DIRECT INTERACTION OF ROTAVIRUS NON STRUCTURAL PROTEIN 4 WITH HEAT SHOCK PROTEIN 56 PROTEIN

A Senior Scholars Thesis

by

SOON YOUNG MOON

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

May 2012

Major: Biology

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Approved by:

Research Advisor: Judith Ball
Associate Director, Honors and Undergraduate Research: Duncan MacKenzie

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ABSTRACT

Direct Interaction of Rotavirus Non Structural Protein 4 with Heat Shock Protein 56 Protein. (May 2012)

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Rotavirus (RV) causes more than 2 million diarrhea incidents and more than 600,000 deaths around the world every year. In order to prevent and treat this fatal disease, we must study, in depth, the mechanisms and pathogenesis of the individual viral proteins. It is known that RV non-structural protein 4 (NSP4) binds to several host-cell proteins, including cycophilin A and 40, which are immunophilins. In this study, we will specifically study another immunophilin/chaperone protein, Heat Shock Protein 56 (FKBP4). Previous studies suggest that FKBP4 plays an important role in transporting cholesterol from the ER to caveolae. During this study, we will use the yeast 2-hybrid assay (Y2H) to ascertain direct binding between NSP4 and FKBP4. After completion of this project, FKBP4 may be interacting weakly or does not interact with NSP4.

DEDICATION

I would like to dedicate this project to science community who studies Rotavirus and other proteins that are involved in this research.

ACKNOWLEDGMENTS

I would like to thank Dr. Judith Ball and Krystle Yakshe for assisting me throughout this whole project process. I would also like to thank Undergraduate Research Scholar program for allowing me to participate in their events to present my project and guidance along the way as well as Texas A&M University by providing this education opportunity.

NOMENCLATURE

Cav-1	Caveolin-1
CypA	Cyclophilin A
Cyp40	Cycolophilin 40
CPRG	Chlorophenol Red Beta D-Galactopyranoside
FKBP4	Heat Shock Protein 56
HSP 56	Heat Shock Protein 56
NSP4	Non-Structural Glycoprotein 4
RED	Restriction Enzyme Digestion
RV	Rotavirus
Y2H	Yeast 2-Hybrid
MID1IP1	Mid 1 Interacting Protein 1

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CHAPTER I

INTRODUCTION

Rotavirus (RV)

Rotavirus (RV) is a major cause of viral gastroenteritis in children, the elderly and immunocompromised patients. Annually, RV causes more than 2 million diarrhea incidents and more than 600,000 deaths around the world. Although much is known about the epidemiologoy and genetics of RV, further understanding of RV-induced fluid loss is needed to reduce RV mortality and morbidity. To enhance the knowledge of the mechanisms by which RV infection affects the host, we will determine how the RV enterotoxin, NSP4, traffics to the plasma membrane by determining which host-cell proteins are directly interacting with NSP4 and contributing to this trafficking event. We will use the yeast two-hybrid assay to determine if NSP4 is directly interacting with specific cellular proteins.

RV consists of a triple layered protein coat encasing 11 strands of double stranded RNA (dsRNA) that code for 12 proteins. The complete virion, the triple layer particle (TLP), is infectious. The spike protein, viral protein 4 (VP4), is cleaved by intestinal proteases to activate the virus and allow attachment of the virus to sialic acid and other receptors and co-receptors on intestinal epithelial cells to enter the cell. (Estes) Once

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This thesis follows the style of Virus Research.

the virus enters the cell, the low calcium environment of the cytoplasm causes the outer layer,composed of VP7, to disassociate and the virus becomes a double layered particle (DLP). This double-layered particle is transcriptionally active, but not infectious because it lacks the receptor molecule that allows RV to enter the cell. Once in the cytoplasm, the dsRNA genome of RV is transcribed into mRNA within the DLP. The mRNA exits the DLP through specific DLP channels and travels to ribosomes to be translated into viral proteins. Most of the viral proteins aggregate into a replication center near the ER called the viroplasm. It is here that the enterotoxin NSP4 plays its first major role in RV pathogenesis. (Cubitt 1980 and Holzel, Jayaram et al 1980)

NSP4

NSP4 is encoded by RV gene 10, is translated by ER membrane bound ribosomes, and is co-translationally inserted into the ER membrane. Traditionally, it has become known as a resident ER glycoprotein. NSP4 traverses the ER membrane such that a short N-terminal region is in the ER lumen and an extended C-terminal domain extends into the cytosol. There is a binding site in the C-terminus that attaches to VP6 (middle layer protein) in the viroplasm and recruits the DLP into the ER. Ca^{++}-dependent VP7 is added in the ER and possibly the VP4 spike protein to complete viral maturation. (Ball 1996, Cohen et al 1979) NSP4 then travels from the ER to the plasma membrane via an unconventional trafficking pathway that bypasses the Golgi. It has been shown that NSP4 binds to several host-cell proteins, including tubulin, caveolin-1, cholesterol,

cyclophilin A, cyclophilin 40 and HSP56, which may contribute to NSP4 secretory transport.

NSP4 also is released from cells early post infection, giving it access to surface receptors. Thus, in addition to playing a key role in RV morphogenesis, NSP4 induces diarrhea by itself when bound to surface molecules. Purified NSP4 or peptides administered intraperitoneally (IP) to mouse pups induces Cl^- secretion from cells via a calcium-mediated pathway. NSP4 also plays key role in the immune response. Hence, NSP4 is a multifunctional protein that has distinct properties when expressed inside the cell and when secreted. (Ball 1996, Taylor and Bellamy 2003)

Caveolin chaperone complex and FKBP4

The caveolin chaperone complex (CCC) is a complex consisting of several proteins that plays an important role in transporting cholesterol from the ER to caveolae microdomains on the plasma membrane. Previous studies showed that the CCC consists of heat shock protein 56 (FKBP4), caveolin-1 (cav-1), cyclophilin 40 (cyp40), cyclophilin A (cypA), and cholesterol. Studies in Dr. Judith Ball's lab have shown that NSP4 directly binds with caveolin-1 using immunoprecipitation and yeast two-hybrid assays (Parr et al 1996). We reported that NSP4 and peptides preferentially bind highly curved, cholesterol-rich model membranes that resemble caveolae. (Schroeder 2001) We then isolated NSP4 from caveolae fractions acquired from infected cells (Parr 2006). Our recent data shows that NSP4 directly binds to cholesterol (Schroeder, 2011). Taken together, we have shown an interaction between NSP4 and, caveolin-1 (cav-1),

cyclophilin 40 (cyp40), cyclophilin A (cypA) and cholesterol. Other labs have found additional binding partners for NSP4. (Gibbons, 2011). We were unable to discern the binding of NSP4 to FKBP4 due to the lack of a reactive antibody. Thus we propose to determine if NSP4 directly binds to FKBP4 using the Yeast 2 Hybrid (Y2H) system.

CHAPTER II

METHODS

In order to study protein-protein interactions, we used the Pro Quest Y2H method. In order to perform Y2H, first we must isolate, amplify and identify the proper gene of interest (in this experiment NSP4 and FKBP4). Then we must transform the yeast with these genes linked to the activating domain of GAL4 (FKBP4) and the binding domain of GAL4 (NSP4). If the two proteins interact, the activating and binding domains will be brought together, the yeast will recruit transcription factors to the GAL4 binding site and transcription will occur. If the 2 proteins fail to interact, the activating and binding domains will remain separated and transcription will not occur. As yeast transcribe the genes, leucine, tryptophan or various reporter genes, such as LacZ, HIS3, URA3, will be expressed depending on the protein:protein interaction level. We will analyze various plates that are missing the appropriate amino acids, that are supposed to be expressed by yeast, to qualitatively observe the protein:protein interactions. In order to quantify the strength of the interaction, we will utilize the CPRG technique.

Designing the vector

We purchased a plasmid containing the FKBP4 sequence from OriGene, pCMV6-XL5-FKBP4. The plasmid was made with a CMV promoter, and has the gene of interest cloned between two Not1 sites. The FKBP4 gene was cloned into a Gateway entry vector, pENTR11 (Invitrogen) by cutting the FKBP4 gene out of the plasmid Pcmv6-

XL5-FKBP4 using FastDigest enzymes (fermentas), BamHI, XhoI and XmnI. After running a restriction enzyme digestion (RED), the samples were run in 1% agrose gel. We then isolated a fragment corresponding to the correct size of our gene of interest using UltraClean GelSpinKit (MoBio). After we isolated the gene it was amplified via PCR and then it inserted into pENTR11 with T4 ligase. The ligation was checked using another RED and running a gel. Once we established that the the entry vector was correct, we amplified the gene using E. coli transformation. The same protocol was followed for NSP4.

E. coli transformation

We individually transformed these plasmids into competent E coli that we purchased (DH5alpha). Competent E coli will take up the surrounding plasmid. In this experiment we transformed with NSP4 and FKBP4. Transformation of the plasmids was aided by heat shock and incubating at 37 degrees with shaking overnight. We pellet the cells and isolated the plasmid using a miniprep isolation kit. To check for the correct plasmid, we utilized the RED technique. FastDigest enzymes (Fermentas) were used in this experiment. Bam HI, EcoRV, HindIII was used for NSP4, while BamHI, XhoI and XmnI was used for FKBP4. After RED, the samples were run in a 1% agrose gel. Based on the fragment sizes we can identify whether or not our gene was transformed correctly.

Recombination of plasmid to proper vector

Both entry level plasmids, NSP4 and FKBP4, were ready to examine by Y2H using the destiny vectors PDEST22 and pDEST32 respectively. The destiny vectors PDEST22 and pDEST32 contain the gene for leucine and tryptophan, respectively, and both contain reporter genes (HIS3, URA3 and lacZ). The LR Clonase enzyme kit was used for the recombination. The recombination was checked by restriction enzyme digestion and then to ensure that we have the correct sequence, the sample was sequenced at the Texas A&M University core laboratory and cross-checked with the sequence that was provided by the company.

Y2H

After the genes were cloned into the proper vector, they were transformed into yeast. As we mentioned earlier, each construct contained a gene that expressed a specific amino acid when the yeast translated the plasmid. Thus only correctly transformed yeast can grow on the plate with deficient media that was prepared without the specific amino acids. Table 1 set up we used.

Table 1 Transformation Setup

Transformation	Plasmid 1	Plasmid 2	Plate	Purpose
1	pDBLeu	None	SC-Leu	Transformation control
2	None	None	SC-Leu, SC-Leu, -Trp	Negative transformational control
3	pDBLeu	Pexp-AD502	SC-Leu, -Trp	Self activation control
4	pD32 FKBP4	Pexp-AD502	SC-Leu, -Trp	Self activation of PDEST32
5	pDBLeu	pD22-NSP4	SC-Leu, -Trp	Self activation of pD22-NSP4
6	pD32 FKBP4	pD22-NSP4	SC-Leu, -Trp	Interaction test for pD32-FKBP4 and pD22-NSP4
7	pD32 FKBP4	None	SC-Leu	Transformational control

pDBLeu is plasmid provided by company that allows yeast to express leucine
Pexp-AD502 is plasmid provided by company that allows yeast to express tryptophan
SC=Synthetic Complete Medium

Qualifying a protein protein interaction

After the colonies grow in plate 6, which is testing the protein protein interaction between NSP4 and FKBP4, is grown in various plates to determine how strong of interaction these proteins are by comparing with controls that were provided by company that contains either two interaction protein or two proteins that do not interact (Control A,B,C,D,E; A has no interaction, E have very strong interaction). Also utilizing reporter gene we can eliminate false positive because these expression require interaction of two proteins in order to be expressed.

When HIS3 is activated, yeast will produce imidazole glycerol phosphate dehydratase that allows histidine biosynthesis. But 3AT (3 Amino 1,2,4 Triazole) is an inhibitor of this enzyme. Thus stronger the two protein interacts; more growth of yeast on a plate that lacks histidine will be observed. As concentration of 3AT increase, we should see less growth.

When URA3 is activated, yeast will synthesize uracil, thus only when two proteins interact, there will be a growth on a plate. Also when URA3 is activated, yeast convert 5FOA (5 fluorooratic acid) to 5 fluorouracil, which is toxic. Thus stronger the interaction of two proteins, less growth is observed in 5FOA plate.

When lacZ is activated, yeast will express beta galactosidase. Thus when we run X-Gal (5-bromo-4-chloro-indolyl-β-D-galactopyranosid) assay, colonies will change color to blue if positive. X-Gal assay is completely by lysing the yeast by repeating freeze-thawing, and adding X-Gal solution to observe color change.

After qualitatively observe the protein protein interactions, we have to quantify our data.

Quantifying protein protein interaction

To quantify, we used CPRG (Chlorophenol red D galactopyranoside) technique. CPRG act as a substrate to beta glycosidase that is expressed by the yeast if there was protein protein interaction. Using time and precipitation observed using spectrophotometer, we can calculate how much of beta galactosidase unit is present, which allows us to determines whether interaction is weak, moderate or strong.

CHAPTER III

RESULTS

Y2H with NSP4 and Cav1

We know from our previously reported data that NSP4 interacts with Cav1 by Y2H (Parr 2006). Consequently, this interaction was utilized to develop the necessary reagents and techniques to evaluate NSP4 with heat shock protein 56 (hsp56) using Cav1 as the positive control. Both the NSP4 and Cav1 plasmids previously were constructed, so learning and utilization of the technique was pursued . Individual transformation of the yeast with the controls, NSP4 and Cav1 was successful. We verified the transformation success by additional controls in which we have set up. In this experiment, yeast (S. cerevisiae MAV203 strand) was genetically modified by the company yeast requires all essential amino acids in order to grow. To take advantage of this characteristic, we set up our experiments with various plasmids that allow yeast to express the missing or deleted amino acid(s) to test transformation success.

Table 2 Transformation Controls of NSP4 and Cav1

Transformation	Plasmid 1	Plasmid 2	Plate	Purpose	Results
1	pDBLeu	None	SC-Leu	Transformation control	Growth
2	None	None	SC-Leu, SC-Leu, -Trp	Negative transformational control	No growth
3	pDBLeu	Pexp-AD502	SC-Leu, -Trp	Self activation control	Growth
4	pD32 Cav1	Pexp-AD502	SC-Leu, -Trp	Self activation of PDEST32	Growth
5	pDBLeu	pD22-NSP4	SC-Leu, -Trp	Self activation of pD22-NSP4	Growth
6	pD32 Cav1	pD22-NSP4	SC-Leu, -Trp	Interaction test for pD32-FKBP4 and pD22-NSP4	Growth
7	pD32 Cav1	None	SC-Leu	Transformational control	Growth

In Table 2, transformation 1 was set as the transformation control. The yeast was transformed with the pDBLeu plasmid, which is a necessary gene for yeast to synthesize Leucine (leu) amino acid. We plated the yeast in transformation 1 in complete synthetic (SC) media without leu. Thus if transformation was successful and the yeast expressed the pDBLeu gene, then these yeast will synthesize leu and demonstrate growth in the plate that lacks leu. After incubating for 96 hours, growth was present. This confirmed success in transformation. Transformation 2 was the negative control. We transformed yeast with no plasmid, then plated in SC without leu and SC without leu and tryptophan (trp). Due to the lack of essential amino acids that are required for yeast growth, there should not be any growth, and there was no growth after 96 hours of incubation. Transformation 3 was the positive control. The yeast were transformed with pDBLeu and Pexp-AD502 plasmids, which are necessary genes for yeast to synthesize leu and trp

amino acids, respectively. We plated transformation 3 yeast in SC media that lacked leu and trp. Thus if transformation was successful and the yeast expressed pDBLeu and Pexp-AD502 gene, then yeast will synthesize leu and trp and show growth in the plate that lacks leu and trp. After incubating for 96 hours, growth was present. This confirmed success in transformation. Transformation 4 was set up to test the activation of pD32 Cav1. The vector pD32 contains a gene that expresses leu, and we recombined this vector with the Cav1 gene. If plasmid pD32 Cav1 was transformed successfully, the yeast will synthesize leu as well as express Cav1. The yeast was transformed with the pD32 Cav1 and Pexp-AD502 plasmids. We plated transformation 4 yeast in SC media without leu and trp. Thus if transformation was successful and yeast expressed pD32 Cav1 and the Pexp-AD502 gene, then yeast will synthesize leu and trp and show growth in the plate that lacks leu and trp. After incubating for 96 hours, growth was present as anticipated. This confirmed success in transformation. Transformation 5 was a self activation test. The yeast were transformed with pDBLeu and pD22 NSP4. The vector pD22 contains the gene that expresses trp, and we recombined it with this vector encoding the NSP4 gene. If plasmid pD22 NSP4 was transformed successfully, the yeast will synthesize trp. We plated transformation 5 yeast in SC media without leu and trp. Thus if transformation was successful and the yeast expressed pDBLeu and pD22 NSP4 genes, then the yeast will synthesize leu and trp and permit growth in the plates that lacks leu and trp. After incubating for 96 hours, growth was present. These results again confirmed success in transformation. Transformation 6 evaluated the interaction between Cav1 and NSP4. Yeast were transformed with pD32 Cav1 and pD22 NSP4. If the

transformation was successful, the yeast should synthesize leu and trp and demonstrate growth in the plate that lacks leu and trp. After incubating for 96 hours, growth was present. This confirmed success in transformation.

In the final transformation control, transformation 7, yeast was transformed with pD32 Cav1 plasmid which includes a necessary gene for yeast to synthesize leu amino acid. We plated transformation 7 yeast in SC media without leu. Thus if transformation was successful and yeast expressed then pD32 Cav1 gene, then yeast will synthesize leu and there will be growth in the plate that lacks leu. After incubating for 96 hours, growth was present. This confirmed success in transformation.

Interaction controls

Controls A, B, C, D, and E were provided by the company that have different interaction strengths. As shown in Table 3, Control A has no interaction and the interaction strengths increase as we go to Control E, which has very strong interaction.

Table 3 Interaction Controls

Control Strain	Interaction Strength
A	None
B	Weak
C	Moderately Strong
D	Strong
E	Very Strong

Controls A, B, C, D, E were plated with transformation 5 and 6 yeast grown from previous steps onto the following media plates: SC that lacks leu, trp, and histamine (his) but contains 3AT (We tested three concentrations, 12.5mM, 50mM and 100 mM), SC that lacks leu, trp, and uracil (ura), SC that lacks leu, trp, but contains 0.2% 5 fluoroorotic acid (5FOA), and Yeast Extract - Peptone - Dextrose plus Adenine medium (YPAD). These medias are used to obtain qualitative data as well as to test for the presence of false positives. Each gene is recombined with the activating and binding domains. For example pD22 includes the binding domain while pD32 includes the activating domain. Only when the two protein interact will the activating and binding domains recruit the transcription factors that allow expression of the three reporter genes (his3, ura3, and lacZ). Thus, if the two proteins interact, the yeast will express the his3 gene and synthesize his. Thus when yeasts are plated in SC that lacks leu, trp, and his, but contains 3AT, there will be growth since the activating and binding domains recruit transcription factors and express his. 3AT is a his inhibitor, thus the higher the 3AT concentration, the greater the inhibition, and the lower the growth should be observed. These plates are utilized to establish a basal expression level and eliminate false positives. If the proteins do not interact, there will be no growth. If the two proteins interact, yeast also will express the ura3 gene, enabling the synthesis of ura. To test expression of ura3, we plated yeast in SC that lacks leu, trp, and ura. If there was expression of the Ura3 gene, there will be growth. Uracil tends to convert (5FOA) to 5 fluorouracil, an analog of thymine that inhibits dTMP synthase and kills the cells. Thus if yeast that express the ura3 gene is plated in SC that lacks leu, trp, but contains 0.2%

5FOA, there should be no growth. If there is no expression of the ura3 gene, then there is no conversion of 5FOA to 5fluorouracil.

The YPAD plate contains all the necessary nutrients and amino acids, so all of the yeast should grow in YPAD. Colonies that grew on YPAD was used for X gal assay and CRPG assay. If plate SC lacks leu, trp, and his but contains 12.5mM 3AT, then controls A and B will have no growth, but controls C and D would have little growth, while E would have a lot of growth. Transformation 5 and 6 both should have growth in controls D and E. In SC that lacks leu, trp, and his, but contains 50mM 3AT, controls A, B showed no growth, controls C and D showed little growth (less than 12.5mM 3AT), while control E had a lot of growth. Transformation 5 and 6 both had growth between controls D and E. In SC that lacks leu, trp, and his, but contains 100mM 3AT, controls A, B showed no growth, controls C and D showed little growth (less than 50mM 3AT), while control E had a lot of growth. Transformation 5 and 6 both had growth between controls D and E.

In SC that lacks leu, trp, and ura, controls A and B have no growth, but C and D have little growth, while E has a lot of growth. Transformation 5 and 6 both had growth equivalent to control C.

SC that lacks leu, trp, but contains 0.2% 5 fluoroorotic acid (5FOA), controls A, and B have the most growth, but C and D has little growth, while E has no growth. Transformation 5 and 6 both had growth equivalent to that of D.

In YPAD plate, every sample had great growth. To this plate, we performed 5-bromo-5-chloro-3-indolyl-D-galactoside (x gal) assay. X gal assay is used to obtain another qualitative interaction assay. If two proteins are interacting and lacZ gene is expressed, it changes x gal from white to green. In our experiment, control A, B stayed white, C had slight blue hue, D had blue color, E had deep indigo blue. Transformation 5 and 6 changed color similar to D.

To quantify our result, we performed chlorophenol red beta D galactopyranoside (CPRG) assay. If lacZ gene is expressed, it changes color from yellow to maroon color. After color changes, we add stop solution and measure the amount of color change. We used FLOUstar Omega Microplate Reader to measure amount of color change. We are in progress of analyzing the significance of color change.

In CPRG assay, we obtained result on Figure 1.

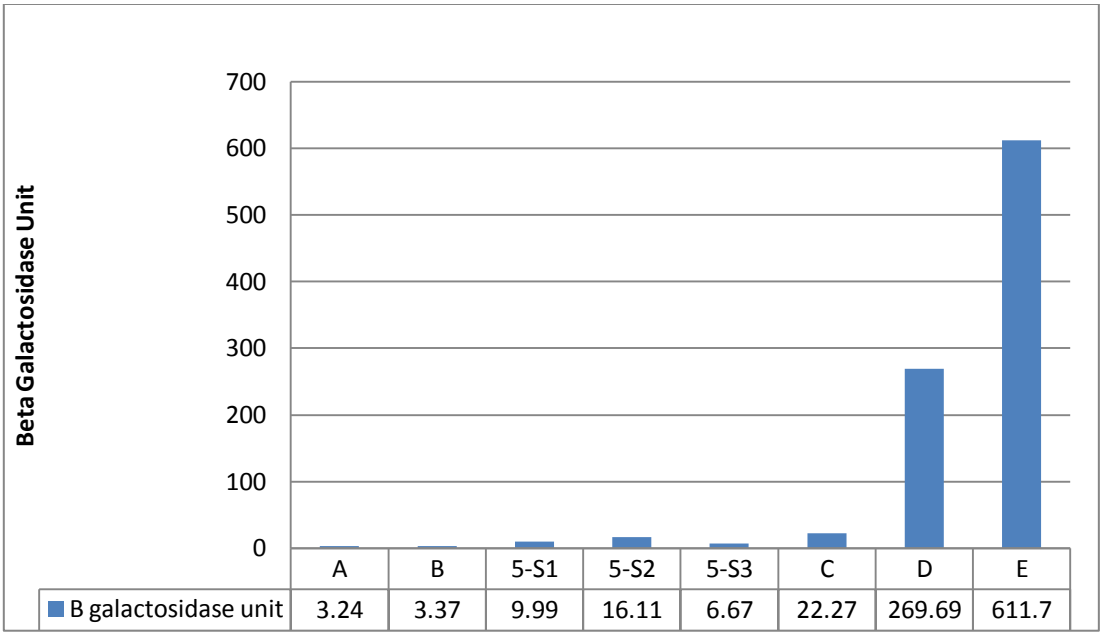

Figure 1 Beta Gal Unit in Cav1 and NSP4 Interaction Test

After comparing values from Figure 1, we concluded that Cav1 and NSP4 have a weak interaction.

Y2H with NSP4 and MID1IP1

We know from previous data that NSP4 does not interact with Mid1 interacting protein 1 (MID1IP1) (Parr 2006). NSP4 and MID1IP1 protein interaction was utilized to develop the necessary techniques and better understanding of Y2H as negative control. Both plasmids were constructed in previous experiment, thus Y2H was performed. Transformation of yeast with controls, NSP4 and MID1IP1, was successful as well. Using the same Y2H method as with NSP4 and Cav1, we showed successful transformations of the yeast (Table 4).

Table 4 Transformation Control of NSP4 and MID1IP1

Transformation	Plasmid 1	Plasmid 2	Plate	Purpose	Results
1	pDBLeu	None	SC-Leu	Transformation control	Growth
2	None	None	SC-Leu, SC-Leu, -Trp	Negative transformational control	No growth
3	pDBLeu	Pexp-AD502	SC-Leu, -Trp	Self activation control	Growth
4	pD32 MID1IP1	Pexp-AD502	SC-Leu, -Trp	Self activation of PDEST32	Growth
5	pDBLeu	pD22-NSP4	SC-Leu, -Trp	Self activation of pD22-NSP4	Growth
6	pD32 MID1IP1	pD22-NSP4	SC-Leu, -Trp	Interaction test for pD32-FKBP4 and pD22-NSP4	Growth
7	pD32 MID1IP1	None	SC-Leu	Transformational control	Growth

Based on the data from Table3, transformation 1 was set as the transformation control using the same logic as previously utilized with Y2H analyses of NSP4 and Cav1. After incubating for 96 hours, growth was present, confirming success in transformation. Transformation 2 served as the negative control for the same reason as previous experiments of Y2H with NSP4 and Cav1. After incubating for 96 hours, no growth was present, confirming success in transformation. Transformation 3 was positive control for same reason as previous experiment on Y2H with NSP4 and Cav1. After incubating for 96 hours, growth was present, confirming success in transformation. Transformation 4 was testing activation of pD32 MID1IP1. Yeast was transformed with pD32 MID1IP1 and Pexp-AD502. Since pD32 MID1IP1 contains pD32 vector, if transformation was

successful, yeast will synthesize leu. After incubating for 96 hours in SC without leu and trp, growth was present. This confirmed success in transformation. Transformation 5 was self activation test for same reason as previous experiment on Y2H with NSP4 and Cav1. After incubating for 96 hours, no growth was present, confirming success in transformation. Transformation 6 was our testing interaction between MID1IP1 and NSP4. We transformed yeast with pD32 MID1IP1 and pD22 NSP4. If transformation was successful, yeast should synthesize leu and trp itself, thus growth in the plate that lacks leu and trp. After incubating for 96 hours, growth was present. This confirmed success in transformation. Transformation 7 was set up as another transformation control. The yeast was transformed with Pd32 MID1P1. If transformation was successful, yeast will synthesize leu and grow in SC that lacks leu. After incubating for 96 hours, growth was present. This confirmed success in transformation.

To obtain qualitative data to test NSP4 and MID1IP1 interaction, same protocol of NSP4 and Cav1 was used. In plate SC that lacks leu, trp, and his but contains 12.5mM 3AT, 50mM 3AT and 100mM 3AT control A, B has no growth, but C and D has little growth while E had a lot of growth (As 3AT concentration increased, less growth was observed). Transformation 5 and 6 both had no growth.

Transformation 5 and 6 both had no growth. SC that lacks leu, trp, and ura, control A, B has no growth, but C and D has little growth while E had a lot of growth. Transformation 5 and 6 both had growth amount as much as control C. SC that lacks leu, trp, but

contains 0.2% 5 fluoroorotic acid (5FOA), control A, B has most growth, but C and D has little growth while E had no growth. Transformation 5 and 6 both had growth amount as much as B.

In YPAD plate, every sample had great growth. To this plate, we performed 5-bromo-5-chloro-3-indolyl-D-galactoside (x gal) assay. X gal assay is used to obtain another qualitative interaction assay. If two proteins are interacting and lacZ gene is expressed, it changes x gal from white to green. In our experiment, control A, B stayed white, C had slight blue hue, D had blue color, E had deep indigo blue. Transformation 5 and 6 changed color similar to A and B.

In CPRG assay, we obtained result on Figure 2.

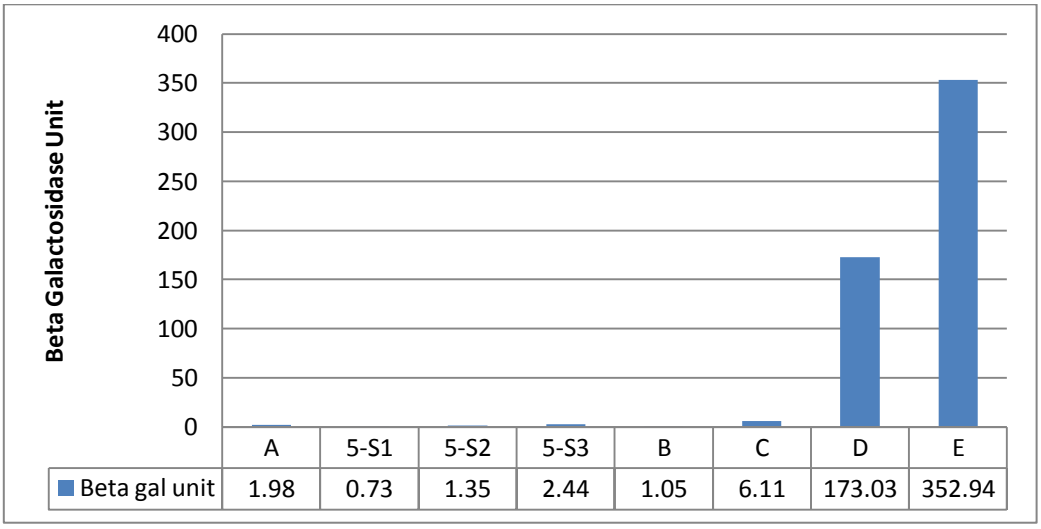

Figure 2 Beta Gal Unit in MID1IP1 and NSP4 Interaction Test

After comparing values from Figure 2, we concluded that MID1IP1 and NSP4 does not interact.

Y2H with NSP4 and FKBP4

This project is testing the protein protein interaction between NSP4 and FKBP4. The NSP4 plasmid was constructed in previous experiments, but we had to construct FKBP4. We recombined pD32 vector with FKBP4. The recombination was done by using Not1 site that are constructed within pD32 vector that has high recombination rate. After recombination, we performed the PCR to amplify and sequenced the amplified gene to check if FKBP4 has recombined correctly. The sequence result showed that recombination was successful. Then Y2H was performed. Transformation of yeast with NSP4 and FKBP4 was not successful. Even though we used the same Y2H method as with NSP4 and Cav1, we had trouble with growth of yeast in any plate. We also had trouble with fungus contamination. In order to eliminate these troubles, we spend numerous months to check for media, reagents, yeast stock, lab environment. After eliminating all the possible source of error, we were able to continue with Y2H on NSP4 and FKBP4. (Table 5)

Table 5 Transformation of NSP4 and FKBP4

Transformation	Plasmid 1	Plasmid 2	Plate	Purpose
1	pDBLeu	None	SC-Leu	Growth
2	None	None	SC-Leu, SC-Leu, -Trp	No Growth
3	pDBLeu	Pexp-AD502	SC-Leu, -Trp	Growth
4	pD32 FKBP4	Pexp-AD502	SC-Leu, -Trp	Growth
5	pDBLeu	pD22-NSP4	SC-Leu, -Trp	Growth
6	pD32 FKBP4	pD22-NSP4	SC-Leu, -Trp	Growth
7	pD32 FKBP4	None	SC-Leu	Growth

After success of transformation, we plated transformation 5 and 6 colonies with control A,B,C,D, and E in selective plates.

Table 6 Selective Plate Growth Comparison of NSP4 and FKBP4 Interaction Test

Selective Plate	**A**	**E**	**5-1**	**5-2**	**5-3**	**5-4**	**5-5**
-Ura	-	Most	A	A	A	A	A
5FOA	-	Most	C	D	D	D	A
12.5mM 3AT	-	Most	A	A	A	A	A
50Mm 3AT	-	Most	A	A	A	A	A
100Mm 3AT	-	Most	A	A	A	A	A
X-Gal	No change	Blue	No change	No change	No change	No change	No change

Control A to E, the growth is in gradient. Growth was observed in comparison of control CPRG assay is still in progress, but from result in Table 6, we believe there is either no interaction or very weak interaction between NSP4 and FKBP4 protein. But we did not finish experiment; further study is required for further conclusion.

CHAPTER IV

SUMMARY AND CONCLUSIONS

From this experiment, we did verify that Cav1 and NSP4 interacts weakly, because selective media experiment confirmed expression of lac Z, Ura 3, and His 3 genes after Y2H. We verified that MID1IP1 does not interact with NSP4, confirmed by no expression of lac Z, Ura 3, and His 3 gene after Y2H. We are in progress of verifying interaction between FKBP4 and NSP4. Due to incomplete data, we cannot conclude there is an interaction between these two proteins, but from what we have, FKBP4 and NSP4 have very weak or no interaction.

Also from this experience, I have learned how a research laboratory is organized and operates the importance of controls, lab notebook and organization. I also learned that correct preparation of solutions and media is critical in any experiment and each step must be verified. I mastered the technique of preparing of plasmids, performing the multi-step Y2H assay, polymerase chain reaction, restriction enzyme digestion, Western blot analyses to confirm protein expression.

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