The dissertation committee for Tonya Michelle Colpitts certifies that this is the approved version of the following dissertation:

Characterization of the Entry Mechanisms Utilized by the Alphavirus Venezuelan Equine Encephalitis Virus to Infect Mosquito Cells

Committee:

Robert A Davey, Supervisor

Christopher C Broder

Lisa A Elferink

Peter W Mason

Scott C Weaver

Dean, Graduate School

Characterization of the Entry Mechanisms Utilized by the Alphavirus Venezuelan Equine Encephalitis Virus to Infect Mosquito Cells

by

Tonya Michelle Colpitts, B.A.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas Medical Branch

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas Medical Branch

October, 2007

Dedication

This work is dedicated to my loving family.

Acknowledgements

I would first and foremost like to acknowledge my mentor, Dr. Robert Davey, whose patience and intelligence were a great help. I could not have begun to do this without him. I would also like to thank the members of my supervisory committee, Dr. Lisa Elferink, Dr. Peter Mason, Dr. Scott Weaver and Dr. Christopher Broder. The impact of their insights and suggestions is inestimable. The help of Eugene Knutson from the optical imaging core lab was invaluable, as much of my work was done with the confocal microscope and the figures would not have turned out so great without him. Mark Griffin from the flow cytometry core lab was also extremely helpful and available to answer any questions during my research. Finally, I would like to acknowledge my family and friends, whose love and support really kept me going during this demanding process.

Characterization of the Entry Mechanisms Utilized by the Alphavirus Venezuelan Equine Encephalitis Virus to Infect Mosquito Cells

Tonya Michelle Colpitts, PhD

The University of Texas Medical Branch, 2007

Supervisor: Robert A Davey

Venezuelan equine encephalitis eirus (VEEV) is a New World alphavirus that can cause fatal encephalitis in humans. VEEV is an enveloped, positive-strand RNA virus that is transmitted by a mosquito vector. Most research on alphavirus entry was done with the Old World alphavirus Semliki Forest virus (SFV) in mammalian cells. Not much is known about the entry of New World alphaviruses, especially in cells of the viral vector, the mosquito. Work with SFV has shown that Old World alphaviruses enter mammalian cells via receptor-mediated, clathrin-mediated endocytosis. This endocytic pathway utilizes several proteins in the mammalian cell, including the clathrin protein, the small GTPases known as Rab proteins and the large GTPase dynamin. These proteins have been shown to play a role in the entry of several viruses and are thought to be involved in alphavirus entry in mammalian cells. Here mosquito homologs of these proteins are identified, isolated and characterized in the mosquito cell. Rab5, Rab7 and dynamin are shown to be involved and necessary for VEEV entry and infection in mosquito cells. A novel entry assay is used to confirm that VEEV requires a low pH to enter mosquito cells. This work represents the first characterization of the involvement of mosquito endocytic pathways for infection of a New World alphavirus and sheds light on an important aspect of virus infection in an insect vector. The role of actin in VEEV internalization was also examined. Actin is known to be involved in the mammalian endocytic pathway and to act together with dynamin to coordinate endocytosis. Here mosquito actin is identified and shown to colocalize with mosquito dynamin. Both proteins also colocalize with internalized VEEV. Inhibiting actin polymerization prevents entry of the virus both by microscopic examination as well as utilizing the luciferase entry assay. This work shows that VEEV enters the mosquito cell via a pH-dependent endocytic pathway that requires functional endocytic proteins including Rab5, Rab7 and dynamin. It is also shown that F-actin must be present for VEEV to enter mosquito cells and that actin and dynamin act together during virus internalization.

Table of Contents

List of Tables

List of Figures

List of Illustrations

Hypothesis

The New World alphavirus Venezuelan equine encephalitis virus enters and infects mosquito cells via a pH-dependent, endocytic pathway that requires the participation of functional mosquito endocytic proteins. In this study we will i) use a chimeric virus expressing VEEV envelope proteins as well as a novel luciferase entry assay and VEEV pseudotypes to test the hypothesis that VEEV enters mosquito cells via an endocytic pathway, ii) create mosquito cell lines that express the wild-type and dominant negative forms of human genes that are thought to be important in endocytosis, iii) create mosquito cell lines that express the wild-type and dominant negative forms of these same genes from the mosquito genome, iii) use these cell lines to establish which proteins and pathways are necessary for VEEV entry into mosquito cells, iv) use fluorescently labeled VEEV particles to show colocalization with the proteins thought to be important for the endocytosis of the virus in mosquito cells.

Specific Aim 1: Show that VEE enters mosquito cells via an endocytic pathway

The effects of ammonium chloride, monensin and chloroquine (established endocytic acidification inhibitors) on VEEV entry into and infection of mosquito cells will be explored using the entry assay and the results should point to entry via an endocytic pathway. First, a chimeric virus containing the nonstructural proteins of Sindbis virus (SINV) and the structural proteins and capsid of VEEV and also encoding green fluorescent protein (GFP) will be used to show that infection of mosquito cells requires low pH. Next, an entry assay will be used to illustrate that the block in infection after inhibition of acidification is at the point of viral entry into the cell. Viral pseudotyping has proven to be a useful tool for isolating the functions of viral envelope proteins. A retroviral pseudotype system has already been established for the study of VEEV glycoproteins using both MLV and HIV core proteins. Retroviral pseudotypes will be made that have an MLV core and VEEV envelope proteins that also contain the gene for luciferase protein fused to the HIV Nef protein, which inserts itself into the viral membrane upon budding. Control pseudotypes will also be made that express the envelope proteins of SFV (an Old World alphavirus), VSV (known to enter mosquito cells via pH-dependent endocytosis) and MLV 10A1 (known to enter cells at the surface without dependence on pH). These will be used in a novel luciferase entry assay that will first be optimized for an *Aedes albopictus* cell line, C710.

Specific Aim 2: Establish mosquito cells lines that express both wild-type and dominant negative versions of specific genes that are expected to have a required function in an endocytic pathway.

The wild-type (WT) and dominant negative (DN) forms of several human genes, chosen for their specific roles in endocytosis, will be fused to GFP and inserted into plasmids that have the insect ACTIN 5C promoter so that they will express in mosquito cells. These genes include Rab5, Rab7, dynamin and clathrin. The DN forms will be made by inserting a mutation previously reported to cause a DN phenotype into the gene sequence via Quikchange mutagenesis. The plasmids will be transiently transfected into the C710

cells. Using the NCBI website and the BLAST program, similar protein sequences have been found for these same genes in the *Anopheles gambiae* and *Aedes aegypti* mosquito genomes. Using mosquito-cell derived RNA, cDNA will be made for each mosquito type and these genes will be amplified using the PCR reaction. The genes will also be fused to GFP and inserted into the same plasmid mentioned above. The same mutations that resulted in DN forms of the human genes will be inserted into the mosquito gene sequences. These plasmids will also be transfected into the mosquito cells to establish wild-type and dominant negative cell lines.

Specific Aim 3: Elucidate the pathway used for VEEV entry and infection in mosquito cells and what specific proteins are involved and/or necessary.

A chimeric virus encoding the nonstructural proteins of Sindbis virus (SINV) and the structural proteins and capsid of VEEV will be used to study infection in the WT and DN cell lines for the genes mentioned in Aim 2. The plasmids will be transfected into C710 cells and the effects of gene expression and protein production on VEEV infection will be assessed. Fluorescently labeled virus particles will also be made to show colocalization with certain mosquito wild type proteins in the mosquito cells. The fluorescent particles will be added to the mosquito cells established in Aim 2, which may need to be transiently transfected for each infection study. It is expected that the particles will colocalize with some or all of the proteins that are required for endocytosis. In addition, the virus particles will be added to the dominant negative cell lines and it is expected that they will remain on the cell surface in some or all of these cells. These experiments will point out important proteins in the specific endocytic pathway that VEEV uses to enter and infect mosquito cells as well as where the viral genome enters the cell cytoplasm. The cell lines expressing the DN form of the Rab5 gene will block release from the early endocytic vesicle and the cells expressing the DN Rab7 gene will block release from the late endocytic vesicle. Dynamin DN protein should prevent entry and infection if VEEV utilizes a dynamin-dependent pathway to infect mosquito cells.

Introduction

Chapter 1: Alphaviruses

Venezuelan equine encephalitis virus (VEEV) is an enveloped, positive-strand RNA virus that is a member of the alphaviruses, in the *Togaviridae* family. This includes 29 arthropod-borne viruses that have wide geographic distribution and can cause serious human and veterinary disease. The current approved treatment for alphaviral infection is supportive care and there is no specific antiviral therapy available. Alphaviruses have a very broad host range and can infect several different vertebrates and invertebrates. Most human infections result in signs and symptoms that include fever, rash, joint pain and headaches (Peters and M., 1990; Strauss and Strauss, 1994). A subset of alphaviruses is also associated with fatal encephalitis. Alphaviruses have been divided into two groups according to their phylogeny and geographic distribution: Old World and New World alphaviruses. The encephalitic alphaviruses are only found in the New World category and include VEEV, Eastern Equine Encephalitis Virus (EEEV) and Western Equine Encephalitis Virus (WEEV). Members of the Old World alphavirus lineage include Sindbis Virus (SINV) and Semliki Forest Virus (SFV). A phlyogenetic tree showing the relationships between representatives of both Old and New World alphaviruses is shown in Illustration 1. The reasons for this difference in encephalitic pathogenicity between the two lineages points to distinct molecular characteristics in the alphavirus that have yet to be defined in detail (Paredes et al., 2001; Strauss and Strauss, 1994; Weaver et al., 2004b).

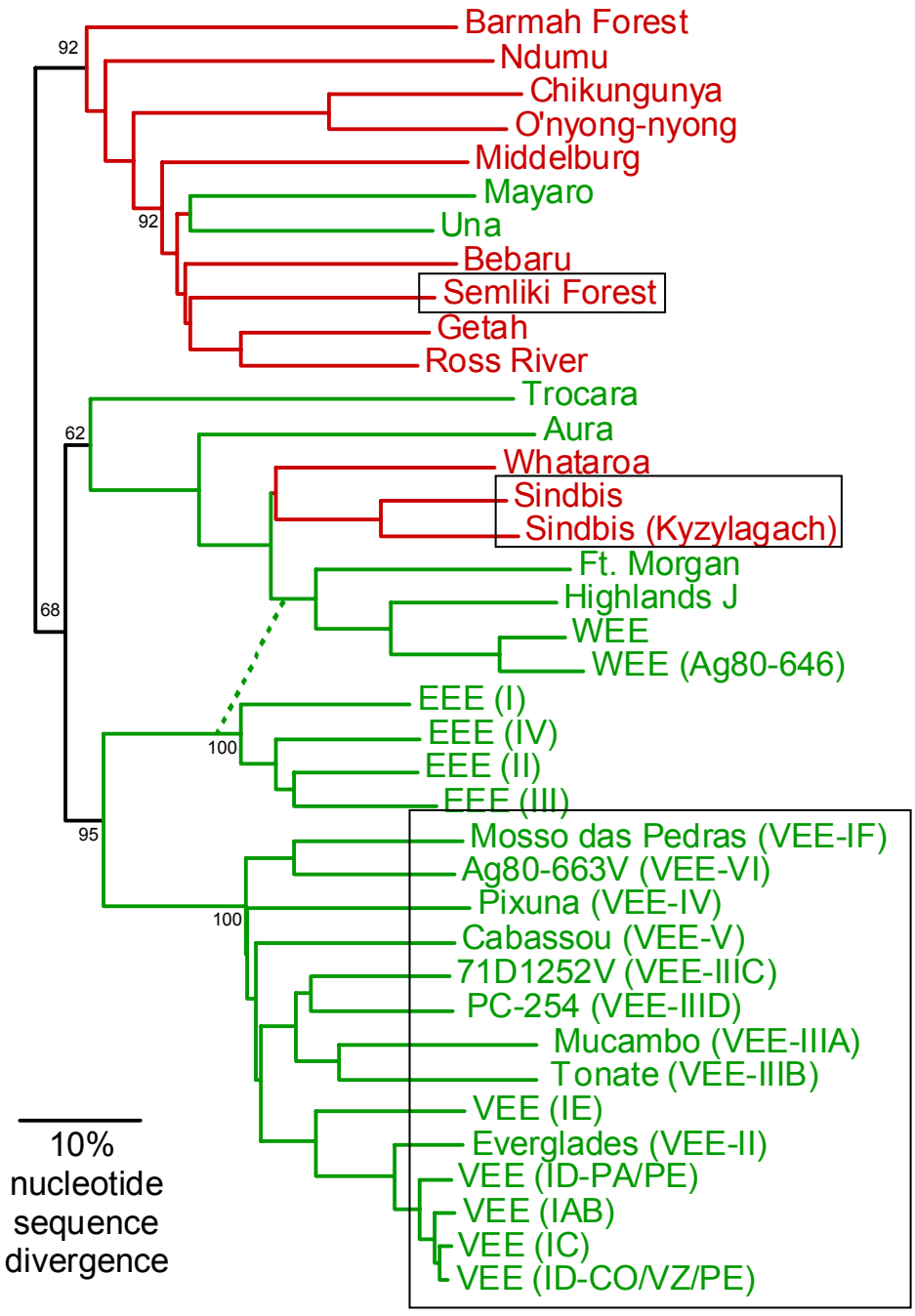


Illustration 1: Alphavirus Phylogenetic Tree

A phylogenetic tree illustrating the relationships between various Old World (red) and New World (green) alphaviruses. The locations of Semliki Forest virus, Sindbis virus and Venezuelan Equine Encephalitis virus (VEEV) complex are highlighted by black boxes. Illustration courtesy of Dr. Scott Weaver, UTMB, Galveston, TX

There have been several outbreaks of VEEV in the past two decades in both South America and Mexico. In 1995, one of the largest VEEV epidemics on record occurred in Venezuela, involving over 75,000 people. These recent outbreaks indicate that the virus is a reemerging pathogen. It is also known that the virus is a highly developed biological weapon. There is no licensed vaccine available for public human use and the concern that VEEV may be used for biological terrorism indicates the need for development of effective antivirals and an improved vaccine. There is a live-attenuated vaccine strain, TC-83, that was created by passaging the virulent subtype IAB strain and this is commonly used to vaccinate equines in the Americas. This experimental vaccine is occasionally used for vaccination of human military or laboratory personnel at risk for VEEV exposure but is often accompanied by a high rate of adverse side-affects as well as failure to seroconvert (Weaver et al., 2004a; Weaver et al., 2004b).

Alphaviruses are maintained in an enzootic cycle in nature between a vertebrate host and a mosquito vector (Illustration 2). They produce a lifelong persistent infection in arthropods while infection with the virus will usually result in an acute, short-lived infection in vertebrates (Strauss and Strauss, 1994). Though alphaviruses can be found worldwide, each specific virus is usually found in one location and utilizes one host in nature (Illustration 3.) VEEV is found in parts of South America, persists in nature within small rodents and is usually transmitted to equines and humans via *Culex* or *Aedes* mosquitoes (Brault et al., 2002; Strauss and Strauss, 1994). VEEV can readily infect cells derived from an *Aedes* mosquito and can infect *Anopheles* mosquitoes, although to a lesser extent. Infection of the mosquito begins with ingestion of a viremic blood meal which will then result in infection of the midgut epithelial cells. This is followed by virus dissemination into secondary tissues including the salivary glands and then salivation during blood feeding results in viral particles passaging through the salivary ducts and into a vertebrate host (Strauss and Strauss, 1994; Weaver et al., 2004b).

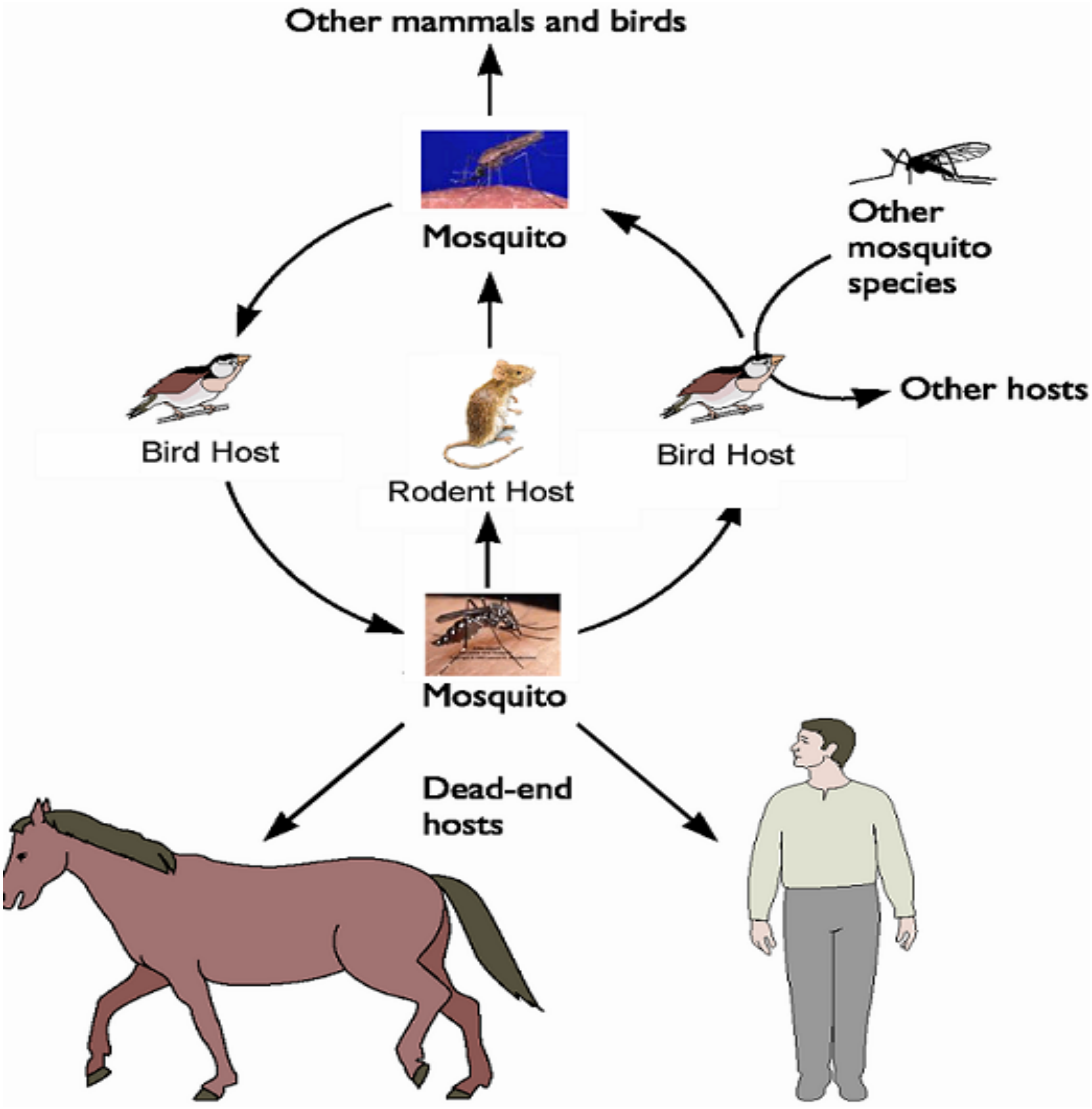


Illustration 2: Alphavirus Host/Vector Cycle

VEEV is transmitted by mosquito vectors and maintained in nature through a rodent host/mosquito vector cycle. The mosquito vectors may also transmit virus to humans and equines, termed dead-end hosts, although VEEV strains IAB and IC can utilize equines for viral spread.

Illustration 3: Geographical Distribution of Select Alphaviruses with Host Animal

The New World virus VEEV is found only in the Americas whereas the Old World viruses such as SINV and SFV are found in Europe, Asia, Africa and Australia. VEEV is maintained in nature in rodent hosts whereas the Old World viruses are maintained in avian hosts.

Chapter 2: Virus Structure, Genome Organization & Protein Function

Alphaviruses are membrane-coated spherical viruses approximately 70 nm in diameter that contain an icosahedral core. The genome consists of a positive-sense, single stranded RNA that is approximately 12 kilobases in length. The genome encodes one open reading frame that is translated into a polyprotein. This is then cleaved by host and virus proteases into the four nonstructural proteins (nsPs). The nsP1 protein functions to create the negative-strand RNA copy of the genome (Wang, Sawicki, and Sawicki, 1991). nsP2 encodes a protease at its C-terminal domain that aids in processing the polyprotein (Ding and Schlesinger, 1989). nsP2 is also involved in the initiation of subgenomic RNA synthesis as well as the regulation of the negative-strand RNA synthesis (Sawicki and Sawicki, 1993; Suopanki et al., 1998). nsP3 is involved in viral genome replication and may function in plus-strand synthesis (De et al., 2003). nsP4 functions as the viral polymerase (Lemm et al., 1998). The next step in the viral lifecycle is the replication of the genome into a negative sense RNA strand. This RNA serves as a template for the production of additional genomic RNA as well as subgenomic RNA. The subgenomic RNA is produced from the 3' end of the minus strand and serves as an mRNA that encodes the four structural proteins of the alphavirus as well as the capsid protein (Paessler et al., 2003; Strauss and Strauss, 1994) (Illustration 4). The nucleocapsid protein contains a serine protease that functions to cleave itself from the structural polyprotein. E3 and 6K serve as signal sequences for the translocation of the two glycoproteins E2 and E1, respectively, and are usually cleaved off by the signalase in the lumen of the endoplasmic reticulum (Strauss and Strauss, 1994). A schematic of the VEEV lifecycle is shown in Illustration 5.

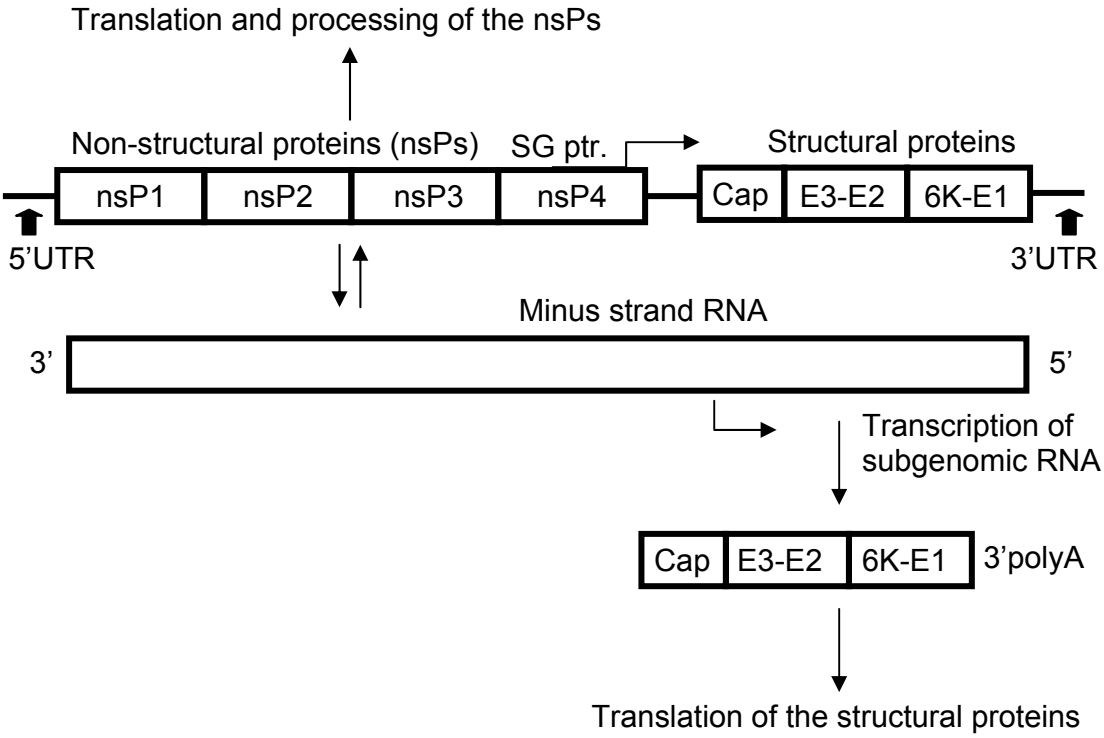


Illustration 4: Organization of the VEEV genome with replication strategy

The VEEV genome is organized into two regions: the four nonstructural proteins (nsPs) are located immediately after the 5' untranslated region (UTR) and the three structural proteins are located immediately after the subgenomic promoter (SG ptr) and just before the 3' untranslated region (UTR). The nsPs are translated directly from the genome while the structural proteins (capsids, E3, E2 and E1) are translated from subgenomic RNA that is made from a minus strand RNA.

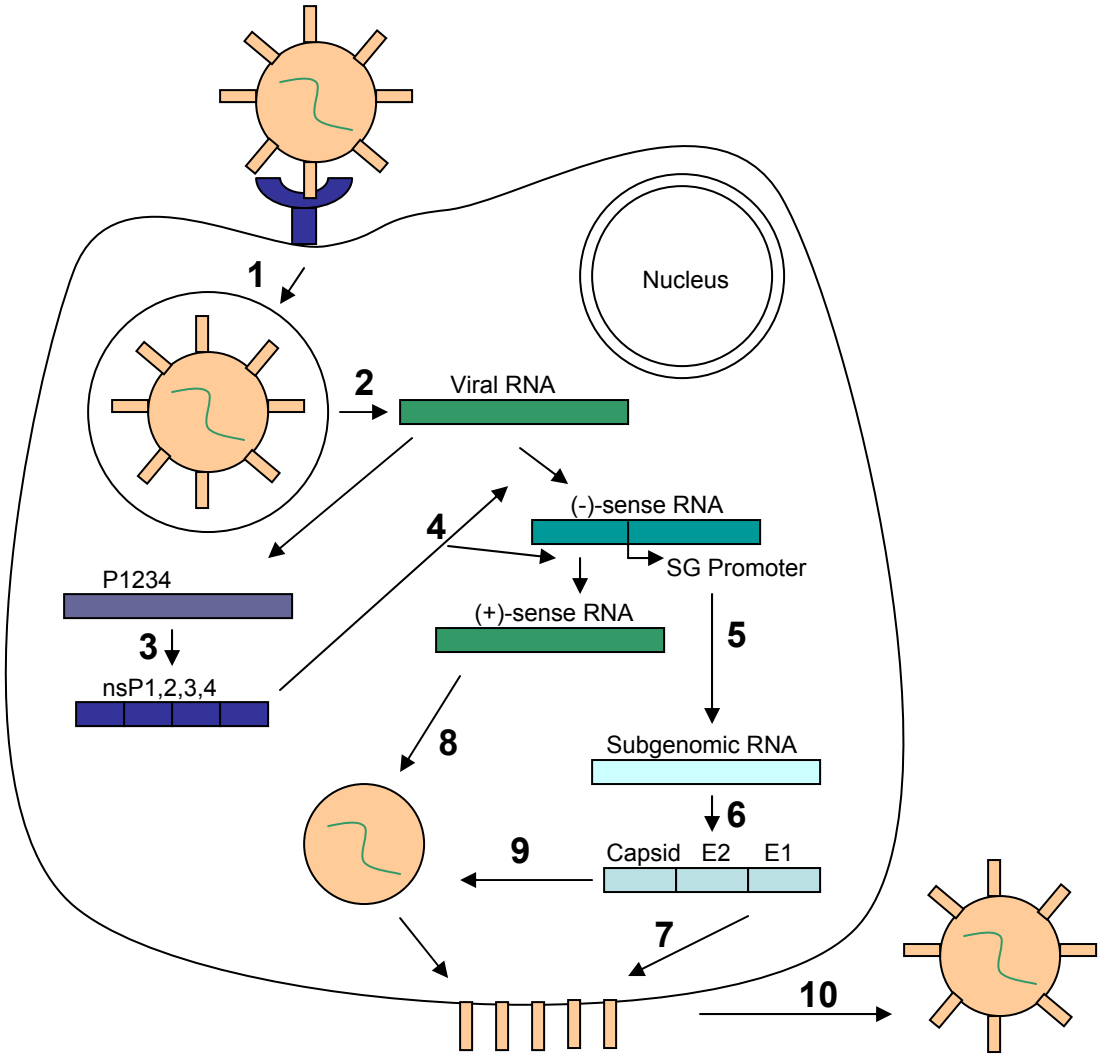


Illustration 5: Lifecycle of VEEV

1) After the virus binds a receptor on the cell surface, it is internalized into an endocytic vesicle. 2) Fusion of the viral membrane with the cellular membrane is triggered by a lowering of pH and the viral RNA genome is released into the cytoplasm. 3) The nonstructural proteins (nsPs) 1-4 are then translated from the viral genomic RNA. 4) nsPs aid in minus-strand RNA synthesis as well as new genomic RNA synthesis. 5) The subgenomic promoter transcribes the subgenomic (SG) RNA from the minus-strand.
6) The structural proteins are translated from the SG RNA. 7) Processed E1 and E2 glycoproteins are embedded in the cell membrane. 8+9) Newly synthesized viral genomic RNA associates with capsid protein and travels to the membrane. 10) New virions bud from the plasma membrane to the extracellular space.

Early biochemical work revealed that generally only three structural proteins are incorporated into a mature virion, and these are the glycoproteins E2 and E1 as well as the capsid protein. The capsid protein coats the viral RNA genome and protects it from degradation in addition to providing the structural core of the mature virion. The viral glycoproteins are embedded in the lipid bilayer that surrounds the nucleocapsid and RNA genome (Strauss and Strauss, 1994). Later, structural studies indicated that the E1 protein lies flat against the host-derived membrane and the E2 protein protrudes from the viron surface in spike-like projections (Mukhopadhyay et al., 2006). Detailed structural analysis by cryoelectron microscopy has also revealed the arrangement of the capsid and envelope proteins as well as the icosohedral structure. The C-terminus of the E2 protein also appears to contact the capsid protein. An example of a VEEV virus particle as well as a cross-section exposing the internal proteins is shown in Illustration 6. In each virion, there are 240 copies of the capsid protein as well as 240 copies of each mature glycoprotein, E2 and E1. The nucleocapsids form in the cell cytoplasm and bud through the cell plasma membrane to acquire the lipid envelope that contains the two viral glycoproteins (Strauss and Strauss, 1994; Strauss and Strauss, 2001; Strauss, Strauss, and Kuhn, 1995). As mentioned earlier, these glycoproteins are made as a single polyprotein, (Capsid-E3-E2-6K-E1), which is cleaved by cellular proteases to release the smaller subunits. The polyprotein production is driven by the 26S subgenomic promoter located just before the gene encoding the polyprotein. The E3 protein acts as a signal sequence for the E2 glycoprotein and 6K acts as a signal sequence for E1 during protein maturation. These proteins assist with the translocation of the glycoproteins within the cell. There is some evidence that in certain cases mature virions may also contain small amounts of E3 protein. The capsid protein is cleaved first and then the signal sequence peptides and their corresponding glycoproteins are cleaved by the cellular proteases. E2 and E1 associate to form a heterodimer and are exported to the plasma membrane through the cellular secretory pathway. These heterodimers form a trimer during either transport or budding and mature virions are coated with these trimers of E2/E1 heterodimers

embedded in the lipid bilayer (Strauss, Strauss, and Kuhn, 1995; Yao, Strauss, and Strauss, 1996).

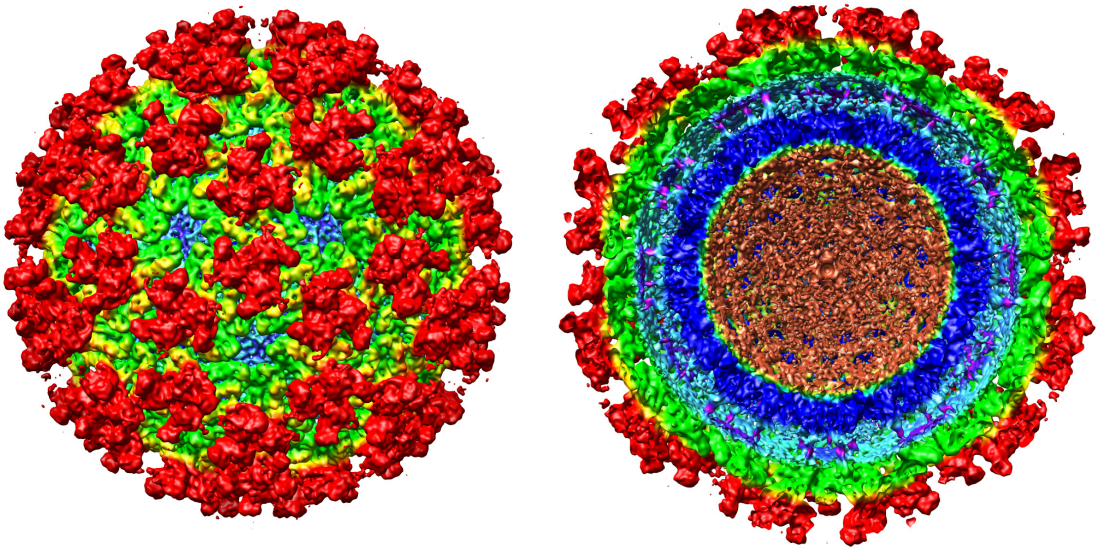


Illustration 6: Surface projection model of VEEV virus particle and cross-section

An example of a VEEV particle (left) along with cross-section of VEEV (right) showing the trimers of heterodimers of E1 & E2 (red), protein skirt region (green), lipid bilayer (light blue) and nucleocapsid (dark blue). Picture courtesy of Dr. Stan Watowich and Dr. Scott Weaver, UTMB, Galveston, TX

Chapter 3: Receptor Binding & Entry

The glycoproteins are the first point of contact the virus has with the cell and the binding of a cellular surface receptor is a crucial step in the ability of these viruses to infect cells (Strauss et al., 1994). Virus enters mammalian cells by first attaching to the cellular surface when the viral glycoprotein E2 binds a cellular receptor. Amino acid changes in the E2 protein can lead to the usage of new receptors, altered host range and altered pathogenicity (Strauss et al., 1994). The region of E2 amino acids from 170 to 220 are thought to be especially important for binding of an alphavirus to a host cell (Strauss and Strauss, 1994). After binding, the virus is generally thought to enter mammalian cells via receptor-mediated endocytosis. Another viral glycoprotein, E1, mediates fusion with the host cell membrane upon activation by low pH. The viral RNA genome is then released into the cytoplasm where replication can begin (Strauss and Strauss, 1994). The E1 protein of alphaviruses is known as a class II fusion protein; these proteins have an internal fusion peptide, contain mostly beta-strand secondary structures and are not predicted to form coiled-coils. This structure allows for faster fusion with the host membrane that is less leaky that fusion mediated by class I proteins (like those found in influenza virus) and is less sensitive to temperature changes (Zaitseva et al., 2005). It is also important to note that only a small portion of activated E1 proteins are necessary to cause the fusion or merging of contacting membrane monolayers; the remaining activated proteins are responsible for the opening and expansion of a fusion pore after lipid mixing (Zaitseva et al., 2005). A schematic of alphavirus-cell membrane fusion is shown in Illustration 7.

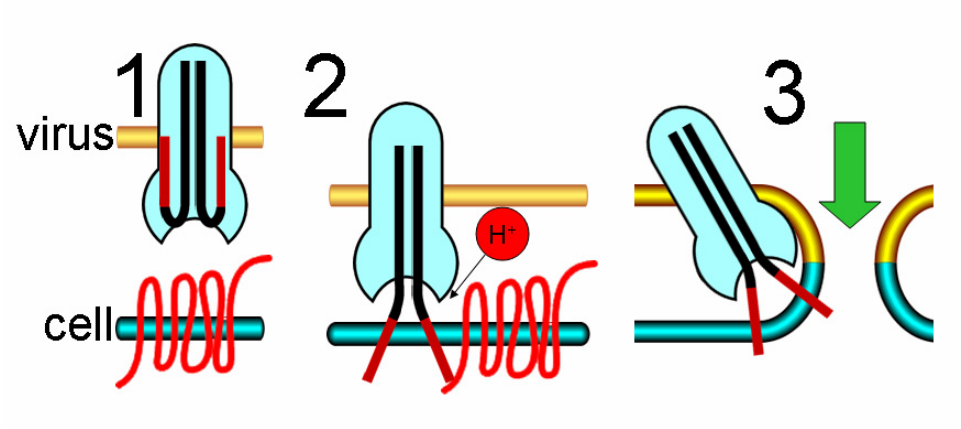


Illustration 7: Viral membrane-cell membrane fusion

Fusion of the alphaviral membrane with a host cell membrane occurs after the virus binds to a cellular receptor (1). The cellular membrane is labeled cell and is in blue while the receptor is in red. The viral membrane is labeled virus and is in tan with the viral glycoprotein in light blue. After the viral E2 protein binds with a cellular receptor, the virus is internalized into an endosome into the interior of the cell. The presence of low pH in the cellular endosome will cause a conformational change in the viral E1 glycoprotein that triggers fusion of the membranes (2). This creates a pore through which the viral RNA genome travels to reach the cellular cytoplasm (3).

Chapter 4: Receptors

Alphaviruses have a wide host range comprised of both invertebrate, arthropod vectors and vertebrate hosts, including birds, rodents, equines and humans, among others. They are also able to infect and replicate in a variety of cell types within their hosts, including neurons, muscle cells, synovial cells, epithelial cells and brown fat cells. These facts have led researchers to question whether the virus is using the same receptor for all cell types and organisms; if this is the case, then this receptor must be expressed on a number of different mosquito, avian and mammalian cells. It is generally accepted that alphaviruses bind to protein receptors; early research with SINV demonstrated that treatment of cells with proteases abolished virus binding but treatment of cells with phospholipases or neuraminidases had little or no effect (Strauss and Strauss, 1994). Charge could also be important for binding to cells; treatment of cells with heparin can increase binding of alphaviruses which could mean that a charge repulsion must be overcome in order for the virus to bind tightly to its receptor (Smith and Tignor, 1980; Strauss et al., 1994). Changes in the amino acid sequence of the alphavirus glycoprotein, which could also increase or decrease charge, may lead to the utilization of different receptor sets. Research on particle competition between SINV and EEEV has suggested that different alphaviruses can use the same receptor. However, since each only partially competed for binding it also suggested that each virus can use more than one receptor type (Smith and Tignor, 1980). Attachment cofactors are thought to be important in enhancing efficient viral binding to cells and it has been shown that lectin molecules may aid in the attachment of alphaviruses. DC-SIGN and L-SIGN are C-type lectins that are highly expressed on the surface of dendritic cells and on some epithelial cells. These lectins may be required for effective infection of mammalian dendritic cells by mosquito-derived alphavirus particles (Klimstra et al., 2003). Carbohydrate modifications of alphaviral glycoproteins could alter the ability of the virus to bind to and enter target cells.

In efforts to identify receptors, antibodies against cell proteins were used to block SINV binding to hamster cells. A monoclonal antibody was identified that inhibited binding by up to 80%. It was discovered that this antibody bound to the high affinity laminin receptor. Transfection of cells with the laminin receptor expression constructs increased virus binding by as much as 4.5 times and antisense RNA to the protein decreased binding. It was concluded that the high affinity laminin receptor is a functional receptor for SINV in mammalian cells (Wang et al., 1992). The protein is highly conserved among mammals and is widely expressed in many cell types. Interestingly, the antibody against this receptor had very little effect on virus binding to chicken cells even though the chicken cells expressed the high affinity laminin receptor and the amino acid sequence is 98% identical to the mammalian protein (Wang et al., 1991) . This suggests that the chicken cell must contain another receptor with higher affinity for the virus and that the broad host range of alphaviruses could be due in part to utilization of more than one receptor in a single cell type.

The question of which receptor the virus was using to gain entry to mosquito cells was the next to be addressed since alphaviruses utilize mosquitoes as their transmission vectors. A direct binding assay was used to find receptors in mosquito cells by probing a blot of cell extracts with radiolabeled VEEV. A 32 kDa protein was identified that expressed on the surface of the mosquito cells and bound to the virus. Both antibodies against the protein as well as antibodies to VEEV E2 (receptor-binding glycoprotein) interfered with viral binding. In competition experiments with the protein, it was found that SINV competed with VEEV for binding but poliovirus did not, suggesting that the protein was fairly specific for alphavirus attachment and entry. The mosquito cell protein cross-reacted with the high affinity laminin receptor in immunological assays and also bound laminin protein. The exact identity of the protein was not determined (Ludwig, Kondig, and Smith, 1996).

Chapter 5: Alphavirus Entry into Mammalian Cells

Once a virus particle attaches itself to the surface of a host cell, it must find a way to deliver its genome to the cellular cytoplasm without damaging the cell and without sending too strong a signal to the immune system. Since the membrane of a cell is not permeable to macromolecules, the virus must utilize cellular machinery to cross the membrane. Viral entry consists of multiple steps which are well orchestrated and may depend on exact timing and location with regards to cellular components. Factors such as interaction with cellular receptors and resulting cellular signaling may also have an impact on the possibility of viral entry and infection (Marsh and Helenius, 1989; Marsh and Helenius, 2006).

Three mechanisms for enveloped virus entry into mammalian cells were proposed from early electron microscopy data: 1) the virus enters via phagocytosis with the aid of cellular microfilaments, 2) the virus enters via a particle-induced mechanism independent of cellular microfilaments or 3) the virus enters via endocytosis in coated vesicles, which is generally considered particle and microfilament-independent (Sieczkarski and Whittaker, 2002a). Four well-known mechanisms of enveloped virus entry into a cell are shown in Illustration 8, including phagocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis and caveolin- and clathrin-independent endocytosis. The first three pathways have been well characterized in recent years while the last pathway is not yet well understood.

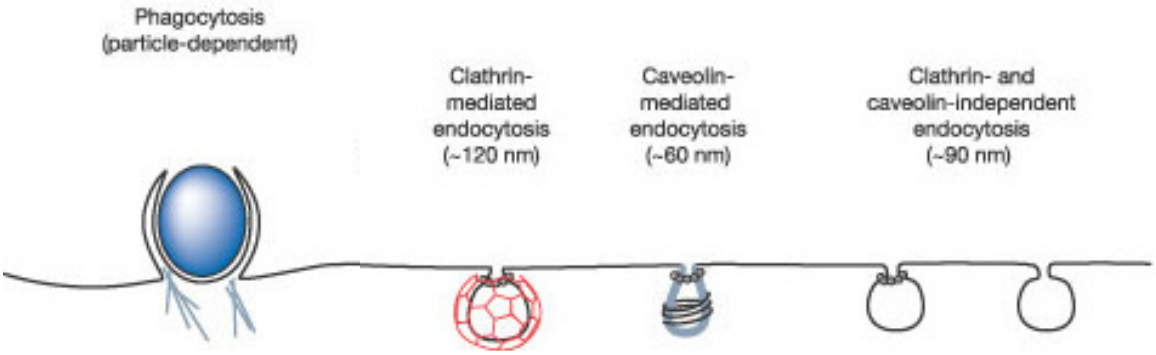


Illustration 8: Examples of various mechanisms of entry into a cell.

Four well-known mechanisms of entry into a cell: phagocytosis, which is particle-dependent, is shown on the far left. This is followed by clathrin-mediated endocytosis, which requires the clathrin protein, and caveolin-mediated endocytosis, which requires the caveolin protein. The final mechanism of entry shown is clathrin- and caveolin-independent endocyotis. The last three methods of entry can only accommodate cargo up to approximately 120, 60 and 90 nanometers in diameter, respectively.

In 1980 Helenius *et al.* found that Semliki Forest virus (SFV), an Old World alphavirus, entered BHK-21 cells via an endocytic pathway. They proposed that the viral membrane must fuse to a lysosomal membrane and this fusion resulted in the release of the viral nucleocapsid into the cell cytosol (Helenius et al., 1980). This group as well as others went on to look at the biochemical requirements of SFV entry and found that virus uptake occured by absorptive endocytosis in a manner similar to the receptor-mediated endocytosis of extracellular proteins. The uptake was rapid, blocked by low temperature, required that the virus bind a cellular receptor and occured via coated pits and coated vesicles (Kielian and Jungerwirth, 1990; Marsh and Helenius, 1980). Endosomal acidification inhibition via chemicals also prevented the entry and infection of SFV by inhibiting the release of the genome into the cytoplasm, presumably through inhibition of fusion (Helenius, Marsh, and White, 1982). These studies and others pointed to the fact that alphaviruses must use endocytosis to enter cells and entry occurs in coated vesicles, but the exact mechanisms and required proteins for virus uptake remained unknown.

In contrast to the work on SFV, research by one group on Sindbis virus consistently suggested that this alphavirus may be able to enter mammalian cells by absorption on the cell surface rather than by utilizing an endocytic pathway. Cassell *et al*. found that inhibitors of endosomal acidification did not block SINV entry into cells but may prevent synthesis of viral RNA (Cassell, Edwards, and Brown, 1984). The same group found that SINV can infect hamster cells defective in endosomal acidification (Edwards and Brown, 1991). This created some controversy as to the mechanism that an alphavirus takes to enter a cell on way to establishing productive infection but it is generally accepted that at least Old World alphaviruses enter mammalian cells via pH-dependent, clathrin-mediated endocytosis.

Once virus uptake has occured by endocytosis, there may be other factors required in order for the viral membrane to fuse with the endocytic vesicle membrane in order to release the nucleocapsid and viral genome into the cytoplasm. For SFV, this fusion was found to require both cholesterol and sphingolipids in the target membrane as well as acidic pH (Lu and Kielian, 2000). The dependence of SFV fusion on both

cholesterol and sphingolipids suggested that microdomains in the membrane known as "lipid rafts" may be involved in the fusion event. Lipid rafts had been shown to play a role in the entry of SV40 virus, which enter cells via caveolae that are rich in lipid rafts. Work with model membranes showed that neither SFV nor SINV required lipid rafts to be present in order for fusion to occur. In addition, sphingolipids and cholesterol found together in lipid raft domains may actually be inhibitory for alphaviral fusion (Waarts, Bittman, and Wilschut, 2002). The role of lipid rafts in alphavirus infection in mammalian cells remains unclear.

Chapter 6: Endocytic Proteins

A. Clathrin

As mentioned earlier, alphaviruses are generally thought to enter mammalian cells via clathrin-mediated endocytosis. In this process, the clathrin proteins form a coat around invaginations at the cell surface by polymerizing over the cytoplasmic face of a region of membrane. Functional clathrin is comprised of both heavy (CHC) and light chain clathrin proteins (of which there are two isoforms, LCa & LCb), where each heavy chain is randomly associated with one light chain in the triskelion (Royle, 2006). The assembly state of the triskelion is thought to be regulated by the light chain. This three-legged structure comprised of three heavy chains and three lights chains is the assembly unit of a clathrin coat (Ybe et al., 1998). The polymerized clathrin coat is a basket-like structure composed of pentagons and hexagons and can easily be visualized in electron micrographs (Heuser, 1980). The coat is formed from clathrin lattices that build along the plasma membrane and coated pits bud laterally through the lattices (Heuser et al., 1987). Clathrin does not link to the plasma membrane directly when forming the coated vesicles but does so via adaptor proteins. These adaptor proteins may act to secure clathrin molecules in close proximity to one another, as new clathrin-coated pits bud from large clathrin patches found on the plasma membrane (Royle, 2006).

B. Dynamin

The assembly of clathrin is thought to initiate vesicle formation. The protein dynamin then polymerizes over the neck of membrane-derived clathrin-coated pits. The hydrolysis of GTP by dynamin drives membrane vesicle scission, pinching off the vesicle from the plasma membrane. This turns the membrane invagination into a clathrin-coated vesicle. Since scission is required for the movement of endocytic vesicles from the cell surface into the cell, the large GTPase dynamin is essential for clathrin-mediated endocytosis (Song, Leonard, and Schmid, 2004). Dynamin is also one of few GTPases that can self-phosphorylate, inducing the formation of dynamin ring structures via the self-assembly of many dynamin molecules (Shpetner and Vallee, 1992; Warnock, Hinshaw, and Schmid, 1996). It is these ring structures that wrap around the neck of the clathrin-coated membrane invaginations and induce membrane scission, rendering dynamin unique among GTPases in its ability to act as a mechanochemical enzyme (Sweitzer and Hinshaw, 1998; Warnock, Hinshaw, and Schmid, 1996). The hydrolysis of GTP and dynamin binding of GTP in exchange for GDP has been shown to cause dynamin to constrict and vesiculate under physiological conditions. Thus, GTP hydrolysis thought to be the regulating step in the activation of dynamin to close the neck of clathrin-coated pits during endocytosis (Sweitzer and Hinshaw, 1998). Although dynamin can self-assemble through dynamin-dynamin interaction, interaction with microtubules can activate the rate of GTP hydrolysis 16-fold. This supports the mechanochemical role of the enzyme, much like the proteins kinesin and dynein (Shpetner and Vallee, 1992).

There are three forms of mammalian dynamin: dynamin I is found mostly in the brain, dynamin II is ubiquitously expressed and dynamin III is found predominantly in the testes (Liu and Robinson, 1995). The dynamin protein contains several domains that contribute to the GTPase activity, including guanine-binding and hydrolysis. Amino acid mutations in these domains render the protein nonfunctional by either preventing GTP binding or hydrolysis and are termed ‘dominant negative’ mutations. Three well-characterized mutations (K44A, S45N, T65F) render the dynamin protein defective in

GTP hydrolysis and binding and result in impaired endocytosis. There is a strong correlation between the rate of endocytosis and the rates for dynamin assembly-stimulated GTP-hydrolysis (Song, Leonard, and Schmid, 2004). A complex relationship most likely exists between this cycle of GTP binding and hydrolysis and the role of dynamin in endocytic clathrin-coated vesicle formation. Overexpression of the mutant dynamin proteins will result in the accumulation of distinct intermediates along the pathway of clathrin-coated pit and vesicle formation (Damke et al., 2001). These mutations in the GTP-binding domains also alter the distribution of plasma membrane- but not Golgi-derived clathrin assembly proteins such as alpha-adaptin and clathrin (Herskovits et al., 1993). Both of these observations suggest that dynamin plays a major role in the early steps along the clathrin-mediated endocytic pathway at the plasma membrane.

C. Actin

There is increasing evidence that dynamin may act together with the actin cytoskeleton to coordinate its function as a mechanochemical enzyme during endocytosis. It has been known for some time that actin is an important participant in endocytosis in yeast but this was thought to be a specialized function in yeast and not in mammalian cells. Recently it has become increasingly clear that the role of actin in endocytosis is most likely evolutionarily conserved (Smythe and Ayscough, 2006). Invagination of clathrin-coated pits occurs immediately following a burst of dynamin recruitment to the invaginations followed by transient actin assembly. This indicated that dynamin may trigger actin polymerization or act as an upstream factor that regulates polymerization at the plasma membrane where coated pits are forming (Mousavi et al., 2004; Zhu et al., 2005). In addition, several actin cytoskeleton-associated proteins have been found to interact with dynamin in cells including profiling, syndapin, mAbp1, intersectin and cortactin (Cao et al., 2003; Yarar, Waterman-Storer, and Schmid, 2005). Cortactin assembles over the surface of clathrin lattices as well as along actin filaments associated with pits and was found to play an important role in endocytosis. Dynamin was found to colocalize with cortactin only in areas where active actin assembly was occurring. The interaction of dynamin with cortactin at actin branching sites is most likely responsible for the recruitment of dynamin to clathrin-coated vesicles (Cao et al., 2003). One proposed model is that after the formation of a clathrin-coated pit, dynamin is recruited to the neck of the pit where it is oligomerized into a ring structure. At the same time, the ligand-receptor interaction that triggered the pit formation also triggers assembly of actin resulting in a tight assembly of actin and cortactin at a branched site along the neck of the pit. The elongation of actin filaments along with the interaction of cortactin and dynamin along this neck will result in the movement of the vesicle away from the cell surface and eventually end in separation of the vesicle from the membrane (Zhu et al., 2005).

In addition to the proposed mechanical role, actin is also thought to have an important structural role in clathrin-mediated endocytosis, controlling the localization of clathrin and other endocytic machinery on the plasma membrane. Actin cytoskeletal assembly and disassembly may have critical effects on clathrin-coated pit formation, lateral movement and internalization (Yarar, Waterman-Storer, and Schmid, 2005). The formation of new clathrin-coated pits requires the assembly of accessory proteins on the plasma membrane and the actin cytoskeleton acts as a scaffold to ensure close proximity of these proteins (Qualmann, Kessels, and Kelly, 2000). Latrunculin A, a drug that binds actin filaments and prevents further elongation, completely blocks endocytosis (Ayscough et al., 1997). Disrupting the actin cytoskeleton or inhibiting polymerization may therefore interfere with scaffolding assembly or other functions of actin during endocytosis and prevent the assembly of proteins necessary for new clathrin-coated pit and vesicle formation.

D. Rab Proteins

The endocytic pathway that a virus takes into a cell may consist of a variety of vesicles and specific endosome populations. Rab proteins are small GTPases that target the movement of endocytic vesicles and determine endosome specificity by their localization. They organize effector proteins into membrane subdomains and direct intracellular transport. Rab5 regulates the functions of early endocytic vesicles whereas Rab7 is found in late endosomes. Cargo that is first localized in Rab5 domains on early endosomes can later be found in Rab7 domains on late endosomes. As endosomes migrate from the cell surface inward with their cargo, they become larger through fusion and Rab5 is quickly replaced with Rab7. Thus, the Rab proteins control cargo transport between endosomes as well as endosome maturation by their presence on endosomal membranes (Rink et al., 2005).

D1. Rab5

There are three isoforms of mammalian Rab5 and they are thought to act cooperatively to regulate endocytosis. Increased levels of Rab5 increase both early endosome fusion and the rate of endocytosis; decreased levels will decrease endocytosis. This and other observations have led to the opinion that the GTPase activity of Rab5 is a rate-limiting factor along the endocytic pathway (Bucci et al., 1992). Mutations in any of the GTP-binding domains of Rab5 will lead to altered function of the protein. Two well-characterized mutations of the Rab5 protein are Q79L, leading to a constitutively active (CA) phenotype, and S34N, leading to a dominant negative (DN) phenotype. The CA mutant will result in large early endosomes, indicative of the protein having an enhancing effect on early endosome fusion. Endocytosis in these mutant cells will often result in an accumulation of protein in these enlarged endosomes. The DN mutant has an opposite inhibitory effect on endosome fusion and blocks endocytic uptake of ligands from the plasma membrane (Bucci et al., 1992). The level of Rab5 protein fluctuates on separate

early endosomal membranes and fusion/fission events along with GTP-hydrolysis link these fluctuations (Rink et al., 2005). Rab5 is also thought to act at the level of clathrin-vesicle internalization, as overexpression of GDP-bound Rab5 will delay receptor endocytosis by retaining receptors in clathrin-coated pits at the plasma membrane (Galperin and Sorkin, 2003).

D2. Rab7

The Rab7 protein is a GTPase very similar to Rab5 yet it is known to function in a later step along the endocytic pathway compared to the Rab5 protein. It has a distinct role in regulating transport from early to late endosomes, from endosomes to lysosomes and also plays an important part in late endosome organization (Bucci et al., 2000; Feng et al., 2001; Vitelli et al., 1997). Evidence that Rab7 functions in the late steps of endocytosis has come from the use of a mutant protein, T22N, which acts as a dominant negative much like the Rab5 S34N protein. Expression of this DN mutant does not effect transferrin internalization, which should only be inhibited by blocking early steps of endocytosis such as the Rab5 proteins since it is recycled back to the plasma membrane from the early endosome. A study using the endocytic marker LDL showed that the DN Rab7 protein inhibited the degradation of internalized protein while the initial internalization rate remained unchanged (Vitelli et al., 1997). This data suggested that Rab7 played a fundamental role in regulating endocytosis at the level of late endosomes and could be by controlling fusion, much like Rab5 and its function in early endosomes (Vitelli et al., 1997). Another study using the glycoprotein of vesicular stomatitis virus (VSV) as a transport marker showed that overexpression of the DN protein resulted in accumulation of protein in early endosomes while expression of the WT Rab7 did not. In addition, it was found that internalization of horseradish peroxidase (another endocytic marker) was unchanged in cells expressing DN Rab7, indicating that the Rab7 protein did not affect early steps in endocytosis (Feng, Press, and Wandinger-Ness, 1995). Rab7 is also thought to be an important protein in lysosome biogenesis from the late endosome. There are continuous fusion events between late endosomes and lysosomes and Rab7 has

been shown to be a key regulatory protein for these events as well as for the maintenance of the lysosomal compartment. In the absence of functional Rab7, lysosomes become dispersed while overexpression of Rab7 results in the accumulation of large endocytic structures near the perinuclear region. This would suggest that Rab7 also acts to regulate the aggregation and/or fusion of late endocytic vesicles/lysosomes (Bucci et al., 2000).

Chapter 7: Involvement of Endocytic Proteins in Viral Infection

Endocytosis offers many advantages to a virus, including being transported inside the host cell bypassing many barriers present at the cell surface while at the same time not leaving behind any proteins that may elicit an immune response. In addition, viruses that require low pH for membrane fusion can use the decreasing pH of the endosomal pathway as a cue for uncoating at specific time points or locations (Marsh and Helenius, 2006). Since many viruses gain access to the cell via endocytosis, endocytic proteins play an important role in virus infection. In 1998, DeTulleo *et al.* explored whether SFV entered HeLa cells through the clathrin-mediated pathway. They used cells expressing a dominant negative form of dynamin, which prevents the formation of clathrin-coated pits and vesicles by inhibiting the 'pinching-off' stage of vesicle formation. SFV as well as Sindbis virus (SINV), another alphavirus, required an active dynamin-dependent and clathrin-dependent endocytic pathway for productive infection (DeTulleo and Kirchhausen, 1998). This was clear evidence that blocking a protein-specific trafficking pathway could inhibit alphaviral infection in mammalian cells. SFV fusion with the cellular membrane occurs at a pH of approximately 6.2, which can be found in early endosomes within the cell (White and Helenius, 1980). This led to the conclusion that SFV fusion occurs at the early endosome. In 2003, Sieczkarski *et al.* used dominant-negative forms of Rab5 and Rab7 to explore the endosome populations required for SFV infection via the endocytic pathway. Results of these experiments showed that Rab5 is required for SFV infection but Rab7 is not, suggesting that SFV infection requires only early endosome function (Sieczkarski and Whittaker, 2003). Interestingly, studies with green fluorescent protein-tagged Rab5 and Rab7 showed that SFV transport occurred to Rab7 positive vesicles. Vonderheit *et al.* observed virus transport by confocal microscopy and saw that SFV was transferred from an early endosome that contained both Rab5 and Rab7 to a vesicle that contained only Rab7. They also observed that Rab7 and SFV were transferred together and that labeled transferrin as well as Rab5 was left behind in the early endosome (Vonderheit and Helenius, 2005). This data along with the

dominant-negative data suggests that Rab7 may not be required for infection but that SFV will associate with this protein if it is present during transport to late endosomes.

Other viruses have been shown to require only Rab5, only Rab7 or both GTPases to be functionally present on their respective endosomal vesicles in order to enter and productively infect cells. Using dominant negative mutants of both Rab5 and Rab7, it was found that influenza virus must access both early and late endosomes before fusion can occur and infection can be established. In contrast, another enveloped virus, vesicular stomatitis virus (VSV), was shown to require a functional Rab5 protein but infection was not inhibited by dominant negative Rab7 protein. This suggested that VSV must only access the early endosome when infecting cells and that fusion may occur at a higher pH than with influenza virus, since pH drops as the endosomes mature from early to late (Sieczkarski and Whittaker, 2003; Zerial and Stenmark, 1993). These data were in agreement with each viral glycoprotein's fusion abilities with respect to pH; the VSV G protein fuses at a pH of 5.6-6.3 while the HA glycoprotein of influenza is thought to undergo fusion at a pH of 5.5 (Sieczkarski and Whittaker, 2003). Entry of two flaviviruses, West Nile virus (WNV) and dengue virus, was also found to require functional Rab5 but not Rab7 protein. This would suggest that flaviviruses must access an early endosome in order to establish infection but not a late endosome. By use of chemical inhibitors, both viruses were also found to require endosomal acidification as well as functional clathrin-mediated endocytic pathway (Krishnan et al., 2007). Interestingly, HIV-1 also requires functional Rab proteins for infection yet does not require a functional clathrin-mediated endocytic pathway for entry. It was found that dominant negative Rab5 expression reduced infection with HIV-1 but did not have an effect on internalization. In addition, dominant negative Rab7 almost completely blocked HIV-1 gene expression yet once again did not have an effect on internalization of the virus. The conclusion was that the Rab proteins must be playing a later role in infection beyond entry, such as having a part in the replication of the virus at some point beyond the late endosome (Vidricaire and Tremblay, 2005).

Functional dynamin has been found to be required in order for a number of viruses to enter and infect cells. The dominant negative dynamin mutant K44A has been used to show that two alphaviruses, SFV and SINV, utilize this protein to gain access to cells and establish infection (DeTulleo and Kirchhausen, 1998). Other viruses that require dynamin for infection are human rhinovirus 14, adenovirus, parvoviruses and influenza virus (Bartlett, Wilcher, and Samulski, 2000; DeTulleo and Kirchhausen, 1998; Roy et al., 2000; Wang et al., 1998). It is interesting to note that for one retrovirus, avian leukosis virus, infection is greatly reduced by the expression of DN dynamin yet the protein has no effect at all on the ability of another retrovirus, murine leukemia virus, to enter and infect cells (Lee, Zhao, and Anderson, 1999; Mothes et al., 2000). This suggests that related viruses can have different needs for dynamin to productively infect cells.

To determine if clathrin is required for virus entry and infection, a mutant form of a clathrin accessory protein, Eps15, is often used. Eps15 plays an important early role in clathrin-mediated endocytosis and is constitutively associated with the AP-2 adaptor protein. Deleting a portion of the gene known as an EH (Eps15 homology) domain results in a dominant-negative protein that inhibits this pathway (Benmerah et al., 1999). Sun *et al.* looked at whether or not VSV used a clathrin-dependent endocytic pathway by using a dominant-negative form of the Eps15 gene. Expression of the dominant-negative protein inhibited transferrin internalization, which is indicative of inhibition of clathrin-dependent endocytosis. Dominant-negative (DN) Eps15 also inhibited VSV infection. Furthermore, RNAi suppression of the clathrin heavy chain was used to block clathrin mediated endocytosis before infecting cells with VSV. The RNAi suppression reduced VSV infection to 35% of the control infection with normal clathrin expression (Sun et al., 2005). Taken together, these results indicated that VSV enters mammalian cells via a clathrin-mediated endocytic route.

Others have used the same dominant-negative Eps15 to show that influenza A virus does not have to enter mammalian cells via a clathrin-dependent pathway. They concluded that influenza may infect cells via a non-clathrin, non-caveolae-dependent

endocytic pathway in addition to the traditional clathrin-dependent route. That evidence pointed to the fact that other viruses may also enter cells via several routes depending on receptor-binding or cell type (Sieczkarski and Whittaker, 2002b). WNV was also shown to enter mammalian cells via clathrin-mediated endocytosis by utilizing the DN Eps15 protein. Viral particles were observed to attach and accumulate on the plasma membrane but failed to enter in cells transfected with the mutant protein. In cells expressing control GFP, a great deal of virus was internalized to the cytoplasm. This provided strong evidence that WNV enters cells via a clathrin-mediated pathway (Chu and Ng, 2004). The requirements for endocytosis and functional clathrin for entry and infection of Hepatitis C virus (HCV) was examined using siRNA against the heavy chain of the clathrin protein. A reduction in infection of about 80% was seen in cells transfected with this siRNA when compared with control cells. This suggested that HCV entered cells via clathrin-mediated endocytosis. The results were confirmed using the drugs chlorpromazine, an inhibitor of the clathrin-mediated pathway, and chloroquine, an inhibitor of endosomal acidification, both which also greatly reduced viral infection (Blanchard et al., 2006). A diagram showing the use of endocytic proteins along the steps of cellular trafficking during virus entry is shown in Illustration 9. Once the virus binds a cellular receptor, both virus and receptor are internalized into an invagination that then becomes a cellular vesicle, which will then mature along the endocytic pathway with the aid of various proteins. Illustration 10 demonstrates at which step along the endocytic pathway two viruses, SFV and influenza, are known to fuse with cellular membranes and release their genomes into the cell. The SFV viral membrane is thought to fuse with the membrane of the early endosome at a pH of approximately 6.2, while influenza will not release its genome into the cytoplasm until the late endosome after reaching a pH of approximately 5.5 (Sieczkarski and Whittaker, 2002a).

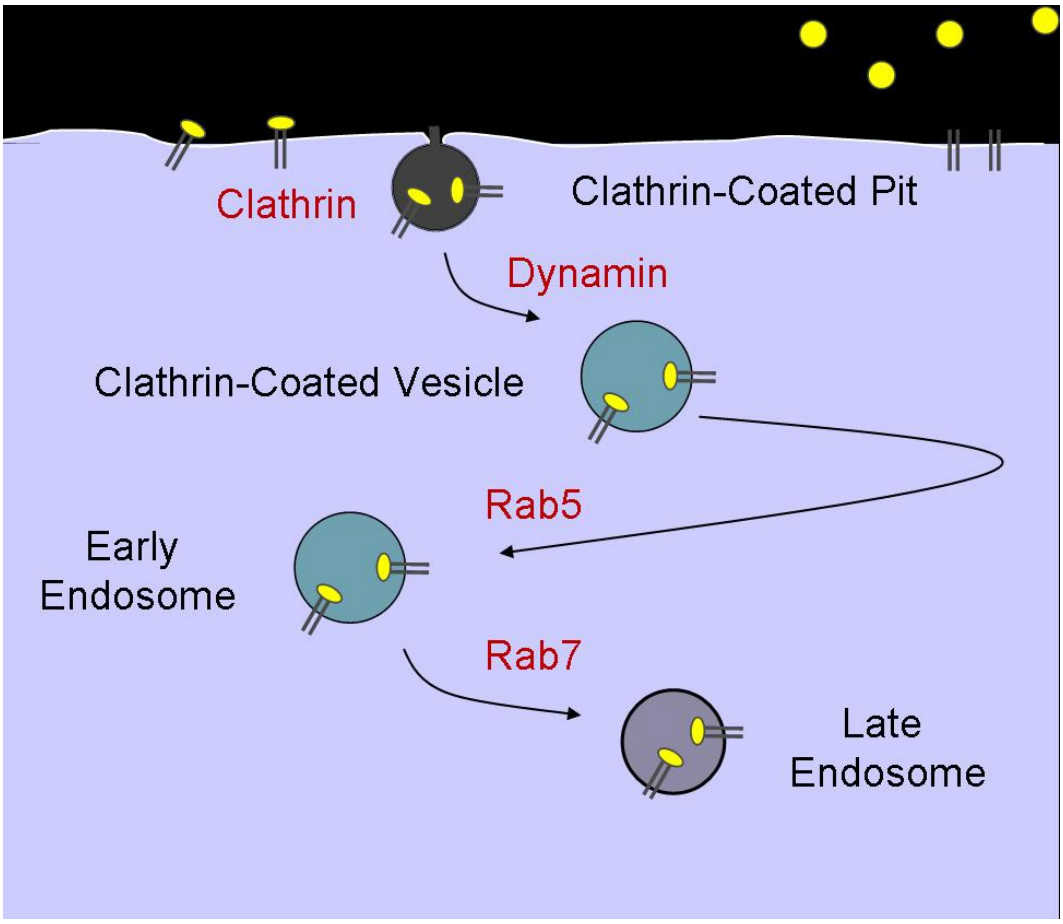


Illustration 9: Use of endocytic proteins in virus entry.

Endocytic proteins are named in red and are placed along the endocytic pathway at the location where they function in the cell. Endocytic compartments are named in black. Virus is represented in yellow; also shown with receptor (double black lines).

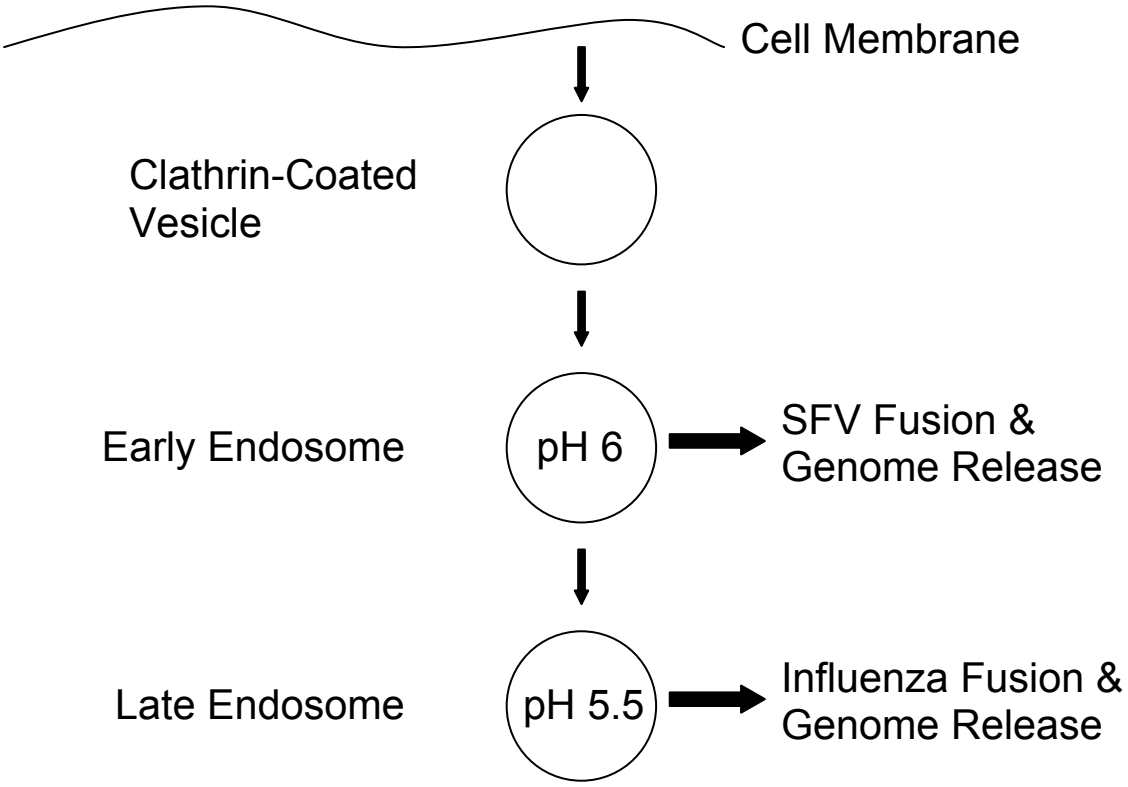


Illustration 10: Endosomal pH and the release of viral genomes

Viruses exit the endocytic pathway at different points depending on what pH is required for fusion of the viral membrane with the cellular membrane. SFV fuses with the membrane of the early endosome at a pH of approximately 6.2 and influenza virus fuses with the membrane of the late endosome at a pH of approximately 5.5.

Chapter 8: Role of the Cytoskeleton in Viral Infection

A virus will utilize the host actin and microtubule systems for several steps during its lifecycle, including attachment, internalization, endocytosis, replication, transport, assembly and cell-to-cell spread (Radtke, Dohner, and Sodeik, 2006). The ability of a virus to enter and exit the cell is a very important aspect of its life cycle and many viruses have evolved mechanisms to use elements of the host cytoskeleton, such as actin polymerization, to aid in these movements (Frischknecht and Way, 2001). Viruses often induce rearrangement of cytoskeletal proteins to move aside barriers or so they can utilize the filaments to assist with movement through the cell. Infectious particles of many viruses also actually contain components of the cytoskeleton such as actin (Radtke, Dohner, and Sodeik, 2006). Though many viruses utilize the host cytoskeleton for entry and infection, requirements for the various proteins involved may differ. Vaccinia virus is the only virus currently thought to use actin-powered motility to aid in cell-to-cell spread. By mimicking components of a tyrosine-kinase receptor signal transduction pathway, vaccinia is able to induce actin tails to achieve actin-based motility (Frischknecht et al., 1999). Vaccinia also utilizes microtubules within the cell for expression of mRNA (Mallardo, Schleich, and Krijnse Locker, 2001) and for intracellular enveloped virus transport to the cellular membrane (Ward and Moss, 2001). Movement of cell-associated enveloped vaccinia (CEV) that is bound to the cell surface is entirely actin-based and polymerization is required for viral travel along the cell (Rietdorf et al., 2001). These CEVs can often be seen on the tips of filopodia and are thought to arrive there by actin-based movement. This process may be responsible for cell-to-cell transmission of infectious virus (Rietdorf et al., 2001; Ward and Moss, 2001). Human parainfluenza virus RNA is replicated in association with ribonucleoproteins bound to actin filaments in order to replicate and actin depolymerization will inihibit viral RNA synthesis (Gupta et al., 1998). Influenza virus enters cells via receptor-mediated endocytosis after binding a sialic acid receptor (Marsh and Helenius, 1989). Actin dynamics are necessary for influenza virus entry into polarized epithelial cells but disruption of the cytoskeleton does not affect entry or infection of non-polarized cells

(Sun and Whittaker, 2007). The actin cortex maintains the structure of lipid rafts and therefore actin polymerization is also required for influenza virus budding since the virus incorporates lipid rafts into the viral envelope (Simpson-Holley et al., 2002).

Respiratory syncytial virus (RSV) requires both actin and microtubules in order to productively infect cells, with both proteins cooperatively aiding in replication and microtubules having a large role in the production of cell-associated virus (Kallewaard, Bowen, and Crowe, 2005). Actin and the actin-binding protein profilin are both required for transcription of RSV RNA and actin also plays a dominant role in the release of RSV particles to the outside of the cell (Burke et al., 2000; Kallewaard, Bowen, and Crowe, 2005). Murine leukemia virus (MLV) enters NIH 3T3 fibroblasts via an endocytic route while the virus enters rat XC cells at the cell surface. This difference in entry mechanisms provided a unique opportunity for researchers to distinguish which proteins may be linked to a specific route of entry for the one virus. They found that MLV entry via endocytosis required both functional actin and microtubules while entry at the cell surface required actin only (Kizhatil and Albritton, 1997). HIV is known to require actin for entry and disruption of the cytoskeletal network will reduce infection significantly, presumably due to actin-dependent receptor colocalization at the cell surface (Iyengar, Hildreth, and Schwartz, 1998). Imaging of viral particles with GFP fused to the Vpr protein revealed HIV moving along microtubules immediately following entry and infection of a cell (Chicurel, 2000). HIV also requires actin to exit the cell since actin depolymerization will significantly inhibit viral budding (Sasaki et al., 1995). Retroviruses are also known to surf along filopodia towards the cell body before internalization. Viral particles pseudotyped with vesicular stomatitis viral glycoproteins surf along filopodia in a similar manner but will continue to move along the plasma membrane until reaching a clathrin-containing region (Lehmann et al., 2005). During surfing, viral particles are thought to be coupled to an actin filament inside the filopodium through the cytosolic domain of its receptor. They can then be pulled down to the base of the filopodium in order to gain access to the appropriate region of the cell surface for internalization (Jay, 2000). The movement of clathrin-coated pits along the

cell membrane is increased upon actin depolymerization and actin may act as a scaffold to hold the pits in place (Yarar, Waterman-Storer, and Schmid, 2005). Many viruses have taken advantage of this function of actin and utilize the filaments to both as guides to reach the areas of endocytic activity as well as to aid them in keeping the necessary components in place until the virus arrives.

Entry of an Ebola virus glycoprotein-pseudotyped retrovirus is inhibited by the destabilization of actin filaments by cytochalasin D, suggesting that Ebola virus requires activated actin for ingress into the cell. The same Ebola pseudotype was used to show that the virus is transported inside vesicles along microtubules, another type of cellular filament, until reaching the microtubule organizing center (Yonezawa, Cavrois, and Greene, 2005). Influenza virus is also thought to move along microtubules immediately upon entering a cell (Lakadamyali et al., 2003). Microtubules are required for the movement of herpes simplex virus type 1 (HSV-1) capsids from the cell surface to the nucleus. During this microtubule-based retrograde transport, the capsids colocalize with microtubules and microtubule-disrupting drugs reduce transport (Sodeik, Ebersold, and Helenius, 1997). Herpes simplex virus is also thought to require actin polymerization as well as functional mysosin, another component of the cellular cytoskeleton, in order for its capsid protein to travel to the nuclear membrane for primary budding (Forest, Barnard, and Baines, 2005).

Chapter 9: Viral Entry and Infection: Mosquito Cells

While endocytosis in mammalian cells has been well studied, much less is known about these processes in the cells of the arthropod vectors of arboviruses, especially the role endocytosis plays in viral entry and infection. Since mosquitoes serve as viral vectors in nature for a great deal of viruses, including alphaviruses and flaviviruses, it is important to understand both viral infection of mosquito cells as well as the molecular mechanisms of these cells. Since VSV can readily infect many different cell lines, it was a good virus to use in the exploration of enveloped-virus entry into mosquito cells. In 1987 Superti *et al.* concluded that VSV enters mosquito cells via absorptive endocytosis in a similar manner as entry into mammalian cells. Ammonium chloride (NH_4Cl)was used to raise lysosomal pH and was shown to block VSV infection in *Aedes albopictus* mosquito larvae cells. They also saw VSV entering these cells in coated pits and vesicles via electron microscopy (Superti et al., 1987). California encephalitis virus (CEV), a member of the bunyavirus family that is transmitted by *Aedes* mosquito vectors, was also shown to enter mosquito cells via an endocytic pathway that was inhibited by the addition of NH_4Cl, which prevented the release of virus from endosomes. The CEV G1 envelope protein was shown to undergo conformational changes upon exposure to low pH that may be necessary for fusion and nucleocapsid entry into cell cytoplasm (Hacker and Hardy, 1997).

Hase *et al.* looked at flavivirus infection of C6/36 cells, which are also derived from *Aedes albopictus* mosquito larvae. They observed both Japanese encephalitis virus (JEV) and dengue-2 viruses entering the cells through the plasma membrane by electron microscopy and concluded that, in contrast to VSV, both of these viruses entered at the cell surface without a need for endocytosis. They did not observe the formation of coated pits at the virion attachment site and saw no evidence of viral entry by receptor-mediated endocytosis (Hase, Summers, and Eckels, 1989). Hase *et al.* then looked at SFV entry into mosquito cells and concluded that, unlike the flaviviruses, the alphavirus entered via receptor-mediated endocytosis at physiological pH. After lowering the pH to 5.8, SFV was seen entering the cells at the surface via the plasma membrane. They concluded that

the SFV fusion protein must be inactive at physiological pH and needs a drop in pH to become active, while the JEV fusion protein is active at physiological pH and can fuse directly to the plasma membrane (Hase, Summers, and Cohen, 1989). Since these experiments were done with electron microscopy, it was unknown if the virus observed fusing with the cell membrane was that which produced infection and it was generally not accepted that these viruses entered the mosquito cells at the surface only. In 1998, JEV entry into mosquito cells was reexamined by looking at the effects of bafilomycin A1, which is a specific inhibitor of vacuolar type H+-ATPase that prevents vesicles from reaching their usual low pH. The results of these experiments suggested that JE enters mosquito cells via an endocytic pathway that involves intracellular acidic compartments and this entry can be inhibited by the addition of bafilomycin A1 (Nawa, 1998).

Recently, the entry of WNV, a medically important flavivirus, into mosquito cells was examined using both chemical inhibitors as well as mutant human proteins, which seemed to function in the mosquito cells in a manner similar to their mammalian function. A dominant negative mutant form of Eps15 (an adaptor protein which blocks clathrin-coated pit formation) was used to show that WNV enters mosquito cells via a clathrin-mediated endocytic pathway. Specific blocking antibodies against clathrin as well as inhibitory drugs were also used to confirm that the virus requires the participation of clathrin as well as functional endocytic machinery in order to establish productive infection in mosquito cells (Chu, Leong, and Ng, 2006). These experiments showed that both the clathrin-mediated endocytosis as well as a low-pH step required for membrane fusion during WNV entry is conserved in both mammalian and insect hosts. However, the endocytic pathways of mosquito cells are not well understood and the consequences of overexpressing human protein in these cells were not fully examined. Therefore, the conclusions of this work may be premature.

In general, virus entry in mosquito cells remains poorly understood and little is known about the infection mechanisms of pathogenic arboviruses. In addition, the entry pathway of alphaviruses into mosquito cells needs to be characterized with respect to specific proteins and endocytic processes. Furthermore, electron microscopy alone can

not be used alone to define the productive infection pathway. As in mammalian cells, the proteins required for productive infection in mosquito cells along these pathways need to be isolated and their specific roles elucidated. In 2003, Mizutani *et al.* detected a JNK-like protein in C6/36 cells. JNK is a mitogen-activated protein kinase that responds to extracellular stimulation and stress. The mosquito cell JNK-like protein was shown to be activated in response to bacteria as well as LPS and this group concluded that it may play an important role in phagocytosis. An inhibitor of JNK inhibited phagocytosis of bacteria and also inhibited acidification of intracellular compartments. Treating mosquito cells with this inhibitor blocked the entry of West Nile virus (WNV), a flavivirus. This suggested that WNV enters cells via a JNK-dependent signaling pathway (Mizutani et al., 2003). It remains to be seen what other signaling pathways and proteins are important for virus entry and infection into mosquito cells and is the subject of the work herein.

Chapter 10: Alphavirus Infection of Mosquitoes

Although the present work addresses the role of mosquito proteins in alphaviral entry and infection in cells, it is important to keep in mind the mechanisms involved in virus infection in the mosquito itself. The first step in viral infection of the mosquito is the ingestion of a viremic blood meal, which will then result in infection of the midgut epithelial cells. Infection of the midgut is followed by virus dissemination into secondary tissues, including the salivary glands. Salivation during blood feeding results in viral particles passaging through the salivary ducts and into a vertebrate host (Strauss and Strauss, 1994; Weaver et al., 2004b). Much of the research investigating alphaviral infection of mosquitoes has been with western equine encephalitis virus (WEEV). Using this virus, it has been shown that there are natural barriers to alphavirus infection in certain species of live mosquitoes (Hardy et al., 1983; Houk et al., 1986; Kramer et al., 1981). Researchers have shown that there is a mesenteronal infection (MI) barrier in mosquito species that are refractory for infection with WEEV. When the virus was injected directly into the hemocoel of these refractory mosquitoes, they then became susceptible to infection (Kramer et al., 1989). This MI barrier is thought to be due to the inability of the virus to penetrate into the midgut epithelial cells. Without midgut infection, the virus can not disseminate into secondary tissues and can not be transmitted to a new host (Kramer et al., 1989; Weaver et al., 2004b). It was demonstrated that there is a significant difference in viral binding to epithelial cell brush borders from susceptible versus refractory mosquito species. Viral binding to susceptible mosquito cell brush borders was specific while the binding to the brush borders of epithelial cells from refractory mosquito species was nonspecific (Houk et al., 1990). This may indicate that the MI infection barrier could be due to an alternation in receptor or other portion of the epithelial cell brush border (Houk et al., 1990). In addition to the MI barrier, barriers to viral dissemination have been found within mosquitoes, especially if they are initially infected with a low dose of virus. The ability of the mosquito to transmit the virus after infection seems to depend on several mechanisms which may limit the multiplication of

virus when the mosquito is not infected with a high dose of infectious virus (Kramer et al., 1981). When interpreting results from research done on alphavirus infection *in vitro,* it is important to remember that these natural infection barriers are not present and that infection *in vivo* may have very different results. While the present work is very significant in showing which proteins are required for alphavirus infection of mosquito cells, it would be prudent to confirm the results in live mosquitoes in the future.

Chapter 11: Endocytic Proteins in Insect Cells

The large GTPase dynamin was first discovered in *Drosophila melanogaster*, where it is encoded by the gene *shibire*. Mutations in this gene result in temperature-sensitive paralysis in adult flies and also have distinct effects on vesicular traffic in cells. The paralysis is thought to result from a reversible block to endocytosis which prevents membrane cycling and depletes synaptic vesicles (van der Bliek and Meyerowitz, 1991). It was determined that the dynamin protein expressed from the *shibire* gene motorizes intracellular traffic but the action is limited to endocytosis and interaction with microtubules. The *shibire* gene was also found to encode more than one form of dynamin, with alternative C termini that differ in length and amino acid composition (van der Bliek and Meyerowitz, 1991). Many Rab proteins have also been identified in *Drosophila* through cDNA cloning techniques. The proteins were found to have high amino acid identity (>80%) to those of corresponding mammalian proteins and bound GTP in a similar manner (Satoh, Tokunaga, and Ozaki, 1997). A dominant-negative mutant of Rab1 protein has recently been described for *Drosophila* and used in the organism to show that the protein functions in a manner similar to previous reports of the mammalian protein (Satoh et al., 1997). It remains unclear if other Rab proteins have similarly conserved functions in insect cells compared with their mammalian counterparts.

Chapter 12: Viral Pseudotypes

In the present work, viral pseudotypes are used extensively. As used herein, a viral pseudotype is defined as a viral core surrounded by the envelope/glycoproteins of a different virus. Pseudotypes have been a useful tool for exploring the functions of envelope proteins, altering the cell tropism of viral vectors and for exploring novel gene therapy approaches. Benefits of pseudotyping include the ability to isolate the function of viral envelope proteins, needing less amounts of envelope protein and the fact that pseudotypes are not replication-competent, which makes the experiment safer than using whole virus. Pseudotypes are produced by transiently transfecting two or more DNA plasmids that code for a viral core, viral glycoproteins and some type of marker. Most pseudotypes currently made use a retroviral vector (human immunodeficiency virus type 1 – HIV-1 or murine leukemia virus – MLV) for the viral core. This core is produced from a DNA plasmid containing the retroviral *gag* and *pol* genes, which code for the viral polymerase and proteins that make up the viral core, including the capsid protein (Soneoka et al., 1995). An example of a viral pseudotype is shown in Illustration 11.

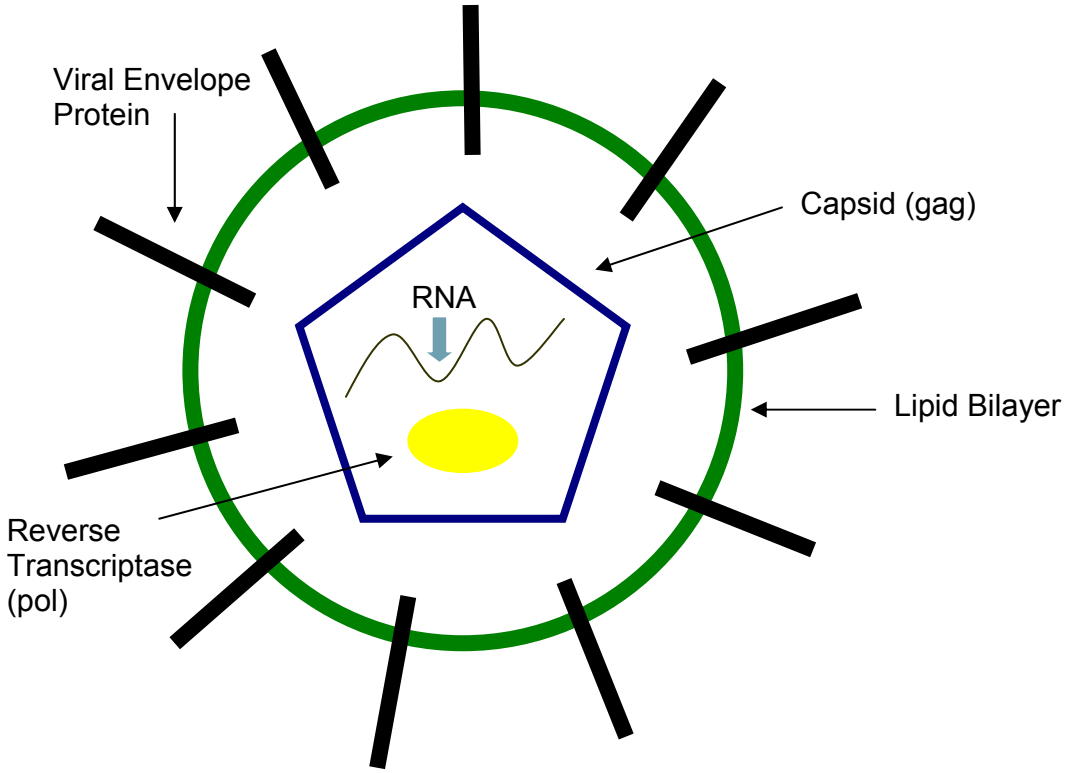


Illustration 11: Example of a viral pseudotype showing the various components

A viral pseudotype consists of a retroviral core comprised of the reverse transcriptase (yellow) and the capsid protein (blue) along with the RNA encoding a marker gene. The lipid bilayer (green) surrounds this core and viral envelope proteins (black) are embedded in this membrane.

The plasmid used for the production of viral envelope proteins often codes for the vesicular stomatitis virus glycoprotein (VSV-G) but many examples of other envelope proteins used exist. This glycoprotein has a broad host range, allowing the pseudotype to transduce most cell types. Pseudotypes produced using this glycoprotein also reach very high titers and can be concentrated by centrifugation. These are often used for gene therapy (Qiao et al., 2006). The drawback with these pseudotypes is that they induce syncytium formation due to the fusogenic properties of VSV-G, and they are also recognized by human complement (Kahl et al., 2004). This has led investigators to explore other envelope proteins that may be useful in producing lentiviral pseudotypes. Among the envelope proteins presently being used are alphavirus glycoproteins, including SFV, RRV, SINV and VEEV (Kahl et al., 2004; Kolokoltsov, Weaver, and Davey, 2005; Sharkey et al., 2001). Pseudotypes can also be made using another virus as the core/vector in place of a retro- or lentivirus. The core of vesicular stomatits virus is a common example (Hanika et al., 2005). Another good alternative to putting a retroviral core in the pseudotype is to use the same type of virus for both the core and the envelope proteins. For example, use of the nonstructural genes from one alphavirus to produce the viral core and the glycoprotein genes of another alphavirus for the structural proteins.

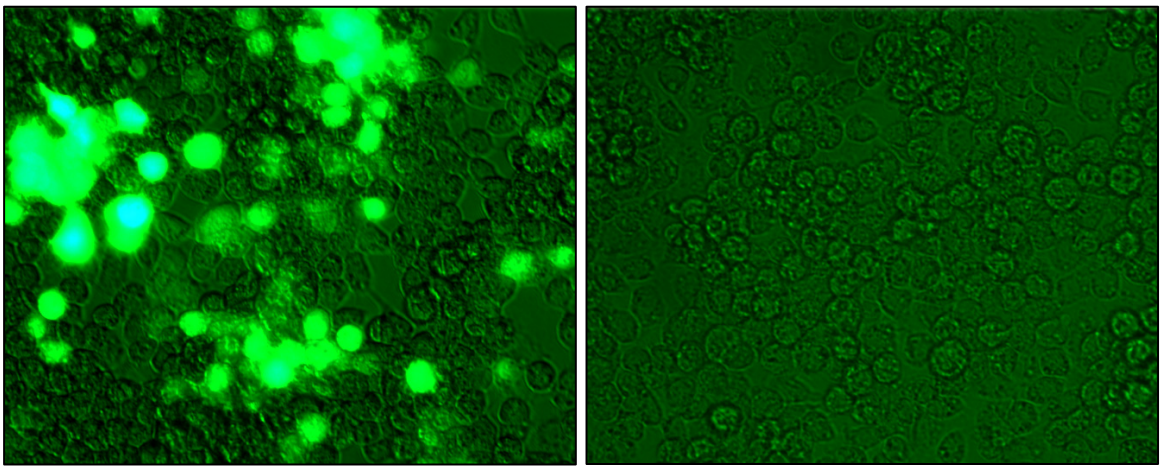
In the present work, a chimeric alphavirus is also used. For these, the structural proteins of one alphavirus (VEEV) are encoded in the genome of a related virus (SINV). Thus the progeny viruses mimic the behavior of the structural protein donor virus (VEEV) for entry pathway used and membrane fusion mechanism but share the replication pathway of the virus donating the remainder of the genome (SINV). The chimeric virus used in the present work is SIN83 which has the nonstructural genes from SINV and the VEEV structural genes derived from the vaccine strain of the virus, TC83 (Paessler et al., 2003). This virus is nonpathogenic but yields relevant information on the entry mechanisms of its pathogenic parent, VEEV.

Results

Chapter 13: VEEV Infects Mosquito Cells Via a pH-dependent Mechanism

It was previously shown that SFV and VEEV enter mammalian cells through a pH-dependent endocytic pathway (Helenius, Marsh, and White, 1982). However, SINV, another alphavirus, was shown to infect both mammalian and mosquito cells at neutral pH (Paredes et al., 2004). It remains unclear why such a difference exists but could be explained by experimental design differences or differences in entry mechanism of each alphavirus. This led us to first determine if VEEV, a New World alphavirus, enters mosquito cells via a pH-dependent endocytic pathway. Monensin, chloroquine and ammonium chloride (NH_4Cl) were used to treat mosquito cells and then the cells were challenged with virus. Monensin is a cationic ionophore which binds potassium ions and uncouples the sodium/potassium gradient across endosomal membranes. This ion gradient supplies the energy required to pump protons into the endosomes. Ammonium chloride and chloroquine are primary amines that cross the endosomal membrane and buffer against acidification. All experiments were done with C710 *Aedes albopictus* mosquito cells since VEEV can infect *Aedes* mosquitoes. SIN83-GFP virus was used for initial infection studies. This virus is composed of the VEEV structural proteins but contains a recombinant SINV genome encoding GFP and can be used at a BSL-2 level. It infects cells identically to VEEV, and has been characterized elsewhere (Paessler et al., 2003). At 8 h post-infection, more than 60% of untreated cells became infected. In contrast, none of the drug treated cells were infected (Fig. 1). Even though a range of drug concentrations was tested, the block to infection was similar with 1, 5, 10 or 50 μM monensin, 10, 20 or 30 mM NH_4Cl and 5, 10 or 20 mM chloroquine and is therefore not shown. These observations indicated that VEEV requires acidification of endosomes in order to infect mosquito cells.

A.



B.

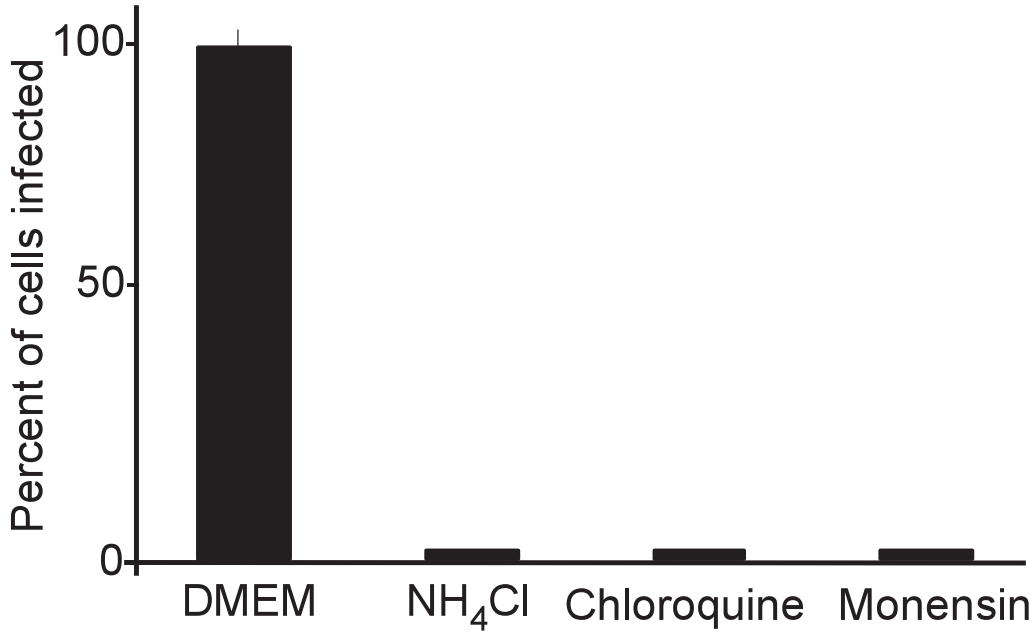


Figure 1. Infection of C710 cells with SIN83 is inhibited by lysomotropic agents. A. SIN83 virus encoding a GFP reporter gene was used to measure infection in untreated C710 cells (left) or cells treated with 20 mM ammonium chloride for 1 h at RT (right). MOI of 1. B. Virus titer for cells treated with each indicated chemical agent, expressed as a percentage of the titer observed for untreated cells. The agents were used at the following concentrations: 20 mM ammonium chloride, 20 mM chloroquine, 10 μM monensin. The average +/- standard deviation for a triplicate sample is shown.

While VEEV infection was effectively blocked by each of the agents used, it was unclear which step in infection was affected. It has been reported that chloroquine, monensin and ammonium chloride block SFV uncoating and nucleocapsid release into the cytoplasm in mammalian cells (Helenius, Marsh, and White, 1982). Studies done with SINV, another Old World alphavirus, indicated that chloroquine and ammonium chloride instead inhibited infection of mammalian cells by blocking the synthesis of viral RNA (Cassell, Edwards, and Brown, 1984). In another study using mosquito cells, ammonium chloride also inhibited SINV RNA synthesis while chloroquine increased infection (Hernandez, Luo, and Brown, 2001). However, low pH induces fusion of both SFV and SINV viruses to membranes and ammonium chloride inhibits this fusion in mammalian cells (Glomb-Reinmund and Kielian, 1998). Since the acidification inhibitors prevented VEEV infection, the next step was to determine where this block occurred. To determine if the block in VEEV infection was at the point of entry rather than at a downstream step, the inhibitors were tested using an entry assay.

Chapter 14: VEEV Enters Mosquito Cells Via a pH-dependent Mechanism

Recently, a novel entry assay was developed to monitor penetration of viruses into the cellular cytoplasm in real-time. The entry assay permits blocks at the level of virus entry to be quantitatively identified with high sensitivity. The assay utilizes viral pseudotypes that contain a murine leukemia retroviral (MLV) core and have viral envs on the surface. A protein expressed from the HIV *nef* gene is used to create a nef-luciferase fusion protein that is encapsulated into the virus particle after transfection and budding of the pseudotype. This assay measures virus entry by directly detecting the release of luciferase that will occur after fusion of cellular and viral membranes. It overcomes limitations imposed by infection assays, allowing the functions of the virus envs for entry to be assessed in live cells without interference from downstream steps such as replication (Fig. 2).

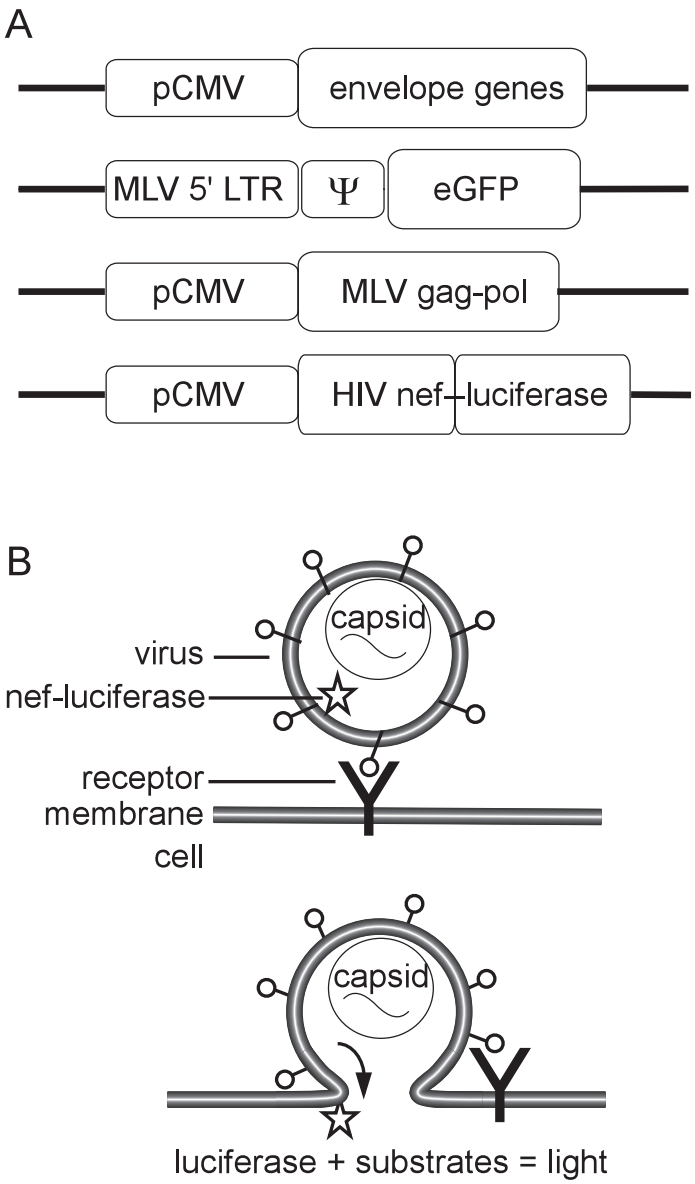


Figure 2. Production of pseudotype viruses for use in luciferase virus entry assay
A. 293FT cells were transfected with plasmids encoding the indicated recombinant genes. The first plasmid codes for viral envelope proteins (VEEV, SFV, VSV or 10A1 MLV were used), the second codes for infection GFP marker (Ψ-GFP), the third encodes the MLV core proteins and polymerase (gag-pol) and the fourth for the *nef*-luciferase fusion protein. After 48 h, virus containing encapsulated luciferase was collected, purified and used for virus entry assays. B. Schematic of the luciferase-based virus entry assay. Virus entry occurs after fusion of virus and cell membranes. Luciferase encapsulated inside the virus particle (star) is then released into the cell cytoplasm. The release is measured by perfusing the cell with luciferin, the substrate for luciferase which enters through high activity cell membrane permeases. Emitted light is then measured.

The entry of VEEV, SFV and VSV env pseudotyped viruses into C710 mosquito cells was compared using the luciferase release assay. Plasmids used in the production of these pseudotypes are shown in Figure 2A. VEEV and SFV were used to compare the entry behaviour of New to Old World alphaviruses, respectively. The VSV pseudotype was included as a positive control since it is well established that VSV enters cells through a pH-dependent, clathrin-dependent endosomal route and utilizes an endocytic pathway in both mammalian and mosquito cells (Marsh and Helenius, 1989; Matlin et al., 1982; Superti et al., 1987). Since virus entry kinetics in mosquito cells was poorly understood, a time course of entry was first established. The pseudotyped viruses were incubated with either 293 or C710 cells and samples were tested for luciferase activity at ten time points, up to 135 min. The 293 cells were incubated at 37°C while the C710 cells were incubated at 27°C since these are the optimal growth temperatures for mammalian and insect cells, respectively. At 75 min the entry signal for the VEEV pseudotype peaked in both C710 and 293 cells. The signal for the VSV pseudotype entry peaked later at 105 min in both cell types. In 293 cells, the SFV pseudotype entry signal peaked at 105 min while in C710 cells the signal peaked earlier, at approximately 90 min (Fig. 3). Based on these findings, an incubation time of 60 min was used for the remaining entry studies. The decrease in signal following the plateau was likely the product of luciferase degradation in the cells ($T_{1/2}$ of luciferase is 30 min in mammalian cells).

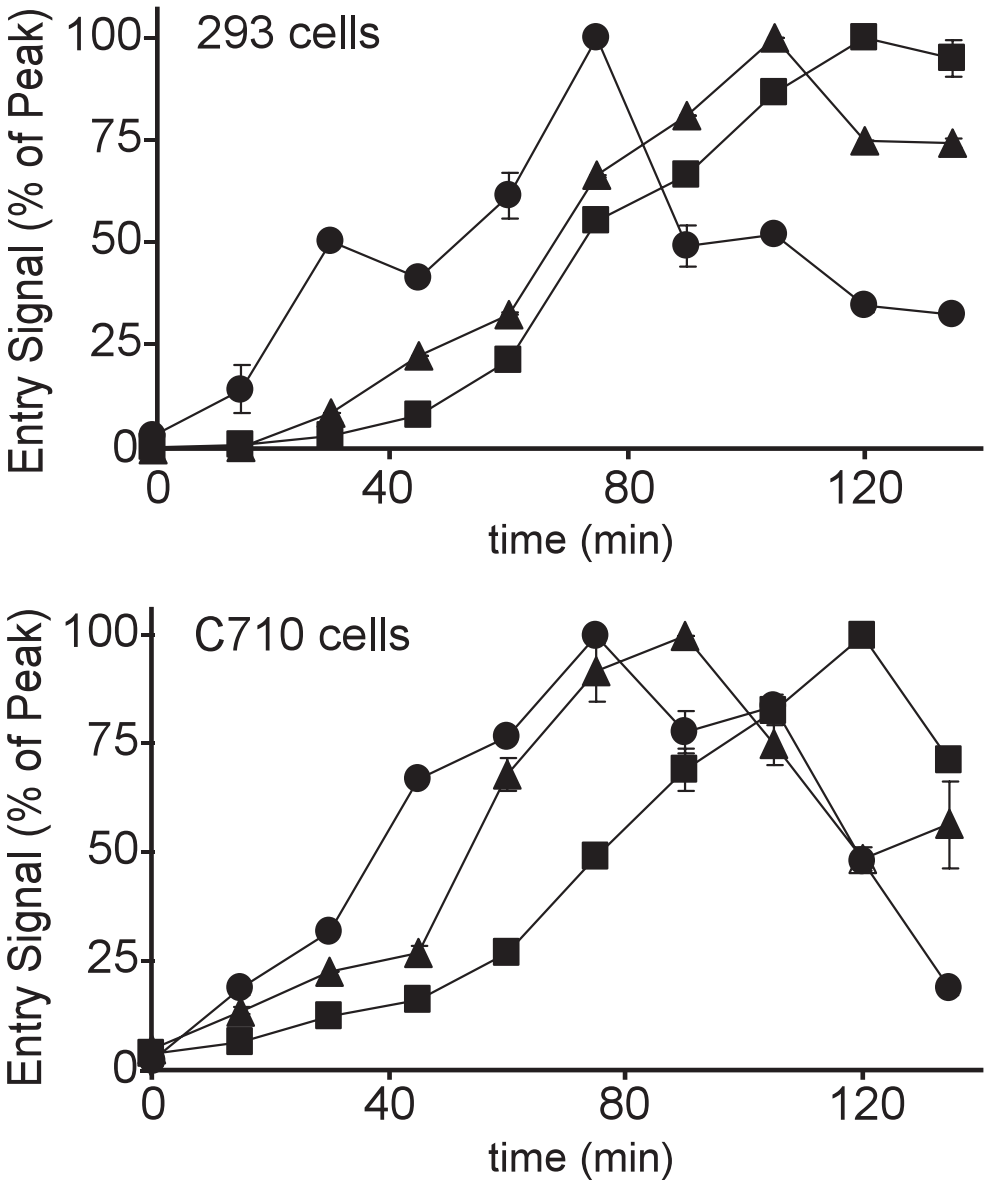


Figure 3. Kinetics of pseudotyped virus entry
HEK293 cells (top panel) or C710 cells (bottom panel) were incubated with viral pseudotypes bearing the envs of VEEV (circles), VSV (squares) or SFV (triangles). Excess virus was washed free of cells. At the times indicated, entry was measured and was expressed as a percentage of the maximum signal obtained for each viral pseudotype. Entry was measured at ten time points, from time zero to 135 minutes. Assay was done in duplicate and the average +/- standard deviation was plotted.

Viral pseudotypes were also made with each envelope protein that also encoded for GFP as a marker of infection. These were used to determine viral titer in mammalian cells and to then compare this titer with the luciferase activity of each viral pseudotype in mammalian cells. This assay was done in an attempt to determine if an equal amount of luciferase was encapsulated within the particles for different types of pseudotyped virus. The viruses were added to 293 cells and allowed to infect for a period of 24 hours and titer was counted by analyzing GFP expression and is given as colony forming unit per mL. As shown in Table 1, the viral titers and their corresponding luciferase activities were comparable. The virus stocks were diluted accordingly so that each amount of viral pseudotype used in the entry assay would have roughly the same amount of luciferase activity per mL of virus.

Table 1. Comparison of virus pseudotype titer to entry assay luciferase activity

Viral pseudotype	**Pseudotype titer (CFU/mL)[a]**	**RLU(x1000)/10^6 Cells[b]**
VSV	2.3×10^8	3,241 +/- 318
VEE	1.5×10^5	9.22 +/- 0.8
SFV	2.5×10^6	17.39 +/- 1.2
10A1 MLV	8.0×10^6	52.21 +/- 4.8

[a] A GFP-encoding MLV pseudotyped virus was used. Virus titer was determined by limiting dilution and counting colonies of GFP-expressing cells 48 h after addition of virus to HEK293 cells.
[b]One milliliter of viral supernatant was added to 10^6 cells and the relative luciferase activity (RLU) was measured after 1 h incubation. The average of three experiments +/- standard deviation is shown for each viral pseudotype.

To determine the effects of inhibiting endosomal acidification on virus entry, C710 cells were preincubated with monensin, chloroquine and ammonium chloride at the same concentrations used for infection studies for 1 h at 27˚C (1, 5, 10 and 50 μM monensin, 10, 20 and 30 mM ammonium chloride and 5, 10 and 20 mM chloroquine.) The luciferase-containing virus particles were then added to the cells for an hour and entry signals were determined via measurement of luciferase activity. Each of the chemical inhibitors reduced the entry signal to a similar low level for the viral pseudotypes used and the effect of a middle dose is shown (Fig. 4). VSV and VEEV signals were completely abolished while low levels were still detected in the presence of the inhibitors for the SFV pseudotype (Fig. 4, lower panel), with a small portion of the virus fusion from untreated cells remaining in the presence of each inhibitor. However, SFV entry pathway or mechanism may differ from VEEV since a component of entry still took place in the presence of the inhibitors. A pseudotype was also made bearing the envs of the 10A1 strain of MLV, which is known to enter cells at the cell surface through a pH-independent mechanism (Blanchard et al., 2006), and was used in the entry assay as a control for both cell viability and assay function. The 10A1 MLV pseudotype efficiently entered the C710 cells in the presence of all three inhibitors (Fig. 4) and indicated that cells were competent for virus infection and that the assay was unaffected by each chemical treatment. These findings indicated that the inhibitors block VEEV infection by acting at the level of virus entry and that acidification, likely in an endocytic compartment is required to allow VEEV entry to occur in a mosquito cell.

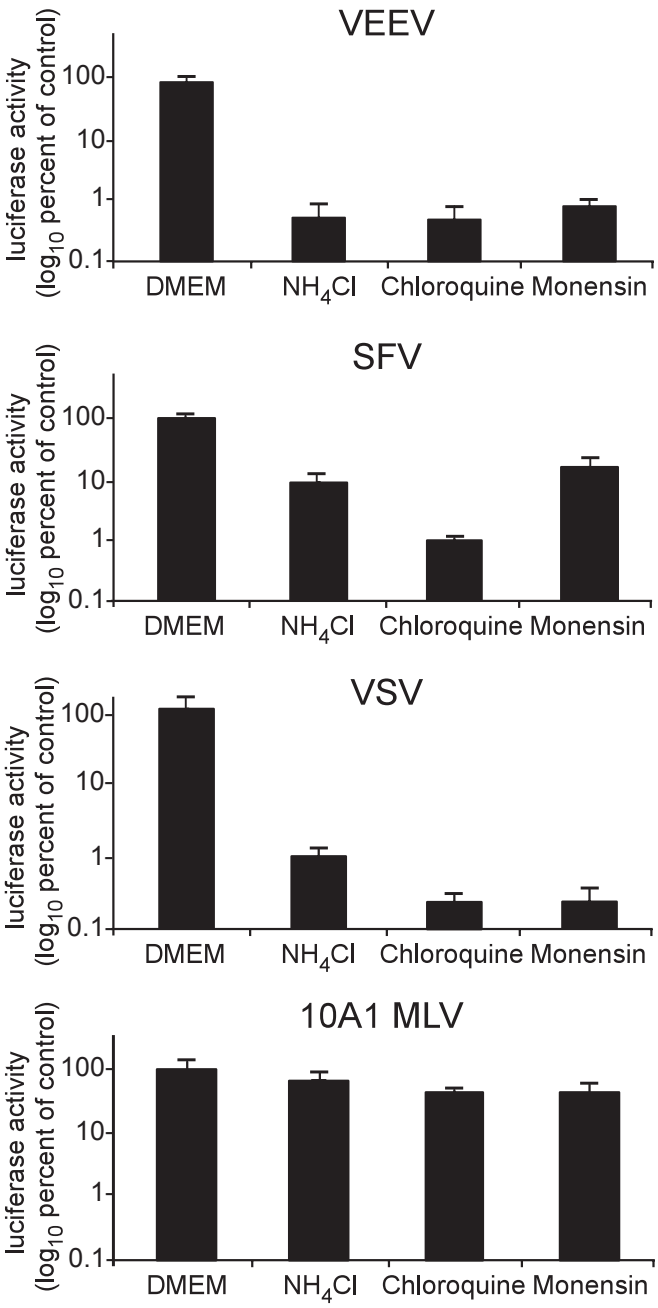


Figure 4. Lysomotropic agents prevent alphavirus entry into mosquito cells. C710 cells were incubated for 1 h at 27°C with each agent. Inhibitors were used at the following concentrations: 20 mM NH_4Cl, 20 mM chloroquine and 10 μM monensin. Entry was measured with pseudotypes bearing the envs of VEEV, VSV, SFV, VSV and 10A1 MLV as indicated. Entry was measured at 1 h after addition of virus to cells. Average luciferase activity (log percent of control) is shown for assays performed in triplicate +/- standard deviation.

Chapter 15: Identification of Rab5, Rab7 & Dynamin in Mosquito Cells

Having established that VEEV uses a pH-dependent route to enter mosquito cells, we then wanted to identify if an endocytic pathway was involved. Recently it was shown that VEEV needs both functional Rab5 and Rab7 endosomal GTPases for entry in mammalian cells (Kolokoltsov, Fleming, and Davey, 2006). Rab5 is found on early endosomes and Rab7 is found on late endosomes. Both are involved in endosomal fusion, trafficking and maturation (Bucci et al., 1995; Bucci et al., 1992; Bucci et al., 2000; Feng et al., 2001; Feng, Press, and Wandinger-Ness, 1995). Dynamin is involved in many aspects of endocytosis and is specifically required for clathrin-mediated endocytosis, which is the mechanism utilized by the alphavirus Semliki Forest virus to enter mammalian cells (DeTulleo and Kirchhausen, 1998). If VEEV follows a similar pathway in mosquito cells as in mammalian cells, then entry and infection should have a similar dependence on mosquito homologs of these proteins.

The genome of *Anopheles gambiae* was recently sequenced and published and the sequencing of the *Aedes aegypti* genome is near completion (Holt et al., 2002; Kaufman, Severson, and Robinson, 2002; Loftus, 2005). By sequence comparison, mosquito protein homologs were identified that showed 82%, 88% and 70% amino acid identity to human Rab5, Rab7 and dynamin genes, respectively. The aligned mosquito sequences had E values of $< 2e^{-69}$ for human Rab5, $1e^{-83}$ for human Rab7 and $2e^{-54}$ for human dynamin. Since the E value represents the chance of obtaining a similar alignment strictly by chance these low E values indicated that the genes identified were highly likely to be related genes and possibly functional homologs. Interestingly, the human genome contains three Rab5 isoforms (Bucci et al., 1995) but only one mosquito equivalent was identified within each sequence database. Both humans and mosquitoes appear to only have one form of the Rab7 protein. There are three separate dynamin proteins in mammalians, dynamin 1, 2 & 3, yet the mosquito genome apparently contains only one gene coding for a dynamin protein.

cDNA encoding related Rab5, Rab7 & dynamin mosquito genes was isolated from both *Anopheles gambiae* and *Aedes albopictus* cDNA libraries. The sequences of the isolated Rab5 & Rab7 DNA matched those identified in the databases except for several silent mutations and have been submitted to Genbank (NCBI) with accession numbers: *Aedes albopictus* Rab7 – 858647 and *Anopheles gambiae* Rab5 - 863568. The dynamin sequence also matched that identified in the database but had several mutations that altered the amino acid charge in the protein and the sequence has yet to be submitted. Alignments of the mosquito Rab5, Rab7 and dynamin to the human protein amino acid sequences are shown in Figures 5, 6 & 7, respectively.

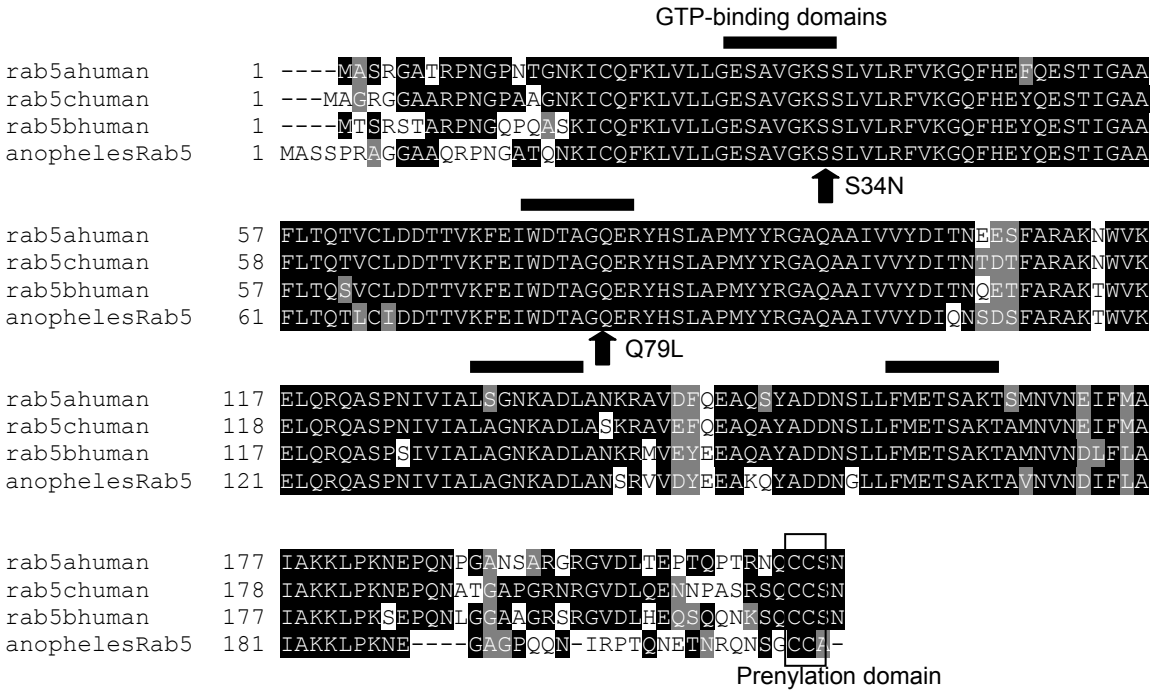


Figure 5. Amino acid alignment of human Rab5 a, b, c and mosquito Rab5 proteins
Amino acid alignment of the three isoforms of the human Rab5 GTPase (Rab5ahuman, Rab5bhuman, Rab5chuman) and an *Anopheles gambiae* mosquito homolog (AnophelesRab5) isolated from a cDNA library. Sequences were aligned using ClustalW (Thompson, Higgins, and Gibson, 1994) and regions of homology shaded using Boxshade software. The GTP-binding domains are indicated by horizontal bars and the amino acid substitutions used to create the dominant negative (S34N) and constitutively active (Q79L) forms of Rab5 are indicated by vertical arrows. The conserved prenylation domain is also indicated.

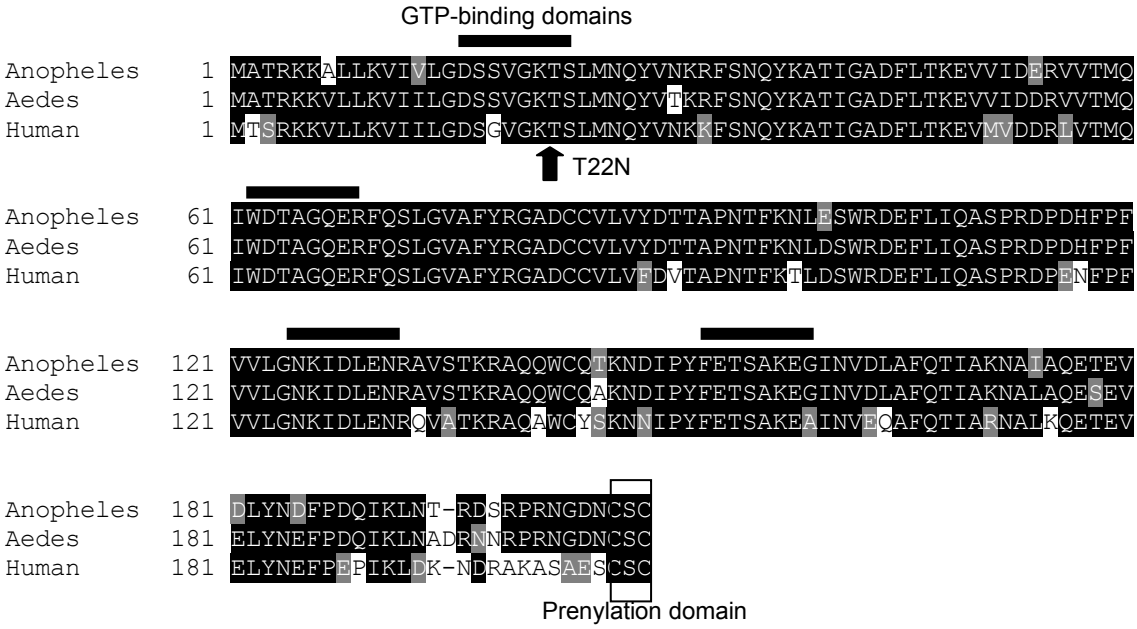


Figure 6. Amino acid alignment of human and mosquito Rab7 proteins
Amino acid alignment of the human Rab7 GTPase (Human), an *Anopheles gambiae* mosquito homolog (Anopheles) and an *Aedes aegypti* mosquito homolog (Aedes.) isolated from a cDNA library. Sequences were aligned using ClustalW (Thompson, Higgins, and Gibson, 1994) and regions of homology shaded using Boxshade software. The GTP-binding domains are indicated by horizontal bars and the amino acid substitution used to create the dominant negative (T22N) form of Rab7 is indicated by a vertical arrow. The conserved prenylation domain is also indicated.

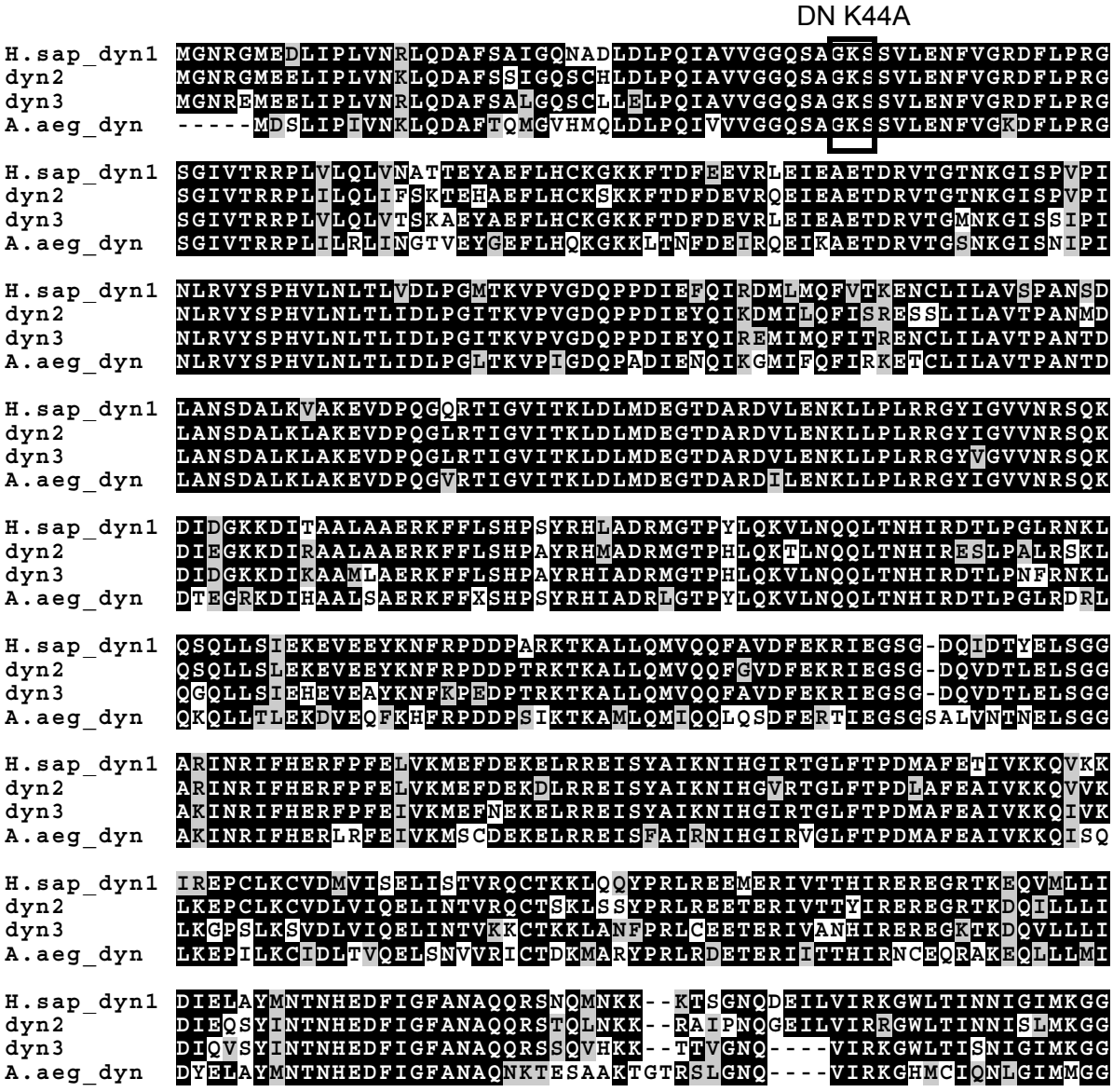


Figure 7. Amino acid alignment of human and mosquito dynamin proteins
The first half of an amino acid alignment of the three human (H. sap.) dynamin GTPases (dyn1, 2 & 3) and an *Aedes aegypti* mosquito homolog (A. aeg_dyn) isolated from a cDNA library. Sequences were aligned using ClustalW (Thompson, Higgins, and Gibson, 1994) and regions of homology shaded using Boxshade software. The first GTP-binding domain is where the dominant negative mutation was made and the amino acid substitution is indicated by a black box and is designated DN K44A.

Chapter 16: Cloning & Expression of Rab5, Rab7 & Dynamin Mosquito Genes

Using the sequences retrieved from the BLAST search, primers were designed to extract the Rab5, Rab7 and dynamin mosquito genes from the cDNA and amplify with the polymerase chain reaction. First, RNA was extracted from C710 mosquito cells using RNAqueous (Ambion, TX) and cDNA was made by reverse transcription with the Superscript kit (Invitrogen, CA) according to the manufacturer's instructions. Then, the cDNA and specific primers were used in the PCR reaction with Accutaq polymerase (Sigma, CA). The Rab genes were obtained with one reaction as they are relatively small amplicons at 650 base pairs in length. The dynamin gene was amplified in a two-step process since it is rather large at 2600 base pairs in length. First, two reactions were done simultaneously to obtain a 5' and a 3' portion of the dynamin gene (two halves that together equal the entire gene). Once the two halves of dynamin were obtained and purified, additional PCR was done with these amplicons as templates for full-length dynamin. The PCR amplicons are shown on a 1.2% agarose gel in Figure 8.

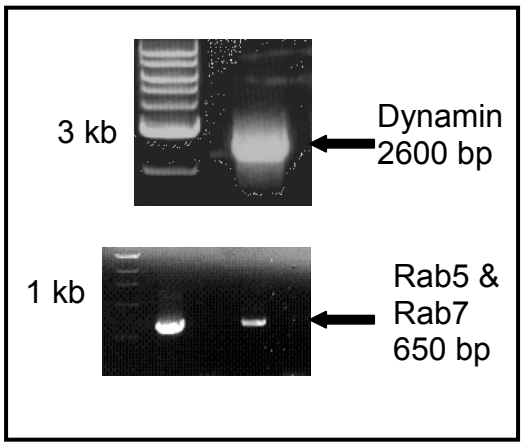


Figure 8. PCR amplicons for mosquito Rab5, Rab7 & dynamin genes
Photograph of agarose gel with DNA bands of the PCR products for the putative mosquito Rab5, Rab7 & dynamin genes. The amplicons were run alongside a 1000 kb ladder (New England Biolabs). Rab5 & Rab7 are approximately 650 base pairs and dynamin is 2600 base pairs of DNA.

The PCR amplicons were then cloned into the TOPO cloning vector, which will rapidly ligate to any PCR product with a 5' overhang. The mosquito genes were then excised from the TOPO vector using restriction endonucleases and inserted into an expression vector for protein production. For expression of the mosquito genes, the pAc5.1/V5-HisA insect expression plasmid (Invitrogen, CA) was modified by insertion of the GFP coding sequence (pAc5.1-GFP.) Two different pAc5.1-GFP vectors were made, one for the insertion of the Rab genes and one for the insertion of the dynamin gene. The GFP tag was placed in the expression vector so that it would be at the N-terminus of the Rab5 and Rab7 mosquito proteins and at the C-terminus of the dynamin mosquito protein, corresponding to the location of GFP in mammalian expression of these proteins. The mosquito genes were then cloned into the pAc5.1-GFP vector using DNA ligase. Figure 9 illustrates the resulting plasmids for the mosquito genes. The human Rab5 genes, each similarly tagged with GFP, were also cloned into the same mosquito expression construct for comparison of the expression of these proteins in human cells with the expression of mosquito proteins in mosquito cells.

To confirm that mosquito Rab5, Rab7 & dynamin genes were functionally homologous to their mammalian counterparts in endosome formation and maturation, amino acid substitutions at conserved residues were made in both proteins that give well characterized dominant negative (DN) and constitutively active (CA) phenotypes in the corresponding mammalian proteins (Bucci et al., 2000; Feng, Press, and Wandinger-Ness, 1995; Stenmark et al., 1994). To characterize the function of each protein bearing these substitutions, plasmids containing the mosquito genes were transfected into mosquito cells and those coding for human proteins were transfected into human cells. The cells were examined by confocal microscopy to compare expression and localization within the cell. The human cell transfection efficiency was consistently over 90% by GFP expression. Unfortunately, the mosquito cells were more difficult to transfect and transfection efficiencies did not reach very high levels. Typically, approximately 5% of the mosquito cells expressed detectable fluorescent protein, which was enough for the cells to be analyzed by microscopy.

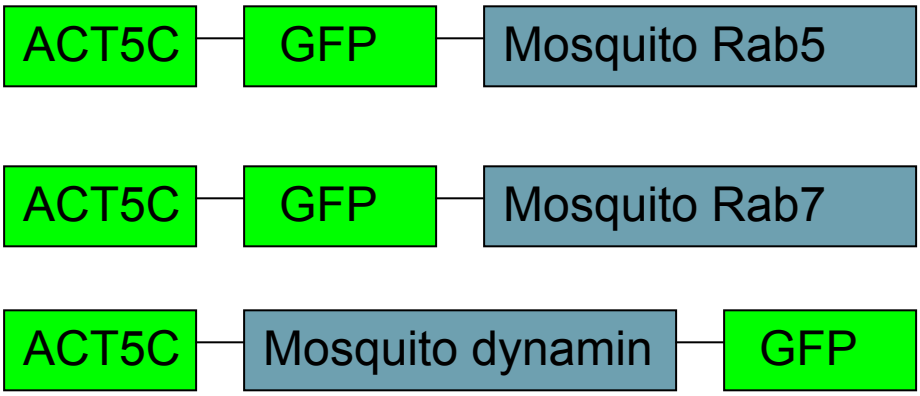


Figure 9. Mosquito gene position in the pAc5.1GFP vector

The mosquito genes Rab5 and Rab7 were cloned into the expression vector with GFP at the N-terminus of the protein while the dynamin gene was cloned in with GFP at the C-terminus. The insect promoter ACT5C is located just before the GFP-mosquito protein fusion and will drive expression of the proteins.

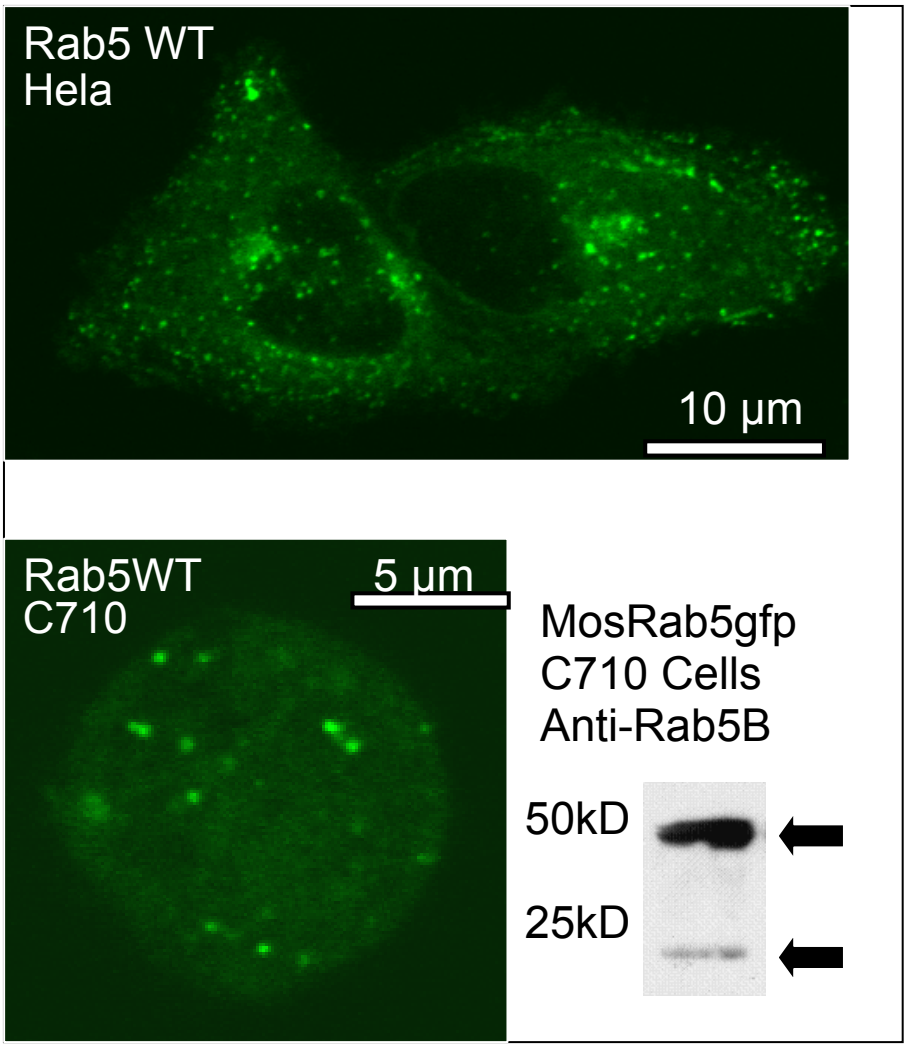


Figure 10. Comparison of Rab5 WT human and mosquito proteins
After transfection of human and mosquito cells with expression plasmids encoding genes for human and mosquito GFP-Rab5 wild-type, respectively, cells were examined by confocal microscopy. A representative slice through the center of each cell type is shown, with human protein expression in a Hela cell in the top panel and mosquito protein expression in a C710 cell in the bottom panel. The lower right-hand corner shows a Western blot with C710 mosquito cell lysate expressing mosquito GFP-Rab5 protein (MosRab5gfp) probed with an antibody against human Rab5B (Santa Cruz, CA). Mosquito cell lysate was boiled in SDS-PAGE buffer with 2% β-mercaptoethanol and run on a 12% SDS-PAGE gel. The lysate was then transferred to nitrocellulose. The arrows highlight the protein band, with the lower band representing endogenous protein (25kD) and the upper protein representing the GFP-Rab5 fusion protein (50kD).

The Rab5 wild type (WT) protein from both humans and mosquitoes shared a similar expression pattern in human (Hela) and mosquito (C710) cells, respectively. Each protein was visible as punctate, vesicular staining within the cellular cytoplasm (Fig. 10). The human protein expression in Hela cells is shown in the top panel while the mosquito protein expression in C710 cells is shown in the bottom panel. The mosquito protein appears to express in larger and fewer vesicles than the human protein but the reasons for this remain to be determined. A Western blot was also done on mosquito cell lysate to confirm that the cells were in fact expressing Rab5 protein. The expression plasmid containing the mosquito GFP-Rab5 gene was transfected and cell lysate was obtained after 24 h. The lysate was run on a 12% SDS-PAGE gel and transferred to nitrocellulose. The blot was then probed with a polyclonal antibody against the human isoform Rab5B (Fig.10). Two bands were visible on the blot, one representing native Rab5 in the mosquito cell (approximately 25kD) and one representing the GFP-Rab5 fusion protein (approximately 50kD). Interestingly, the 50kD band is much darker than the 25kD band, suggesting that mosquito cells may have relatively low amounts of Rab5 expression in the cell since transfection of the protein seemed to have raised the amount of protein visible on the blot considerably.

Expression of the mutant Rab proteins was then examined after transfection of both human and mosquito Rab5 DN & CA into human and mosquito cells, respectively. Again they shared similar expression patterns, with the DN proteins very diffuse and spread throughout the cellular cytoplasm and the CA proteins located on large vesicular bodies, which would be expected if the early endosomes can not fuse due to constitutively active Rab5 (Fig.11).

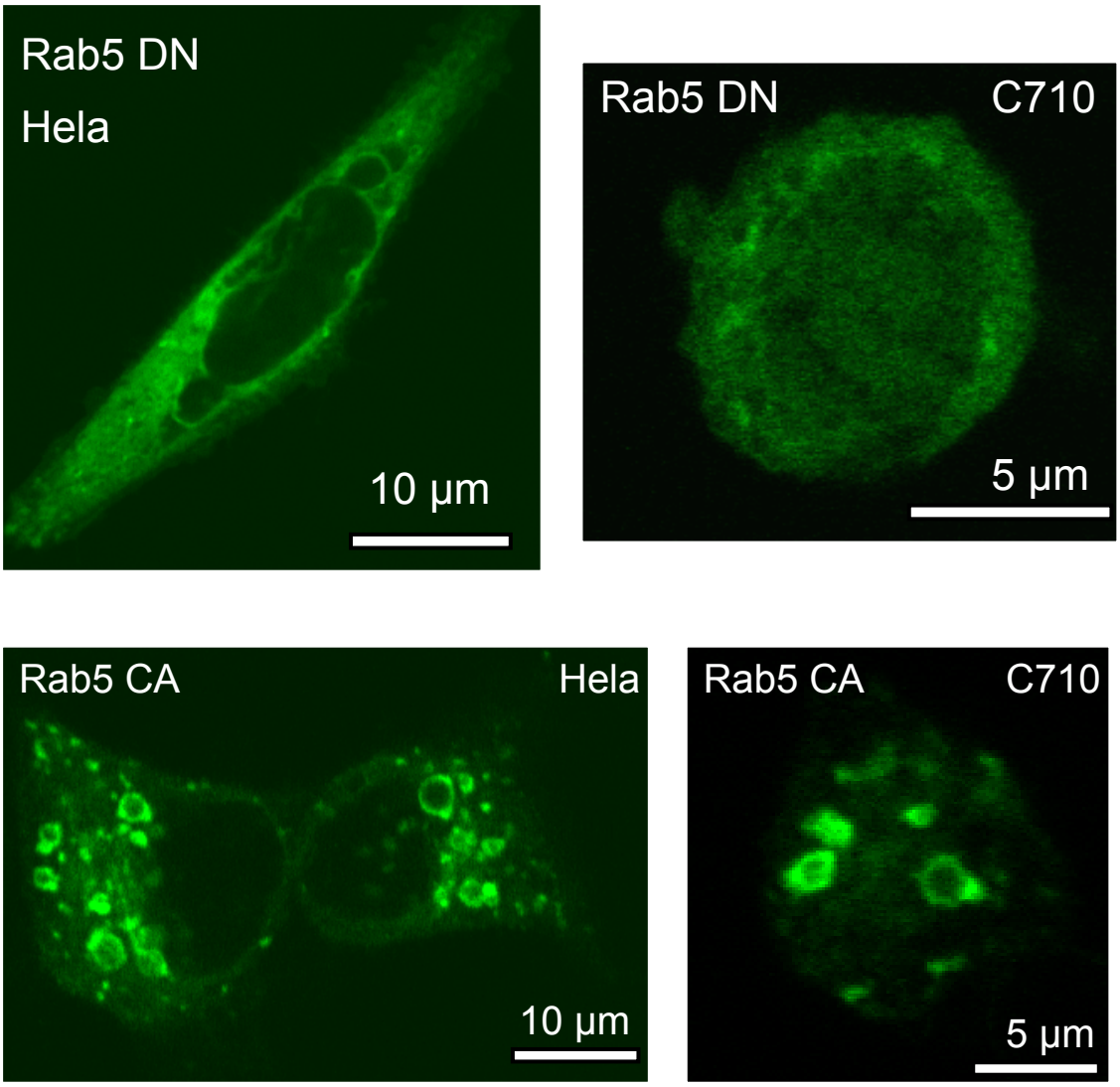


Figure 11. Comparison of Rab5 DN & CA human and mosquito proteins
After transfection of human and mosquito cells with expression plasmids encoding genes for GFP-Rab5 dominant negative and constitutively active mutant proteins from human and mosquito, respectively, cells were examined by confocal microscopy. A representative slice through the center of each cell type is shown, with human protein expression in Hela cells in the left panels and mosquito protein expression in C710 cells in the right panels.

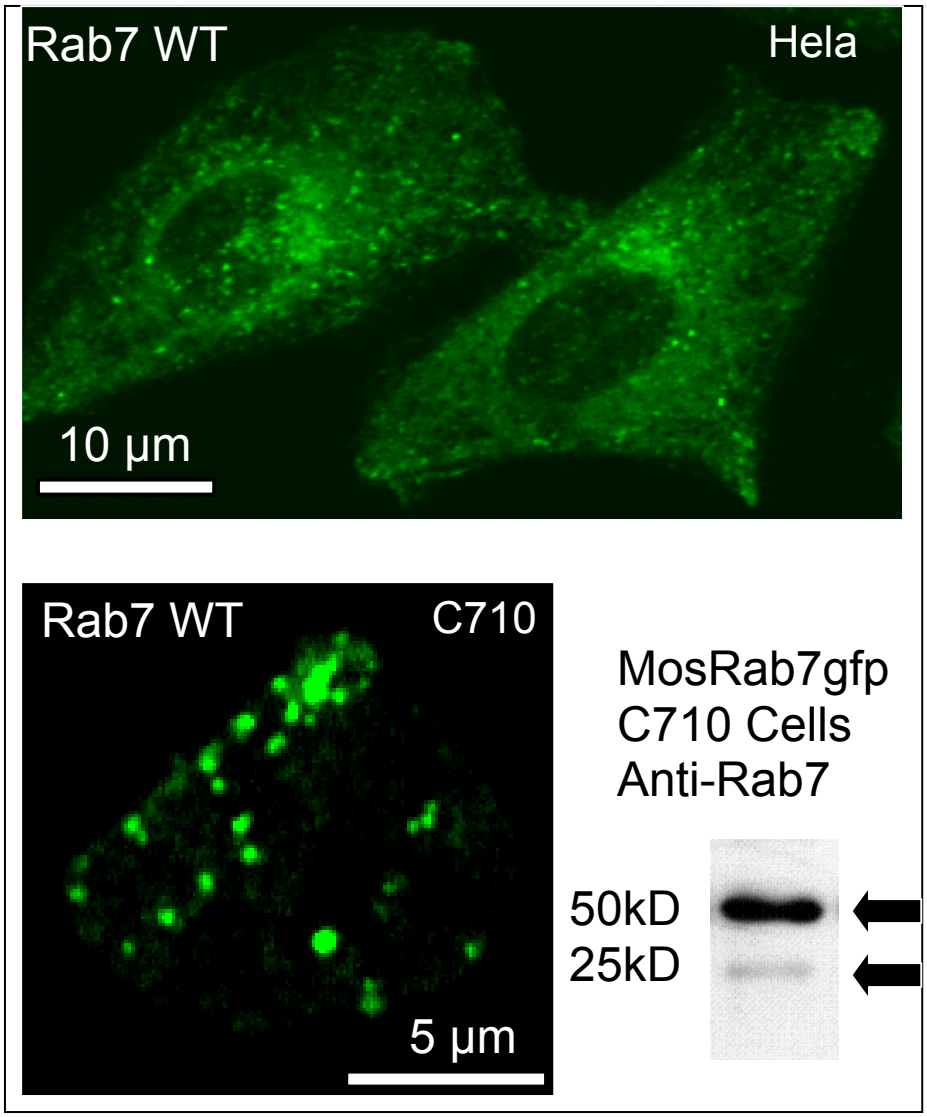


Figure 12. Comparison of Rab7 WT human and mosquito proteins
After transfection of human and mosquito cells with expression plasmids encoding genes for human and mosquito GFP-Rab7 wild-type, respectively, cells were examined by confocal microscopy. A representative slice through the center of each cell type is shown, with human protein expression in a Hela cell in the top panel and mosquito protein expression in a C710 cell in the bottom panel. The lower right-hand corner shows a Western blot with mosquito cell lysate expressing mosquito GFP-Rab7 protein probed with an antibody against human Rab7 (Santa Cruz, CA). Mosquito cell lysate was boiled in SDS-PAGE buffer with 2% β-mercaptoethanol and run on a 12% SDS-PAGE gel. The lysate was then transferred to nitrocellulose. The arrows highlight the protein band, with the lower band representing endogenous protein (25kD) and the upper protein representing the GFP-Rab7 fusion protein (50kD).

Similarly, both the human and mosquito WT Rab7 proteins expressed equally well in human and mosquito cells and gave similar staining, though slightly less punctate and more diffuse than the Rab5 expression in the human cell (Fig. 12). Again, a Western blot was done on mosquito cell lysate to confirm that the cells were in fact expressing Rab7 protein. The expression plasmid containing the mosquito GFP-Rab7 gene was transfected and cell lysate was obtained after 24 h. The lysate was run on a 12% SDS-PAGE gel and transferred to nitrocellulose. The blot was then probed with a polyclonal antibody against the human Rab7 (Fig.12). Two bands were again visible on this blot, one representing native Rab7 in the mosquito cell (approximately 25kD) and one representing the GFP-Rab5 fusion protein (approximately 50kD). In a manner similar to the blot probing the Rab5 protein, the band representing transfected Rab7 contains considerably more protein than the band for native protein, suggesting that mosquito cells may also have relatively low amounts of Rab7 expression in the cell.

The mutant forms of human and mosquito Rab7 (DN) also expressed well in the human and mosquito cells, respectively, and gave similar phenotypes as has been reported in the literature for human protein in mammalian cells (Fig.13). The protein is dispersed throughout the cytoplasm and considerably less punctate than the Rab7 WT proteins for both the human and mosquito genes in each cell type.

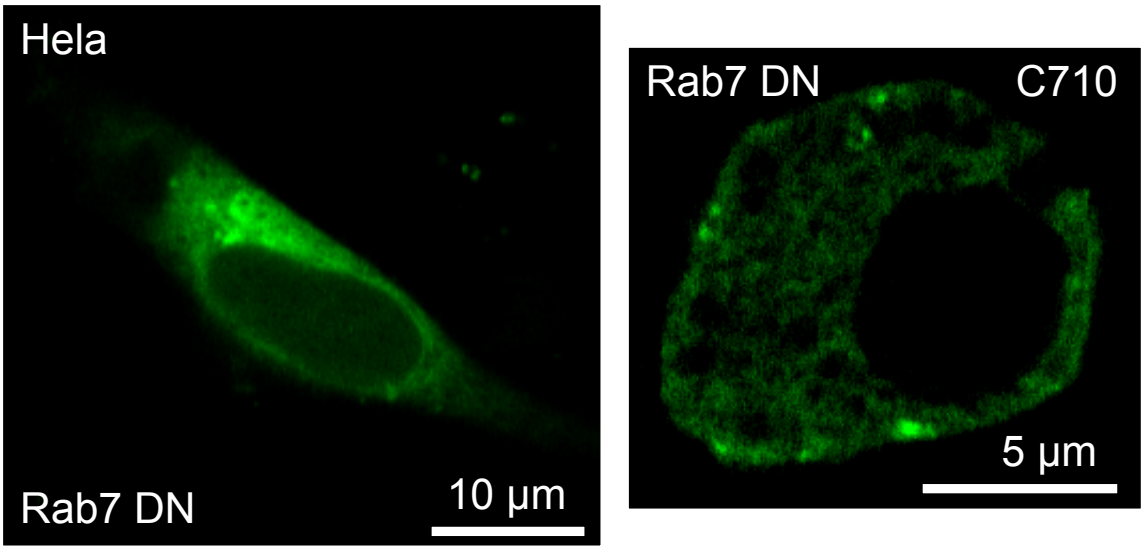


Figure 13. Comparison of Rab7 DN human and mosquito proteins
After transfection of human and mosquito cells with expression plasmids encoding genes for GFP-Rab7 dominant negative mutant protein from human and mosquito, respectively, cells were examined by confocal microscopy. A representative slice through the center of each cell type is shown, with human protein expression in a Hela cell in the left panel and mosquito protein expression in a C710 cells in the right panel.

Since the human antibodies against Rab5 & Rab7 functioned well in a Western blot using mosquito protein, the next step was to use the antibodies in an immunofluorescence assay to determine if native and transfected Rab proteins localized to similar locations within the mosquito cell. C710 cells were again transfected with the expression plasmids encoding Rab5 & Rab7 WT proteins and then fixed and incubated with the same antibodies used in the Western blots, i.e. polyclonal antibodies against human Rab5B and Rab7, respectively. A labeled secondary was used to visualize where the antibodies bound and confocal microscopy was done to examine the cells. Figure 14 clearly demonstrates that the mosquito proteins and the antibodies against human protein colocalized quite well. The top panel shows the Rab5 colocalization while the bottom panel shows Rab7. Transfected GFP-protein is shown in the left panel for both proteins, antibody is shown in the middle panel and a merged image is shown on the far right. Two points of colocalization are highlighted by white circles for each protein. These results again pointed to the fact that the transfected mosquito proteins were actual Rab proteins as well as that they were expressing in the same locations within the cell as the native proteins, therefore they should be able to function along the appropriate endocytic pathway.

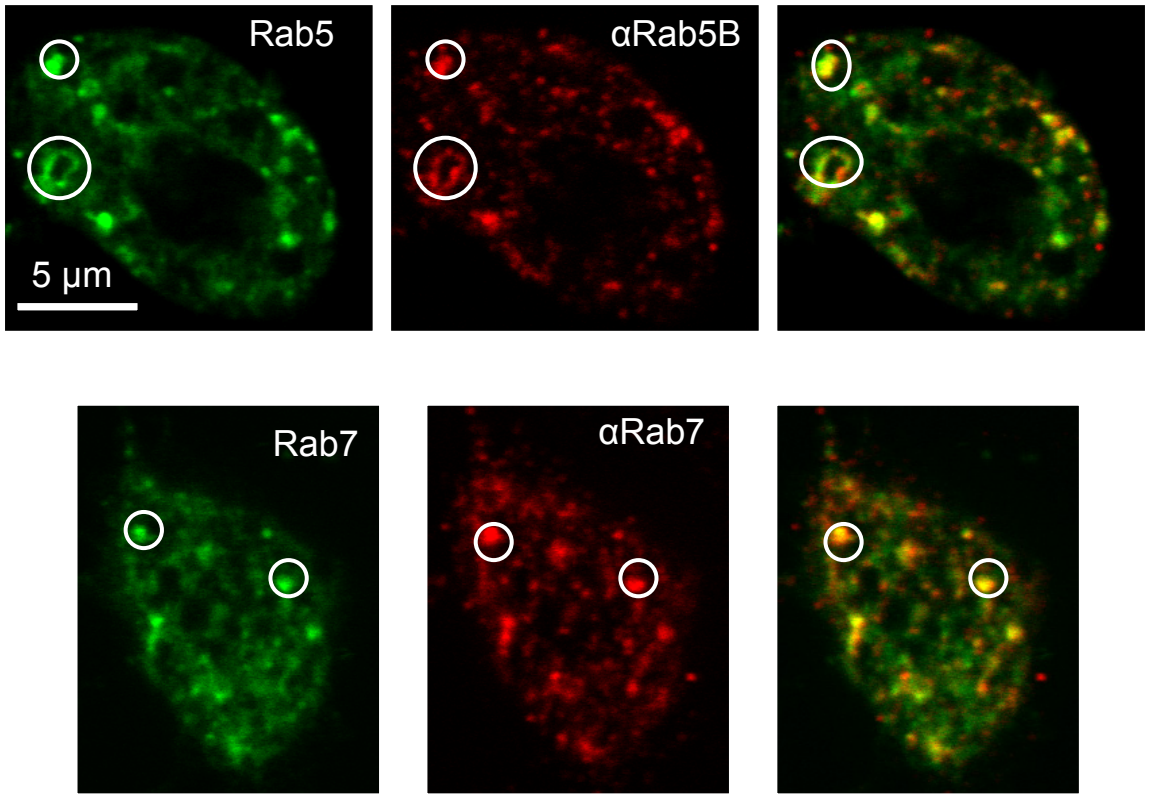


Figure 14. Colocalization of Rab5/Rab7 mosquito proteins with human antibodies
After transfection of C710 mosquito cells with expression plasmids encoding genes for GFP-Rab5 & GFP-Rab7 WT (left panels), cells were fixed and incubated with polyclonal antibodies against human Rab5B & Rab7 (middle panels), respectively (Santa Cruz, CA). Cells were then examined by confocal microscopy. A representative slice through the center of each cell type is shown, with colocalization indicated by white circles. Merged images are in the far right panel.

After confirming that the Rab proteins expressed in a similar manner to their human counterparts, it was then time to examine expression of the mosquito dynamin proteins. The dynamin wild type (WT) gene gave a phenotype that was similar to previous reports of the mammalian protein in human cells (Fig.15). The protein was visible as punctate, vesicular staining mostly along the cellular membrane, as was expected since dynamin functions at a very early step along endocytosis. The mosquito dynamin dominant negative (DN) protein expressed as larger spots through the cell, with bright accumulations of protein found along the membrane (Fig.15). A Western blot was also done on the mosquito cell lysate to confirm that the cells were in fact expressing dynamin protein. The expression plasmid containing the mosquito GFP-dynamin gene was transfected and cell lysate was obtained after 24 h. The lysate was run on an 8% SDS-PAGE gel and transferred to nitrocellulose. The blot was then probed with a polyclonal antibody against human dynamin (Fig.15). Presence of a strong band where the dynamin protein should be was evidence of the mosquito dynamin protein in the cell lysate. Two bands were visible on this blot, one representing endogenous dynamin in the mosquito cell (approximately 100 kD) and one representing the recombinant GFP-dynamin fusion protein (approximately 125 kD). Interestingly, in this case both bands had similar intensity, suggesting that the native dynamin protein is expressed at a relatively high level in the mosquito cell and transfection of the protein may not alter levels or phenotype. Unfortunately, the dynamin antibody did not function well in the immunofluorescence assay, though the detection of the protein by Western blot suggested that the mosquito protein expressed is clearly the counterpart to human dynamin.

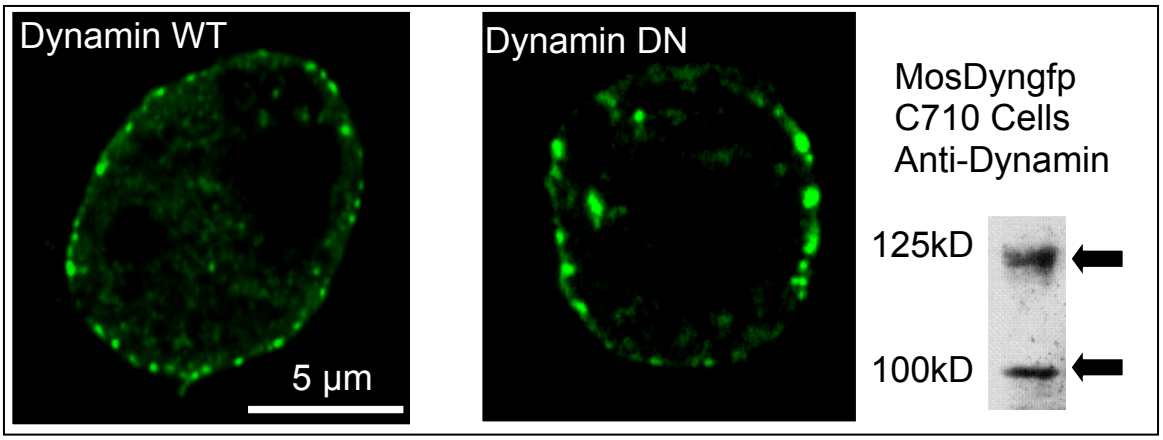


Figure 15. Comparison of mosquito dynamin WT & DN proteins
After transfection of C710 mosquito cells with expression plasmids encoding genes for mosquito GFP-dynamin wild-type & dominant negative proteins, cells were examined by confocal microscopy. A representative slice through the center of the cell is shown, with dynamin WT expression in the left panel and dynamin DN expression in the middle panel. The right panel shows a Western blot with mosquito cell lysate from cells expressing mosquito GFP-dynamin WT protein probed with an antibody against human dynamin (Santa Cruz, CA). Mosquito cell lysates were boiled in SDS-PAGE buffer with 2% β-mercaptoethanol and ran on an 8% SDS-PAGE gel. The lysates were then transferred to nitrocellulose. The arrows highlight the protein band, with the lower band representing endogenous protein (100kD) and the upper protein representing the GFP-dynamin fusion protein (125kD).

In each case the pattern of expression of the human protein matched that previously described for mammalian cells and the mosquito proteins gave similar expression patterns. Western blots with mosquito cell lysate probed with antibodies against human proteins clearly showed the presence of all three endocytic proteins in the mosquito cell. The protein amount was also greatly increased upon expression of the cloned mosquito Rab5 & Rab7 genes, which strongly supported that the proteins found in the mosquito genome and expressed in the cells were in fact the mosquito counterparts to the human proteins. The dynamin protein seemed to have a high expression level before transfection but this remains to be proven. In addition, an immunofluorescence assay clearly showed that the transfected Rab proteins were expressed in similar locations within the cell as the native proteins. Together, these results indicated that each protein likely served similar roles in endosome biogenesis and function in both human and mosquito cells.

Chapter 17: Characterization of Rab5, Rab7 & Dynamin Mosquito Proteins

To further examine if the mosquito Rab5, Rab7 & dynamin genes encoded for protein with similar function to their human counterparts, an assay was chosen to test the role the proteins played in endocytosis. After human and mosquito protein expression in transfected cells, both human and mosquito cells were then incubated with labeled human transferrin. Transferrin uptake in mammalian cells occurs primarily through clathrin-mediated endocytosis and disruption of this pathway results in accumulation of transferrin prior to the block (Damke et al., 2001; van Dam and Stoorvogel, 2002). Human transferrin has been successfully used in drosophila cells for study of endocytosis (Blitzer and Nusse, 2006) but it was unknown if it would function in mosquito cells. Human transferrin was incubated with the mosquito cells and then the cells were fixed and stained with antibody against cytoplasmic tail of the human transferrin receptor (this domain is highly conserved across many species including mosquitoes). In most cases, staining for transferrin and the transferrin receptor overlapped or was adjacent and the assay confirmed that the mosquito transferrin receptor was involved in the endocytic uptake of the human transferrin protein (Fig. 16).

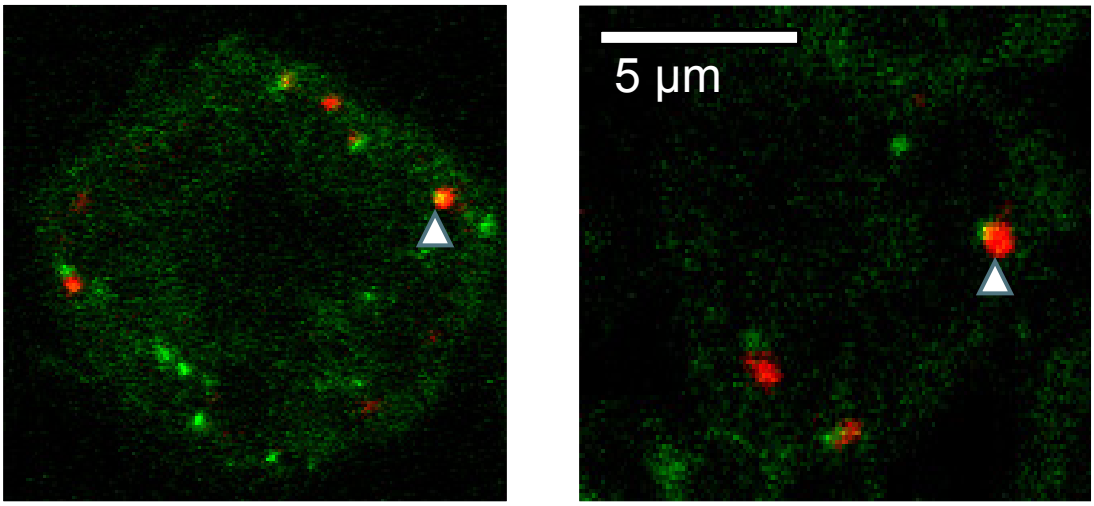


Figure 16. Colocalization of human transferrin & mosquito transferrin receptors
After incubation with human transferrin (red dots), C710 mosquito cells were fixed and probed with an antibody against a conserved region of the human transferrin receptor that is also present in the mosquito transferrin receptor (green). A representative slice through the center of the cell is shown, with points of colocalization highlighted with white arrowheads.

After confirming that human transferrin likely enters mosquito cells via an endocytic route, it could then used in an uptake assay with the transfected proteins. Since Rab5 controls early endosome formation, disrupting Rab5 function in mammalian cells results in inhibition of rapid transferrin uptake and sequestration of transferrin close to the cell surface (Bucci et al., 1995; Stenmark et al., 1994; Trischler, Stoorvogel, and Ullrich, 1999). The assay was first performed with human proteins in human cells to establish a phenotype for the Rab5 WT, DN and CA proteins that could then be used to compare the mosquito protein function. After transfection of the plasmids coding for the human genes, cells were incubated with labeled transferrin for 15 minutes at 37°C. Cells were then fixed and examined by confocal microscopy. In the cells expressing human GFP-Rab5 WT, transferrin uptake was rapid and is visible throughout the cell. There was abundant colocalization of the transferrin with the Rab5 protein and both proteins are visible as punctate and vesicular (Fig.17, upper panel). Expression of the Rab5 DN protein clearly prevented transferrin from entering the cell beyond the membrane or perhaps early endosome (Fig. 17, lower left panel). This is similar to the dominant negative phenotype reported for this protein in the literature, with the mutant protein preventing trafficking beyond the cell membrane. The constitutively active mutant form of human Rab5 also gave the expected phenotype, expressing as large early endosomes that clearly contain a great deal of trapped transferrin (Fig. 17 lower right panel.) This indicated that the endosomes took up transferrin but prevented it from leaving the fusing early endosomes to continue on the endocytic pathway through the cell.

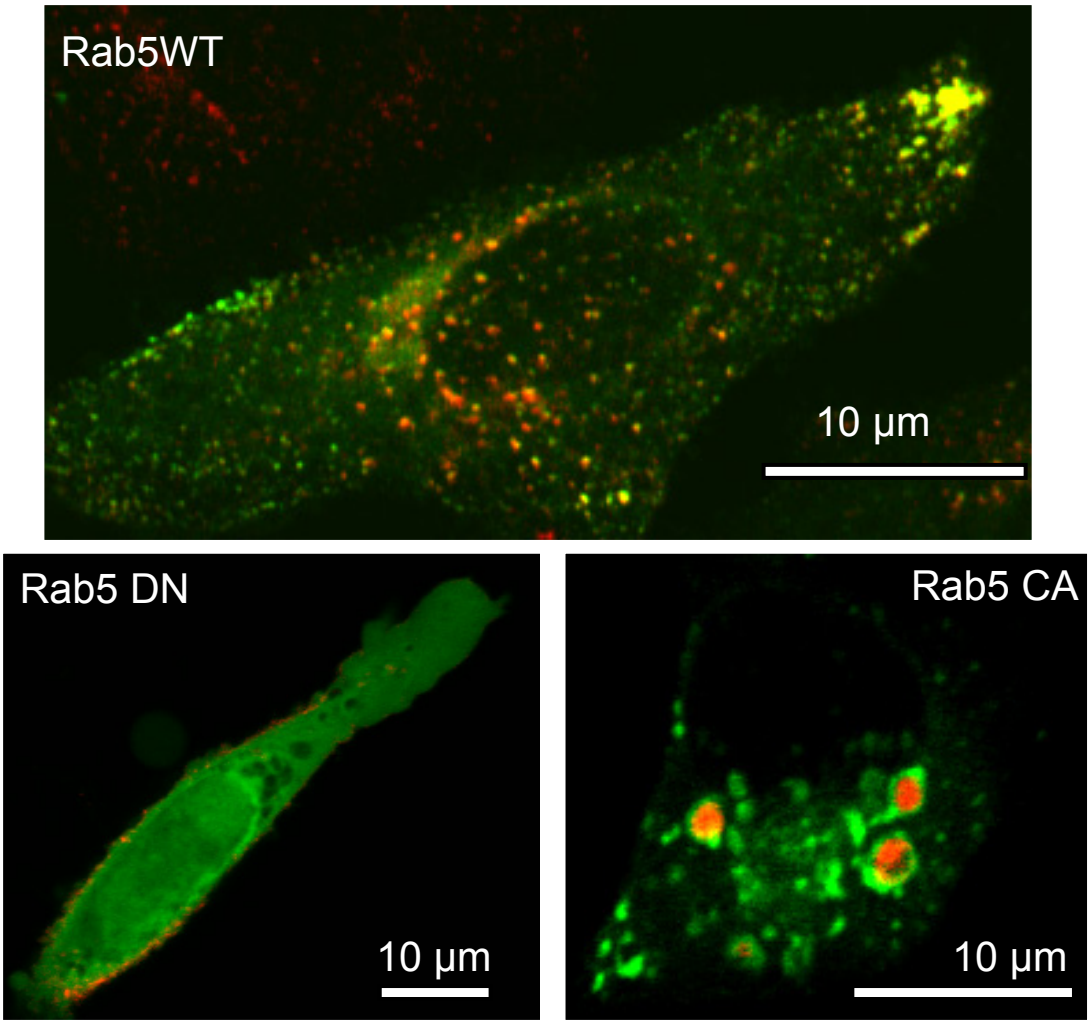


Figure 17. Characterization of human Rab5 WT, DN & CA
Human Hela cells were transfected with expression plasmids encoding either human GFP-Rab5 wild-type, dominant negative or constitutively active protein. Cells were then incubated with labeled human transferrin (red) for 15 min at 37°C, fixed and analyzed by confocal microscopy. A representative slice through the center of the cell clearly shows cytoplasm vs. membrane.

Once the mammalian protein phenotypes were established using the transferrin uptake assay, the mosquito Rab5 genes were then tested. In mosquito cells, transferrin uptake was rapid and evident at 15 min after the addition of labeled transferrin, consistent with the rapid endocytic uptake seen in mammalian cells. Confocal microscopy revealed similar patterns of staining for transferrin in the mosquito GFP-Rab5 WT –expressing C710 cells with transferrin often associated with Rab5-containing vesicles (Fig. 18 top and bottom left panels). Again, the Rab5 proteins seemed to express in larger and fewer vesicles and the transferrin uptake mimicked this vesicular pattern. This could indicate that mosquito cells are less endocyticly active than human cells or that they simply have larger vesicles with more capacity. The activity of the mosquito Rab5 DN mutant was also determined by transferrin uptake. As seen earlier, expression of mosquito Rab5 DN protein presented as diffuse staining throughout the cellular cytoplasm with punctate, vesicular staining along the cell membrane. The DN protein expression blocked transferrin internalization into the C710 mosquito cells much as it did in the human cells, with the transferrin remaining at the cell surface in small vesicles and possibly coated pits (Fig.18 top and bottom middle panels). This is consistent with the function of Rab5 in early endosome formation and trafficking. The mosquito GFP-Rab5 CA was again present on large fluorescent vesicles that contained trapped transferrin (Fig. 18 top and bottom right panels). This is consistent with the CA form of Rab5 causing accumulation of early endosomes by preventing fusion of early with late endosomes and inhibiting the maturation of endosomes beyond the early endosome.

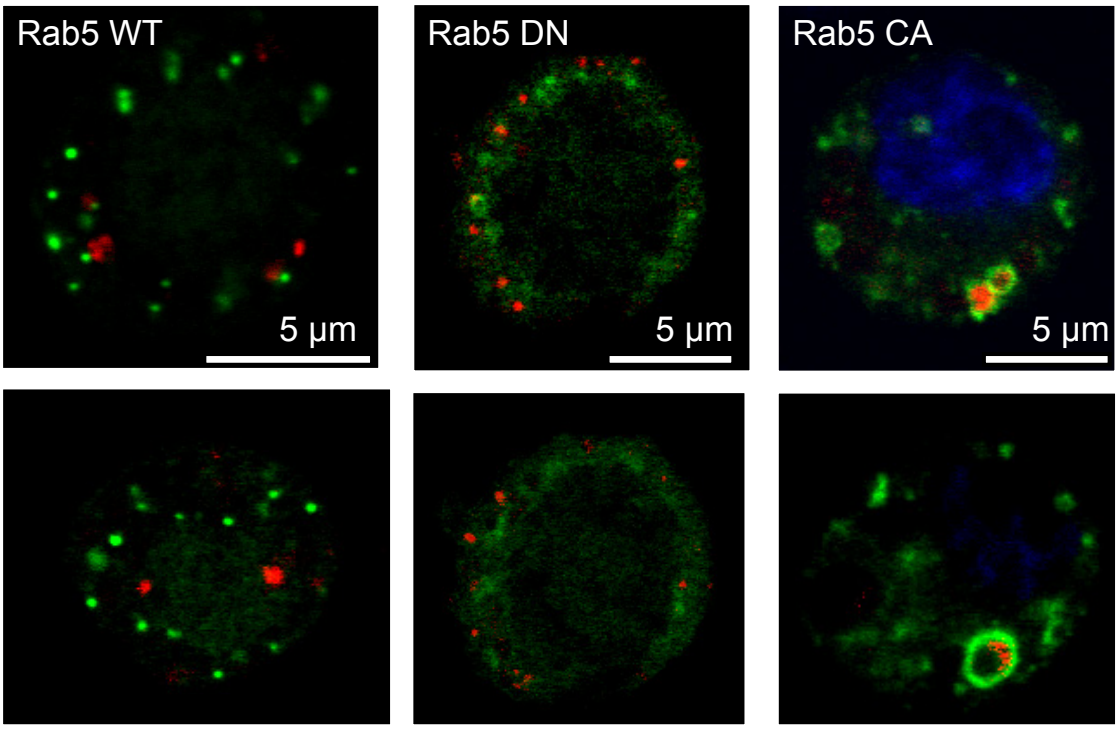


Figure 18. Characterization of mosquito Rab5 WT, DN & CA
Mosquito C710 cells were transfected with expression plasmids encoding either mosquito GFP-Rab5 wild-type, dominant negative or constitutively active protein. Cells were then incubated with labeled human transferrin (red) for 15 min at 27°C, fixed and analyzed by confocal microscopy. A representative slice through the center of the cell is shown. A DAPI stain was also done to illustrate location of nucleus in the mosquito cell (top right corner). The lower pictures have the same scale bars as the picture located directly above.

In each case, the mosquito gene functioned equivalently to the human gene indicating that the mosquito Rab5 protein is similar to that found in human and other mammalian cells. The pattern of transferrin uptake and the phenotypes of each mutant appeared consistent with a conserved role in insect cell endosome formation and maturation.

The function of the mosquito dynamin WT and DN proteins were then tested in the same transferrin uptake assay. As seen before, the dynamin protein expressed in a vesicular pattern throughout the cell with a concentration of protein along the cell membrane. Transferrin uptake can clearly be seen in the cells expressing mosquito dynamin WT protein (Fig.19 left panel). In the cells expressing the DN protein, there was hardly any transferrin entry into the cell and there was even a lack of transferrin bound to the cell surface, indicating that the DN protein may influence binding (Fig.19 right panel). These results are consistent with what has been seen in mammalian cells expressing mammalian WT and DN dynamin proteins, indicating that the mosquito dynamin is functioning in a similar manner.

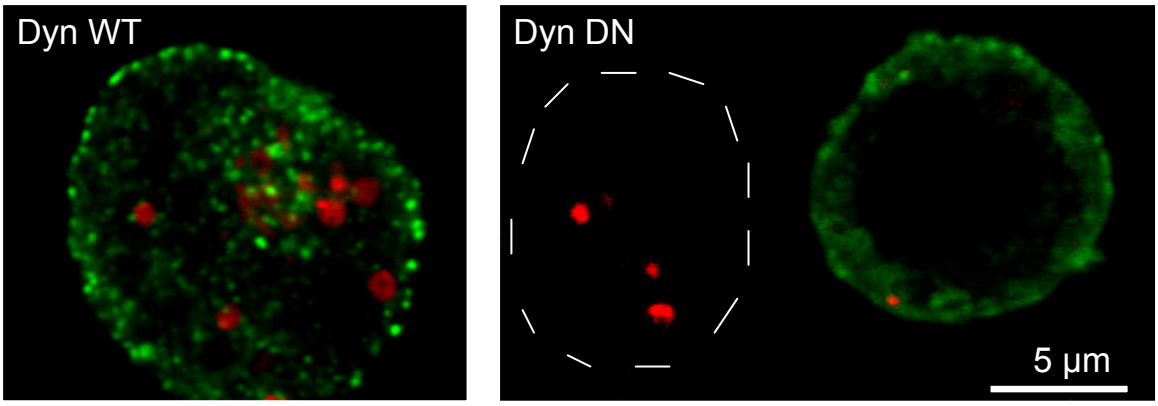


Figure 19. Characterization of mosquito dynamin WT & DN proteins
Mosquito C710 cells were transfected with expression plasmids encoding either mosquito GFP-dynamin wild-type or dominant negative protein. Cells were then incubated with labeled human transferrin (red) for 15 min at 27°C and fixed for confocal microscopy. A representative slice through the center of the cell is shown. The picture of the dynamin DN-expressing cell (right panel) also shows an untransfected cell for comparison (outline of cell is shown by dashed lines).

After confirming that both Rab5 and dynamin proteins functioned as they were expected to along the endocytic pathway, the function of the Rab7 mosquito proteins were then tested. Transferrin is recycled back to the cell surface from the early endosome and therefore its pathway should be unaffected by the mosquito Rab7 DN mutant, since it is located on and influences late endosomes. Indeed, expression of both GFP-Rab7 WT and DN proteins allowed transferrin uptake into the mosquito cells (picture not shown) as expected, though no colocalization between transferrin and Rab7 protein was seen. Rab7 has been reported as important in late endosome formation and lysosome biogenesis (Bucci et al., 2000; Feng, Press, and Wandinger-Ness, 1995). To assess if Rab7 associates with vesicles in mosquito cells, markers for late endosomes and lysosomes must be identified in mosquitoes. Antibodies recognizing late endosome and lysosome markers have yet to be made for mosquito or insect cells, so an alternative method was chosen to test Rab7 function. It was decided to test the cells in an assay with Lyso Tracker reagent (Invitrogen, CA). This weak base accumulates and reaches peak fluorescence in highly acidic compartments, typically late endosomes and lysosomes, and has previously been used to characterize Rab7 function in mammalian cells (Bucci et al., 2000; Gutierrez et al., 2004). To confirm the function of Lyso Tracker and establish a phenotype for the Rab7 proteins, the reagent was first tested using human proteins in Hela cells. Cells were transfected with either human GFP-Rab7 WT or DN and then incubated with Lyso Tracker reagent for 1 h at 37°C. The reagent colocalized nicely with the Rab7WT in the Hela cells while the Rab7 DN-expressing cells did not seem to accumulate any Lyso Tracker (Fig.20). This confirmed that the DN mutant was functioning to prevent endocytosis beyond the late endosome.

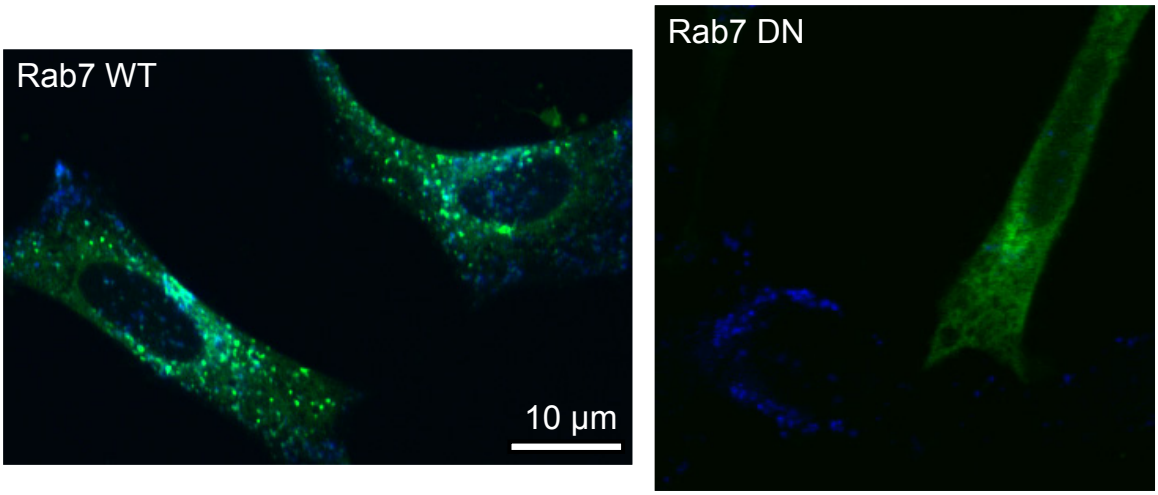


Figure 20. Characterization of human Rab7 WT & DN proteins
Hela cells were transfected with expression plasmids encoding either human GFP-Rab7 wild-type or dominant negative protein. Cells were then incubated with labeled Lyso Tracker (blue) 1 h at 37°C and fixed for confocal microscopy. A representative slice through the center of the cell is shown in all cases.

Since the reagent seemed to function as expected in human cells, the Lyso Tracker was then used to characterize the mosquito Rab7 proteins in mosquito cells. C710 cells were transfected with either GFP-Rab7 WT or DN and then incubated with Lyso Tracker reagent for 1 h at room temperature. It was observed that cells expressing GFP-Rab7 WT accumulated Lyso Tracker (red) in perinuclear vesicles and that the Rab7 co-localized with the Lyso Tracker (Fig. 21 top panels). The cells that expressed the DN protein did not accumulate any Lyso Tracker which suggested that the Rab7 mutant protein was not allowing the reagent to reach the late endosome/lysosome as it did in the WT-expressing cells (Fig.21 bottom panels).

Together with the lack of association between labeled Rab7 with transferrin, the co-localization with Lyso Tracker indicated that the Rab7 homolog was present in late endocytic vesicles and/or lysosomes as expected. In summary, this portion of the work indicates that mosquito Rab5 is necessary for early endosome formation and Rab7 likely has a role in endocytic trafficking towards lysosomes. In addition, mosquito dynamin plays an early role in endocytosis near the cell membrane. These observations indicate that Rab5, Rab7 & dynamin all have conserved roles for endosome function and maturation in insect cells as in mammalian cells and will be useful for study of virus entry pathways.

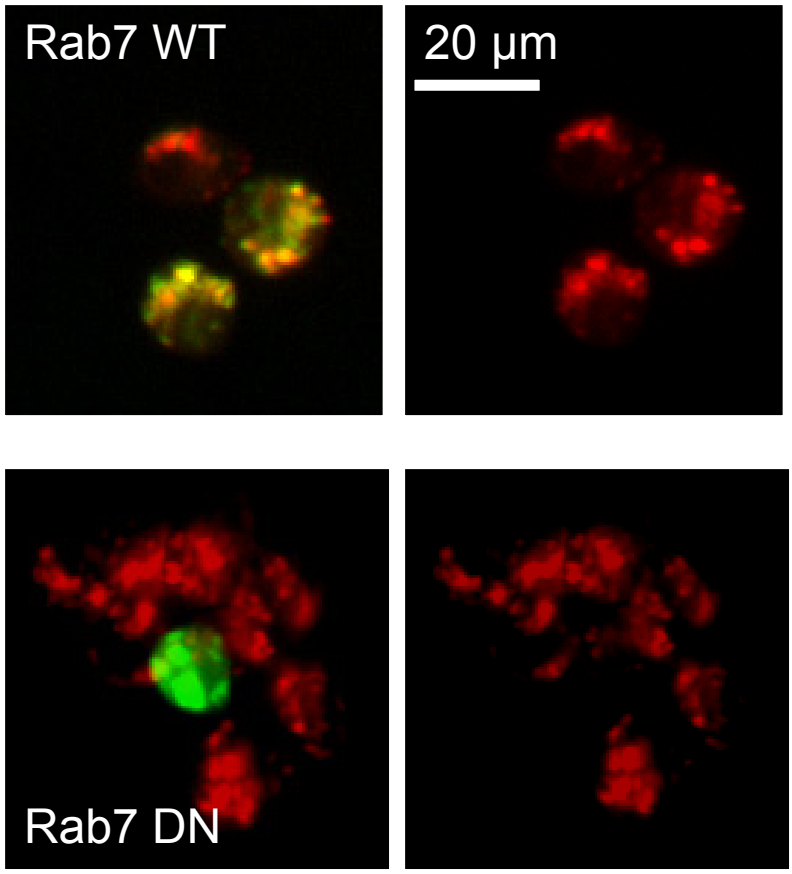


Figure 21. Characterization of mosquito Rab7 WT & DN proteins
Mosquito C710 cells were transfected with expression plasmids encoding either mosquito GFP-Rab7 wild-type (top two panels) or dominant negative (bottom two panels) protein (green). Cells were then incubated with labeled Lyso Tracker (red) for 1 h at 27°C and fixed for confocal microscopy. A representative slice through the center of the cell is shown. Colocalization will appear yellow in color.

Chapter 18: Functional Rab5 and Rab7 are Required for VEEV Infection of Mosquito Cells

Given the important roles of Rab5, Rab7 & dynamin in endosomal trafficking, it was expected that expression of the DN and CA proteins in mosquito cells should disrupt VEEV infection if endocytosis was required for productive infection. Rab5 function will be necessary if VEEV enters the cell and utilizes early endosomes for trafficking from the membrane. Rab7 function will be important if the late endosome must be accessed by the virus before fusion can occur and the genome can be released. Dynamin will almost certainly be required if VEEV enters mosquito cells via clathrin-mediated endocytosis, as it is a crucial part of the mechanism by which clathrin coated pits become vesicles.

Flow cytometry was used to get a quantitative and accurate measurement of how expression of each protein affected subsequent infection of the mosquito cells by VEEV. After transfection of the plasmids encoding for the various endocytic proteins, cells were infected with the SIN83 chimeric virus. This is the same as the virus used earlier with the chemical inhibitors except that it lacks the second subgenomic promoter as well as the GFP marker. After infection, cells were fixed and incubated with an antibody against VEE (711, ATCC) and then incubated with a labeled secondary antibody for detection. Cells were analyzed by flow cytometry to detect expression of the GFP fusion proteins and subsequent infection with the SIN83 virus (detected by the antibody). Infection of cells that were not expressing the indicated protein was compared to infection of cells expressing the transfected gene fused to GFP. Controls were virus alone, expression of GFP alone and virus infection of cells expressing GFP alone. This analysis by flow cytometry allowed a detailed analysis of the effects of expressing the wild-type versus the mutant endocytic genes on VEEV infection of mosquito cells and gave internal controls for variation in cell number, MOI and transfection efficiency.

First, the mosquito cells were transfected with human Rab5 and Rab7 wild-type and mutant genes to determine how their expression impacted subsequent virus infection in mosquito cells. The wild-type human Rab5 had little effect on infection but both the

DN S34N and the CA Q79L forms of human Rab5 significantly decreased the SIN83 infection (Fig. 22, upper panel) by 45 and 30%, respectively ($P<0.01$). This not only suggested a function for Rab5 in VEEV infection of mosquito cells but also indicated that the human and mosquito Rab5 proteins are similar in function. Next, the mosquito Rab5 genes were tested in the same infection assay with flow cytometry. Interestingly, as the amount of mosquito Rab5 WT homolog expression increased, the infection by SIN83 also increased, rising 2.5-fold over the infection seen for cells transfected with only GFP. This suggested that Rab5 activity in mosquito cells is normally limiting for infection. Similar to the human Rab forms, the expression of the mosquito Rab5 DN S34N and CA Q79L proteins also decreased infection but to a greater extent, by an average of 53 and 57%, respectively ($P<0.01$) (Fig. 22, middle panel). Neither the human nor the mosquito Rab5 DN S34N protein expression and subsequent infection with SIN83 differed significantly from the Rab5 CA Q79L protein expression and infection ($p>0.05$). The results of the flow cytometry assay clearly illustrated that Rab5 function and therefore early endosomes are necessary for mosquito cell infection with the SIN83 virus and therefore VEEV.

The experiment was repeated with mosquito Rab7 WT and DN T22N proteins. After transfection, the C710 cells were infected with the SIN83 virus and cells were fixed and analyzed by flow cytometry. Unlike the Rab5 WT-expressing cells, the transfection of the WT Rab7 did not have a significant impact on SIN83 infection, instead increasing infection by a small amount (20%). In contrast, expression of the Rab7 DN T22N mutant significantly lowered the infection of the cells (Fig.22, lower panel), by 45% ($P<0.05$). Together, these results suggest that functional early as well as late endosomes are important for VEEV infection of mosquito cells. The raw flow data for GFP alone, virus alone, GFP with virus and the five mosquito Rab proteins with virus infection is shown in Figure 23.

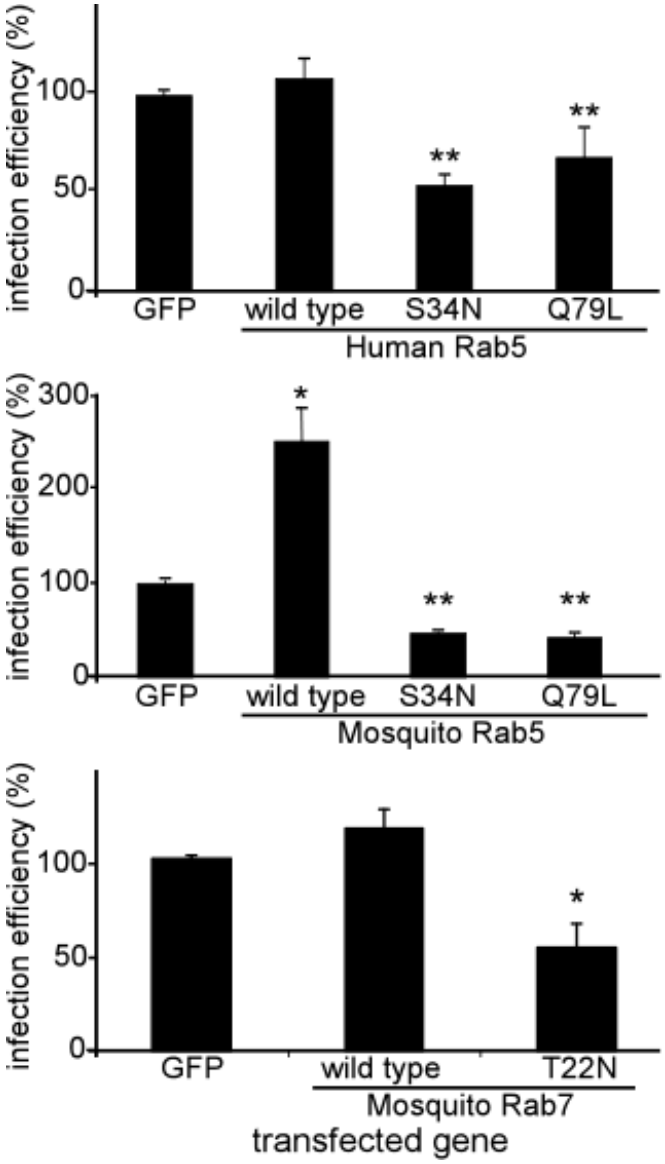


Figure 22. Rab5 and Rab7 are necessary for infection of mosquito cells with SIN83. C710 cells were transfected with an insect expression plasmid containing either GFP alone or genes for either the human or mosquito proteins (Rab5 WT, Rab5 DN S34N, Rab5 Q79LCA, Rab7 WT, Rab7 DN T22N) fused to GFP. After 24h, cells were challenged with SIN83 virus. Cells were fixed 12 h post-infection and stained with an anti-VEEV env antibody (ATCC, VA), as a marker of infection. Flow cytometery analysis was used to determine the impact of gene expression on infection and infection efficiency was calculated as described in Fig. 21. The effects of both human Rab5 expression (upper), mosquito Rab5 homolog expression (middle) and mosquito Rab7 homolog expression (bottom) are shown. The average of three separate experiments is shown +/- standard deviation. * $p<0.05$, ** $p<0.01$

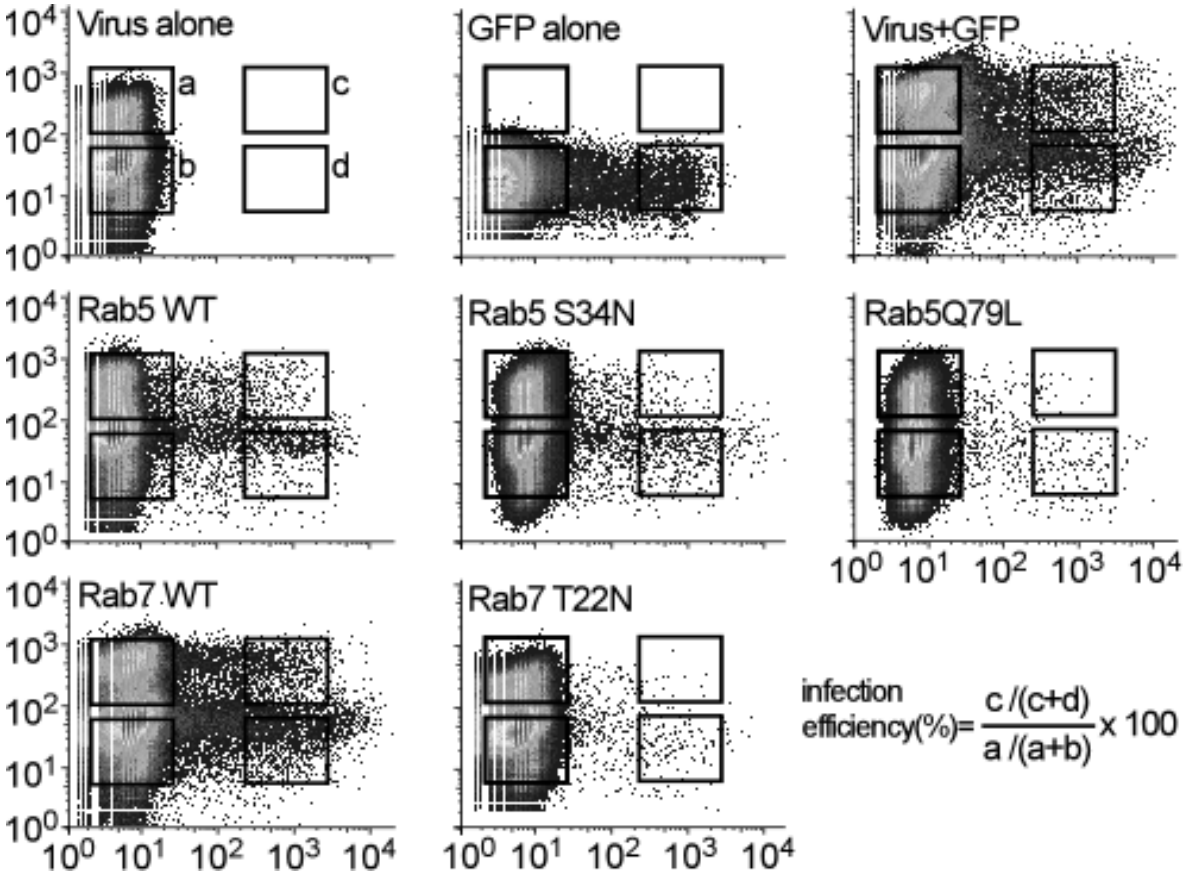


Figure 23. Flow cytometry analysis of infection in mosquito cells. C710 cells were transfected with each indicated gene fused to GFP. After 1 d cells were infected with SIN83 virus. Cells were then stained for VEEV env expression. Cells were analyzed by FACS flow cytometry and divided into 4 quadrants (a,b,c,d, shown top left panel) where a=cells infected but not expressing the GFP-fusion protein, b= cells not infected and not expressing the GFP fusion protein, c= cells infected and expressing the GFP fusion protein at moderate to high levels and d=cells not infected but expressing the GFP fusion protein. The impact of gene expression on infection was calculated as shown at lower right where the proportion of infected cells expressing the GFP fusion protein is expressed as a percentage of uninfected cells expressing the GFP fusion protein. This analysis internally controls for experiment to experiment variation in: virus infectivity, expression plasmid transfection efficiency and total cell number. The top three panels serve as controls showing staining with virus alone, GFP alone (no added virus) and virus infection in the presence of GFP. The lighter shades indicate higher numbers of cells.

Chapter 19: Functional Dynamin is Required for VEEV Infection of Mosquito Cells

The infection assay was then repeated to test the impact of mosquito dynamin on VEEV infection of mosquito cells. C710 cells were transfected with either GFP-dynamin WT or DN expression plasmids and then infected with the SIN83 virus. The cells were then fixed and analyzed by flow cytometry as they were previously after expression of the Rab proteins. Expression of the mosquito dynamin WT protein had a slight effect on virus infection, with SIN83 infection increasing approximately 20% after in expressing cells. The dynamin DN protein reduced infection by 25% ($P<0.05$) (Fig.24). This indicated that the dynamin protein may be important for virus entry but a fair amount of virus is entering the cell even in the presence of inactive dynamin. A complete knockdown of the protein would be useful in determining of the protein is absolutely required for VEEV infection. However, these results along with the requirement for Rab5 & Rab7, clearly illustrate that VEEV is utilizing an endocytic pathway to enter and infect mosquito cells and that the virus has a distinct requirement for endosomal acidification and endosomal trafficking in mosquito cells.

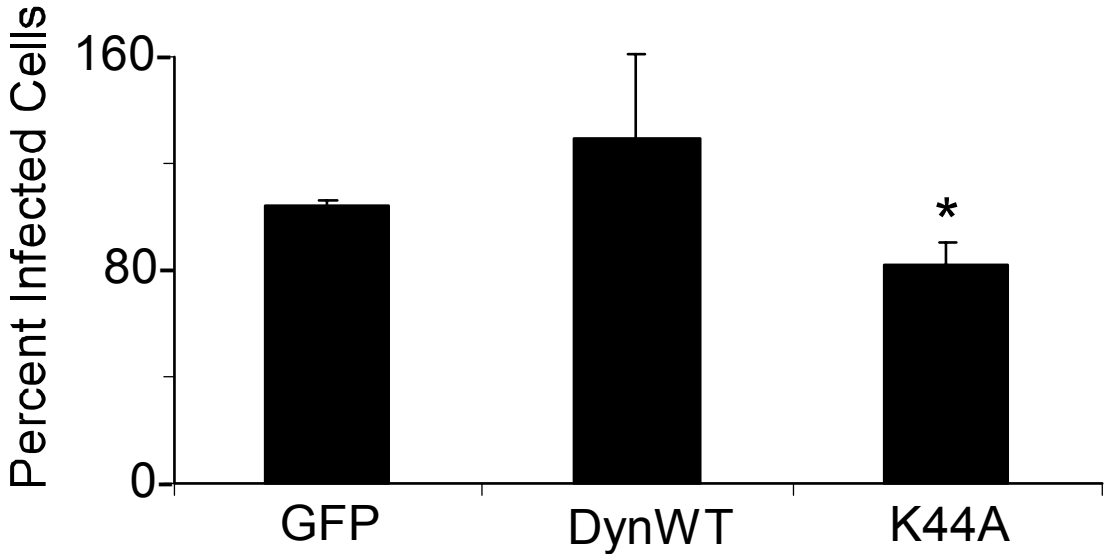


Figure 24. Dynamin is necessary for infection of mosquito cells with SIN83.
C710 cells were transfected with an insect expression plasmid containing either GFP alone or genes for mosquito GFP-dynamin WT or DN K44A. After 24h, cells were challenged with SIN83 virus. Cells were fixed 12 h post-infection and stained with an anti-VEEV env antibody (ATCC, VA), as a marker of infection. Flow cytometery analysis was used to determine the impact of gene expression on infection and infection efficiency was calculated as described in Fig. 21. The average of three separate experiments is shown +/- standard deviation. * $p<0.05$

Chapter 20: Identification & Characterization of Mosquito Clathrin

Once it was clear that VEEV was entering the mosquito cell via endocytosis, the next question was which endocytic route was being taken to gain access to the cell. Since SFV has been reported to utilize clathrin-mediated endocytosis to enter mammalian cells, that pathway was a likely candidate for the route being used by VEEV. Going back to the *Aedes aegypti* genome and doing another BLAST search revealed a putative mosquito clathrin protein that showed 77% amino acid identity to human clathrin heavy chain, with both human and mosquito proteins containing 1675 amino acids in length. Primers were designed against the mosquito clathrin DNA sequence and PCR was performed using the *Aedes* cDNA previously made from the C710 RNA. The clathrin gene is large, with over 5,000 base pairs and attempts to isolate the gene using flanking oligos failed. It was decided to do the PCR in two steps with two sets of oligos, flanking and internal. First, two reactions were done separately to obtain a 5' and a 3' portion of the clathrin gene (two halves that together equal the entire gene). Oligos were designed to give an overlapping region at the junction of the two segments. Once the individual halves of the putative clathrin gene were obtained and purified, additional PCR was done with these amplicons as templates for full-length clathrin. The PCR amplicons are shown after running them on a 1% agarose gel (Fig.25).

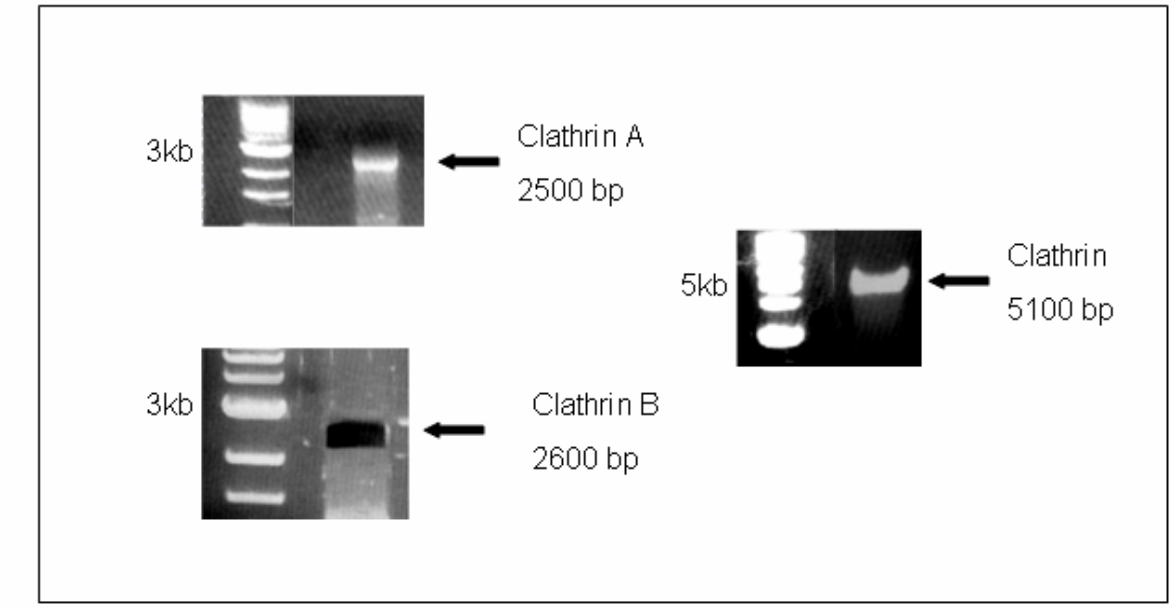


Figure 25. PCR amplicons for the mosquito clathrin gene
Photograph of agarose gel with DNA bands representing the PCR products for the putative mosquito clathrin gene. The amplicons were run alongside a 1 kb ladder (New England Biolabs). Full-length clathrin is approximately 5100 base pairs of DNA. The amplicon for Clathrin B was excised from the gel for cloning purposes prior to the photograph.

While the PCR reaction to obtain mosquito clathrin yielded a DNA amplicon of appropriate size, cloning into a suitable expression vector proved extremely difficult. Attempts to use TOPO cloning-based methods, as was done with the other mosquito endocytic proteins, were unsuccessful. This could be partly due to the large size of the clathrin gene. In addition, the gene contained many useful restriction endonuclease sites so the digestion and ligation of the gene into a plasmid was highly problematic. Unfortunately, the gene was never successfully cloned into a plasmid and was therefore not expressed in mosquito cells. To overcome this obstacle, two antibodies were identified, an antibody against human clathrin heavy chain as well as an antibody against human clathrin light chain A, that bound mosquito clathrin. These were used to help demonstrate the function and location of clathrin within the mosquito cells. First, a Western blot was done with each antibody to establish that the mosquito cell did constitutively express clathrin proteins. A solid band representing both clathrin heavy chain (left) as well as clathrin light chain (right) were clearly be seen in the blot (Fig.26).

Since mammalian clathrin-specific antibodies reacted with mosquito cell proteins and the PCR done with oligos designed from alignment with mammalian clathrin produced putative mosquito clathrin DNA of appropriate size, this suggested that the mosquito cells did indeed contain clathrin protein. The next step was to demonstrate that the mosquito clathrin had a homologous function to mammalian clathrin in endocytosis. The C710 cells were incubated with the human transferrin used in the previous uptake assays and then fixed as before. With no GFP- clathrin expression plasmid to mark location of the mosquito protein in the cell, the antibody against human clathrin light chain A (CLTA) was used to stain mosquito clathrin after fixation and a labeled secondary antibody was used to visualize the protein during examination by confocal microscopy. The mosquito clathrin clearly colocalized with transferrin at several points throughout the cell, indicating that clathrin is somehow functioning in the endocytic uptake of transferrin and that its function may not be limited to coated pits at the cell surface (Fig.27, colocalization indicated by white circles, merged images at far right).

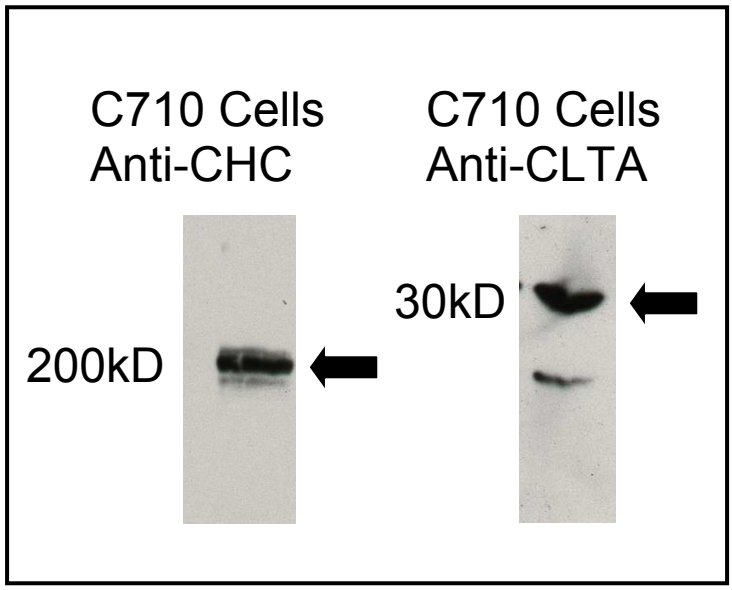


Figure 26. Identification of mosquito clathrin via Western blot
Mosquito cell lysates were boiled in SDS-PAGE buffer with 2% β-mercaptoethanol and run on a 6% SDS-PAGE gel. They were then transferred to nitrocellulose and the subsequent Western blot was probed with monoclonal antibodies against human clathrin heavy chain (CHC) (left blot) and human clathrin light chain A (CLTA) (right blot.) Black arrows indicate the location of the clathrin protein.

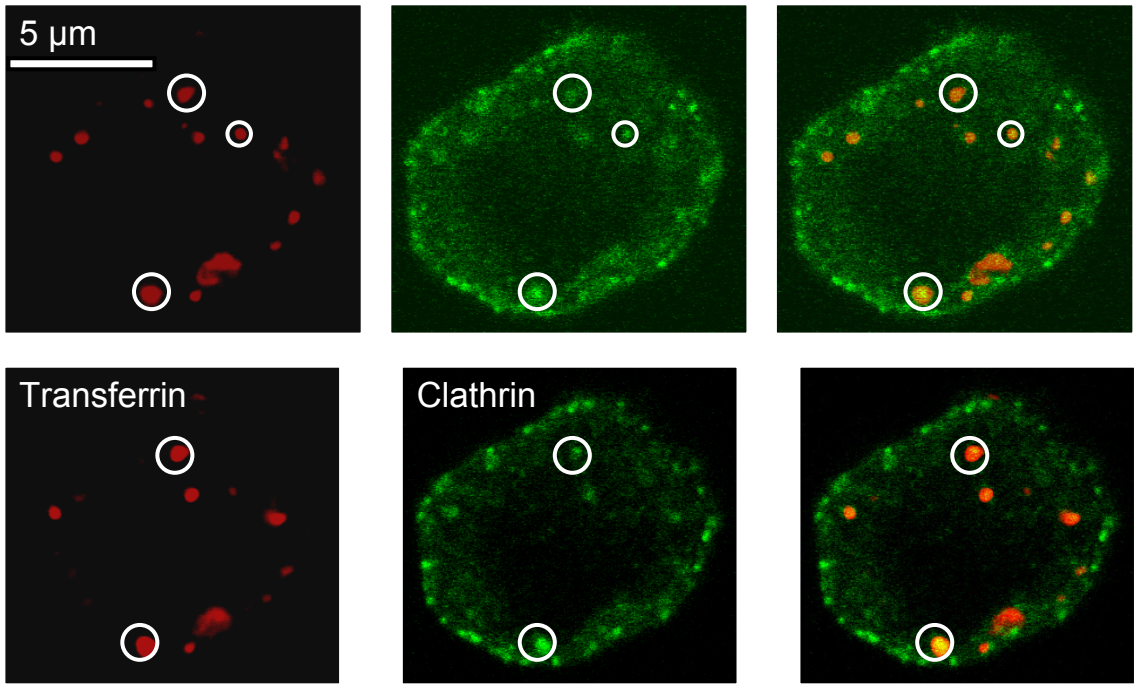


Figure 27. Colocalization of transferrin with mosquito clathrin
After incubation of C710 mosquito cells with labeled transferrin (red – far left), cells were fixed and incubated with a monoclonal antibody against human clathrin light chain (green – middle). Cells were then examined by confocal microscopy. The top and bottom rows show two individual cells and a representative slice through the center of each cell is shown. Two areas of colocalization are indicated by white circles. The transferrin protein can be seen in the left panel (red), the antibody-bound mosquito clathrin can be seen in the middle panel (green) and merged images are in the far right panel.

After confirming that the mosquito cells contained clathrin and that the protein had a function along the endocytic pathway, at least in uptake of transferrin, the interaction of clathrin with dynamin was investigated. Dynamin is known to mediate the movement of endocytic vesicles from the cell surface into the cell and is essential for clathrin-mediated endocytosis since it is involved in vesicle release from the plasma membrane (Song, Leonard, and Schmid, 2004). It therefore would be expected to interact with clathrin, especially at the cell surface. C710 cells were transfected with GFP-dynamin WT plasmid and fixed after protein expression was seen. The fixed cells were then incubated with the human clathrin light chain antibody and examined by confocal microscopy. Expression of both the transfected dynamin protein and the clathrin (indicated by bound antibody) is greatest at the cell surface, as expected (Fig.28). There was almost complete colocalization between the two proteins, suggesting that clathrin and dynamin may work together in a similar manner as they do during endocytosis in mammalian cells. More information about the mosquito clathrin protein will be revealed once it is successfully cloned and mutant proteins made as they were for the other endocytic proteins. It will also be interesting to investigate the role clathrin plays in virus entry, if any.

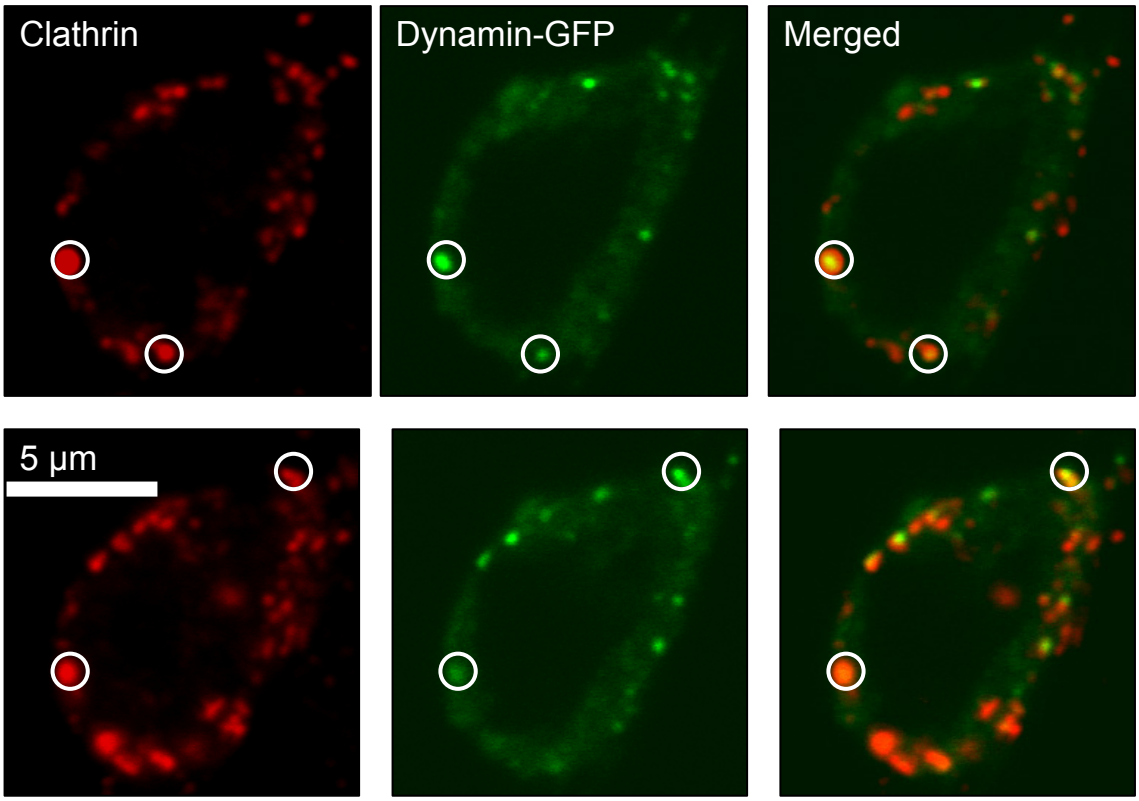


Figure 28. Colocalization of mosquito dynamin with mosquito clathrin
After transfection of C710 mosquito cells with dynamin-GFP WT, cells were fixed and incubated with a monoclonal antibody against human clathrin light chain A and a labeled secondary antibody (red). Cells were then examined by confocal microscopy. The top and bottom rows show two individual cells and a representative slice through the center of each cell is shown. Two areas of colocalization are indicated by white circles. The antibody-bound mosquito clathrin can be seen in the left panel (red), the dynamin-gfp protein can be seen in the middle panel (green) and merged images are in the far right panel.

Chapter 21: Mosquito Actin

Given the important role that actin plays in mammalian endocytosis, it was important to investigate what function, if any, the actin protein played in mosquito cell endocytosis as well as in the internalization of virus particles. Actin is known to play major roles within the cytoskeleton of many different cell types yet it was important to confirm the presence of the protein and examine its function in the mosquito cells. The first step in determining actin function was to identify actin protein in mosquito cells. For this, phalloidin-594 was used to stain the mosquito actin after fixation of the cells. Phalloidin is a toadstool toxin from *Amanita phalloides* that is widely used to visualize filamentous or polymerized actin (F-actin) in fixed cells (Lengsfeld et al., 1974). Figure 29 shows the pattern of phalloidin staining in the mosquito cells, which is presumably mosquito actin. The mosquito actin was distributed throughout the mosquito cell, with a high concentration of the protein close to the cell membrane. The cells displayed a similar staining pattern as is seen in mammalian cells with two notable exceptions; the mosquito cells seemed to have fewer filaments and surface projections away from the cell body than is normally seen in mammalian cells.

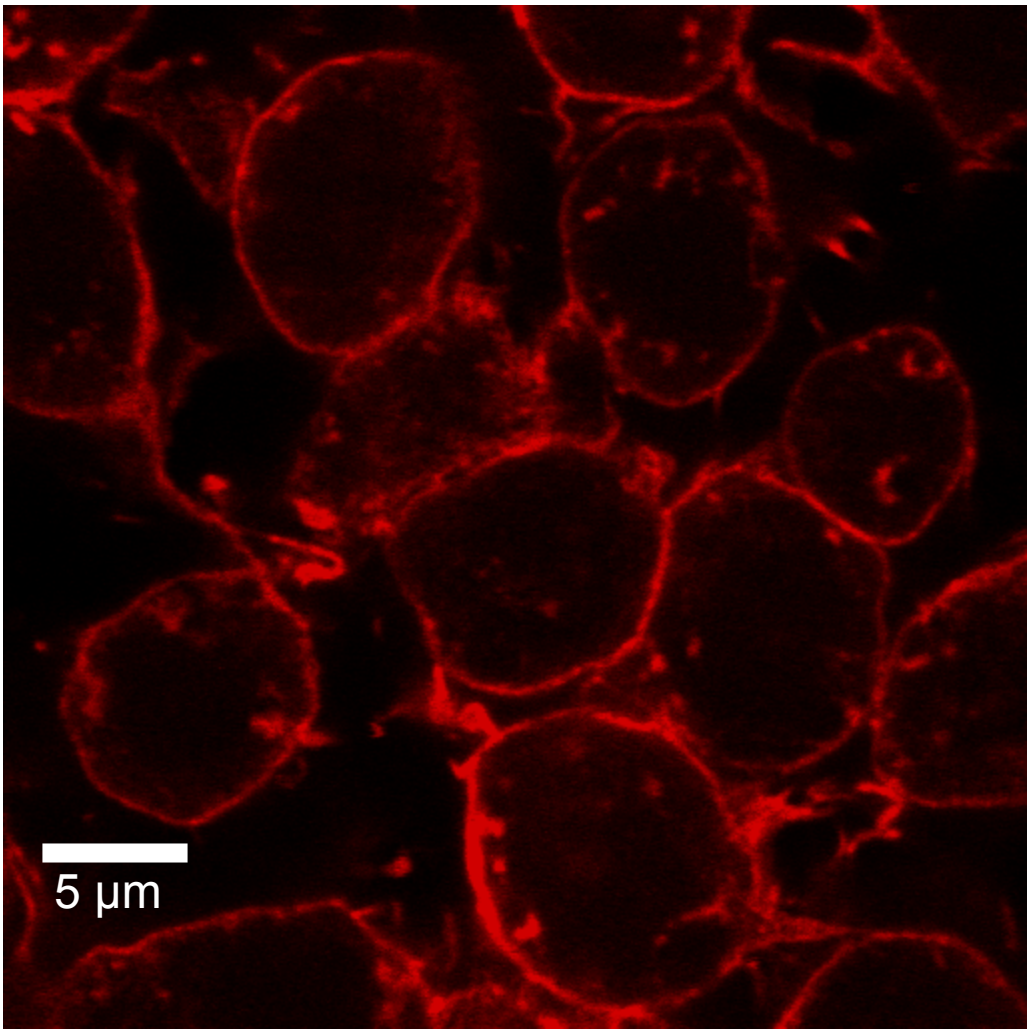


Figure 29. Visualization of mosquito actin
C710 cells grown on coverslips were fixed and incubated with a phalloidin-594 stain (red) for 20 min at room temperature. Excess stain was washed off and cells were then examined by confocal microscopy. A representative slice through the cells near the bottom of the cell body (where they attach to the slide) is shown for a wide field of cells.

To confirm that the phalloidin was staining the actin protein in the mosquito cells, a second approach was used involving the moesin protein. Moesin is an actin-binding protein that is abundant in actin-containing structures such as microvilli, lamellipodia and membrane ruffles (Franck, Gary, and Bretscher, 1993). Moesin is also one of several proteins responsible for linking actin filaments to the cell membrane and is known to colocalize with F-actin in the cytoskeleton (Niggli and Rossy, 2007). We obtained an insect moesin from *Drosophila melanogaster* that was fused to GFP. This construct has been used with success to illustrate the distribution of actin filaments in live Drosophila flies and has provided an excellent model for cell shape and cytoskeletal pattern *in vivo* (Edwards et al., 1997). Its behavior in mosquito cells was previously unknown. Using this construct together with the phalloidin stain would confirm that we were staining F-actin in the mosquito cells should the two proteins localize to similar places within the cell and along the membrane. After transfection of this moesin-GFP construct into C710 cells, the cells were fixed and stained with phalloidin-594 (red) to visualize the actin. The moesin protein had almost complete colocalization with the phalloidin staining, confirming that the phalloidin was staining a mosquito actin protein in the cells (Fig.30).

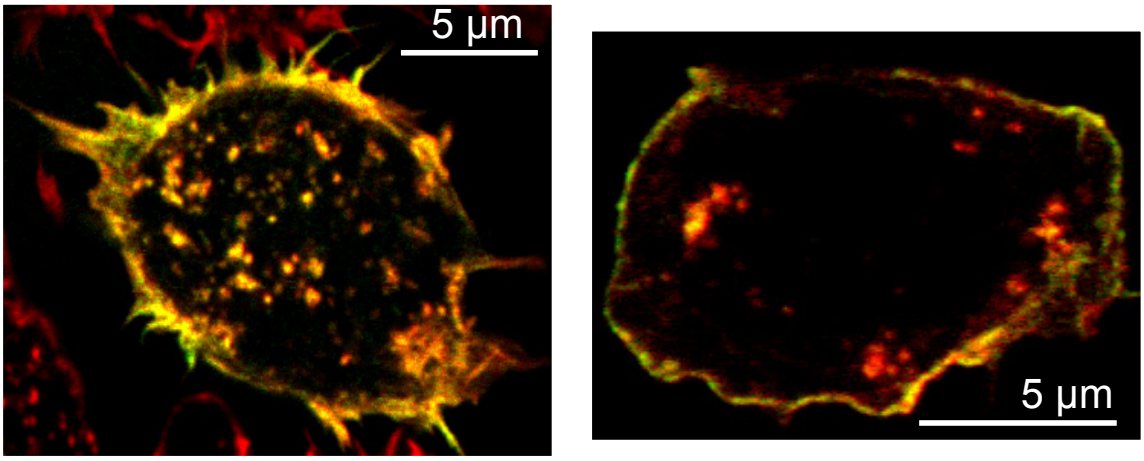


Figure 30. Colocalization of moesin-gfp with mosquito actin
After transfection of C710 cells with Drosophila moesin-gfp protein (green), cells were fixed and incubated with a phalloidin-594 stain (red) for 20 min at room temperature. Excess stain was washed off and cells were then examined by confocal microscopy. A representative slice through the cell is shown for two individual cells. Areas of colocalization will be yellow. The left panel shows a slice at the bottom of the cell body where it attaches to the slide (thus the filopodia and actin elongation) and the right panel shows a slice through the middle of a cell.

Chapter 22: Actin in VEEV Internalization & Infection

There are many examples of viral interactions with the cellular cytoskeleton and in many cases these interactions are crucial components of the viral lifecycle. Viruses utilize both actin and microtubules for many different functions, including attachment, entry, for intracellular transport, as cofactors for replication and for return to the cell surface for budding. Some viruses even alter the cytoskeleton by inducing the depolymerization of actin to aid in diffusion through the cellular cytoplasm (Smith and Enquist, 2002). The entry and endocytosis of several viruses has been shown to require actin polymerization, including adenovirus and vaccinia (Smith and Enquist, 2002).

To probe the involvement of actin in VEEV internalization, labeled virus was used along with the phalloidin stain. The SIN83 virus was labeled using AlexaFluor 350 carboxylic acid, succinimidyl ester, which is an intense blue dye that will covalently bind to primary amines. The dye will be covalently linked to the virus through the lysines located on the viral glycoproteins. The virus was designated SIN83-blue in the present work. Cells were incubated with the SIN83-blue for 30 min at RT, fixed and then stained with phalloidin-488 (green). Numerous virus particles were seen colocalized with F-actin bundles within the cellular cytoplasm (Fig. 31). This suggests that F-actin plays a key role in virus uptake in mosquito cells.

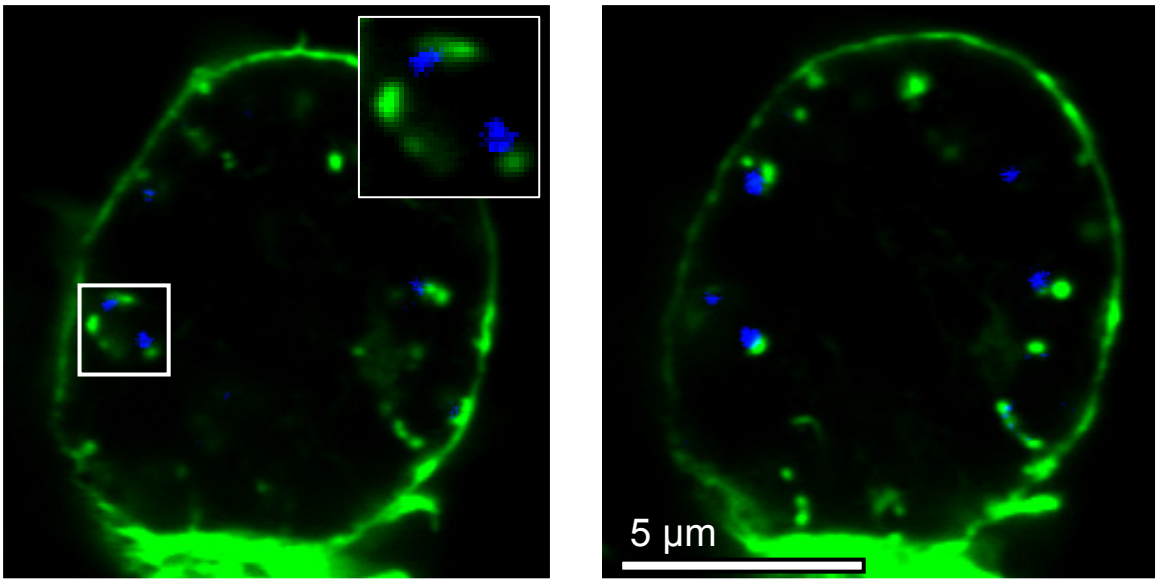


Figure 31. Association of VEEV with F-actin in the mosquito cell
After incubation of C710 cells with SIN83-blue for 30 min at RT, cells were fixed and incubated with a phalloidin-488 stain (green) for 20 min at room temperature. Excess stain was washed off and cells were then examined by confocal microscopy. A representative slice through the center of the cell is shown for two individual cells. The white square highlights an area of colocalization cropped in.

It was also observed that the internalization of VEEV appeared to cause the formation of actin rings in the mosquito cells (Fig.32). Actin rings are known to be involved in several cellular functions, including cytokinesis and osteoclastic bone resorption and their production is often stimulated by cortactin (Albertson, Riggs, and Sullivan, 2005; Hurst et al., 2004). Cortactin is thought to be one of the primary proteins responsible for linking actin to dynamin during endocytosis (Cao et al., 2003). Therefore, the virus could be inducing the formation of actin rings during endocytosis by linking dynamin and cortactin. It will be interesting to see if the actin rings have a function in virus internalization or trafficking though the cell. Alternatively, the actin may be present on the membranes of the endocytic vesicles during endocytosis of the virus, which would present as ring-like structures under analysis by microscopy. More studies must be done in order to confirm the presence of actin rings and determine the functions, if any, they play in the internalization of VEEV.

Again, to confirm that the phalloidin was staining the actin protein in the mosquito cells once infected with virus, a second approach was used involving the moesin protein. After transfection of the moesin-GFP expression construct into C710 cells, the cells were incubated with the SIN83-blue for 30 min at RT. The cells were then fixed and stained with phalloidin-594 (red) for 20 min at RT to visualize the actin. Again, the moesin protein had almost complete colocalization with the phalloidin staining after incubation with the virus, confirming that the phalloidin was staining a mosquito actin protein in the cells (Fig.33). Both proteins colocalized with the virus in the cellular cytoplasm. This also suggests that moesin may play a role in virus internalization since it was found in close proximity to both virus and actin well within the cell after incubation with the SIN83. Future work with mosquito moesin protein would help determine the role this protein may play in both endocytosis and virus infection.

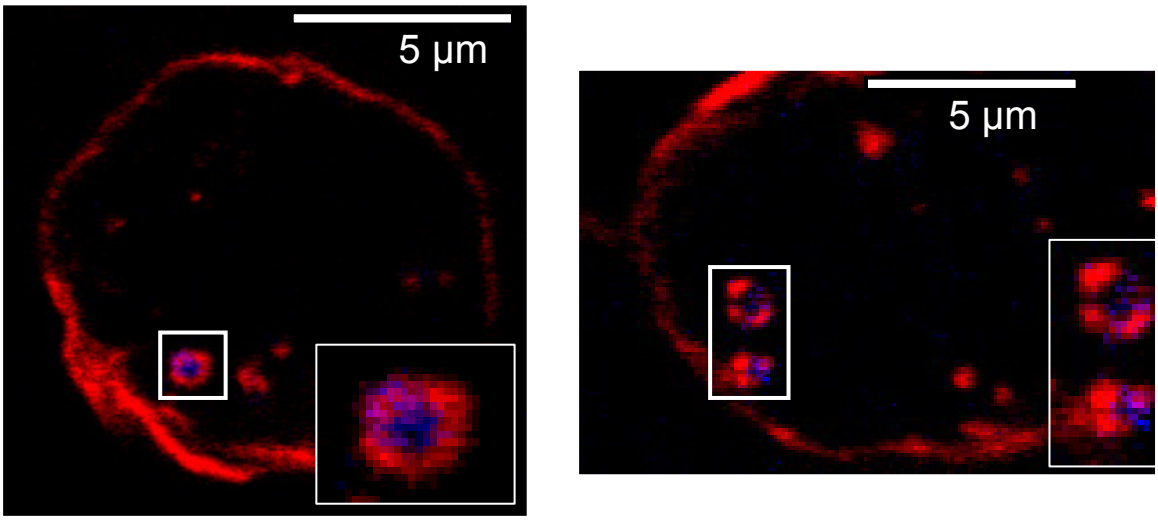


Figure 32. VEEV may initiate the formation of F-actin rings in the mosquito cell
After incubation of C710 cells with SIN83-blue for 30 min at RT, cells were fixed and incubated with a phalloidin-594 stain (red) for 20 min at room temperature. Excess stain was washed off and cells were then examined by confocal microscopy. A representative slice through the center of the cell is shown for two individual cells. The white squares show areas of colocalization and actin rings cropped in.

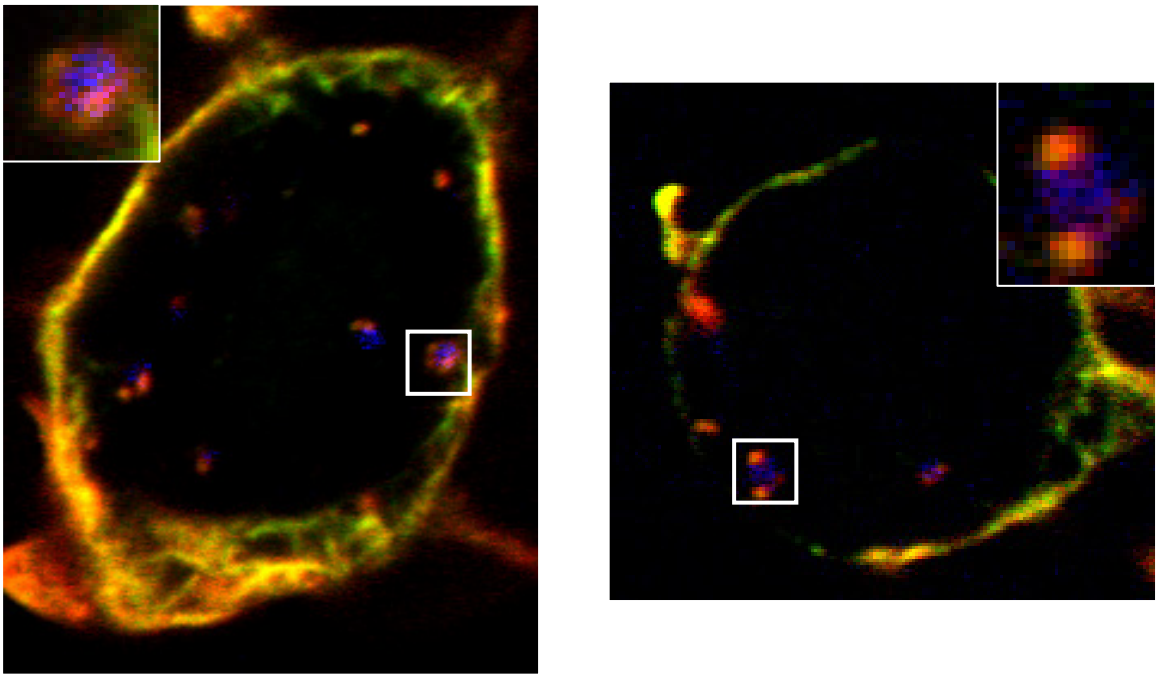


Figure 33. Colocalization of moesin-gfp with mosquito actin with SIN83-blue
After transfection of C710 cells with Drosophila moesin-gfp protein (green), cells were incubated with SIN83-blue for 30 min at RT, fixed and stained with phalloidin-594 (red) for 20 min at room temperature. Excess stain was washed off and cells were then examined by confocal microscopy. A representative slice through the cell is shown for two individual cells. Areas of actin and moesin colocalization will be yellow. The white squares show areas of interest that were enlarged at top left and right for each image respectively.

Since actin seemed to be involved in some manner in the entry of VEEV, the next question was if dynamic actin was necessary for VEEV internalization. The involvement of actin polymerization in virus internalization is often investigated using cytochalasin-D, which is a drug that inhibits actin polymerization and prevents the formation of F-actin (Lee and Song, 2007). Cytochalasin D is also known to inhibit the formation of actin rings in fission yeast (Gabriel et al., 1998).

To investigate if actin polymerization was required for the entry VEEV, C710 cells were incubated with 1μg/mL cytochalasin-D for 1 h at RT and incubated with SIN83-blue for 30 min at RT. After fixation, the cells were examined by confocal microscopy. When actin was prevented from polymerizing by the drug, blue virus was seen on membrane structures but not within the cell (Fig.34). This confirmed that actin polymerization was involved in VEEV internalization, whether it was during endocytosis or for the formation of actin rings or other structures is unclear. Since actin polymerization seems to be necessary for the early stages of VEEV infection, it may have a role in other steps of the viral lifecycle, such as replication or budding.

Though the microscopy studies indicated that VEEV internalization may require actin polymerization, a quantitative assay was necessary to determine if dynamic actin was crucial for virus entry. To confirm the necessity of actin polymerization for VEEV internalization, the luciferase entry assay was used with the VEEV pseudotyped MLV particles containing the luciferase protein. Cells were incubated with various doses of cytochalasin D for 1 h at RT and then supernatant containing viral pseudotypes were added to the cells for an additional hour at 27˚C. Virus entry was determined via measurement of luciferase activity. Cytochalasin-D inhibited VEEV entry in a dose-dependent manner (Fig.35). This assay validated the microsopy studies, confirming that actin polymerization must occur in the mosquito cell in order for the virus to enter. The exact role actin plays in VEEV entry, for example whether the actin serves to localize the virus over an active endocytic region of the cell or aids in vesicle fission from the cell surface, remains to be determined.

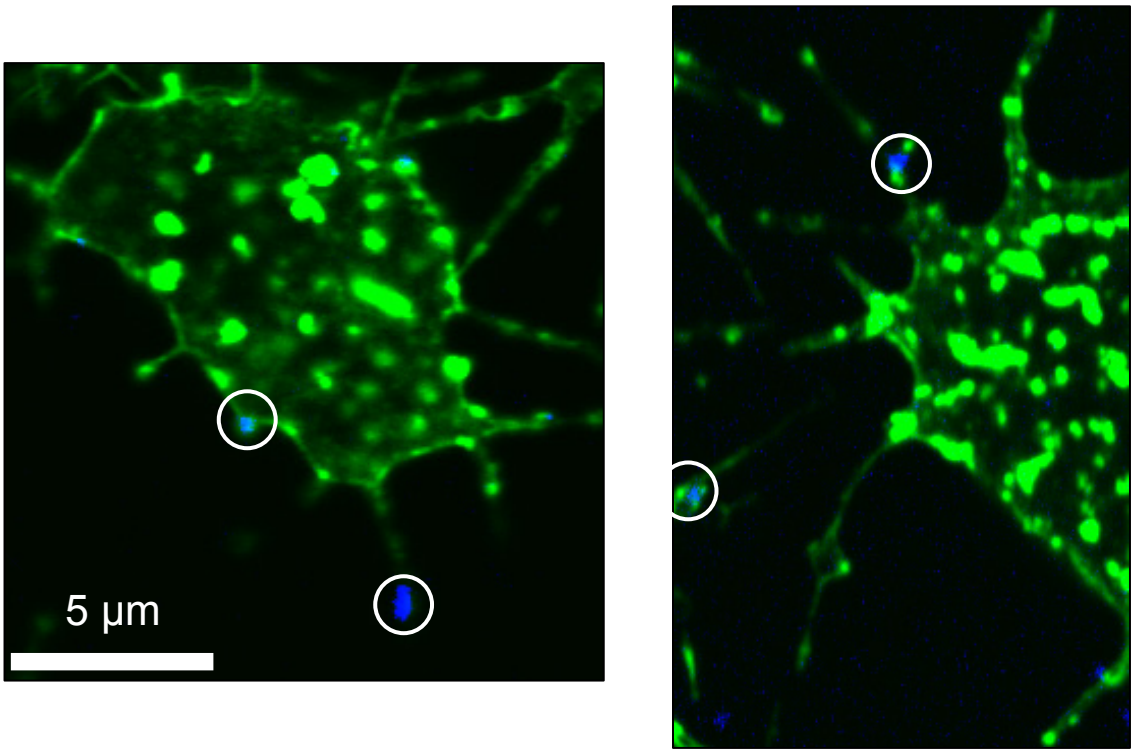


Figure 34. Actin polymerization required for internalization of VEEV
After incubation with cytochalasin-D for 1 h at RT, C710 cells were infected with SIN83-blue for 30 min at RT. Cells were then fixed and incubated with a phalloidin-488 stain (green) for 20 min at room temperature. Excess stain was washed off and cells were then examined by confocal microscopy. A representative slice through the cell near the bottom of the cell body where the cell attaches to the slide is shown for two individual cells. White circles illustrate the location of the SIN83 blue particles.

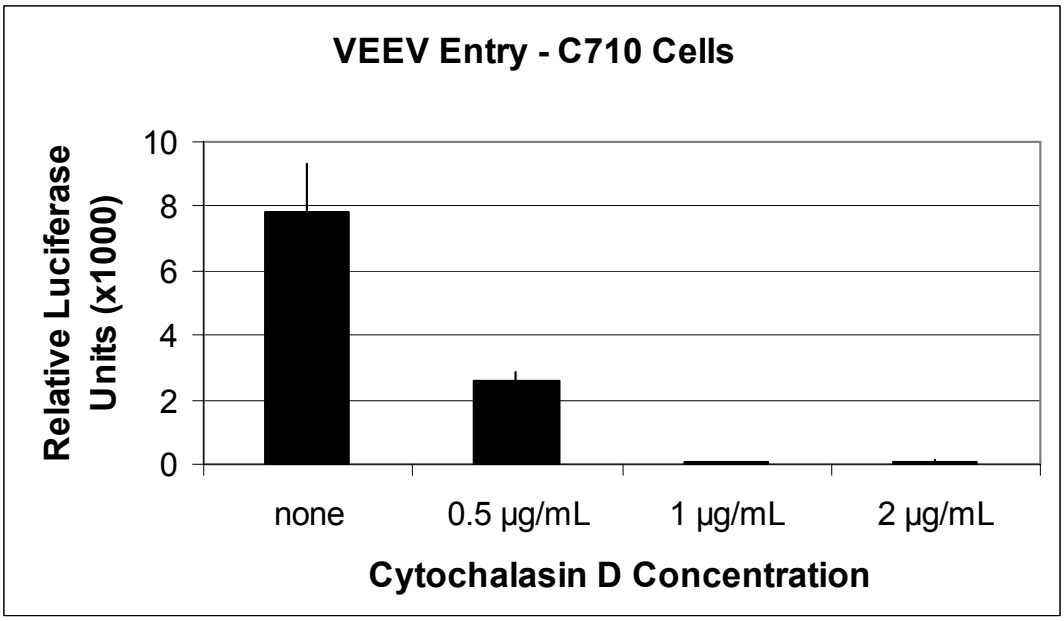


Figure 35. Actin polymerization is required for VEEV to enter the mosquito cell
After incubation with cytochalasin-D for 1 h at RT, C710 cells were incubated at 27°C with VEEV pseudotypes containing the luciferase protein. Entry was measured at 1 h after addition of virus to cells.

Chapter 23: Actin and Dynamin

Given that actin and dynamin both play important roles in the early stages of endocytosis in mammalian cells, it was predicted that they should have similar localizations along the cell surface as well as a number of interactions with one another in the mosquito cells. To visualize the interaction of the two proteins, C710 cells were transfected with the mosquito dynamin-GFP wild-type expression plasmid, fixed and incubated with phalloidin-594 to stain for F-actin. Many areas of colocalization of the two proteins in the mosquito cells were apparent, especially at the plasma membrane and along membrane extensions (Fig.36). This suggested that actin and dynamin are functioning at similar locations at the cell surface and may be interacting with one another in the mosquito cell. Interestingly, there seemed to be an abundance of dynamin protein throughout the cell while the F-actin remained along the plasma membrane.

Since actin colocalized with both dynamin protein in an uninfected cell as well as with the SIN83-blue virus in an infected cell, the next step was to see if actin colocalized with dynamin in an infected cell. C710 cells were transfected with the wild-type mosquito dynamin-GFP expression plasmid and then incubated with SIN83-blue for 30 min at RT. Cells were then fixed and incubated with phalloidin-594 to stain for F-actin. Virus can clearly be seen inside a vesicle within the cell and both actin and dynamin were found in close proximity to the virus, seemingly on the outside of the vesicle (Fig. 37). In this case, both F-actin and dynamin protein were found within the cell along with virus whereas in uninfected cells the actin remained mostly near the plasma membrane (Fig.36). The reasons for this behavior have yet to be determined but it suggests that actin is recruited to have an active role in virus internalization beyond the first steps of entry. Dynamin is known to play a crucial role in the early steps of endocytosis and the present work has shown that the protein is important for VEEV infection. Here it appears that dynamin may also have a function in virus internalization beyond entry.

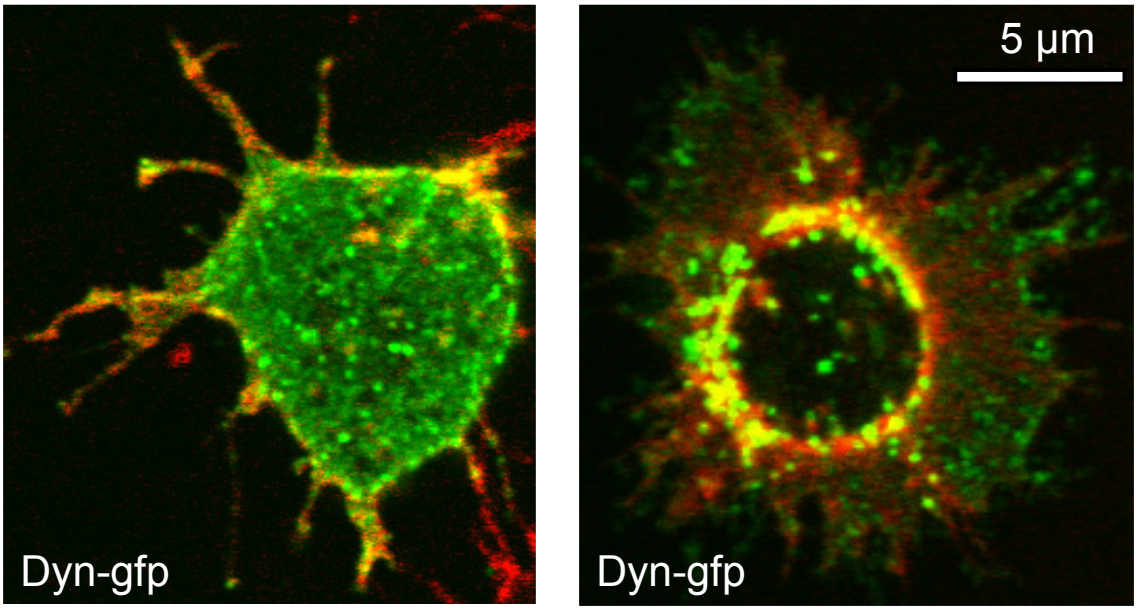


Figure 36. Colocalization of mosquito dynamin with mosquito actin
After transfection of C710 mosquito cells with GFP-dynamin WT (green), cells were fixed and incubated with a phalloidin-594 stain (red) for 20 min at room temperature. Excess stain was washed off and cells were then examined by confocal microscopy. A representative slice through the cell near the bottom is shown for two individual cells. Both slices were taken very near the bottom of the cell body where the cell attaches to the slide to illustrate the presence of both proteins in filopodia. Areas of colocalization are yellow.

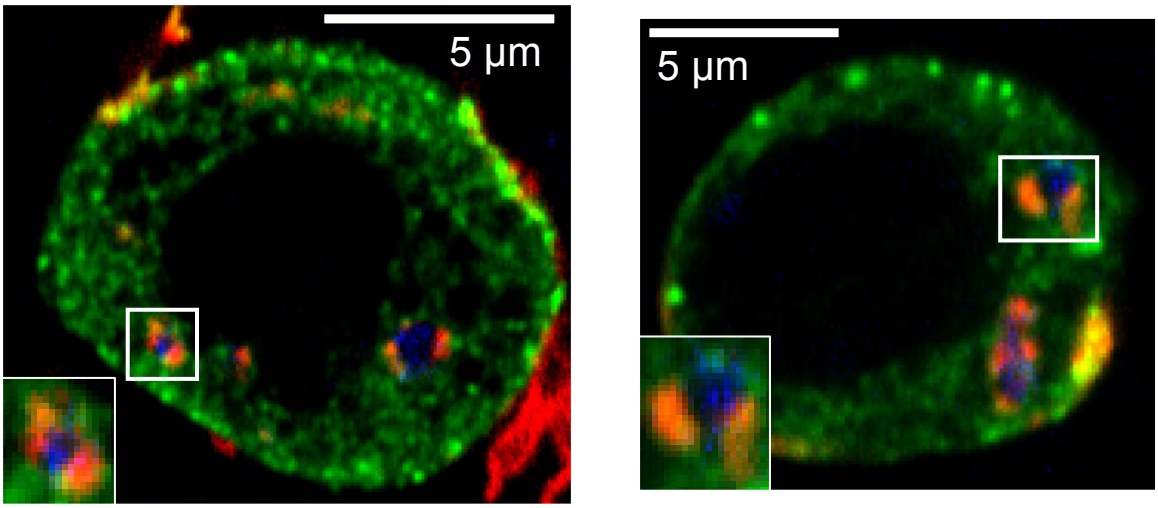


Figure 37. Colocalization of dynamin with mosquito F-actin and SIN83-blue
After transfection of C710 mosquito cells with GFP-dynamin WT (green), cells were incubated with SIN83-blue for 30 min at RT. Cells were then fixed and incubated with phalloidin-594 stain (red) for 20 min at room temperature. Excess stain was washed off and cells were then examined by confocal microscopy. A representative slice through the center of the cell is shown for two individual cells. Areas of F-actin and dynamin colocalization are yellow and the SIN83 virus is blue. The white squares show areas of interest that were enlarged at bottom left.

Given that the transfected wild-type mosquito dynamin protein colocalized with F-actin in an infected mosquito cell, this suggested that they may act together to aid in virus internalization. To test this, the dominant-negative (DN) dynamin protein was used to investigate whether it colocalized with actin and virus and if this affected virus penetration into the cell. C710 cells were transfected with the DN mosquito dynamin-GFP expression plasmid and then incubated with SIN83-blue for 30 min at RT. Cells were then fixed and incubated with phalloidin-594 to stain for F-actin. In cells expressing the DN dynamin-GFP protein (green), virus can clearly be seen on the cell surface but not within the cell (Figs. 38, 39). In neighboring cells that are not expressing DN dynamin protein, virus can clearly be seen well inside the cell and associated with the mosquito F-actin (red). It is interesting to note that even though the dynamin protein is not functional with respect to GTP hydrolysis, virus was still seen to colocalize with the DN dynamin as well as with F-actin close to the cell surface. Further study may reveal the relationship between the dynamin and actin proteins in the mosquito cell and how there interactions affect virus internalization.

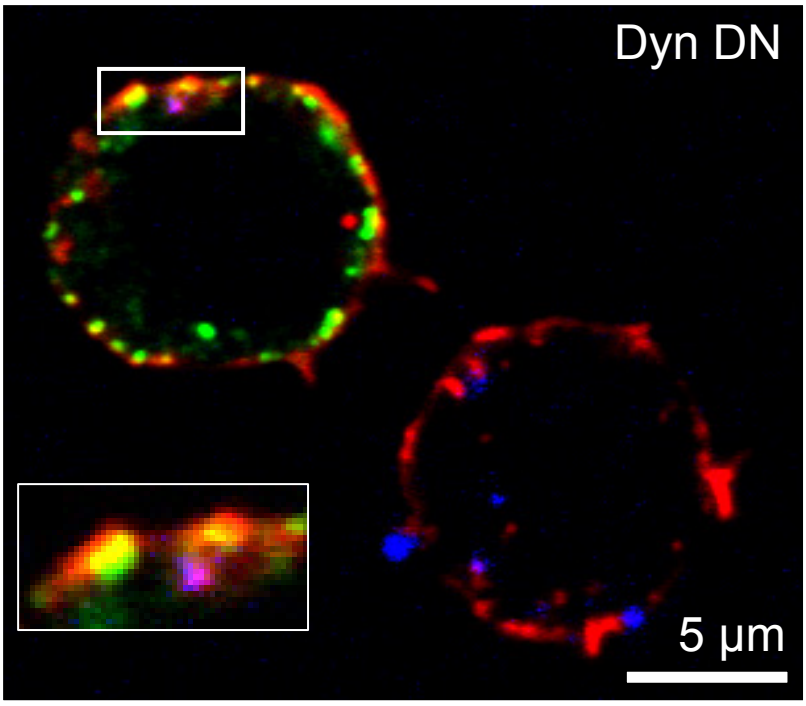


Figure 38. DN mosquito dynamin with mosquito F-actin and SIN83-blue
After transfection of C710 mosquito cells with GFP-dynamin DN (green), cells were incubated with SIN83-blue for 30 min at RT. Cells were then fixed and incubated with a phalloidin-594 stain (red) for 20 min at room temperature. Excess stain was washed off and cells were then examined by confocal microscopy. A representative slice through the center of the cell is shown for two individual cells. Areas of F-actin and dynamin colocalization are yellow and the SIN83 virus is blue. The white square shows an area of interest that was enlarged at bottom left.

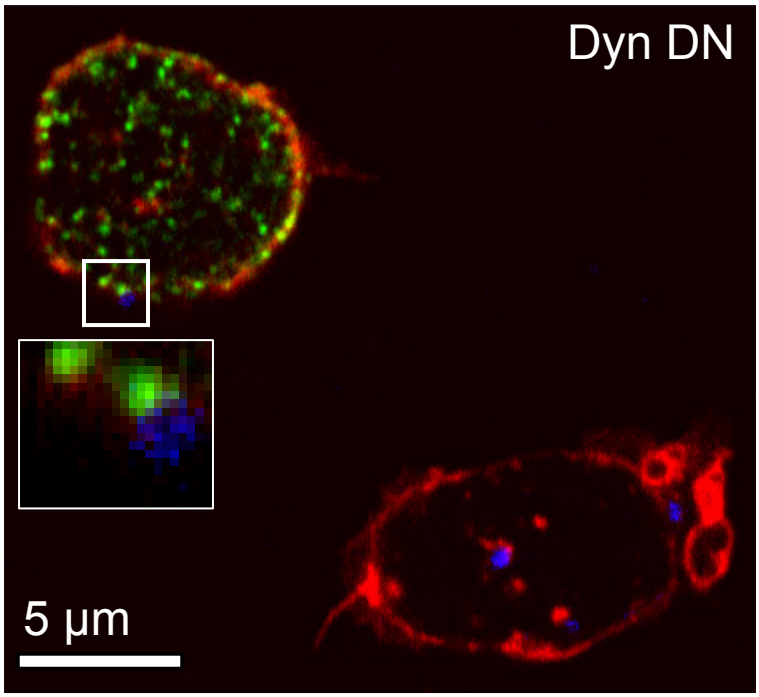


Figure 39. DN mosquito dynamin with mosquito F-actin and SIN83-blue
After transfection of C710 mosquito cells with GFP-dynamin DN (green), cells were incubated with SIN83-blue for 30 min at RT. Cells were then fixed and incubated with a phalloidin-594 stain (red) for 20 min at room temperature. Excess stain was washed off and cells were then examined by confocal microscopy. A representative slice through the center of the cell is shown for two individual cells, one expressing DN GFP-dynamin and one untransfected cell. Areas of F-actin and dynamin colocalization are yellow and the SIN83 virus is blue. The white square shows an area of interest that was enlarged at bottom left of the DN dynamin-GFP-expressing cell.

Discussion

Virus entry into a host cell is a key first step in the pathogenesis of a viral infection. Many families of viruses enter host cells by an endocytic pathway rather than at the cell surface. Endocytosis is an important mechanism in virus entry, especially for pH-dependent enveloped viruses. After initial binding to the host cell receptor, these viruses require low pH-induced fusion to occur between the viral and cellular membranes in order to release the viral genome (Marsh and Helenius, 2006; Sieczkarski and Whittaker, 2002a). Several viruses, including West Nile, Vesicular Stomatitis and Influenza A viruses, require a functioning endocytic pathway in order to enter and infect cells and disabling steps along this pathway causes a marked reduction in virus entry (Chu and Ng, 2004; Sieczkarski and Whittaker, 2002a; Sieczkarski and Whittaker, 2002b; Sieczkarski and Whittaker, 2003). Recent work has shown that many pH-independent viruses can also enter via an endocytic route. Although HIV has been shown to fuse at the plasma membrane of cells, inhibitors of endosomal acidification partially inhibit entry and infection of the virus in certain cell types (Vidricaire and Tremblay, 2005). Many studies have shown that the endocytic process is important for the internalization of most animal viruses and inhibiting this pathway will significantly block virus entry (Sieczkarski and Whittaker, 2002a). The present work is the first investigation into the endocytic pathway in a mosquito cell and the first demonstration that this process is important for virus entry and infection of mosquito cells.

Most of the information on alphavirus attachment and entry into host cells has come from work with the Old World alphaviruses such as SFV and SIN in mammalian cell types. Little is known about the infection pathway taken by New World alphaviruses in mammalian cells and especially in the cells of their transmission vector, the mosquito. In addition, there has been little agreement in the literature as to the mechanism of alphavirus entry in both mammalian and mosquito cells. Previous studies have suggested that alphaviruses can enter but not infect cells in the presence of lysosomotropic agents, which inhibit the acidification of the endosome. The addition of these agents prevented

the fusion of SFV with the cellular membrane and therefore inhibited the release of the viral genome (Marsh et al., 1982). Working with SIN, it was shown that viral RNA synthesis is blocked after treatment of cells with lysosomotropic agents soon after infection and that later addition of the drugs blocked subsequent steps in virus replication. It was concluded that virus may be able to enter cells but not productively infect them in the presence of these agents (Cassell, Edwards, and Brown, 1984). By using a novel luciferase entry assay, the present work concludes that entry of both VEEV and SFV requires endosomal acidification in mosquito cells. Our data supports the hypothesis that both Old and New World alphaviruses enter insect cells via a pH-dependent pathway and that infection of mosquito cells with VEEV requires endosomal acidification for membrane fusion to take place.

In mammalian cells, endocytic vesicle trafficking and fusion is partly controlled by small GTPases known as Rab proteins. Rab5 is responsible for early endosome function and regulation, while Rab7 is responsible for the maturation and regulation of late endosomes (Sieczkarski and Whittaker, 2003). There has been a great deal of research on these proteins in mammalian cells and this has led to a greater understanding of the endocytic mechanisms of the cell. Several Rab proteins have been identified in the fruit fly Drosophila, including Rab5 and Rab7, and they have been shown to play similar roles in endocytosis and trafficking as their mammalian counterparts (Guha et al., 2003; Satoh et al., 1997; Satoh, Tokunaga, and Ozaki, 1997; Wucherpfennig, Wilsch-Brauninger, and Gonzalez-Gaitan, 2003). The Drosophila Rab5 was shown to be expressed from multiple splice variants of a single gene, whereas the three human isoforms of Rab5 (A, B & C) are derived from completely separate genes (Wucherpfennig, Wilsch-Brauninger, and Gonzalez-Gaitan, 2003). Prior to the current study, it was unknown if these proteins existed in other insects and functioned in endocytosis. By identifying and isolating these proteins in mosquito cells, we have illustrated their importance for endocytosis in mosquitoes as well as created new tools for investigating this important molecular process. The mosquito genes that were identified had over 80% amino acid sequence identity to the human genes. Interestingly, the

mosquito Rab5 appears to be coded for by only one gene, much like the Drosophila homolog. Additional gene expression experiments and subsequent gene sequencing must be done in order to determine if there are multiple splice variants of the protein that can be expressed from this one Rab5 gene.

Dynamin is a large GTPase that drives the scission of clathrin-coated pits from the cell surface, turning the pit into a clathrin-coated vesicle. Since these invaginations can not move into the cell without scission, dynamin is essential for clathrin-mediated endocytosis (Song, Leonard, and Schmid, 2004). Mammalian dynamin has three distinct forms, dynamin I, dynamin II and dynamin III (Liu and Robinson, 1995). Interestingly, dynamin was first discovered in Drosophila, where it is encoded by the gene *shibire*. Mutations in this gene had distinct negative effects on vesicular traffic in cells (van der Bliek and Meyerowitz, 1991). Researchers concluded that the Drosophila dynamin protein expressed from the *shibire* gene drives intracellular traffic but the action was limited to endocytosis and interaction with microtubules. The Drosophila *shibire* gene was also found to encode more than one form of dynamin, with alternative C termini that differ in length and amino acid composition (van der Bliek and Meyerowitz, 1991). Here, a putative mosquito dynamin was identified and isolated from mosquito cells. The mosquito dynamin that was characterized in the present work had approximately 70% sequence identity to the mammalian gene by amino acid alignment and the GTP-binding domains were especially conserved. This indicated that dynamin may serve a similar function in the endocytic pathway in mosquito cells as it is known to in mammalian cells.

In mammalian cells, there are well-characterized dominant negative (DN) and constitutively active (CA) mutant forms of these endocytic proteins that have been widely used to investigate the endocytic process (Bucci et al., 1992; Song, Leonard, and Schmid, 2004; Vitelli et al., 1997). By expressing the wildtype and DN/CA Rab5, Rab7 and dynamin mosquito proteins in mosquito cells, we have shown that the mosquito cell utilizes these proteins for endocytosis much like the mammalian cell does. Both Rab5 and Rab7 wild type human proteins presented as punctate vesicular proteins while the dominant negative mutants were more diffusely distributed in the cytosol, as seen

previously (Gutierrez et al., 2004). This was also the case with the mosquito Rab proteins and mosquito dynamin in the mosquito cells. Interestingly, though mosquito cells are typically smaller than mammalian cells, they had disproportionately fewer vesicles than the mammalian cells when counted by Rab protein expression, though the dynamin proteins seemed to express more abundantly. Functionally, the mosquito Rab5 and dynamin proteins behaved similarly to the human proteins, locating to endocytic vesicles that rapidly took up transferrin. The Rab7 protein did not associate with transferrin but did colocalize with Lyso Tracker, indicating that it was not present on early endosomes but instead on late endosomes to lysosomes, as is mammalian Rab7.

The mosquito Rab5, Rab7 and dynamin proteins share almost completely conserved GTP-binding domains with their human counterparts and identical amino acid substitutions within these domains act as dominant negatives to disrupt normal endosome maturation and trafficking. Expression of the dominant negative mutant forms of both the mosquito Rab5 and dynamin proteins prevented the entry of transferrin, consistent with their function in mammalian cells. The constitutively active Rab5 protein formed large vesicles in the mosquito cell that accumulated transferrin, which is also consistent with the mammalian phenotype of this mutant. The mosquito cells seemed to take in less transferrin than the mammalian cells, which could be due to the lower number of vesicles or due to a less active endocytic pathway. Expression of the dominant negative form of the Rab7 protein did not prevent transferrin from entering the cell, which was expected since transferrin recycles from the early endosome back to the cell surface (Li et al., 1994). The transferrin assay confirmed that both Rab5 and dynamin are functional within the mosquito endocytic pathway and that expression of the mutant proteins has an effect on endocytosis in these cells as it does in mammalian cells. Our results indicate that Rab5 has an evolutionary conserved role in endosome biogenesis that is shared between mosquitoes and humans. This assay also confirms that the mosquito dynamin has a role in the movement of cargo from the cell surface towards the center of the cell. Both the transferrin and Lyso Tracker assays also suggested that Rab7 does not have a function in the early stages of endocytosis but may act on later steps. Prospective late endosomal

and lysosomal markers are currently being sought and tested in mosquito cells in order to confirm the function of mosquito Rab7.

Both Rab5 and Rab7 proteins have been shown to be important for entry and infection for several viruses. Entry of HIV and influenza viruses requires functional Rab5 as well as Rab7, while SFV and VSV only need functional Rab5 present for productive infection (Sieczkarski and Whittaker, 2003). SFV was shown to require Rab7 function in mammalian cells in order to reach the late endosome but it is unclear if access to this compartment leads to productive infection (Sieczkarski and Whittaker, 2003; Vonderheit and Helenius, 2005). In mammalian cells, VEEV infection was shown to require both Rab5 and Rab7, indicating that the virus must access both early and late endosomes for productive infection (Kolokoltsov, Fleming, and Davey, 2006). In addition to regulating endocytosis, Rab5 and Rab7 may act as signaling molecules to create the appropriate environment in the mosquito cell for virus entry and infection. Here, disruption of the endocytic pathway by expression of DN & CA forms of Rab5 and Rab7 were used to determine the importance of this pathway for VEEV infection of mosquito cells. The expression of the wildtype form of the mosquito Rab5 homolog increased infection with VEEV (SIN83) virus showing that endocytosis may be limiting for infection. In contrast, the expression of dominant negative and constitutively active mutant forms greatly and significantly reduced mosquito cell infection. This indicates that functional Rab5 is required for VEEV entry and infection in mosquito cells. The infection studies with wild-type Rab7 indicated that the increased expression of the protein over endogenous levels does not increase infection as greatly as did the Rab5 protein. However, dominant negative Rab7 expression decreased infection with VEEV, indicating a need to access a late endosome after the early endosome in order to infect mosquito cells. Interestingly, VEEV also requires access to this compartment in mammalian cells (Kolokoltsov, Fleming, and Davey, 2006). This indicates that a conserved pathway is utilized by VEEV to infect both its mammalian and insect host.

Dynamin is known to be necessary for many viruses to enter and infect cells. Two Old World alphaviruses, SFV and SINV, can not enter cells without the presence of

functional dynamin. It was found that both viruses utilize this protein to establish productive infection in mammalian cells (DeTulleo and Kirchhausen, 1998). Several other viruses require dynamin for infection, incuding human rhinovirus 14, adenovirus, parvoviruses and influenza virus (Bartlett, Wilcher, and Samulski, 2000; DeTulleo and Kirchhausen, 1998; Roy et al., 2000; Wang et al., 1998). In the present work, expression of dominant-negative (DN) dynamin protein was used to determine the importance of this protein for VEEV infection of mosquito cells. The expression of the wildtype form of the mosquito dynamin did not greatly increase infection but did have a slight impact. Expression of the DN dynamin reduced virus infection by approximately 25% indicating that the protein is utilized by the virus to enter the cell but it is still unknown to what extent. The existence of additional forms of dynamin, as is the case in Drosophila, or the high level of endogenous dynamin protein could explain the ability of the virus to infect cells during overexpression of the DN dynamin. Further work must be done in order to conclusively determine the requirements of VEEV infection for functional dynamin protein in mosquito cells.

An important aspect of the present work was the development of a novel tool to measure virus entry into mosquito cells. The assay was shown to be rapid, quantitative and specific. Using this fusion assay, it was shown that both VEEV and SFV enter mosquito cells via a pH-dependent pathway that requires endosomal acidification. This assay is based on viral pseudotypes and can be used with any pseudotyped virus. It enables researchers to precisely detect entry by measuring the fusion of the viral membrane with the cellular membrane in real-time. In addition, mosquito homologs of human Rab5, Rab7 and dynamin proteins were identified and characterized. It was shown that they each were essential for endocytosis in mosquito cells much as they are in mammalian cells. This is the first identification of a GTPase in a mosquito cell. These results demonstrate for the first time that mosquitoes have counterparts to mammalian proteins that are essential for the functioning of the endocytic pathway. These proteins can be used to further study both endocytosis and virus entry into mosquito cells. By using flow cytometry, it was demonstrated that Rab5, Rab7 and dynamin proteins are

involved and necessary for VEEV infection in mosquito cells. Mosquito clathrin was also identified and was found with transferrin and dynamin protein along the endocytic pathway.

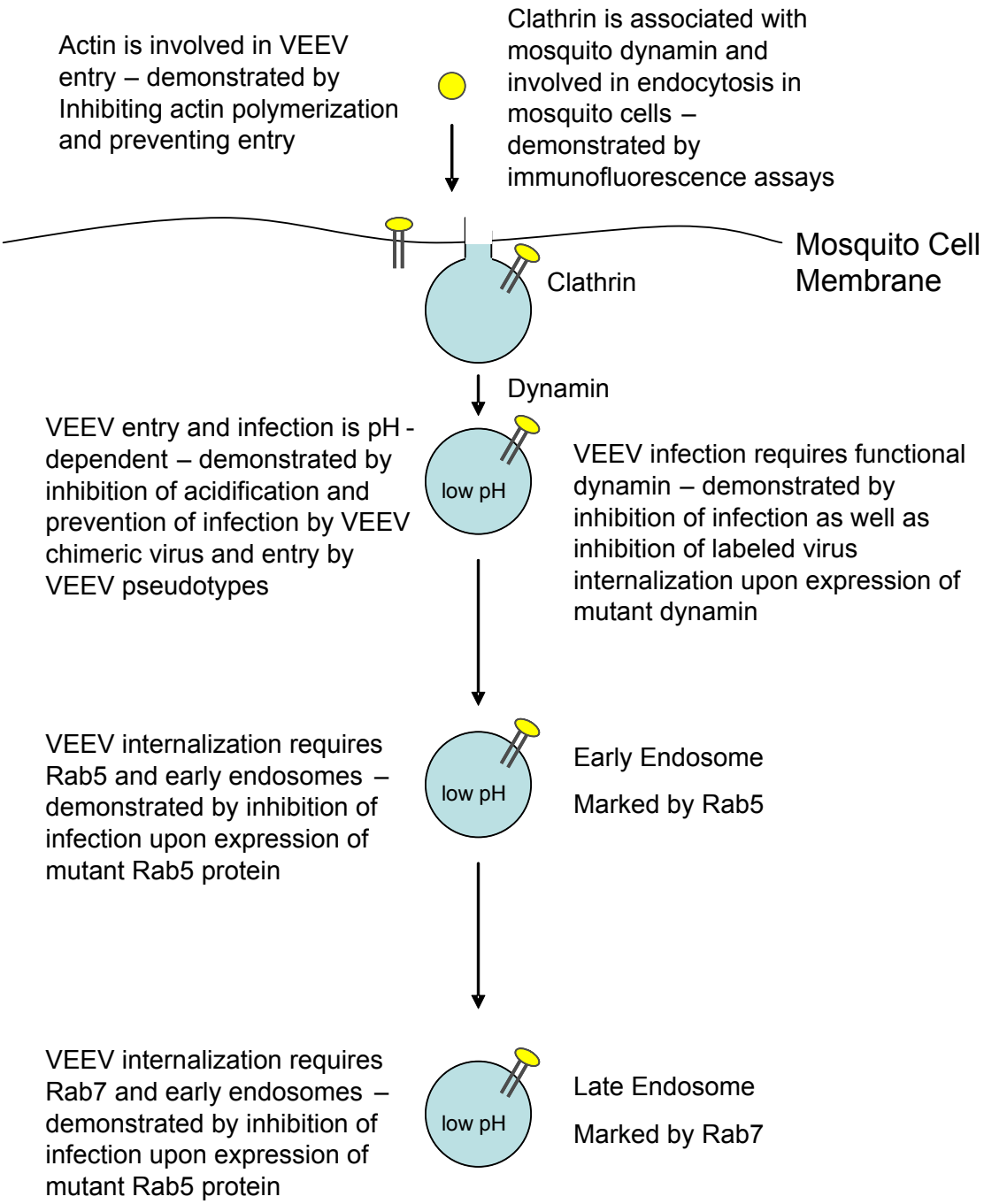
It is becoming clear that the cytoskeletal protein actin is acting together with dynamin and other endocytic proteins to aid in vesicle scission as well as trafficking in mammalian cells (Smythe and Ayscough, 2006). Dynamin may trigger actin polymerization during endocytosis or may regulate the polymerization of actin at the plasma membrane where coated pits are forming (Mousavi et al., 2004; Zhu et al., 2005). In the present work, actin was identified in the mosquito cell by phalloidin staining and it was shown to colocalize with mosquito dynamin, especially along the plasma membrane. This presence of both proteins in the same cellular locations suggested that actin interacts with dynamin and plays a role in the early steps of endocytosis in the mosquito cell. The functions of actin during endocytosis as well as the reasons for the interaction between mosquito actin and dynamin remain to be determined.

Several viruses have been shown to require actin polymerization for entry and infection (Frischknecht and Way, 2001). Here, the role of mosquito F-actin for VEEV entry and internalization was investigated. Inhibiting the polymerization of actin with cytochalasin-D prevented the entry and internalization of VEEV, both in the entry assay and in microscopy studies. This indicated that actin polymerization is required for VEEV entry into the mosquito cell. In addition, incubation with virus seemed to stimulate the formation of actin rings, though this may be due to the presence of actin on the membrane of vesicles during endocytosis. It will be important to test whether VEEV can directly stimulate actin polymerization in cells, as do vaccinia and other viruses (Frischknecht et al., 1999). F-actin and mosquito dynamin also colocalized with one another as well as with virus during VEEV internalization into the mosquito cell. It is important to note that in both infected and uninfected mosquito cells, mosquito dynamin was often found throughout the cell while in uninfected cells F-actin was usually found at the cell surface. Actin was found well within the cellular cytoplasm only after incubation with virus and only in close proximity to the virus during internalization. Further

investigation will be necessary to determine the function of F-actin during virus entry as well as during virus internalization from the plasma membrane to the interior of the cell. It will also be interesting to examine the function of the dynamin and F-actin interactions during the internalization of a VEEV particle.

There are many proteins involved in the mammalian endocytic pathway that most likely have homologs within the mosquito genome. The recent sequencing of both the *Anopheles gambiae* and *Aedes aegypti* mosquito genomes provides a novel opportunity to identify these proteins and determine how they function in the mosquito endocytic pathway as well as what their roles may be in virus entry and infection. Many viruses that are highly infectious and sometimes fatal to humans are transmitted by mosquitoes or other insect vectors. Knowledge of the molecular mechanisms of the endocytic pathway in mosquito and other insect cells could greatly improve our abilities to break the human-vector infection studies in highly pathogenic arboviruses. This research represents the first identification and characterization of mosquito endocytic proteins and, importantly, the involvement of these proteins in virus entry. Mosquito Rab5, Rab7, dynamin and actin are most likely involved in the entry of other alphaviruses and they may prove useful in investigating arbovirus entry as well as preventing arbovirus infection of mosquitoes, including VEEV. The full impact of these proteins could be determined with a complete knockdown, either using dsRNA/siRNA methods or creating knock-out mosquitoes. In addition, there are likely additional Rab proteins or endocytic proteins in the mosquito cell that will prove to be important for viral infection. It would also be beneficial to investigate the roles of these proteins in alphavirus infection *in vivo*, as this will most closely imitate the natural infection cycle. A final illustration is below outlining the conclusions reached in this work as well as the methods used to demonstrate the results.

Summary


Actin is involved in VEEV entry – demonstrated by Inhibiting actin polymerization and preventing entry
Clathrin is associated with mosquito dynamin and involved in endocytosis in mosquito cells – demonstrated by immunofluorescence assays
Mosquito Cell Membrane
Clathrin
Dynamin
VEEV entry and infection is pH - dependent – demonstrated by inhibition of acidification and prevention of infection by VEEV chimeric virus and entry by VEEV pseudotypes
low pH
VEEV infection requires functional dynamin – demonstrated by inhibition of infection as well as inhibition of labeled virus internalization upon expression of mutant dynamin
VEEV internalization requires Rab5 and early endosomes – demonstrated by inhibition of infection upon expression of mutant Rab5 protein
low pH
Early Endosome
Marked by Rab5
VEEV internalization requires Rab7 and early endosomes – demonstrated by inhibition of infection upon expression of mutant Rab5 protein
low pH
Late Endosome
Marked by Rab7

Conclusions

- **Characterized for the first time the endocytic pathway and endocytic proteins in mosquito cells**
- **Characterized mutant forms of these proteins that are widely used as tools to investigate endocytosis in mammalian cells**
- **Demonstrated that both VEEV and SFV utilize a pH-dependent endocytic pathway to enter mosquito cells**
- **Demonstrated that VEEV requires both Rab5 & Rab7 GTPases in order to establish productive infection in mosquito cells**
- **Illustrated that VEEV utilizes dynamin, which colocalizes with clathrin, to enter and infect mosquito cells**
- **Showed that actin is involved in mosquito endocytosis and required for VEEV entry and infection of mosquito cells**

Materials & Methods

Cell lines and cultivation.

C710 *Aedes* cells (kindly provided by Dr. Ilya Frolov, UTMB, TX) were used for entry assays as well as all transfection and infection studies. The cells were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bioproducts, CA), 1% penicillin-streptomycin and 1% tryptose phosphate broth (Sigma, MO). 293FT cells (Invitrogen, CA) were used to generate envelope pseudotyped virions and were grown in the same medium with 0.5 mg/ml of Geneticin (Invitrogen, CA) for selection.

Chemicals.

Chloroquine and monensin were from Calbiochem (San Diego, CA). Ammonium Chloride was from Sigma (St. Louis, MO). All other chemicals used were Sigma Ultragrade (St. Louis, MO).

Expression plasmid constructs.

All plasmids were prepared using Qiagen miniprep kits (Valencia, CA) after standard transformation into DH5α competent bacterial cells. A pcDNA3 (Invitrogen, CA) expression plasmid containing the VEEV envelope proteins from E3 to E1 from subtype IC strain 3908 was used to make VEEV env pseudotypes (Kolokoltsov, Weaver, and Davey, 2005). Similarly, a pcDNA3 expression plasmid containing the SFV envelope proteins was used for SFV pseudotype production and was provided by Dr. D. Sanders (Indiana University Medical School, IN) and is described elsewhere (Kahl et al., 2004). The MLV 10A1 envelope was expressed from the p10A1 vector (Clontech, CA) and was used to make MLV pseudotypes. A plasmid encoding VSV-G (pVSV-G, BD

Biosciences, CA) was used to make vesicular stomatitis virus (VSV) pseudotypes as described previously (Kolokoltsov, Weaver, and Davey, 2005). pGAG-POL which encodes the murine leukemia virus (MLV) gag and polymerase was a gift of Dr. J. Cunningham (Harvard Medical School, MA). The marker gene encoding plasmid was based on pFB (Stratagene, CA) and encodes enhanced green fluorescent protein (GFP) under control of the MLV LTR and virus packaging sequence (pFB-GFP).

Production of envelope pseudotyped MLV.

MLV particles bearing the envelope (env) proteins of VEEV, VSV, SFV and MLV 10A1 were made according to previous work (Kolokoltsov, Weaver, and Davey, 2005). Human kidney cells that constitutively express the SV40 large T antigen (293FT cells) (Invitrogen, CA) were used for the transient transfection. These cells allow for increased protein production from plasmids containing an SV40 origin of replication. 293FT cells were transiently transfected using the calcium phosphate method (Chen and Okayama, 1987) with pGAG-POL, pFB-GFP and plasmids encoding appropriate env proteins. All transfections were done in $10cm^2$ tissue culture plates with cells at 80% confluence. The VSV-G-encoding plasmid was used at 1 μg per transfection and other plasmids were used at 5 μg each. The plasmids were first mixed with 0.1% TE in dH_20 to a final volume of 500 μL. Next, 500 μL of 2X BES buffer (50 mM BES, 280 mM NaCl, 1.5 mM $Na_2HPO_4 \cdot 2H_2O$) was added and the mixture was vortexed. Then, 65 μL 2 M calcium chloride was added slowly while vortexing and the solution was allowed to incubate at room temperature for 10 min. After incubation, the solution was added to the cells dropwise and the cell plate was placed at 37°C overnight. After overnight incubation, the medium was replaced. When virus production peaked after a total of 48 h, the supernatants were collected and filtered through a 0.45 μm cellulose acetate filter. The filtrate was used directly, or else viral pseudotypes were purified and concentrated by pelleting through 20% (w/v) sucrose in 20 mM Tris-HCl, pH 7.4.

Luciferase-based entry assay.

The rapid entry assay requires that luciferase enzyme is enclosed within an intact viral envelope. This was achieved by fusing the luciferase protein to the HIV *nef* protein. The *nef*-luciferase encoding plasmid (pcDNA3-Nef-luc) used in making virus for entry assays was made by amplifying the *nef* gene and cloning it into the pcDNA3 vector that contained the firefly luciferase gene. This plasmid is described elsewhere (Saeed, Kolokoltsov, and Davey, 2006). After transient transfection of plasmids encoding viral structural proteins and the *nef*-luciferase fusion construct (1 μg), virions budding from the cell surface incorporate the *nef* protein inside the viral envelope. The *nef* protein will insert into the cellular membrane and therefore be encapsulated inside the viral pseudotype until the viral membrane fuses with another cellular membrane. Previously this method was used exclusively for mammalian cells but here we show it can be applied to mosquito cells as well. Entry assays were performed on C710 cells and involved incubating virus-containing supernatant with 10^5 cells in suspension for 1 h at room temperature at an MOI of ≥1. Cells were then pelleted, washed with PBS and lysed by freeze/thaw to ensure that luciferin could permeate subcellular compartments. This treatment does not disrupt virus particles. The thawed cells were then resuspended in luciferase assay buffer (Promega, #E2510) and light emitted by luciferase was detected after 12 sec using a luminometer (Turner Biosystems model 20/20).

Use of inhibitors of endosomal acidification.

Ammonium chloride and chloroquine were dissolved directly in DMEM. Monensin was made as a 50 mM stock in ethanol. All were diluted in DMEM immediately before use. For entry assays, cells in suspension were pre-incubated with drug in DMEM for 1 h at room temperature and then virus was added as described above. For infection assays, cells were plated and then incubated with drug in DMEM for 1 h at room temperature and

then infected with virus, making sure to keep the same final concentration of drug in the media throughout the infection process.

Use of human endocytic trafficking proteins to identify entry pathway of viruses.

Rab5 is required for early endosome formation and function, and Rab7 is necessary for late endosome maturation. A dominant negative (DN) recombinant form of mammalian Rab5, S34N, blocks early endosome formation in mammalian cells. This mutation results in a Rab5 protein that can not hydrolyze GTP. The constitutively active (CA) mutant, Q79L, of Rab5 prevents endosome fusion and results in accumulation of early endosomes in mammalian cells. This mutation results in a Rab5 protein that can not exchange GDP for GTP. Both Rab5 S34N and Q79L encoding plasmids were provided by Dr. P. Stahl at Washington University Medical School. Rab7 is required for formation of late endosomes and expression of the mutant form, T22N (Dr. Wandinger-Ness, UNM), blocks this step and prevents lysosome biogenesis in mammalian cells (Bucci et al., 2000; Feng et al., 2001). Each wild-type protein fused to GFP retains function and permits detection by fluorescent microscopy and by flow cytometry. For expression in insect cells, human Rab5 and Rab7 genes were fused to GFP and cloned into an insect expression vector pAc5.1/V5-HisA (Invitrogen, CA). This vector contains the insect actin 5C promoter which allows for gene expression in mosquito as well as mammalian cells (Zhao and Eggleston, 1999).

Identification and use of mosquito homologs to human Rab5, Rab7, dynamin & clathrin.

Human Rab5, Rab7, dynamin and clathrin are all important for clathrin-coated endocytosis as well as vesicle fission from the plasma membrane during endocytosis in mammalian cells. Corresponding mosquito genes were identified using sequence alignment within the recently published *Aedes aegypti* and *Anopheles gambiae* genome sequences using the human gene amino acid sequences as a template. The human amino

acid sequences were aligned against all mosquito genome open reading frames (ORFs) using TBLASTN (Altschul et al., 1997) and putative mosquito gene sequences were returned. Exons were identified within these ORFs and deduced amino acid sequences were then aligned back to the human amino acid sequences. The mosquito amino acid sequences had between 70 and 80% similarity to the human genes. This level of similarity suggested analogous protein function between the two species. RNA was extracted from C710 mosquito cells using RNAqueous (Ambion, TX) and cDNA made by reverse transcription with a Superscript kit (Invitrogen, CA) according to the manufacturer's directions. The Rab5, Rab7, dynamin and clathrin mosquito genes were amplified from the cDNA library by PCR using Accutaq polymerase (Sigma, MO). The PCR amplicons were then cloned into TOPO 2.1 cloning vector (Invitrogen, CA), which uses topoisomerase I enzyme to ligate DNA. The vector has 3' T-overhangs for direct ligation of PCR products made with Accutaq polymerase.

PCR oligos used to amplify the Rab5, Rab7 and dynamin genes are as follows:

mosquito Rab5 – 5'-CCTAAGCTTCATATGGCATGAGTCCGCGAG-3'

and 5'-AAGGATCCGCGGCCGCTCAAGCACAGCAGCCGCTG-3';

mosquito Rab7 –5'-CCTAAGCTTCATATGGCAACTAGGAAAAAGGTC-3'

and 5'- GAAGGATCCGCGGCCGCTTAGCACGAGCAGTTGTCTCC-3';

mosquito dynamin – 5' – CCTGGATCCGCTAGCCATATGGATTCGCTCATTCCC-3'

and 5' – GAACTCGAGGATGGAATGAGAGGTGCAGG-3'

eGFP 5'– GACTCGAGCACGGTGAGCAAGGGCGAGGAG-3'

and 5'-CTGTCTAGAGCGGCCGCTTACTTGTACAGCTCGTCCATGCCG-3'.

Restriction endonuclease sites used for cloning are underlined. For expression of insect genes, the pAc5.1/V5-HisA insect expression plasmid (Invitrogen, CA) was modified by insertion of the GFP coding sequence (pAc5.1-GFP.) The amplicons were then cut out of the TOPO 2.1 vector and cloned into the premade pAc5.1-GFP vector using BamHI and EcoRI. All of the human genes were also cloned into this vector after cutting them out of their original expression plasmids. The DN or CA mutant forms of the mosquito genes were made by making amino acid substitutions at conserved residues important for

GDP/GTP exchange previously characterized in mammalian homologs, i.e. Rab5 S34N and Q79L and Rab7 T22N and dynamin K44A. The site directed mutagenesis was performed at the Recombinant DNA Core Facility at UTMB and changes confirmed by DNA sequencing.

The clathrin PCR was done in two steps since the clathrin gene is over 5000 base pairs.

For clathrin both forward and reverse primers were made as follows:

5-CCTGCGGCCGCCAATATGACTCAACCTCTACCTATTAGGTTTCAGGAGCAC CTGCAGCTCACCAACATCAACATC-3' and

5'-GAAGTTTAAACAGCTGTTACATACCACCATATCCCGG-3'.

The forward primer contains a Not I restriction endonuclease site and the reverse primer contains a Pme I restriction endonuclease site. Internal forward and reverse primers were also made for mosquito clathrin to give two PCR amplicons that could then be used as templates for the whole clathrin gene.

The internal primers contain PacI restriction sites and are as follows:

5'-CAAAGGCTTTAATTAATTTGTTCGAG-3' and

5'-CTCGAACAAATTAATTAAAGCCTTTG-3'.

Unfortunately, the cloning of mosquito clathrin was unsuccessful, most likely due to its large size.

Transfection of plasmids into mosquito cells.

The pAc5.1-GFP plasmid containing either the human or mosquito genes were transfected into C710 cells using Effectene (Qiagen, CA) according to manufacturer's instructions. Briefly, for a 10 cm^2 plate, 4 μg of DNA was mixed with 500 μL buffer EC and 32 μL enhancer was added. This was allowed to incubate for 5 min on the benchtop. Then 30 μL Effectene reagent was added and the solution vortexed briefly. After 10 min incubation, the solution was added to the cells. Expression was observed after 24 h and peaked at 48 h. For microscopy studies, human transferrin-594 (Invitrogen, CA) was added to DMEM at a 1/200 dilution and incubated with cells for 30 min at 27°C. The

cells were then pelleted, washed with PBS and fixed in 2% paraformaldehyde for 10 min at room temperature before imaging. Transfection assays were also done with a Drosophila moesin-GFP construct, generously donated by Dr. Dan Kiehart, Duke University. This plasmid utilizes the Drosophila spaghetti squash promoter for expression, which is functional in mosquito cells. This plasmid was transfected in exactly the same way as the mosquito gene plasmids.

Western blots.

After the pAc5.1-GFP plasmids containing the mosquito genes were transfected into C710 cells, cells were incubated at 27°C until protein expression could be detected by microscopy. Cells were then incubated with cell lysis buffer (Promega, CA) for 5 min at RT. The cells were spun down to remove debris and the cell lysate was pipetted out. The mosquito cell lysate was boiled in SDS-PAGE buffer with 2% β-mercaptoethanol and run on either an 8 or 12% SDS-PAGE gel for 1.5 h at 15 milliamps per gel. The proteins were then transferred to nitrocellulose. The nitrocellulose was blocked with 5% milk in 1% TBST for 1 h at RT and then incubated with the appropriate antibody overnight at 4°C. The nitrocellulose was washed and then incubated with the appropriate horseradish peroxidase secondary antibody for 1 h at RT and then washed again. The protein blots were incubated with ECL substrates (Amersham, NJ) for 5 min at RT and then detected on Kodak film.

Immunofluorescence Assays.

For immunofluorescence assays, the C710 cells were fixed in 2% formaldehyde for 15 min at RT and then permeablilized for 1 min at room temperature before incubation with appropriate antibody. Antibodies used were against human clathrin heavy chain (clone X22, Affinity Bioreagents, CO), clathrin light chain (clone CON.1, Lab Vision Corporation, CA), as well as two rabbit polyclonals against human Rab5B & Rab7 (Santa

Cruz Biotechnology, CA). Cells were either grown in suspension or grown on Lab-Tek chamber slides (Nunc, Fisher Scientific, PA). Cells were first blocked in 1% BSA (Fisher Scientific, PA) in PBS (-) for 20 min at room temperature and were then incubated with antibody diluted in 1% BSA 1/100 for 20 minutes at room temperature. Cells were washed three times in PBS (-) and then incubated with labeled secondary antibody (either anti-mouse or anti-rabbit) diluted 1/500 in 1%BSA. After three additional washes, cells were allowed to sit in PBS (-) before examination by confocal microscopy. For actin staining, fixed cells were incubated with labeled phalloidin (Invitrogen, CA) for 20 minutes at room temperature and then washed three times with PBS (-) before analysis by microscopy.

Labeled SIN83.

Labeled virus was made to track location in the cell after entry in comparison to mosquito proteins. SIN83 virus was concentrated and purified by pelleting through 20% (w/v) sucrose in 20 mM Tris-HCl, pH 7.4 and the viral pellet was resuspended in PBS (-). The virus was labeled using AlexaFluor 350 carboxylic acid, succinimidyl ester (Invitrogen, CA), which is an intense blue dye that will covalently bind to primary amines. This means that the dye will be linked to the virus through the lysines located on the viral glycoproteins. The dye was added to virus according to manufacturer's instructions. The dye powder was resuspended in water at 1 mg/mL and aliquots of 10 µL were added to 100 µL of viral supernatant. The mixture was incubated for 2 h at RT and then 25 µL of 0.1 M glycine pH 7.0 was added to stop the reaction. The labeled virus was then dialyzed (10kD MWCO membrane, Spectrapor, CA) in PBS overnight and then an additional dialysis was done in DMEM, again overnight. The virus was then aliquoted in 10 µL volumes and stored at -80°C until used on cells as detailed in the text.

Infection with SIN83.

When studying infection, SIN83, a virus comprising the structural proteins of VEEV but containing a SIN genome, was used as described elsewhere (Paessler et al., 2003). It is antigenically and structurally identical to VEEV but can be safely used at BSL-2. This virus has the receptor specificity and entry mechanism of VEEV dictated by the VEEV envelope proteins (TC-83 vaccine strain). It was used as a safe alternative to wild-type VEEV, which is a BSL-3 restricted virus and select agent. SIN83 was used in flow cytometry and microscopy experiments and was a gift of Dr. Ilya Frolov (UTMB, Galveston, TX). Another form of the virus, SIN83-GFP, had GFP as an infection marker under control of a second subgenomic 26S promoter and was used when indicated. Virus titer was determined by plaque formation and GFP expression, respectively.

Infection analysis by flow cytometry.

Insect expression vectors encoding either the human or mosquito genes were transfected into C710 cells using Effectene (Qiagen, CA) according to manufacturer's instructions and detailed above under 'plasmid transfection'. Plasmids were used at 4 μg DNA per 10^6 cells. After 24 h, cells were infected with SIN83 virus at an MOI of 1. At 12 h post-infection when viral proteins were expressed in cells, the cells were removed, pelleted at 350 x g, washed with PBS, pelleted again and fixed in 2% paraformaldehyde for 10 min at room temperature. The cells were washed and resuspended in PBS containing 5% BSA. Cells were then incubated with polyclonal serum raised against VEEV (VEEV immune ascetic fluid 711, ATCC #VR-1250AF) for 20 min at room temperature. Cells were again pelleted, washed, and incubated for 20 min with a secondary antibody labelled with Alexa Fluor 647 (Invitrogen, CA). Cells were washed and pelleted before analyzation. Cells were analyzed using a Becton-Dickinson FACSCanto instrument in the Flow Cytometry Core Facility at UTMB.

References

Albertson, R., Riggs, B., and Sullivan, W. (2005). Membrane traffic: a driving force in cytokinesis. *Trends Cell Biol* 15 (2)**,** 92-101.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25 (17)**,** 3389-3402.

Ayscough, K. R., Stryker, J., Pokala, N., Sanders, M., Crews, P., and Drubin, D. G. (1997). High rates of actin filament turnover in budding yeast and roles for actin in establishment and maintenance of cell polarity revealed using the actin inhibitor latrunculin-A. *J Cell Biol* 137 (2)**,** 399-416.

Bartlett, J. S., Wilcher, R., and Samulski, R. J. (2000). Infectious entry pathway of adeno-associated virus and adeno-associated virus vectors. *J Virol* 74 (6)**,** 2777-2785.

Benmerah, A., Bayrou, M., Cerf-Bensussan, N., and Dautry-Varsat, A. (1999). Inhibition of clathrin-coated pit assembly by an Eps15 mutant. *J Cell Sci* 112 (Pt 9)**,** 1303-1311.

Blanchard, E., Belouzard, S., Goueslain, L., Wakita, T., Dubuisson, J., Wychowski, C., and Rouille, Y. (2006). Hepatitis C virus entry depends on clathrin-mediated endocytosis. *J Virol* 80 (14)**,** 6964-6972.

Blitzer, J. T., and Nusse, R. (2006). A critical role for endocytosis in Wnt signaling. *BMC Cell Biol* 7**,** 28.

Brault, A. C., Powers, A. M., Holmes, E. C., Woelk, C. H., and Weaver, S. C. (2002). Positively charged amino acid substitutions in the e2 envelope glycoprotein are associated with the emergence of venezuelan equine encephalitis virus. *J Virol* 76 (4)**,** 1718-1730.

Bucci, C., Lutcke, A., Steele-Mortimer, O., Olkkonen, V. M., Dupree, P., Chiariello, M., Bruni, C. B., Simons, K., and Zerial, M. (1995). Co-operative regulation of endocytosis by three Rab5 isoforms. *FEBS Lett* 366 (1)**,** 65-71.

Bucci, C., Parton, R. G., Mather, I. H., Stunnenberg, H., Simons, K., Hoflack, B., and Zerial, M. (1992). The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. *Cell* 70 (5)**,** 715-728.

Bucci, C., Thomsen, P., Nicoziani, P., McCarthy, J., and van Deurs, B. (2000). Rab7: a key to lysosome biogenesis. *Mol Biol Cell* 11 (2)**,** 467-480.

Burke, E., Mahoney, N. M., Almo, S. C., and Barik, S. (2000). Profilin is required for optimal actin-dependent transcription of respiratory syncytial virus genome RNA. *J Virol* 74 (2)**,** 669-675.

Cao, H., Orth, J. D., Chen, J., Weller, S. G., Heuser, J. E., and McNiven, M. A. (2003). Cortactin is a component of clathrin-coated pits and participates in receptor-mediated endocytosis. *Mol Cell Biol* 23 (6)**,** 2162-2170.

Cassell, S., Edwards, J., and Brown, D. T. (1984). Effects of lysosomotropic weak bases on infection of BHK-21 cells by Sindbis virus. *J Virol* 52 (3)**,** 857-864.

Chen, C., and Okayama, H. (1987). High-efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol* 7 (8)**,** 2745-2752.

Chicurel, M. (2000). Virology. Probing HIV's elusive activities within the host cell. *Science* 290 (5498)**,** 1876-1879.

Chu, J. J., Leong, P. W., and Ng, M. L. (2006). Analysis of the endocytic pathway mediating the infectious entry of mosquito-borne flavivirus West Nile into Aedes albopictus mosquito (C6/36) cells. *Virology* 349 (2)**,** 463-475.

Chu, J. J., and Ng, M. L. (2004). Infectious entry of West Nile virus occurs through a clathrin-mediated endocytic pathway. *J Virol* 78 (19)**,** 10543-10555.

Damke, H., Binns, D. D., Ueda, H., Schmid, S. L., and Baba, T. (2001). Dynamin GTPase domain mutants block endocytic vesicle formation at morphologically distinct stages. *Mol Biol Cell* 12 (9)**,** 2578-2589.

De, I., Fata-Hartley, C., Sawicki, S. G., and Sawicki, D. L. (2003). Functional analysis of nsP3 phosphoprotein mutants of Sindbis virus. *J Virol* 77 (24)**,** 13106-13116.

DeTulleo, L., and Kirchhausen, T. (1998). The clathrin endocytic pathway in viral infection. *Embo J* 17 (16)**,** 4585-4593.

Ding, M. X., and Schlesinger, M. J. (1989). Evidence that Sindbis virus NSP2 is an autoprotease which processes the virus nonstructural polyprotein. *Virology* 171 (1)**,** 280-284.

Edwards, J., and Brown, D. T. (1991). Sindbis virus infection of a Chinese hamster ovary cell mutant defective in the acidification of endosomes. *Virology* 182 (1)**,** 28-33.

Edwards, K. A., Demsky, M., Montague, R. A., Weymouth, N., and Kiehart, D. P. (1997). GFP-moesin illuminates actin cytoskeleton dynamics in living tissue and demonstrates cell shape changes during morphogenesis in Drosophila. *Dev Biol* 191 (1)**,** 103-117.

Feng, Y., Press, B., Chen, W., Zimmerman, J., and Wandinger-Ness, A. (2001). Expression and properties of Rab7 in endosome function. *Methods Enzymol* 329**,** 175-187.

Feng, Y., Press, B., and Wandinger-Ness, A. (1995). Rab 7: an important regulator of late endocytic membrane traffic. *J Cell Biol* 131 (6 Pt 1)**,** 1435-1452.

Forest, T., Barnard, S., and Baines, J. D. (2005). Active intranuclear movement of herpesvirus capsids. *Nat Cell Biol* 7 (4)**,** 429-431.

Franck, Z., Gary, R., and Bretscher, A. (1993). Moesin, like ezrin, colocalizes with actin in the cortical cytoskeleton in cultured cells, but its expression is more variable. *J Cell Sci* 105 (Pt 1)**,** 219-231.

Frischknecht, F., Moreau, V., Rottger, S., Gonfloni, S., Reckmann, I., Superti-Furga, G., and Way, M. (1999). Actin-based motility of vaccinia virus mimics receptor tyrosine kinase signalling. *Nature* 401 (6756)**,** 926-929.

Frischknecht, F., and Way, M. (2001). Surfing pathogens and the lessons learned for actin polymerization. *Trends Cell Biol* 11 (1)**,** 30-38.

Gabriel, M., Horky, D., Svoboda, A., and Kopecka, M. (1998). Cytochalasin D interferes with contractile actin ring and septum formation in Schizosaccharomyces japonicus var. versatilis. *Microbiology* 144 (Pt 8)**,** 2331-2344.

Galperin, E., and Sorkin, A. (2003). Visualization of Rab5 activity in living cells by FRET microscopy and influence of plasma-membrane-targeted Rab5 on clathrin-dependent endocytosis. *J Cell Sci* 116 (Pt 23)**,** 4799-4810.

Glomb-Reinmund, S., and Kielian, M. (1998). The role of low pH and disulfide shuffling in the entry and fusion of Semliki Forest virus and Sindbis virus. *Virology* 248 (2)**,** 372-381.

Guha, A., Sriram, V., Krishnan, K. S., and Mayor, S. (2003). Shibire mutations reveal distinct dynamin-independent and -dependent endocytic pathways in primary cultures of Drosophila hemocytes. *J Cell Sci* 116 (Pt 16)**,** 3373-3386.

Gupta, S., De, B. P., Drazba, J. A., and Banerjee, A. K. (1998). Involvement of actin microfilaments in the replication of human parainfluenza virus type 3. *J Virol* 72 (4)**,** 2655-2662.

Gutierrez, M. G., Munafo, D. B., Beron, W., and Colombo, M. I. (2004). Rab7 is required for the normal progression of the autophagic pathway in mammalian cells. *J Cell Sci* 117 (Pt 13)**,** 2687-2697.

Hacker, J. K., and Hardy, J. L. (1997). Adsorptive endocytosis of California encephalitis virus into mosquito and mammalian cells: a role for G1. *Virology* 235 (1)**,** 40-47.

Hanika, A., Larisch, B., Steinmann, E., Schwegmann-Wessels, C., Herrler, G., and Zimmer, G. (2005). Use of influenza C virus glycoprotein HEF for generation of vesicular stomatitis virus pseudotypes. *J Gen Virol* 86 (Pt 5)**,** 1455-1465.

Hardy, J. L., Houk, E. J., Kramer, L. D., and Reeves, W. C. (1983). Intrinsic factors affecting vector competence of mosquitoes for arboviruses. *Annu Rev Entomol* 28**,** 229-262.

Hase, T., Summers, P. L., and Cohen, W. H. (1989). A comparative study of entry modes into C6/36 cells by Semliki Forest and Japanese encephalitis viruses. *Arch Virol* 108 (1-2)**,** 101-114.

Hase, T., Summers, P. L., and Eckels, K. H. (1989). Flavivirus entry into cultured mosquito cells and human peripheral blood monocytes. *Arch Virol* 104 (1-2)**,** 129-143.

Helenius, A., Kartenbeck, J., Simons, K., and Fries, E. (1980). On the entry of Semliki forest virus into BHK-21 cells. *J Cell Biol* 84 (2)**,** 404-420.

Helenius, A., Marsh, M., and White, J. (1982). Inhibition of Semliki forest virus penetration by lysosomotropic weak bases. *J Gen Virol* 58 Pt 1**,** 47-61.

Hernandez, R., Luo, T., and Brown, D. T. (2001). Exposure to low pH is not required for penetration of mosquito cells by Sindbis virus. *J Virol* 75 (4)**,** 2010-2013.

Herskovits, J. S., Burgess, C. C., Obar, R. A., and Vallee, R. B. (1993). Effects of mutant rat dynamin on endocytosis. *J Cell Biol* 122 (3)**,** 565-578.

Heuser, J. (1980). Three-dimensional visualization of coated vesicle formation in fibroblasts. *J Cell Biol* 84 (3)**,** 560-583.

Heuser, J. E., Keen, J. H., Amende, L. M., Lippoldt, R. E., and Prasad, K. (1987). Deep-etch visualization of 27S clathrin: a tetrahedral tetramer. *J Cell Biol* 105 (5)**,** 1999-2009.

Holt, R. A., Subramanian, G. M., Halpern, A., Sutton, G. G., Charlab, R., Nusskern, D. R., Wincker, P., Clark, A. G., Ribeiro, J. M., Wides, R., Salzberg, S. L., Loftus,

B., Yandell, M., Majoros, W. H., Rusch, D. B., Lai, Z., Kraft, C. L., Abril, J. F., Anthouard, V., Arensburger, P., Atkinson, P. W., Baden, H., de Berardinis, V., Baldwin, D., Benes, V., Biedler, J., Blass, C., Bolanos, R., Boscus, D., Barnstead, M., Cai, S., Center, A., Chaturverdi, K., Christophides, G. K., Chrystal, M. A., Clamp, M., Cravchik, A., Curwen, V., Dana, A., Delcher, A., Dew, I., Evans, C. A., Flanigan, M., Grundschober-Freimoser, A., Friedli, L., Gu, Z., Guan, P., Guigo, R., Hillenmeyer, M. E., Hladun, S. L., Hogan, J. R., Hong, Y. S., Hoover, J., Jaillon, O., Ke, Z., Kodira, C., Kokoza, E., Koutsos, A., Letunic, I., Levitsky, A., Liang, Y., Lin, J. J., Lobo, N. F., Lopez, J. R., Malek, J. A., McIntosh, T. C., Meister, S., Miller, J., Mobarry, C., Mongin, E., Murphy, S. D., O'Brochta, D. A., Pfannkoch, C., Qi, R., Regier, M. A., Remington, K., Shao, H., Sharakhova, M. V., Sitter, C. D., Shetty, J., Smith, T. J., Strong, R., Sun, J., Thomasova, D., Ton, L. Q., Topalis, P., Tu, Z., Unger, M. F., Walenz, B., Wang, A., Wang, J., Wang, M., Wang, X., Woodford, K. J., Wortman, J. R., Wu, M., Yao, A., Zdobnov, E. M., Zhang, H., Zhao, Q., Zhao, S., Zhu, S. C., Zhimulev, I., Coluzzi, M., della Torre, A., Roth, C. W., Louis, C., Kalush, F., Mural, R. J., Myers, E. W., Adams, M. D., Smith, H. O., Broder, S., Gardner, M. J., Fraser, C. M., Birney, E., Bork, P., Brey, P. T., Venter, J. C., Weissenbach, J., Kafatos, F. C., Collins, F. H., and Hoffman, S. L. (2002). The genome sequence of the malaria mosquito Anopheles gambiae. *Science* 298 (5591)**,** 129-149.

Houk, E. J., Arcus, Y. M., Hardy, J. L., and Kramer, L. D. (1990). Binding of western equine encephalomyelitis virus to brush border fragments isolated from mesenteronal epithelial cells of mosquitoes. *Virus Res* 17 (2)**,** 105-117.

Houk, E. J., Kramer, L. D., Hardy, J. L., and Presser, S. B. (1986). An interspecific mosquito model for the mesenteronal infection barrier to western equine encephalomyelitis virus (Culex tarsalis and Culex pipiens). *Am J Trop Med Hyg* 35 (3)**,** 632-641.

Hurst, I. R., Zuo, J., Jiang, J., and Holliday, L. S. (2004). Actin-related protein 2/3 complex is required for actin ring formation. *J Bone Miner Res* 19 (3)**,** 499-506.

Iyengar, S., Hildreth, J. E., and Schwartz, D. H. (1998). Actin-dependent receptor colocalization required for human immunodeficiency virus entry into host cells. *J Virol* 72 (6)**,** 5251-5255.

Jay, D. G. (2000). The clutch hypothesis revisited: ascribing the roles of actin-associated proteins in filopodial protrusion in the nerve growth cone. *J Neurobiol* 44 (2)**,** 114-125.

Kahl, C. A., Marsh, J., Fyffe, J., Sanders, D. A., and Cornetta, K. (2004). Human immunodeficiency virus type 1-derived lentivirus vectors pseudotyped with envelope glycoproteins derived from Ross River virus and Semliki Forest virus. *J Virol* 78 (3)**,** 1421-1430.

Kallewaard, N. L., Bowen, A. L., and Crowe, J. E., Jr. (2005). Cooperativity of actin and microtubule elements during replication of respiratory syncytial virus. *Virology* 331 (1)**,** 73-81.

Kaufman, T. C., Severson, D. W., and Robinson, G. E. (2002). The Anopheles genome and comparative insect genomics. *Science* 298 (5591)**,** 97-98.

Kielian, M., and Jungerwirth, S. (1990). Mechanisms of enveloped virus entry into cells. *Mol Biol Med* 7 (1)**,** 17-31.

Kizhatil, K., and Albritton, L. M. (1997). Requirements for different components of the host cell cytoskeleton distinguish ecotropic murine leukemia virus entry via endocytosis from entry via surface fusion. *J Virol* 71 (10)**,** 7145-7156.

Klimstra, W. B., Nangle, E. M., Smith, M. S., Yurochko, A. D., and Ryman, K. D. (2003). DC-SIGN and L-SIGN can act as attachment receptors for alphaviruses and distinguish between mosquito cell- and mammalian cell-derived viruses. *J Virol* 77 (22)**,** 12022-12032.

Kolokoltsov, A. A., Fleming, E. H., and Davey, R. A. (2006). Venezuelan equine encephalitis virus entry mechanism requires late endosome formation and resists cell membrane cholesterol depletion. *Virology* 347 (2)**,** 333-342.

Kolokoltsov, A. A., Weaver, S. C., and Davey, R. A. (2005). Efficient functional pseudotyping of oncoretroviral and lentiviral vectors by Venezuelan equine encephalitis virus envelope proteins. *J Virol* 79 (2)**,** 756-763.

Kramer, L. D., Hardy, J. L., Houk, E. J., and Presser, S. B. (1989). Characterization of the mesenteronal infection with Western equine encephalomyelitis virus in an incompetent strain of Culex tarsalis. *Am J Trop Med Hyg* 41 (2)**,** 241-250.

Kramer, L. D., Hardy, J. L., Presser, S. B., and Houk, E. J. (1981). Dissemination barriers for western equine encephalomyelitis virus in Culex tarsalis infected after ingestion of low viral doses. *Am J Trop Med Hyg* 30 (1)**,** 190-197.

Krishnan, M. N., Sukumaran, B., Pal, U., Agaisse, H., Murray, J. L., Hodge, T. W., and Fikrig, E. (2007). Rab 5 is required for the cellular entry of dengue and West Nile viruses. *J Virol* 81 (9)**,** 4881-4885.

Lakadamyali, M., Rust, M. J., Babcock, H. P., and Zhuang, X. (2003). Visualizing infection of individual influenza viruses. *Proc Natl Acad Sci U S A* 100 (16)**,** 9280-9285.

Lee, K., and Song, K. (2007). Actin dysfunction activates ERK1/2 and delays entry into mitosis in mammalian cells. *Cell Cycle* 6 (12)**,** 1487-1495.

Lee, S., Zhao, Y., and Anderson, W. F. (1999). Receptor-mediated Moloney murine leukemia virus entry can occur independently of the clathrin-coated-pit-mediated endocytic pathway. *J Virol* 73 (7)**,** 5994-6005.

Lehmann, M. J., Sherer, N. M., Marks, C. B., Pypaert, M., and Mothes, W. (2005). Actin- and myosin-driven movement of viruses along filopodia precedes their entry into cells. *J Cell Biol* 170 (2)**,** 317-325.

Lemm, J. A., Bergqvist, A., Read, C. M., and Rice, C. M. (1998). Template-dependent initiation of Sindbis virus RNA replication in vitro. *J Virol* 72 (8)**,** 6546-6553.

Lengsfeld, A. M., Low, I., Wieland, T., Dancker, P., and Hasselbach, W. (1974). Interaction of phalloidin with actin. *Proc Natl Acad Sci U S A* 71 (7)**,** 2803-2807.

Li, G., Barbieri, M. A., Colombo, M. I., and Stahl, P. D. (1994). Structural features of the GTP-binding defective Rab5 mutants required for their inhibitory activity on endocytosis. *J Biol Chem* 269 (20)**,** 14631-14635.

Liu, J. P., and Robinson, P. J. (1995). Dynamin and endocytosis. *Endocr Rev* 16 (5)**,** 590-607.

Loftus, B. J. (2005). Aedes aegypti Sequencing Project. Broad Institute of Harvard & MIT (http://www.broad.mit.edu) and The Institute for Genomic Research (TIGR) (http://www.tigr.org) *In* "x", Vol. x.

Lu, Y. E., and Kielian, M. (2000). Semliki forest virus budding: assay, mechanisms, and cholesterol requirement. *J Virol* 74 (17)**,** 7708-7719.

Ludwig, G. V., Kondig, J. P., and Smith, J. F. (1996). A putative receptor for Venezuelan equine encephalitis virus from mosquito cells. *J Virol* 70 (8)**,** 5592-5599.

Mallardo, M., Schleich, S., and Krijnse Locker, J. (2001). Microtubule-dependent organization of vaccinia virus core-derived early mRNAs into distinct cytoplasmic structures. *Mol Biol Cell* 12 (12)**,** 3875-3891.

Marsh, M., and Helenius, A. (1980). Adsorptive endocytosis of Semliki Forest virus. *J Mol Biol* 142 (3)**,** 439-454.

Marsh, M., and Helenius, A. (1989). Virus entry into animal cells. *Adv Virus Res* 36**,** 107-151.

Marsh, M., and Helenius, A. (2006). Virus entry: open sesame. *Cell* 124 (4)**,** 729-740.

Marsh, M., Wellsteed, J., Kern, H., Harms, E., and Helenius, A. (1982). Monensin inhibits Semliki Forest virus penetration into culture cells. *Proc Natl Acad Sci U S A* 79 (17)**,** 5297-5301.

Matlin, K. S., Reggio, H., Helenius, A., and Simons, K. (1982). Pathway of vesicular stomatitis virus entry leading to infection. *J Mol Biol* 156 (3)**,** 609-631.

Mizutani, T., Kobayashi, M., Eshita, Y., Shirato, K., Kimura, T., Ako, Y., Miyoshi, H., Takasaki, T., Kurane, I., Kariwa, H., Umemura, T., and Takashima, I. (2003). Involvement of the JNK-like protein of the Aedes albopictus mosquito cell line, C6/36, in phagocytosis, endocytosis and infection of West Nile virus. *Insect Mol Biol* 12 (5)**,** 491-499.

Mothes, W., Boerger, A. L., Narayan, S., Cunningham, J. M., and Young, J. A. (2000). Retroviral entry mediated by receptor priming and low pH triggering of an envelope glycoprotein. *Cell* 103 (4)**,** 679-689.

Mousavi, S. A., Malerod, L., Berg, T., and Kjeken, R. (2004). Clathrin-dependent endocytosis. *Biochem J* 377 (Pt 1)**,** 1-16.

Mukhopadhyay, S., Zhang, W., Gabler, S., Chipman, P. R., Strauss, E. G., Strauss, J. H., Baker, T. S., Kuhn, R. J., and Rossmann, M. G. (2006). Mapping the structure and function of the E1 and E2 glycoproteins in alphaviruses. *Structure* 14 (1)**,** 63-73.

Nawa, M. (1998). Effects of bafilomycin A1 on Japanese encephalitis virus in C6/36 mosquito cells. *Arch Virol* 143 (8)**,** 1555-1568.

Niggli, V., and Rossy, J. (2007). Ezrin/radixin/moesin: Versatile controllers of signaling molecules and of the cortical cytoskeleton. *Int J Biochem Cell Biol*. In Press

Paessler, S., Fayzulin, R. Z., Anishchenko, M., Greene, I. P., Weaver, S. C., and Frolov, I. (2003). Recombinant sindbis/Venezuelan equine encephalitis virus is highly attenuated and immunogenic. *J Virol* 77 (17)**,** 9278-9286.

Paredes, A., Alwell-Warda, K., Weaver, S. C., Chiu, W., and Watowich, S. J. (2001). Venezuelan equine encephalomyelitis virus structure and its divergence from old world alphaviruses. *J Virol* 75 (19)**,** 9532-9537.

Paredes, A. M., Ferreira, D., Horton, M., Saad, A., Tsuruta, H., Johnston, R., Klimstra, W., Ryman, K., Hernandez, R., Chiu, W., and Brown, D. T. (2004). Conformational changes in Sindbis virions resulting from exposure to low pH and interactions with cells suggest that cell penetration may occur at the cell surface in the absence of membrane fusion. *Virology* 324 (2)**,** 373-386.

Peters, C. J., and M., D. J. (1990). Alphaviruses. *In* "Virology" (B. N. Fields, and D. M. Knipe, Eds.), pp. 713-761. Raven Press, New York.

Qiao, J., Moreno, J., Sanchez-Perez, L., Kottke, T., Thompson, J., Caruso, M., Diaz, R. M., and Vile, R. (2006). VSV-G pseudotyped, MuLV-based, semi-replication-competent retrovirus for cancer treatment. *Gene Ther* 13 (20)**,** 1457-1470.

Qualmann, B., Kessels, M. M., and Kelly, R. B. (2000). Molecular links between endocytosis and the actin cytoskeleton. *J Cell Biol* 150 (5)**,** F111-116.

Radtke, K., Dohner, K., and Sodeik, B. (2006). Viral interactions with the cytoskeleton: a hitchhiker's guide to the cell. *Cell Microbiol* 8 (3)**,** 387-400.

Rietdorf, J., Ploubidou, A., Reckmann, I., Holmstrom, A., Frischknecht, F., Zettl, M., Zimmermann, T., and Way, M. (2001). Kinesin-dependent movement on microtubules precedes actin-based motility of vaccinia virus. *Nat Cell Biol* 3 (11)**,** 992-1000.

Rink, J., Ghigo, E., Kalaidzidis, Y., and Zerial, M. (2005). Rab conversion as a mechanism of progression from early to late endosomes. *Cell* 122 (5)**,** 735-749.

Roy, A. M., Parker, J. S., Parrish, C. R., and Whittaker, G. R. (2000). Early stages of influenza virus entry into Mv-1 lung cells: involvement of dynamin. *Virology* 267 (1)**,** 17-28.

Royle, S. J. (2006). The cellular functions of clathrin. *Cell Mol Life Sci* 63 (16)**,** 1823-1832.

Saeed, M. F., Kolokoltsov, A. A., and Davey, R. A. (2006). Novel, rapid assay for measuring entry of diverse enveloped viruses, including HIV and rabies. *J Virol Methods* 135 (2)**,** 143-150.

Sasaki, H., Nakamura, M., Ohno, T., Matsuda, Y., Yuda, Y., and Nonomura, Y. (1995). Myosin-actin interaction plays an important role in human immunodeficiency virus type 1 release from host cells. *Proc Natl Acad Sci U S A* 92 (6)**,** 2026-2030.

Satoh, A., Tokunaga, F., Kawamura, S., and Ozaki, K. (1997). In situ inhibition of vesicle transport and protein processing in the dominant negative Rab1 mutant of Drosophila. *J Cell Sci* 110 (Pt 23)**,** 2943-2953.

Satoh, A. K., Tokunaga, F., and Ozaki, K. (1997). Rab proteins of Drosophila melanogaster: novel members of the Rab-protein family. *FEBS Lett* 404 (1)**,** 65-69.

Sawicki, D. L., and Sawicki, S. G. (1993). A second nonstructural protein functions in the regulation of alphavirus negative-strand RNA synthesis. *J Virol* 67 (6)**,** 3605-3610.

Sharkey, C. M., North, C. L., Kuhn, R. J., and Sanders, D. A. (2001). Ross River virus glycoprotein-pseudotyped retroviruses and stable cell lines for their production. *J Virol* 75 (6)**,** 2653-2659.

Shpetner, H. S., and Vallee, R. B. (1992). Dynamin is a GTPase stimulated to high levels of activity by microtubules. *Nature* 355 (6362)**,** 733-735.

Sieczkarski, S. B., and Whittaker, G. R. (2002a). Dissecting virus entry via endocytosis. *J Gen Virol* 83 (Pt 7)**,** 1535-1545.

Sieczkarski, S. B., and Whittaker, G. R. (2002b). Influenza virus can enter and infect cells in the absence of clathrin-mediated endocytosis. *J Virol* 76 (20)**,** 10455-10464.

Sieczkarski, S. B., and Whittaker, G. R. (2003). Differential requirements of Rab5 and Rab7 for endocytosis of influenza and other enveloped viruses. *Traffic* 4 (5)**,** 333-343.

Simpson-Holley, M., Ellis, D., Fisher, D., Elton, D., McCauley, J., and Digard, P. (2002). A functional link between the actin cytoskeleton and lipid rafts during budding of filamentous influenza virions. *Virology* 301 (2)**,** 212-225.

Smith, A. L., and Tignor, G. H. (1980). Host cell receptors for two strains of Sindbis virus. *Arch Virol* 66 (1)**,** 11-26.

Smith, G. A., and Enquist, L. W. (2002). Break ins and break outs: viral interactions with the cytoskeleton of Mammalian cells. *Annu Rev Cell Dev Biol* 18**,** 135-161.

Smythe, E., and Ayscough, K. R. (2006). Actin regulation in endocytosis. *J Cell Sci* 119 (Pt 22)**,** 4589-4598.

Sodeik, B., Ebersold, M. W., and Helenius, A. (1997). Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. *J Cell Biol* 136 (5)**,** 1007-1021.

Soneoka, Y., Cannon, P. M., Ramsdale, E. E., Griffiths, J. C., Romano, G., Kingsman, S. M., and Kingsman, A. J. (1995). A transient three-plasmid expression system for the production of high titer retroviral vectors. *Nucleic Acids Res* 23 (4)**,** 628-633.

Song, B. D., Leonard, M., and Schmid, S. L. (2004). Dynamin GTPase domain mutants that differentially affect GTP binding, GTP hydrolysis, and clathrin-mediated endocytosis. *J Biol Chem* 279 (39)**,** 40431-40436.

Stenmark, H., Parton, R. G., Steele-Mortimer, O., Lutcke, A., Gruenberg, J., and Zerial, M. (1994). Inhibition of rab5 GTPase activity stimulates membrane fusion in endocytosis. *Embo J* 13 (6)**,** 1287-1296.

Strauss, J. H., and Strauss, E. G. (1994). The alphaviruses: gene expression, replication, and evolution. *Microbiol Rev* 58 (3)**,** 491-562.

Strauss, J. H., and Strauss, E. G. (2001). Virus evolution: how does an enveloped virus make a regular structure? *Cell* 105 (1)**,** 5-8.

Strauss, J. H., Strauss, E. G., and Kuhn, R. J. (1995). Budding of alphaviruses. *Trends Microbiol* 3 (9)**,** 346-350.

Strauss, J. H., Wang, K. S., Schmaljohn, A. L., Kuhn, R. J., and Strauss, E. G. (1994). Host-cell receptors for Sindbis virus. *Arch Virol Suppl* 9**,** 473-484.

Sun, X., and Whittaker, G. R. (2007). Role of the actin cytoskeleton during influenza virus internalization into polarized epithelial cells. *Cell Microbiol* 9 (7)**,** 1672-1682.

Sun, X., Yau, V. K., Briggs, B. J., and Whittaker, G. R. (2005). Role of clathrin-mediated endocytosis during vesicular stomatitis virus entry into host cells. *Virology*.

Suopanki, J., Sawicki, D. L., Sawicki, S. G., and Kaariainen, L. (1998). Regulation of alphavirus 26S mRNA transcription by replicase component nsP2. *J Gen Virol* 79 (Pt 2)**,** 309-319.

Superti, F., Seganti, L., Ruggeri, F. M., Tinari, A., Donelli, G., and Orsi, N. (1987). Entry pathway of vesicular stomatitis virus into different host cells. *J Gen Virol* 68 (Pt 2)**,** 387-399.

Sweitzer, S. M., and Hinshaw, J. E. (1998). Dynamin undergoes a GTP-dependent conformational change causing vesiculation. *Cell* 93 (6)**,** 1021-1029.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22 (22)**,** 4673-4680.

Trischler, M., Stoorvogel, W., and Ullrich, O. (1999). Biochemical analysis of distinct Rab5- and Rab11-positive endosomes along the transferrin pathway. *J Cell Sci* 112 (Pt 24)**,** 4773-4783.

van Dam, E. M., and Stoorvogel, W. (2002). Dynamin-dependent transferrin receptor recycling by endosome-derived clathrin-coated vesicles. *Mol Biol Cell* 13 (1)**,** 169-182.

van der Bliek, A. M., and Meyerowitz, E. M. (1991). Dynamin-like protein encoded by the Drosophila shibire gene associated with vesicular traffic. *Nature* 351 (6325)**,** 411-414.

Vidricaire, G., and Tremblay, M. J. (2005). Rab5 and Rab7, but not ARF6, govern the early events of HIV-1 infection in polarized human placental cells. *J Immunol* 175 (10)**,** 6517-6530.

Vitelli, R., Santillo, M., Lattero, D., Chiariello, M., Bifulco, M., Bruni, C. B., and Bucci, C. (1997). Role of the small GTPase Rab7 in the late endocytic pathway. *J Biol Chem* 272 (7)**,** 4391-4397.

Vonderheit, A., and Helenius, A. (2005). Rab7 associates with early endosomes to mediate sorting and transport of Semliki forest virus to late endosomes. *PLoS Biol* 3 (7)**,** e233.

Waarts, B. L., Bittman, R., and Wilschut, J. (2002). Sphingolipid and cholesterol dependence of alphavirus membrane fusion. Lack of correlation with lipid raft formation in target liposomes. *J Biol Chem* 277 (41)**,** 38141-38147.

Wang, K., Huang, S., Kapoor-Munshi, A., and Nemerow, G. (1998). Adenovirus internalization and infection require dynamin. *J Virol* 72 (4)**,** 3455-3458.

Wang, K. S., Kuhn, R. J., Strauss, E. G., Ou, S., and Strauss, J. H. (1992). High-affinity laminin receptor is a receptor for Sindbis virus in mammalian cells. *J Virol* 66 (8)**,** 4992-5001.

Wang, K. S., Schmaljohn, A. L., Kuhn, R. J., and Strauss, J. H. (1991). Antiidiotypic antibodies as probes for the Sindbis virus receptor. *Virology* 181 (2)**,** 694-702.

Wang, Y. F., Sawicki, S. G., and Sawicki, D. L. (1991). Sindbis virus nsP1 functions in negative-strand RNA synthesis. *J Virol* 65 (2)**,** 985-988.

Ward, B. M., and Moss, B. (2001). Vaccinia virus intracellular movement is associated with microtubules and independent of actin tails. *J Virol* 75 (23)**,** 11651-11663.

Warnock, D. E., Hinshaw, J. E., and Schmid, S. L. (1996). Dynamin self-assembly stimulates its GTPase activity. *J Biol Chem* 271 (37)**,** 22310-22314.

Weaver, S. C., Anishchenko, M., Bowen, R., Brault, A. C., Estrada-Franco, J. G., Fernandez, Z., Greene, I., Ortiz, D., Paessler, S., and Powers, A. M. (2004a). Genetic determinants of Venezuelan equine encephalitis emergence. *Arch Virol Suppl* (18)**,** 43-64.

Weaver, S. C., Ferro, C., Barrera, R., Boshell, J., and Navarro, J. C. (2004b). Venezuelan equine encephalitis. *Annu Rev Entomol* 49**,** 141-174.

White, J., and Helenius, A. (1980). pH-dependent fusion between the Semliki Forest virus membrane and liposomes. *Proc Natl Acad Sci U S A* 77 (6)**,** 3273-3277.

Wucherpfennig, T., Wilsch-Brauninger, M., and Gonzalez-Gaitan, M. (2003). Role of Drosophila Rab5 during endosomal trafficking at the synapse and evoked neurotransmitter release. *J Cell Biol* 161 (3)**,** 609-624.

Yao, J. S., Strauss, E. G., and Strauss, J. H. (1996). Interactions between PE2, E1, and 6K required for assembly of alphaviruses studied with chimeric viruses. *J Virol* 70 (11)**,** 7910-7920.

Yarar, D., Waterman-Storer, C. M., and Schmid, S. L. (2005). A dynamic actin cytoskeleton functions at multiple stages of clathrin-mediated endocytosis. *Mol Biol Cell* 16 (2)**,** 964-975.

Ybe, J. A., Greene, B., Liu, S. H., Pley, U., Parham, P., and Brodsky, F. M. (1998). Clathrin self-assembly is regulated by three light-chain residues controlling the formation of critical salt bridges. *Embo J* 17 (5)**,** 1297-1303.

Yonezawa, A., Cavrois, M., and Greene, W. C. (2005). Studies of ebola virus glycoprotein-mediated entry and fusion by using pseudotyped human immunodeficiency virus type 1 virions: involvement of cytoskeletal proteins and enhancement by tumor necrosis factor alpha. *J Virol* 79 (2)**,** 918-926.

Zaitseva, E., Mittal, A., Griffin, D. E., and Chernomordik, L. V. (2005). Class II fusion protein of alphaviruses drives membrane fusion through the same pathway as class I proteins. *J Cell Biol* 169 (1)**,** 167-177.

Zerial, M., and Stenmark, H. (1993). Rab GTPases in vesicular transport. *Curr Opin Cell Biol* 5 (4)**,** 613-620.

Zhao, Y. G., and Eggleston, P. (1999). Comparative analysis of promoters for transient gene expression in cultured mosquito cells. *Insect Mol Biol* 8 (1)**,** 31-38.

Zhu, J., Zhou, K., Hao, J. J., Liu, J., Smith, N., and Zhan, X. (2005). Regulation of cortactin/dynamin interaction by actin polymerization during the fission of clathrin-coated pits. *J Cell Sci* 118 (Pt 4)**,** 807-817.