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Mutant Nipah Virus-like Particles as a Protein Delivery Technology

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ABSTRACT

Gene and protein delivery technologies are a critical therapeutic in the treatment of genetic diseases. Proteins like CRISPR-Cas9 could eventually be delivered to edit genes and treat a wide variety of disorders. Virus-like particles (VLPs) are one promising method to deliver proteins. These non-infectious particles are composed of viral components and are capable of enclosing and transporting a variety of functional proteins. Paramyxoviruses, a class of enveloped RNA viruses, have been used for VLP research. Interactions between the matrix (M) and nucleocapsid (NP) proteins drive the packaging of cargoes and budding of particles. Using parainfluenza virus 5 (PIV5), previous research has shown that a 30 amino acid sequence from the C-terminal end of PIV5 NP protein can confer packaging efficiency to a protein of interest when bound to it. PIV5 M protein amino acid substitutions made to areas critical for M-NP interactions resulted in increased VLP production. However, a PIV5-based VLP therapeutic may meet challenges in practice due to preexisting immunity in a significant proportion of the global population. Nipah virus (NiV), a zoonotic paramyxovirus that is genetically homologous to PIV5, can also create VLPs and has less risk of preexisting immunity. In this study, Nipah virus M protein mutants analogous to those that improved PIV5 VLP formation were generated and tested for increased VLP production compared to wild-type Nipah virus M protein. From a group of 22 proposed mutants, only one, Y187L, showed improvement in VLP production compared to wild type. These results are important for future efforts to maximize the yield of VLPs in mammalian cell lines to produce therapeutics.

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Chapter 1

Introduction

Genetic diseases in humans are caused by mutations in genes. In the United States, about 30 million people are affected by such diseases, two-thirds of whom are children (1). Gene therapies, which seek to treat these diseases, typically introduce genetic material into a patient's cells that mediate a genetic change to alleviate the patient's condition (2). Protein-based therapeutics offer a different approach. Instead of delivering genetic material to cells, these therapeutics send proteins to directly mediate benefits in cells, thus treating patients. Some of these proteins can perform beneficial genetic changes. For example, CRISPR-Cas9 is an enzyme used in gene editing whose use could benefit millions of people, such as those with sickle cell disease or muscular dystrophy (3). However, these proteins must be properly delivered into target cells. Despite the emergence of many promising delivery technologies, including adeno-associated viruses, lipid nanoparticles, and inorganic nanoparticles, the delivery of gene therapies remains a major challenge (3). Thus, vehicles for safe and effective protein delivery are critically needed to unlock life-changing therapeutics.

Paramyxoviruses provide one promising avenue to address the critical need for protein delivery platforms. Paramyxoviruses are a diverse group of non-segmented, negative-sense RNA genome viruses. This includes human and zoonotic pathogens such as measles virus, mumps virus, Nipah virus, and parainfluenza virus 5 (PIV5) (4). These viruses propagate infection through the creation of infectious particles. The matrix (M) protein is one of the most critical proteins for viral assembly. It helps the nucleocapsid (NP)-encapsidated genome, in the form of

ribonucleoprotein (RNP), associate with glycoproteins on the surface of viral particles (5). Some paramyxoviruses, like PIV5, require both NP and M proteins for efficient particle budding. Others, like Nipah virus, can bud efficiently while expressing M protein alone (4). Paramyxoviruses find their utility in protein delivery platforms through their capability of producing virus-like particles (VLPs). VLPs are noninfectious, delivery capable particles that can be produced in a variety of systems including mammalian cell lines, insect cell lines, and plants. Their ability to avoid lysosomal degradation of their contents and thus deliver their cargo intracellularly make VLPs attractive therapeutic delivery platform (6).

Leveraging this knowledge of M-NP interactions, PIV5 was used as a model virus to examine viral budding and particle formation. PIV5's NP protein C-terminal end was discovered to be crucial for virus assembly. Mutations were made to viruses in this C-terminal region that affected virus particle formation. These debilitated viruses were passaged in cells and analyzed for compensatory mutations that were likely intended to rescued particle formation (7). In subsequent experiments, separate DLD-containing sequences from the C-terminal ends of both PIV5 and Nipah NP protein were used as an appendage to package foreign cargos into VLPs based on both viruses. However, these particles lacked the viral fusion protein and thus were not delivery capable (8). Improving upon these findings, work by Panthi et al. created delivery capable particles by expressing viral proteins required for attachment and entry to cells, in addition to those necessary for particle formation. In both PIV5 and Nipah virus-based experiments, efficient delivery of cargos to cells was achieved, including the delivery of Cre recombinase to cell nuclei. This platform hosts a variety of benefits, including minimal modification or damage to cargoes, a wide array of viable cargo proteins, and direct delivery of cargos to the cytoplasm (9). However, the PIV5-based platform may have issues with preexisting

immunity. VLP surfaces display surface proteins similar to the wild type virus, and thus can trigger immune responses (10). Therefore, infection with a virus corresponding to the one used as the basis for VLP production could create preexisting immunity that limits the effectiveness of VLP treatment. PIV5 may already have widespread exposure in the global population due to its many recorded hosts (11), which may limit the effectiveness of PIV5 VLPs.

In this study, Nipah virus M protein mutants were predicted based on structural and sequential homology to those second-site mutants found to be beneficial for VLP formation in PIV5. Plasmids for these mutant M proteins were transfected into cells to view their effect on Nipah VLP budding. One mutant, Y187L, showed a 1.5-fold improvement in total VLP production compared to wild type M protein. These results indicate that Nipah virus M mutations analogous to those that can increase VLP production in PIV5 are generally poor at improving Nipah virus VLP production. This may indicate that Nipah virus's M protein interactions may already be fine-tuned to maximize VLP production. Furthermore, this may show that PIV5 M and Nipah virus M are too distant for this strategy of mutation alignment.

Chapter 2

Review of Literature

Paramyxoviruses

Paramyxoviruses are a family of enveloped, non-segmented, negative-sense RNA viruses. They include many pathogens of note to both animals and humans, including measles virus and mumps virus. While measles and mumps virus have vaccines, most paramyxoviruses do not have any approved treatments or vaccines (12).

Paramyxoviruses produce particles that propagate infection to additional hosts as well as within the same host. These form by organizing proteins at selected sites at host cell membranes, where enveloped particles will develop and bud (4). A variety of proteins encoded in the paramyxovirus genome facilitate particle formation and budding. The glycoprotein (G) and fusion protein (F) help form the viral envelope and are critical for viral particle attachment and entry to host cells. Nucleocapsid protein (NP) binds the RNA genome. This interaction creates helical ribonucleoproteins (RNPs) (4). Bridging the RNP with the particle membrane is the matrix (M) protein. Matrix proteins lie just below the particle envelope, ensuring the proper organization of viral particles (5). For some paramyxoviruses, the expression of only M protein can still facilitate particle formation (4), highlighting the importance of this protein.

Nipah virus and Parainfluenza virus 5

Nipah virus, a Henipavirus, is a zoonotic paramyxovirus of note. The virus was first characterized after an outbreak in people involved in pig farming in Malaysia in 1999 (13). Subsequent outbreaks occur regularly in Southeast Asia, particularly in Bangladesh. Fruit bat species belonging to the genus *Pteropus* transmit this virus to humans, either directly or indirectly. These bats have an expanding range into Africa, potentially increasing the prospects of spillover into humans for additional populations (14). Transmission takes place through an intermediate host, such as pigs, or through contaminated fruit or raw date palm sap (15). This virus has a high fatality rate of >40%, mediated by severe encephalitis (16, 17). This makes the live pathogen a Biosafety Level 4 virus, requiring the highest level of precautions in laboratory settings. While human-to-human transmission has been observed in Nipah virus outbreaks, the number of cases has not been particularly high. This means that the virus, while deadly, carries less concern of becoming highly transmissible (12). This means that most of the global population has not been exposed to Nipah virus.

Parainfluenza virus 5 (PIV5) is another paramyxovirus critical to this study. This virus is an Orthorubulavirus, a genus within the paramyxovirus subfamily *Rubulaviridae*. Like other paramyxoviruses, it has a non-segmented, negative-sense RNA genome. The virus has been found in a variety of species, including humans, dogs, pigs, and cats (18, 19). PIV5 has been implicated in contributing to canine infectious respiratory disease (CIRD), also known as kennel cough (20). However, PIV5 has also been used as a component of vaccines against CIRD (21). Given the similarity of PIV5 strains isolated from various hosts, the viruses' lack of virulence in humans (11), and potential human exposure to PIV5 through vaccinated dogs (21), there is

reason to believe that many people could be exposed to PIV5 globally. This exposure may result in an immune response.

Virus-like Particles

Virus-like particles (VLPs) are structures containing the proteins required for viral particle formation but lacking the viral genome (22). Thus, these particles resemble a wild-type virus without the risk of establishing an infection. VLPs are especially promising in therapeutics as they can be created at a large scale and in a cost-effective manner through a variety of expression systems, including bacteria, plants, and mammalian cells (23, 24). VLPs are being investigated for use against a variety of ailments. They have already been approved by the Food and Drug Administration in vaccinations against hepatitis B virus (HBV) and human papillomavirus (HPV) (25). Importantly, VLPs can be utilized as drug delivery systems, capable of carrying functional proteins to cells (24). In this capacity, VLPs have had promising results delivering gene editing proteins in an efficient and targeted manner, while avoiding viral genome integration risks as seen with adeno-associated virus (AAV) vectors (22).

Virus-like Particles as a Protein Delivery Platform

Some paramyxoviruses, like Nipah virus and measles virus, can bud efficiently with only functional M protein. Others, like PIV5, require interactions with other components of the viral genome for efficiency, such as NP protein (4). In previous research conducted at the Dr. Anthony P. Schmitt Lab, PIV5 was found to have a region in the extreme C-terminal end of the NP protein that was required for M protein interaction and VLP production. NP protein mutants

were created by substituting amino acids, creating PIV5 viruses with defects in particle formation. These viruses were passaged several times in Vero or MDBK cells. Second-site mutations were then discovered in multiple viral proteins, including several found in the PIV5 M protein. These adaptive mutations may have had the function of repairing impaired M-NP interactions to recover normal particle formation (7). In further work by the same lab, a 15-residue sequence DLD-containing sequence from the C-terminal end of PIV5 NP protein was transplanted onto *Renilla* luciferase. This allowed the enzyme to efficiently package into VLPs, despite being a foreign protein. The same outcome was observed when using Nipah virus nucleocapsid (N) protein in place of PIV5 NP protein (8). Subsequently, a VLP-based protein delivery platform was developed leveraging paramyxovirus M-NP interactions. Cargo proteins are outfitted with an amino acid appendage that allows the foreign cargo to interact with M proteins. This interaction packages the cargo into a VLP. This creates VLPs that act as delivery-capable vehicles for proteins (9).

Panthi et al. note several potential advantages to the use of VLPs as protein delivery vehicles. The platform is applicable to a wide variety of cargo proteins, including CRISPR-Cas9 proteins. The technology is industrially scalable, opening the possibility for this system to be used for the mass production of future therapeutics. VLPs do not risk incorporation into cellular DNA. Furthermore, cargo proteins are sent directly to the cytoplasm of target cells and require minimal modification (9).

However, viral vectored therapies, such as a VLP-based delivery platform, can be limited by preexisting immunity. If people are naturally exposed to the virus used as a vector for a treatment, their immune response would be stimulated as if they were reinfected with wild type virus (10). This could damage the therapeutic being delivered, worsening the effectiveness of the

treatment. PIV5 was the model virus for much of the previous work on VLPs in protein delivery. As a pathogen capable of infecting many animals and humans worldwide (11), PIV5 may have a significant degree of exposure in humans. This may limit its use as a vector for future VLP-based therapies.

Chapter 3

Materials and Methods

Virus Sequence Alignment

Second-site mutations acquired by PIV5 that improved VLP production were discovered in previous work (7). To discover sequences analogous to those in PIV5 (accession number: AF052755) for Nipah virus (accession number: AF212302), two separate methods were utilized. In one method, a pairwise sequence alignment was conducted directly between PIV5 M protein and Nipah M protein. This was performed via GGSEARCH2SEQ. The other method consisted of three discrete steps. First, a pairwise sequence alignment was conducted between PIV 5 M and Newcastle disease virus (NDV) M protein (PDB: 4G1O). Second, a Pairwise Structural Alignment via the RCSB Protein Data Bank was performed between the resultant NDV M sequence and Hendra Virus (HeV) M protein (PDB: 6BK6). Lastly, another pairwise sequence alignment was conducted between the resultant HeV M and NiV M. Given the structural and sequential similarities between the two viruses (26), this method of alignment was feasible. Alignment yielded mutations analogous to the original PIV5 M mutations in Nipah virus M protein.

Antibodies

Nipah virus M proteins were detected via Myc-tag using an anti-Myc antibody 9E10, number 12-2500 (Thermo-Fisher).

Plasmids

Plasmid pCAGGS-Myc-NiVM was the only plasmid encoding a protein used for Nipah VLP production and has been described before (27). The wild type Nipah virus M sequence was modified via PCR to generate each of the mutants. A list of mutants and their associated plasmid is shown in Table 1.

Table 1. Plasmids generated by mutant.

Mutant	**Plasmid Generated**
V16L	pCAGGS.Myc.NiVM.V16L
V16M	pCAGGS.Myc.NiVM.V16M
T120K	pCAGGS.Myc.NiVM.T120K
I150R	pCAGGS.Myc.NiVM.I150R
W141R	pCAGGS.Myc.NiVM.W141R
C157R	pCAGGS.Myc.NiVM.C157R
Y187L	pCAGGS.Myc.NiVM.Y187L
Y187F	pCAGGS.Myc.NiVM.Y187F
R191D	pCAGGS.Myc.NiVM.R191D
M236L	pCAGGS.Myc.NiVM.M236L
H238N	pCAGGS.Myc.NiVM.H238N
G247I	pCAGGS.Myc.NiVM.G247I
G248I	pCAGGS.Myc.NiVM.G248I
S251I	pCAGGS.Myc.NiVM.S251I
H227M	pCAGGS.Myc.NiVM.H227M

S317D	pCAGGS.Myc.NiVM.S317D
P329D	pCAGGS.Myc.NiVM.P329D
G347R	pCAGGS.Myc.NiVM.G347R
G347W	pCAGGS.Myc.NiVM.G347W
G352R	pCAGGS.Myc.NiVM.G352R
G352W	pCAGGS.Myc.NiVM.G352W
Q291E	pCAGGS.Myc.NiVM.Q291E

Sequence identity was confirmed through agarose gel electrophoresis. After ligation into plasmids, restriction enzyme digest and agarose gel electrophoresis confirmed plasmid identity in DNA preparations.

Cell Line

Human embryonic kidney 293T (HEK-293T) cells were used as VLP producer cells. These cells were used as they are highly efficient in transfection and process proteins very well (28). These cells were the generous gift of Dr. Robert Lamb of Northwestern University. Cells were maintained at 37°C with 5% carbon dioxide in Dulbecco's Modified Eagle's Medium (DMEM) (Corning, USA) with 10% fetal bovine serum (FBS) (Sigma-Aldrich) and 1% penicillin-streptomycin (Life Technologies).

VLP Production and Detection

In 6 well plates, cells were grown to 80% confluence. This was analyzed via Nikon Eclipse TS100 microscope (Nikon Inc., Melville, New York). Cells were transfected using PEI Max (Polysciences) transfection reagent per manufacturer instructions. VLP-producing cells were transfected with M plasmid pCAGGS-NiV M, 0.5 μg. Cell media was DMEM with 2% FBS, which was changed to fresh media of the same type after 6-8 hours. VLPs were collected at 48 hours post transfection. Cell lysates and VLPs were detected from proteins as previously described (9). VLPs were collected and then purified via microcentrifugation through a 20% sucrose cushion. VLPs and cell lysates were fractionated via 10% SDS-PAGE gels. Polyvinylidene difluoride (PVDF) membranes were used for protein detection with antibodies. The primary antibody was an anti-Myc monoclonal antibody. Following this, alkaline phosphatase (AP)-conjugated secondary antibodies were used. Blots were imaged using a Fuji FLA-7000 laser scanner (FujiFilm Medical Systems, Stamford, Connecticut). Total VLP production was analyzed in reference to VLP production of wild type controls.

Statistics

Statistical analysis was performed by one way ANOVA in Graphpad Prism 8 (San Diego, CA). *P* values ≤0.05 were considered statistically significant. (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$).

Chapter 4

Results

To determine which mutant residues in PIV5 M corresponded to those in NiV M, the proteins were aligned using pairwise sequence alignment and pairwise structural alignment. Two separate methods were developed to translate mutations from PIV5 to Nipah virus, and the outcomes were compared. The direct sequence alignment and three-step hybrid sequence and structural alignment often produced similar results. Due to differences in viral sequence and method, some mutants had multiple outcomes for Nipah virus M alignment. Sequences from both methods were considered for further study. Results are summarized in Table 2.

Table 2. Sequence and structural alignments from PIV5 M to NiV M. PIV5 M residue denotes amino acid number of the observed mutation in PIV5. Frequency denotes how many PIV5 mutants developed that mutation out of 100 rescued viruses in previous study (7). Fold increase in VLP production shows PIV5 mutant increase in VLP production over wild type virus (++, higher than wild type, +++ much higher than wild type) (9). Proposed mutations are outcomes of both methods of sequence alignment from PIV5 M to Nipah virus M.

PIV5 M residue	**Frequency**	**2nd-site mutation(s)**	**Fold increase in VLP Production**	**Proposed mutations to NiV M**
17	5	I17L (3); I17M (2)	++	V16L, V16M
117	3	T117K	++	T120K
123	2	E123K	+++	N/A
147	2	C147R	+++	I150R, W141R, C157R
184	9	F184L	++	Y187L, Y187F
188	4	G188D	+++	R191D, T192D
235	1	W235L	+++	M236L
237	1	H237N	+++	H238N
250	1	S250I	++	K248I, S251I
278	5	I278M	+++	H277M
318	1	G318D	+++	S317D
330	3	E330D	+++	P329D
357	5	G357W (3); G357R (2)	+++	G347W, G347R

Not listed in table 2 is mutant Q291E. This mutant was shown by Norris et al. to increase budding by ~60% relative to wild type (29), and thus was added to the study later.

To generate Nipah virus M mutant plasmids for transfection, mutant Nipah M DNA was generated via PCR. Experiments were split between undergraduate lab members and the graduate student, with the author as the primary facilitator. PCR success was confirmed by agarose gel electrophoresis. PCR products underwent restriction enzyme digest and ligation into plasmids to encode Nipah M protein. Candidate bacterial colonies were tested by agarose gel electrophoresis. A representative agarose gel electrophoresis for PCR products is shown in Figure 1.

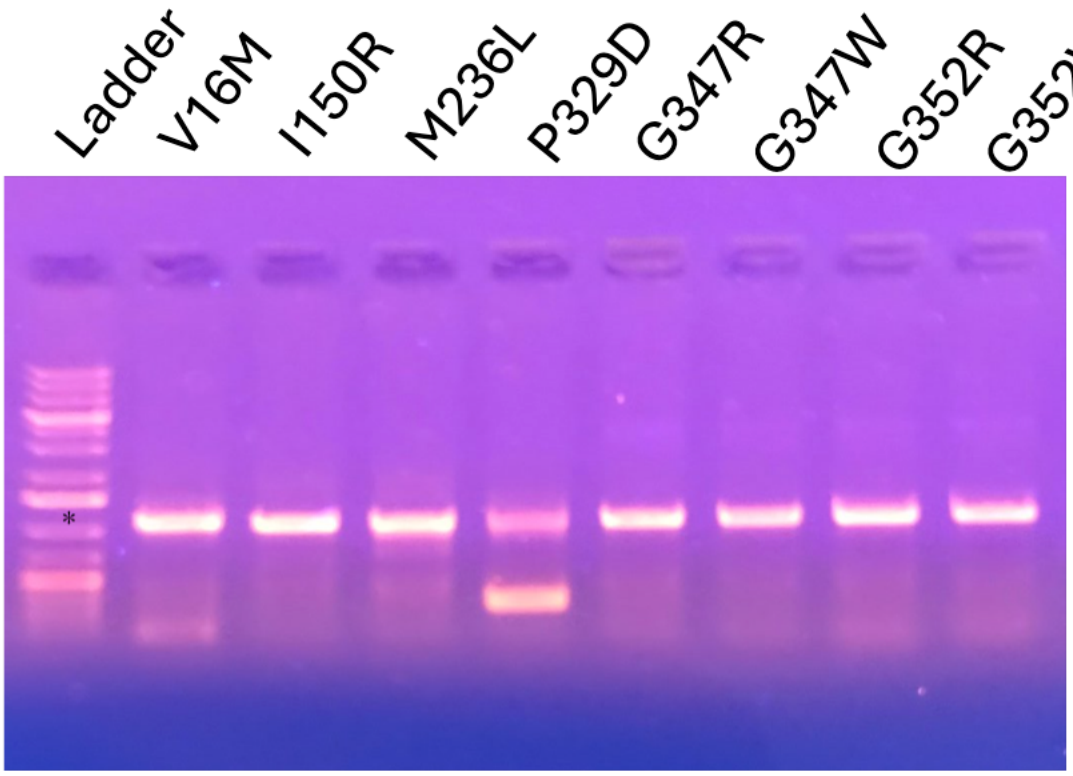

Figure 1. Agarose gel electrophoresis for PCR products select mutants. Expected size of PCR products were calculated per each product and range between 1074-1099 base pairs. Range is marked by the * symbol. All mutants except P329D match this size (~450bp). Ladder indicated is a 2-log DNA ladder (New England Biolabs). Courtesy of Srijana Adhikari.

To determine the effects of the M mutants on VLP production, the plasmids encoding the altered Nipah M proteins were transfected into HEK-293T cells to express altered Nipah M proteins. VLPs were harvested from the cell culture medium. VLPs were purified by pelleting through a sucrose cushion. Western blotting was used to quantify VLP production as shown in Figure 2.

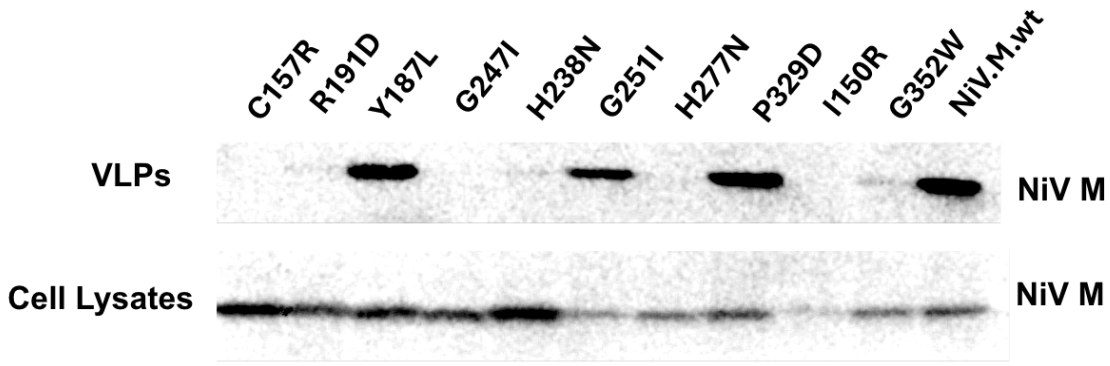

Figure 2. VLPs and cell lysates on western blots for select mutants. Courtesy of Srijana Adhikari.

Using multiple western blotting results, total VLP production for mutants was pooled and analyzed relative to wild type VLP production (Figure 3).

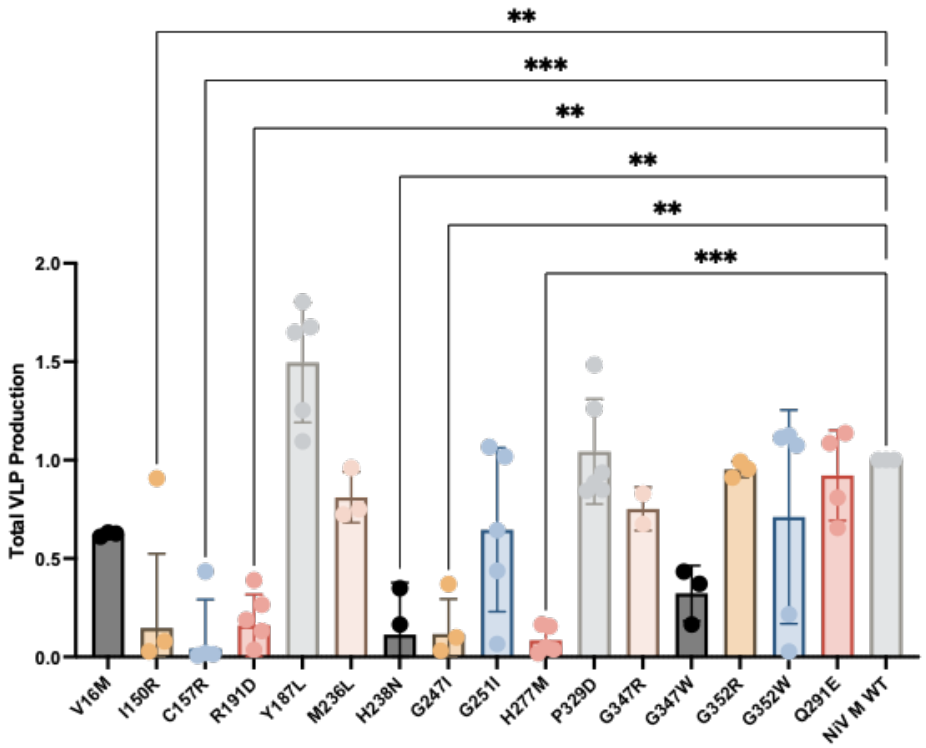

Figure 3. Total VLP production of NiV M mutants compared to wild type NiV M. Error bars indicate standard deviations. (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$).

One group of mutants had detrimental effects on VLP production compared to control, reducing production below half of normal wild type total VLPs. This group included mutants I150R, C157R, R191D, H238N, G247I, H227M, and G347W. The reduction for mutant G347W was the only result of this group that was not statistically significant. Another group of mutants had total VLP production ranging between 50% of control production and equal to control production. These mutants included V16M, M236L, G251I, P329D, G347R, G352R, G352W, and Q291E. Of these mutants, only P329D exhibited a marginal improvement (roughly 4%) over wild type control. Mutants V16L, T120K, W141R, Y187F, G248I, and S317D were tested for VLP production in separate experiments, yet none were able to improve VLP production more than wild type Nipah M (data not shown). The mutants in these three groups do not improve VLP production. One mutant, Y187L, improved VLP production to roughly 1.5 times that of NiV M wild type control. While this was not a statistically significant finding, Y187L consistently resulted in increased VLP production. In five separate experiments with Y187L, VLP production was increased compared to NiV M wild type control by 1.25 times, 1.80 times, 1.68 times, 1.65 times, and 1.09 times. While these are modest gains, the general trend of this mutant increasing VLP production is promising.

Chapter 5

Discussion

Gene therapies represent an exciting class of treatments for those with genetic disorders. Protein-based therapeutics are one way that the benefit of gene therapies could be realized for patients. VLPs, such as those based on PIV5, are a promising delivery platform for protein-based therapeutics (6). One potential limitation of such therapies is the large-scale production of VLPs from mammalian cell lines. This issue was addressed when substitutions discovered in the M protein of PIV5 increased VLP production over VLPs based on wild type virus (7, 9). However, this platform may be limited by preexisting immunity to PIV5 in humans, given the range of organisms it can infect and its previous isolation from humans (11). Therefore, a related virus with less predicted human exposure, such as Nipah virus, would be a promising candidate for this platform. Experiments linking substitutions in PIV5 M protein that increased VLP production to analogous changes in Nipah virus M protein had yet to be completed.

In this study, Nipah virus M mutants analogous to those beneficial to VLP production in PIV5 M protein were found using sequential and structural alignments. Mutant Nipah virus M plasmids encoding each of the proposed mutations were synthesized via PCR and ligation. These were confirmed via restriction enzyme digest and sequencing. Western blotting was used to measure VLP production of each mutant relative to wild type Nipah virus.

The mutant Y187L was the only mutant found to have increased VLP production compared to wild-type virus (~50%), although this was not significant. The reason why this mutant mediated the greatest effect is unknown. The switch from tyrosine to leucine mediates

some benefit for particle component recruitment or association. This may be due in part due to the transition from an aromatic side chain to a less bulky hydrophobic structure.

The remaining mutants were either close to control VLP production levels or were found to have a deleterious effect on VLP production. Mutants V16M, M236L, G251I, P329D, G347R, G347W, G352R, G352W, Q291E were all not significantly worse than control VLP production and did not show any consistent increase compared to control. Mutants I150R, C157R, R191D, H238N, G257I, and H227M had statistically significant decreases in VLP production compared to control. The reason for these negative effects is unknown. M protein secondary or tertiary structure may be damaged by these amino acid substitutions. Furthermore, proteins may be somewhat functional but much less stable with these changes, which would hamper VLP production.

The homology of PIV5 M and Nipah virus M may be implicated in the lack of success for many mutations. A protein Basic Local Alignment Search Tool (BLAST) query comparing Nipah M protein (Accession: NP_112025.1) to PIV5 M protein (Accession: YP_138514.1) yields 23.96% identity. Therefore, a 24% degree of similarity may not be enough homology between these two proteins to effectively link changes in PIV5 M protein to Nipah virus M.

Mutants in different viruses from PIV5 may relate more closely to those in Nipah virus M. Cedar virus (CedV) is both a paramyxovirus and a Henipavirus and is therefore related to Nipah virus. However, CedV lacks the pathogenicity of NiV, and is recognized as a Biosafety Level 2 pathogen (30, 31). Given the safety of working with this live virus, experiments performed by P. T. Schmitt, Ray, and A. P. Schmitt could be translated to CedV. The C-terminal end of the CedV NP protein could be altered to disrupt particle formation in viruses. These viruses could then be passaged. Second-site mutants that seek to rescue budding function could

therefore be mapped as was done in PIV5 (7). These mutations would be paired to Nipah virus via sequence alignments. Nipah virus M mutants could then be constructed and transfected into producer cells to test VLP production. A protein BLAST between Nipah virus M protein (Accession: NP_112025.1) and Cedar virus M protein (Accession: AJP33318.1) indicates ~63% similarity between the proteins. Due to the closer relation of these two proteins, the mutations in CedV M mapped to Nipah virus M may have a greater chance of resulting in improved budding efficiency.

Chapter 6

Conclusion

Protein-based therapies have great potential to treat genetic diseases. VLP-based delivery vehicles are one avenue of treatment in this category. Paramyxoviruses, like PIV5 and Nipah virus, are one class of viruses capable of producing VLPs adequate for this function. Mutations to the M protein of these viruses can modulate VLP production quantity to be greater than that of the wild-type virus, an important factor for future industrially scalable treatment technologies.

The goal of this study was to elicit and create Nipah virus M mutants derived from mutations beneficial to VLP production seen in a homologous virus, PIV5. Both structural and sequential alignments were used to find consensus sequences in Nipah virus M that matched PIV5 mutations. Once mutant plasmids were generated, Nipah virus mutants were assessed for their effect on VLP production.

Of the mutants assessed for VLP production, only one produced budding that was increased compared to wild-type virus. The mutant Y187L had total VLP production roughly 1.5 times that of the wild-type control, although this was not statistically significant. Therefore, this mutation changed some aspect of the viruses' balance between genome replication and particle production that allowed for a greater production of VLPs. While this increase may seem modest, the ramifications of this finding could be important in practice. VLP production in mammalian expression systems is heavily limited by high costs, low yields, and long expression times compared to bacterial or plant expression systems (6). Increasing the production of VLPs by roughly 50% in this mammalian cell line could therefore boost the feasibility of this production

system in a future industrial setting. In terms of gene therapies, this would mean more access to treatments for patients due to decreasing production costs and decreasing the time it takes to yield an adequate amount of VLPs.

The rest of the mutants, while also unsuccessful in significantly increasing VLP production, still provide critical insights. Collectively, the lack of increased VLP production in mutants may suggest that Nipah virus, in its wild-type form, may be maximally optimized for VLP production. More likely, however, is that the limited homology between PIV5 M protein and Nipah virus M protein is not sufficient to effectively target regions in Nipah M protein where mutations could be beneficial to VLP production. Thus, other methods will have to be pursued to accurately connect PIV5 M mutations to Nipah virus M.

Multiple avenues of study remain with this technology. Cedar virus could undergo the same studies as were conducted with PIV5 to generate second-site M mutants. These mutants could be mapped onto Nipah virus, and those Nipah virus M mutants could then be tested for VLP production. The exact molecular basis of the VLP budding benefit conferred by mutants are still unknown. Future studies that discover the underlying mechanism could help understand benefits previously seen in PIV5 or elicit new targets for future studies. Working with other zoonotic paramyxoviruses with a likelihood of low preexisting immunity in the global population should also be explored. The primary candidate for this line of inquiry would be Newcastle disease virus.

The results of this study helped elucidate M protein particle budding dynamics in Nipah virus. This adds to a body of research seeking to improve the VLP delivery platform for future therapeutics requiring functional protein delivery to mediate benefits.

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