

**The Role of Receptor-Induced Conformational Changes in Feline Calicivirus Infectious  
Entry**

A Thesis

Presented to the Faculty of the Graduate School

Of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

By

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January 2014

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## ABSTRACT

Keywords: Calicivirus biology, Calicivirus lifecycle, Non-enveloped virus cell entry

Despite 30 years of vaccination the prevalence of feline calicivirus (FCV) infection remains the same, at 30%. This is due to the high variability of the virus and the high mutation rate of RNA viruses. FCV is a non-enveloped, positive sense RNA virus of the genus Vesivirus. Previously, FCV-5 mutants that are resistant to inactivation by incubation with the fJAM-A soluble receptor were identified. The native FCV-5 virus and capsid mutants underwent an increase in hydrophobicity after incubation with fJAM-A, and it was proposed that this increase in hydrophobicity is due to a conformational change in the viral capsid that is important for membrane association and genome release. The specific mechanism of entry for FCV is not well understood. I produced the resistant capsid mutants in a genomic background expressing the FCV-5 capsid proteins and the FCV-Urbana non-structural proteins. I found that the FCV5-Urbana virus was inactivated by incubation with fJAM-A. Capsid mutants of FCV5-Urbana were shown to be resistant to inactivation by fJAM-A when incubated for 30 minutes at 37°C. Pre-incubation at 4°C for 3 hours led to inactivation of FCV-Urbana and the FCV5-Urbana capsid mutants. These findings suggest that a tectonic effect occurs after pre-incubation of FCV with fJAM-A at 4°C for 3 hours. Further characterization of these mutations and their effect on the virus's ability to retain infectivity after incubation with soluble fJAM-A could further our understanding of infectious entry for FCV. Identifying protein interactions necessary for genome release and association with membranes would provide greater understanding of infectious entry for members of the family *Caliciviridae*.

## BIOGRAPHICAL SKETCH

Amanda Fischer obtained her Bachelor of Science degree in Biological Sciences from Cornell University's College of Agriculture and Life Science in 2010. In 2010 she joined the doctoral program in Veterinary Medicine at Cornell University in Ithaca, NY. In 2013 she started her Master of Science degree in the field of Comparative Biomedical Sciences in the graduate school at Cornell University.

As a veterinary student, Amanda Fischer received funding from the Veterinary Investigator Program, sponsored by Merial and the NIH, for two summers of research under the supervision of Dr. John Parker. Her work was presented at the Merial-NIH Veterinary Scholars Symposium in Orlando, FL in 2011. She presented a poster titled "Expression and Purification of Feline Calicivirus P Domain Protein". This work inspired her to apply for an NIH training grant for veterinary students interested in biomedical research. Upon obtaining this funding, Amanda started as a graduate student in the laboratory of Dr. John Parker.

As a graduate student, Amanda Fischer presented a poster in the 12<sup>th</sup> annual Biological and Biomedical Sciences Symposium at Cornell University, titled "The Importance of Feline Calicivirus Capsid Proteins in Cellular Receptor Binding and Genome Release". She also presented in the Baker Institute for Animal Health's seminar series with a talk, titled "The Role of Receptor-Induced Conformational Changes in Feline Calicivirus Infectious Entry". Amanda Fischer plans to return to the College of Veterinary Medicine to complete her veterinary degree.

Amanda Fischer's thesis, *The Role of Receptor-Induced Conformational Changes in Feline Calicivirus Infectious Entry*, was supervised by Dr. John Parker.

## ACKNOWLEDGEMENTS

I would like to express my deep appreciation to my committee chair, Dr. John Parker, who has continually encouraged me in my veterinary and research careers. Without him I would not have the confidence to continue to pursue these endeavors and would certainly never had thought to apply for the NIH training grant that has provided me with such valuable experience. Without his guidance and assistance this thesis would not have been possible.

I would also like to thank Dr. Colin Parrish for his support and for allowing me to assist in the veterinary student virology labs as a graduate student. It was an enriching experience to help teach students and to learn how to explain topics in virology from a new perspective. I enjoyed the opportunity to share what I had learned in veterinary school, and felt like I had a positive influence on their perspective of virology.

I would like to thank the members of the Parker lab, past and present. Dr. Robert Ossiboff has helped with research related and career focused questions with equal ease. Current members, Dr. Zhengchun Lu and Dr. Emily Desmet, provided much needed encouragement through periods of frustrated optimization. Caroline Solomon commiserated with my first year grad student frustration and kept me company on many Saturday afternoons. I would also like to thank the Baker Institute for their overwhelming support and the encouraging learning environment they provide. Knowing I was driving towards a positive work environment every day made me feel so welcome when I initially lacked so much faith in my abilities and research experience.

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## **Feline Calicivirus**

Feline calicivirus (FCV) is a non-enveloped, positive sense RNA virus in the genus *Vesivirus* in the family *Caliciviridae*. The RNA genome is composed of three major open reading frames (ORFs), surrounded by short non-coding regions on each end of the genome. The non-coding regions of the RNA genome are conserved and likely provide important RNA secondary structure that may regulate translation, transcription, or replication. The 5' end of the RNA genome is capped by the virally encoded VPg protein. VPg acts as a 5' cap replacement allowing for ribosomal binding and translation [1]. Open reading frame (ORF) 1 encodes a 195 kDa polyprotein that undergoes proteolytic processing to produce the viral non-structural proteins. These include the RNA dependent RNA polymerase, the 2C-like protein, the 3C-like protease, and the VPg protein [2]. ORFs 2 and 3 encode the major and minor capsid proteins VP1 and VP2, respectively. ORF2 is divided into 6 regions, denoted A-F, indicating distinct regions of the encoded protein. Region E is the most variable and has been used in the phylogenetic analysis of FCV strains. ORF3 appears to be more conserved than ORF2, as ORF2 encodes the antigenic site recognized by neutralizing antibodies [3]. The major viral capsid protein encoded by ORF2 is the primary antigen recognized by the host's immune system [4], [5]. The capsid of FCV is composed of 180 copies of the major capsid protein which forms 90 dimers. This capsid protein is made of three parts: the N-terminal arm, the protruding (P) domain, and the shell (S) domain (Figure 1). The P domain can be examined in two parts, the P1 and the P2 domains [6].

FCV is an important viral pathogen of cats. Infections with FCV can lead to different clinical presentations, including mild upper respiratory infections, severe pneumonia, or systemic disease. Common clinical signs associated with mild FCV infection are fever, rhinitis,

conjunctivitis, chronic stomatitis, and oral ulceration. FCV has a short incubation time; between 2-10 days, and clinical signs have typically resolved in 1-2 weeks. Without proper disinfection feline calicivirus can persist for up to 28 days in the environment [7], [8]. All isolates of FCV in the United States are members of one antigenically diverse serotype [4]. The infection can be acquired from acutely presenting animals, persistent carriers, or fomites, contaminated surfaces in the environment. Previously infected cats can act as carriers causing persistence of the disease in a population, especially where cats are housed in large numbers. Virus can be shed from oropharyngeal ulcers in apparently healthy cats, and these carrier cats can be the source of new cases of FCV infection. Persistence is also affected by genetically distinct strains of FCV circulating in the environment, re-infecting cats in the population. Due to the circulation of multiple genetically distinct viruses within a population, genetic recombination between strains is possible, but does not seem to be important to the genetic diversity of FCV [4], [5], [9],[3].

FCV virulence is believed to be related to changes in capsid binding or the efficiency of genome replication in cells. Interest in understanding the determinants of FCV virulence stems from the fact that the current vaccine does not prevent infection or the persistent carrier state, but may reduce the severity and duration of clinical signs associated with infection [4]. Although vaccines against feline calicivirus have been available for 30 years, the prevalence of feline calicivirus infection has remained unchanged, at ~ 30%. The high prevalence is related to the variability of the viral genome and the high mutation rate associated with RNA viruses [3]. Random mutations in the FCV genome can result in viral isolates associated with more severe clinical signs, such as strains that cause severe pneumonia and mortality in kittens. When viruses isolated from an outbreak in Missouri were sequenced, it was found that 7 amino acid changes in the VP1 capsid protein resulted in the altered clinical syndrome [3]. Within the past decade highly virulent

strains of FCV have emerged that vary significantly from the vaccine strain (F-9). Virulent systemic (VS) strains of FCV grew significantly faster than non-virulent F-9 [5]. These VS strains of FCV are associated with a systemic, hemorrhagic fever and high mortality among cats (vaccinated and un-vaccinated). Infection with VS strains of FCV is associated with ulcerative facial dermatitis, cutaneous edema, alopecia, crusting, and anorexia. Current treatment of these infections is supportive (broad spectrum antibiotics and steroids), and affected cats should be put under strict quarantine [5].

Studies of new vaccine candidates have provided more insight into the *in vivo* response to FCV infection. Neutralizing antibody levels are not necessarily indicative of protection against FCV. Because of this it has been suggested that cellular immunity may be more important in protecting animals from clinical disease than antibody responses, and that serum neutralization may not be the best determinant of protection against FCV [9]. Small changes in certain areas of the genome, specifically the P domain of the capsid, have a significant impact on clinical outcome. Interactions between the virus and cellular receptor are very important in determining morbidity and mortality in clinical cases [4]. Greater understanding of the virus is necessary to protect cats from developing severe disease.

Virulent systemic strains exhibit increased transmission via fomites and resistance to disinfection procedures. Removing virus from a contaminated environment requires strict and specific disinfection procedures. As a non-enveloped virus, FCV is stable in the environment and is not inactivated by most commonly used veterinary disinfectants. Sodium hypochlorite will inactivate the virus when used appropriately, but chlorhexidine and quaternary ammonia compounds do not. Because of its stability and close relationship, feline calicivirus is frequently used as a

surrogate in the study of Norwalk and Norwalk-like viruses, which cause gastroenteritis in humans [10].

### **Feline calicivirus cell surface receptor**

The feline junctional adhesion molecule A (fJAM-A) is the cellular receptor for FCV [11]. JAM-A is important for maintaining tight junctions between epithelial and endothelial cells. The fJAM-A protein is a type I transmembrane glycoprotein and a member of the immunoglobulin superfamily (IgSF). IgSF proteins are the cellular receptors for multiple viruses, including reovirus, coxsackievirus, poliovirus, and adenovirus. JAM-A contains an N-terminal signal peptide, two immunoglobulin-like domains, a transmembrane domain, and a short cytoplasmic tail containing a PDZ-binding motif [12], [13]. JAM-A is widely distributed in feline tissues, including the tongue, skin, liver, spleen, colon, and bone marrow [14]. The fJAM-A protein is also found on the surface of leukocytes and platelets in addition to epithelial and endothelial cells [14]. The protein has three main proposed functions: (i) assembly of intercellular junctions, (ii) angiogenesis, and (iii) leukocyte transmigration [5][15]. Infection of cells with FCV leads to the loss of fJAM-A from the cell surface, potentially weakening the affected epithelial or endothelial barriers [14]. This redistribution of fJAM-A may play a role in the disease signs typically seen with FCV infection.

Mammalian orthoreoviruses, members of *Reoviridae*, also use JAM-A as a cellular receptor in human and mouse cells. Reoviruses attach at the dimer interface of JAM-A [13]. In contrast, the dimer interface is not involved in FCV binding to fJAM-A [16]. The regions of fJAM-A responsible for viral binding were identified by mapping point mutations in the fJAM-A sequence using the known structure of hJAM. The D1 domain is necessary for binding, but is

insufficient for infection. Both the D1 and D2 Ig-like domains of fJAM-A are necessary for normal binding and infection of feline cells by FCV. The cryogenic electron microscopy (cryo-EM) structure of FCV bound to fJAM-A shows that fJAM-A binds to the face of the P2 domain of the FCV major capsid protein. It has been proposed that FCV-fJAM-A interactions prepare the capsid for another structural change that occurs in the endosome (Figure 2), allowing genome release from the capsid [13]. Understanding the details of the interactions between FCV and the fJAM-A ectodomain may be important for understanding VS-FCV infections, as virus attachment and entry are critical stages in the pathogenesis of infection. The clinical signs associated with VS-FCV are related to the breakdown of epithelial barriers, which are partially maintained by JAM proteins [6], [15].

Limitations in our knowledge of cellular entry for *Caliciviridae* are preventing the development of an *in vitro* tissue culture system for members of this family. Human noroviruses (HuNVs) bind histo-blood group antigens (HBGA) on the surface of cells, while murine norovirus (MNV) binds sialic acid moieties [1]. However, the presence of HBGA molecules on cells does not confer susceptibility to infection by HuNV [13]. HuNV can be produced *in vitro* by transfecting human cells with the HuNV RNA genome showing that replication isn't the limiting factor in viral infection. Gaps in our knowledge base have led to great difficulty in cultivating HuNV *in vitro* [17]. This challenge has been related to a lack of understanding concerning the molecules needed for viral binding and entry of HuNV [18]. Currently, FCV is the only member of *Caliciviridae* for which a functional receptor has been identified [6], [15] allowing the use of *in vitro* assays to study virus-receptor interactions.

## **Model for *Caliciviridae***

FCV is an excellent model for members of the family *Caliciviridae*. Like FCV, other members of the *Caliciviridae* family cause disease, including human norovirus (HuNV), murine norovirus (MNV), and rabbit hemorrhagic disease virus (RHDV). Gnotobiotic piglets can be infected with HuNV and have been used as a model for human infection, however the use of this model organism is labor intensive and time consuming. When infected with certain strains of HuNV viral capsids and non-structural proteins are seen within enterocytes of infected gnotobiotic piglets. However, this model has not helped clarify what is missing from the *in vitro* culture model that prevents HuNV entry of cultured cells [1]. There are currently no *in vitro* tissue culture systems for the cultivation of HuNV [17], or RHDV. Although, MNV can be cultivated in tissue culture, a functional cellular receptor has not yet been identified. Because of these barriers it has been difficult to gain an understanding of the mechanisms of cell entry for members of the *Caliciviridae* family. Gaining a greater understanding of how feline calicivirus binds to its receptor may help to improve our understanding of HuNV infectious entry [10]. FCV can be reliably grown in CRFK cells and is thus an ideal model system to understand the entry mechanisms of *Caliciviridae* [4].

Cellular entry for enveloped viruses is better understood than cellular entry for non-enveloped viruses. Enveloped viruses initiate a membrane fusion event where the viral envelope becomes incorporated into a cellular membrane, releasing the viral nucleocapsid into the cytoplasm. Different factors that can trigger this fusion event are known. These include altered pH or exposure to specific cellular proteins. For viral entry of non-enveloped viruses, their capsid protein(s) must come into close association with hydrophobic cell membranes without the

assistance of a membrane bilayer. A conformational change in the virus capsid is believed to be required for membrane association. This change may make the capsid more hydrophobic, allowing membrane association, or may release a viral lytic factor that will disrupt the limiting membrane. This conformational capsid change can be initiated through a variety of factors, similar to enveloped viruses (i.e. pH changes and proteolytic cleavages). Once the conformational change has occurred, the virus forms a close association with the membrane and either transports the capsid across the membrane or releases the genome across the membrane by means of a pore or through membrane lysis. It has been suggested that viruses entering through pore formation induce osmotic swelling, destroying the vesicle, and causing release of the virus into the cytoplasm [19], [20].

Conformational changes in the capsids of non-enveloped viruses are important for association of the capsid with host cell membranes. Polioviruses undergo a conformational change after interaction with specific cellular receptors that causes a change in antigenicity and a change in infectivity [21], [22]. These structural changes initiate the exposure of specific regions of the viral capsid that are inaccessible prior to the conformational change. To understand these changes, viruses that are not inactivated by the soluble cellular receptor are selected. Poliovirus soluble receptor resistant (SRR) mutants have specific amino acid mutations in the capsid protein that affect receptor-induced conformational changes and infectivity. Some of these mutations lie within a hydrophobic pocket of the capsid at the base of the five-fold canyon that surrounds the five-fold axis of symmetry. The canyon is where the poliovirus receptor (Pvr) binds the virus [22]. Movement in this hydrophobic pocket during virus-receptor interactions may be required for conformational changes within the poliovirus capsid that mediate viral entry. Different antiviral drugs have been shown to prevent the production of the altered viral particle [6], [10].

The steps that initiate early infection are very specific to each virus and help determine tissue tropism. The method by which some non-enveloped viruses, such as poliovirus, associate with cellular membranes is partially understood. However, little is known about how *Caliciviridae* penetrates the limiting cellular membrane. Other viruses, like picornaviruses are believed to form a pore in cellular membranes, while adenoviruses lyse endosomal membranes [19].

Feline calicivirus likely uses a process similar to that proposed for poliovirus. It is known that for poliovirus uncoating is initiated by interaction with the Pvr [23]. Upon binding the canyon region of the capsid, a structural change is initiated that results in the formation of a new capsid conformation (135S). This new capsid conformation exposes the N-terminus of VP1 and releases VP4 from the interior of the 135S particle. The N-terminus of VP1 interacts with the cellular membrane to allow genome transport. Recent studies suggest that VP1 is important for binding the cellular membrane, while VP4 is important for pore formation [24]. Similarly, the interaction of fJAM-A with FCV induces a conformational change in the capsid [6]. However, the subsequent pathway is poorly understood for FCV. The interaction of viral proteins with the membrane is believed to enable the transport of viral RNA (vRNA) into the cytosol [19].

Some viruses require transport through the endosomal pathway to achieve productive infections. For viruses that require endosomal transport there are two main pathways used, the clathrin-dependent pathway or the caveolae/lipid raft-dependent pathway. Some viruses that use the clathrin-dependent pathway are canine parvovirus, adenovirus, and possibly papillomaviruses. Caveolae-mediated endocytosis is used by murine polyomavirus and Simian vacuolating virus (SV40) to access the endoplasmic reticulum (ER) [19]. For viruses using the endosomal pathway, interaction with endosome specific components initiates release into the cytoplasm.

This endosomal requirement may be the low pH environment within endosomes or the action of specific catalytic enzymes that require low pH to cleave viral proteins. For example exposure of reovirus to low pH within endosomes is required for entry. Proteases cleave the outer capsid proteins  $\sigma 3$  and  $\mu 1$  to prime the virus particle for membrane interaction. The exposed portions of the capsid initiate membrane transport through release of the  $\mu 1N$  peptide, the lytic factor for reovirus.  $\mu 1N$  is believed to play a role in altering membrane integrity [20].

Membrane rafts in the caveolae-dependent pathway have been implicated in the life cycle of a variety of viral families, enveloped and non-enveloped alike. Their importance has been shown for members of the picornaviridae and papovaviridae families. For Coxsackievirus B4, the viral receptors are recruited to the lipid rafts after viral binding, allowing transport of the virus via the caveolae-dependent pathway. Different virus strains may be transported using different endosomal pathways. Different strains of human papillomavirus used either caveola-dependent or clathrin-dependent endocytic pathways depending on the cell type used for *in vitro* growth. For these strains of human papillomavirus, time to viral entry was associated with the type of endocytic pathway used [25]. For FCV, we know that clathrin-mediated endocytosis and low pH are important to properly transport the virus into the cell [26]. How the virus travels to endosomes and how the genome is released are poorly understood [19]. It has been proposed that virus-receptor interactions prepare the capsid for structural changes that occur within the endosome, allowing genome release from the capsid [6], [16].

Release of the viral genome is a critical step in the lifecycle of a virus. The mechanism of action for this step depends on where the virus is located at this point in the cellular entry pathway, and may be a multistep process. The three main proposed mechanisms for this membrane disruption

are transient membrane modification, pore formation, and total disruption. For some viruses, membrane disruption involves proteolytic cleavage and the exposure of a previously buried portion of the capsid (i.e. Papillomavirus, adenovirus). Some viruses need low pH or other endosomal factors (i.e. Adenovirus, parvovirus). Drugs that raise endosomal pH or prevent the normal acidification of endosomes inhibit infection by viruses dependent upon exposure to low pH, e.g. influenza. Lowered pH may alter capsid conformation allowing for the exposure of specific buried peptide sequences that can then bind the membrane or become susceptible to cleavage by endosomal proteases. Parvoviruses use phospholipase A2 activity present in the N-terminus of the VP1 capsid protein to modify membrane lipids, disrupt the membrane, and initiate genome release. For reovirus there is a myristoyl switch that initiates proteolytic and autolytic changes that result in the exposure of  $\mu$ 1N, which binds the endosomal membrane enabling viral transport through a pore [27]. For poliovirus, it is believed that the genome is injected through a pore formed in the cellular membrane by the viral capsid proteins [28]. The initial interactions of poliovirus with Pvr results in an altered viral particle (135S), which then undergoes a second conformational change resulting in genome release [19].

Different types of membrane lytic factors have been identified including amphipathic  $\alpha$ -helices, myristoyl groups, and lipid-remodeling domains. For adenovirus (AdV), an amphipathic  $\alpha$ -helix in the interior capsid protein VI (pVI) facilitates membrane disruption and allows escape from the endosome. Mutations in this  $\alpha$ -helix result in a change in protein structure and decreased disruption of lipid membranes. For the amphipathic  $\alpha$ -helix of AdV, membrane disruption is likely mediated by induction of positive curvature (a stress to the membrane) that allows the release of the relatively large particle from the endosome [29]. The exact mechanism by which AdV disrupts the membrane could be either large pore formation or total disruption [20]. The

ability of some viral proteins to traverse the membrane is dependent on interaction with hydrophobic membranes to achieve a new conformation. An example of this is Flock house virus (FHV), which has a relatively unstructured  $\gamma$  protein *in vitro* that alters conformation upon interaction with lipid membranes. As with AdV, it is unclear if FHV induces total disruption of the membrane or large pore formation [19], [20].

The intermediates in the membrane transport pathway are poorly understood for most non-enveloped viruses. The transport of viruses across the membrane could be better understood with specific assays to monitor small molecule transport across cellular membranes. *In vitro* experiments examining the release of molecules from liposomes may not accurately reflect what is seen in endosomal compartments. There are likely unidentified factors playing a role in membrane disruption. One example is uncertainty over how much protein is required for pore formation for viruses that release lytic factors [29]. These simple mechanistic questions remain unanswered for many viruses. For viruses for which receptors are known, it would be helpful to get high-resolution structural models of the complexed molecules to understand structural changes that occur in response to viral-receptor interactions. Knowing the changes in angle between secondary and tertiary structures would provide insight into conformational changes required for cellular entry.

### **Conformational change in FCV**

Certain details are known regarding the mechanism of cellular entry for FCV, but many details are missing from this picture. FCV binding to permissive cells leads to permeabilization of the cell membrane to small molecules, such as the toxin  $\alpha$ -sarcin [16]. This observation suggests that FCV forms a pore in the membrane that may be necessary for genome release. Additionally, the

atomic structure of the FCV-5 capsid is known [6]. Conservation of tertiary structure for viral capsids in *Caliciviridae* suggests that these viruses may use similar mechanisms of entry [13]. To elucidate this mechanism of entry, differences in infectivity for FCV isolates were identified. VS-FCV viral isolates, such as FCV-5 are inactivated by incubation with the purified soluble fJAM-A ectodomain at 37°C. This property allowed for the selection of SRR mutants as was done for poliovirus [21]. Using this approach 20 unique capsid mutants were identified in the capsid of FCV-5 that are not inactivated by incubation with soluble fJAM-A. One proposed mechanism of action for this inactivation involves structural changes in the capsid allowing membrane association and genome release. The FCV-5 virus and SRR mutants undergo an increase in hydrophobicity after incubation with the soluble receptor, potentially initiating genome release [3], [6]. This study used mutant viruses selected in tissue culture, so it remains possible that mutations in other parts of the genome played a role in the altered neutralization capacity. Based on this previous work, we believe that conformational changes increasing the hydrophobicity of the FCV capsid are required for release of the viral genome from the capsid and association of the capsid with membranes (Figure 3) [3]. The three soluble receptor resistant mutants I am working with are G329D, K572E, and V516A. All of the mutations are located within the FCV5 capsid VP1 domain. The G329D mutation is located at the P domain-S domain hinge in an amino acid that is highly conserved among caliciviruses. The K572E mutation is located in the antigenic domain and is buried at the dimer interface of the capsid structure. The V516A mutation is buried between two P domains in a surface exposed loop [6]. All of these mutations produce viable virus that undergoes a change in hydrophobicity when incubated with fJAM-A.

A cryo-EM study of the interaction between FCV and fJAM-A confirmed that movement occurs in the viral capsid in response to fJAM-A binding [13]. The movement was determined to be in the S and P domains, but poor resolution prevented the identification of a specific secondary structure participating in the move. I hypothesize that a conformational change in the FCV capsid exposes previously buried hydrophobic regions. This conformational change might allow the virus to associate with the cellular membrane. A second conformational change may occur that causes the capsid to insert into the membrane and form a pore, releasing the genome. The trigger for conformational change in FCV has not been discovered, but may be an important factor in the development of VS-FCV. Viral isolates causing virulent systemic (VS) disease were found to have a 1000-fold decrease in infectivity after pre-incubation with soluble fJAM-A at 37°C, while non-VS isolates showed no significant effect. This decrease in infectivity was not as pronounced at other temperatures and incubation at 4°C showed modest changes in infectivity. The ability of this conformational change to have a significant effect on infectivity is both temperature and concentration dependent [6]. Our understanding of the interactions between FCV and its cellular receptor are still incomplete. Knowing the significance of specific amino acid interactions involved in viral binding will improve our knowledge of virus-cell interactions for members of *Caliciviridae*.

**Figures:**

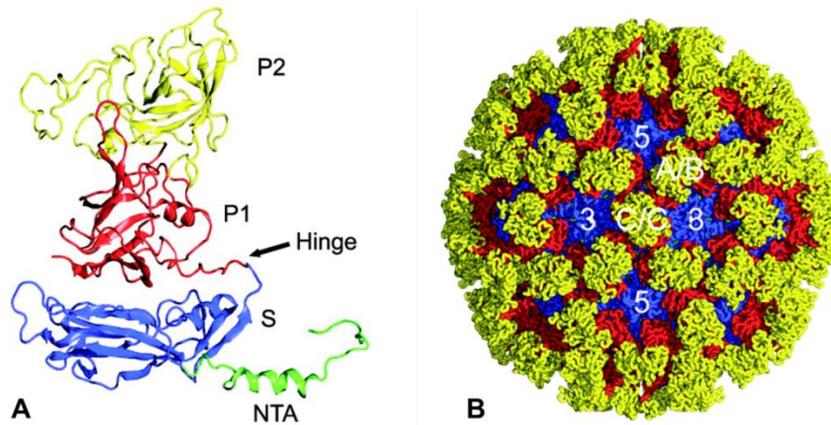


Figure 1: The FCV capsid protein. (A) The feline calicivirus capsid protein has an N-terminal arm (NTA) in green, a shell domain (S) in blue with a hinge domain between the S and protruding (P) domain. The P domain is divided into two subdomains P1 and P2. (B) The complete cryogenic electron microscopy (EM) structure of the FCV capsid shows two types of capsid protein dimers, A/B and C/C. The image is along the 2-fold axis and shows the 3-fold and 5-fold axes of the capsid [6].

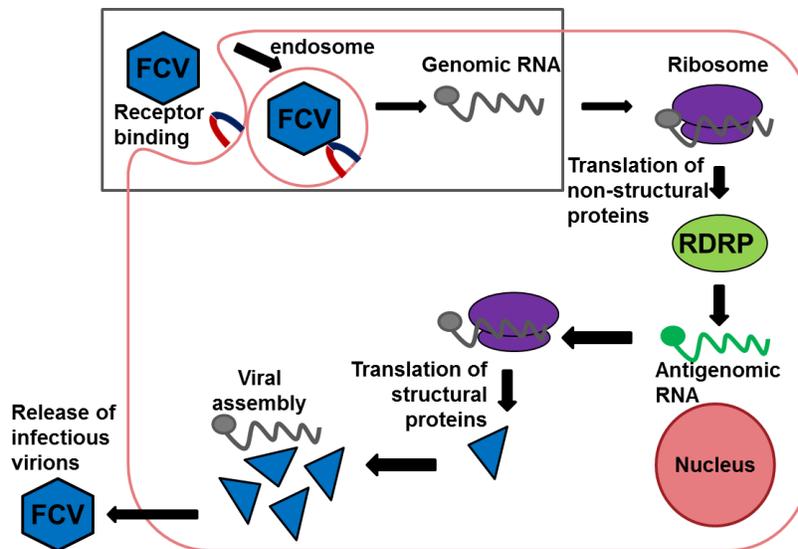


Figure 2: FCV viral lifecycle. Receptor binding is followed by clathrin-mediated endocytosis and genome release. The 5' VPg binds to the translational machinery and translates the non-structural proteins. The viral RNA dependent RNA polymerase (RDRP) transcribes the antigenomic RNA which is used to produce more genomic RNA. The RNA and structural proteins are assembled into infectious virions, which were released from cells.

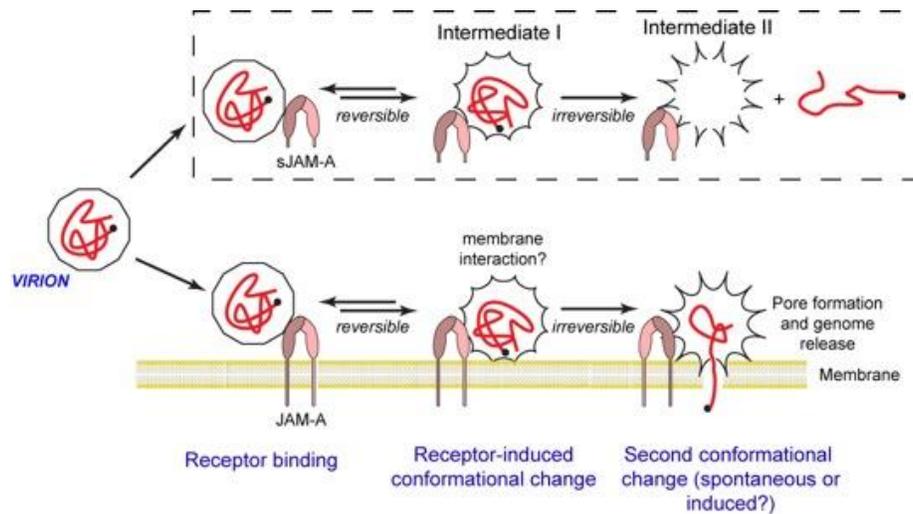


Figure 3: Model for FCV conformational change. The virion interacts with both soluble and bound cellular receptor. We know the capsid undergoes a reversible change in hydrophobicity after this interaction. We propose that there is a second conformational change resulting in genome release across the membrane. (Parker et al)

## Methods

### *Cells*

Crandell-Reese feline kidney (CRFK; ATCC CCL-94) cells were grown in Eagle's essential medium (EMEM; CellGro) supplemented with 5% fetal bovine serum (FBS; Optima), 100 U ml<sup>-1</sup> penicillin, 100g ml<sup>-1</sup> streptomycin, 0.25g ml<sup>-1</sup> amphotericin B, 1 mM sodium pyruvate, and nonessential amino acids (CellGro) at 37°C with 5% CO<sub>2</sub>. Baby hamster kidney cells expressing T7 RNA polymerase (BHK-T7) were maintained in Glasgow minimal essential medium (GMEM) at 37°C with 5% CO<sub>2</sub> supplemented with 5% fetal bovine serum (FBS; Optima), 100 U ml<sup>-1</sup> penicillin, 100g ml<sup>-1</sup> streptomycin, 0.25g ml<sup>-1</sup> amphotericin B, 1 mM sodium pyruvate, and nonessential amino acids. Geneticin selection agent (G418S) was added every other day at a concentration of 1mg ml<sup>-1</sup> to maintain expression of the T7 RNA polymerase.

### *Plasmids and primers for the production of viral mutants*

FCV-Urbana was prepared using the cDNA infectious clone of FCV (pQ14) as previously described [6] [26], [30]. An infectious cDNA clone that contains the non-structural genes of FCV-Urbana fused with the structural genes of FCV-5 was prepared by Dr. Emily Desmet and used to prepare mutations in the capsid of an FCV-5-Urbana chimeric virus. Mutations were first introduced into the FCV-5 capsid sequence within the PF151-FCV5-VP1 shuttle vector by site-directed mutagenesis (Phusion site-directed mutagenesis kit, Thermo Scientific), and sequenced for correctness. The mutated segment of the viral genome (pF151) was then subcloned into a plasmid containing the genome for the recombinant FCV5-Urbana virus (pQ14). Control viruses used were the FCV5-Urbana chimeric virus and FCV-Urbana.

Table 1: Primers used to produce FCV-5 capsid mutants

<u>T<sub>A</sub></u> (°C)	<u>Mutation</u>	<u>Orientation</u>	<u>Sequence</u>
65.2	V516A	Sense	*5'-AG ACA ATC ACG <u>CCA</u> ATC GCG AT-3'
	V516A	Antisense	5'-TGG AAA ATT GCA ATC TTT GTT TG- 3'
69.3	G329D	Sense	5'- TAT TGA AAC CTC CTG <u>ATT</u> CTA TGT TAA CTC A-3'
	G329D	Antisense	5'- AAT GGA ATT TGA AGT CAG GAC CCG -3'
55.8	K572E	Sense	5'- TTC TAT AGG AAT TCT ATG <u>GAG</u> TTA GGT TAT GTT ATC - 3'
	K572E	Antisense	5'- GAT TGG ATG GTT GCC ACC G -3'

\*all primers were phosphorylated on their 5' end as required for the Phusion site-directed mutagenesis kit. Underlined letters are mutated from the known sequence in the FCV5 capsid.

### *Production of f-JAM*

Glutathione-S-transferase (GST)-tagged fJAM-A ectodomain protein was purified from *E. coli* [15]. The pET-41A-fJAM-A (pC43) plasmid was transformed into BL21 *E. coli*. Successfully transformed bacteria were selected with kanamycin and grown in large cultures as previously described. Protein production was induced with 0.4 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). Bacteria were centrifuged, resuspended in phosphate-buffered saline (PBS) (137 mM NaCl, 3 mM KCl, 8 mM Na<sub>2</sub>HPO<sub>4</sub> [pH 7.5]) containing 0.2% Triton X-100, 1 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride, and lysed by sonication. The protein was purified using affinity chromatography with GSTrap Fast Flow columns and elution using a reduced glutathione solution containing 50mM TrisHCl and 10mM glutathione in PBS (GE Healthcare).

In some samples the GST-tag was cleaved from the fJAM-A ectodomain using the HRV 3C protease (Novagen), but the purification protocol for cleaved ectodomain resulted in a much lower yield of protein [15]. Because of this, GST-fJAM-A was used for all experiments. The

amount of protein produced was quantified using a BCA assay kit (Bio-Rad) and a spectrophotometer.

#### *Electrophoresis and immunoblot of recombinant fJAM-A*

The purity and size of GST-fJAM-A ectodomain was confirmed by gel electrophoresis on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gels (Bio-Rad). Specificity was confirmed by immunoblot on polyvinylidene fluoride (PVDF) membrane. The GST-tag was identified using an anti-GST-horseradish peroxidase (HRP) conjugated antibody at a dilution of 1:5000 (GE Healthcare). The presence of bound antibody was detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce). The production of GST-fJAM-A was compared to the results of uninduced bacteria expressing the pC43 plasmid and the results from bacteria expressing a different plasmid (pV41). Samples containing GST-fJAM-A were pooled for purification with an overnight precipitation at 4°C using saturated ammonium sulfate (AS) solution at a ratio of 2:1 (AS solution to protein). Precipitated protein was resuspended in PBS and quantified using the BCA assay kit [15].

#### *Transfection of BHK-T7 cells and CRFK cells with cDNA clones*

FCV amplification and purification was achieved by transfecting a co-culture of cells with the infectious clones of FCV isolates. The transfection was done using a modified protocol used to recover recombinant norovirus [31][6]. This co-culture contained BHK-T7 and CRFK cells grown in GMEM. For transfection each cell type was plated at  $2.5 \times 10^5$  cells/well and incubated overnight. Cells were grown to approximately 80% confluency before transfection using FuGene6 transfection reagent (Promega) according to standard protocol. The culture was

transfected with our wild-type (WT) and mutant FCV5-Urbana encoding plasmids. Initially 2.5  $\mu\text{g}$  of DNA was used per well for the transfection.

Wells showing cytopathic effect (CPE) were harvested, freeze-thawed three times, and diluted for plaque assay as previously described [5]. Plaques were picked and freeze-thawed three times in 500  $\mu\text{l}$  Dulbecco's modified Eagle's medium (DMEM). Confluent CRFK cells grown in EMEM were infected with 300  $\mu\text{l}$  plaque isolated virus and 700  $\mu\text{l}$  DMEM rocking at room temperature for 1 hour. Four ml of EMEM was added and the flask was moved to an incubator at 37°C until CPE was approximately 80%. Then flasks were freeze thawed three times. This process was repeated two times to achieve second passage virus.

Purification of FCV was achieved by infecting T150 flasks of confluent CRFK cells with second passage virus, incubating the cells with virus, and lysing the cells as previously described [6].

Third passage viral stocks were prepared from twice-plaque purified viruses that were amplified in CRFK cells. Recovered viruses were sequenced for correctness. Plaque assays to determine viral titer were performed as previously described [5]. Concentrated viral titers were achieved for all recovered viruses.

Table 2: FCV5-Urbana and SRR mutant stocks

Virus	Stock Titer (PFU ml <sup>-1</sup> )
FCV5-Urbana	$3.5 \times 10^8$
Urbana	$3.75 \times 10^8$
G329D	$1.5 \times 10^9$
V516A	$5.2 \times 10^8$
K572E	$3.67 \times 10^8$

### *Sequencing viral mutants*

Viral mutants were purified as described above. To determine the stability of point mutations viral RNA (vRNA) was sequenced. Viral RNA was purified using the QIAmp Viral RNA Mini kit (Qiagen) according to standard protocol. RNA was eluted in RNase-free water and stored at -80°C. The vRNA was used in the One-Step RT-PCR kit (Invitrogen) to obtain cDNA fragments corresponding to the region of the genome encoding the capsid mutation. The reaction was completed using 125ng RNA and the sequencing primers (2.5µM) for that mutation. DNA fragments produced from the RT-PCR reaction were purified using the Wizard SV Gel and PCR cleanup kit and sent to the Cornell sequencing service as specified in their handbook.

### PCR protocol for RT-PCR

50°C 30 min, 94°C 2 min, 94°C 15s, 46°C 30s, 72°C 1 min 30s, 72°C 10 minutes

\*underlined region repeated for 25 cycles

### *Viral inactivation assays*

Inactivation of FCV after incubation with GST-fJAM-A was assessed by change in viral titer with increasing concentrations of GST-fJAM-A. It was previously shown that increasing concentrations of GST-fJAM-A incubated with FCV-5 resulted in ~250-fold loss of titer. Assays assessing this concentration-dependent decrease were conducted similarly to previous protocols [6]. Increasing concentrations of GST-fJAM-A were incubated with  $1 \times 10^8$  PFU of virus for 30 minutes at 37°C. A concentration of GST-fJAM-A that produced ~200-fold loss of titer was used to conduct viral inactivation assays with FCV5-Urbana and FCV5-Urbana capsid mutants.

Inactivation assays were also conducted with a 3 hour pre-incubation at 4°C and an increased

concentration of GST-fJAM-A, similar to that described in the identification of the temperature dependent inactivation of FCV-5 [6]. Control reactions were incubated with PBS and DMEM plus 0.1% bovine serum albumin (BSA; Calbiochem). Incubated solutions were diluted for standard plaque assay. Change in virus yield was calculated by subtracting the  $\log_{10}$  (PFU ml<sup>-1</sup>) of virus incubated without GST-fJAM-A from the  $\log_{10}$  (PFU ml<sup>-1</sup>) of virus incubated with GST-fJAM-A.

#### *Single and multiple cycle growth assessment for FCV5-Urbana viruses*

To examine single and multiple cycle growth kinetics for the FCV5-Urbana SRR mutants, glass vials were inoculated with  $2 \times 10^5$  CRFK cells in 1 ml of EMEM and allowed to grow for 16 hours [5], [6]. The next day media was aspirated and cells were infected at a multiplicity of infection (MOI) of 5 or 0.01 for single or multiple cycle growth curves, respectively. Cells were incubated at room temperature for 1 hour while rocking. Then 900  $\mu$ l of warmed EMEM was added and samples were allowed to incubate to their respective time points. Samples were stored at -80°C and frozen and thawed three times before plaque assay. Virus yield was calculated by subtracting the  $\log_{10}$  (PFU ml<sup>-1</sup>) at T=0 from the  $\log_{10}$  (PFU ml<sup>-1</sup>) measured for each time point.

#### *Data Analysis and Interpretation*

Assays were performed a minimum of three times with 3 replicates per experiment. Differences between groups were analyzed for statistical significance using Students T test or ANOVA as was appropriate. Statistical analysis was done using R [32].

## Results

**Transfection of BHK-T7-CRFK co-culture was improved through optimization of cell and transfection reagent concentration.** Recovery of recombinant FCV requires synthesis of the positive-sense full-length FCV genomic RNA within the cytosol of cells permissive to viral replication. The original approach used a vaccinia virus to deliver the T7 RNA polymerase to CRFK cells transfected with the cDNA infectious clone of FCV-Urbana [6], [33]. However, this approach is inefficient because of cytotoxicity induced by vaccinia infection and low numbers of transfected CRFK cells. Recovery of recombinant reoviruses uses baby hamster kidney cells engineered to constitutively express the T7 RNA polymerase (BHK-T7 cells). For reoviruses this system is ideal as BHK cells are easily transfected and permissive for reovirus growth [34]. BHK-T7 cells are not permissive to FCV infection. One method of overcoming this obstacle is the use of a co-culture system where BHK-T7 cells are grown with CRFK cells. This system was successfully developed in the Parker lab, allowing production of recombinant FCV (unpublished data).

I optimized the original BHK-T7-CRFK co-culture protocol used because of initial difficulty in obtaining the FCV capsid mutants. Different ratios (1:1, 2:1, and 3:1) of BHK-T7 to CRFK cells were mixed and plated in six well plates, and then transfected using Fugene6 and 2.5 µg of pEGFP-N1 DNA (pV41) at a ratio (vol:wt) of 3:1 or 6:1. The results of the transfection show that plating  $3.4 \times 10^5$  BHK-T7 cells and  $1.6 \times 10^5$  CRFK cells per well 16 hours prior to transfection with 15 µl Fugene6 and 2.5 µg of DNA resulted in the maximal amount of 15% transfected cells (Figure 4E). These cell ratios and Fugene6:DNA ratios for transfection of infectious cDNA clones of FCV led to successful recovery of recombinant viruses.

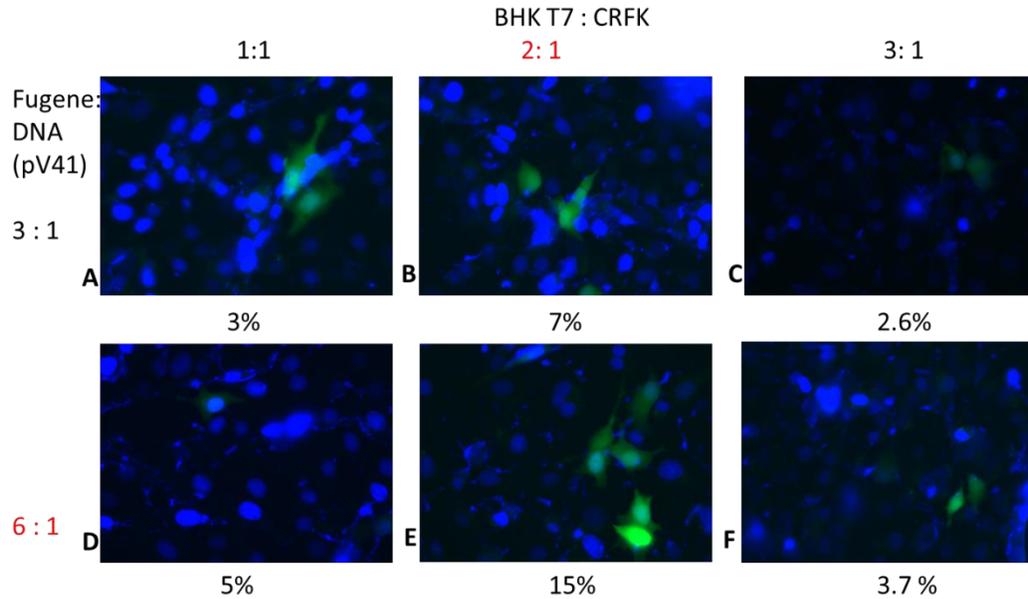


Figure 4: Transfection of BHK-T7-CRFB co-culture. For samples with a 1:1 ratio, each cell type was plated at  $2.5 \times 10^5$  cells/well (A,D). For samples with a 2:1 ratio,  $3.4 \times 10^5$  BHK-T7 cells and  $1.6 \times 10^5$  CRFB cells were plated (B,E). For samples with a 3:1 ratio  $3.75 \times 10^5$  BHK-T7 cells and  $1.25 \times 10^5$  CRFB cells were plated (C,F). For each experiment 2.5  $\mu$ g of DNA (pV41) was used per well for the transfection. 7.5  $\mu$ l (A-C) or 15  $\mu$ l (D-F) Fugene6 was used for transfection. Percentages listed below the images represent the average number of fluorescent cells per field. An average of 4 fields was counted for each treatment.

**Recovery of FCV5-Urbana chimeric viruses and mutants was successful.** The SRR mutant FCV-5 viruses previously described were selected from an *in vitro* culture system and only the capsids were sequenced [6]. The selection process may have produced other variations in the genome contributing to the differences in infectivity that were described. To control for this variation, recombinant viruses were used. We know that FCV-Urbana is not inactivated by fJAM-A incubation and has slower growth kinetics relative to FCV-5 [6], [5]. Use of a chimeric

FCV5-Urbana virus eliminates the effect of differences in the non-structural proteins between FCV-5 and FCV-Urbana to influence infectivity. All of the SRR mutants I am studying are located within the FCV-5 capsid VP1 domain. The G329D mutation is located at the junction of the protruding and shell domains. The K572E mutation is located at the dimer interface of the capsid structure and is buried between two individual capsid proteins. The V516A mutation is located in a surface exposed loop in the proposed receptor binding site [6]. All of these mutants undergo a change in hydrophobicity after incubation with fJAM-A, but are not inactivated by this interaction. Using the optimized protocol for transfecting BHK-T7-CRFK co-cultures, I was able to recover the wild type and mutant recombinant viruses. The capsid sequences of these viruses were verified by sequencing.

**The growth kinetics of the recombinant G329D mutant and the parental FCV5-Urbana chimeric virus were similar.** The SRR mutant G329D virus in the FCV-5 parental background was originally selected by incubation with soluble fJAM-A ectodomain. When growth of the G329D mutant was compared to FCV-5 a decrease in infectivity at 4 hours post-infection was noted [6]. However, when I compared the growth kinetics of G329D and FCV5-Urbana, I found that the growth kinetics and final yield of virus for the parental and mutant viruses were similar under single (MOI=5) and multiple (MOI=0.01) cycle growth conditions (Figure 5). The original SRR mutant in FCV-5 was only sequenced for capsid mutations, and was not examined for mutations in non-structural proteins [6]. These findings suggest that there were other mutations in the G329D SRR mutant outside of the capsid region influencing growth.

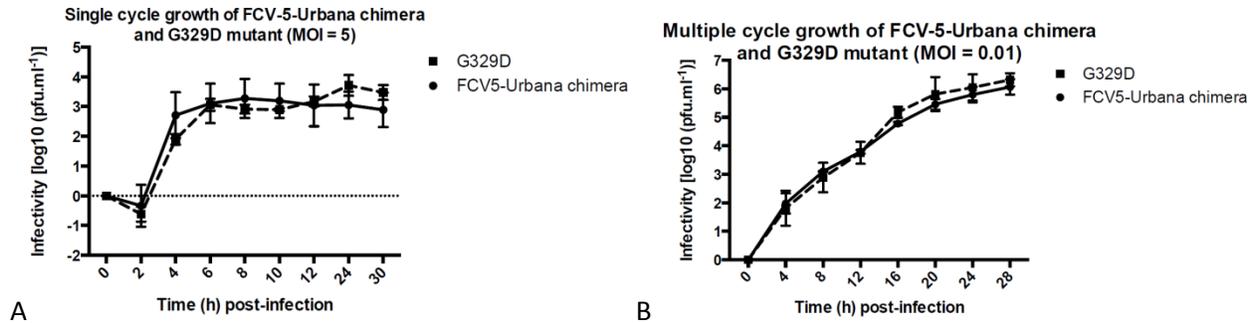


Figure 5: Growth of G329D mutant. CRFK cells were infected with FCV5-Urbana and G329D under single cycle (MOI=5) (A) or multiple cycle (MOI=0.01) (B) conditions. The change in viral titer was determined by plaque assay. The mean log<sub>10</sub> titer (log<sub>10</sub> titer at each point – log<sub>10</sub> titer at T=0) for each time point is shown. Error bars represent the standard deviation for three replicates. Significance was determined by Students T test.

**The recombinant V516A mutant had a lower viral yield than the parental FCV5-Urbana.**

The V516A mutation is present on the surface of the P2 domain close to the dimer interface between two P-domains of the FCV capsid protein. I found this mutant had a lower viral yield under both single (MOI=5) and multiple (MOI=0.01) cycle growth conditions at 24 hours post-infection (Figure 6). This difference was more pronounced at infections with a low MOI, becoming apparent at 16 hours post-infection. A difference in early growth kinetics was not seen as previously described for the V516A SRR mutant and FCV-5 [6], suggesting that there may be other mutations in the original SRR mutants contributing to the delay in growth. The V516A mutation lies within the proposed receptor binding site for fJAM-A [6], [15], suggesting this mutation may affect receptor binding efficiency. The V516A SRR mutant had modestly increased binding affinity for soluble fJAM-A receptor when compared to the parental FCV-5 [6]. The decrease in efficiency of growth for V516A may be explained by altered receptor binding or by effects on subsequent receptor-induced conformational changes.

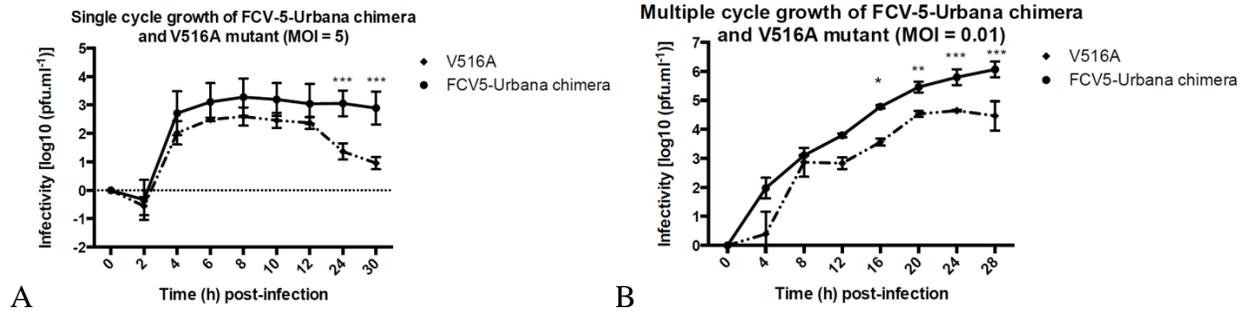


Figure 6: Growth of V516A mutant. CRFK cells were infected with FCV5-Urbana and V516A under single cycle (MOI=5) (A) or multiple cycle (MOI=0.01) (B) conditions. The change in viral titer was determined by plaque assay. The mean log<sub>10</sub> titer (log<sub>10</sub> titer at each point – log<sub>10</sub> titer at T=0) for each time point is shown. Error bars represent the standard deviation for three replicates. Asterisks indicate significant differences between the mutant and parental strain as determined by Students T test (\* p<0.05, \*\* p< 0.01, \*\*\* p<0.001).

**The recombinant K572E mutant had slower growth kinetics and decreased viral yield in comparison to the parental FCV5-Urbana.** The K572E mutation is within the P2 domain of the FCV-5 capsid protein, but it is not readily accessible, being mostly buried within the dimer interface. K572E exhibited significantly decreased viral yield under multiple cycle (MOI=0.01) and single cycle (MOI=5) growth curve conditions (Figure 7). At low MOI it was also evident that there were slower growth kinetics at 4 hours post-infection compared to parental FCV5-Urbana. This is similar to what was previously described with mutations in the native FCV-5 SRR isolate [6]. Previous work showed that the binding affinity of the SRR K572E mutant for soluble fJAM-A was comparable to the parental FCV-5 [6]. The difference in growth noted is more likely due to causes other than changes in receptor binding affinity. One explanation is the K572E mutation alters the ability of the FCV-5 capsid to undergo the conformational changes necessary for infection.

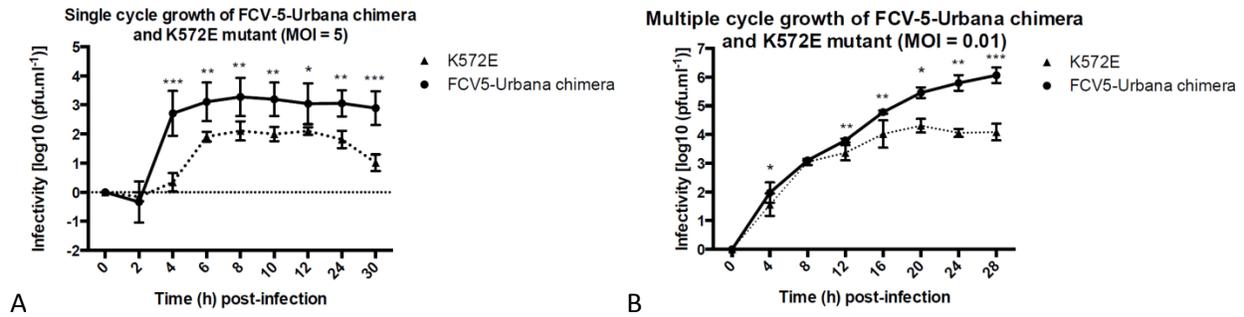


Figure 7: Growth of K572E mutant. CRFK cells were infected with FCV5-Urbana and K572E under single cycle (MOI=5) (A) or multiple cycle (MOI=0.01) (B) conditions. The change in viral titer was determined by plaque assay. The mean log<sub>10</sub> titer (log<sub>10</sub> titer at each point – log<sub>10</sub> titer at T=0) for each time point is shown. Error bars represent the standard deviation for three replicates. Asterisks indicate significant differences between mutant and parental strain as determined by Students T test (\* p<0.05, \*\* p< 0.01, \*\*\* p<0.001).

**FCV5-Urbana is inactivated by preincubation with GST-fJAM-A at 37°C.** Previously Ossiboff et al. showed that preincubation with GST-fJAM-A at 37°C resulted in a significant loss of viral infectivity for FCV-5. In contrast, FCV-Urbana was mostly unaffected by incubation with GST-fJAM-A at 37°C [6]. FCV-5 is a virulent systemic isolate of FCV, while the FCV-Urbana virus is a tissue culture adapted strain [3]. I found that like FCV-5, the FCV5-Urbana chimeric virus was inactivated by incubation with GST-fJAM-A at 37°C. To quantify viral inactivation by GST-fJAM-A ~10<sup>8</sup> PFU of virus was incubated with increasing amounts of purified GST-fJAM-A for 30 minutes at 37°C. I showed a similar saturable, concentration dependent decrease in FCV-5 infectivity. The FCV5-Urbana virus showed a maximum ~10,000-fold loss of viral titer (Figure 8). This decrease in viral titer is greater than was previously seen for FCV-5 [6]. This could be due to the differences in growth kinetics between the two parental

viruses used in the chimeric virus [30]. From these findings, I conclude that FCV5-Urbana is inactivated by GST-fJAM-A in a dose dependent manner.

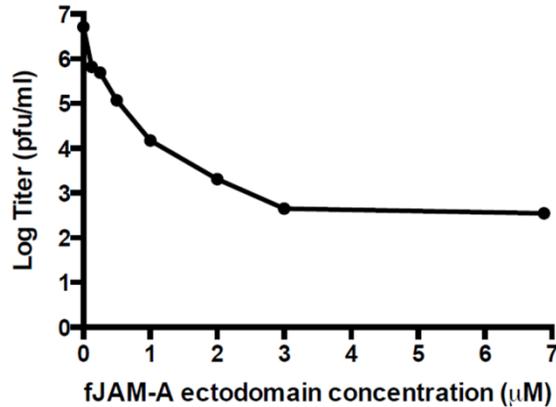


Figure 8: Inactivation of FCV5-Urbana by GST-fJAM-A. FCV5-Urbana ( $\sim 1 \times 10^8$  PFU) was incubated with increasing concentrations of soluble GST-fJAM-A at 37°C for 30 minutes. Samples were plaque titrated on CRFK cell monolayers. Each point represents the average of three replicates.

**The recombinant isogenic SRR mutants are resistant to inactivation by GST-fJAM-A at 37°C.** I examined the inactivation of three specific FCV-5 capsid mutants using the FCV5-Urbana chimeric virus background. These capsid mutants in the background of FCV-5 were previously shown to be resistant to soluble receptor inactivation [6]. I found that the three FCV5-Urbana mutant viruses were significantly resistant to inactivation by incubation with GST-fJAM-A at 37°C. As expected, I found that FCV5-Urbana showed a greater than 2 log loss of titer after incubation with GST-fJAM-A at 37°C (Figure 9). The changes in titer for the recombinant SRR mutants were significantly different from the change in titer seen for the parental strain

( $F_{3,4}=11.81$ ,  $p=0.01$ ). I conclude from these findings that single point mutations within the capsid protein of FCV-5 significantly alter the effect of soluble fJAM-A on virus infectivity.

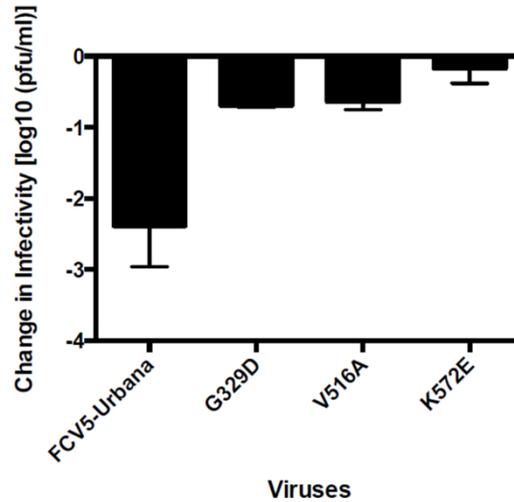


Figure 9: Inactivation of SRR mutants by GST-fJAM-A incubation at 37°C. 0.37  $\mu$ M of GST-fJAM-A was incubated with  $1 \times 10^8$  PFU of virus at 37°C for 30 minutes. The infectivity of samples was assayed by plaque titration. The change in log<sub>10</sub> titer was calculated by subtracting the titer of samples incubated with GST-fJAM-A from samples incubated without GST-fJAM-A. The mean change in log<sub>10</sub> titer and standard deviation of at least three replicates is shown. Significance of differences observed were determined by ANOVA.

**FCV-5-Urbana, FCV-Urbana, and recombinant SRR mutants are inactivated by preincubation with GST-fJAM-A at 4°C.** It was previously shown that incubating FCV-5 with GST-fJAM-A at 4°C for 3.5 hours resulted in only a modest loss in infectivity [15]. However, preincubation of FCV-5 at 4°C for 3 hours prior to incubation for 30 minutes at 37°C resulted in a significant ~250-fold decrease in viral infectivity for FCV-5 [6]. Preincubating FCV5-Urbana for 3 hours at 4°C prior to warming to 37°C resulted in a greater than 4 log decrease in viral titer. Similarly, the recombinant G329D mutant was significantly inactivated under the same

conditions and did not differ significantly from parental FCV5-Urbana in its behavior. In contrast, the recombinant K572E and V516A mutants show a modest decrease in infectivity, which was significantly less than that seen for FCV5-Urbana and G329D. Incubation of FCV-Urbana virus for 3 hours at 4°C prior to incubation for 30 minutes at 37°C led to an average 1 log decrease in titer (Figure 10). The changes in infectivity for FCV5-Urbana and G329D were significantly different from that seen with FCV-Urbana, V516A, and K572E ( $F_{4, 14}=25.59$ ,  $p<0.001$ ). These findings suggest a difference between the SRR mutants in their response to preincubation with soluble receptor at 4°C for 3 hours. One explanation for this observation is that multiple copies of the receptor binding to the virion saturated the receptor binding sites. Conformational changes induced by the receptor upon warming to 37°C may cause global destabilization of the viral capsid. These changes may not occur for SRR mutants when saturating levels of receptor are bound to the capsid.

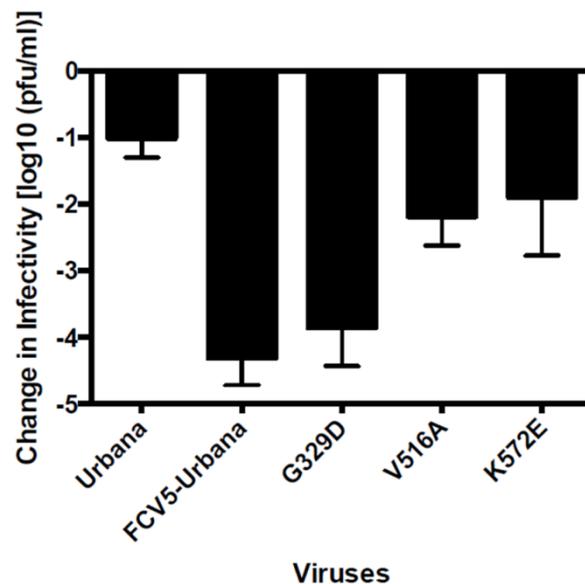


Figure 10: Inactivation of FCV5-Urbana, FCV-Urbana, and FCV5-Urbana mutants by GST-fJAM-A at 4°C. 6.88µM of GST-fJAM-A was incubated with  $1 \times 10^8$  PFU of virus for a pre-

incubation at 4°C for 3 hours before warming to 37°C for 30 minutes. The infectivity of samples was assayed by plaque titration. The change in log<sub>10</sub> titer was calculated by subtracting the titer of samples incubated with GST-fJAM-A from samples incubated without GST-fJAM-A. The mean change in log<sub>10</sub> titer and standard deviation of at least three replicates is shown.

Significance of differences was determined by ANOVA.

## Summary and Future Directions

A major finding of my thesis is that soluble receptor resistant (SRR) mutants in the context of the FCV5-Urbana genetic background are similar to the original SRR mutants in that they are not inactivated by incubation with GST-fJAM-A at 37°C. Inactivation of FCV-5 following interaction with GST-fJAM-A at 37°C is likely caused by a conformational change that takes place in the FCV capsid [6]. The three SRR mutations I studied are in different regions of the capsid protein, but are resistant to inactivation. The results of my experiments confirm that specific point mutations in the capsid are responsible for the differences in susceptibility to receptor inactivation.

One interesting observation I made was that preincubation of viruses with soluble receptor at 4°C prior to warming samples to 37°C resulted in some degree of viral inactivation for all of the viruses tested. Previous work showed that incubation of FCV-5 at 4°C for 3.5 hours resulted in a modest loss of infectivity (average of 0.6 logs). At 4°C virus can bind receptor but presumably no conformational changes occur. However, warming to 37°C after a 4°C preincubation resulted in ~200-fold loss of viral titer for FCV-5. Unlike FCV-5, FCV-Urbana is resistant to receptor induced inactivation when incubated with receptor for 30 minutes at 37°C with no preincubation [6]. I found that FCV5-Urbana and the G329D mutant in the FCV5-Urbana parental background lost ~4 logs of viral titer when warmed to 37°C after a 3 hour preincubation at 4°C. The effect of these conditions on the V516A and K572E mutants was less pronounced with ~100-fold loss of titer. FCV-Urbana showed a 10-fold loss of titer. Differences between the capsid mutants are likely due to the location of these mutations and the length of time that they were incubated with the soluble receptor (Figure 11). The G329D mutation is located at the hinge between the P and S domains. It was shown by cryo-EM that the P domain rotates relative to the S domain in

response to the binding of soluble fJAM-A [16]. This amino acid likely plays an important role in maintaining the flexibility in this region to allow capsid conformational changes. The K572E mutation is located at the dimer interface and is not exposed to the capsid surface. A conformational change in the capsid could result in the exposure of this amino acid, allowing an interaction with the receptor. The significant difference in inactivation behavior between the parental and mutant viruses suggests that this amino acid plays a role in the proposed conformational changes. The resistance of this mutant to inactivation suggests the mutation may stabilize the capsid against conformational changes and may create a requirement for an additional trigger for genome release. The V516A mutation is located in a surface exposed loop in the proposed receptor binding region [6]. The exposure of this amino acid suggests it is likely involved in facilitating the first step of the capsid conformational change. Alternatively, this amino acid may facilitate virus-receptor interactions that are required for the second conformational change in the capsid structure.

The model I have proposed for FCV conformational change involves at least two distinct steps (Figure 3). For other viruses, like poliovirus, different regions of the capsid are exposed in a multi-step process [21], [28]. I propose that under the conditions in which the SRR mutants were selected (incubation for 30 minutes at 37°C) only the initial conformational step is induced by receptor binding. However, I speculate that when capsids are incubated with soluble receptor for 3 hours at 4°C, the receptor binding sites on the capsid become saturated. Subsequently, when the solution is warmed to 37°C a ‘tectonic’ effect occurs inducing both conformational steps leading to viral inactivation [35], [24]. The degree to which this ‘tectonic’ effect occurs depends on the particular SRR mutant.

Some SRR capsid mutants had decreased viral yield and slower growth kinetics compared to the FCV5-Urbana parental virus. Significantly lower viral yields were seen for the V516A and K572E mutants under single and multiple cycle growth conditions. Slower growth kinetics under single and multiple cycle growth curves were seen for the K572E mutant. Differences between FCV-5 and the SRR mutants seen in single and multiple cycle growth curves from previous experiments [6] could be due to the differences in the non-structural proteins contained within these viruses [5] or mutations in ORF1 that were not sequenced in the original SRR mutants [6]. Mutations in the non-structural proteins of the SRR mutants were potentially contributing to the differences seen in growth kinetics and virus yield. However, the differences in growth between the SRR mutants in the FCV5-Urbana background and the parental FCV5-Urbana virus are solely based on the single mutations in the capsid protein. These findings suggest a relationship between FCV infectivity and receptor-induced conformational changes in the viral capsid. This supports my hypothesis that conformational changes are required for membrane penetration and/or genome delivery.

Conformational changes in the FCV capsid likely facilitate membrane association leading to genome release. Further work to assess the ability of FCV5-Urbana mutants to interact with membranes following receptor-induced conformational changes is needed to confirm that receptor-induced virus inactivation is related to functional changes in virus conformation. One approach to determine if receptor-altered capsids can associate with membranes would be to incubate virus with liposomes in the presence or absence of fJAM-A. Standard liposome floatation assay protocols would be used to quantify viral association with membranes [27],[10], [21]. The liposome fractions would be assayed for the presence of viral protein using immunoblot procedures and negative-stain electron microscopy [27]. Based on my hypothesis, I

expect viral capsids to associate with liposomes after interaction with soluble fJAM-A. Given the differences in growth kinetics between the parental FCV5-Urbana and the SRR mutants, it would be interesting to see if membrane association is related to the receptor inactivation properties observed. FCV5-Urbana may associate more strongly with liposomes upon interaction with fJAM-A, and the SRR mutants may associate to a lesser degree. Alternatively, if the two-step model is correct, the first conformational change may be all that is required for membrane association. If this is the case, I would expect FCV5-Urbana and the SRR mutants to associate with membranes to a similar degree following incubation with fJAM-A.

It is possible that the viral capsid needs to be in close proximity to the membrane following interaction with fJAM-A in order to bind membranes. If this is true then no association with liposomes would occur when FCV5-Urbana was incubated with soluble cellular receptor. This possibility could be tested by incorporating His-tagged fJAM-A into liposomes. In order to observe genome release with membrane association, the receptor ectodomain could be linked to liposomes using a protocol similar to that described in studies of poliovirus [22]. I would expect that the G329D, V516A, and K572E mutants will associate with the liposomes under these conditions, but that they will not release their genome. These mutants are resistant to soluble receptor inactivation, suggesting that another trigger is necessary to fully inactivate these viruses. The nature of that trigger is unknown. Conditions within endosomes may be required, as exposure to low pH has been shown to be required for FCV infection [26]. It would be interesting to investigate the effect of low pH on receptor-inactivation of the SRR mutants.

For other non-enveloped viruses the induction of membrane leakage was seen following membrane association [29], [37]. The capacity of FCV to induce membrane leakage could be

assessed using fluorescent dye release from liposomes. ANTS/DPX (fluor/quencher) loaded liposomes could be used to quantify liposome leakage, as previously described [21], [29]. Incubation of FCV5-Urbana with fluorophore-filled liposomes would be expected to result in greater fluorophore leakage than FCV5-Urbana SRR mutants. I hypothesize that SRR mutants need another trigger to initiate genome release. It is unlikely that capsid binding would initiate membrane disruption. Incubation of virus with fluorophore filled liposomes for different amounts of time could elucidate details regarding genome release with capsid mutants. We know these mutants have delayed growth kinetics, which could be due to a delay in genome release.

FCV-5 is an isolate capable of infecting transiently transfected fJAM-A expressing Chinese Hamster Ovary (CHO) cells [15]. The ability of mutant viruses to bind fJAM-A ectodomain could also be assessed using flow cytometry as previously described [6], [15]. These experiments have been performed previously with different FCV isolates. This method would show the association of the FCV5-Urbana SRR mutants with fJAM-A expressed on cells. Modifying the protocol used to isolate vRNA from virus incubated with soluble GST-fJAM-A could allow us to examine genome release in response to cellular membrane bound fJAM-A. Viral RNA can be isolated from pelleted, infected CRFK cells [7]. Differences in genome release between liposomes and cellular membranes would support that there is a trigger from the cellular membrane required for genome release.

Specific mutants of fJAM-A are known to affect viral infectivity (unpublished data). These mutants could be used to further clarify the specific interactions needed to initiate capsid conformational changes and genome release. The use of mutated soluble receptors would aid in the characterization of the interactions between fJAM-A and FCV related to genome release and

membrane association. The differences in infectivity seen with the fJAM-A mutants may be due to altered protein interactions. It is possible that amino acid changes in the receptor may create stronger interactions between the capsid and the receptor, preventing genome release. Another possibility is that after the initial conformational change in the capsid a second interaction between virus and receptor is formed using a different region of the receptor. Facilitation of this second interaction may require low pH to initiate the second conformational step. In this regard, FCV-5 may not require exposure to low pH during virus entry. Experiments to test the pH-dependence of FCV-5 entry may reveal interesting differences.

The current cryo-EM structure of FCV bound to fJAM-A does not provide a clear enough picture of the structures participating in receptor induced conformational change [16], [13]. Improving our understanding of the specific secondary structures participating in the capsid conformational changes would greatly improve our understanding of viral infection. For adenovirus a specific  $\alpha$ -helix in the pVI protein is required for the virus to initiate membrane disruption [29].

Identification of changes in the secondary or tertiary structures in the FCV capsid might allow us to understand the nature of the receptor-induced conformational changes. Similarly, structures of the mutants I studied here both alone and in combination with fJAM-A could reveal important structural intermediates.

Further information regarding the viral entry kinetics of FCV could be learned from the use of RT-qPCR assays [38]. Due to a lack of time, I was unable to begin these assays; however, this avenue of investigation should be further developed. Genome release is a poorly understood aspect of non-enveloped virus entry. A clearer view of early entry kinetics relative to the timing of genome release would help determine the mechanism of entry used [19]. I would expect the

absolute and relative amounts of positive and negative strand vRNA extracted from cells to differ across the growth curve timeline. The two step RT-qPCR assay developed will likely provide greater specificity at low copy numbers than the one step RT-QPCR assay previously described for FCV [7].

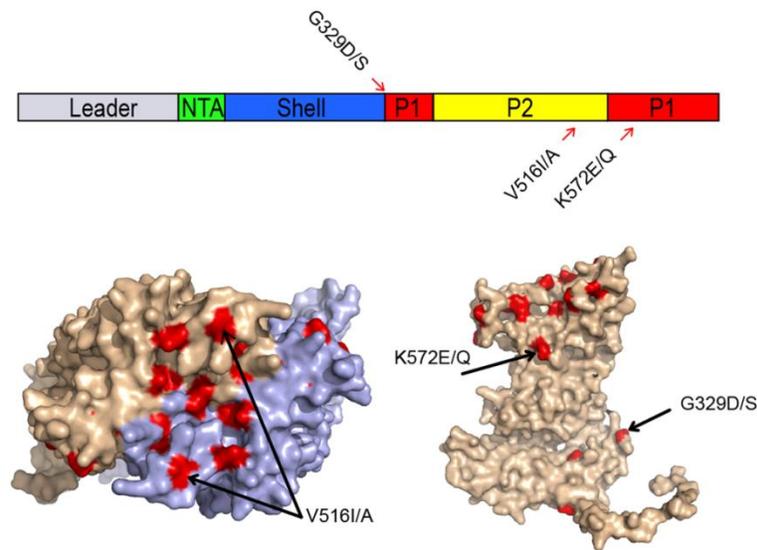


Figure 11: Location of SRR mutants. The G329D mutation is located at the protruding (P) domain-shell (S) domain hinge. The V516A mutation is located in the P2 domain in a surface exposed loop in the proposed receptor binding region. The K572E mutation is located in the antigenic region of the P1 domain and is buried at the dimer interface of the capsid structure. The structure on the left shows a top-down view of a capsid dimer. The structure on the right shows a single capsid protein in a cutaway of the dimer interface (Parker et al).

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