows a similar approach. However, there are limits to expressing viral proteins within insect cells due to the failure of RSV/CMV promoters being compatible with insect producer-cell transcription machinery. Expression of viral pseudotypes in insect cells utilizes a different expression system designed around insect cell-derived polyhedrin promoters using a baculovirus core capsid (Liu 2013). Baculovirus engineered to lack the native envelope glycoprotein (gp64) is rendered replication-incompetent (Tani 2001; Kitagawa 2004). These gp64-null core constructs can be further engineered with a sequence encoding the green reporter protein fluorescent signal (GFP) from *Aequorea Victoria* (Kitagawa 2005). Baculovirus cores can then be co-transfected with helper plasmids containing sequences encoding foreign viral envelope proteins under the control of a late-stage polyhedrin promoter into producer Sf9 cells to create pseudotype baculovirus that is replication-incompetent and bearing foreign envelope proteins (Westenberg 2012). These pseudotyping techniques provide a unique opportunity to study the interactions between the pseudotyped viral envelope proteins and their receptors on the cell surface of targets. These interactions must take place in order for transduction of a reporter gene to occur, while avoiding *in vitro* culturing artifacts that can arise from serial passaging virus in different cell types.
CHAPTER 2. MATERIALS & METHODS

2.1. Cell Lines & Cell Culturing

HEK293T-based ΦNXgp (Phoenix CRL-3215) producer mammalian cells were obtained from the American Type Culture Collection (ATCC) in Rockville, MD. These cells are engineered for the stable expression of replication-incompetent, envelope-deficient MuLV core proteins (Pear 1993; Swift 2001).

Baby hamster kidney epithelial cells (BHK, CCL-10 – ATCC) were the mammalian target cells for all assays. Sf9 insect cells used for both insect cell virus production and cell targeting were graciously donated by Andy Mesecar (Purdue University, West Lafayette, IN.). CHO 22/18.4 mammalian cells that express HS or are engineered to lack HS at the cell surface, respectively, were graciously donated by Richard Kuhn (Purdue University, West Lafayette, IN.).

All mammalian cells were incubated at 37°C with 5% CO₂ supplementation in DMEM media (Gibco – Invitrogen) supplemented with 10% heat-inactivated FBS. All insect cells were incubated at 28°C with no CO₂ in Sf900III media (Gibco – Invitrogen) supplemented with 3% heat-inactivated FBS. No antibiotics were used in cell culturing. Mammalian cell passaging was
performed every 2 days and required washing cells with 1% PBS and treatment with 0.25% Trypsin/EDTA for 1 minute before suspending cells into solution with fresh DMEM/10% FBS media for splitting. Moderately adherent Sf9 cells were passaged every 3 days by removing old media and replacing with fresh Sf900III/3% FBS media and shaking the monolayer into suspension for splitting. All culturing was performed in flasks treated with poly-L-lysine for cell attachment (Sarstedt).

2.2. Alphavirus Production Plasmids

Producer ΦNXgp cells were stably transfected with pJ6Ωpuro and MFG.S-nls/lacZ to select for retroviral packaging-producer cells expressing the nuclear-localized lacZ gene (ΦNXgpLacZ). This gene is used in assays of infected target cells for the expression of β-galactosidase following transduction (Morgenstern 1990; Ory 1996). Mammalian expression of the VEEV alphavirus envelope proteins of the wild type TC-83 strain or a mutant sequence with a R58-59E deletion in the furin cleavage motif in E3 was performed using the pcDNA3.1 vector plasmid with the sequence encoding E3-E2-6K-E1 polyprotein under the CMV promoter. Expression of the VEEV envelope proteins in insect cells was achieved by subcloning the sequence encoding the wild type and mutant R58-59E E3-E2-6K-E1 polyprotein into the insect cell helper plasmid vector pBACgus-1 under control of a polyhedrin promoter (Novagen). Gene sequences were digested from pCDNA3.1 using HindIII and XbaI. The pBACgus-1 vector was digested with HindIII and AvrII for a complementary end ligation using T4 Ligase.
to create pBACgusVEEV and pBACgus58-59 for the wild-type and mutant sequence, respectively (New England Biolabs – NEB). Sequences underwent diagnostic digestion with EspI for validation before sequencing.

2.3. Transduction Assay

Pseudotyped virus produced from either mammalian or insect cells under standard conditions of pH or adjusted pH was collected from the supernatant media of cultured cells and filtered through a 0.45 µM membrane to remove cell debris. Virus was either concentrated from media with ultracentrifugation through a 5 mL 30% sucrose cushion at 28,000 RPM @ 4°C for 2 hours or directly applied onto target cells with 5 µg/mL polybrene (Sigma-Aldrich) for 3 hours before being removed and replaced with fresh media. Pseudotyped virus binds and enters cell targets dependent on the protein interactions mediated by the viral envelope glycoproteins. Entry allows the transduction of the reporter gene and subsequent β-galactosidase activity for assaying in mammalian cells after 48 hours. The observation of GFP fluorescence was performed after 72 hours in insect cells.

Traditional multiplicity of infection (MOIs) or plaque assays cannot be performed with replication-incompetent virus. Virus titer quantities are directly assayed from transduced reporter signal of β-galactosidase activity (mammalian cell infection) using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Gold Bio) or GFP (insect cell infection) fluorescence detection under the microscope. Depending on the amount of supernatant media applied to target
cells for infection and the percentage of cells expressing the reporter from the total amount of cells plated, the transducing units per milliliter (TU/mL) of media can be computed to yield the virus titer from the producer cell source that was transiently transfected to express pseudotyped virus.

2.4. SDS-PAGE Immunoblot Assay

Virus was produced from twelve, 10-cm plates each containing 8x10^6 ΦNXgp cells that were transfected with a pcDNA3.1 mammalian expression vector carrying the sequence coding for the structural proteins E3-E2-6K-E1 of VEEV under the CMV promoter and incubated at 37°C with 5% CO₂ (Fig. 6). The cell cultures had their pH adjusted to 6.5 with 50 mM MES buffer in fresh DMEM media 44 hours later. Virus was collected after 48 hours and filtered through a 0.45 μM membrane. The sample was divided and pH adjusted to 7.4 or 6.1 using pre-titrated volumes of 50 mM NaOH or 50 mM MES before being concentrated through a 30% sucrose buffer adjusted to pH 7.4 (50 mM HEPES, 100 mM NaCl, and 1.8 mM CaCl₂) or pH 6.1 (25 mM HEPES, 100 mM NaCl, 1.8 mM CaCl₂, and 25 mM MES) at 4°C for 2 hours at 28,000 rpm.

The isolated pellets were recovered in 2x loading buffer and separated through 12.5% SDS-PAGE gel electrophoresis. The samples were transferred onto a 0.45 μM nitrocellulose membrane and blocked with a 5% bovine albumin washing buffer for 30 mins before being probed for 24 hours at 4°C with primary rabbit-pAb αVEEV E3 at 1:1000 concentration. The membrane was washed 2x with washing buffer and then probed with secondary goat αRabbit-pAb
conjugated to HRP at 1:2,500 concentration for 4 hours at 25°C. The membrane was washed again 2x with washing buffer before development solution was applied.

2.5. **Neutralization Assay**

Antibody neutralization experiments were performed with virus produced from six, 10-cm plates each containing 8x10^6 ΦNXgp cells as previously described. Adjustments in pH of virus cultures to 6.5 were performed using pre-titrated volumes of 50 mM MES 44 hrs following transfection. Virus was collected 4 hrs later and filtered through a 0.45 µm membrane. The sample was divided, and the pH was adjusted to 7.4 or 6.1 using pre-titrated volumes of 50 mM NaOH or 50 mM MES, respectively. Samples were then concentrated as previously described before being recovered in 6ml DMEM/10% FBS media adjusted to pH 7.4 or pH 6.1 and divided into 1 ml aliquots. Aliquots were incubated with a rabbit-pAb antibody αVEEV E3 at 1:50 and 1:500 concentrations or rabbit serum at 1:50 concentration for 1 hr at 25°C. Hexadimethrine bromide (Polybrene – Sigma) was added to each sample at a final concentration of 5 µg/ml before being applied to target BHK cells for 4 hrs at 37°C. Samples were then aspirated and fresh DMEM/10% FBS media was applied. The transduction of a β-galactosidase reporter gene was assayed using X-gal 48 hrs later.
2.6. Indirect-Immunofluorescence Assay

ΦNXgp cells that were transfected and expressing virus had their culture pH adjusted for 4 hrs before being fixed. Fixation methods were performed with membrane permeating 100% methanol or 4% paraformaldehyde/0.1% glutaraldehyde with or without triton X-100 treatment to compare extracellular and intracellular E3. Primary rabbit-pAb αVEEV E3 was added for 1 hr @ RT. Samples were then washed and probed with a secondary αRabbit antibody conjugated to Alexa488 (Parker 2010). All samples were treated with Hoechst 33342 stain. Qualitative results using multi-wavelength, inverted fluorescent microscopy (Olympus IX 81 with MetaMorph v7.6) were performed to support the data from western blot and neutralization studies. Quantitative observations using the corrected total cell fluorescence across triplicate assays were calculated using ImageJ processing software (NIH) to account for variance in fluorescence brought on through membrane permeabilization.

2.7. Heparan Sulfate Binding Assay

Two cell lines, CHO 22 and CHO 18.4, were used to test the importance of HS presence to E3 presentation on mature VEEV virus. CHO 22 expresses HS at the cell surface however CHO 18.4 does not. CHO 22 and CHO 18.4 cells were each plated into separate 6-well plates at 5x10^5 cells per well with DMEM/10%FBS media. Prior to incubation with virus, 3 wells were treated with 6ug/ml heparinase I (Sigma-Aldrich) for 1 hour at room temperature while 3 wells were left untreated. Virus was produced as previously described using the
Phoenix system in environmental conditions of pH 7.4 or pH 6.1, concentrated through a pH-adjusted 30% sucrose cushion at 28,000 RPM for 2 hours and applied to target CHO 22 or CHO 18.4 cell sets in triplicate for both sets. Virus was removed after 3 hours and replaced with fresh DMEM/10%FBS media and incubated at 37°C with 5% CO₂. Transduction was assayed after 48 hours.

2.8. NRAMP Transduction Assay

Cells were passaged in separate T25 flasks and were treated with 160µM exogenous ammonium iron citrate III (Sigma-Aldrich) for 72 hours or standard DMEM/10% FBS media as a control. 24 hours prior to applying virus, all cells were treated with 0.25% trypsin and plated in 6-well plates to produce 3 sets of triplicate wells for samples. Cells treated with 160µM exogenous ammonium iron citrate III were labeled “72 hours” and cells treated with standard DMEM/10% FBS media were labeled “24 hours” or “No Iron Control.” The cells labeled “24 hours” then had their media supplemented with 160µM final concentration ammonium iron citrate. Cells had virus applied the next day with supplementation of 160µM final concentration ammonium iron citrate for 3 hours after which media was removed and replaced with fresh DMEM/10% FBS media. After 48 hours, for all labeled samples, one set was assayed for transduction of the reporter gene. The second set was fixed in 4% paraformaldehyde/0.1% glutaraldehyde while the third set had the cells removed to undergo lysis for immunoblots to assay intracellular production of NRAMP. The second set of fixed cells were probed with primary rabbit-αNRAMP2 at 1:50 in 1x PBS/1%BSA for 4 hours before being
washed and probed with secondary goat anti-rabbit IgG-Alexa488 at 1:200 for 1 hour. The supernatant extracts from cells from the third set that underwent lysis had 2x laemmli buffer added at 1:1 v/v ratio and used in 12.5% SDS-PAGE before being transferred to a 0.45µM membrane that was probed with primary rabbit-αNRAMP2 at 1:50 for 4 hours, washed, and then probed with secondary anti-rabbit IgG HRP at 1:1,000 for 1 hour before being developed and imaged. These sets allowed for direct comparison of virus entry to intracellular and cell-surface levels of NRAMP.
CHAPTER 3. INVESTIGATING THE ROLE OF THE ALPHAVIRAL E3 GLYCOPROTEIN IN LOW pH

3.1. Abstract

Despite the growing evidence supporting a crucial role for E3 association during intracellular transport, direct evidence of E3 glycoprotein still attached to budding virus without the use of mutants in any species of the alphavirus genus remains elusive (Parker 2010; Sjöberg 2011; Zhang 2011; Uchime 2013). The pseudotyping of viral constructs was performed to produce virus to investigate the retention of E3 on mature virus particles. Expression of VEEV envelope proteins with mammalian MuLV packaging vectors that are envelope-deficient and replication incompetent permits the ability to safely work with a BSL3 agent in a BSL2 environment. Furthermore, mutations in strains corresponding to in vitro cell passage adaption in replicating virus do not effectively exist as artifacts here, allowing this system to observe the chemistry of envelope proteins without selection (Bernard 2000; Smit 2002).

Production and retention of E3 protein at low pH was assayed directly in immunoblots and using immuno-fluorescence microscopy (IFA) using a polyclonal
antibody specific to VEEV E3 generated in the lab. Indirect analysis of E3 was carried out in antibody neutralization studies. Since plaque assays cannot be performed on replication-incompetent virus, transduction assays served in quantifying virus through viral entry and a successfully transduced β-galactosidase gene. The data strongly supports that E3 is retained at low pH and absent at higher, physiological pH levels on mature virus outside of the cell. This retention is present on both virus particles as well as on the cellular surface of producer cells.

3.2. Introduction

In alphaviruses the translation of the E2 and E3 envelope glycoproteins occurs as the polyprotein precursor pE2. While the roles of E2 and E1 have been studied and well characterized over the years, the role of E3 has only been recently observed as crucial in the success of infectious mature virus particles (Lobigs 1990; Uchime 2013; Fields 2015). The E3 glycoprotein is a small, cysteine-rich 7kD protein present in all the alphaviral species members with approximately 50% sequence similarity between them. It remains a part of pE2 until a late-stage cleavage event by a cellular subtilisin-like furin protease between the trans-golgi and cell surface (Nakayama 1997). It is believed that upon cleavage at the furin motif the β-ribbon linker between domains A and B of E2 is destabilized, priming the spike for low pH activation and subsequent disassociation of E2 from E1 to permit the conformational changes necessary for
fusion within the endosome (Jose 2009; Li 2010; Voss 2010). The association of the E3 glycoprotein, including after cleavage, is exclusively with that of the E2 glycoprotein. This association with E2 protects the E1 fusion motif through highly conserved residues and intracellular, pH-dependent electrostatic associations (Zhang 2011; Uchime 2013; Zeng 2015). For many years this E3-E2 association was assumed to disassociate with E3 lost to the extracellular environment after leaving the mature spike complex of E2-E1. With increasing data supporting an intracellular role in stabilizing E2 and protecting E1 from premature fusion during transport, it is possible that this role of E3 can extend to that of mature virus outside the cell.

Alphaviruses have a temporal strategy for the processing of structural proteins to ensure successful transport within a cell before assembly. The pE2 protein sequence is translated first and is retained through lectin-mediated chaperones to form heterodimers with E1 proteins translated thereafter within the endoplasmic reticulum (Andersson 2003). However, in low pH, the association of E3 with the E2-E1 heterodimer as a part of pE2 is crucial to protect against premature fusion and maintain stability of the spike complex. The requirement for protection helps explain the late-stage intracellular cleavage of pE2. Following cleavage, however late, E3 is subject to retention due to compartmental pH levels while within cell. This suggests that the association of E3 must be able to accommodate a variable level of pH throughout the infectious pathway including that of the environment the virus is budding into (Kim 1998).
The appearance of E3 on the mature particle is limited to structural evidence for SFV and a best-fit model predicting its association with E2 for VEEV, supported by cleavage mutants and cell-based studies involving SIND and SFV mutants (Lescar 2001; Sjöberg 2011; Zhang 2011). However, it is unclear as to the extent or the mechanism that the appearance of E3 on mature virus is observed with SFV or if the appearance of E3 on mature virus could include other alphavirus members (Paredes 1998; Lescar 2001). Given the variance in sequence similarity and cellular tropisms across the various species of alphaviruses, properties affecting the processing and retention of the E3 glycoprotein need to be considered.

Direct evidence of VEEV E3 on mature virus has remained elusive given its inherent size and orientation on the spike complex of virions. The only published data showing the E3 glycoprotein along with the other structural envelope proteins, E2 and E1, came from using overexpressed viral protein from bacterial cells to determine the structure of the spike complex of VEEV at 4.4 angstroms resolution. This required large amounts of culture followed by an intensive purification protocol and shown unmodified viral E3 protein (Zhang 2011). Currently, there has been no work observing expressed E3 glycoprotein from virus-producing cells. Strain-specific neutralization of VEEV using generated monoclonal antibodies against E3 has been observed suggesting E3 was present on mature virus, but the same study was unable to successfully show recognized E3 on immunoblots or through indirect-immunofluorescence assays with the same antibodies (Parker 2010). Less than a year later, it was observed
that the E3 glycoprotein could remain associated with the E2-E1 heterodimer spike following cleavage in acidic compartments within infected cells using mutants for SFV (Sjöberg 2011). Consideration of these findings suggests VEEV expresses E3 on mature virus particles and a potential pH-dependent retention mechanism exists that could be occurring with VEEV E3 and shared across other species of alphavirus.

In order to investigate whether the E3 glycoprotein can be retained on mature virus in a pH-dependent manner, we utilized the Phoenix mammalian expression system to produce non-replicative virus that avoids culturing adaptions. Adaptive mutations in viral studies have played a large role in revealing critical residues responsible for infectious roles. A considerable amount of epidemiological research has focused on the various species members and their variants that demonstrate an increased virulence or tropism range. Serological isolates recovered from outbreaks and individual cases for CHIKV has shown that a key A226V amino acid substitution in the E2 glycoprotein allows virus to bypass the cholesterol requirement considered crucial to alphavirus entry (Strauss 1994; Chatterjee 2000; Lu 1999; Tsetsarkin 2007). This capacity for virus to mutate in vivo has been studied in vitro for prototypic members SIND and SFV in the cell passaging of wild-type strains AR339/SFV4 to map key nucleic-acid mutations and amino-acid-residue changes that confer increased virulence, expanded tropism, or rescued infectivity of the species in novel mutants (Klimstra 1998, 1999; Smit 2002; Ryman 2007; Knight 2009). This in vitro approach was also applied to structural studies for SFV to map
conformational changes necessary for fusion by E1 (Tubulekas 1998; Liao 2005; Liu 2009). The caveat to these studies is that in vitro replicating virus can evolve into a product of the experimental conditions that it is being studied, and these artifacts should be considered when making predictions and applying findings to in vivo behaviors outside the laboratory setting (Klimstra 1998; Smit 2002; Hafer 2009). Ultimately, the specialized design and non-replicating nature of pseudotype virus creation is the best approach to investigate virus and host cell protein interactions without adaption and cultural artifacts affecting nucleotide sequences.

In order to probe for and determine the location of the E3 glycoprotein, our lab had successfully generated from rabbits one of the first polyclonal antibodies specifically against the E3 glycoprotein for alphaviruses using a novel expression method for purified VEEV E3. Characterization of the antibodies showed that recognition was species specific, VEEV pE2 from virus-producing cell lysate and overexpressed E3 protein from bacterial cells was recognized in immunoblots whereas RRV or CHIKV pE2 and E3 were not (Laura Hughes-Baker thesis work). We attempted to investigate if E3 was retained in a pH-dependent manner using these polyclonal antibodies in efforts to explain the protection from VEEV of mice immunized with monoclonal antibodies against E3 using the capability of E3 to bind the spike complex at low pH. Determining the fate of the E3 glycoprotein following cleavage could help clarify whether protection in mice was coming from antibody recognition of pE2 or retained E3.
3.3. Results

3.3.1. Production of VEE Pseudotype Virus Retaining E3

Immunoblot analysis was performed utilizing polyclonal antibodies to visualize expressed E3 directly from virus-producing cells using virus budding from twelve, 10-cm plates each containing $8 \times 10^6$ ΦNXgp cells (Phoenix – ATCC) that were transfected with a pcDNA3.1 mammalian expression vector carrying the sequence coding for the structural proteins E3-E2-6K-E1 of VEEV. Samples were adjusted to either pH 6.1 or 7.4 using 50mM MES or NaOH, respectively. For virus produced at pH 7.4, it was observed that E3 was not retained on budded mature virus, whereas for virus produced at pH 6.1, it was observed that E3 was retained on budded mature virus. There was comparable expression of pE2 and E1 envelope protein between samples at both pH 7.4 and 6.1 in immunoblots (Figure 7).
Figure 7. Immunoblot Analysis of VEEV at pH 7.4 vs pH 6.1

Immunoblot of a 12.5% polyacrylamide gel of VEEV pseudotypes. The media of cells producing virus was adjusted to pH 6.1 or pH 7.4 with 50mM MES or NaOH, respectively. Virus was concentrated through a 30% sucrose cushion buffered to pH 6.1 or pH 7.4. A) Membrane was blotted sequentially with two primary antibodies, rabbit pAbE3 and pAbE2, at 1:650 and 1:1,250 dilutions, respectively, for 4 hrs. Goat anti-rabbit secondary conjugated to HRP was used for 2 hrs. Lane 1) Producer cells that were lysed. Lane 2) Purified virus isolated at pH 7.4. Lane 3) Purified virus isolated at pH 6.1. Lane 4 & 8) Lysate from producer cells expressing VEEV R58-59E cleavage mutant with E3 covalently linked to E2 on the mature virus. Lane 5) Purified virus of R58-59E cleavage mutant. Lanes 6 & 7) Purified virus isolated at broad pH range to observe any differences in protein expression B) Immunoblot of a 12.5% polyacrylamide of VEEV pseudotypes produced from twelve, 10-cm plates. Membrane was probed overnight at 4°C with primary rabbit pAbE3 at a 1:650 dilution before being probed with secondary anti-rabbit HRP for 4 hrs. The membrane was washed 2x with 1N NaOH for 5 minutes before a sequential incubation with primary pAbE1 at 1:2,500 dilution and secondary anti-rabbit HRP. Lanes 1 & 2) Purified virus isolated at pH 6.1 or pH 7.4, respectively. Lane 3) Control R58-59E mutant lysate.
3.3.2. pH-Dependent Neutralization of VEEV Using E3 Polyclonal Antibodies

It is hypothesized that virus treated with an antibody against E3 will significantly diminish the transduction efficiency of virus budding off into an environment with lower pH and retaining E3 through an electrostatic association with E2. Antibody neutralization experiments were performed in separate pH conditions of 7.4 and 6.1 seen in immunoblots as influencing the retention of E3 on budded VEEV pseudotyped virus. Initial neutralization of pseudotype virus prepared at pH 7.4 or 6.1 was performed using an increasing concentration of polyclonal antibodies against E3. Transduction efficiency was measured in transducing units per milliliter of applied media (TU/ml). There was significant neutralization of VEEV expressed at pH 6.1 that was absent in virus expressed at pH 7.4. The difference in pH between pseudotype virus samples did not cause any significant reduction of transduction. The neutralization increased with the concentration of antibody for virus samples expressed at pH 6.1 with no observable decrease in transduction efficiency by virus treated with rabbit serum only (Figure 8).
Figure 8. Neutralization Assays of VEEV Pseudotypes

Neutralization assays of VEEV pseudotypes were performed in triplicate. Producer φNXlacz cells were transfected with plasmids encoding the envelope proteins of VEEV before the media was adjusted to pH 6.5 after 44 hours. Virus was collected and filtered 4 hours later before the media was further adjusted to pH 7.4 (A) or pH 6.1 (B). Samples were incubated with antibodies against VEEV E3 at dilutions of 1:500, 1:150, or 1:50. Antibodies against VEEV E2 and CHIKV E2 were used as positive and negative controls, respectively. Error bars show standard deviation. Statistical analysis performed on triplicate assays using the student’s t-test for a two-tailed distribution and assuming equal variances comparing neutralization by αE3 at 1:50 dilution to rabbit serum at 1:500 show significant neutralization of virus by E3 antibodies at pH 6.1 (B) compared to samples at pH 7.4 (A). *, significance of difference by p value = p < 0.05.

Despite low virus titers, preliminary data were promising in that it showed a significant neutralization of VEEV by E3 antibodies at pH 6.1 versus rabbit serum controls that was absent from sample at pH 7.4 with no significant impact to transduction levels brought on by pH adjustment. Another set of neutralization assays were performed scaling up the amount of producer cells as described in Chapter 2. Harvested virus was incubated with pAb αVEEV E3 at 1:50 and 1:500 dilution before being applied to target BHK cells and assayed for the presence of the gene reporter β-galactosidase. Transduction levels of the reporter show a statistically significant trend of neutralization proportionate to antibody
concentration in samples treated at low pH 6.1 that is absent at higher pH 7.4 levels. Rabbit serum was used at a concentration of 1:50 of total buffer volume for samples expressed at pH 7.4 and pH 6.1 to observe any difference in virus production brought on by the serum itself. Transduction efficiencies of pseudotype virus, like the preliminary assays, show no significant difference between viruses prepared at either pH. These data support the hypothesis that virus budding off into low pH can be neutralized by antibodies against the E3 glycoprotein, and the absence of neutralization at pH 7.4 supports the observed pH-dependent retention of E3 on mature virus seen in immunoblots (Figure 9).
Figure 9. pH-Dependent Neutralization of VEEV with Polyclonal E3 Antibodies

Neutralization assays were performed on virus produced from twelve, 10cm plates. Virus was concentrated using a pH adjusted sucrose gradient at pH 7.4 or pH 6.1 before incubation with antibodies against VEEV E3 at 1:50 and 1:500 dilutions or rabbit serum control. Statistical analysis performed on triplicate assays using the student’s t-test for a two-tailed distribution and assuming equal variances comparing neutralization by αE3 at 1:50 concentration to rabbit serum at 1:50 concentration. *, significance of difference by p value = p < 0.05. Error bars show standard deviation. Following incubation of virus with polyclonal antibodies against VEEV E3, a significant amount of neutralization of virus was observed for samples prepared at pH 6.1 versus rabbit serum control. This neutralization was absent for virus prepared at pH 7.4.

3.3.3. Localization of pH-Dependent Retention of E3 in Producer Cells

The data from immunoblots and neutralization assays using polyclonal antibodies against the E3 glycoprotein support the hypothesis that E3 is retained in a low pH-dependent manner on budded virus particles. However, the amount of virus budding with glycoprotein spikes of VEEV containing pE2 independent of the extracellular pH needed to be considered. In order to distinguish the localization of the E3 glycoprotein in ΔNXgp producer cells, as a part of pE2 or
as cleaved E3, ΦNXgp cells transfected with the wild-type sequence encoding the envelope proteins for TC-83 VEEV to express VEEV enveloped MuLV pseudotype virus (WT) were fixed with formaldehyde and permeated or not permeated to observe cell surface versus intracellular E3 following probing with pAb αVEEV E3. In producer cells fixed with formaldehyde, the cell-surface localization of extracellular E3 is significantly higher in fixed but not permeabilized cells that have been incubating in media at pH 6.1 (Figure 10A) than it is in cells that had been incubated at pH 7.4 (Figure 10B). Once treated with the permeating agent Triton X-100, there is an increase of E3 signal for both pH samples as intracellular pE2 and cleaved E3 are labeled (Figure 10C & D).
Figure 10. pH-Dependent Localization of VEEV E3 Using IFA

Indirect immunofluorescence assays (IFA) on ΦNXgp cells expressing VEEV pseudotype virus (WT). Producer cells were incubated in media at pH 6.1 or pH 7.4 before being fixed with 4% formaldehyde without permeation (A-B) or with permeation (C-D) by Triton X-100 (TX). Samples were probed with rabbit-pAb αVEEV E3 primary before a secondary goat-pAb αRabbit conjugated to Alexa488 was added. Hoechst 33342 stain (blue) to label cellular DNA was then added to fixed cells.

Since minor permeation can occur after fixing cell monolayers with formaldehyde or paraformaldehyde, a mutant construct with the amino acid substitution R58-59E in the furin cleavage motif of pE2 was transfected into ΦNXgp cells. This mutation prevents recognition and cleavage by furin protease so producer cells express virus that buds with E3 covalently associated to the envelope spike. Cells were fixed with 4% formaldehyde without permeabilization (Figure 11A & B) or with permeabilization (Figure 11C & D) in order to observe any difference in E3 signal on the surface of producer cells with formaldehyde
fixation. Levels of cell surface E3 signal after probing were similar between samples produced at pH 6.1 and pH 7.4.

![Image](image_url)

**Figure 11. Localization of VEEV E3 in Cells Producing R58-59E Mutant**

Indirect immunofluorescence assays on ΦNXgp cells expressing VEEV pseudotype virus cleavage mutant (R58-59E). Producer cells were incubated in media at pH 6.1 or pH 7.4 before being fixed with 4% formaldehyde without permeation (A-B) or with permeation (C-D) by Triton X-100 (TX). Cells were probed with rabbit-pAb αVEEV E3 primary before a secondary goat-pAb αRabbit conjugated to Alexa488 (green) was added. Hoechst 33342 stain (blue) to label cellular DNA was then added to fixed cells.

Cells were also fixed in 100% v/v methanol to observe any discrepancy in signal between fixatives (Jamur 2010). The intracellular signal from these cells was comparable to samples fixed with formaldehyde for both wild type VEEV (Figure 12A & B) and R58-59E cleavage mutant (Figure 12C & D) samples at pH 6.1 and pH 7.4.
Figure 12. pH-Dependent Localization of VEEV E3 After Methanol Fixation

Indirect immunofluorescence assays on φNXgp cells expressing VEEV pseudotype MuLV virus following transfection with a plasmid containing the wild type (A-B) or a cleavage mutant (R58-59E) sequence (C-D) encoding the envelope proteins of VEEV. Producer cells were incubated in media at pH 6.1 or pH 7.4 before being fixed with 100% methanol before being probed with rabbit-pAb αVEEV E3 primary before a secondary goat-pAb αRabbit conjugated to Alexa488 (green) was added. Hoechst 33342 stain (blue) to label cellular DNA was then added to fixed cells.

All IFA experiments were performed in triplicate assays in 6-well plates for 4% formaldehyde and methanol fixed φNXgp cells expressing pseudotype virus with WT or R58-59E cleavage mutant envelope proteins of VEEV at pH 6.1 and pH 7.4. The IFA figures shown are only representative examples since three random images were acquired per experimental condition, so qualitative data were supported with quantitative observations. The corrected total cell fluorescence (CTCF) across the triplicate assays for each experimental condition
averages the intensity of fluorescence of a given image over one square pixel area against the averaged lowest intensity per image using Image J (NIH). This allows the statistical comparison of the ratios of intensity of fluorescence between the acquired images of cell samples. These values, where the higher the number equals greater signal, accounts for variance in fluorescence brought on through membrane permeabilization or artifacts from pH adjustment. Fluorescence intensity ratios were determined for all sample sets and averaged (Table 1).

Using a two-tailed student t-test assuming equal variances between IFA samples producing VEEV pseudotypes fixed with 4% formaldehyde with NO TX, there is a significant increase of cell surface E3 in producer cells budding VEEV at pH 6.1 versus pH 7.4. There is also a comparable signal of intracellular E3 once cells are permeated using methanol fixation or with Triton X-100. *, significance of difference by p value = p < 0.05.

Table 1. Quantitative Fluorescence Analysis of Phoenix Producer Cells

<table>
<thead>
<tr>
<th></th>
<th>pH 6.1</th>
<th>±</th>
<th>pH 7.4</th>
<th>±</th>
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<tr>
<td>4% Form NO TX (R58-59E)</td>
<td>248667.6</td>
<td>± 49028.4</td>
<td>159685.8</td>
<td>± 54855.8</td>
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<tr>
<td>4% Form w TX (R58-59E)</td>
<td>218523.9</td>
<td>± 36920.9</td>
<td>197285.9</td>
<td>± 54872.5</td>
</tr>
<tr>
<td>4% Form w TX (WT)</td>
<td>130946.6</td>
<td>± 25237.4</td>
<td>129986.7</td>
<td>± 16960.9</td>
</tr>
<tr>
<td>*4% Form NO TX (WT)</td>
<td>181118.4</td>
<td>± 40927.9</td>
<td>96616.86</td>
<td>± 43679.9</td>
</tr>
<tr>
<td>Methanol (R58-59E)</td>
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<td>222007.9</td>
<td>± 44531.8</td>
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<tr>
<td>Methanol (WT)</td>
<td>131415.4</td>
<td>± 16514.3</td>
<td>78558.2</td>
<td>± 16220.2</td>
</tr>
</tbody>
</table>
3.4. Discussion

The retention of the E3 glycoprotein on budded, mature virus in a pH-dependent manner was investigated using a species-specific polyclonal antibody against VEEV E3. The media of producer cells budding pseudotype VEEV was adjusted in pH to investigate if a low extracellular pH would promote the retention of the E3 glycoprotein on the envelope spike following furin cleavage. Immunoblots show the presence of E3 in virus samples prepared from cells adjusted to pH 6.1 that is absent in virus samples prepared for cells at pH 7.4. We also show the presence of the E3 glycoprotein in expressed virions. In neutralization assays, there was a significant decrease in transduction of the lacZ reporter gene resulting from entry by VEEV pseudotype virus produced at pH 6.1 in the presence of polyclonal antibodies against E3 that was absent for the same virus prepared at pH 7.4. This neutralization increased with antibody concentration and was not affected by the difference in pH. Overall transduction levels of virus exiting at pH 6.1 were lower, but this is not statistically significant. This could be due to a few possibilities. First, the percentage of virus budding and retaining E3, even transiently, could be noninfectious due to the structural hindrance the presence of E3 still associated with the E2-E1 heterodimer spike could pose on receptor binding and conformational changes. Second, a percentage of virus budding with retained E3 could potentially be binding something at the producer cell surface and fail to successfully disassociate from the producing cell.
In the immunofluorescent microscopy studies, the location of E3 was investigated in virus-producing cells at pH 7.4 vs pH 6.1. The presence of intracellular E3, as a part of pE2 or cleaved, was compared to cell-surface levels of E3 of producer cells in pH 7.4 vs pH 6.1 both qualitatively and quantitatively. The fluorescence signal represents the amount of E3 glycoprotein retained on mature virus and shows a qualitative increase on fixed producer cell samples at pH 6.1 compared to fixed cells at pH 7.4. This increase is statistically significant between the differences in pH when comparing the averaged total cell fluorescence of each well in triplicate assays. In fixed producer cells that have been treated to permeate the cellular membrane, total E3 signal is comparable across all producer cell sets independent of fixation method. This is due to intracellular E3 being accessible and recognized by antibody, thus raising the overall signal of pH 7.4 samples comparable to pH 6.1 samples and suggesting intracellular production of virus proteins is not affected by the changes in pH outside the producing cell.

Interestingly, the use of a furin cleavage mutant (R58-59E) that covalently retains E3 as part of the envelope spike on budding VEEV pseudotypes showed an increase of E3 fluorescence at pH 6.1 compared to pH 7.4 from cells fixed with 4% formaldehyde that were not permeated. It is not clear why this is, however, large deviations for those samples budding virus with the R58-59E substitution can be seen for both formaldehyde and methanol fixation methods. This range of variation could possibly be due to differences in production of the mutant particles versus virus containing the wild type sequence for furin
cleavage. However, once permeated with triton X-100, the intensity of E3 signal is more comparable between pH 6.1 and 7.4 once intracellular E3 is recognized by antibody. This suggests the differences of signal between pH 6.1 and pH 7.4 for furin cleavage mutants could be stemming from differences in virus assembly and exit and not from levels of protein production. The difference in signal intensity for R58-59E between the two pH levels is not seen in cells fixed in methanol. Furthermore, the signal for cells budding virus with the wild-type sequence for furin cleavage at pH 6.1 before being fixed with methanol is greater than those cells at pH 7.4 before fixation. While this level of signal between these comparisons is not equal despite intracellular production of E3 being accessible to antibody, fixation with methanol can remove a considerable amount of protein at the cell surface, including E3, and potentially explain the differences between signals.

The presence of the E3 glycoprotein on mature virus introduces another aspect of the protein’s role following virus processing and budding from infected cells. The crystal structure for the entire glycoprotein spike has been solved at physiological pH and low pH for CHIKV and SIND, respectively, showing E3 associating exclusively with E2. This is supported by the cryo-EM structure of the entire VEEV virion at 4.4Å and in mutagenesis studies of key residues between E2 and E3 in VEEV and cleavage-impaired SIND and SFV mutants (Parades 1998; Li 2010; Voss 2010; Zhang 2011; Uchime 2013). As a member of the alphavirus genera with over 26 separate species, it is likely that after leaving the cell, VEEV has adapted the ability to protect the fusion epitope of E1 while
budding into acidic environments. As virus exits cells in environments where cells are potentially undergoing apoptosis or necrosis, the same mechanism by which E3 prevents premature fusion by E1 during intracellular transport would be required to prolong protection to that of the varying degrees of pH outside a budding cell. This capability to extend the association of E3 to the E2 glycoprotein, probably through an electrostatic association in the presence of abundant protons from low pH, can also explain how antibody neutralization is possible of virus retaining E3 on its surface. Indeed, the structural data for VEEV E3 shows that it is favorably positioned distal to the viral membrane associating with E2 and likely to participate in binding if present. Given binding associations with such motifs like heparan sulfate have already been identified for alphaviruses and in lab adapted strains of the E2 glycoprotein, the retention of E3 adds another potential site for protein interactions.
4.1. Abstract

The transient retention of the E3 glycoprotein on mature virus particles offers a new avenue for protein interactions between virus and extracellular proteins. It has been observed that the furin cleavage motif between E3 and E2 binds to heparan sulfate (HS) in lab-adapted strains of SIND and cleavage mutants presenting E3 as a part of pE2 for SFV. This increased binding capacity is believed to be due to the similarities in the residues known to participate in binding one of the four known sequences of HS binding (XBBXBX) and those in pE2 recognized by the subtilisin-like furin protease (XBXBBX) where X, hydrophobic residue; B, basic residue (Klimstra 1998, 1999; Zhu 2010). It has also been observed that the natural resistance-associated macrophage protein (NRAMP) is a definitive receptor of the prototypic alphavirus member SIND (Rose 2011). This receptor was targeted for downregulation in its cell surface expression using exogenous iron supplementation to probe the impacts of pH-dependent, transiently-associated E3 on virus entry. Our data show a significant decrease in entry and reporter gene transduction by VEEV pseudotype virus
following the downregulation of NRAMP expression for BHK cell targets. This decrease is absent in RRV pseudotype controls. This suggests that VEEV utilizes NRAMP as a receptor. We also found for VEEV pseudotypes retaining E3 at low pH an ability to bind HS as an attachment factor which rescues infection and results in a significant increase in entry and gene reporter transduction in BHK cells lacking NRAMP on the cell surface. Taken together, these findings suggest that the ability to bind HS as an attachment moiety by E3 on mature virus impacts the tropism and entry of particles, permitting the virus to bypass the negative effects of NRAMP receptor downregulation.

4.2. Introduction

The processing of pE2 has been observed to vary across cell types budding mature virus. A percentage of particles do retain pE2 on the virus coat, which has been shown to inhibit infectivity despite not being disruptive of the assembly and budding processes (Zhang 2002). The importance of pE2 cleavage is supported by work with mutant CHO-K1 cell lines protected from SIND infection due to a cleavage-deficiency for pE2 by a lack of furin protease. Indeed, cell-dependent infection requires successful processing of pE2 across mammalian cells with a stricter requirement in insect cells (Watson 1991; Heidner 1996). Mutants in which E3 is covalently associated to E2, show significant attenuation of entry by VEEV and SFV particles due to E3 blocking spike activation (Swapna Apte thesis work; Sjöberg 2011). The contacts made by
E3 to the β-ribbon region of E2 are disordered after cleavage priming E3 for disassociation following a low pH trigger. This permits the conformational changes within the E2 linker region for proper disassociation of E2 from E1 to allow orientation of the E1 trimer assembly necessary for fusion (Li 2010; Voss 2010). In order for a mature virus particle to be optimally infectious, E3 must be removed from E2 on the virus at basic pH in order to permit the conformational changes necessary for E1 to initialize fusion. Covalently associated E3 prevents these structural changes, whereas cleaved E3 does not.

Despite the presence of E3 glycoproteins on mature particles, production and release of virus is not inhibited (Tubulekas 1998). Work with SFV cleavage mutants retaining E3 on budded, mature virus suggested a low-pH requirement for its retention outside the cell. However, a similar low-pH environment exists within the cell during transport and assembly of the virus structural proteins. It is unclear how much of the virus retains the E3 glycoprotein for the protection of the E1 fusion peptide region when the association of E3 with the spike complex outside the cell is detrimental to infectivity (Sjöberg 2011). The successful neutralization of wild-type TC-83 strain VEEV virus with monoclonal antibodies against E3 following challenge in mice suggests that budding virus retains enough E3 glycoprotein to be significantly inhibited (Parker 2010). Furthermore, the furin cleavage site within pE2 allows for the retention of the furin motif sequence on E3 which itself is suggested to be topologically positioned and favorable for binding interactions (Klimstra 1998, 1999; Parker 2010; Zhang 2011; Uchime 2013). Transiently associated E3 in low pH environments outside
the cell could potentially explain these observations and offer new binding capabilities to mature virus.

Our previous findings suggest a pH-dependent mechanism that could explain how a transient association of E3 with the E2-E1 spike would be possible, as E3 would continue its protective role through electrostatic association. Knowing this, we investigated what potential purpose would exist that would select for virus retaining an otherwise detrimental protein association outside the cell. Since no known receptors that bind the E2 glycoprotein have been shown to interact with E3 directly, an ancillary role for transiently-associated E3 in binding attachment factors is possible (Wang 1992; Linn 2005; Kielian 2010; Rose 2011). Of the identified cell-surface moieties known to facilitate virus attachment, heparan sulfate (HS) has been implicated before in virus binding by the E2 glycoprotein in RRV and SIND through cell passage studies and investigations of binding sites on E2 using Cryo-EM (Heil 2001; Zhang 2004; Zhu 2010). Tissue culture adapted strains of VEEV have been shown to be capable of binding HS from mutations within E2 to positively charged amino acids. This ability to bind HS decreased entry for VEEV in CHO cell lines lacking cell surface HS (Bernard 2002).

Heparan sulfate is a ubiquitous glycosaminoglycan (GAG) moiety with sulfonated disaccharide chains present on most cell types that permits an electrostatic association with oppositely charged residue side-chains of proteins. Its role in biological processes includes angiogenesis, blood coagulation, and in
negative regulation of cell-cell attachment (O'Donnell 2009; Meneghetti 2015). HS has been implicated as an attachment factor for various viruses besides alphaviruses over the years. Binding studies with respiratory syncytia virus and with the flavivirus Dengue (DENV) have shown virus capable of utilizing HS to enter cell targets (Hallak 2000; Dalrymple 2011). Cell culture adaption and work with mosquito salivary glands have also shown HS to possess the capacity to bind strain-specific SIND isolates (Ciano 2014). This capacity of binding HS moieties appears only limited to the ability of the virus to acquire adaptive mutations to charge-favorable residues. Indeed, studies with point mutations on the E2 glycoprotein of SIND, CHIKV, and SFV have all shown changes in virulence based on the acquired attachment to HS moieties (Bernard 2000; Heil 2001; Smit 2002; Gardner 2013, 2014).

The only evidence suggesting E3 on mature virions comes from structural studies on SFV, which also has been shown to bind HS (Lescar 2001; Smit 2002). The similarities between the furin-like protease cleavage motif and the residues observed capable of binding cell surface HS offer an opportunity to investigate the impacts of transient E3 retention on viral entry as it can potentially serve in binding an attachment factor and impact virus infectivity (Klimstra 1998, 1999). Indeed, with lab-adapted strains and natural variants, it was shown that association with attachment factors could guide the receptor interaction and tropism of alphavirus members. Work with North American eastern equine encephalitis virus (NA-EEV), Eastern equine encephalitis (EEEV), and SIND have shown that these specific alphavirus species can use E2 glycoprotein
binding to HS moieties to gain neurovirulence (Ryman 2007; Gardner 2011, 2013). For the first time, it was shown that association with attachment factors could potentially explain variance in tropism across the alphavirus species apart from receptor binding, and the varying degrees of virulence within the same virus.

We first wanted to observe if VEEV retaining E3 in a pH-dependent manner can bind HS. Second, we attempted to show if this transient binding of HS by E3 could allow the virus to alter tropism or impact entry. Taking advantage of a recent putative receptor identified for SIND in the natural resistance-associated macrophage protein (NRAMP2), we attempted to observe whether VEEV was affected by downregulation of this receptor and whether virus retaining E3 could then mitigate the effect (Rose 2011). Found in both mammalian and insect cells, NRAMP is an iron transporter belonging to a class of proteins that includes lactoferrin which influence the innate immune response to viral infection through iron cation withdrawal (Nevo 2006; Johnson 2011).

There are two expressed types of NRAMP: The well-studied, primarily intracellular NRAMP1, which functions in the immune response, and the ubiquitously expressed homolog NRAMP2 located on cellular membranes. There are 4 identified isoforms of NRAMP2 that arise from alternative splicing, and the variant expressed is tissue specific and dependent on the presence of iron. The prevalence of NRAMP2 in kidney epithelia and its upregulation in situations of iron deficiency is well studied (Zhao 2012). In contrast, there is no known
physiological sequestration of iron in mammalian cells so regulation of iron uptake in instances when iron is abundant comes from the regulation of the presence of NRAMP2 itself. Through supplementation of exogenous iron, cell-surface NRAMP2 is decreased through iron binding and uptake and initiates feedback inhibition of the protein synthesis pathway for NRAMP production to prevent excess uptake of cytotoxic levels of iron (Canonne-Hergaux 1999; Rose 2011). This novel mechanism of downregulating cell-surface NRAMP allows for the investigation of the effects of VEEV E3 on entry using the NRAMP receptor.

4.3. Results

4.3.1. Entry of VEEV Retaining E3 in Cells Expressing Heparan Sulfate

The presence of HS moieties on the cell surface of targets was directly investigated. Two cell lines, CHO 22 and CHO 18.4, were used to test the importance of HS presence on E3 retention on mature VEEV virus. CHO 22 expresses HS at the cell surface whereas CHO 18.4 does not (Heil 2001). Virus was produced as previously described in Chapter 2 using the aforementioned Phoenix system in environmental conditions of pH 7.4 or pH 6.1 and applied to target CHO 22 or CHO 18.4 cells in triplicate transduction assays. Viruses transiently retaining E3 in a low pH-dependent manner are hypothesized to convey an enhanced level of cellular transduction resulting from attachment of E3 to heparan sulfate. This increased level of transduction is expected to be minimized in experiments with target cells treated with heparinase.
Preliminary data show that the pH-dependent presence of E3 on pseudotype virus yielded higher levels of transduction and thus entry of virus into CHO 22 cell targets with HS moieties than that of CHO 18.4 cell targets lacking cell-surface HS. This suggests that HS plays a role in entry of virus transiently retaining the E3 glycoprotein (Figure 13).

![Bar chart](image)

**Figure 13. Transduction of CHO 22 & CHO 18.4 By VEEV Retaining E3**

Transduction efficiencies of VEEV pseudotype virus prepared at pH 7.4 or 6.1 performed in triplicate for CHO cell targets. Error bars are standard deviation. Samples prepared to retain E3 (pH 6.1) or lacking E3 (pH 7.4) on mature virus were produced and concentrated before being applied to target CHO cell lines expressing HS (CHO 22) or lacking HS (CHO 18.4).

These data prompted the experiments to be repeated with CHO 22 and CHO 18.4 cells treated with 6μg/ml heparinase for 1 hour prior to incubation with virus. This cleaves away heparan sulfate at the α(1-4) glycosidic linkage and resulted in a decrease in transduction levels for CHO 22 to that of samples
infected with virus prepared at pH 7.4 lacking E3. This level is comparable to the transduction levels observed in CHO 18.4 cells, which lack HS. Furthermore, transduction levels of CHO 18.4 cells treated with heparinase by virus presenting E3 on its surface were not affected. It can be concluded that E3 plays a role in binding HS and can mediate entry as an attachment factor (Figure 14).

![Graph showing transduction levels](image)

*Figure 14. Transduction of CHO cells Following Heparinase Treatment*

Transduction efficiencies of target CHO cells following entry by pseudotype VEEV that was either retaining E3 (pH 6.1) or lacking E3 (pH 7.4) performed in triplicate. Error bars are standard deviation. Target cells were treated with heparinase for 1 hr or untreated before virus was applied. Transduction was increased in CHO 22 cells with HS that was absent in CHO 18.4 lacking HS. This increase in entry in CHO 22 cells was abolished after treated by heparinase with no significant change observed in CHO 18.4 cells.

As previously stated, SFV is the only other prototypic alphavirus species with data suggesting the presence of E3 on mature virus, and with the capacity for E3 to bind to the heterodimer spike at low pH (Lescar 2001; Sjöberg 2011).
As controls, VSVG and SFV were used to measure any adverse effects of pH adjustment on entry and of E3 retention amongst alphavirus species, respectively (Figure 15).

Figure 15. Transduction of CHO 22 and CHO 18.4 cells by SFV Pseudotypes

Transduction efficiencies of SFV and VSVG pseudotype controls in CHO target cells performed in triplicate. Error bars are standard deviation. SFV was produced following the protocol shown to produce VEEV pseudotypes retaining E3 glycoprotein on mature particles and applied to cells with or without treatment by heparinase.
There was no observed significant change in transduction following adjustment of pH for VSVG pseudotypes, however, there is a significant decrease of transduction observed for SFV pseudotypes following treatment of target CHO 22 cells by heparinase that is absent from CHO 18.4 cells lacking native HS expression. This difference does not appear to be dependent on pH as there is comparable levels of transduction for virus produced at pH 6.1 and pH 7.4 for both CHO cell lines.

4.3.2. Transduction of Target Cells Following Downregulation of NRAMP

Experiments targeting the downregulation of the NRAMP receptor were performed in efforts to further observe whether the presence of the E3 glycoprotein improved the ability of virus to bind to and enter target cells lacking a putative receptor. The hypothesis was that if E3 on mature VEEV could bind HS and thus increase entry as seen in previous data, any observed decrease in transduction levels due to a decrease in cell surface expression of NRAMP could potentially be rescued by E3. Cells were treated with exogenous supplementation of iron citrate prior to virus being applied. A rabbit polyclonal antibody against mammalian NRAMP2 (Santa Cruz Biotech) was utilized in assays to determine the successful downregulation of cell surface NRAMP in target BHK cells that were treated for 72 hours as described in Chapter 2. Validation of NRAMP2 expression was carried out alongside transduction experiments with indirect-immunofluorescence and SDS-PAGE immunoblot
assays and shows successful downregulation and overall decrease in both cell-surface and intracellular levels of NRAMP2 (Figure 16).
Target BHK and CHO cells were treated with 160μM ammonium iron citrate for 72 hours before virus was applied. Extra cells were prepared to validate the downregulation of both extracellular and intracellular NRAMP2. Target BHK, CHO 22 (HS+), and CHO 18.4 (HS−) cells treated with iron for 1 hour or 72 hours have a decreased cell-surface NRAMP2 signal from polyclonal antibodies in IFA versus control untreated cells (A). Target cells treated with iron for 72 hours (Lanes 1-3) and untreated controls (Lanes 4-6) were lysed for immunoblotting with polyclonal antibodies against NRAMP2 and show a decreased intracellular signal for two species of NRAMP2 at 90kD and 65kD (B).
Interestingly, the data show that entry of pseudotype VEEV virus produced at pH of 7.5 – 8 for standard transduction assays was significantly affected by a downregulation of the NRAMP2 receptor with a decrease in transduction of both target CHO 22 and 18.4 cells observed, suggesting that VEEV potentially utilizes NRAMP2 as a receptor (Figure 17).

![Graph showing VEEV-NRAMP2 interaction](image)

**Figure 17. Transduction of CHO Cells by VEEV Following Iron Treatment**

**Top**) Transduction of VEEV pseudotypes in CHO 22 and CHO 18.4 cells following incubation of target cells with 160 μM iron citrate for 1 hour or 72 hours performed in triplicate. Error bars are standard deviation. Virus was applied in the presence of iron supplementation throughout infection before cells were fixed and assayed for reporter gene β-galactosidase activity. **Bottom**) Percentage of transduction with relation to control samples with no iron treatment.
Transduction assays using both SIND and RRV as controls supported the data observed for VEEV and the use of pseudotypes for this goal. When the NRAMP receptor was downregulated, entry of SIND was significantly decreased whereas RRV entry was only affected for CHO 22 cells. This supports what was reported for wild-type virus (Rose 2011) (Figure 18).

*Figure 18. Transduction of CHO Cells by RRV & SIND Following Iron Treatment*

**Top** Transduction of RRV (left) and SIND (right) pseudotypes in CHO 22 and CHO 18.4 cells following incubation of target cells with 160 μM iron citrate for 72 hours performed in triplicate. Error bars are standard deviation. Virus was applied in the presence of iron supplementation throughout infection before cells were fixed and assayed for reporter gene β-galactosidase activity. **Bottom** Percentage of transduction with relation to control samples with no iron treatment.
Transduction assays of both CHO 22 and CHO 18.4 target cells were performed with VSVG pseudotype virus following iron treatment of target cells for 72 hours to test for any significant effects of iron citrate supplementation to virus entry by a virus not belonging as a species to the alphavirus family. There were no significant differences between either cell type with or without HS following iron treatment (Figure 19).

![VSVG - NRAMP Graph](image)

**Figure 19. Transduction of CHO Cells by VSVG Following Iron Treatment**

Transduction of VSVG pseudotypes in CHO 22 and CHO 18.4 cells following incubation of target cells with 160 μM iron citrate for 72 hours performed in triplicate. Error bars are standard deviation. Virus was applied in the presence of iron supplementation throughout infection before cells were fixed and assayed for reporter gene β-galactosidase activity.
4.3.3 Retention of E3 on VEEV Increases Entry after NRAMP2 Downregulation

Following downregulation of cell-surface NRAMP, it was found that entry was rescued by the presence of the E3 glycoprotein on the surface of virus targeting CHO 22 cells expressing HS on their surface. This increase in virus entry is not seen for CHO 18.4 cells that lack HS and there is no significant difference between CHO 22 and BHK cells for the heparinase experiments. These findings were considered for target cells that had been treated with iron citrate and thus underwent a downregulation of both cell-surface and intracellular NRAMP2. The previous data showing a significant decrease in entry for VEEV in target CHO cell lines provided an opportunity to use VEEV prepared as previously described to retain E3 on the surface of mature virus. VEEV retaining E3 in a pH-dependent manner was applied to target CHO 22 and CHO 18.4 cells following treatment with 160 µM iron citrate for 72 hours (Figure 20).
Figure 20. Transduction of CHO Cells by All Viruses Following Iron Treatment

Percentage of observed transduction from triplicate assays of target cells CHO 22 (A) and CHO 18.4 (B) that were treated with 160 μM iron citrate for 72 hours before being incubated with VEEV, RRV, SIND, and VSVG pseudotypes produced at pH 7.4 or pH 6.1. Error bars are standard deviation.

Transduction assays of target BHK cells following iron treatment were performed with VEEV, RRV, SIND, and VSVG pseudotypes produced at pH 7.4
or pH 6.1 to compare entry levels with that of what was observed for the CHO cell lines (Figure 21). The levels of entry were comparable to what was seen in previous experiments involving heparan sulfate. Decreases in entry for virus following downregulation of NRAMP2 that was previously observed for BHK cells was rescued by virus transiently retaining E3 for VEEV.

![Graph](image)

**Figure 21. Transduction of BHK Cells by VEEV Following Iron Treatment**

Percentage of observed transduction of target BHK cells that were treated with 160 μM iron citrate for 72 hours prior by VEEV, RRV, SIND, and VSVG pseudotypes produced at pH 7.4 or pH 6.1 performed in triplicate. Error bars are standard deviation.

### 4.4. Discussion

The impacts of retaining the E3 glycoprotein on mature virus were investigated using VEEV pseudotype retrovirus. Despite a structural detriment to
optimum infectivity or cell-dependent inhibition, the data suggests VEEV has evolved a dual-role for the E3 glycoprotein. First, to protect the E1 fusion envelope motif while maintaining stability during transport through an infected cell. Second, to extend this electrostatic interaction and pH protection into the extracellular matrix surrounding a cell as needed. Given the acidic environment of viral infections that virus particles are budding into, it is plausible that this auxiliary role for E3 has been selected for due to its additional binding capabilities.

The results involving the increase of entry into cells containing heparan sulfate support the hypothesis that the presence of E3 significantly affects the entry of VEEV into CHO 22 cells expressing HS on their surface. The decrease in transduction in CHO 18.4 cells by virus retaining E3 suggests, even transiently associated E3 to E2, can inhibit entry and it is possible that the presence of HS on CHO 22 cells rescues this inhibition by HS binding to E3 and potentially strips it away from the virus. These findings support previous observations that pE2 cleavage-deficient SIND can establish HS binding and entry with resuscitating mutations in E3 or E2 (Ryman 2004). Overall, the data shows an increase in entry for virus retaining E3 to that of virus where E3 is absent in cell targets expressing HS on their cell surface which is neutralized with heparinase to match levels of entry observed for CHO 18.4 cells lacking cell-surface HS.

The utilization of the iron transporter protein NRAMP2 was an attempt to use a defined receptor for alphavirus prototypic member SIND. Establishing its
use in experiments to probe any effects of pH-dependent retention of the E3 glycoprotein on mature virus was supported first by the significant inhibition of SIND pseudotypes that was not seen for RRV pseudotypes, and finally the observed decrease in transduction of VEEV pseudotypes into target BHK and CHO 22/18.4 cell lines. This decrease in entry levels was shown to be rescued by virus prepared at pH 6.1 shown to retain E3 on mature virus particles. This return of infectivity following downregulation of cell-surface and intracellular NRAMP2 in CHO 22 (HS+) and CHO 18.4 (HS-) suggests that the retention of E3 in low pH environments on mature virus particles could be selected for by having an advantageous role in binding attachment factors such as HS.

These findings could explain the observed range of tropisms for alphavirus members and also the expansion by some members into cells that leads to increased virulence (Ryman 2007; Gardner 2011, 2013). A closer look at critical residues within the sequence encoding E3 needs to be performed. Given the sequence similarity between the various species of alphavirus, it is still not clear if the increase in entry by cells with HS is occurring between E3 exclusively, by residues shown to bind HS in E2, or a combination of both. Looking at the direct binding of the E3 envelope glycoprotein to HS is necessary to identify the binding characteristics of having it transiently associated.
5.1. Abstract

Cell type is an important factor that should be considered when investigating glycoprotein retention on mature arbovirus. The construction of novel baculoviral pseudotypes was performed to express a replication-incompetent baculoviral core construct lacking the native gp64 envelope protein that incorporates the alphaviral VEE envelope proteins on its surface. This was to investigate the impacts of insect cell processing and membrane composition on the low pH-dependent retention of VEEV E3 previously seen in mammalian cell studies. Immunoblot analysis of envelope protein production and neutralization studies with polyclonal antibodies against VEEV envelope proteins show similar translation and envelope protein modification in Sf9 cells of infectious virus comparable to mammalian cells, and a significant decrease in GFP reporter in targeted Sf9 cells resulting from the inhibition in entry of virus produced at low pH by E3 antibodies that is absent at higher pH for insect cells. These results support previous findings showing infectious particles budding from insect cells with differential processing of the pE2 glycoprotein from that of mammalian cells.
while remaining comparably infectious. This suggests that the higher level of pH-dependent neutralization by polyclonal antibodies against E3 could potentially be due to the more efficient cleavage of the pE2 polyprotein in insect cells and thus more E3 readily available for retention at lower pH.

5.2. Introduction

Comparative studies across mammalian and insect cells have identified differences in virus production and composition brought on by differences in the cellular physiology of vectors. Epidemiological evidence of encephalitic viruses like VEEV and arthralgia-causing pathogens like CHIKV and RRV has supported a pattern of developing serological isotypes of enveloped viruses due to the type of environment their emergence occurs in based on the vectors available for production and spread (Kuno 2005; Solignat 2009). Phylogenic studies for adaptive evolution contingent on hosts have identified selection occurring for CHIKV and other Class II fusion viruses such as DENV that produces virus capable of taking advantage of insect vector availability through key mutations in the viral envelope spike broadening the range of tropism and membrane fusion criteria (Bennett 2002; Tsetsarkin 2007; Dubrulle 2009). This selection potentially differs amongst alphaviruses, and has been seen to be impacted by whether virus can be transmitted vertically, but has the capacity to produce low serological variance due to the ubiquitous prevalence of some of these viruses in highly selective environments (Schuffenecker 2006; Vazeille 2009; Jones 2010).
The importance of cellular membrane composition has also been considered and well-studied for enveloped alphaviruses, encompassing individual species member tropisms and cell-specific permissiveness. This stems from the inherent differences between the insect vectors that have low levels of sterol content used for transmission, and the incidental mammalian cell hosts which are susceptible to the various species that are sterol-rich. The need for cholesterol and sphingolipid in target cell membranes for both fusion and successful budding has been observed for SFV, SIND, RRV, and CHIKV for mammalian cell hosts with key mutations bypassing this need being observed in both E1 and E2 glycoproteins (Strauss 1994; Lu 1999; Chatterjee 2000, 2002; Kielian 2006; Tsetarkin 2007; Umashankar 2008). In cell passaging studies, the differential incorporation of cholesterol into the lipid bilayer of budding alphavirus species has been observed in mammalian and insect cells. This suggests that the impact of target membrane composition on the success of virus entry and exit is cell-type specific owning to the importance of cellular identity of the producing cell to the viral envelope that buds from it (Hafer 2009).

The successful expression of proteins from vectors for gene payload delivery or immunity priming have to consider the cell type in order to be optimally effective due to differences in protein modification capabilities. Consequentially, the differences between host cell types and their effect on viruses are considered for medicinal aims of vectorology for both vaccine development and gene therapy strategies (Kang 2002; Kahl 2005; Akahata 2010). For alphaviruses, differential processing of pE2 has been observed across
mammalian and arthropod cell lines in that within arbovirus vectors, seen in *A. albopictus* C6/36 cells, cleavage of pE2 in insect cells is more efficient than mammalian cell lines (Heidner 1996). Indeed, this particular efficiency in pE2 cleavage is crucial for insect cells in order for virus to be infectious (Watson 1991). To further distinguish the neutralization of E3 in prior mammalian cell studies; of retained E3 glycoprotein versus the percentage of virus particles failing to cleave the pE2 polyprotein and presenting E3 a part of the spike complex, we needed to investigate the retention of E3 in insect cells.

The PhoenixGP expression system employed for the mammalian cell studies is unable to express within insect cells due to the specific mammalian promoters RSV and CMV engineered within the genomic sequences that code the gag/pol and alphaviral envelope protein genes, respectively. Previous attempts with filovirus, rhabdovirus, and recently CHIKV E2-E1/Capsid proteins have successfully demonstrated processing and infectivity of viral proteins and establish the baculovirus expression system, a well-studied protein expression system for both bacterial and insect cells, as a viable pseudotype vector system in insect cell lines (Kitagawa 2004; Cho 2008; Westenberg 2010; Metz 2011; Kuo 2011; Laura Hughes-Baker thesis work). The utilization of an insect cell-based expression system for pseudotype virus in insect cells would allow us to probe the impacts of the differential processing of viral envelope proteins in insect cells on the pH-dependent retention of the E3 glycoprotein.
5.3. Results

5.3.1. Creation of VEEV Pseudotype Baculovirus

Bacmid constructs encoding the baculoviral core capsid either lacking the native gp64 envelope protein (MW024) or containing gp64 as a wild-type control (MW033) were graciously donated by the Dolphin group (Kings College, London, UK). Virus budding from producer insect cells in either pH 7.4 or pH 6.1 conditions could potentially yield differences in E3 glycoprotein retention accounting for cleavage and lipid membrane composition differences between mammalian and insect cells. Following the transfection of producer Sf9 cells, virus was harvested and the validation of viral envelope proteins from the baculoviral pseudotypes was performed using SDS-PAGE and immunoblot analysis with antibodies against VEEV E2/E1, E3, and the major baculoviral capsid vp39 (a generous gift from Loy Volkman UC Berkeley) (Figure 22).
Figure 22. SDS-PAGE of VEEV Pseudotype Baculovirus

SDS-PAGE immunoblot of samples from concentrated virus prepared at pH 7.4 or pH 6.1 and producer cell lysate 96 hours after transfection of Sf9 cells. The 0.45μm nitrocellulose membrane was probed with 1:500 rabbit pAbE3 and 1:1,250 dilutions of rabbit pAbE2/E1 for 4 hrs. A mouse mAb against baculoviral capsid vp39 was used at 1:100 for 4 hours. Goat anti-rabbit and rabbit antimouse secondary conjugated to HRP were used for 2 hrs. A) (Lane 1 & 3) Virus pellet at pH 7.4 and pH 6.1, respectively. Lane 5 & 6) Control MW033 bacmid pellet and Sf9 producer cell lysate, respectively. B) Samples were probed only with 1:500 rabbit pAbE3 before a goat anti-rabbitHRP secondary. (Lane 1) Control MW033 bacmid pellet. (Lane 2) Sf9 producer cell lysate. (Lane 3) ϕNXgp producer cell lysate expressing R58-59E mutant for VEEV producing cleavage-deficient pE2.

Antibodies against E3 failed to recognize the glycoprotein from protein samples of budded virus for neither pH nor pE2 in Sf9 producer cell lysates. Translation and modification of the E2 and E1 glycoproteins are present on mature virus as well as in Sf9 producer cell lysate. However, there is a considerable amount of production of baculovirus core capsid protein being made within the Sf9 producer cells as seen in the lysate (Figure 22A, Lane 6) that is not budding into mature baculovirus pseudotypes as seen in the signal for capsid protein for virus pellet samples at either pH 7.4 or pH 6.1 (Figure 22A, Lanes 1 & 3).
5.3.2. Neutralization of VEEV Pseudotype Baculovirus by E3 Antibodies

VEEV pseudotype baculovirus produced following the transfection of producer Sf9 cells was harvested after 96 hours and incubated with polyclonal antibodies against E3, E2, or rabbit serum before being applied to target Sf9 insect cells. Target cells were assayed for reporter GFP expression 72 hours later. There was a significant pH-dependent neutralization of infectious particles incubated with polyclonal antibodies against E3 for virus prepared at pH 6.1 that is absent for virus prepared at pH 7.4. This neutralization is significantly more than that seen for samples treated with polyclonal antibodies against E2. There was no significant effects on infection by pH adjustment or neutralization of virus by rabbit serum at either pH (Figure 23).
Figure 23. pH-Dependent Neutralization of VEEV Pseudotype Baculovirus

Fluorescence microscopy of both producer and target Sf9 insect cells using VEEV pseudotype baculovirus. A) Producer Sf9 cells were transfected with a bacmid construct encoding the entire genome of baculovirus (033), or gp64-null baculovirus (024) along with a helper plasmid encoding the envelope glycoproteins pE2-6K-E1 of VEEV. Media containing VEEV pseudotype baculovirus was collected 96 hours later and had its pH adjusted to 7.4 or 6.1. B) Virus samples were incubated with antibodies against VEEV E2, E3, or rabbit serum for 1 hour before being applied to target Sf9 cells. The pH-dependent neutralization of virus at pH 6.1 by polyclonal antibodies to VEEV E3 is significant compared to virus at pH 7.4. The inhibition of virus by polyclonal αVEEV E2 at 1:50 dilution is comparable to previous mammalian studies while polyclonal αCHIKV E2 and rabbit serum show no cross-reactivity by antibodies nor detriment to infectivity by pH adjustment and background serum.

The pH-dependent neutralization of VEEV pseudotype baculovirus was reflected in the transduction of target Sf9 cells following quantitative analysis that supports the qualitative data showing significant neutralization of VEEV in a pH-dependent manner (Figure 24).
**Figure 24. Neutralization of VEEV Pseudotyped Baculovirus in Sf9 Cells**

The pH-dependent neutralization of VEEV pseudotype baculovirus was performed in triplicate assays and is shown for target Sf9 insect cells transduced by a GFP reporter following incubation of polyclonal antibodies at specified dilutions. Error bars are standard deviation. Levels of inhibition are comparable to mammalian studies.

5.4. Discussion

The differences in translational processing of the pE2 polyprotein for alphaviruses between mammalian and insect cells was considered for VEEV. Despite differences in the pH-dependent retention and neutralization of the E3 glycoprotein observed in mammalian producer cells using the Phoenix expression system, those experiments cannot adequately distinguish between species of pseudotype virus budding off with cleaved E3 that is retained through the electrostatic association brought on by a low pH extracellular environment from that of virus budding with a portion of the spike complexes as pE2-E1
heterodimers (Figure 8). Indeed, a necessary distinction since the polyclonal antibodies against E3 recognize both VEEV E3 and pE2. Insect cells have been shown to contain a stricter requirement of pE2 cleavage for producing infectious particles, and thus an efficient furin processing event of pE2. The amount of pH-dependent neutralization of VEEV pseudotypes produced from insect cells would therefore coincide with virus inhibited by antibodies binding cleaved E3 retained at low pH and not of virus that contains pE2. The adoption of a baculovirus expression system for expressing VEEV envelope proteins to investigate the differences inherent between mammalian and insect cell translational machinery shown here, for the first time, the successful expression of VEEV pseudotype baculovirus.

The immunoblots of protein samples from VEEV pseudotype baculovirus did not show recognition of E3 by antibody in virus pellets. Furthermore, unlike what was observed for mammalian cells, there was a lack of signal for E3 as a part of pE2 in producer cell lysate after probing with polyclonal antibodies against E3 (Figure 22B, Lane 2). This could explain the absence of E3 in mature virus at pH 6.1. Given the small 7kD size of E3, it is possible that the E3 glycoprotein is at levels of production too low or the pseudotype virus titer is below the sensitivity of the polyclonal antibodies. Despite this, the recognition of E2 and E1 by polyclonal antibodies in virus pellets at either pH show virus production, and coincide with the infectivity of these pseudotype particles seen by the fluorescence of a GFP reporter following entry. This suggests that even with levels of E3 protein too low to be recognized, infectious particles are still
produced. The significant pH-dependent neutralization of viral entry by polyclonal antibodies against E3 suggests that even though protein levels are low, it is enough to bind to E2 and be neutralized by antibody.
CHAPTER 6. CONCLUSIONS & FUTURE DIRECTIONS

6.1. Conclusions

The ability of Venezuelan equine encephalitis virus to retain the E3 glycoprotein on mature virus particles presents a unique capability by the virus to protect the fusion epitope of E1 from the pH in the extracellular environment outside of the producing cell it is budding from. The importance of this small, 7kD glycoprotein has been slowly gaining attention. Data from recent studies investigating key residue contacts between E2 and E3 support the critical association of E3 required for the prevention of premature fusion and the successful maturation of the E1 glycoprotein during intracellular transport (Lobigs 1990; Lescar 2001; Parrott 2009; Uchime 2013; Fields 2015; Swapna Apte thesis work). This strategy is shared with other viruses using Class II fusion mechanisms such as those belonging to flavivirus family. Dengue contains the analogous prM and E proteins in which protection from premature fusion is achieved by glycoprotein association extending on with mature virus (Guirakhoo 1992; Konishi 1992; Vazquez 2002; Mukhopadhyay 2005).

The association of E3 extending onto mature virus particles has limited evidence supporting it. The data showing mice protected from a lethal challenge
by VEEV following inoculation with monoclonal antibodies against E3 was the first to show neutralization of an alphavirus by antibodies against the E3 glycoprotein (Parker 2010). The ability for pH-dependent association between the E3 glycoprotein and the E2-E1 heterodimer spike further supported the importance of E3 processing and release from the envelope spike for SFV (Sjoberg 2011). However, this work indirectly suggests that the extracellular environment of the producing cell has influence on whether budding virus retains the small 7kD E3 protein. As the viral pE2-E1 proteins are transported for assembly and budding, the intracellular environment they move through becomes progressively acidic. The temporal strategy for priming the furin cleavage motif located between E3 and E2 is a necessary step to remove E3 and prepare budding virus for infectivity while maintaining protection from low pH until exit. Upon exit, however, despite the critical importance of a basic pH maintenance in the lumen, the physiological relevance of a similar range of acidity existing extracellularly during chronic, lytic infections or other disease states with high occurrence of inflammation is considerable (Kim 1998; Sanchez-San 2009). This could explain why neutralization of mature virus by antibodies against E3 had been observed or why requirements of pE2 cleavage have been found to differ by cell type (Heidner 1996; Parker 2010).

For years it was assumed that following a late-stage cleavage event by cellular furin, E3 disassociates from E2, but specifically where and when this separation takes place has not been fully understood. Recent work supports the disassociation of E3 from mature virus particles outside the cell at neutral pH
Structurally, major advances in the determination of the conformational changes that the E2 and E1 glycoproteins undergo during fusion at either neutral and acidic pH using CHIKV, SIND, and VEEV has shed more light on the location of E3 with the envelope spike on mature virus (Li 2010; Voss 2010; Zhang 2011). However, retaining E3 on mature virus is detrimental to infectivity (Bernard 2000; Sjoberg 2011; Gardner 2013; Swapna Apte thesis work). Despite this detriment, cleaved E3 associated with E2 through pH-dependent electrostatic associations could allow the virus to present new binding sites for attachment, while permitting its removal from the envelope spike at neutral pH to allow E2 to bind a receptor (Klimstra 1998, 1999; Li 2010; Parker 2010; Zhang 2011).

The utilization of pseudotyped virus in the studies described throughout have allowed us to avoid the replication processes within the infectious pathway of various viruses, including VEEV. This permitted the study of viral entry only and prevented culture artifacts that have in the past been utilized as a tool to investigate point mutations and amino acid adaptations from serial passaging (Heidner 1996; Klimstra 1998; Tubulekas 1998; Heil 2001). Here, adaptation is counter to the goal of identifying whether the wild-type TC-83 sequence of VEEV envelope spike protein can facilitate the pH-dependent retention of the E3 glycoprotein at low pH. We show that indeed, at pH 6.5 or lower, MuLV pseudotyped with VEEV envelope proteins retains E3 on budded, mature particles as observed in neutralization assays and indirect-immunofluorescence assays. This ability to bind to the envelope spike at low pH supports the
observations with SFV mutants with E3 covalently associated to E2 (Sjoberg 2011). However, unlike the attenuation observed in permanent E3-E2 associations, this pH-dependent retention does not significantly affect the level of entry for budded virus particles with E3 still associated. Furthermore, virus budding into this extracellular pH transiently present residues in E3 for binding interactions to occur, and should be considered at the same level of importance as those interactions between E2 and receptors and attachment factors.

6.2. Future Directions

The ability of VEEV pseudotyped MuLV to retain the E3 glycoprotein on budded particles in a pH-dependent manner is supported throughout the body of work detailed, and is the first reported data showing E3 from infectious virus. The significance of this retention is reflected in the studies investigating the effects of E3 on mature virus as an attachment factor to target cells containing heparan sulfate moieties. Mutations in the E2 glycoprotein have been observed to confer attachment to heparan sulfate and permit an increase in both virulence and tropism range for various alphaviruses (Klimstra 1998, 1999; Heil 2001; Ryman 2007; Zhu 2010; Gardner 2011, 2013). Essentially, the incorporation of positively-charged residues within ectopic regions of the envelope spike protein permits attachment to the negatively-charged sulfate groups on glycosaminoglycans.
The capability of the pE2 precursor protein to bind heparan sulfate has been demonstrated using direct-binding studies with agarose beads investigating the importance of the furin cleavage motif given its similarity to the consensus sequences identified in heparan-sulfate binding (Klimstra 1998, 1999). Partial deletions to the cleavage sequence on pE2 still showed binding to heparan sulfate beads which has yet to be addressed in HS binding studies with virus budding from mammalian cells with a low extracellular pH (Watson 1991; Heidner 1996). In our work, we found that the neutralization of VEEV pseudotypes by antibodies against E3 is much more pronounced in Sf9 insect cells, which have a stricter cleavage requirement for infectious-capable virus and thus will only retain E3 following cleavage if conditions of low pH are present (Heidner 1996). This efficiency for furin cleavage seen in insect cells is absent for mammalian cells that bud mature virus with an indefinite amount of pE2, therefore it is necessary to determine which region of pE2 is responsible for binding heparan sulfate if virus is budding in an extracellular environment of low pH.

6.2.1. Results

Preliminary pull-down assays were performed using purified E3 protein either containing (VEEV5A) or lacking (VEEV7B) the furin cleavage motif, and purified VEEV virus produced from ΦNXgp cells at either pH 6.1, known to retain E3, or pH 7.4 in which E3 disassociates. Samples were incubated with heparan-
agarose beads (Sigma-Aldrich), washed, and eluted before being used in immunoblots probing for E3 to observe direct binding between E3 and heparan sulfate (Figure 25).

![Diagram showing immunoblot results with lanes labeled 1 to 5 and 6.1, 7.4, and corresponding protein bands labeled E1, E3, and 5A, 7B.]

**Figure 25. Direct Binding of E3 to Heparan Sulfate**

Immunoblots of pull-down assays using heparan agarose beads. **A)** Samples of purified E3 protein with (5A) or without (7B) the furin motif were incubated with heparan beads before being washed (*Lanes 1 - 2*) and eluted (*Lanes 4 - 5*) with 1.5M of NaCl. **B)** Purified virus with E3 (6.1) or without E3 (7.4) were incubated with heparan beads before being washed (*Lanes 1 & 3*) and eluted (*Lanes 2 & 4*) with 1.5M of NaCl. Membranes were probed with pAbE1 and pAbE3 at 1:2,500 and 1:500, respectively.

The data suggest that it is the furin cleavage motif that mediates attachment to heparan sulfate, because removal of the motif in purified protein samples eliminates binding and recovery in elution samples. Furthermore, binding to heparan sulfate beads is seen for VEEV purified from ΦNXgp cells that had been incubated in media at pH 6.1 that is absent for VEEV purified from the same cells incubated at pH 7.4. Together, these initial results support the
conclusion that virus transiently retaining E3 in a pH-dependent manner can utilize heparan sulfate as an attachment factor in mammalian cells. This work supports prior studies where infections from virus budding with a percentage of pE2 can also bind heparan sulfate and significantly change the virulence (Klimstra 1999; Ryman 2004).


VITA

Jason Segura was born in Oak Lawn, Illinois on September 20th, 1980 to Jose and Susan Segura. He graduated from high school with distinction from Bloom Trail with a focus in life sciences. He first attended Purdue University Calumet while working within industry as a technician studying electrical engineering before changing majors in 2006 and graduating with a B.S. in Biology in 2008. His experience conducting undergraduate research on epilepsy in the lab of Dr. Ramila Sarac compelled him to continue his education into graduate school. A drive to study diseased states, including cancer and those acquired via pathogens, led him to join the lab of Dr. David Sanders at Purdue University in West Lafayette working on alphavirus entry and attachment. He obtained his M.S. in biology specializing in virology in 2011, and his Ph.D. in molecular biosciences specializing in molecular virology in 2016. Jason continues in his endeavors to pursue a research career studying diseased states as a postdoctoral fellow at the National Institute of Allergies and Infectious Diseases within the National Institutes of Health in Bethesda, Maryland.