MAPPING THE INTERACTION DOMAIN OF NSP4 WITH THE CAVEOLIN CHAPERONE COMPLEX COMPONENTS

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

The Intracellular Transport of NSP4: A Viral Enterotoxin. (May 2014)

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Rotaviruses are a leading cause of gastroenteritis in children worldwide. Rotavirus non-structural protein 4 (NSP4), a protein coded for by the viral genome, is known to play a key role in viral production and has been found to be able to escape the cell without being part of the virus. Being highly communicable, rotaviruses are transmitted fecal-orally, and in food and water. Symptoms accompanying disease include fever, vomiting and diarrhea, resulting in dehydration and the loss of electrolytes in the absence of hydrating fluids. Non-structural protein 4 (NSP4), encoded by the viral genome segment 10, is known to contribute to this pathogenicity as a viral enterotoxin, causing secretory diarrhea in the absence of histological alterations. NSP4 has recently been shown to traffic from the ER to the plasma membrane (PM) and to subsequently exit the cell. Discerning how NSP4 traffics to the PM and released is a focus of the laboratory. To date, several domains have been mapped to the C-terminus of NSP4. This study has begun to elucidate the transport domain responsible for transport of NSP4 to the PM. Using this knowledge, more effective therapeutics and vaccines can be developed.

DEDICATION

I would like to dedicate this research to my parents. They were hesitant when they found out I would be working in such close proximity with a prevalent virus, but their desire to support me in all my endeavors overrode their concern. They listened patiently, with the rapt attention only a parent is capable of, anytime I explained my project and any new developments, no matter how insignificant. Their unwavering confidence in my abilities inspired my personal confidence in my abilities as well. I owe to them all the achievements of my past, my present and my future.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Judith Ball. She allowed me to enter her laboratory as an undergraduate despite my lack of qualifications in the scientific field. Without her faith in me, I would not have had the opportunity to become involved in the research field outside of the standard course laboratory work. I am taking away not only knowledge of various laboratory techniques and experimental protocols, but invaluable lessons about research, life, and most importantly, patience. I would also like to thank Zachary Franklin. His constant support and encouragement gave me strength on the days I needed it the most. He reminded me to give everything my best effort, no matter how challenging the situation. His faith in my success parallels only that of Dr. Ball's in me. Most importantly, I would like to thank Krystle Yakshe. When she decided to take me on as a student researcher, she became my first true mentor of my undergraduate career. However, she went above and beyond when she befriended me outside of the laboratory as well. These past two years, I have come to deeply appreciate her guidance both in the lab and in my life. I would truly not have been able to make it this far without her.

CHAPTER I

INTRODUCTION

Rotavirus

Rotaviruses (RV) are a major cause of life-threatening diarrhea and gastroenteritis in infants, young children, elderly and immunocompromised individuals. According to the Global Burden of Disease 2004 Update, diarrheal diseases causes approximately 17% of the 10.4 million deaths globally among children under five years (9). In addition, diarrheal diseases serve as the fifth most common cause of death in all ages and the third most common in low-income countries. In Africa alone, 45% of deaths in children are due to diarrheal diseases, compared to 35% in Asia and 20% in the remainder of the world (9). Though rotaviruses are not the only cause of these diarrheal diseases, the ability to protect against rotavirus infection would result in reduction in the number of those infected and subsequent disease symptoms. As of now, only two vaccines are approved for worldwide usage, Rotatrix ® and RotaTeq ®, despite their limited efficacy against new viral strains and the possibility of intussusception (20). Further study of rotavirus is imperative in creating better vaccines and therapeutics for protection of children in the United States and worldwide.

Rotavirus belongs to the family Reoviridae, and contains 11 double-stranded RNA (dsRNA) segments enclosed in a triple-layered, concentrically arranged capsid coat with outer protein spikes. This triple-layer protein coat comprises the infectious viral particle and is believed to stabilize the virus, which allows for fecal-oral transmission from person to person, through water, or through inadequately heated food. The RV genome codes for six structural proteins

(VP) and six non-structural proteins (NSP); the core of the virus holds NSP3, which aids in mRNA circularization, NSP2, a helicase, NSP5, a phosphoprotein with kinase activity, NSP1, a RNA binding protein, and NSP6, a protein that binds to NSP5 (7, 12). The inner core is composed of 60 dimers of VP2, a protein required for replicase activity, which encases VP1, a RNA-dependent polymerase and VP3, a guanylyltransferase and methyltransferase (7, 12). The middle protein layer is composed of 60 dimers of VP6 while the outer protein layer consists of 260 trimers of VP7. The 60 intermittent spikes penetrate the outer protein coat and are composed of asymmetric dimers of VP4 (7, 12). The main domains of the spikes lie in the innermost layer, continue through the middle layer and protrude just above the outer layer. These spikes bind to receptors on the cell surface and allow for attachment of the viral particle to the cell. The spikes undergo enzymatic cleavage to produce VP5* and VP8* which aid in receptor recognition, thus increasing infectivity in some rotavirus strains (4, 12).

The virus is able to enter the cell by initially attaching to outer membrane molecules via the VP4 spikes on the capsid coat. The virus then, is able to be brought into the cell where it loses its calcium-dependent VP7 outer layer creating a double-layered particle (DLP). A conformational change in the DLP opens 12 aqueous channels that allow for nucleotides and hydrolysable ATP to enter the DLP core while newly synthesized 5'-capped, but not polyadenylated, RNA strands leave the core (18). Viroplasms, or "virus factories", containing VP2, NSP2, NSP5, and NSP6, and other viral proteins form adjacent to the endoplasmic reticulum (ER) (10, 17). There, DLPs are assembled with viral proteins and dsRNA segments (8). NSP4, originally classified as a resident ER protein, aids in the DLP's budding into the calcium rich environment of the ER lumen by binding to VP6 (1). Once inside the ER lumen, the DLP acquires transient lipid

membrane and VP7 is assembled to form the TLP (1). Whether the VP4 spikes are added in the lumen with VP7 or at the plasma membrane before the virion leaves the cell remains unclear.

NSP4

NSP4 is a key protein in the morphogenesis and pathogenesis of RV. It contains 175 amino acids, with one transmembrane domain, 2 N-terminal hydrophobic domains, 2 N-terminal, N-linked glycosylation sites and protein binding domains in the extended cytoplasmic C-terminus region (1, 2).

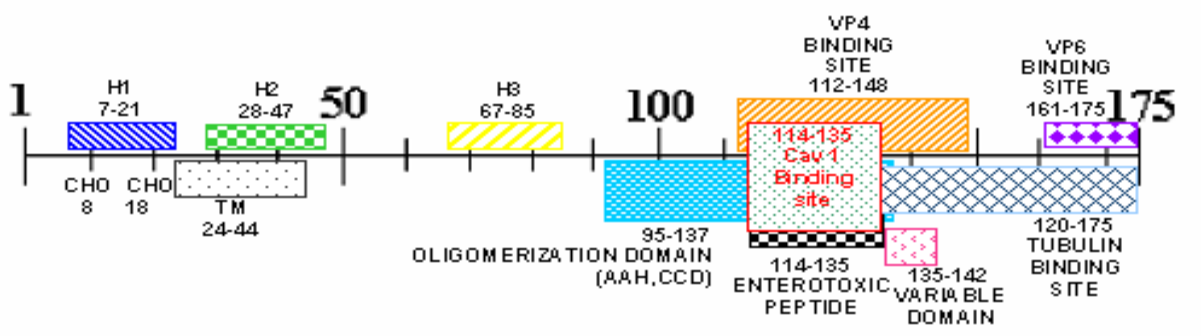

Figure 1. NSP4 structure and binding sites (Taken from Parr, et al., ref. (15))

NSP4's main purpose is to function as an intracellular viral receptor and aid in the morphogenesis and maturation of the viral particle. Residing in the ER membrane, NSP4 mediates the budding of the DLP into the ER by attaching to the VP6 outer layer of the DLP on the cytosolic side of the reticulum. In this way, NSP4 plays a regulatory role in the maturation process of the viral particle. Experiments in which NSP4 was silenced resulted in a 75-80% reduction in the amount of infectious progeny, a notable accumulation of empty viral particles, an intracellular redistribution of viral proteins, and in increase in viral RNA transcription (5, 13,

19). These results indicate NSP4 plays an active role in replication and morphogenesis. Furthermore, proteins commonly found in the viroplasm were dispersed throughout the cytoplasm.

NSP4 is known to interact with intracellular host cell proteins such as caveolin-1 (Cav-1), cyclophilin A (CypA), cyclophilin 40 (Cyp40), heat shock protein 56 (HSP56) and many other host cell and viral proteins (3, 14, 15, 22, 23). These interactions provide a possible explanation for NSP4 transport through the cell to the plasma membrane by an unconventional mechanism. We have shown that NSP4 remains sensitive to endoglycosidase H, an enzyme used by the golgi apparatus to modify proteins destined to leave the cell, and thus, does not go through oligosaccharide processing in the golgi before leaving the cell (2, 6, 21). Therefore, we predict NSP4 reaches the plasma membrane and exits the cell by more than one mechanism.

NSP4 also plays a role as a viral enterotoxin, contributing to the pathogenicity of the virus. Enterotoxins are defined as toxins that stimulate net secretion in ligated segments in the absence of histological alteration and/ or promote secretory currents as measured in Ussing chambers (1). In an experiment involving NSP4, the protein alone causes age-dependent diarrhea in mice by promoting chloride secretory currents in intestinal mucosa cells, without alterations of the histological compositions of the cells (1). Therefore, it is also necessary to evaluate the intracellular transport mechanism of NSP4 to better understand how to generate effective treatments for its enterotoxic activity.

We have previously found that NSP4 interacts with a complex known to transport cholesterol from the ER to the PM, called the caveolin chaperone complex (CCC). The objective of this project is to map the domain of NSP4 responsible for its interactions with the CCC. Entry vectors using the pCR®8/GW/TOPO® (Invitrogen) backbone were constructed using several NSP4 truncations. The LR Clonase enzyme was then used to transfer the truncations into the destination vector plasmid, pcDNA6.2™/V5-DEST by recombination using *att* sites. The resulting NSP4 truncations contained a V5 tag on the C-terminal end for easy detection of fragments. HT29.f8 and MA104 cell lines were then transfected with the plasmid using transfection reagents. Immunoprecipitations were performed using magnetic DynaBeads conjugated to antibodies against the proteins of the CCC. The precipitated proteins were dissociated from the beads and separated by SDS-PAGE followed by a Western blot on to a nitrocellulose membrane. An anti-V5 antibody was used to detect immunoprecipitated NSP4. Once it is clear which domain associates with the CCC components, we will determine how transport is affected when the interaction of the CCC components and NSP4 is ablated using confocal microscopy and surface biotinylation.

CHAPTER II

METHODS

Oligonucleotide Design

PCR primers were designed to amplify truncations of NSP4 from RV strain SA11.4f using the TA TOPO Cloning method (Life Technologies, Grand Island, NY). Briefly, forward and reverse oligonucleotides were designed to amplify portions of NSP4 with a single A overhangs, and nucleotides were added to ensure that NSP4 was inserted in-frame with the V5 tag in the destination vector, pcDNA6.2™/V5-DEST. The oligonucleotides used are listed in **Table 1** and were ordered from Integrated DNA Technologies (Coralville, IA).

Table 1. Primer sequences and corresponding names for NSP4 constructions

Primer Order	Primer Name	Primer Sequence
Forward from 1	CO4	accatggaaaagcttaccgac
Forward from 138	CO6	accatgctacataaagcatccattcc
Forward from 241	CO7	accatgaaattggcaggttataaagag
Forward from 270	CO8	accatgactaaagatgagatagaaaagc
Forward from 304	CO9	accatggtagtcaaagaaatgagac
Forward from 336	CO10	accatgattgacaaattgactacacg
Reverse from 150	CO13	ggaatggatgctttatgtag
Reverse from 270	CO14	agttatctgctctttataacctgcc
Reverse from 321	CO11	tctcatttctttgactactctgtccat
Reverse from 420	CO12	gcctgtcgtttgcaccg
Reverse from 525	CO5	cattgctgcagtcacttctcttg

Media and Reagents

Luria Broth media was prepared for growth of DH5α E. coli; 10 g of Bacto-tryptone, 5 g of yeast extract, and 5 g of NaCl were added per liter of autoclaved distilled water and the pH of the solution was adjusted to 7.0. To prepare plates, 15 g of Bacto-agar were added. A spectinomycin stock was prepared by adding 50 mg of spectinomycin dihydrochloridepentahydrate to 5 ml of distilled water (10mg/ml). The solution was then sterile filtered and aliquoted into 0.5 ml portions in Eppendorf tubes and stored at -20°C. Carbenicillin stocks were prepared by dissolving 1 g carbenicillin (GoldBio, St. Louis, MO) in 10 ml milli-Q dH_2O (100mg/ml). The solution was sterile filtered, aliquoted, and stored as above.

Polymerase Chain Reaction

PCR was performed to amplify NSP4 truncations with TA overhangs using MyTaq DNA Polymerase (Bioline, Taunton, MA). The vector pENTR11-FLNSP4 served as the template for the truncation constructions, which contains the entire RV SA11.4f NSP4 sequence. The appropriate forward and reverse primers were used according to the following table:

Table 2. Forward and reverse primers used in tubes for experiment.

Tube Order	**Forward Primer**	**Reverse Primer**
Tube 1-525	CO4	CO5
Tube 1-420	CO4	CO12
Tube 1-321	CO4	CO11
Tube 1-270	CO4	CO14
Tube 1-150	CO4	CO13

Taq polymerase, which catalyzes the addition of the nucleotides to the expanding chains and dNTP mix, which provides the necessary nucleotides, were added to each tube. The cycling parameters were set up as following:

Table 3. PCR cycling parameters used

Stages:	**Temperature:**	**Time:**
Stage 1	95°	2 minutes
Stage 2	95°	15 seconds
	52°	15 seconds
	72°	10 seconds
Stage 3	72°	10 minutes
Hold	4°	Hold

The PCR enzymes were removed using the UltraClean PCR Cleanup Kit (Mo Bio Laboratories, Carlsbad, CA) following manufacturer's instructions. The reaction products were run on a 1% agarose gel to verify the correct PCR fragments were constructed.

The PCR fragments were cloned into the pCR®8/GW/TOPO® vector using the pCR®8/GW/TOPO® TA Cloning® Kit (Life Technologies) according to manufacturer's instructions. The resulting products were transformed into DH5α E. coli as described below and plated on LB media plates with 40 μg/mL carbenicillin (GoldBio).

E. Coli Transformation and Expansion

Plasmids and E. coli strains were thawed on ice for 15 min. Plasmids (1 μg) were gently mixed with E. coli and incubated for 15 min on ice. The E. coli were heat shocked for 45 sec at 42°C and incubated at R.T. for 5 min. 250 μL of warm Super Optimal Broth with Catabolite Repression (SOC) media was added to each tube and tubes were shaken at 250 rpm for 1 h at

37°C. E. coli was plated on agar plates with LB and incubated overnight at 37°C. Individual colonies were picked and placed into 5 ml LB media with selection antibiotics and shaken at 250 rpm for 12-16 hours. If further expansion was necessary, 2 ml of overnight culture was added to 100 ml of LB media with selection antibiotics and shaken at 250 rpm until the O.D. 600 reached 1.

Plasmid Isolation

Plasmids were isolated with the Fermentas GeneJet Miniprep Kit (Fisher Scientific, Pittsburg, PA) or the Promega Wizard Maxiprep Kit (Promega, Madison, WI) per manufacturer's instructions. Briefly, tubes containing expanded E. Coli were centrifuged at 3000 rpm for 10 min and the supernatant discarded. The pellets were resuspended in proprietary Resuspension buffer. Lysis solution was added and the tubes mixed by inversion 6 times. Neutralization solution was added and the tubes inverted 6 times and centrifuged at 12,000 rpm for 2 min. The supernatant was transferred to a GeneJet spin column and centrifuged at 12,000 rpm for one minute and the flow through discarded to a waste container. The Wash solution was added to each filter and centrifuged at 12,000 rpm 1 min. The flow through was discarded and each tube centrifuged for 2 min to dry. The spin columns were transferred to a clean Eppendorf tube and Elution buffer was added. The tubes were incubated at room temperature and centrifuged for two minutes to collect the resulting plasmid DNA and was stored at -20°C.

LR Clonase Reaction

The NSP4 gene was transferred from the pCR®8/GW/TOPO® to the destination vector, pcDNA6.2™/V5-DEST (Life Technologies) using the LR Clonase II reaction (Life

Technologies). Briefly, the pCR®8/GW/TOPO® vector containing an NSP4 truncation, the pcDNA6.2™/V5 destination vector, 1X TE buffer and LR Clonase II were added to a microcentrifuge tube. The mixture was incubated at room temperature (25°C) for 1 hour. One microliter of Proteinase K solution was added to each sample, the samples were vortexed briefly, and then incubated for 10 minutes in the 37°C water bath. One microliter of each reaction was transformed into DH5α E. coli as described above.

Restriction Enzyme Digestion

Restriction enzyme digestions were performed with Fast Digest Restriction Enzymes (Fermentas) on pcDNA6.2™/V5-DEST plasmids containing NSP4 by mixing 0.5-1 μg of plasmid with 5X Green Buffer, DNAse-free water and the necessary restriction enzymes. The samples were incubated at 37°C in a water bath for fifteen minutes. A 1% agarose gel was made by adding 1 g of High Melt Agar (CLP, Oakland, California) to 100 mL of TAE Buffer and heating for 2 minutes in a microwave with intermittent stirring. The samples were mixed with 6X loading buffer and added to the cooled 1% agarose gel containing Ethidium Bromide (10 ug/mL). The gel was run at 150 volts and the bands were detected using the AlphaImager® gel imaging machine under UV light.

Transfection of Mammalian Cells with pcDNA6.2/V5-DEST-NSP4 Fragments

MA104 and HT29.f8 cells were grown in a 48-well plate to 90% confluency. Briefly, Lipofectamine (Life Technologies) or plasmid DNA was added to serum-free DMEM and equilibrated for ten minutes at 25°C Media containing the Lipofectamine and the plasmid DNA were mixed together and incubated for 5 min. The plasmid-Lipofectamine mixture was added to

the wells containing cells and incubated at 37°C for 4 hours to allow for uptake of the plasmid DNA. Serum was added to the cells and the cells were incubated for 44 more hours at 37°C. Transfection was verified by epifluorescence using the control plasmid pEGFP-C2, which expresses GFP.

Co-Immunoprecipitation

Dynabeads® (Life Technologies) coated with Protein G were prepared by aliquotting into microcentrifuge tubes, removing the storage buffer, and resuspending in PBS. The tube was placed on a magnet in order to separate the beads from solution and the supernatant removed. The antibody to be conjugated to the beads was diluted in PBS with 0.05% Tween-20 and added to the beads. The tube was incubated at room temperature with rotation for 10 minutes to allow for binding of the antibody to Protein G. The tube was placed on the magnet then the supernatant removed. The beads with attached antibodies (Ab) were washed by resuspending in PBS with 0.05% Tween-20 and pipetting gently. The tube was placed on the magnet and the supernatant removed once again. The cell lysates were added to the tube and the Dynabeads-Ab complex resuspended by pipetting. The tubes were incubated overnight at 4°C with rotation. The tubes were placed on the magnet and the supernatant saved for further analysis. The Dynabeads-Ab-Ag complex was washed three times with Washing Buffer and the supernatant discarded.

Polyacrylamide Gel Electrophoresis

To prepare an acrylamide gel, 12.5% NEXT GEL® (Amresco, Solon, OH), 10% ammonium persulphate (w/v) (APS), and TEMED were combined in a small beaker and mixed well. The gel apparatus was set up and the mixture added until it polymerized. The gels were placed in the gel

running apparatus and 1X NEXT GEL Running Buffer was added and the solution poured over the entire apparatus. Samples were individually loaded in the wells and the gel was run at 120V for 2 hours to separate the proteins.

Western Blot

The polyacrylamide gel was removed from the gel running apparatus and sandwiched between two filter papers and two foam pads next to a nitrocellulose membrane. The sandwich was placed into the blot apparatus and run at 300 mA for 2 hours at 4°C. The blot was blocked with 10% milk in PBS for 1 hour and incubated with antibodies overnight at 4°C. The blot was washed with PBS, PBS Tween-20, and PBS again. Secondary antibody was added and incubated for 1 hour. The blot was washed and detected using chemiluminescent substrate (Millipore, Billerica, MA). The chemiluminescence was captured on a film and developed.

CHAPTER III

RESULTS

Truncations of NSP4 Cloned Into pCR®8/GW/TOPO® Backbone

NSP4 truncations were amplified with Taq Polymerase to produce A overhangs on the 3' ends according to **Table 2**. The correct fragments were identified by resolution on a 2% agarose gel. The PCR fragments were inserted into the pCR®8/GW/TOPO® backbone using the TA-TOPO Cloning Kit. Restriction enzyme digestion with XhoI and EcoRI indicated the correct fragments were incorporated into the plasmid prior to continuing with the next step as shown in **Figure 2**. The staggered pattern and migration to the correct molecular weights indicate that the correct fragments were generated. Markers (DNA Hind III) are shown on the left.

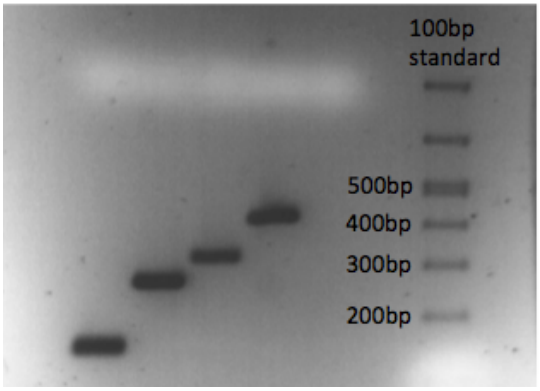

Figure 2. NSP4 PCR products (1-150, 1-270, 1-325, 1-420)

NSP4 Truncations Were Transferred From Entry Vector to Destination Vector

Using vectors from the Gateway system by Invitrogen, the NSP4 truncations were transferred from the pCR®8/GW/TOPO® entry vector to the pcDNA6.2™/V5-DEST destination vector using LR Clonase II. The vector maps of the pCR®8/GW/TOPO® and pcDNA6.2™/V5-DEST are shown in **Figure 3**.

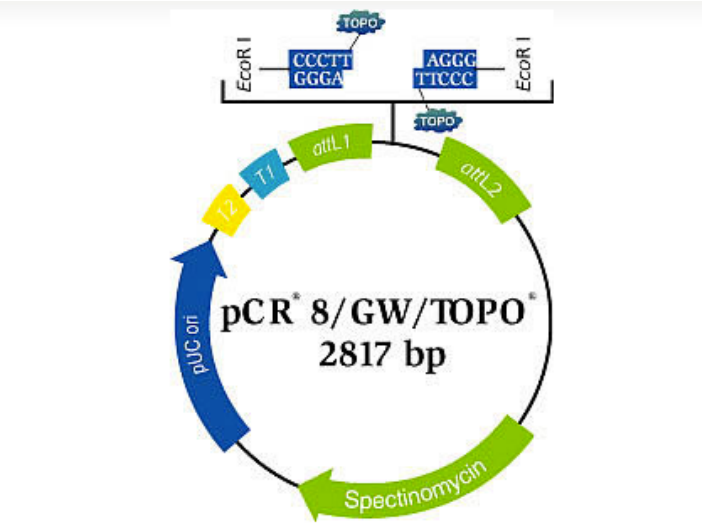

Figure 3a. Diagram of pCR®8/GW/TOPO® vector backbone showing Spectinomycin resistance gene and *attL* sites used in LR Clonase II reaction.

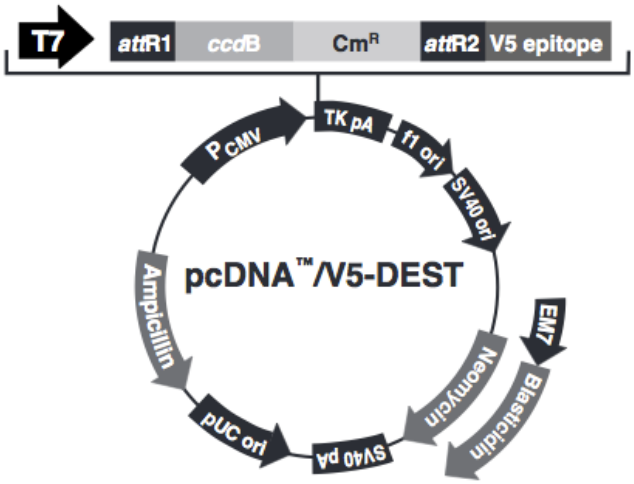

Figure 3b. Diagram of pcDNA6.2™/V5-DEST vector backbone showing Ampicillin resistance gene and *attR* sites used in LR Clonase II reaction

The plasmids are resistant to either spectinomycin or ampicillin, respectively, and empty vectors contain a ccdB gene. Recombination with the LR Clonase II transfers the fragment from the

TOPO vector to the pcDNA6.2™/V5-DEST vector. The products were transformed into E. coli and growth was monitored. The bacteria grew indicating they contained the carbenicillin resistance gene and that the transformation was successful with the pcDNA6.2™/V5-DEST vector containing the NSP4 truncations.

Plasmids were isolated and subjected to restriction endonucleases digestion to verify the correct constructs were incorporated into the vectors. NdeI, ApaI or BglII were used for restriction enzyme digestion. The resulting gel verified the correct plasmid was isolated and contained the appropriate truncations. To further verify that the plasmids contained the correct NSP4 truncation they were sequenced using forward and reverse primers mentioned in the methods section.

Transfected Mammalian Cells Express NSP4 Truncations With V5 tag

Transfection parameters for MA104 and HT29.f8 cells were determined using the pEGFP-12.2-DEST plasmid with MDCK and HT29.f8 cell lines. Intracellular fluorescence using epi-fluorescence microscopy indicated a successful transfection **(Figure 5)**. This experiment revealed that Lipofectamine 2000 and PolyPlus JetPrime® produced the best transfection results in MDCK and HT29.f8 cells, respectively. Following these parameters, MA104 cells (in lieu of MDCK cells) and HT29.f8 cells were transfected with the pcDNA6.2™/V5-DEST plasmids containing the NSP4 truncations. The cells were incubated for 48 h, lysed and subjected to SDS-PAGE and Western blot. Detection with an antibody targeting the V5 tag showed that protein expression occurred at 48 h post transfection (hpt). However, HT29.f8 cells transfected with the plasmid containing $NSP4_{138\text{-}525}$ was toxic to the cells, and caused significant CPE at 48 hpt.

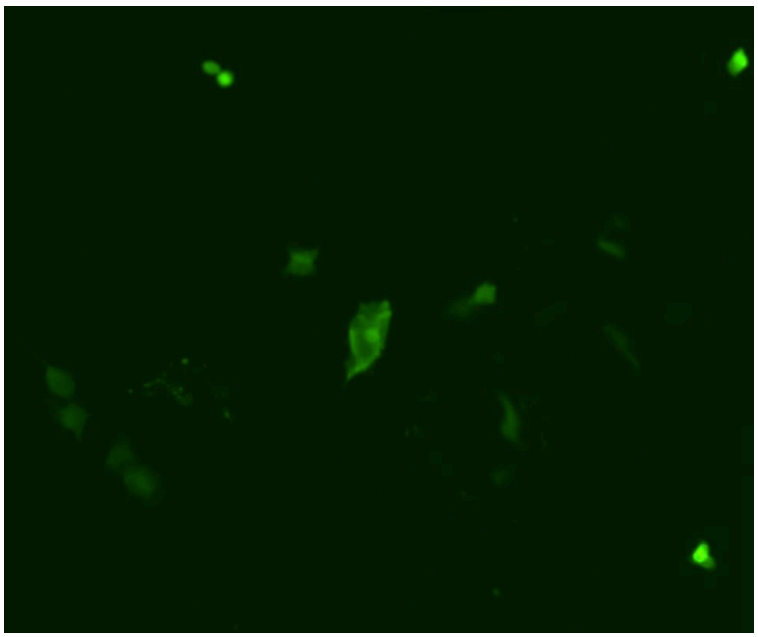

Figure 5a. Epi-fluorescence of MDCK cells transfected with pDEST-12.2 expressing a GFP tag.

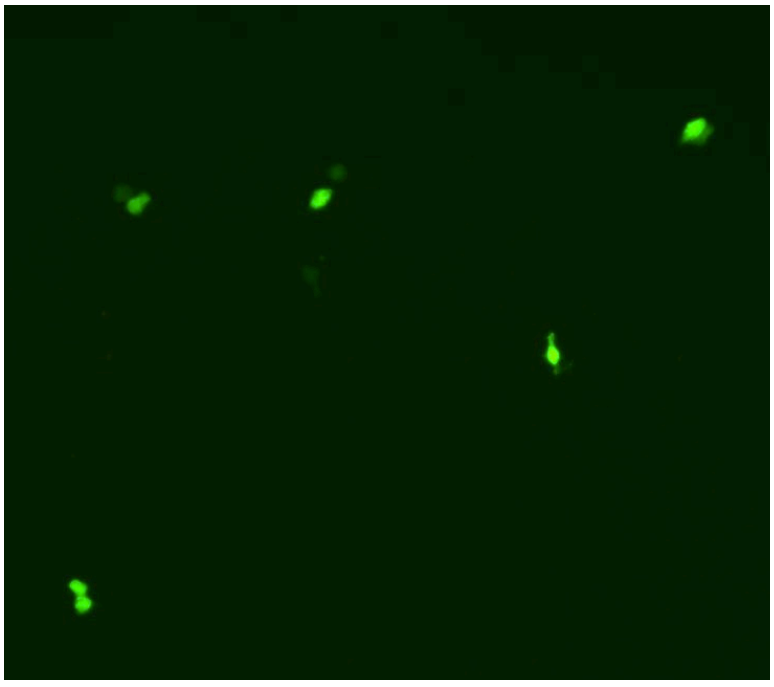

Figure 5b. Epi-fluorescence of HT-29.f8 cells transfected with pDEST-12.2 expressing a GFP tag.

Immunoprecipitation of NSP4 Truncations Using Antibodies Directed Against the V5-Epitope Tag

To map the binding domain of the CCC proteins, antibodies directed against the V5 tag were coupled to the Dynabeads® for co-immunoprecipitations. Cells were transfected with plasmids containing either full-length or the NSP4 truncations, lysed, and subjected to SDS-PAGE and silver stain or Western blot. The silver stain showed the monomeric and dimeric forms of full-length NSP4 in addition to higher molecular weight proteins that co-immunoprecipitated with the V5 tag (**Figure 6**).

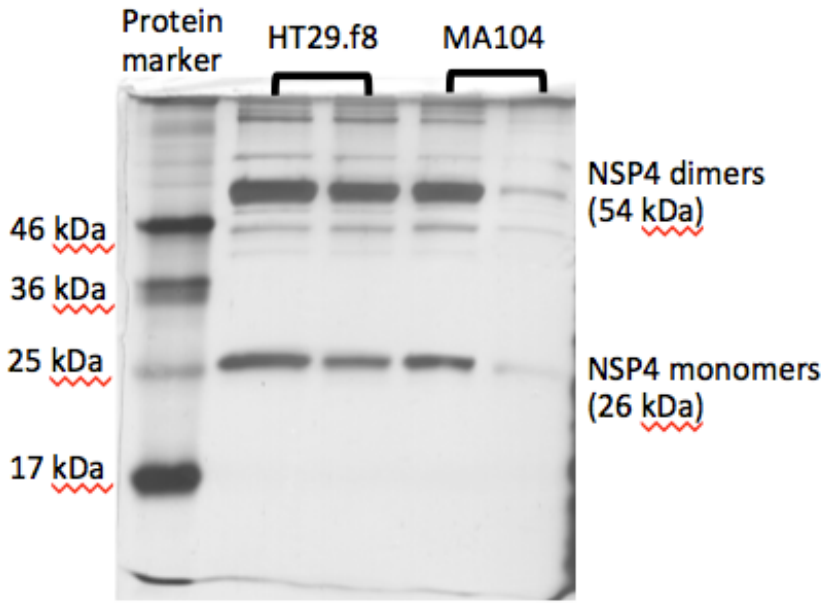

Figure 6. Silver stain showing the monomeric and dimeric forms of full length NSP4

To further verify that the proteins were indeed NSP4, a western blot was performed using a rat antibody directed against the C-terminus of NSP4. Using an antibody generated in rats allowed

us to avoid cross reactivity with the antibody generated in rabbits used for the pull down assay. The developed film shows the monomeric and dimeric forms of NSP4 were immunoprecipitated, verifying that the bands are full-length NSP4 (**Figure 6**).

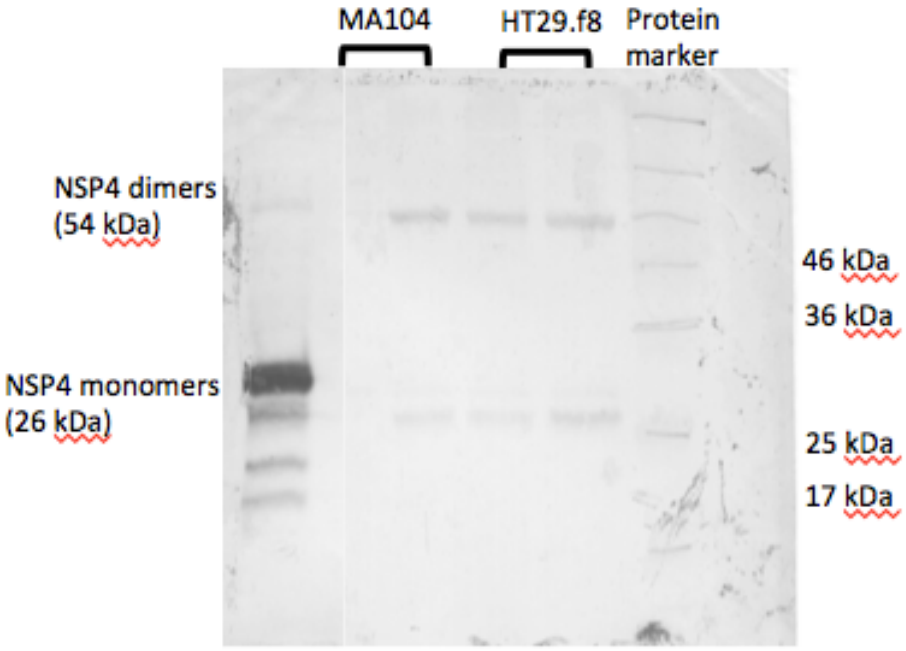

Figure 7. Western blot showing monomeric and dimeric forms of NSP4 of full length NSP4

CHAPTER IV

DISCUSSION

To prepare the NSP4 truncations, seven plasmids containing a NSP4 truncation mutant and lacking a stop codon were generated and verified by RE digestion and sequencing. The truncations were cloned into the pCR®8/GW/TOPO® entry vector using TA TOPO® cloning. The inserts were transferred to the pcDNA6.2™/V5-DEST entry vector backbone containing the V5 epitope at the C-terminus. Using liposome-mediated transfection, the plasmids were transfected into both HT29.f8 and MA104 mammalian cells. NSP4-V5 truncations were expressed and immunoprecipitated. Additional experiments are now ready to be completed.

At this point, we have shown that the constructs have been made and full length NSP4 can be transfected into mammalian cells and immunoprecipitated with a V5 tag antibody. The next step is to verify expression of the correct NSP4 deletion mutants by western blot. After verification of the correct truncation, immunoprecipitations and surface biotinylations will be performed with the truncated NSP4 to determine if NSP4 is associating with the soluble CCC and whether it is reaching the PM. Once the transport domain has been delineated, then small molecules can be designed to block transport of NSP4 out of the cell. By blocking this domain, secretory diarrhea would be prevented, alleviating the severity of symptoms associated with RV infection.

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