Mutating the Hantavirus glycoprotein to determine amino acid residues required for virus entry

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Abstract

Hantaviruses are negative sense RNA viruses that can be transmitted from rodents to humans in certain cases. There are two major types of Hantavirus, Hantavirus Pulmonary Syndrome (HPS), which has a mortality rate greater than 50%, and Hemorrhagic Fever with Renal Syndrome (HFRS), which has a mortality rate less than 10% but affects a greater population. This study focused on a species of Hantavirus causing HPS that has been found to be the only Hantavirus capable of human-to-human transmission, as well as the Hantavirus with the most reported cases in the United States, the Andes virus (ANDV). An essential component of the Hantavirus entry process is the glycoprotein (GP) which mediates entry and fusion. This study focused on the GP of the ANDV to help determine what factors are necessary for infection to occur. In order to accomplish this, we made six mutations of various residues thought to be significant to the function of the GP via PCR mutagenesis. Cells were then transfected with the mutant GPs and found to express them sufficiently.

Table of Contents

Introduction/Review of Literature.....1

Methodology.....6

Results.....12

Conclusion.....14

Figures and Tables

- Figure 1: Hantavirus Entry Process, pg.3
- Figure 2: Glycoprotein Conformational Changes, pg.4
- Figure 3: pcDNA 3.1 ANDV GP ΔMluI, pg.6
- Figure 4 :pcDNA 3.1 ANDV GP ΔMluI + BsiWI, pg.12
- Figure 5: Glycoprotein Mutations, pg.13
- Figure 6: Western Blot, pg.13
- Table 1: PCR Reaction-Six Mutations, pg.8
- Table 2: Western Blot, pg.11

Introduction/Review of Literature

In the spring of 1951, soldiers fighting in Korea became inexplicably ill with symptoms of nausea, anorexia, acute headaches, and high fever; these grim symptoms were subsequently followed by shock and eventually renal failure. This strange new disease initially killed 10% of all those infected. Throughout the course of the Korean War in the early 1950s, 3,000 United Nation troops became infected with this mysterious disease known as Korean Hemorrhagic Fever. The discovery of this disease precipitated a search to determine the etiological agent for its transfer to humans. In 1978, Dr. Colleen B. Jonsson and her team at the Center for Predictive Medicine discovered what they called the Hantaan virus and its carrier, the striped field mouse. (Jonsson, 2010)

Today, the Hantaan virus is more commonly known as Hemorrhagic Fever with Renal Syndrome (HFRS). It is one of the two major types of hantavirus disease, an enveloped RNA virus which is a genera of the family *Bunyaviridae*. The outbreak of hantavirus during the Korean War marked the first major eruption of HFRS, caused by the Old World strain of the virus. In 1993, there was a similar outbreak in the Four Corners region in the United States, where 24 people became infected and 12 died, indicating a 50% mortality rate(CDC, 2012). This was the first outbreak of the second principal type of hantavirus, hantavirus pulmonary syndrome, or HPS. HPS has only ever infected people in the New World, as HFRS has only ever infected in the Old World. These two also differ in their symptoms, HPS mainly affecting the lungs whereas HFRS impacts kidney function. Hantavirus diseases are known for being highly fatal and damaging, with mortality rates ranging from 10% (HFRS) to up to 50% (HPS). Each year, there are an estimated 150,000

cases of HFRS in Asia, and since its discovery in 1993 there have been 2,000 cases of HPS (Jonsson, 2010),(CDC, 2012).

Humans can become infected with the hantavirus by inhaling aerosolized infected rodent excrement, urine, or saliva. This is why most people that become infected are in areas where there is a significant rodent population. The initial symptoms of the hantavirus disease develop around 1 to 5 weeks after contact with the viral agent, and typically the victim will develop a fever, muscle aches and headaches, nausea, and fatigue. The late symptoms vary depending on which disease the person contracted, HPS or HFRS. Victims with HPS typically will have shortness of breath as their lungs begin to fill with fluids. HFRS' late symptoms include acute shock, vascular leakage, and acute kidney failure (CDC, 2011).

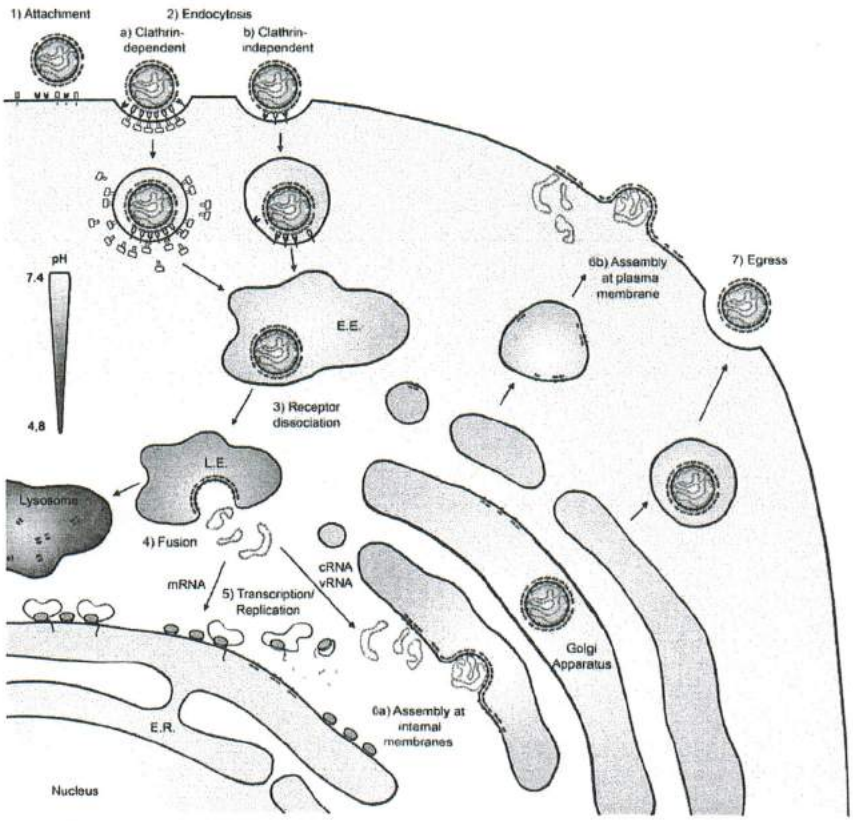
There is no definitive method of treating hantavirus, but patients with HPS are typically set up with intubation and mechanical ventilation; those infected with HFRS need monitoring of fluid levels and more severe cases require dialysis. As a result of hantaviruses' tendency to mutate quickly, it is more likely that developing a treatment that inhibits a specific process or factor in viral entry would be more effective than an antiviral drug which the virus could become resistant to quickly. Therefore, it is immensely important to understand the elements that the hantavirus is dependent upon for cell entry.

Like many other diseases, the hantavirus relies on the function of its GPs, Gn and Gc, for the fusion of membranes, and eventual entry into a cell(Tischler, 2014). Because of the importance

of the GP to viral entry, various studies have been done and are currently taking place in order to determine what is required for the GPs to operate correctly so that a treatment may inhibit the function of the GP, thus inhibiting the entire viral entry process. The human immunodeficiency virus, HIV, is also dependent on the function of its GPs for entry and infection purposes; recently researchers have developed new drugs that are able to block a certain interaction necessary for the operation of the GP, rendering the GP useless and unable to enter the cell(Goodsell, 2015).

The Hantavirus enters the cell through clathrin-mediated endocytosis; the virus binds to the exterior of a cell where Gn and/or Gc interact with cell surface receptors, proposed receptors are β3 integrins, delay-accelerating factor (DAF)/CD55, and the receptor for the globular head domain of complement C1q. (Tischler, 2014) After internalization, the virus remains inside an endosomal sack where the virus is not able to replicate. To overcome this obstacle, the virus will fuse with the endosomal membrane which in turn releases it into the cytoplasm

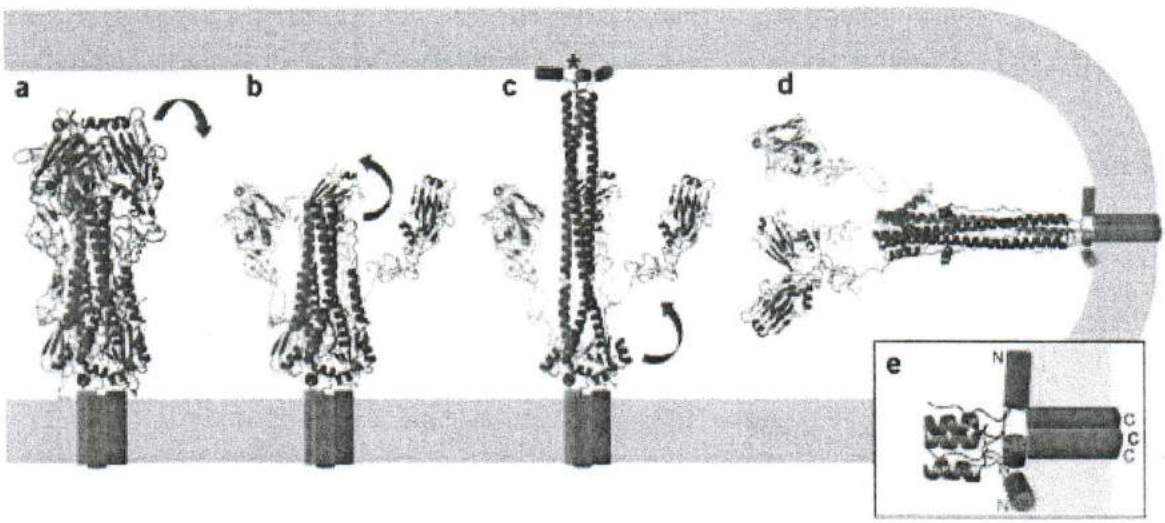
Figure 1.: Hantavirus Entry Process



http://lspolicy3.lhric.org/access/web?id=fd1acc3c-87d5-11e5-b327-002590c53f26

where it can copy its RNA (See Figure 1). This fusion process is mediated by the Gc GP after it undergoes various conformational changes. Conformational changes are vital to the function of Gc and are believed to be triggered by a drop in pH, as long as there is cholesterol in the target membrane, as well as other additional unidentified triggers. This structural change is essentially a rearranging of the protein and results in the GP forming a hairpin conformation with a segment of the GP that was initially concealed within the protein to now be exposed at the surface. (Nour, et al., 2013) This portion of the GP is extremely important because it contains the fusion loop, which inserts itself into the membrane of the endosome to prompt fusion of the membranes (See Figure 2).

Figure 2.: Glycoprotein Conformational Changes



http://www.ncbi.nlm.nih.gov/pubmed/18596815

Based on its molecular structure and *in vitro* binding assays performed, hantavirus GPs can be classified as class II viral fusion proteins. (Tischler, 2005) This is important for the

understanding of the hantavirus GP because one can make assumptions regarding the function and requirements for it based on how other class II fusion proteins act.

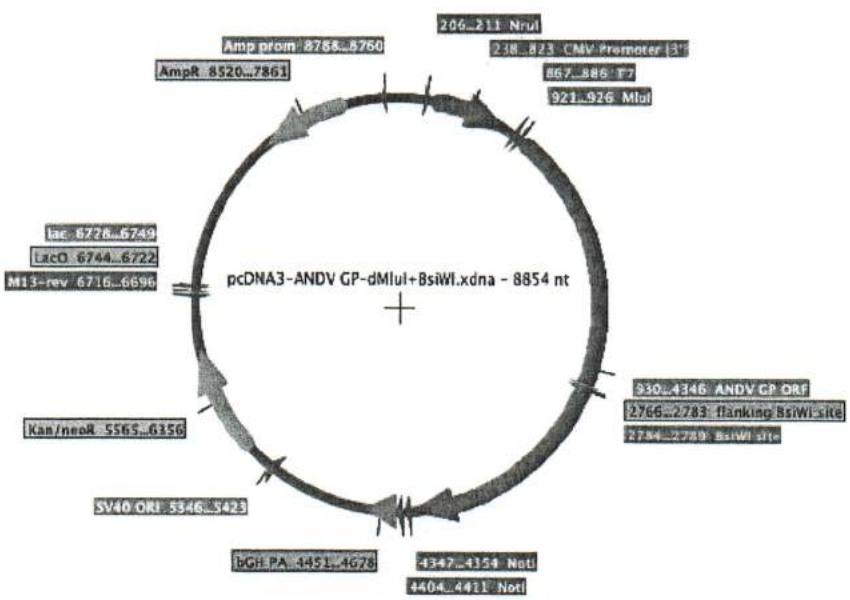
Another important finding was the discovery that hantaviruses are dependent on the sterol regulatory pathway for entry (Petersen, et al., 2014; Kleinfelter, et al., 2015). Identifying pathways rather than individual molecules that are needed for virus replication could lead to the development of multiple therapeutic targets. Moreover, common pathways used by multiple viruses within a family would represent ideal candidates for therapeutic development. High levels of cholesterol were shown to be required for the hantavirus GP to mediate fusion, thus safe, effective cholesterol-lowering drugs may be a viable option for treatment of hantaviruses (Petersen, et al., 2014). It is still not known why cholesterol is needed for ANDV infection, and more studies are warranted to determine why this is.

The aim of this study was to determine what residues are necessary for the function of the andes virus GP during fusion. Various residues were chosen based on their location on the GP, the majority found in the predicted fusion loop. PCR mutagenesis was conducted to mutate specific amino acids in the sequence to alanine, a hydrophobic amino acid. Alanine is commonly used for experiments like this because, once mutated, it will retain the protein's natural secondary structure. Unlike other amino acids, alanine won't form unnecessary bonds with others that could drastically alter the molecular structure.

Methodology

Plasmid pcDNA 3.1 ANDV GP ΔMluI was chosen as a template for this study (See Figure 3.). To generate mutations within this plasmid for analysis, primers had to be designed. The first round of PCR mutagenesis was done to create a BsiWI restriction enzyme site in the GP sequence of the plasmid. After this was created, six different mutations in the GP sequence of the plasmid were created using various forward and reverse primers. E.Coli were transformed with the mutant plasmids to increase quantity; E.Coli colonies that took up the plasmid were selected so the plasmid could be purified. Mutant samples were sent out for DNA sequencing to ensure the correct modifications to the DNA were made. 293Tkc cells were transfected with the mutant DNA and a western blot analysis was performed to test for expression of the viral protein.

Figure 3.: pcDNA 3.1 ANDV GP ΔMluI



BsiWI site PCR mutagenesis

The first two PCR reactions were run to make the GP segments with the BsiW1 site. The third overlapping PCR reaction joined these fragments together. Each reaction was accomplished by mixing the primer, template, and master mix. For the first PCR reaction, 1µl of plasmid was mixed with 2µl of CMV-F primer and 1µl of BsiWI-R primer along with 10µl master mix and 6µl H_2O. The second PCR reaction entailed 1µl of the plasmid as well as 1µl BsiWI-F primer, 2µl BGH-R primer, 10µ master mix, and 6µl H_2O. The PCR samples were then run in a thermocycler at 98°C in order to denature the DNA and separate the strands, then 59°C because this is the optimal annealing temperature for the primers used, and finally 72°C because this is the temperature at which the polymerase works best. The third PCR performed involved 2µl each of the first and second PCR products along with CMV-F primer, BGH-R primer, and H_2O, and then 10µl of master mix. This sample was run in the thermocycler for the same temperatures and durations.

Plasmid Digest

In order to replace the wild type GP with the mutant GP, we first had to remove the wild type sequence. To remove the wild type GP sequence from the new BsiWI plasmid, 23.1µl of the plasmid was mixed with 5µl 3.1 NEB buffer, 1.2µl of both NotI and MluI, and 19.5µl of sterile H_2O. NotI and MluI are restriction enzymes that specifically cut at the beginning and end of the GP sequence in the plasmid. This solution was incubated at 37°C for 1.5 hours and then 3µl of rapid dephosphatase was added to the mixture before it continued its incubation for another 30 minutes. Dephosphatase was added to remove the phosphates from the free ends of the DNA in

order to ensure that the backbone would not be able to close on itself, preventing the insert from being placed properly.

Six mutation PCR mutagenesis

Six mutations were made in this experiment, four in or nearby the predicted fusion loop (W115, N118, G128, and Y786), and two in the predicted ij fusion loop (F249 and P253). These mutations were made to residues that are known to confer cholesterol sensitivity in other viruses. Prior to the PCR reaction, all 12 primers (for the six mutations) were hydrated with water to create a 100μM stock. 2μl of that hydrated stock was then mixed with 18μl of H_2O to create a working stock.

Table 1:PCR Reaction-Six Mutations	
W776A-F + BGH-R	Y786A-F + BGH-R
W776A-R + 1112-F	Y786A-R + 1112-F
N769A-F + BGH-R	F900A-F + BGH-R
N769A-R + 1112-F	F900A-R + 1112-F
G779A-F + BGH-R	P904S-F + BGH-R
G779A-R + 1112-F	P904S-R + 1112-F

The primers were mixed as shown in Table 1. In the first reaction, 1μl of plasmid was mixed with 1μl of the hydrated forward primer and 2μl of BGH-R. The BGH-R primer sits at the end of the andes virus GP sequence. This solution was added to 10μl of master mix and 6μl of sterile H_2O. For the second reaction, 1μl of the plasmid was combined with 1μl of 1112-F primer, 1μl hydrated primer, 10μl master mix, and 7μl sterile H_2O. The 1112-F primer sits upstream of the mutations that were being made. The PCR tubes were then run in the thermocycler for 1 minute at 98°C and then an additional 15 seconds at 98°C for denaturation; next, the temperature was lowered to 59°C for 15 seconds to allow for annealing; finally the temperature was raised again to 72°C for 1 minute to allow for elongation; then an additional 3 minutes at the same temperature. These steps were repeated 35 times with the exception of the first and last steps.

Gel Electrophoresis

Gel electrophoresis was performed after each PCR reaction. This was done to ensure that there were the right amount of DNA segments and that they were the correct size. Gel electrophoresis was also employed to separate segments of DNA from one another. PCR products were run on a 1% agarose gel. 10µl of a 10kb ladder was used and 20µl of each PCR product with 2µl 10x loading dye were added into the wells. 10µl of ethidium bromide was added to the end of the gel in the 1x TAE buffer because ethidium bromide migrates on the gel in the opposite direction from the DNA. Adding extra ethidium bromide at the end helped to visualize the DNA better when it migrated further down on the gel. The products were run at 110-120 V and the time frame depended on the size of the DNA being tested.

Gel purification

Bands cut from gel electrophoresis were then purified following the instructions of the QIA quick Gel Extraction Kit.

Gibson Assembly

The Gibson Assembly was employed in order to ligate the mutant GP segment to the plasmid backbone. The amount of sample used for each Gibson Assembly was dependent on the concentration of it found by using a nanodrop. Backbone and insert were mixed in proportion (1:3 insert to vector) along with 4µl of master mix. The samples were run in a thermocycler at 50°C for 1 hour.

E.Coli Transformation

4µl of gibson product was added to C2992 *E.Coli* cells (54µl per tube); then the cells were

incubated with the gibson product for 30 minutes on ice (this is when the cells take in the plasmid); next they were heat shocked at 42°C for 30 seconds. Following the heat they were placed on ice again for 5 minutes. After, 100µl of nutrient-rich SOC media was added near a flame to prevent contamination. They were then incubated for an hour at 37°C and subsequently 11.6µl of the mixture was spread onto LB Agar Plates with Carbenicillin and incubated at 37°C overnight. Carbenicillin was on the plates because the plasmid contains a resistance gene to this antibiotic, thus only the cells that successfully transformed would be able to survive. A few colonies were picked off the plate and grown in flasks of LB media and Carbenicillin to amplify the E.Coli and plasmid. The plasmid was then purified from the bacteria using the QIAprep Spin Miniprep Kit.

293T Cell Transfection

293T cells were seeded on 6-well plates for transfection in 10% FBS without antibiotics (1.2 million cells/well). 250ul OptiMEM, 200ng RFP (red fluorescent protein), and 2ug of Gp plasmid were mixed together separate from the cells. OptiMEM was added in order to keep cells in reduced serum conditions during transfection. 8.8uL of PEI, a cheap and effective transfection reagent, was added to each tube; after incubating for 20 minutes this solution was added to the cells. To check that the transfection was successful, the cells were checked for RFP expression under a microscope. Approximately 50% of the cells expressed the RFP, so the transfection was considered successful. The 293T cells were then lysed in RIPA buffer to extract the viral protein.

Western Blot Analysis

To test for expression of the mutated GPs from the transfection, a western blot analysis was

employed. The concentrations noted in Table 2 were added to the mutated PCR samples and pipetted into the polyacrylamide gel. The gel was run at 70V for the stacking gel and 110V for separating. After the the gel had run for a sufficient amount of time, the membrane was taken out of the frame and blocked in milk for 1 hour on a rocker. The membrane was then washed 3x every 5 minutes with 0.25% PBS buffer. The membrane was then stained with a BY antibody which binds specifically to the hantavirus GP as well as 5ml of milk for 1 hour on the rocker to reduce nonspecific binding. To remove excess antibody the membrane was washed in 0.25% PBST and then stained with HRP antibody, which binds to the first antibody. Next, the

Table 2: Western Blot

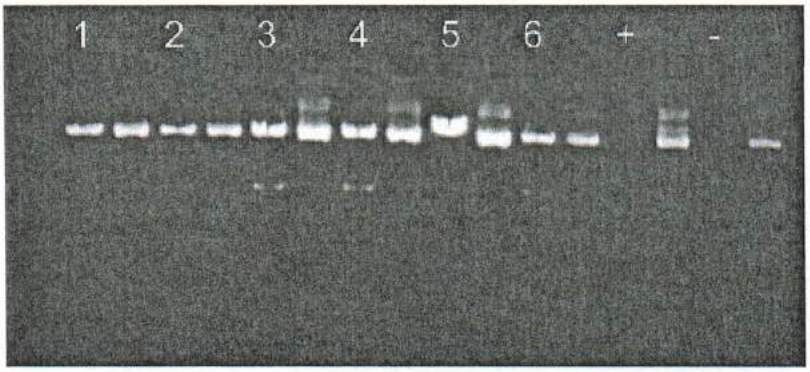
Sample	3x SB	Sample vol. (30 ug)	PBS	Total Volume
1-1	10ul	16.1ul	3.9ul	30ul
1-3	10ul	9.78ul	10.22ul	30ul
2-3	10ul	6.25ul	13.75ul	30ul
2-4	10ul	7.27ul	12.73ul	30ul
3-3	10ul	6.99ul	13.01ul	30ul
3-4	10ul	6.72ul	13.28ul	30ul
4-1	10ul	10.46ul	9.54ul	30ul
4-2	10ul	6.35ul	13.65ul	30ul
5-3	10ul	3.65ul	16.35ul	30ul
5-4	10ul	6.19ul	13.81ul	30ul
6-2	10ul	5.69ul	14.31ul	30ul
6-4	10ul	12.53ul	7.47ul	30ul
+(WT)	10ul	6.77ul	13.23ul	30ul
-	10ul	3.56ul	16.44ul	30ul
1x SB	80ul	0	160ul	240ul

membrane was washed in the same manner as before, then incubated with HRP substrate, the result of which is light production. The illumination was captured on film in a darkroom and developed.

Results

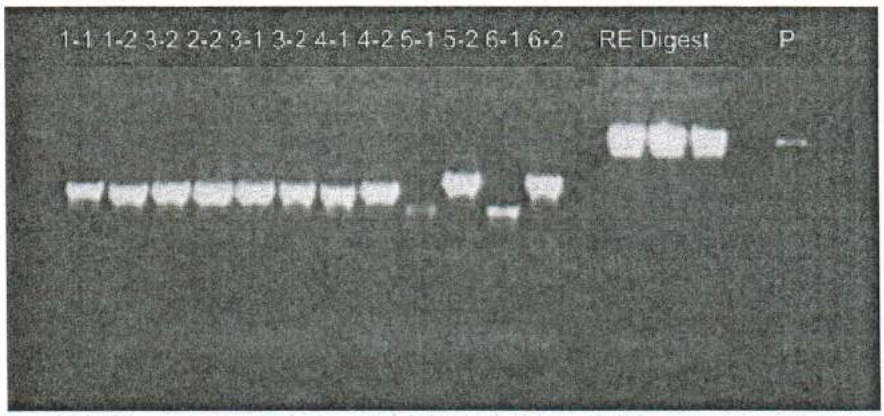
The initial set of PCR reactions were designed to create a new restriction enzyme site in the GP sequence. Six different samples of this vector were run on a gel to test if they were the correct size. The gel results show that all vectors with the exception of the fifth were the correct size. The third and fourth vectors appeared to be the brightest and most accurate so they were chosen for the next round of PCR mutagenesis (See Figure 4).

Figure 4.: pcDNA 3.1 ANDV GP ΔMlul + BsiWI Site



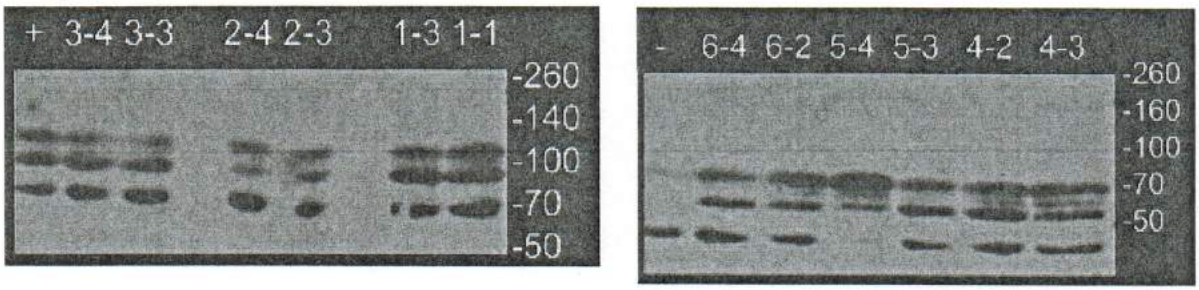
Following the PCR mutagenesis designed to create the six GPmutations, each sample was run twice on a gel to find the best sample. It was found that all samples were the correct size so that they could be used for further testing(See Figure 5). The gel electrophoresis shows us that the plasmid is the right size, but to ensure that these mutations were correct samples,they were sent out for DNA sequencing.

Figure 5.: Glycoprotein Mutations



After receiving the DNA sequencing results and seeing that all six mutations made the proper amino acid changes, 293T cells were transfected with the mutated plasmids. To test for expression of these mutations in the cells, a Western Blot analysis was employed. The Western Blot results showed that there was expression of the mutated plasmids in the human cells, therefore the cell transfection was successful (See Figure 6.).

Figure 6.: Western Blot



Based on the western blot analysis (Figure 6), the cells were successfully expressing all six mutations in the ANDV GP. The GP runs between the 50 and 70kDa bands. The smaller band

between 35 and 50kDa is Beta-actin. This is a common protein in cells and is regularly used as a control when running western blots to show that the same amount of cellular protein was added to each well. This shows that there are truly similar amounts of GP being expressed in most of the cells we transfected.

Conclusion

The purpose of this study was to determine what components of the Hantavirus GP are necessary for its entry into a cell. Several residues were selected to be mutated in the GP to assess whether they are essential for the entry process. By looking at these factors more can be understood about the functioning of the GP as well as determining factors that could become possible targets for treatment in the future.

To establish which of the mutated GPs is unable to enter cells, a cell infection experiment would have to take place. Since the ANDV is a lethal pathogen, infection of cells would have to take place in a biosafety level 4 laboratory. If it was found after this point that one of the mutations created in this experiment has a significant impact on viral entry, experiments would be warranted to further determine whether it is possible to inhibit that portion of the GP for treatment purposes.

This experiment lays the foundation for determining whether these mutations are necessary for ANDV infection. This project indicates that it is possible to make these mutations and still successfully have cells function successfully. This allows future studies to make viruses with these mutant GPs and carry out fusion and infection assays.

While there is yet to be a critical outbreak of ANDV, this does not discount this disease from

being a serious threat to people living in the Western Hemisphere, as well as all over the world. If there is anything to be learned from the Ebola Virus outbreak of 2014, ignoring problems that only affect the few until they affect the many can lead to the death of thousands in a short amount of time. The probability of rodents carrying ANDV is far too great for this issue to not receive the attention it deserves; steps must be taken to develop a treatment for ANDV, before it is too late.

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