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Respiratory Syncytial Virus (RSV) is a leading cause of bronchopneumonia in infants and elderly. Knowledge of viral and host protein interactions is important for better understanding of the viral pathogenesis and may lead to development of novel therapeutic drugs. Here, we show that RSV Matrix (M) protein interacts with cellular adaptor protein complex (AP) 3 and its medium (µ) subunit (AP3µ1). A yeast two-hybrid assay indicated a novel protein-protein interaction that was then further confirmed in a mammalian system by colocalization between the RSV M and AP3µ1 proteins in a cytoplasmic defined region via Confocal Laser Scanning Microscopy (CLSM) analysis. Further evidence of this novel interaction was indicated via the presence of a known adaptor protein µ subunit sorting signal sequence, YXXL, which is conserved across various animal RSV M proteins. Subsequent studies also showed a specific up-regulation in the amount of AP3µ1 protein found in the cell during RSV infection, while corresponding subunits of the AP3 complex were unaffected. The interaction of AP3µ1 with RSV M represents a critical insight into the life cycle of this important virus protein.

Key Words: Respiratory Syncytial Virus, Matrix protein, Adaptor protein 3, Adaptor protein µ subunit, protein-protein interactions

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# Interaction of the Respiratory Syncytial Virus Matrix Protein and the Cellular Adaptor Protein Complex 3μ Subunit

by

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Dean, University Honors College

I understand that my project will become a part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

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Casey J Ward, Author

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## Introduction

### Respiratory Syncytial Virus as a Pathogen of Humans

Respiratory Syncytial Virus (RSV) is the leading cause of lower respiratory tract infection in infants and to date there is no vaccine or effective therapeutic treatment (Lee and Barton, 2007). In addition to being the leading cause of lower respiratory tract infection in infants, RSV causes significant morbidity and mortality in elderly (Mullooly et al., 2007; Thompson et al., 2003) and immunocompromised patients (Small et al., 2002). The impact of this disease is greatly muted in the United States due to excellent medical care, but throughout the world RSV infections result in a significant cause of morbidity and mortality leading to approximately one million deaths annually. Despite continued efforts there are no effective therapeutic treatments for this pathogen and no vaccine. Early efforts to produce a formalin inactivated RSV vaccine in the mid-1960's though resulted in an exacerbated immune response upon subsequent natural infection, leading to the removal of the vaccine from the market (Zhan et al., 2007). Currently, Synagis (palivizumab; MedImmune, Gaithersburg, MD), an antibody targeted against the RSV Fusion (F) and Attachment (G) surface glycopeptide epitopes, is licensed for the immuno-prophylactic treatment of at-risk individuals such as transplant recipients or chemo patients (Chavez-Bueno et al., 2007). Steady progress has been made in understanding the viral life cycle and the consequences of infection, but continued

research is still greatly needed in understanding the pathogenesis of RSV and the role of individual gene products in these mechanisms.

**RSV Biology**

Human respiratory syncytial virus (RSV) belongs to the subfamily *Pneumovirinae* within the *Paramyxoviridae* family, order *Mononegavirales (Collins PL, 2001)*. Other common *Paramyxoviridae* family viruses include measles, mumps, Newcastle disease, human parainfluenza virus, and Sendai virus. RSV is an enveloped virus with a contiguous single stranded, negative sense, 15.2 kb RNA genome, containing 10 genes coding for 11 proteins (Figure 1). There are four nucleocapsid proteins (nucleocapsid N protein, phosphoprotein P, large polymerase subunit L and transcription elongation factor M2-1), three transmembrane envelope glycoproteins (fusion F protein, attachment G protein and small hydrophobic SH protein), two non-structural proteins (NS1 and NS2), the Matrix (M) protein, and M2–2, an RNA regulatory factor (Ghildyal et al., 2006).

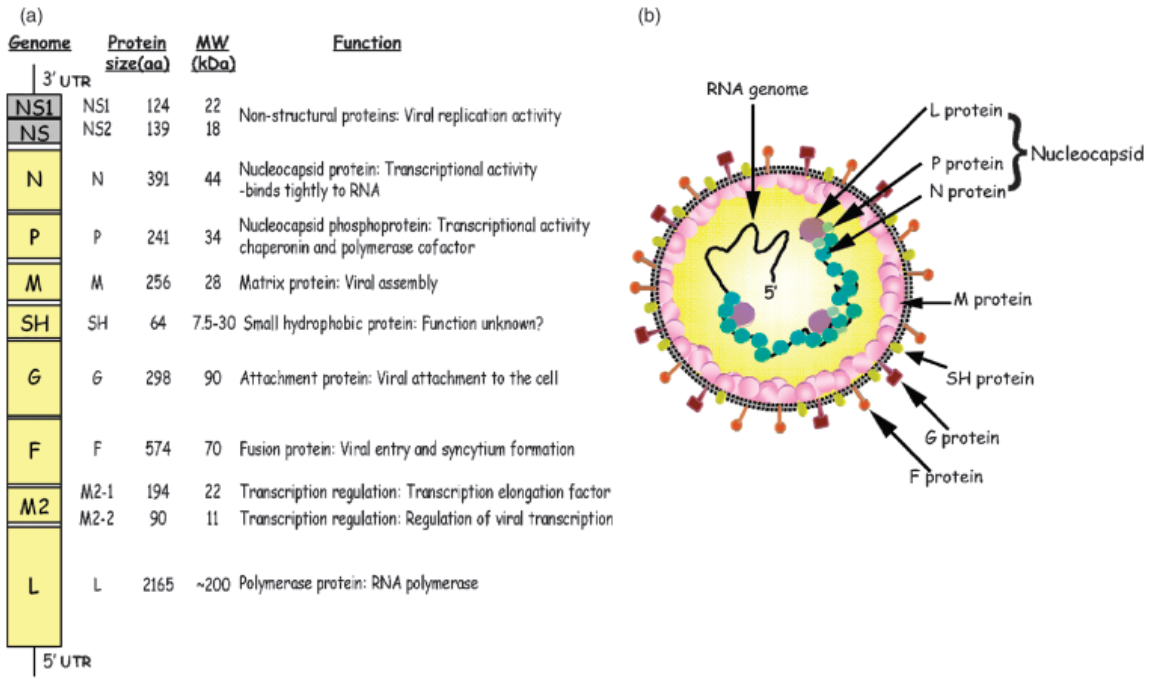



**Figure 1. RSV genome organization.** RSV is an enveloped virus with a contiguous single stranded, negative sense, 15.2 kb RNA genome, containing 10 genes and 11 proteins (Ghildyal et al., 2006).

The first step in RSV replication is attachment of the virus particle to a host cell, generally a ciliated epithelial cell in the nasal epithelium, mediated by G. RSV enters the cell by fusion with the plasma membrane mediated by F (Srinivasakumar et al., 1991). The viral envelope is then incorporated into the cell membrane, and the nucleocapsid is released into the cytoplasm. Virus transcription and replication are mediated by and at nucleocapsids.

**Matrix Protein**

The primary known function of the Matrix (M) protein of respiratory syncytial virus is to provide structure to the virion. Recent studies demonstrate that RSV M is present on the cytoplasmic side of the plasma membrane of infected cells, associating peripherally with

the plasma membrane rather than inserting into the bilayer (Marty et al., 2004). The interaction of M and the membrane appears to be largely electrostatic, though the hydrophobic C-terminal domain of M may play a role as well.

The Matrix protein has unique characteristics for an RNA virus protein in that it has been shown to localize to the nucleus early in infection and export late in infection. As shown in figure 2, this observation has important implications in understanding the life cycle of RSV because, unlike DNA viruses, there is no explicit need to manipulate any of the nuclear machinery for viral RNA or mRNA transcription and translation. Thus, it has been proposed that the M protein may function as a type of biological clock for the virus by entering the nucleus early in infection to inhibit host cell transcription, thereby focusing the efforts of the infected cells machinery on viral replication, then late in infection by exiting the nucleus and terminating viral replication in preparation for virus assembly only once sufficient viral protein has accumulated in cells.

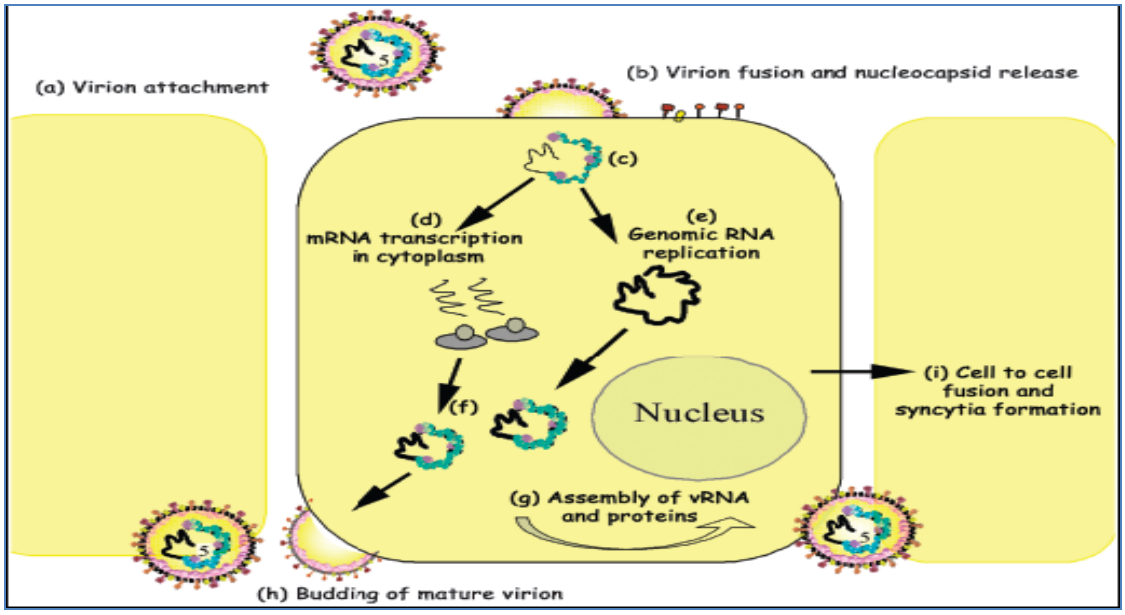



**Figure 2. RSV life cycle.** The respiratory syncytial virus life cycle, beginning with virion attachment and fusion and ending with mature virion exit is shown, with all viral stages indicated (Ghildyal et al., 2006).

The observations that support this hypothesis are that the M protein is known to bind RNA in a sequence non-specific manner (Rodriguez et al., 2004). A decrease in host cell transcription has been observed during RSV infection and the M protein was shown to localize to the nucleus early, approximately six hours after entry (Ghildyal et al., 2003).

The M protein also plays an important role in virus assembly and budding from the plasma membrane. The M protein accomplishes this through specific interactions with RSV nucleocapsids and envelope glycoproteins in the cytoplasm as well as with the host cell membrane. Assembly is coordinated by M, where the envelope glycoproteins are translocated to the cell membrane, forming patches that exclude cellular membrane proteins. The cytoplasmic tails of the viral proteins interact with M, which forms a layer under the plasma membrane. The preformed nucleocapsids are recruited to these sites

through interaction with M, facilitating final assembly and budding (Ghildyal et al., 2006).

**Protein-Protein Interactions and Predicted Motifs of Matrix Protein**

Protein-protein interactions exist in virtually all biological processes. Any major process in biology and virology involve protein complexes. An understanding of protein-protein interactions helps elucidate important biological processes and functions.

Interactions of M with cellular proteins are not well defined and appear to vary between different paramyxoviruses species. The present understanding of protein-protein interactions of the RSV Matrix protein concentrate primarily on viral protein-protein interactions between the RSV Matrix proteins and subsequent RSV virus proteins. Ghildyal et al have demonstrated that the Matrix protein associates with the RSV G protein first at the Golgi, followed by RSV F at the plasma membrane (Ghildyal et al., 2005b). It is possible that M interacts independently with both G and F, where either protein is sufficient for virus assembly, but this has only been confirmed by limited supporting data. In addition, Li et al showed that the association of the M protein with viral nucleocapsids is mediated by the M2-1 protein (Li et al., 2008). Ghildyal et al. have also demonstrated that the nuclear import of the M protein is mediated by importin Beta1 independently of importin alpha and the nuclear export of the M protein is mediated by a Crm-1 nuclear export mechanism (Ghildyal et al., 2009; Ghildyal et al., 2005a). Finally, Evans et al. have also showed that the Matrix protein interacts with the NS1 protein, however, its functional significance is unknown (Evans et al., 1996). These interactions

are significant in helping elucidate the full function of the Matrix protein during natural infection.

RSV M has critical conserved motifs that help elucidate some of its unique functions and allow comparison to other *Paramyxovirdae* Matrix proteins. These include a common region of low hydrophobicity at the N-terminus followed by a gradual increase in hydropathy towards the C-terminus, which includes a highly hydrophobic region. RSV M contains a predicted Zinc-finger domain, putative residues approximating nuclear localization signals (NLS) and nuclear export signals (NES). The M protein also includes many predicted phosphorylation sites and has shown to be phosphorylated in infected cells (Peeples, 1991). It is not known whether phosphorylation regulates its function but it has been suggested that phosphorylated and non-phosphorylated forms of M may have different specific interactions with the plasma membrane and viral proteins. Alternatively, M may be de-phosphorylated during viral maturation, perhaps in order to drive the budding of new virions.

To date, the research being done on the M protein has focused on trying to describe the downstream implications of these interactions as they may relate to viral transcription, assembly or budding of RSV virions.

**Adaptor Protein Complexes 1 and 3**

There exists four heterotetrameric adaptor protein complexes, AP-1, AP-2, AP-3, and AP-4, which act as scaffolds bringing together membrane lipids, sorting signals present in the cytosolic domains of membrane proteins, components of vesicle fusion machinery

and additional components of vesicle formation apparatus (Newell-Litwa et al., 2007). The function of these adaptors is linked to the donor organelles in which they perform their function. AP-1 generates vesicles from the trans-Golgi network that transports cargoes bound for late endosome and lysosome compartments whereas AP-1 adaptors found in early endosomes generates vesicles routed to the cell surface or back to the Golgi complex. The other adaptor protein of interest in this study is AP-3 which is present in endosomes as well but delivers proteins to late endosomes and lysosomes. The adaptor protein complexes contain four distinct structures consisting of two large subunits (λ and β-1, in AP-1 and δ and β-3, in AP-3) together making up the hinge and ear structures, a medium-sized μ subunit and a small σ subunit that together make up the trunk or core of the heterotetramer (see figure 3) (Robinson and Bonifacino, 2001).

The mechanisms that control the recruitment of AP-3 to membranes and AP-3 dependent membrane protein sorting include GTPases, kinases, intermediate filament proteins, accessory proteins, and clathrin. One mechanism that is used to study interactions of the adaptor-protein complexes is to treat cells with Brefeldin-A which interferes with GTPase's function in recruitment and release of the adaptor proteins from the membrane (Newell-Litwa et al., 2007). Through disrupting the recruitment and release of the adaptor proteins from the membrane, researchers are able to study the downstream effects in cellular function or pathogenesis from the inability of AP-3 mediated interactions to take place.

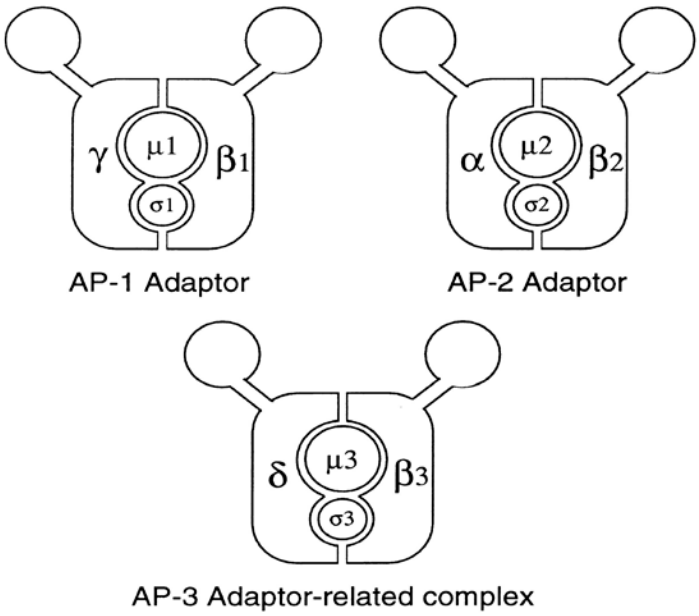



**Figure 3. Adaptor protein complexes.** The adaptor protein complexes are made up of a core region consisting of the μ and σ subunits, and a hinge region made up by the β and an adaptor-specific (γ, α,or δ) adaptin subunits. (Simpson et al., 1997).

**Function of μ Subunit in Protein-Protein Interactions with Other Viruses**

The μ subunit has been implicated in cargo selection of proteins containing three distinct sorting signals in the cytoplasmic tails of certain transmembrane proteins for selection into clathrin-coated vesicles. These sorting signals include NPXY, YXXL and di-leucine. Of these, the best characterized is the YXXL, as this sequence binds to both the AP-1 μ and AP-3 μ subunits (Robinson and Bonifacino, 2001). Proteins containing NPXY, YXXL and di-leucine sorting signals can have very different steady state distributions in the cell and can be localized at the plasma membrane, recycling endosomes, late endosomes, lysosomes and the trans-Golgi network (TGN), as well as at more specialized organelles such as melanosomes and synaptic vesicles. These sorting signals not only act as internalization signals when the proteins are at the plasma membrane, they also help to

determine the proteins' trafficking itineraries and steady state distributions inside the cell (Bonifacino and Dell'Angelica, 1999).

Both AP-1 and AP-3 μ subunits have been shown in interactions with human viral proteins. It has been shown that the clathrin-adaptor complex AP-1 binds Human Immunodeficiency Virus (HIV) 1 and Murine Leukemia Virus Gag and facilitates their budding (Camus et al., 2007). It has also been demonstrated that the AP-3 adaptor complex directs the intracellular trafficking of HIV-1 Gag and is required for efficient transport of Vesicular Stomatitis Virus-G from the TGN to the cell-surface(Coleman et al., 2005) (Dong et al., 2005; Nishimura et al., 2002). Furthermore, the HIV-1 Nef protein has been shown to interact via di-leucine based sorting motifs with the adaptor protein complex mu subunits of AP-1 and AP-3 (Coleman et al., 2005; Craig et al., 2000). With every novel interaction that is determined between viral proteins and an adaptor complex protein, more light is shed on the many functions of these intricate trafficking complexes' role during viral infection.

**Preliminary Data and Research Goals**

Some of the cellular proteins required for trafficking of the M protein have been discovered but many others are still unknown (Ghildyal et al., 2005a). Our hypothesis is that interactions between the Matrix protein and cellular proteins are necessary for localization of the M protein and its functions in virus assembly and budding. Discovery of the cellular proteins that interact with the M protein, elucidation of their functional significance and determination of interaction domains will increase the general

understanding of this integral viral protein. It may also provide information necessary for the development of attenuated viruses for vaccines and development of antiviral drugs that can target the conserved nature of the M protein.

In order to discover novel interactions between the Matrix protein and host proteins, a yeast-two hybrid analysis was performed resulting in six potential cellular interactions with the RSV Matrix protein (Figure 4).

| Gene |
| --- |
| **H. sapiens euk. translation elongation factor 1 alpha 1 (EEF1α1)** |
| **H. sapiens ribosomal protein S20 (RPS20)** |
| **H. sapiens ubiquitin-conjugating enzyme E2I (UBE2I)** |
| **H. sapiens ubiquitin C (UBC)** |
| **H. sapiens phosphoglucomutase 1 (PGM1)** |
| **H. sapiens nucleic acid binding protein sub2.3 (NABP2.3)** |
| **H. sapiens adaptor-related protein complex 1, μ1 subunit (AP1μ1)** |
| **H. sapiens adaptor-related protein complex 3, μ1 subunit (AP3M1)** |

**Figure 4. Yeast-two hybrid results.** Results of BLAST search performed on novel interactions screened using pre-transformed HeLa cDNA library as prey against RSV M protein; RPS20 and NABP2.3 are common false positives and were not explored further.

Based on the knowledge of significant roles played by these cellular protein interactions with the Matrix protein of other viruses, these potential interactions deserve further investigation. To date, our lab has researched the interaction between the RSV M protein

and ubiquitin C (UBC) and it was determined that ubiquitination was critical in RSV pathogenesis. My thesis proposal was to continue validating the yeast-two hybrid results by characterizing the interactions between the cellular adaptor proteins AP1µ1 and AP3µ1 and the RSV Matrix protein in a mammalian system. The reason behind characterizing these interactions in a mammalian system is because proteins encoded by mammalian cDNAs are more likely to be in their native conformation; therefore, post-translational modifications and experimental results are more likely to represent biologically significant interactions.

Based upon previous data with other human viruses interacting with the adaptor protein 1 and 3 µ subunits via a YXXL binding motif, a sequence alignment was performed using Clustal W from the Oregon State University Center for Genome Research and Biocomputing (CGRB) bioinformatics site comparing the various animal RSV Matrix proteins to see if a homologous binding motif was seen. Based upon figure 5, a clearly defined YXXL homologous region was established, providing further evidence that an interaction with the adaptor protein µ subunits is critical for RSV to maintain M's function in pathogenesis across various animal viruses.

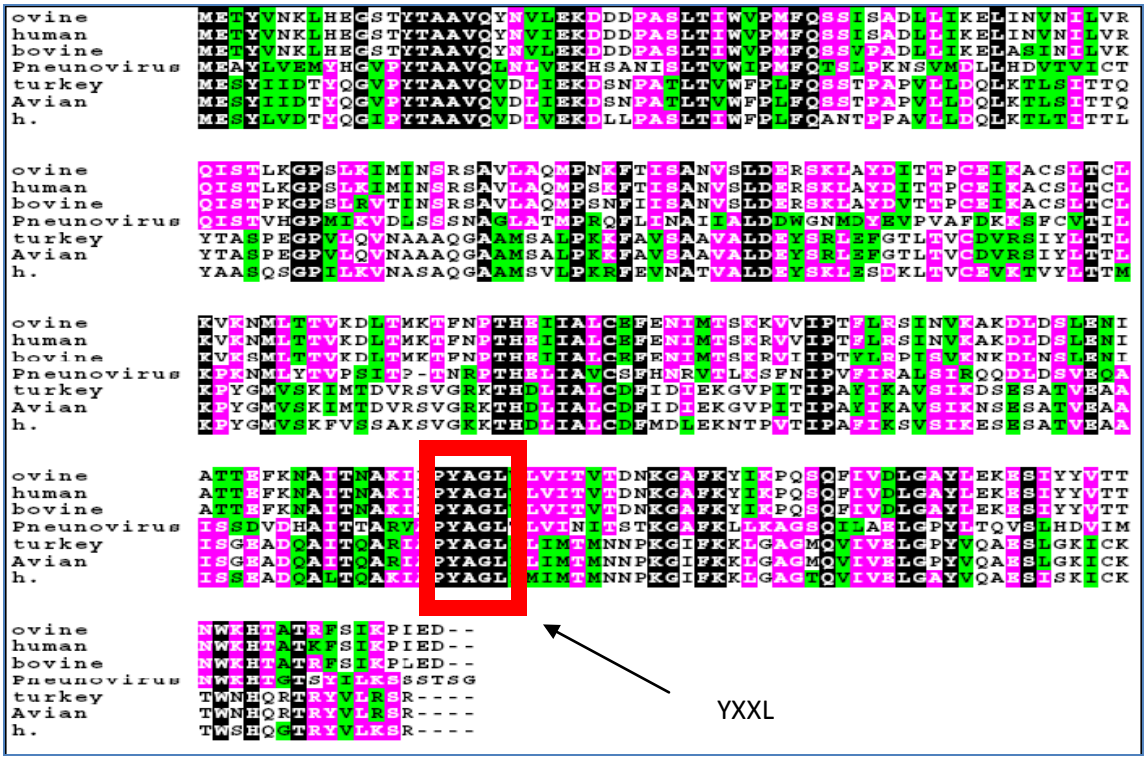



**Figure 5. RSV Matrix sequence alignment.** ClustalW sequence alignment of Matrix protein comparing various animal respiratory syncytial virus strains**.**

To characterize the interactions between the adaptor proteins 1 and 3 µ subunits and the RSV M protein, I used five common molecular biology methods. The first procedure performed was to determine if the RSV Matrix protein colocalized with the adaptor proteins of interest in a virally-infected cell and via plasmid transfection by CLSM analysis. The second method was to confirm the interaction through a mammalian-two hybrid system. The third protocol was to determine if the interactions could be detected via co-immunoprecipitation (CIP) or via pull-down assay. Fourth, site-directed mutagenesis of the vector pEPI DESTC RSVM-GFP C1 (a kind gift from Dr. Reena Ghildyal of Monash University, Australia (Ghildyal et al., 2005a)) was performed to mutate the wild-type YXXL motif of RSV M to AXXL. Subsequent co-localization studies were performed to see if this mutation resulted in a quantifiable alteration in

colocalization from the wild-type interaction seen via the plasmid transfection colocalization assay. Lastly, I determined if there was any subsequent up or down regulation in the amount of the adaptor proteins of interest as a result of the presence of the Matrix protein at various time points in RSV infected cells.

## Materials and Methods

**Yeast-Two-Hybrid.** Yeast-two-hybrid was performed using Clontech's Matchmaker 2 system with a pre-transformed HeLa cDNA library as prey and the RSV Matrix protein as bait. Several novel interactions were detected using dropout medium lacking leucine, tryptophan, and histidine in addition to beta-galactosidase assays. Plasmids from positive interactions were isolated using the E.Z.N.A yeast plasmid kit (Omega) then sequenced at the Center for Genome Research and Biocomputing (CGRB) core facility at Oregon State University. Gene identity was determined by performing a BLAST search of the insert sequence.

**Antibodies.** The primary specific antibodies that were used are the following (see Results section for specific dilutions used): monoclonal mouse anti-Matrix (a kind gift from Erling Norrby and Mariethe Ehnlund, Karolinska Institute, Sweden), polyclonal goat anti-AP3µ1 (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal rabbit anti-AP1µ1b (Proteintech, Chicago, IL), rabbit anti-δ adaptin (SA4) (a kind gift from Andrew Peden, Cambridge University, UK), rabbit anti-GAPDH, mouse anti-GAPDH (a kind gift from Kathy Magnusson, Oregon State University).

The secondary specific antibodies that were used are the following (see results for specific dilutions used): Donkey anti-mouse Alexa-Fluor 546 (Molecular Probes, Eugene, OR), chicken anti-mouse Alexa-Fluor 546 (Molecular Probes), goat anti-rabbit Alexa-Fluor 488 (Molecular Probes), rabbit anti-goat Alexa-Fluor 488 (Molecular Probes), donkey anti-rabbit IR Dye 800 (Rockland, Gilbertsville, PA), donkey anti-rabbit IR Dye

700 (Rockland), donkey anti-goat IR Dye 800 (Rockland), donkey anti-mouse IR Dye 800 (Rockland), donkey anti-mouse IR Dye 700 (Rockland).

**Cell Culture and Transfection**. HEp-2 cells grown at 37°C and 5% $CO^2$ in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 100U penicillin, 100µg/mL streptomycin, 2.5µg/mL amphotericin B and 10% fetal bovine serum (P/S/A/FBS) were transfected using Lipofectamine 2000 (Invitrogen) according to plasmid optimization (described specifically for each method) and manufacturer's protocol .

**Mammalian-Two Hybrid Assay.** Mammalian-two hybrid was performed using Mammalian Matchmaker system (Clontech, Mountain View, CA). Primers were designed for the amplification of the Matrix protein as a C-terminal fusion protein from the vector pCDNA 3.1-RSV Codon-Optimized Matrix (a kind gift from Dr. James Crowe, Vanderbilt Medical School, Nashville,TN) in-frame with proper restriction sites (BamHI/EcoRI) for cloning into supplied pVP 16 plasmid. Primers with gene appropriate restriction sites (BamH1/EcoR1) were used to amplify the cellular adaptor protein 1 µ1 subunit (AP1µ1) and cellular adaptor protein 3 µ1 subunit (AP3µ1) from pCDNA4c His Max vectors (Invitrogen, Carlsbad, CA) to be cloned into a supplied pM vector containing a GAL4 DNA binding domain. Competent DH5α E. coli were then transformed and plated on LB agar with 100ug/mL ampicillin. Colonies were then screened for correct inserts by using gene specific primers to perform colony PCR of lysed bacteria. Positive colonies were grown overnight in 5mL LB broth with 100ug/mL ampicillin followed by plasmid purification using the PureLink quick plasmid miniprep

kit (Invitrogen). Vectors were sequenced to confirm proper insertion at Oregon State University's Center for Genome Research and Biocomputing core facility.

Further work is in progress. The AP1µ1-pM, AP3µ1-pM, Codon-Optimized RSV Matrix-pVP 16, and a PG5 CAT reporter gene plasmid will be concurrently transfected into a 6-well plate of HEp-2 cells using Lipofectamine 2000 reagent (Invitrogen), as per the manufacturer's recommendation, in triplicate. An ELISA assay will then be performed to determine the amount of CAT reporter activity by using the CAT ELISA Kit (Roche) as per the manufacturer's recommendations. The ELISA plate will be read using an Ultramark Microplate Reader.

**Colocalization.** Subconfluent (80%) HEp2 cell monolayers grown on glass coverslips were infected with RSV or transfected with various plasmid cDNAs and cultured for the indicated times after infection or transfection. Cells were washed with ice-cold PBS and fixed with 4% paraformaldehyde for 10 min at room temperature, followed by the permeabilization of membranes with 0.2% Triton X-100 for 5 min. Fixed cells were washed thoroughly in PBS and incubated for one hour in bovine serum albumin (BSA)-PBS (1% BSA in PBS) as a preliminary blocking step. Subsequently, fixed cells were incubated for one hour in primary, specific antibody (or a mix of antibodies) diluted in Rockland IR blocking buffer. Fixed cells were extensively washed with a Tween-20 containing wash buffer and bound antibodies were detected with species-specific fluorochrome-conjugated secondary antibodies via one hour incubation in dark. Coverslips were mounted in fluorescent mounting medium containing DAPI (Vectashield, Vector Laboratories, Burlingame, CA) and analyzed by confocal laser

scanning microscopy (CLSM). Pinhole diameter was 1 um for all analyses. Multi-track configuration was performed on CGRB Zeiss 540 Meta Confocal Microscope. Images of more than 20 cells were analyzed for each sample. The vector pEPI DESTC RSVM-GFP C1 was used in transfection mediated colocalization and subsequent site-directed mutagenesis assays and was a kind gift from Dr. Reena Ghildyal of Monash University, Australia.

**Co-immunoprecipitation.** Subconfluent (80%) HEp2 cell monolayers grown in a 6 well plate were either infected or mock-infected with RSV at an MOI of 3. Post 24 hours infection, 2 ml ice cold MPER (Pierce, Rockford, IL) buffer was added to cell monolayer and incubated at 4° C for 10 minutes. Cells were scraped and cell culture plate was washed with additional 1.0 ml ice cold MPER buffer and combined with original extract. Cellular debris was pelleted by centrifugation at 10,000xg for 10 minutes at 4° C. Supernatant was transferred to new tube and 1.0 µg of the appropriate control IgG (normal mouse IgG for monoclonal mouse anti-RSV M antibody analysis), was added together with 20µl of resuspended volume of Protein A/G PLUS-Agarose (Santa Cruz Biotechnologies) and incubated 4° C overnight. Beads were pelleted by centrifugation at 2,500 rpm (approximately 1,000xg) for 5 minutes at 4° C. 1 ml of the above cell lysate, containing approximately 100–500µg total cellular protein, was transferred to a fresh tube. 2µg of monoclonal mouse anti-RSV M or polyclonal goat anti-AP3µ1 antibody was added and incubated overnight at 4° C. 20µl of resuspended volume of Protein A/G PLUS-Agarose was added and then incubated at 4° C on rotating device for overnight. Immunoprecipitates were collected by centrifugation at 2,500 rpm for 5 minutes at 4° C.

Supernatant was then discarded and pellet was washed 4 times with 1.0 ml PBS, each time repeating centrifugation step above. After final wash, supernatant was aspirated and discarded and pellet was resuspended in 40µl of 1x LDS electrophoresis sample buffer. Samples were boiled for 2–3 minutes (70° C) and 20µl aliquots were analyzed by SDS-PAGE Western Blot. Blots were blocked then probed with specific primary antibodies, washed, and probed with IR labeled secondary antibodies and analyzed on LICOR imager.

**Site-Directed Mutagenesis Plasmid Constructs.** Mutation of the YSGL motif of RSV-Matrix protein to ASGL by PCR ligation protocol. Primers used to design these plasmid constructs are as follows:

RSVM-Y197A forward: TCATCCCTGCCTCAGGATTACTATTAGTCATCACA

RSVM-Y197A reverse: ATCCTGAGGCAGGGATGATTTTTGCATTTGTGATA

pEPI DESTC RSVM-GFP C1 BspE1 forward: TACAAGTCCGGACTCAGATATC

pEPI DESTC RSVM-GFP C1 SacI reverse: TTTCTGAGCTCTACTCTATTTTATCAG

Primer pairs RSVM-Y197A forward, pEPI DESTC RSVM-GFP C1 SacI reverse, and RSVM-Y197A reverse, pEPI DESTC RSVM-GFP C1 BspE1 forward were used to mutate respective regions using Pfx polymerase kit (Invitrogen) and pEPI DESTC RSVM-GFP C1 as template DNA to make initial overlapping mutation fragments. The PCR ligation step used the pEPI DESTC RSVM-GFP C1 BspE1 forward, pEPI DESTC RSVM-GFP C1 SacI reverse primer pairs, PCR Supermix with Taq (Invitrogen), and the gel purified fragments from the previous step to make full length cDNA that was then restriction digested and ligated into original vector and screened as described previously.

**Fluorescence Intensity Analysis.** 6-well plates were seeded with approximately 1.0 x $10^6$ HEp2 cells, mock-infected or infected next day at an MOI of 5, then proteins were extracted using MPER (Pierce) with Protease Arrest and scraping from triplicate wells at respective times post-infection. Cell lysates plus NuPAGE Reducing Agent (Invitrogen)were heated at 70° C for 10 minutes and equal amounts of protein were ran out on 4-12% Bis-Tris gels (Invitrogen) followed by transfer to nitrocellulose. Blots were blocked in Rockland IR blocking buffer overnight and then probed with goat anti-AP3µ1, rabbit anti-δ adaptin, mouse or rabbit anti-GAPDH for one hour. Washed extensively in Tween 20 containing wash buffer, then blotted with species-specific IR labeled antibodies for an hour and then washed extensively. Blots were imaged on Licor IR Imager, fluorescent intensity analysis was performed using Licor imaging program and subsequent data analysis was performed by comparing infected versus uninfected triplicate averages of the integral intensity ratio between GAPDH loading control and AP3µ1 integral intensities via unpaired two tail t-test.

## Results

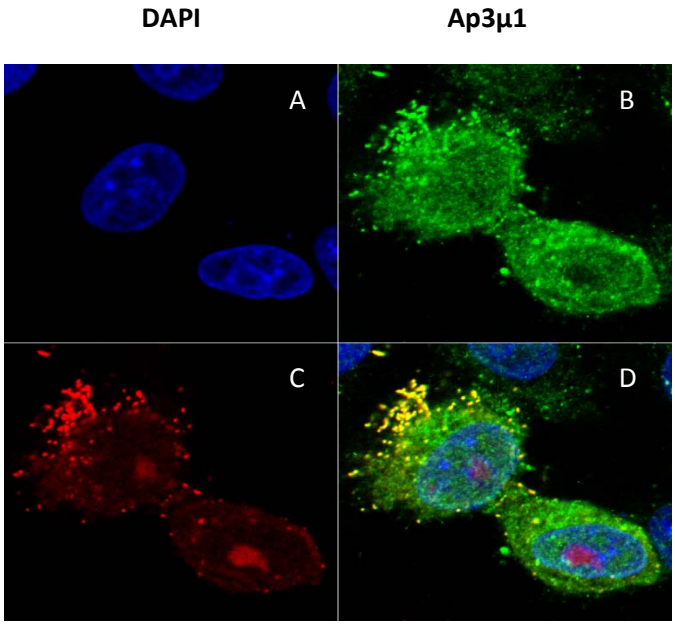
### RSV M protein Associates with AP3µ1 Protein in RSV Infected Cells.

To investigate the association of the M protein with the adaptor protein complex 3 in RSV-infected cells, fixed cells were probed with various dual-antibody concentrations and examined by Confocal Laser Scanning Microscopy (CLSM). Based upon previously shown evidence, the Matrix protein is known to localize to the nucleus predominantly at 6 hours post-infection through an Importin $\beta_1$ mechanism (Ghildyal et al., 2003; Ghildyal et al., 2005a). At subsequent time points of 12 hours and 18 hours, the Matrix protein has been demonstrated to export from the nucleus through a Crm-1 mediated nuclear export mechanism and is seen diffusely throughout the cytoplasm specifically associating with the host cell membrane late in infection (Ghildyal et al., 2009; Marty et al., 2004). At 12 and 18 hours post infection, it has also been previously noted and confirmed in this study, that there is the highest concentration of M protein. Thus, this study choose to perform CLSM analysis of the adaptor protein 1 and 3 µ subunits and Matrix protein at intervals of 6, 12, 18, and 24 hours to determine the time-point of the presumed associations and also as a control to make sure that there was limited to no association occurring in the nucleus based upon the adaptor proteins having their significant functions in cytoplasmic defined regions.

The results of the CLSM assay, seen in figures 6, 7, 8 and 9, showed no significant colocalization between AP3µ1 and the M protein at 6 hours as hypothesized and significant colocalization occurring at 12, 18 and 24 hour post RSV infection. The AP3µ1 displayed a punctuate pattern throughout the cytoplasm with extension to the cell margin.

This fluorescent pattern for the AP-3 adaptor complex is consistent with previously published data (Craig et al., 2000; Dell'Angelica et al., 1997; Robinson, 1987; Robinson and Pearse, 1986; Simpson et al., 1997). While the RSV M protein was seen localized to the nucleus early in infection, then later in infection, it was localized to cytoplasmic inclusions and seen in association with the cytoplasmic side of the cell membrane, consistent with previously published data.

**Figure 6. Colocalization of RSV M and AP3µ1, post 6 hours.**




**Figures 6,7,8,9.** The M protein is localized exclusively in the nucleus of RSV infected cells at 6 hours, and is seen associating with the AP3µ1 protein in distinct cytoplasmic regions at 12, 18, and 24 hours post infection. RSV-infected HEp2 cells were fixed 6, 12, 18, or 24 h after infection and were double stained with various antibody combinations, followed by CLSM analysis. The antibodies used are as indicated: Goat anti-AP3µ1 (1:50), Mouse anti-Matrix (1:100), Rabbit anti-Goat Alexa-Fluor 488 (1:200), Chicken anti-Mouse Alexa-Fluor 546 (1:200) and were incubated for 60 min; cells were washed with Tween 20 wash solution and incubated for 60 min with secondary antibodies. A, is the 405 nm output corresponding to the blue DAPI stained nucleus; B, is the 488nm output corresponding to the green adaptor protein 3 µ subunit; C, is the 546 output corresponding to the red RSV M protein; D, is the computer-generated merged image of all three outputs, with yellow coloration indicating colocalization. Negative control (data not shown) was performed and showed no fluorescence.

**Figure 7. Colocalization of RSV M and AP3µ1, post 12 hours.**

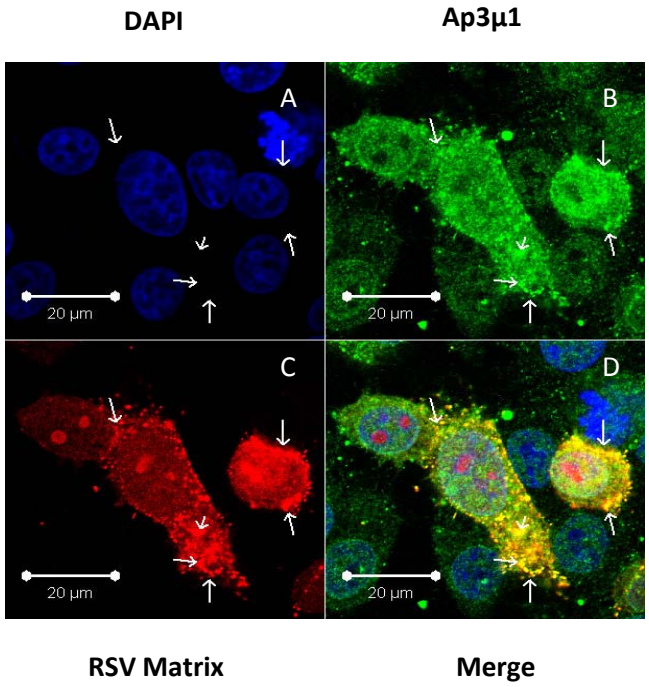



**Figure 8. Colocalization of RSV M and AP3µ1, post 18 hours.**

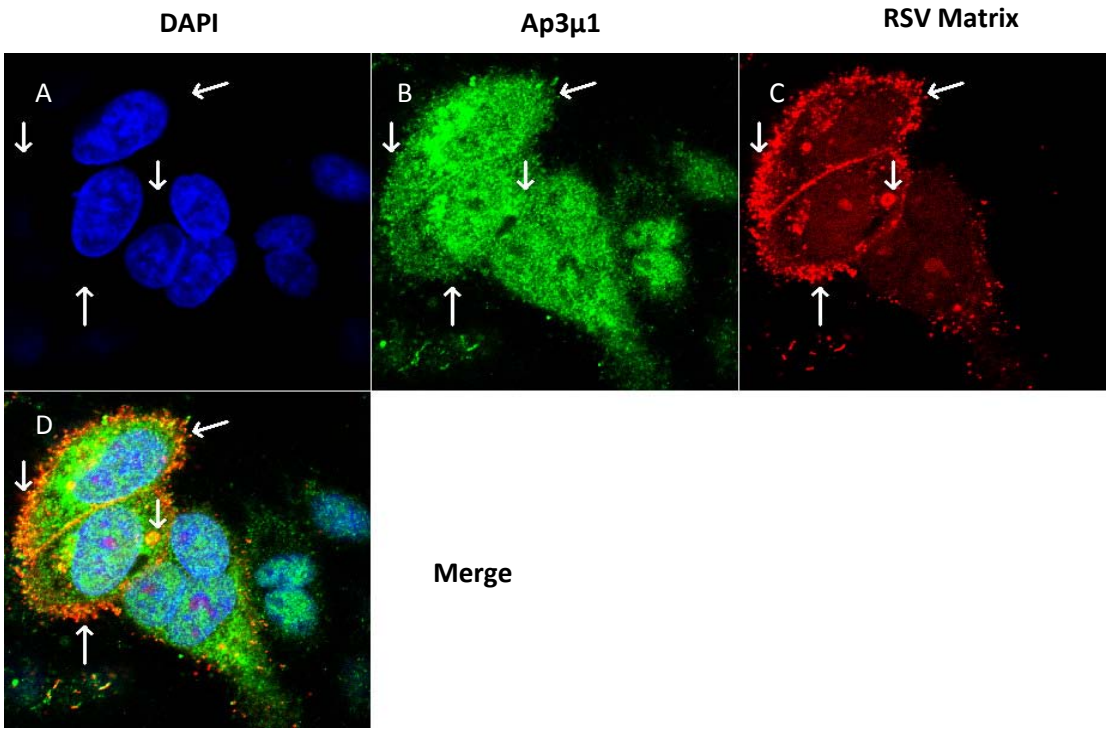
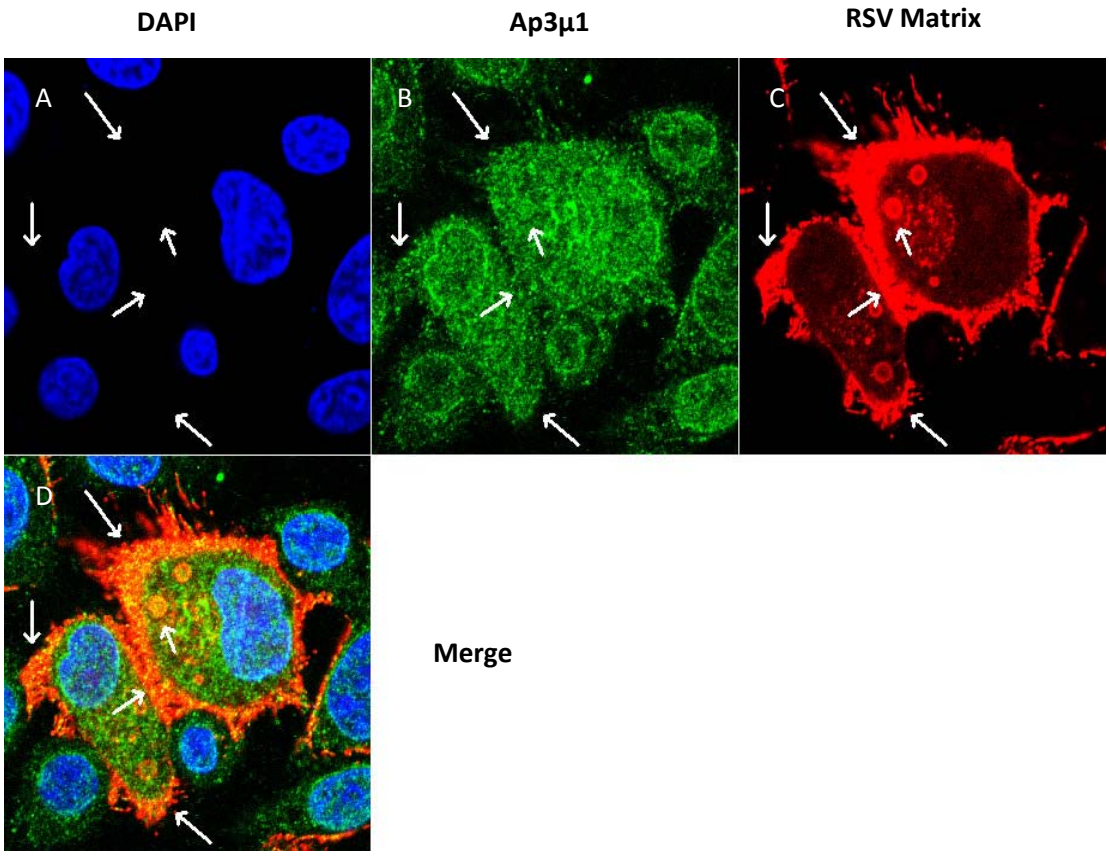


**Figure 9. Colocalization of RSV M and AP3µ1, post 24 hours.**




**RSV Matrix Protein Localizes with AP3µ1 in Transfected Cells**

To determine if the M protein colocalized with the adaptor protein complex 3 in the absence of a natural RSV infection, RSV M was expressed in HEp2 cells by transfection of a C-terminal GFP fusion plasmid, pEPI-DESTC RSVM-GFP C1 and the adaptor protein δ subunit was detected by indirect immunofluorescence (Figure 10). The colocalization occurred in cytoplamsic defined regions as seen during RSV infection using CLSM analysis (Figures 6, 7, 8, and 9). This assay allows for future site-directed mutagenesis experiments for determining the binding motif that regulates the AP3µ1-RSVM interaction.

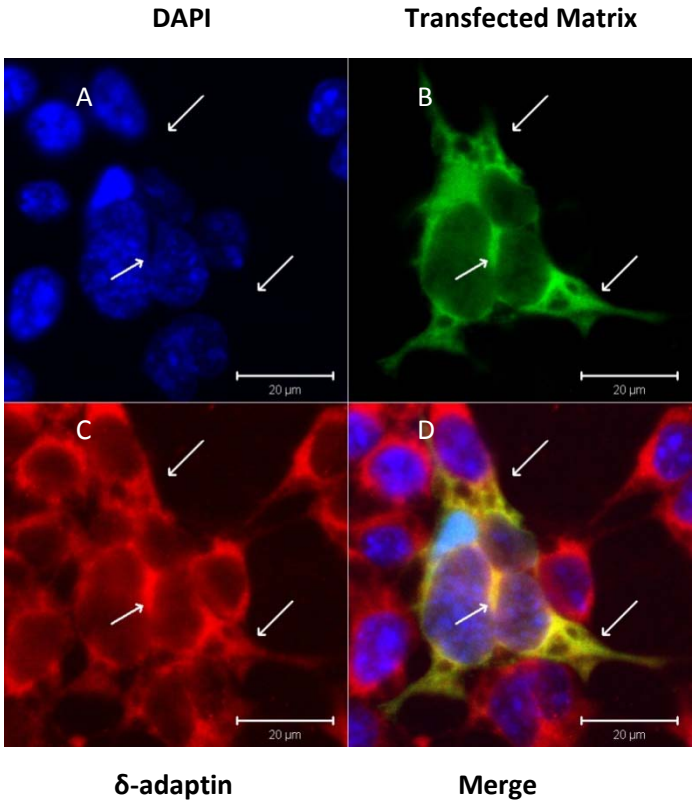


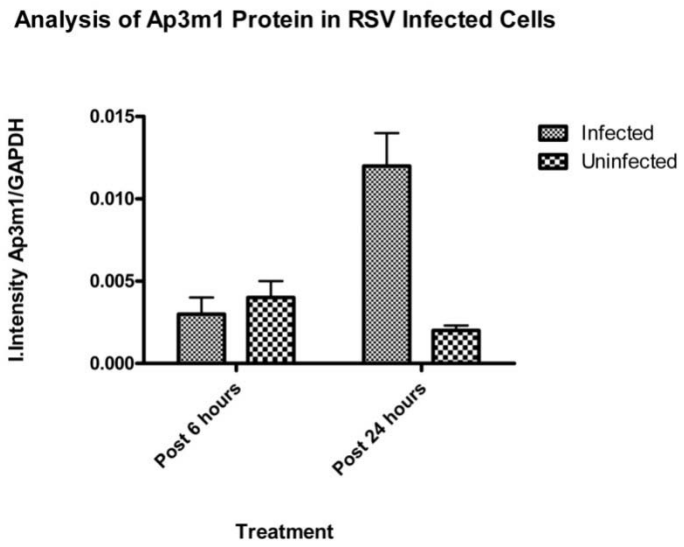

**Figure 10. Colocalization of RSV M and δ-adaptin via transfection of HEp2 cells, post 24 hours.** The M protein is seen associating with the AP3µ1 protein in distinct cytoplasmic regions at 24 hours post transfection. HEp2 cells were fixed 24 h after infection and the δ-adaptin subunit was labeled by mouse anti-δ-adaptin antibody (1:10000) for one hour. Cells were then washed with Tween-20 wash solution and incubated for 60 min with donkey anti-mouse Alex-Fluor 546 (1:200) antibody; followed by extensive washing and CLSM analysis. A, is the 405 nm output corresponding to the blue DAPI stained nucleus; B, is the 488nm output corresponding to the green adaptor protein 3 δ subunit; C, is the 546 output corresponding to the red RSV M protein; D, is the computer-generated merged image of all three outputs, with yellow coloration indicating colocalization. Negative control (data not shown) was performed and showed no fluorescence.

**AP3µ1 is Specifically Up-Regulated Late in RSV Infection.**

One of the interesting qualitative observations that was noticed throughout CLSM analysis was during late infection there seemed to be a significant difference in the amount of AP3µ1 seen in infected cells versus uninfected cells. To understand this, HEp2

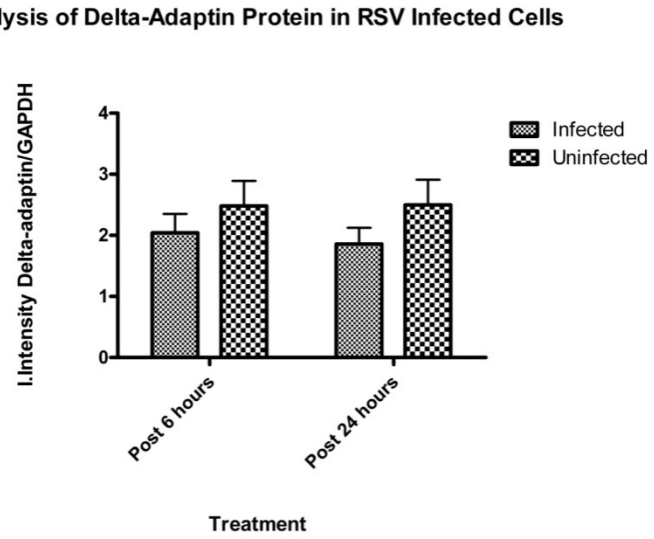
cells were either infected or mock-infected and cell lysates were extracted at 6 and 24 hour post infection time points to be analyzed by Western Blot (Figure 11). Equivalent amounts of cell lysate were loaded and after subsequent transfer of SDS-PAGE gels to nitrocellulose followed by probing with primary and secondary antibodies, as described in figure 10; fluorescent intensities were collected on Licor Imager. Statistical analysis showed that at 6 hours there was no statistical difference between the amount of AP3µ1 in infected versus uninfected cells (p-value=0.5032), while at 24 hours post infection there existed a statistically significant, greater than two fold, increase in the amount of AP3µ1 in infected cells versus uninfected cells (p-value=.0094). To determine if the whole AP-3 adaptor complex was up-regulated, the δ-adaptin subunit was also analyzed via the same protocol as the µ subunit just described. The results of this analysis showed that there was no statistically significant difference between infected and uninfected cells at 6 (p-value=0.4415), and 24 hour post infection (p-value= .2635) (Figure 12). This data seems to suggest that the AP-3 µ subunit is preferentially up-regulated during RSV infection, possibly due to the requirement of the AP3µ1 subunit for proper function of the RSV M protein during RSV infection.

**Figure 11. Analysis of AP3µ1 subunit at 6 and 24 hour post RSV infection.**




**Figures 11,12.** AP3µ1 is up-regulated at 24 hours post-infection in infected cells versus mock infected HEp2 cells while at 6 hours there exists no statistically significant difference. The AP3-δ subunit was also analyzed and it was determined that there existed no statistical significant difference in infected cells versus mock infected cells at both 6 and 24hours post-infection. 15µg of cell lysate, extracted by MPER (Pierce) and scraping, were analyzed by 4-12% Bis-Tris SDS-PAGE gels (Invitrogen) in triplicate and transferred to nitrocellulose. Blots were blocked overnight in Rockland IR blocking buffer, then probed with the following antibodies diluted in 50% Tween-20 Wash Buffer:50% Rockland blocking buffer for one hour incubation periods with extensive wash periods between incubations: Goat anti-AP3µ1 (1:200), Mouse anti-RSV Matrix (1:1000), Rabbit anti-δ adaptin (SA4) (1:1000), Rabbit anti-GAPDH (1:10000), Donkey anti-Goat IR 800 (1:25000), Donkey anti-Rabbit IR 800 (1:25000), Donkey anti-Rabbit IR 700 (1:5000), Donkey anti-Mouse IR 700 (1:5000), Donkey anti-Mouse IR 800 (1:20000). Blots were analyzed on Licor Imager and integral intensities were measured for each band corresponding to the 41kDa GAPDH, 47 kDa AP3µ1, δ-adaptin 160 kDa bands. The statistical analysis was performed on the ratio of the integral intensity measurement of the triplicate average of the protein of interest versus the GAPDH control in infected cells versus mock-infected cells at various time points post RSV infection.

**Figure 12. Analysis of AP3δ subunit at 6 and 24hour post RSV infection.**




**AP1µ2 shows No Colocalization with the RSV M Protein during Infection**

To confirm the data obtained by the yeast-two hybrid analysis, CLSM analysis was performed on the AP1µ1b (more commonly referred to as AP1µ2) variant subunit, expressed in respiratory tissues as compared to the more ubiquitous expression of the AP1µ1a subunit. This analysis was performed at 6, 12, 18, and 24 hour time points as done in the previous analysis of AP3µ1, and at all time points there was little to no evidence of colocalization observed with the RSV Matrix protein (figure 13). The AP1µ2 protein was seen distinctly in cytoplasmic regions extending to the cell margin but was more concentrated in distinct foci at juxtanuclear regions, consistent with previously shown data (Craig et al., 2000).

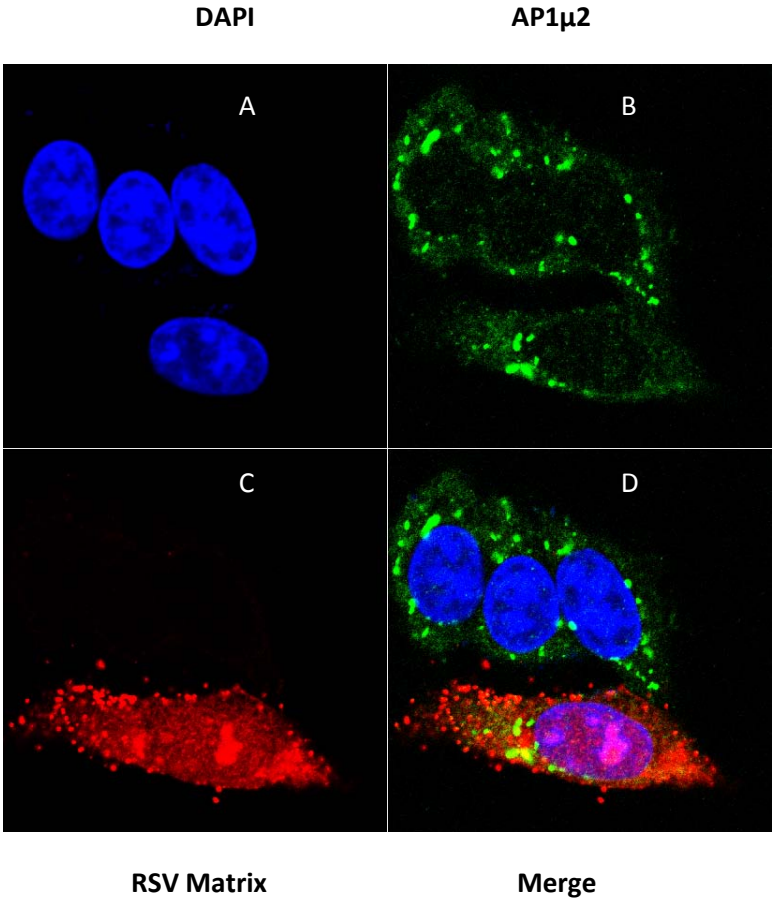



**Figure 13. AP1µ2 and RSV Matrix protein; post 12 hours.** The M protein is not seen associated with the AP1µ2 protein at 6, 12, 18, or 24 hours post infection. RSV-infected HEp2 cells were fixed 6,12,18, or 24 h after infection and were double stained with various antibody combinations, followed by CLSM analysis. The antibodies used are as indicated: Rabbit anti-AP1µ2 (1:50), Mouse anti-Matrix (1:100), Goat anti-Rabbit Alexa-Fluor 488 (1:200), Chicken anti-Mouse Alexa-Fluor 546 (1:200) and were incubated for 60 min; cells were washed with Tween-20 wash solution and incubated for 60 min with secondary antibodies. A, is the 405 nm output corresponding to the blue DAPI stained nucleus; B, is the 488nm output corresponding to the green adaptor protein 3 mu subunit; C, is the 546 output corresponding to the red RSV M protein; D, is the computer-generated merged image of all three outputs, with yellow coloration indicating colocalization. Negative control (data not shown) was performed and showed no fluorescence.

**Discussion**

In this study, we examined the basis of the RSV M association with the adaptor protein complex 3, demonstrating for the first time that the M protein colocalizes with the AP3µ subunit. Through CLSM analysis, the M protein was seen in association with AP3µ1 in specific cytoplasmic regions during natural RSV infection. Based upon CLSM assay data demonstrating colocalization near the cell margin and during later time-points in infection, the interaction presumably aids in the virus assembly and budding functions of RSV M.

The observation that the AP-3M1 subunit is up-regulated at 24 hours post infection but not at 6 hours is also further evidence that the interaction with the AP-3 complex is involved in viral assembly or budding. The finding that the AP-3δ subunit is not up-regulated is evidence that there is a requirement for cells to either maintain AP3µ1 protein from degradation or is up-regulated at a transcriptional level which is currently being explored by RT-PCR analysis. Previous studies with the AP-3 adaptor protein complex have provided evidence for the complex's role in the trafficking itineraries of proteins and their steady state distributions inside the cell. Thus, this evidence may help explain the up-regulation of the AP3µ1 subunit at 24 hours due to increasing amounts of RSV M protein during later points in RSV infection.

The paramyxovirus M proteins have been shown to play a major role in virus assembly and in recombinant paramyxoviruses where M is either mutated or deleted, colocalization of envelope glycoproteins and nucleocapsids are lost, with a consequent reduction in

virus release (Cathomen et al., 1998). For example, in studies demonstrating the interaction between the HIV-1 Nef protein and the AP-1 and AP-3 µ subunits, site-directed mutagenesis of the YXXL motif and di-leucine motif leads to reduced colocalization compared to the wild-type interaction (Craig et al., 2000). To determine if the binding motif between RSV M and AP3µ1 is indeed a YXXL or di-luecine conserved amino acid sequence, our lab is currently mutating the tyrosine residue to an alanine residue at amino acid site 197 in the pEPI-DESTC-RSVMGFP-C1 plasmid and subsequently performing CLSM analysis on HEp2 cells transfected to express the RSV–M-Y197A protein.

To further confirm the interaction between the M protein and AP-3M1 a mammalian-two hybrid (M2H) analysis is also currently underway. The M2H analysis is currently being performed as a further confirmation of the yeast-two hybrid data based on proteins encoded by mammalian cDNAs are more likely to be in their native conformation; therefore, post-translational modifications and experimental results are more likely to represent biologically significant interactions. The M2H system detects protein-protein interactions through the reconstitution of a eukaryotic transcriptional activator that is made up of two functionally and physically separable domains: a DNA binding domain that specifically binds to a promoter or other *cis*-regulatory element, and an activation domain that directs the RNA polymerase II to transcribe the gene downstream of the DNA-binding site. The tether between the two domains is the interaction between two additional proteins, RSVM and target cellular protein that are expressed as protein fusions to the activation domain and DNA-binding domain, respectively. One problem

that had to be addressed during the set-up of this experiment was the inability to express the wild-type M protein from a mammalian plasmid due to the preemptive termination of the polypeptide by a poly-A encoding signal (AAUAA) in the coding region of the RSV M mRNA. To abate this obstacle, our lab obtained a mammalian vector containing a codon-optimized RSV M protein that was subsequently cloned into the pVP16 fusion plasmid to be expressed.

Once an interaction between the proteins of interest has been established, the M2H assay is also a powerful tool for functionally analyzing that interaction using deletional or site-directed mutagenesis. Thus, this current goal of this study is to perform site-directed mutagenesis on the wild-type M protein to make a Y197A mutant codon-optimized RSM-pVP16 fusion plasmid to determine deletional effects on the YXXL motif and its interaction capabilities compared to the wild-type in the M2H assay.

The finding that M-AP3µ1 binding was not detectable using co-immunoprecipitation approaches in transfected and infected cells may be attributable to one or more factors: the interaction of M with AP3µ1 may be transient, followed by other more lasting interactions; or the conditions used during the immunoprecipitation may have led to the disruption of the interaction. To further confirm this interaction, a GST mediated pull-down assay is currently underway. The reasoning behind a pull-down assay is that it allows for a larger quantity of the protein than is typically available under endogenous expression conditions and eliminates confusing results, which could arise from

interaction of the bait with other interacting proteins present in the endogenous system that are not under study.

Although RSV is a prime candidate for early childhood immunization and antiviral drug therapy, to date, the considerable efforts to develop these prevention and treatment modalities have been unsuccessful (Li et al., 2008). The results here indicate that AP3µ1 plays a key role in M's function in either viral assembly or budding ; conceivably, the interaction between AP3µ1 at the proposed YXXL motif may represent a target for the development of antivirals to inhibit M association with nucleocapsids and thereby virus assembly, with a consequent reduction in disease severity. This interaction could also be used to develop attenuating mutations suitable for candidate vaccines. The current focus of this laboratory is to define the key sequences mediating the AP3µ1-M interaction as a prelude to the design of inhibitors of this interaction that may prove to be useful antivirals in the future.

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