THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

INTERACTION OF NIPAH VIRUS MATRIX PROTEIN WITH CELLULAR AP3B1

KHAW, WEI YOUNG

SPRING 2012

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in BIOTECHNOLOGY
with honors in BIOTECHNOLOGY

Reviewed and approved by the following:

Dr. Anthony P. Schmitt
Associate Professor of Molecular Virology
Thesis Supervisor

Dr. Richard Frisque
Professor of Molecular Virology
Co-Thesis Supervisor

Dr. David S. Gilmour
Professor of Molecular and Cell Biology
Honors Advisor

Wendy Hanna-Rose
Associate Department Head for Undergraduate studies
Department of Biochemistry and Molecular Biology

* Signatures are on file in the Schreyer Honors College.
Abstract:

Paramyxovirus matrix (M) protein contributes to viral particle assembly and budding through its ability to interact with viral glycoproteins and ribonucleoprotein (RNP) complexes. For efficient budding to take place, M protein could also be a key player in recruiting host cell factors. Affinity co-purification screening has identified the beta subunit of adaptor protein AP3 complex (AP3B1) as a cellular binding partner of Henipavirus M proteins. Furthermore, short M-binding, AP3B1-derived polypeptides were shown to inhibit M protein function and prevent the release of virus-like particles (VLPs) from transfected cells. Here, we performed a series of biochemical experiments to study the interaction between AP3B1 and Nipah virus (NiV) M protein. We found that M-binding, AP3B1-derived polypeptides impair NiV M protein association with cellular membranes, thereby providing mechanistic insight into the inhibition of VLP production observed earlier. In addition, the binding interface within NiV M protein was mapped to amino acid (aa) residues 191-282, as assessed by a series of co-immunoprecipitation binding experiments. Finally, several mutated versions of M-binding, AP3B1-derived polypeptides were constructed, and these would facilitate further mapping studies in the future.
Table of Contents:

Abstract ........................................................................................................................................... i
List of Figures ................................................................................................................................... iii
Acknowledgment ........................................................................................................................ iv
Introduction ..................................................................................................................................... 1
Materials and Methods .............................................................................................................. 9
Plasmids ........................................................................................................................................ 9
Antibodies ..................................................................................................................................... 9
Membrane Floatation .................................................................................................................. 10
SDS-PAGE & Western Blot ......................................................................................................... 11
Results .......................................................................................................................................... 12
Discussion ..................................................................................................................................... 21
References ..................................................................................................................................... 25
List of Figures:

**Figure 1**: Preliminary data: a partial listing of the proteins identified from the affinity co-purification screening................................................................. 4

**Figure 2**: Preliminary data: inhibition of NiV-like particle production by M-binding, AP3B1-derived polypeptides......................................................... 7

**Figure 3**: Membrane association of NiV M protein in the presence of overexpressed AP3B1 and its derivatives......................................................... 14

**Figure 4**: Membrane association of NiV M protein in the presence of overexpressed Hinge derivatives ................................................................. 16

**Figure 5**: Construction of AP3B1 Hinge 1B mutants ................................................................. 18

**Figure 6**: AP3B1-binding region of NiV M protein was mapped to amino acid residues 191-282................................................................. 20
Acknowledgement:

First and foremost, I would like to express my gratitude to Dr. Anthony Schmitt for accepting me into his research team. I would not have had learned so much had Dr. Schmitt not given me this life-changing opportunity. My life as an undergraduate research assistant in The Pennsylvania State University has been so meaningful, all thanks to Dr. Schmitt’s acceptance.

Next, I would like to thank Weina Sun and Dr. Phuong Schmitt. Like mentors to me, Weina and Dr. Phuong have been very patient in showing me various laboratory skills. I would not have been able to perform many of these experiments independently had Weina and Dr. Phuong not been there for my guidance. Moreover, Weina has been such a helpful mentor to me throughout my time in Dr. Schmitt’s lab. In addition to guiding me along the way in many experiments, she has been providing me many useful comments in making my thesis better in many ways. Besides that, I would also like to thank all my lab mates Tom McCrory, Dr. Zifei Pei and Greeshma Ray for giving me assistance in one way or another.

Last but not least, I would like to extend my gratitude to Dr. Richard Frisque and Dr David Gilmour for being my thesis co-supervisor and honor adviser respectively. My thesis would not have been completed had Dr. Frisque and Dr. Gilmour not provided me with those constructive comments and feedback.
Introduction:

*Paramyxoviridae* forms a relatively large and diverse family of enveloped viruses that harbor single-stranded negative sense RNA genomes. Many of these viruses are significant causes of human and animal disease including human parainfluenza virus, measles virus, mumps virus, human respiratory syncytial virus and Newcastle disease virus. Human respiratory syncytial virus and parainfluenza virus types 1-3 for instance, are significant causes of respiratory infections in young children and the elderly \[^{1-5}\]. Measles virus and mumps virus infections on the other hand, despite the presence of effective vaccines, still run rampant in many developing countries and regions where vaccination remains controversial.

While problems with these pathogenic paramyxoviruses still persist, new members of *Paramyxoviridae* were just discovered about a decade ago in Australia and Malaysia. These new members, now known as Hendra virus (HeV) and Nipah virus (NiV) respectively, are together categorized under the genus *Henipavirus* \[^{6}\]. Both of these newly emerging paramyxoviruses have been identified to be zoonotic, naturally harbored by fruit bats of the genus *Pteropus* \[^{7}\]. Transmitted through intermediary mammalian hosts like horses (for Hendra virus) and pigs (for Nipah virus), Henipaviruses have caused many fatal infections in the forms of vasculitis and encephalitis in humans \[^{8-9}\]. Despite a total of twelve recorded Nipah virus epidemics in South Asia and thirteen known Hendra virus epidemics in Australia since their discoveries in 1990’s, there are still no approved vaccines and effective treatments against these infections hitherto \[^{8,10-12}\]. For this reason in addition to the ease of virus spread, Henipaviruses have been categorized as highly dangerous pathogens by the Centers for Disease Control and Prevention (CDC) and the National Institute of Allergy and Infectious Diseases (NIAID).
Paramyxoviruses in general have a non-segmented, single-stranded negative sense RNA genome about 16 – 20 kb in size. This RNA genome encodes for six principle gene products: nucleocapsids (N or NP), phosphoprotein (P), polymerase (L), matrix (M), fusion (F) and attachment (HN, H or G) proteins [26]. Each of these proteins plays an indispensable role in the virus life cycle. N or NP proteins, for instance, mainly serve to protect the viral RNA genome through encapsidation. In complex with P protein, L protein constitutes a critical part of the viral RNA-dependent RNA polymerase. This polymerase complex performs both the replication and transcription events using the viral ribonucleoprotein (RNP) complex as a template. The two remaining glycoproteins: attachment protein HN, H or G and fusion protein F on the other hand, mediate the binding to the corresponding host surface receptor and the subsequent fusion event respectively. The membrane fusion event that defines viral entry, is likely triggered by the viral attachment protein upon firm binding to the host surface receptor. M proteins organize virus assembly by interacting directly with the RNP complex as well as the glycoprotein cytoplasmic tails at the cellular plasma membrane, allowing for the virus particle assembly and release.

Like adaptors to the other viral components, the highly abundant M proteins physically bring together the viral proteins and enable their proper assembly into virions. For efficient binding to occur however, membrane association of paramyxovirus M protein is required, especially at the early event of the budding process. In fact, previous biochemical studies have almost always indicated the membrane-binding property of the hydrophobic paramyxovirus M proteins [13-16]. Atomic structure study carried out by Money et al. have also proposed that electrostatic interactions could be crucial to M protein-membrane interaction; given the extensive positively charged surface of respiratory syncytial virus M protein [17].
For many (but not all) paramyxoviruses, expression of M protein alone in transfected mammalian cells leads to the budding of virus-like particles (VLPs) from the cells that resemble authentic virions \(^{[25]}\). This is true for the Henipaviruses, in which M protein expression leads to efficient release of Henipavirus-like particles into cell culture supernatants \(^{[12, 27]}\). This however, does not tell the whole story about Henipaviruses budding process. As seen in many other RNA viruses, employment of cellular machineries for the transportation, trafficking, assembly and even the budding of viral particle could be a crucial part of Henipavirus life cycle. Examples include Tsg101 and HIV-1 \(^{[29]}\), Nedd4 and rabies virus \(^{[30]}\), AIP1/Alix and Sendai virus \(^{[31]}\), as well as AmotL1 and parainfluenza virus 5 \(^{[32]}\). We hypothesize that efficient budding of Henipavirus particles may rely upon several cellular factors, and we further hypothesize that host factors critical to Henipavirus budding are likely to be recruited to virus assembly sites through interaction with M protein. Hence, the overall goal of our work is to identify the Henipavirus M protein-interacting host factors and characterize the interactions thereafter.

In preliminary affinity co-purification screening carried out by Weina Sun (W.S.), the beta (\(\beta\)) subunit of the cellular AP3 adaptor complex, indicated here as AP3B1, was identified to interact with Henipavirus M protein. 293T cell lysates were prepared and subjected to FPLC affinity co-purification of Henipavirus M-interacting host proteins. After resolving the eluted polypeptides with SDS-PAGE and in-gel trypsin digestion, analysis by liquid chromatography tandem mass spectrometry was performed to generate the partial listing of the affinity co-purified host proteins (Figure 1).
Figure 1. Preliminary data: a partial listing of the proteins identified from the affinity copurification screening. FPLC co-purified proteins with Henipavirus M were analyzed by liquid chromatography tandem mass spectrometry. The partial listing of identified proteins was shown. Coverage indicates the percentage of amino acid residues within the protein that are present in at least one of the identified peptides. The protein of interest: isoform 1 of AP3 complex subunit beta1 (AP3B1) about 120 kd in size was highlighted.

<table>
<thead>
<tr>
<th>Band ID</th>
<th>Unique peptides</th>
<th>Coverage</th>
<th>Protein name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ~130kd</td>
<td>76 48%</td>
<td>RNA helicases (DHX9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>67 46%</td>
<td>Splicing factors (SF3B1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>27 15%</td>
<td>Treacher Collins-Franceschetti syndrome 1 (TCOF1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9 12%</td>
<td>Death-domain associated protein (DAXX)</td>
<td></td>
</tr>
<tr>
<td>2 ~120kd</td>
<td>67 48%</td>
<td>Heterogeneous nuclear ribonucleoprotein U (HNRPU)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>31 39%</td>
<td>RNA helicases (DDX24)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>29 30%</td>
<td>Splicing factors (SF3B3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>22 22%</td>
<td>Isoform 1 of AP-3 complex subunit beta-1 (AP3B1)</td>
<td></td>
</tr>
<tr>
<td>3 ~115kd</td>
<td>49 37%</td>
<td>Leucine-rich PPR motif-containing protein (LRPRPC)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>38 35%</td>
<td>Pyruvate carboxylase, mitochondrial (PC)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>36 30%</td>
<td>RNA helicases (DHX30)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19 18%</td>
<td>DNA damage-binding protein 1 (DDB1)</td>
<td></td>
</tr>
<tr>
<td>4 ~110kd</td>
<td>43 55%</td>
<td>Cell division cycle 5-like protein (CDC5L)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25 38%</td>
<td>NF-kappa-B-repressing factor (NKR)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19 39%</td>
<td>Zinc finger CCCH-type antiviral protein 1 (ZC3HAV1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 16%</td>
<td>Probable E3 ubiquitin-protein ligase (HERCS)</td>
<td></td>
</tr>
<tr>
<td>5 ~97kd</td>
<td>57 54%</td>
<td>RNA helicases (DDX21)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>56 56%</td>
<td>Isoform 5 of interleukin enhancer-binding factor 3 (ILF3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 20%</td>
<td>Isoform 1 of Exosome component 10 (EXOSC10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 5%</td>
<td>Coatamer, beta subunit (COPB1)</td>
<td></td>
</tr>
<tr>
<td>6 ~90kd</td>
<td>41 35%</td>
<td>RNA helicases (DHX15)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25 36%</td>
<td>Heterogeneous nuclear ribonucleoprotein R (HNRPR)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 8%</td>
<td>Kelch repeat and BTB (POZ) domain containing 6 (KBTBD6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 5%</td>
<td>Cullin 3 (CUL3)</td>
<td></td>
</tr>
<tr>
<td>7 ~50kd</td>
<td>18 52%</td>
<td>Nuclease-sensitive element-binding protein 1 (YBX1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13 30%</td>
<td>Isoform 1 of DNA-binding protein A (CSDA)</td>
<td></td>
</tr>
<tr>
<td>8 ~46kd</td>
<td>37 52%</td>
<td>Ribosomal proteins (RPL4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 10%</td>
<td>DnaJ homolog, subfamily B, member 11 (DNAJB11)</td>
<td></td>
</tr>
<tr>
<td>9 ~44kd</td>
<td>27 40%</td>
<td>Ribosomal proteins (RPL3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13 37%</td>
<td>Interleukin enhancer-binding factor 2 (ILF2)</td>
<td></td>
</tr>
<tr>
<td>10 ~35kd</td>
<td>36 67%</td>
<td>Ribosomal proteins (RPS3A)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8 34%</td>
<td>Exosome complex exonuclease (EXOSC2)</td>
<td></td>
</tr>
<tr>
<td>11 ~30kd</td>
<td>29 73%</td>
<td>Ribosomal proteins (RPS4X)</td>
<td></td>
</tr>
<tr>
<td>12 ~28kd</td>
<td>19 50%</td>
<td>Ribosomal proteins (RPL7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9 19%</td>
<td>Histones (HIST1H1C)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 14%</td>
<td>Casein kinase II subunit beta (CSNK2B)</td>
<td></td>
</tr>
<tr>
<td>13 ~26kd</td>
<td>18 52%</td>
<td>Ribosomal proteins (RPL19)</td>
<td></td>
</tr>
<tr>
<td>14 ~23kd</td>
<td>23 56%</td>
<td>Ribosomal proteins (RPS9)</td>
<td></td>
</tr>
<tr>
<td>15 ~22kd</td>
<td>24 78%</td>
<td>Ribosomal proteins (RPS11)</td>
<td></td>
</tr>
</tbody>
</table>

(Figure courtesy of Weina Sun)
AP3 protein complex consists of 4 subunits (adaptins): a large delta (δ), a large beta (β), a medium mu (µ), and a small sigma (σ) \[33\]. Functionally, the δ subunit has been associated with membrane binding; the β subunit is responsible for clathrin recruitment; the µ subunit recognizes the tyrosine-based sorting signals of cargo; and the σ subunit is proposed to stabilize the protein complex \[33\]. Together, this protein complex has been tightly associated with the formation of secretory lysosomal pathways and the intracellular trafficking of membrane proteins \[33-35\]. In the studies with CD63 (an integral membrane protein present on the surface of hematopoietic cells) for instance, AP3 complex has been shown to be critical for its transport from the trans-Golgi network (TGN) to lysosomes \[18, 40-42\]. Deficiency in AP3 therefore results in abnormal CD63 redistribution onto the plasma membrane, and this in turn, causes defective functions in cells that rely upon this type of secretory lysosomal pathways \[43-45\]. Thus, this important intracellular secretory pathway could potentially be manipulated by Henipavirus for efficient viral release through the M-AP3B1 protein-protein interactions.

After the identification of AP3B1 as Henipavirus M protein-binding partner by affinity co-purification and mass spectrometry, several experiments have been carried out by W.S. for the characterization of this host factor. Up to this point, W.S. has (1) mapped the M protein-binding region of AP3B1 onto the serine-rich, acidic Hinge domain; (2) PCR-constructed AP3B1-derived polypeptides Head, Hinge, Ear, Hinge/Ear, Hinge 1, Hinge 2, Hinge 3, Hinge 1A and Hinge 1B (Figure 2A); and (3) shown that the overexpression of the M protein-binding, AP3B1-derived polypeptides Hinge, Hinge 1, Hinge 3 and Hinge 1B in cells inhibited Henipavirus-like particle production (Figure 2B). Similar inhibition of virus-like particle (VLP) production through the overexpression of viral protein-binding fragments derived from the host protein complexes has also been observed in other RNA viruses. These viral protein-binding
derivatives are thought to function as competitive inhibitors to the endogenous counterparts by upsetting the interaction with viral proteins and subsequently disabling proper virus budding due to the failure of host machinery recruitment [19-24].
Figure 2. Preliminary data: inhibition of NiV-like particle production by M-binding, AP3B1-derived polypeptides. (A) Schematic representation of the AP3B1 domains as well as all the PCR-generated AP3B1-derived fragments, with the polypeptides length indicated in number. (B) Relative VLP production was calculated as the amount of viral M protein detected in purified VLPs divided by the amount of M protein detected in the corresponding cell lysate fractions, normalized to the value obtained in the absence of AP3B1-derived polypeptides. “EV” refers to empty-vector plasmid, i.e., M protein expressed in the absence of any AP3B1-derived polypeptides. Hinge, Hinge 1, Hinge 3, and Hinge 1B polypeptides have all been shown to bind M protein, and all inhibit VLP production. Head, Hinge 2, and Hinge 1A polypeptides all fail to bind M protein, and fail to inhibit VLP production. Hinge 1B is the smallest M-binding, AP3B1-derived polypeptide that has been identified, at 29 amino acid (aa) residues long. Experiment was performed by W.S.
Here, as an extension to W.S.’s work, several experiments were performed to further characterize the Henipavirus M-AP3B1 protein-protein interactions. Given the high similarity between NiV and HeV M proteins (about 92% identical), all experiments were performed using only the NiV M protein. In this study, a membrane-binding assay was employed to examine the molecular mechanism by which M-binding, AP3B1-derived polypeptides inhibit VLP production. The loss of NiV M protein-membrane association in the presence of AP3B1-derived inhibitory polypeptides has been associated to the reduction of Henipavirus-like particle production. In addition, a series of mapping studies had further defined amino acid (aa) residues, both within M protein and within AP3B1 Hinge 1B, that are important for efficient binding.
Materials and Methods:

Plasmids

N-terminally tagged NiV M proteins (GST, Strep-His and Myc tags) were PCR-amplified and ligated into the eukaryotic expression vector pCAGGS. These tags served to facilitate the subsequent binding experiments and immuno-detection assays. The sequences of these tagged versions of NiV M protein were confirmed by DNA sequencing. Similarly, the shorter GST-fused fragments of NiV M protein were engineered by PCR and subcloned into the pCAGGS expression vector. cDNA corresponding to full length human AP3B1 (Open Biosystems accession no. BC038444) was obtained from Thermo Scientific (Lafayette, CO). Using PCR, all AP3B1 polypeptide derivatives (Head, Hinge, Ear, Hinge/Ear, Hinge 1, Hinge 2, Hinge 3, Hinge 1A and Hinge 1B) were generated. All of these polypeptides were Flag-tagged at the N-terminus and subcloned into the expression vector pCAGGS. For the AP3B1 Hinge 1B mutants on the other hand, a 2-step PCR-based mutagenesis procedure was employed as described previously [46]. Once the sequences were verified by DNA sequencing, all the engineered plasmids were amplified in E. coli, and large-scale preparations of plasmid DNA were prepared.

Antibodies

Rabbit polyclonal anti-NiV M antibody has been described previously [47]. Rabbit polyclonal anti-AP3B1 antibodies were purchased from Proteintech Group Inc. (Chicago, IL). Monoclonal antibody specific to the Myc tag (clone 9E10) was purchased from Sigma-Aldrich (St. Louis, MO). Anti-Flag M2 magnetic beads (M8823) were purchased from Sigma-Aldrich (St.
Louis, MO). Alkaline phosphatase-conjugated goat anti-rabbit secondary antibody was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Membrane Floatation

6cm dish of 293T cells were transfected to express NiV M and AP3B1-derived polypeptides using Lipofectamine and Plus reagents. Cells were harvested and lysed in 600ul detergent-free Dounce buffer (25 mM NaCl, 50 mM Na$_2$HPO$_4$, pH 7.4) 24 hrs post transfection (p.t.) by rocking at 4°C for 30 min. The cell lysates were subjected to 40 strokes of Dounce homogenization, and the removal of nuclei and debris was done with centrifugation at 4K rpm for 5 min at 4°C. Supernatants were mixed with 1.5 ml of 80% sucrose in NTE (100 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EDTA). Layers of 50% sucrose (2.4ml) and 10% sucrose (0.6 ml) in NTE were placed on the tops of samples to establish the sucrose gradient. After centrifugation at 40K rpm for 4 hrs in a Beckman AH-650 swinging bucket rotor, six equal fractions were collected from the top of each gradient. The collected fractions were resolved by SDS-PAGE and subsequently analyzed by western blot using the rabbit polyclonal anti-NiV M antibody and alkaline phosphatase-conjugated goat anti-rabbit secondary antibody. Protein bands were detected and quantified with a Fuji FLA-7000 laser scanner (FujiFilm Medical Systems, Stamford, CT). Membrane-bound NiV M protein was calculated as the amount of M protein detected in the top three fractions divided by the amount of M protein detected in all six fractions.
**SDS-PAGE and Western Blot**

Cell lysates prepared from the membrane floatation assay were mixed with protein lysis buffer (PLB) containing dithiothreitol (DTT), boiled for 5 min, loaded and resolved with either 10% or 15% SDS-polyacrylamide gel. PVDF membrane was used for the transfer of electrophoresed proteins. After that, NiV M proteins were probed with primary rabbit polyclonal anti-NiV M antibody at the ratio of 1:1000 after pre-blocking with 5% BSA in PBS-0.3% TWEEN. The membrane was then treated with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody at the ratio of 1:6000 after three washes with PBS-0.3% TWEEN. Again, three washes with PBS-0.3% TWEEN were carried out before the addition of ECF substrate purchased from GE Healthcare (Piscataway, PA). The protein bands were visualized with a Fuji FLA-7000 laser scanner.
Results:

**AP3B1-derived polypeptides impair the ability of Nipah virus M protein to bind cellular membranes.**

Preliminary experiments performed by W.S. have indicated that the overexpression of NiV M protein-binding, AP3B1-derived Hinge polypeptides could remarkably reduce the production of VLPs. One possible mechanism for this inhibitory effect could be an impairment in the ability of M protein to bind cellular membranes. M proteins function for particle formation by inducing various viral components to assemble together at the cell plasma membrane. The cell membrane is then deformed outward, and viral particles are ultimately pinched off and released. M proteins have intrinsic membrane-binding ability, and previous studies have demonstrated that paramyxovirus M protein mutants that fail to bind membranes cannot function for particle production \(^{[25]}\). Thus, we tested the possibility that M-binding polypeptides inhibit VLP production through an effect on M protein membrane-binding function. To measure M protein membrane-binding, a sucrose flotation assay was employed. M proteins were expressed in 293T cells via transient transfection, and detergent-free cell lysates were prepared by Dounce homogenization. The lysates were placed at the bottoms of sucrose flotation gradients. Upon ultracentrifugation, membranes (and proteins that are attached to membranes) will float to the tops of the gradients. Proteins that are not attached to membranes remain at the bottoms of the gradients. Fractions are collected, and M proteins are detected and quantified by SDS-PAGE and western blotting. When M protein is expressed in the absence of any inhibitory polypeptides, about 60% of the protein is detected in the membrane-bound fractions of the gradient (Figure 3). As indicated in Figure 3, expression of AP3B1 Hinge polypeptide impaired the membrane-
binding function of M protein, while expression of the AP3B1 Head polypeptide (which does not bind M protein) had little effect on the membrane-binding function of M protein.
Figure 3. Membrane association of NiV M protein in the presence of overexpressed AP3B1 and its derivatives. (A) Nipah virus M protein and AP3B1-derived polypeptides were expressed in 293T cells. Cells were lysed in detergent-free solution and subjected to sucrose flotation gradients. Samples were collected after ultracentrifugation. The first three left portions are the membrane-bound fraction whereas the three portions to the right (separated by the bar) are the membrane-free fraction. Overexpression of NiV M protein-binding, AP3B1 Hinge fragment reduced membrane association of NiV M protein. (B) Results from three independent experiments performed as in (A) were plotted, with standard deviations indicated as error bars. Plasmid construction for AP3B1 derivatives, transfection of 293T cells, and membrane flotation assay were performed by W.S. SDS-PAGE, transfer of proteins to PVDF membranes, and western blotting were performed by W.Y.K.
To further define the smallest possible inhibitory polypeptides, similar sucrose gradient-based membrane flotation assay was performed on smaller Hinge derivatives. These Hinge derivatives comprised NiV M protein-binding fragments Hinge 1, Hinge 3 and Hinge 1B as well as non-NiV M protein-binding fragments Hinge 2 and Hinge 1A. As described in the legend to Figure 3, transfected cell lysates were fractionated on sucrose flotation gradients, and analyzed by SDS-PAGE and western blot. As expected, the NiV M protein-binding VLP inhibitory fragments Hinge 1, Hinge 3 and Hinge 1B caused reduced membrane association of NiV M protein. In contrast, the non-NiV M protein-binding fragments Hinge 2 and Hinge 1A had little effect on NiV M protein membrane association (Figure 4). In fact, the degree of NiV M protein membrane association in the presence of Hinge 2 and Hinge 1A was not very different from the result when NiV M protein was expressed alone.
Figure 4. Membrane association of NiV M protein in the presence of overexpressed Hinge derivatives. As in Figure 3, the first three left portions are the membrane-bound fraction whereas the three portions to the right (separated by the bar) are the membrane-free fraction. NiV M protein-binding Hinge fragments Hinge 1, Hinge 3 and Hinge 1B all managed to reduce membrane association of NiV M proteins up to 26%, 27% and 29% respectively. The non-NiVM protein-binding fragments Hinge 2 and Hinge 1A had less effect on reduction of NiV M protein membrane association.
**Mutagenesis of AP3B1 Hinge 1B fragment**

Preliminary results obtained by W.S. have shown that the 29 aa M protein-binding, AP3B1-derived Hinge 1B polypeptide, rich in serine and acidic residues, could by itself confer remarkable NiV M protein binding comparable to Hinge 1. These results seemed to point towards the importance of these serine and acidic residues in NiV M-AP3B1 protein-protein interaction. Moreover, when overexpressed in 293T cells, this short polypeptide could effectively inhibit Nipah VLP production. Thus, PCR site-directed mutagenesis experiments were designed in an attempt to single out particular amino acid residue(s) that may be responsible for the interaction with NiV M protein. Mutants of Hinge 1B, in which the serine or acidic amino acid residues (aspartic acid or glutamic acid) were substituted with alanine, were made (Figure 5A). Co-immunoprecipitation experiments were then performed by W.S. using these Hinge 1B mutants. The results indicate that both serine residues and acidic residues are important for the interaction with M protein (Figure 5B). For example, mutation of serine residues 688, 689, and 690 to alanine simultaneously disrupted M protein binding. Mutation of glutamic acid residues 681, 682, and 683 to alanine simultaneously also disrupted M protein binding. However, mutation of glutamic acid residues 678 and 680 to alanine simultaneously did not impair M protein binding. This suggests that there is a specificity to the binding, with certain serine and acidic residues within this cluster being more important than others for M protein binding. Single alanine substitution mutants were not binding-defective (W.S., unpublished observation), suggesting that the binding of Hinge 1B to NiV M proteins is a collective effort of those serine and acidic amino acid residues instead of a particular key residue. Additional mutagenesis experiments will be needed to further define the requirements for binding between Hinge1B and M protein.
Figure 5. Construction of AP3B1 Hinge 1B mutants. (A) The amino acid sequence of the 29 amino acid-residue-long AP3B1 Hinge 1B polypeptide is illustrated, together with amino acid substitution variants that target either serine residues or acidic residues. The substituted amino acid residues are underlined and in bold. Each variant cDNA has been generated through PCR mutagenesis, and plasmids for expression of the altered polypeptides have been constructed. (B) Co-immunoprecipitation of Myc-tagged NiV M protein with the Flag-tagged NiV M protein, the Flag-tagged NiV M protein, the Flag-tagged NiV M protein, and various Hinge 1B mutants (bottom panel). Immunoprecipitation of the AP3B1 Hinge 1B constructs was carried out with the anti-Flag M2 magnetic beads. The top panel showed the expression level of NiV M protein, immunoprecipitated with anti-Myc antibody. Certain serine and acidic residues on AP3B1 Hinge 1B seemed to be more important than others for NiV M-binding. PCR-amplification of the various cDNAs and plasmid construction were performed by W.Y.K. Co-immunoprecipitation was performed by W.S.
Mapping of AP3B1-binding region in Nipah virus matrix protein

To gain insight into the biological relevance of the AP3B1-M protein interaction, it will be important to construct and functionally analyze mutant M proteins that fail to bind AP3B1. Towards this end, we have generated a series of NiV M protein-derived constructs fused to GST. A total of seven NiV M constructs were made using PCR (Figure 6A). These constructs were ligated into the pCAGGS expression plasmid and subsequently expressed in 293T cells. Co-immunoprecipitation assay with the full length AP3B1 was performed by W.S. to examine the binding ability of these NiV M-derived constructs. As indicated in the Figure 6B, GST-fused NiV M protein constructs 1-282 aa, 129-352 aa and 191-352 aa were co-immunoprecipitated with AP3B1. These results narrowed down the AP3B1-binding region on NiV M protein to the 191-282 aa stretch. The binding activity of this mapped region was further confirmed by another co-immunoprecipitation assay including the GST-fused NiV M 191-282 aa construct (Figure 6B).
Figure 6. AP3B1-binding region of NiV M protein was mapped to amino acid residues 191-282. (A) Schematic illustration of NiV M protein-derived polypeptides used to construct GST fusions. The NiV M protein-derived polypeptides represented in green bind independently to AP3B1. (B) 293T cells were transfected to produce GST-fused M protein-derived polypeptides together with Flag-tagged full-length AP3B1, as indicated. AP3B1 was immunoprecipitated using anti-Flag M2 magnetic beads, and co-precipitation of the GST-fused M-derived polypeptides was measured. PCR-amplification of the various cDNAs corresponding to M protein, and plasmid construction to generate GST fusions was performed by W.Y.K. Transfection of plasmids into 293T cells and co-immunoprecipitation were carried out by W.S.
Discussion:

Viral proteins alone are not adequate for the completion of viral life cycle. As an obligatory parasite, the infecting virus requires many of its host factors to ensure successful survival and propagation. Indeed, for members of Paramyxoviridae (and many others), specific host machinery employment is necessary for budding of virions. Unlike many other RNA viruses, however, matrix proteins of Nipah virus generally lack the typical late domain sequences such as PTAP, PSAP, PPxY and YP(x)nL that are responsible for host factor recruitment \cite{25}. This distinction may be an indication that Nipah virus depends upon a different strategy for host factor recruitment. Recently, the cellular motin protein AmotL1 has been identified as parainfluenza virus 5 (PIV5) M protein target. Like Nipah virus, PIV5 M proteins also lack the aforementioned late domain sequences \cite{32}. Moreover, the short M protein-binding fragments derived from this host protein were found to inhibit the production of PIV5 VLP when overexpressed. In the light of this previous study, we set out to identify and characterize Henipavirus M protein-interacting host factor(s) that facilitate the viral budding process. Here, as presented by W.S.’s preliminary results, the beta subunit of cellular protein complex adaptor protein 3 (AP3B1) was shown to be the binding partner of NiV M protein. As a follow-up study to W.S.’s finding that expression of the NiV M protein-binding, AP3B1-derived fragments inhibited the production of NiV-like particles, the molecular mechanism by which this inhibition works was found to be correlated to NiV M protein’s membrane association.

Important in the regulation of the biogenesis of vesicles from the Golgi to the vacuole/lysosomes, homologs of mammalian adaptor protein complex have also been identified in yeast and fruit fly \cite{36}. Studies of the AP3 mutants in mouse models *pearl* and *mocha* have given some insights about the functions of this host factor \cite{37-38}. In *pearl* mice for instance, the mutations result in a truncation of 130 or 233 amino acids in the β3A subunit of AP3 complex
and subsequently affect the cargo selection of lysosomal pathway\textsuperscript{[37]}. Likewise, deletion mutation of the δ subunit in \textit{mocha} mutant leads to a complete disappearance of AP3 complex, which in turn displays similar defective phenotypes as observed in the \textit{pearl} mutant\textsuperscript{[38]}. Together, these studies indicate that the heterotetrameric AP3 complex constitutes a central role in the secretory lysosomal pathways critical for formation of many intracellular granules\textsuperscript{[34-35]}.

In this project, the binding interface between AP3B1 and NiV M protein has been mapped. Amino acid residues 191-282 in NiV M protein were shown to independently interact with the cellular AP3B1 protein in the co-immunoprecipitation assay. For AP3B1, as indicated in W.S.’s preliminary experiments, the Hinge domain (643-809 aa) was identified to be the primary NiV M protein-binding region. The NiV M protein 191-282 fragment harbors a cluster of positively charged residues; AP3B1 Hinge domain, in contrast, comprises several stretches of serine-rich, negatively charged amino acid residues. Thus, it is likely that these two oppositely charged surfaces constitute the binding interfaces of these viral-host proteins via ionic interactions. At this point, however, the exact mechanism in which these two proteins interact is still to be further investigated. Mutation or deletion of these positively charged residues within 191-282 aa is a good starting point of this verification. Similarly, the attempt to find amino acid(s) key to the NiV M protein binding in the AP3B1 Hinge domain is still ongoing. To this end, mutagenesis experiments on the AP3B1 Hinge 1B fragment have shown that its binding ability was compromised when certain serine or acidic residues were substituted with alanine.

Very similar to the case of PIV5, the host-derived viral M protein-binding fragments could inhibit the production of VLPs. In fact, the shortest AP3B1-derived Hinge 1B fragment with only the length of 29 aa was found by W.S. to reduce NiV-like particle production. This inhibition is hypothesized to be the result of competitive binding between the endogenous
AP3B1 and the overexpressed NiV M protein-binding fragments. In the presence of these overexpressed short NiV M protein-binding fragments, NiV M proteins are more likely to bind with these abundant polypeptides instead of the endogenous AP3B1 complex. Upon failure to interact with the endogenous host machinery, the budding (VLP production in this case) efficiency is therefore negatively affected. A similar observation, in which the overexpressed host-derived polypeptides exhibit inhibitory effects on VLPs production, has also been described in other RNA viruses. For instance, the expression of Gag-binding fragments derived from both Tsg101 and Aip/Alix reduced production of the HIV-1 particle [19-24]. All of these discoveries suggest that inhibition of the budding process could potentially be developed into potent antiviral drugs against the respective viruses [39].

The inhibition mechanism of VLP production by NiV M protein-binding fragments has been explored in this project. Through membrane floatation assay, we discovered that the presence of NiV M protein-binding fragments (Hinge, Hinge 1, Hinge 3 and Hinge 1B) reduced the membrane association of NiV M proteins. Hence, the interaction of NiV M protein and AP3B1-derived, NiV M-binding fragments seemed to interrupt the membrane association of NiV M proteins. Once NiV M protein is deprived of its membrane-association ability as the result of binding to the overexpressed NiV M protein-binding fragments, its function in triggering viral particle budding is impaired as well. This helps to explain the reduction of NiV-like particle production as observed in the VLP experiment, although the exact molecular mechanism is yet to be further investigated. It is possible that trafficking of M protein to its final destination at cell plasma membranes is AP3-dependent.

Further verification of the NiV M-AP3B1 protein-protein interaction would be carried out using various other experiments. These experiments include single-point mutagenesis,
fluorescence microscopy and RNA interference. Just like the abovementioned mutagenesis experiment performed on AP3B1 Hinge 1B polypeptide, a similar experiment could be performed to create NiV M mutants that do not bind to the AP3B1 protein complex. This would certainly enable the comparison of functional changes exhibited by both mutants and wild type. And from there, we could better understand the molecular interaction of these two proteins. Besides that, fluorescence microscopy would be employed to observe trafficking and membrane-association of NiV M protein in both the presence and absence of the host derived NiV M protein-binding fragments. Also, the localization of the host factor as well as NiV M proteins could also be assessed with this visualization technique. In addition, siRNA knockdown experiment could be another critical assessment to corroborate the hypothesis that AP3B1 is needed for efficient NiV budding. In fact, siRNA knockdown experiment is currently underway to examine the effect of endogenous AP3B1 deficiency in Nipah VLP production. If AP3B1 is indeed a crucial host factor for Nipah viral budding, we would then expect a reduction of Nipah VLP production upon siRNA knockdown of the endogenous AP3B1. Furthermore, we could couple siRNA experiment to florescence microscopy for phenotype observation. For instance, we could compare the localization and degree of NiV M protein membrane association of the AP3B1-knockdown cells to the siRNA-untreated wild type cells. Together, all of these experiments could hopefully provide clearer evidences that the cellular AP3B1 is indeed recruited through Henipaviruses M proteins for proper particle assembly as well as efficient viral budding.
References:


Academic Vita:

WEI YOUNG KHAW
518 University Drive Apt 111, State College, PA 16801
wyk5026@psu.edu
1-814-753-0307

EDUCATION
The Pennsylvania State University, University Park, PA
B.S. in Biotechnology, anticipated May 2012
Minor in Microbiology, Biochemistry & Molecular Biology

Schreyer Honors College, University Park, PA
Honors in Biotechnology

UNDERGRADUATE RESEARCH EXPERIENCE
Veterinary & Biomedical Science Department, Penn State University, University Park, PA
Spring 2011 - Present
Supervisor: Dr. Anthony P. Schmitt
Graduate Mentor: Weina Sun
- Assist in identifying cellular protein that interacts with viral proteins
- Contribute to the characterization of viral-host protein-protein interactions using biochemical approaches

Biochemistry & Molecular Biology Department, Penn State University, University Park, PA
Summer 2010 - Fall 2010
Supervisor: Dr. Andrey Krasilnikov
- Studied the structures and functions of riboenzymes RNase P/MRP in both prokaryotic and eukaryotic cells
- Facilitated graduate and post-doc colleagues in various experiments by performing transformation, SDS-PAGE, protein purification and media preparation

AWARDS & ACADEMIC ACHIEVEMENTS
- The Evan Pugh Senior Student Awards in acknowledgment of excellent results
- Dean’s List consecutively from Fall 2009 – Present
LEADERSHIP & SERVICE ACTIVITIES
The Penn State IFC/Panhellenic Dance Marathon (THON) Chair of Penn State Malaysian Student Club (MSC)
Fall 2011 - Spring 2012
- Organized various fundraiser events among Malaysians for the pediatric cancer patients
- Reached out to Malaysian communities in the U.S. and Canada through the fundraising events
- Successfully raised a total of $1075.07 for THON on behalf of MSC

Volunteer of the International Student Orientation Program by University Office of Global Program
Fall 2010 - Spring 2012
- Volunteered and helped new international students in their registration with the university office
- Addressed questions from students and/or parents concerning the life, housing, facilities, programs and various orientation activities of the university

Committee and performer of Malaysian Cultural Night by Penn State Malaysian Student Club (MSC)
Spring 2010 & Spring 2012
- Involved in the organization of the event
- Led and presented a live cultural performance to approximately 200 audiences

LABORATORY SKILLS
- Transfection (Liposome-mediated technique)
- Western Blot
- Immunoflourescence Assay
- Agarose Gel Electrophoresis
- SDS-PAGE
- Site-directed Mutagenesis
- PCR
- Molecular Cloning