The Pennsylvania State University
The Graduate School
Department of Veterinary and Biomedical Sciences

FUNCTIONS OF THE RESPIRATORYSYNCYTIAL VIRUS

SH AND P PROTEINS

A Dissertation in
Pathobiology
by
Sandra M. Fuentes

© 2010 Sandra M. Fuentes

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

August 2010
The dissertation of Sandra M. Fuentes was reviewed and approved* by the following:

Biao He
Associate Professor of Infectious Diseases
University of Georgia
Dissertation Adviser
Co-Chair of Committee

Anthony Schmitt
Assistant Professor of Molecular Immunology and Infectious Diseases
Co-Chair of Committee

Avery August
Distinguished Professor of Immunology

Pamela A. Hankey
Professor of Immunology

Michael N. Teng
Assistant Professor of Medicine
University of South Florida

Robert F. Paulson
Associate Professor of Veterinary and Biomedical Sciences
Pathobiology Program Head

*Signatures are on file in the Graduate School.
Abstract

Respiratory syncytial virus (RSV) is the leading cause of pediatric hospitalizations due to lower respiratory tract infections. Immunocompromised and elderly patients can also develop severe respiratory illness. Immunoprophylaxis with monoclonal antibodies against the RSV fusion protein is the only currently available treatment option. Although research is ongoing to develop a safe and effective vaccine against RSV, no vaccine is available yet. Understanding the way RSV uses the cell for survival and proliferation is an important step towards finding effective infection prevention methods.

Some members of the Paramyxoviridae family, which includes RSV, encode for a small hydrophobic (SH) protein of unknown function. Previous studies by our lab show that the SH protein of parainfluenza virus 5 (PIV5) inhibits apoptosis through the TNF-α pathway. The mumps virus SH protein was found to be a functional counterpart of the PIV5 SH despite the lack of sequence homology between these proteins. To understand the function of the RSV SH protein, a recombinant PIV5 containing the RSV SH protein in place of the PIV5 SH protein (PIV5ΔSH - RSV SH) was generated. Analysis showed that the RSV SH could functionally replace the PIV5 SH protein since infection of cells with this virus did not induce apoptosis. Expression of the RSV SH protein in the absence of all other viral proteins was sufficient to inhibit the TNF-α pathway that leads to NF-κB activation. An RSVΔSH mutant was generated in a different approach at understanding the role RSV SH protein during infection. The RSVΔSH virus induced apoptosis in A549 cells, a human lung epithelial cell line. Interestingly the levels of TNF-α in the media of RSVΔSH infected A549 cells were not sufficient to induce
apoptosis in this cell line, suggesting that the RSV SH has a different mechanism of inhibiting apoptosis in A549 cells.

The RSV SH is an accessory protein involved in RSV pathogenesis but not necessary for virus growth. In contrast, the RSV phosphoprotein (P) is necessary for viral RNA synthesis and a P deletion mutant is not viable. The P protein is an integral part of the polymerase complex involved in transcription and replication and it is the major phosphorylated species of the virus. The role of P protein phosphorylation for the proteins function is not completely understood. Recent studies in our lab suggest that the cellular kinase Akt is important for the RNA synthesis of non-segmented negative-sense RNA viruses. To study the role of Akt in RSV RNA synthesis, Akt activity was inhibited with small molecule inhibitors, siRNA and dominant negative constructs. Inhibition of Akt reduced RSV RNA synthesis, protein production and RSV titers. In addition, a small molecule inhibitor of Akt activation also reduced P protein phosphorylation, suggesting that P is a target of Akt. Serine 86 of P was identified as the Akt phosphorylation site in vitro. Mutation of that serine to an alanine significantly reduced P protein phosphorylation by Akt in an in vitro kinase assay. Phosphorylation at serine 86 was also detected in P protein from RSV infected cells. These results suggest that Akt inhibitors could be developed for RSV therapeutics. Identification of the site of Akt phosphorylation in P during RSV infection could lead to a new strategy in the development of an RSV vaccine.
Chapter 1: Introduction ................................................................. 1
  1.1 Respiratory syncytial virus .................................................. 2
  1.2 RSV entry ........................................................................... 3
  1.3 RSV RNA synthesis and protein production .......................... 4
  1.4 RSV assembly and budding .................................................. 7
  1.5 Epidemiology and treatment .................................................. 8
  1.6 The SH protein and apoptosis .............................................. 10
  1.7 The P protein and viral RNA synthesis ................................. 11

Chapter 2: Function of the respiratory syncytial virus small hydrophobic protein 19
  2.1 Abstract .............................................................................. 20
  2.2 Introduction ......................................................................... 21
  2.3 Materials and Methods ........................................................ 24
    2.3.1 Plasmids ........................................................................ 24
    2.3.2 Cells and viruses ............................................................ 24
    2.3.3 RT-PCR and sequencing ................................................. 27
    2.3.4 Production of A2 SH and RSV BI SH antibody ................. 27
    2.3.5 Immunoprecipitation ....................................................... 28
    2.3.6 Analysis of NF-κB activation by immunofluorescence
and ELISA ................................................................. 29

2.3.7 Dual-Luciferase assay ........................................... 30

2.3.8 Trypan blue exclusion assay. ................................. 31

2.3.9 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. ........................................... 31

2.4 Results ................................................................. 32

2.4.1 Generation of PIV5ΔSH-RSV SH viruses .................. 32

2.4.2 Analysis of viral protein production ......................... 32

2.4.3 Growth of PIV5 and RSV SH recombinant viruses ........ 33

2.4.4 Cytopathic effect (CPE) of virus infection ................ 34

2.4.5 NF-κB activation by viruses ................................. 35

2.4.6 Inhibition of TNF-α-induced apoptosis ...................... 36

2.4.7 Induction of apoptosis by RSVΔSH .......................... 37

2.5 Discussion .......................................................... 40

Chapter 3: The role of Akt phosphorylation for RSV P function .......................... 55

3.1 Abstract ............................................................... 56

3.2 Introduction .......................................................... 57

3.3 Materials and Methods ............................................. 62

3.3.1 Viruses, cells and plasmids .................................... 62

3.3.2 Fluorescence Microscopy ...................................... 63

3.3.3 Akt inhibitors, siRNA and dominant-negative mutants .... 63

3.3.4 RT-PCR ......................................................... 64
3.3.5 Luciferase assays ......................................................... 64
3.3.6 Cell viability assays ..................................................... 65
3.3.7 Immunoprecipitation and co-immunoprecipitation ............... 66
3.3.8 Purification of *Escherichia coli* expressed RSV P protein .... 68
3.3.9 *In vitro* kinase assay ................................................ 68
3.3.10 Mass spectrometry ................................................... 69

3.4 Results ........................................................................ 70

3.4.1 Inhibition of Akt reduces RSV protein expression ............... 70
3.4.2 The P protein is a target of Akt .................................... 72
3.4.3 Mapping the Akt phosphorylation site within P ................. 73
3.4.4 P protein phosphorylation in infected cells ....................... 74

3.5 Discussion .................................................................. 75

Chapter 4: Summary and Conclusions ..................................... 94

4.1 Inhibition of apoptosis by the RSV small hydrophobic protein .... 95
4.2 Role of Akt phosphorylation of P in RSV RNA synthesis ........ 97

References ........................................................................ 100
# List of Figures

## Chapter 1

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>Comparison of the <em>Pneumovirinae</em> and <em>Paramyxovirinae</em> genome composition.</td>
<td>14</td>
</tr>
<tr>
<td>1-2</td>
<td>Electron micrograph of RSV virion.</td>
<td>14</td>
</tr>
<tr>
<td>1-3</td>
<td>RSV life cycle.</td>
<td>15</td>
</tr>
<tr>
<td>1-4</td>
<td>Schematic representation of RSV RNA synthesis.</td>
<td>16</td>
</tr>
<tr>
<td>1-5</td>
<td>Extrinsic and intrinsic pathways of apoptosis.</td>
<td>17</td>
</tr>
<tr>
<td>1-6</td>
<td>Akt signaling pathway</td>
<td>18</td>
</tr>
</tbody>
</table>

## Chapter 2

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Generation of PIV5ΔSH-RSV SH.</td>
<td>45</td>
</tr>
<tr>
<td>2-2</td>
<td>Viral protein production in PIV5 and PIV5ΔSH-RSV SH infected cells.</td>
<td>46</td>
</tr>
<tr>
<td>2-3</td>
<td>Growth kinetics and plaque morphology of PIV5 and PIV5ΔSH-RSV SH.</td>
<td>47</td>
</tr>
<tr>
<td>2-4</td>
<td>The PIV5ΔSH-RSV SH viruses inhibit apoptosis induced by PIV5ΔSH.</td>
<td>48</td>
</tr>
<tr>
<td>2-5</td>
<td>Activation of NF-κB by recombinant PIV5.</td>
<td>49</td>
</tr>
<tr>
<td>2-6</td>
<td>The RSV SH protein inhibits TNF-α induced NF-κB activation.</td>
<td>50</td>
</tr>
<tr>
<td>2-7</td>
<td>RSVΔSH virus causes apoptosis in L929 cells.</td>
<td>51</td>
</tr>
<tr>
<td>2-8</td>
<td>RSVΔSH accelerated apoptosis in A549 cells.</td>
<td>52</td>
</tr>
<tr>
<td>2-9</td>
<td>RSVΔSH apoptosis in L929 cells is not mediated through TNF-α.</td>
<td>53</td>
</tr>
</tbody>
</table>

## Chapter 3

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-1</td>
<td>Inhibition of RSV by Akt small molecule inhibitors.</td>
<td>81</td>
</tr>
<tr>
<td>3-2</td>
<td>Inhibition of RSV by Akt siRNA and Akt dominant negative.</td>
<td>84</td>
</tr>
<tr>
<td>3-3</td>
<td>Reduction of Akt phosphorylation by AktIV.</td>
<td>86</td>
</tr>
<tr>
<td>3-4</td>
<td>Interaction between P and Akt1.</td>
<td>87</td>
</tr>
<tr>
<td>3-5</td>
<td>Purification of <em>E. coli</em> expressed RSV P protein and <em>in vitro</em> kinase assay.</td>
<td>88</td>
</tr>
<tr>
<td>3-6</td>
<td>Identification of the Akt phosphorylation site within P.</td>
<td>89</td>
</tr>
<tr>
<td>3-7</td>
<td>Identification of phosphosites in P from infected cells.</td>
<td>91</td>
</tr>
</tbody>
</table>
Chapter 4
Figure 4-1: Working model for the role of Akt in RSV RNA synthesis ............... 99
Acknowledgments

First and foremost I would like to thank my advisor Dr. Biao He for letting me be a part of his lab. He’s believed in my work and has encouraged me to do by best. I thank him for being my mentor and an example of good leadership. I have learned that not much can be accomplished without passion and commitment to your work.

I would also like to thank Dr. Avery August, Dr. Pamela Hankey, Dr. Anthony Schmitt and Dr. Michael Teng for being part of my committee and for their guidance. Special thanks to Dr. Michael Teng and Kim Teng for providing materials and advice for my experiments whenever I needed it. I appreciate all the help. I am also grateful to all past and present members of the He lab: Yuan Lin, Jie Xu, Matt Wolfgang, Jui Patel, Dr. Zhuo Li, Dr. Khalid Timani, Dr. Minghao Sun, Priya Luthra, Dengyun Sun, Pei Xu, Laurie Shuman, Rebecca Wilson, Ping Wang, Jason Aligo and Frank Horvath, for their help with my work and very helpful discussions. It was great to work in such an inviting and positive environment.

Finally, I would like to thank all my friends and family. Special thanks to my parents, Carmen López and Francisco Fuentes, my sister, Laura and my brothers, Paco and Manuel for not getting tired of listening to me complain about experiments that did not work. I have always known I have their love and support and that’s made the difference.
Chapter 1: Introduction
1.1 Respiratory Syncytial Virus

RSV is a member of the Paramyxoviridae family of negative-sense, single stranded, nonsegmented RNA viruses. There are two subfamilies of Paramyxoviridae: the Paramyxovirinae and the Pneumovirinae. While they share 6 common genes, the N, P, L, M, F and G/HN/H, the members of the Pneumovirinae also contain NS1, NS2 and M2 genes (Figure 1-1). In addition some members of the Pneumovirinae and the Paramyxovirinae contain an SH gene. While the Paramyxovirinae have a globular attachment protein with hemaglutinin-neuraminidase (HN) activities, the Pneumovirinae have a non-globular mucin-like attachment protein for cell binding and egress. The Paramyxovirinae include important human and animal pathogens such as mumps virus (MuV), measles virus (MeV), Newcastle Disease virus (NDV), Hendra virus (HeV) and Nipah virus (NiV). This family also includes the prototype paramyxovirus parainfluenza virus 5 (PIV5), formerly known as simian virus 5. The Pneumovirinae includes human, ovine and bovine RSV and the human and avian metapneumoviruses. The genome of RSV contains 10 genes that encode for 11 known viral proteins (Figure 1-2). The fusion (F), attachment (G) and small hydrophobic proteins are the envelope glycoproteins. As the name suggests, the F protein mediates fusion of the virus to the cell and syncytium formation [1, 2]. The G protein aids in viral attachment to the cell, although it is not essential for virus entry in cell culture and RSVΔG virus is able to attach and fuse to the cells, albeit at a lower efficiency than wild type virus [3]. The F and G proteins are the major antigenic determinants of the virus [1]. The small hydrophobic protein is also not necessary for virus infection but it has a role in the pathogenicity of the virus since RSVΔSH is attenuated in an animal model [4]. The nonstructural NS1 and NS2 proteins
have a role in inhibiting the type I interferon response after virus infection [5, 6]. The L, N, P and M2-1 proteins are involved in viral RNA transcription and replication [7]. The large RNA-dependent RNA polymerase or L protein is the major polymerase subunit and has the catalytic activity. The phosphoprotein (P) interacts with L, N, M2-1 as well as itself and provides stability to the polymerase complex [8-13]. The nucleocapsid or N protein tightly binds and encapsidates the viral RNA [1]. M2-1 is the protein synthesized from the first open reading frame of the M2 mRNA and it is a transcription anti-termination factor [14]. The M2-2 protein is encoded from the second open reading frame of the M2 mRNA, it downregulates viral transcription and upregulates viral replication [15]. The matrix protein (M) interacts with the nucleocapsid and envelope glycoproteins, aiding in virion assembly and budding [16-18]. The functions of the small hydrophobic protein and the phosphoprotein will be discussed in Chapter 2 and 3 respectively.

There are two major subtypes or antigenic variants of RSV [19], classified as subtype A and subtype B. Because subtype A is more common, and the availability of a reverse genetics system for the A2 strain of subtype A, this strain was used in this study. The major differences in the genome sequence between the subtypes can be found in the G gene [19, 20]. Overall, there is 80% sequence similarity between both subtypes.

1.2 RSV entry

A schematic representation of the RSV life cycle is shown in Figure 1-3. RSV entry and fusion involves the G and F proteins for binding and fusion of the virus and cell
membranes. Attachment to the cells involves binding of G or F to cell surface glycosaminoglycans (GAGs) [21]. The initial contact step is presumably made by G interacting with heparan sulfate containing glycosaminoglycans (GAGs) in the surface of the cell [22]. This initial contact is not completely necessary since RSV virus lacking the G gene is viable and can be grown in cell culture. However, the G protein is necessary for growth in vivo, as shown by the attenuated phenotype seen in cotton rats and humans [23]. The RSV F protein can also bind heparan sulfate GAGs suggesting that F can mediate the binding step as well [24]. A recombinant RSV with F as its only glycoprotein can bind HEp-2 cells treated with heparinase I or basic fibroblast growth factor (binds to heparan sulfate), suggesting an additional heparin-independent mechanism for attachment [25]. The SH protein has not been found to be involved in virus attachment [3]. It is also not necessary for viral fusion although it may enhance syncytium formation when F and G are both expressed [26]. In the absence of the G protein (RSVΔG) the SH may inhibit syncytia formation [3]. Once at the cell surface the F protein mediates direct, pH-independent fusion of the viral envelope to the cellular plasma membrane. This step releases the virion contents, containing the N-RNA complex and the P, L and M2-1, proteins into the cytoplasm to begin RNA synthesis.

1.3 RSV RNA synthesis and protein production

RSV RNA synthesis involves the L, N, P and M2-1 proteins. RSV transcription is initiated when the polymerase complex recognizes the 3’ extragenic sequence of the genome called the leader. The polymerase transcribes the genes in a stop-start manner controlled by the conserved gene end (GE) and gene start (GS) signals flanking each
gene. When the polymerase encounters the GS signal, transcription starts and continues until it encounters a gene end signal. After the gene end signal the polymerase skips along the intergenic sequence, until it encounters another GS signal [27]. In this manner the genes closer to the promoter are transcribed more than the genes distal to the promoter [1]. Each RSV mRNA corresponding to the 11 known proteins, are capped and polyadenylated by the viral polymerase. Inefficient termination at the GE site can result in readthrough transcription. In this case polycistronic mRNAs are made, where only the first gene in the mRNA will be expressed.

Studies have shown that if the GS is deleted transcription of the downstream gene is almost completely abolished [27]. On the other hand if the GE signal is deleted the upstream and downstream genes are affected resulting in readthrough transcription of the two genes. The gene end signal also controls the polyadenylation of the transcript [27]. The GS signals of the first 9 genes of RSV are highly conserved consisting of 3’-CCCCGUUA-5’; the other nucleotide can be either a U or a C depending on the gene. The GS signal of the L gene is different and consists of 3’-CCCUGUUUUA -5’. The gene end sequences of the 10 RSV genes are 12-13 nucleotides in length and are more diverse in composition than the gene start sequences. They all start with UCA and end with UUUU [28]. Between the GE sequence of a gene and the GS sequence of the next one there is a nontranslated intergenic region. In most cases mutation in these region does not affect viral transcription [29, 30]. However, a single nucleotide deletion at the M/SH intergenic region can decrease SH transcription. Similarly, differences in the F/M2 intergenic region can decrease or increase readthrough transcription, thereby
affecting expression of the M2 gene [30]. The 5’ of the genome contains the trailer sequence. The trailer complement is important for replication since it contains the promoter for antigenome replication. During replication of RSV, the polymerase complex recognizes the promoters in the 3’ leader of the genome or trailer complement of its complementary positive sense copy, called the antigenome. For replication, the polymerase ignores the GS and GE signal by undefined means and produces a complete positive-sense copy of the genome which is encapsidated and used as a template for the production of a complete copy of the negative-sense genome [1]. The phosphorylation of P is speculated to play a role in the switch between transcription and replication complex. For a schematic representation of RSV RNA synthesis see to Figure 1-4.

Studies suggest that RSV RNA synthesis occurs at cytoplasmic inclusion bodies where aggregates of the P, N, L and M2-1 proteins can be found [31, 32]. After synthesis, the mRNAs are translated by ribosomes in the cell. After production, the envelope glycoproteins pass through the ER and Golgi apparatus where they are glycosylated [33-36]. The F protein is produced as F 0, this form of the protein is cleaved by cellular protease furin in the Golgi apparatus, into F 1 and F 2 subunits bound together by disulfide bonds [37, 38]. This cleavage is necessary for the fusion function of the F protein because it exposes the N-terminal hydrophobic portion of F 1. This region, called the fusion peptide, is believed to interact with the cell membrane to mediate fusion during RSV entry events.
1.4 RSV assembly and budding

While RNA synthesis and protein production occur in the cytoplasm, assembly occurs at the plasma membrane. Studies show that the M protein plays a crucial role in this process by facilitating the movement of the viral polymerase complex proteins, also known as nucleocapsid proteins (N, P, L and M2-1) from the cytoplasm to the membrane. M can interact with M2-1 in the cytoplasmic inclusion bodies [39]. Since the members of the polymerase complex interact with each other through P or RNA binding domains, the interaction between M and M2-1 may facilitate the move of these proteins to the membrane. While M can interact directly with the membrane [40], an interaction between M and the cytoplasmic tails of the G and F proteins has also been observed [16, 18]. The structure of the M protein consists of two β sheet-rich domains connected by an unstructured linker region [41]. A positive charge region has been identified that spans both domains and the linker, and has the potential to mediate membrane association and binding to the nucleocapsid proteins. However the specific amino acids involved are still not known [41]. Since the M protein is found in GM1 rich areas of the cell membrane and purified RSV virions contain caveolin, RSV is believed to use lipid rafts structures for assembly and eventually budding [40, 42, 43]. During the budding process the virus uses the plasma membrane as its envelope and pinches off the cell. This process does not appear to be very efficient and RSV virions can sometimes remain cell-associated or be re-adsorbed into the cell [44]. Actin and profilin are also believed to be involved in virion morphogenesis since inhibition of actin stress fiber formation inhibits virus production [45, 46].
1.5 Epidemiology and treatment

The respiratory syncytial virus is the leading cause of serious pediatric respiratory tract infection in infants and young children [47]. Severe respiratory illness due to RSV infection is the cause of over to 100,000 hospitalizations per year in the US [48]. Most children will be infected with RSV by their second year. Since primary infection with RSV does not provide complete immunity, re-infection throughout life is common. These secondary infections are less severe and generally not serious in adults, with the exception of elderly and immunocompromised patients. Normally infection with RSV is restricted to the upper respiratory tract and causes coughing, rhinorrhea, fever and occasionally wheezing. However, certain populations of infants and young children are at high risk of developing severe respiratory disease after RSV infection. These include premature babies, especially those born before 32 weeks of gestational age, and children with chronic lung disease or congenital heart defects with pulmonary complications. These patients may develop lower respiratory tract infection that can lead to pneumonia and broncholitis. The prevention of serious illness in these patients is done by immunoprophylaxis. Palivizumab is the only available immunoprophylaxis method for high risk patients. The previously-used RSV immune globulin intravenous (RSV IGIV), a hyperimmune polyclonal globulin prepared from donors with a high titer of anti-RSV antibodies, is no longer available [49]. Palivizumab is a humanized monoclonal antibody against the RSV surface glycoprotein F that is administered by intramuscular injections every 30 days during the RSV season, which generally lasts 5 months, from November to March. Palivizumab treatment has been proven to reduce the incidence of hospitalization by 45-55% in high risk patients [50, 51]. Severe RSV lower respiratory tract infection
early in life has been associated with higher incidence of recurrent wheezing and asthma later in life [52, 53]. Simoes et al. found that patients treated with palivizumab have a lower incidence of recurrent wheezing 2 years later [54], suggesting that prevention of RSV caused bronchiolitis early in life can reduce the incidence of asthma in these patients. A strong disadvantage of this treatment is its high cost. Treatment with palivizumab for the duration of one RSV season can cost approximately $3600 [55]. Because of this, treatment with palivizumab is restricted to high risk patients, despite the fact that lower respiratory tract infection with RSV is not limited to this population and can sometimes occur in otherwise healthy patients. Recently, some doubts have risen about the cost-effectiveness of treating some patients with palivizumab since hospitalization costs can be less than palivizumab treatment for one RSV season [56]. Another disadvantage of palivizumab is that it does not prevent RSV infection, it only protects against serious disease, therefore a true preventive method against RSV infection is still not available.

Perhaps the best alternative for prevention of RSV is a vaccine. However, although there are several vaccine candidates, currently there is no licensed vaccine against RSV. Many challenges exist in the development of a vaccine against RSV. To be effective, an RSV vaccine would have to be administered to newborns and therefore administered at a time when their immune system is not completely developed. Another challenge is finding a balance between attenuation and immunogenicity. Attenuation is necessary to avoid disease but an over-attenuated virus does not induce protection against subsequent RSV infections. The most common strategy for RSV vaccine development is the use of live
attenuated RSV mutants. As history shows, replication-deficient RSV vaccine or delivery of purified proteins is not a good strategy since it may result in enhanced disease upon subsequent infection [57]. The first attempt at a vaccine, a formalin-inactivated RSV developed in the 1960s, did not protect against RSV and in some cases lead to more severe disease upon infection [58]. Since then, there have been several live attenuated RSV vaccine candidates that have been shown to be attenuated while still inducing the production of RSV neutralizing antibodies [59]. However, none of these candidates are available yet.

1.6 The SH protein and apoptosis

Viruses have developed several mechanisms to evade removal by the host’s defense system. One such mechanism involves disrupting the cell’s apoptotic pathways. Some viruses evade host defenses by prolonging infection in cells through the inhibition of apoptosis. There are many different signaling pathways that induce apoptosis. One of them is the tumor necrosis factor alpha (TNF-α) signaling pathway. Binding of secreted TNF-α to tumor necrosis factor receptor 1 (TNFR1) in the surface of the cell can activate pathways that lead to apoptosis and pathways that lead to the activation of NF-κB and subsequent production of anti-apoptotic proteins. TNF-α is not the only mediator of extrinsically-induced apoptosis. The extrinsic pathway of apoptosis, activated in response to factors outside of the cells, is also known as the death receptor pathway and can also be activated by binding of Fas ligand (FasL) or TNF-related apoptosis-inducing ligand (TRAIL) to its receptors Fas, DR4 or DR5 on the surface of the cells. Like in the TNF-α pathway, binding of FasL to Fas or TRAIL to DR4 or DR5 will induce the
activation of caspases and eventually lead to apoptosis [60]. Apoptosis can also be
induced by an intrinsic pathway that involves the pro-apoptotic and anti-apoptotic
members of the Bcl2 family of proteins and the mitochondria [60]. For a schematic
representation of the extrinsic and intrinsic apoptotic pathways see Figure 1-5.

RSV infected cells do not undergo apoptosis until late during infection, suggesting that
the virus has a mechanism to inhibit this process. Previous studies done in our lab with
another paramyxovirus, PIV5, demonstrated that the SH protein of this virus affects the
TNF-α signaling pathway [61]. L929 cells (mouse fibroblast cell line) infected by
PIV5ΔSH showed increased cytopathic effect, increased apoptosis and increased levels of
TNF-α in the media compared with wild type PIV5 infected L929 cells. More
importantly, induction of apoptosis by PIV5ΔSH was inhibited by the addition of
neutralizing antibodies to TNF-α or TNFR1, suggesting that TNF-α has an important
role in the induction of apoptosis by PIV5ΔSH. Later studies suggested that the SH
protein of mumps virus had a similar anti-apoptotic function [62]. In Chapter 2 of this
thesis, the function of the RSV SH protein is described.

1.7 The P protein and viral RNA synthesis

The respiratory syncytial virus phosphoprotein contains 241 amino acids. Along with the
L and N proteins they are the minimal viral components necessary for transcription and
replication of the encapsidated RSV genome [7, 63]. The P protein is found mainly as a
tetramer. The oligomerization domain of P is in the central part of the protein since
mutation of residues S99, 116, 117, 119, 143, 156 and 161 or deletion of amino acids
120-150 abolishes oligomer formation [8, 11]. The oligomerization domain of P is predicted to have a coiled-coil structure [64]. As an integral part of the polymerase complex the P protein is also able to interact with the L, N and M2-1 proteins [8-10, 12]. Its interaction with the N protein is believed to allow the encapsidation by N of viral specific RNA [8]. N protein expressed alone in bacteria encapsidates bacterial RNA while N and P co-expressed in cells leads to encapsidation by N of viral specific RNA [8]. Evidence suggests that P binds to the RNP complex through its the C-terminal [9, 13]. However some reports have found that the N-terminal of P may also have a role in this interaction [13]. P also serves as a chaperone for N protein to maintain it in a soluble state [1]. The interaction of P with L protein brings the L close to the N-RNA complex to allow transcription and replication. Several studies have also reported an interaction between P and the M2-1 protein. This interaction may help bring the M2-1 protein into the polymerase complex to aid in the elongation of the transcript. Interaction between these proteins was found to be necessary for M2-1-dependent transcription of an RSV minigenome system [12]. An interaction between the P and NS1 proteins has also been reported using the yeast two-hybrid system, although the function of this interaction has not yet been reported [13].

The phosphoprotein is known to be essential for viral RNA synthesis. P is also, as the name suggests, the most heavily phosphorylated protein of the virus [65]. Five serines were originally identified as being the primary sites of P protein phosphorylation in RSV: S116, S117, S119, S232 and S237. More recently other phosphorylation sites have been identified, of these only one, the threonine at position 108, has been found important for
RSV RNA synthesis in a minigenome assay. The role of phosphorylation of the P for its function during viral transcription and replication is not completely understood. While several studies have suggested that phosphorylation of the P protein is important for RSV RNA synthesis \textit{in vitro}, a study by Lu et al suggests that in the context of virus the 5 major phosphorylation sites are not necessary for viral RNA synthesis [66]. They do have an effect however since a virus lacking the 5 major phosphorylation sites of P is attenuated in HEp-2 cells. Previous work from our lab suggests that Akt is important for the gene expression of non-segmented negative sense single strand RNA viruses. Akt is a serine threonine kinase that has multiple roles in the cell, including cell survival, cell proliferation and metabolism (Figure 1-6). In Chapter 3, we investigate the role of Akt phosphorylation for P protein function.
Figure 1-1: Comparison of the Pneumovirinae and Paramyxovirinae genome composition. Members of the Paramyxoviridae family share 6 genes: N, P, L, M, F, G/H/HN, and L. In addition, some members of these family also encode for a small hydrophobic (SH) protein. The members of the Pneumoviridae subfamily encode for 3 additional genes; NS1, NS2 and M2.

Figure 1-2: Electron micrograph of RSV virion. Upper panel shows an electron micrograph of a budding RSV virion. Structural and non-structural proteins are labeled.
Figure 1-3: **RSV life cycle.** The RSV F and G proteins mediate viral attachment by binding to glycosaminoglycans at the cell surface. The F protein aids in the direct fusion of the viral envelope to the cell membrane. Consequently, the virion contents are released to the cytosol where viral RNA synthesis occurs. mRNAs are translated by cell ribosomes and viral envelope proteins are post-translationally modified in the ER and Golgi apparatus. The M protein coordinates assembly by interacting with viral protein in the cytosol and cell membrane.
Figure 1-4: Schematic representation of RSV RNA synthesis. The virus genes are depicted as grey rectangles; the L gene, which comprises almost half of the genome, has been truncated. The GS and GE signals are shown as white and black boxes, respectively. The encoded antigenome and mRNAs are indicated by hatched rectangles. Arrows indicate the location of the promoters [68].
Figure 1-5: Extrinsic and intrinsic pathways of apoptosis. The extrinsic pathway apoptosis can be activated by binding of FasL, TRAIL and TNF-α to its membrane receptors, Fas, DR4 or DR5, and TNFR1. These pathways lead to activation of caspases and eventually apoptosis. The TNF-α pathway can also activate NF-κB and consequently, leads to the production of anti-apoptotic proteins. Apoptosis can also be induced by an intrinsic pathway that involves the pro-apoptotic and anti-apoptotic members of the Bcl-2 family of proteins, and the mitochondria.
Figure 1-6: Akt signaling pathway. Activation of Akt involves binding of a growth factor to a receptor tyrosine kinase (RTK) at the cell surface and RTK autophosphorylation. Phosphatidylinositol 3-kinase (PI3K) can bind to the phosphorylated RTK and phosphorylate phosphatidylinositol 4,5-bisphosphate (PIP₂) to phosphatidylinositol (3,4,5)-trisphosphate (PIP₃). Akt is then recruited to the membrane and binds PIP₃ through its pleckstrin-homology (PH) domain. Once in the membrane Akt is activated by phosphorylation by PDK1 at T308 and mTor complex-2 at S473. Akt has numerous downstream targets and is involved in the inhibition of apoptosis and cell survival, glucose metabolism and protein translation.
Chapter 2: Function of the respiratory syncytial virus small hydrophobic protein
2.1 Abstract

Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract infections in infants. RSV encodes a small hydrophobic (SH) protein of unknown function. RSV lacking SH (RSVΔSH) grows well in cultured cells but is attenuated in animal models, suggesting SH plays a role in viral pathogenesis. Mumps virus and parainfluenza virus 5 (PIV5) and others members of the Paramyxoviridae family, also contain an SH protein. The PIV5 SH protein is necessary for the inhibition of TNF-α-induced apoptosis and NF-κB activation. The mumps virus SH protein, which has no sequence homology to the SH protein of PIV5, has also been reported to inhibit TNF-α pathway induced NF-κB activation. In this study recombinant PIV5 viruses without their own SH but containing RSV SH (from RSV strains A2 or B1) in its place (PIV5ΔSH - RSV SH) were generated and analyzed. Infection of cultured cells with the PIV5ΔSH - RSV SH viruses produced minimal cytopathic effect similar to PIV5 and were able to inhibit apoptosis induced by PIV5ΔSH infection. Furthermore, the SH proteins of RSV strains A2 and B1 were found to inhibit TNF-α pathway induced NF-κB activation in a reporter gene assay. Thus, the small hydrophobic protein of RSV was capable of functionally replacing the PIV5 SH protein by preventing cells from undergoing apoptosis and inhibiting TNF-α pathway induced NF-κB activation. Conversely, cells infected with RSVΔSH virus lost viability and underwent apoptosis faster than RSV infected cells, suggesting that the SH protein of RSV plays a key role in inhibiting apoptosis during RSV infection.
The SH protein of RSV is a type II transmembrane protein of 64 amino acids (RSV subgroup A) or 65 amino acids (RSV subgroup B) [36, 71-73]. Analyses of the SH proteins of the A2 strain and the 18537 strain of subgroup B revealed 76% amino acid sequence identity among these proteins. The major differences between these two SH proteins were found in the C-termini; only 50% sequence homology was found in this region. The putative transmembrane domain and the N-terminal domain of the protein have 84% and ~91% amino acid sequence identity, respectively, between the two strains [71]. For comparison, the F protein is more conserved than the SH protein with 89% amino acid sequence identity between these strains [74], while the amino acid sequence identity of the G protein in the A2 and 18537 strains is 53% [20].

The SH protein from strain A2 is concentrated in the lipid rafts of the Golgi apparatus membranes [75] and is found in four different forms in infected cells: SH₀, SH₉, SHₚ, and SHₗ. SH₀, the 7.5 kDa non-glycosylated form, is the full-length unmodified protein and it is the most common form expressed. SH₉ is the 13-15 kDa N-linked glycosylated form of the protein and it is the precursor of SHₚ. SHₚ (21-40 kDa) is a polylactosaminoglycan-modified form of the protein and it is the precursor of SHₗ (4.8 kDa) that is generated by translation initiation at the second AUG of the SH sequence [36]. SH₀ and SHₚ have been found in virions [71]. Similarly, different glycosylated and non-glycosylated forms of the B1 SH protein have been detected in infected cells [71].
Some studies have suggested that the RSV SH protein may have a role in viral fusion [3, 26] or in changing membrane permeability [76]. However, RSV lacking the SH gene (RSVΔSH) is viable and grows as well as the wild type virus [23, 77, 78], indicating that the SH protein is not necessary for virus entry into host cells. Also the SH protein is not required for syncytium formation [3]. RSVΔSH is attenuated in animals, indicating that RSV plays an important role in viral pathogenesis. Interestingly, PIV5 lacking the SH gene has a similar phenotype: it has normal growth \textit{in vitro} but it is attenuated \textit{in vivo} [79].

PIV5 and mumps virus, members of the \textit{Paramyxoviridae} family, also encode a small hydrophobic (SH) protein. The gene for the SH protein of PIV5 is located between the F and HN genes and encodes for a type II membrane protein of 44 amino acid residues [80, 81]. Studies of a mutant PIV5 virus lacking the SH gene (PIV5ΔSH) have demonstrated that the small hydrophobic protein is necessary for the inhibition of TNF-\(\alpha\)-induced apoptosis in L929 cells [61]. Infection of these cells with PIV5ΔSH virus induces severe cytopathic effect (CPE), whereas wild type infection does not [79]. Further analysis revealed that the cells were dying through TNF-\(\alpha\) mediated apoptosis [61]. Data from additional studies suggest that the SH protein of mumps virus is a functional counterpart of the PIV5 SH protein [62]. The mumps SH gene is also located between the F and HN genes and encodes for a 57 amino acid protein that inhibited TNF-\(\alpha\)-induced apoptosis of cells after infection with a PIV5ΔSH-MuVSH recombinant virus [62]. The PIV5 and mumps SH proteins have no sequence homology but have the same function. Thus, we hypothesize that the SH protein of RSV, even though it does not have sequence homology to the PIV5 or mumps SH protein, may be functionally similar to
other SH proteins from the members of the *Paramyxoviridae* family. To test this hypothesis, PIV5 SH gene was replaced with the RSV SH gene in the PIV5 genome. The recombinant viruses were analyzed and compared. Inhibition of TNF-α signaling by RSV SH in comparison to PIV5 SH was examined using a reporter gene assay. In addition, to determine whether the RSV SH protein plays a role in modulating apoptosis during RSV infection, different cell lines were infected with RSV or RSV△SH virus and induction of apoptosis was analyzed.
2.3 Materials and Methods

2.3.1 Plasmids

All molecular cloning procedures were performed following standard molecular biology techniques [82]. RSV SH genes were cloned from strains A2 and B1 of RSV obtained from Dr. Brian Murphy (NIAID). RSV from strains A2 and B1 were grown in HEp-2 cells, total RNA from infected cells was purified and RT-PCR reaction was carried out with appropriate primers. The PCR product was cloned into an expression vector (pCAGGS [83]), and sequences of SH genes were confirmed by sequencing. PIV5 SH gene in pCAGGS expression vector, plasmids used for recovery of infectious PIV5 virus pCAGGS-NP, pCAGGS-L, pCAGGS-P and the pCAGGS-GFP and pkB-TATA-Luc have been described previously [62, 84]. A plasmid containing the RSV G gene was cloned from strain A2 and used as a control in the luciferase assay. To generate this plasmid, the coding sequence from RSV G gene was inserted into the pCAGGS-MCS. The PIV5 infectious clone used to generate the PIV5∆SH-RSV SH viruses has been described before [85]. The PIV5∆SH-RSV A2 SH and PIV5∆SH-RSV B1 SH plasmids were generated by replacing the PIV5 SH coding sequence with the coding sequences of the RSV SH from strains A2 and B1 between the F and HN genes of PIV5 while maintaining the genome length as a multiple of six.

2.3.2 Cells and viruses

A549, MDBK, HeLa, L929 and L929F cells were cultured in Dubelcco’s Modified Eagle Medium (DMEM) with 10% FBS, 1 % Penicillin-Streptomycin (P/S). For BHK and
BSR-T7 cells, 10% Tryptose phosphate broth (10 % TPB) was added to the medium. In addition, G418 at 400 μg/mL was added to the media of BSR-T7 cells to maintain the expression of T7 RNA polymerase [86]. HEp-2 cells were cultured in Opti-MEM with 10 % FBS, 1% L-Glutamine and 1% P/S.

Recovery of PIV5 and generation of PIV5ΔSH have been described before [85, 87]. To generate the PIV5ΔSH-RSV A2 (or B1) SH virus the PIV5 SH coding sequence from a plasmid that contained an infectious clone of PIV5 (pBH276) was replaced with the coding sequences of the RSV SH gene from strain A2 or B1 while maintaining the genome length to be a multiple of six. BSR-T7 cells were transfected with the plasmid encoding the viral genome along with plasmids encoding NP, P and L of PIV5. Transfected cells were monitored for the appearance of syncytia in BSR-T7 cells as an indication of production of the recombinant virus. A plaque assay was performed using the media from syncytia-positive cells, and plaques were purified in BHK cells [88]. Recovered PIV5ΔSH-RSV A2 SH and PIV5ΔSH-RSV B1 SH were grown in MDBK cells and media collected 4-7 days post-infection (dpi) as described before [88].

Recovery of recombinant RSV from strain A2 (RSV) has been described previously [89]. For RSVΔSH virus, the M gene end, M-SH intergenic region, SH gene start and the SH gene sequence were deleted by PCR, fusing the 3' UTR of the M gene to the SH gene end. This mutation was inserted into a full-length RSV antigenome cDNA clone (D53) and recombinant RSV rescued as described before [89]. RSV and RSVΔSH virus were grown in Vero cells in Opti-MEM 2% FBS, 1% P/S and media collected 3-5 days post-
infection. Titers for the viruses were determined by a plaque assay using RSV antibody followed by immunoperoxidase staining to visualize the plaques [57].

For PIV5 virus infections, cell monolayers were mock infected (DMEM with 1% bovine serum albumin only) or infected at an multiplicity of infection (MOI) of 5, unless indicated otherwise, in DMEM with 1% bovine serum albumin (BSA) for 1-2 hours at 37°C and 5% CO₂. After infection, cells were washed with PBS and placed in DMEM/2% FBS/1% P/S. For co-infection cells were infected with PIV5, PIV5ΔSH-RSV A2 SH, PIV5ΔSH-RSV B1 SH at an MOI of 5 in DMEM/1% BSA. One day after these infections, cells were co-infected with PIV5ΔSH. After each infection cells were washed with PBS and cultured in DMEM/2% FBS/1% P/S.

For RSV infection of L929 and A549 cells, monolayers were mock infected (Opti-MEM, 2% FBS, 1% P/S) or infected with RSV or RSVΔSH at an MOI of 3 for the CPE and Trypan blue exclusion assay and at an MOI of 1 for the TUNEL assay. Cells were incubated with the infection media for 1-2 hours at 37 °C and 5% CO₂. After infection, cells were washed with PBS and fresh Opti-MEM/ 2% FBS/1% P/S was added.

For the single step growth curve, monolayers of MDBK cells in 35 mm diameter plates were infected with PIV5, PIV5ΔSH, PIV5ΔSH-RSV A2 SH or PIV5ΔSH-RSV B1 SH at an MOI of 5. Media was collected at 0, 12, 24, 36 and 48 hours post-infection and frozen at -70°C until use. Viral titers were determined by a standard plaque assay using BHK cells as described before [88].
2.3.3 RT-PCR and sequencing

MDBK cells were infected with the viruses as described above. One day post-infection, RNA from PIV5, PIV5ΔSH, PIV5ΔSH-RSV A2 or PIV5ΔSH-RSV B1 SH infected cells was extracted using the RNeasy kit (QIAGEN, Maryland, USA) following the manufacturer’s protocol. Primer BH191 (sequence: 5’-TATTGACCATTGTCGTTGCTAATCGAAA-3’), which anneals to the vRNA (genome sense RNA), was used in the reverse transcription reaction. The cDNA was then amplified using primers BH191 and BH194 (sequence: 5’TCGAAATAATACTCGGCAAGTGGCC-3’), which anneals to the antigenome sense RNA of PIV5 (Figure 1B). The PCR was carried out at 94 °C for 1 min, 55°C for 1 min, and 72°C for 1 min for 35 cycles. PCR products were analyzed in a 1% agarose gel and the sequences were determined using gel purified products by Davis Sequencing (Davis, CA) with ABI 3730.

2.3.4 Production of RSV A2 SH and RSV B1 SH antibody

Polyclonal antibodies against the RSV SH protein from strains A2 or B1 were produced in rabbits by Harlan (Indianapolis, Indiana) and Sigma-Genosys (The Woodlands, Texas), respectively. Peptides corresponding to the last 17 amino acid residues of the C-termino for both proteins were synthesized using the deduced amino acid sequence (Figure 1A). The amino acid sequence for strain was NVFHNKTFELPRARVNT. For strain B1 the sequence was TFCNNTLELGQMHQINT. Various bleeds were tested before the final production bleed. Specificity of the antibodies was confirmed by
immunoprecipitation of the SH protein expressed from the expression vector (data not shown).

2.3.5 Immunoprecipitation

HeLa cells were mock infected or infected with PIV5, PIV5ΔSH, PIV5ΔSH-RSV A2 SH or PIV5ΔSH-RSV B1 SH at an MOI of 10 as described above. One day post-infection, cells were washed with PBS and cultured in DMEM without cysteine and methionine for 30 minutes at 37 °C with 5% CO₂. Cells were labeled with 110-221 μCi of Pro-mix (³⁵S-Met and ³⁵S-Cys) for 3 hours at 37°C. Labeling medium was removed and cells were lysed with radioimmunoprecipitation assay buffer (RIPA buffer), containing 0.3 M NaCl, 1% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 0.1% (w/v) sodium dodecyl sulfate (SDS), 0.1M Tris-HCl (pH 7.4), 1 mM phenylmethylsulphonyl fluoride (PMSF), 210 ng/ml aprotinin (0.24 trypsin inhibiting U/mL) and 10 mM iodoacetamide. Cell lysates were clarified by centrifugation at 60,000 rpm for 15 min. Aliquots of the clarified lysates were incubated at 4°C with antibodies against the HN, F, V, P, L or SH proteins of PIV5 [80, 90] or antibody against the SH protein of RSV strain A2 or B1. Protein A sepharose beads were added to the lysate-antibody mixture and incubated for 45 min at 4°C. Beads were pelleted and washed. Immunoprecipitated proteins were loaded into a 10% or 17.5% SDS-polyacrilamide gel and electrophoresed. The gel was fixed for 20 minutes in a 7% acetic acid, 20% methanol solution and dried using a BioRad gel dryer. Radioactivity of the gel was examined using a Phosphoimager Storm System (Storm 860, Molecular Dynamics).
2.3.6 Analysis of NF-κB activation by immunofluorescence and ELISA

To detect NF-κB activation, L929 cells grown on glass coverslips were mock infected or infected with PIV5, PIV5ΔSH, PIV5ΔSH-RSV A2 (or B1) SH. One day post-infection, cells were washed with PBS and fixed in 0.5% formaldehyde for 15 min at room temperature. Cells were washed with PBS and permeabilized in 0.1% saponin/PBS solution for 1 hour at 4°C. Subsequently, cells were incubated overnight at 4°C in a 1:100 dilution of anti-p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in 0.1% saponin/1% BSA/PBS. Finally, cells were washed three times with 0.1% saponin/PBS and incubated with FITC labeled anti-mouse IgG antibody (Jackson Laboratory, Bar Harbor, Maine) for 45 min. Unbound antibody was removed by washing, as above, and coverslips placed on glass slides. Fluorescence was examined using an Olympus BX-60 digital microscope with Image Pro plus software.

Immunofluorescence results were confirmed using an ELISA-based NF-κB activation assay. For this experiment L929 cells were mock infected or infected with rPIV5, rPIV5ΔSH, rPIV5ΔSH-RSV A2 SH, or rPIV5ΔSH-RSV B1 SH at an MOI of 10. At 1 dpi, nuclear extracts were obtained as described by Lin et al. (12). One microgram of protein was used for the ELISA from Active Motif (TransAM NF-κB family kit; Active Motif, Carlsbad, CA). The assay was performed according to the manufacturer’s instructions. Briefly, nuclear extracts are added to 96 well plates coated with an oligonucleotide that contains an NF-κB consensus binding site. The activated NF-κB dimmers that bind to the oligonucleotide are identified using a p65 antibody. A
secondary antibody conjugated to horseradish peroxidase (HRP) is added to the well and the active p65 measured with spectrophotometer.

2.3.7 Dual-Luciferase Assay

Eight wells of L929F cells grown in 24-well plates were transfected using FuGene6 (Roche Diagnostics Corp, Indianapolis, Indiana) according to the manufacturer’s recommendations with 250 ng per well of pCAGGS-GFP, pBH462 (pCAGGS-PIV5 SH), pCAGGS-RSV A2 SH, pCAGGS-RSV B1 SH or pCAGGS-RSV G, in addition to 25 ng of pkB-TATA-Luc (an NF-κB-dependent promoter followed by the firefly luciferase reporter gene) and 2.5 ng of phRL-TK (Promega, Madison, WI). Cells were incubated at 37°C with 5% CO$_2$ for 18 to 24 hours; then the medium was replaced with either 500 μl of Opti-MEM alone (four wells) or 500 μl of Opti-MEM containing 10 ng/ml TNF-α (catalog no. 522-009; Alexis, San Diego, CA) (four wells), and cells were incubated for 4 h at 37°C with 5% CO$_2$. Cells were washed with PBS, then 100μl 1X Passive Lysis Buffer (Promega, Madison, WI) was added into each well and the whole plate was put on the shaker at the speed of 100 rpm for 15 minutes at room temperature. Aliquots of 30 μl of cell lysate from each well were transferred to a 96-well plate. Fifty microliters of LAR II (Promega, Madison, WI) and 50 μl of Stop & Glo Reagent (Promega, Madison, WI) were added into each well of the 96-well plate sequentially by the luminometer. The Firefly and Renilla luciferase activities were recorded by a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale CA).
2.3.8 Trypan blue exclusion assay

Confluent monolayers of L929 in 12 well plates were infected with RSV or RSVΔSH at an MOI of 3 as described in Section 2.3.2. At different time points cells were trypsinized and combined with floating cells in the media. Cells were spun at 1,000 rpm in a Beckman Coulter Microfuge® 18 (Fullerton, CA) centrifuge for 15 min. Pellets were resuspended in PBS. Aliquots (10 μL) of the cell suspensions were stained with 10 μL of 0.4% trypan blue (Avocado Research Chemicals Ltd, Ward Hill Massachusetts). Stained cells were loaded into a hemacytometer. Four squares of the hemacytometer were counted and averaged for each triplicate. The triplicate values were averaged and the standard error of the mean was calculated for each sample.

2.3.9 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) Assay

Confluent monolayers of L929 or A549 cells grown in 6 well plates were infected with RSV or RSVΔSH at an MOI of 1. Cells were trypsinized and combined with floating cells in the medium at 1 day post-infection for L929 cells and 2 dpi for A549 cells. The cells were then centrifuged at 200 x g for 7 min at 4°C, washed with PBS and spun again. Subsequently, cells were fixed with 0.5% formaldehyde for 1 h at 4°C. The fixed cells were washed with PBS, spun at 200 x g for 7 min., resuspended in 0.5 ml of 50% DMEM–50% FCS and permeabilized by adding 1.5 ml of 70% ethanol at 4°C for 2 h-2 days. The cells were then incubated with 25 μl of TUNEL reaction mixture (in situ Cell Death Detection Kit, Fluorescein; Roche Diagnostics Corporation, Indianapolis, IN) for 2 hours in a 37°C incubator. Apoptotic cells were identified by flow cytometry.
2.4 Results

2.4.1 Generation of PIV5ΔSH-RSV SH viruses.

We hypothesized that even though there is no sequence homology between the RSV SH and PIV5 SH proteins (Figure 2-1A) the function of these proteins is similar. To test this hypothesis, recombinant viruses that contained the RSV SH gene either of strain A2 or B1, replacing the PIV5 SH gene were produced (Figure 2-1B). To confirm the sequence of the viruses, MDBK cells were infected with the PIV5ΔSH-RSV A2 (or B1) SH viruses and total RNAs were extracted. RT-PCR reactions were performed with primers that annealed to the nucleotides surrounding the SH gene. As shown in Figure 2-1C, the recombinant viruses containing the RSV SH of the A2 or B1 strains produced bands between 700 bp and 800 bp corresponding to the expected sizes of PCR products that contained the RSV SH gene. The RT-PCR products were sequenced and the results confirmed that the desired recombinant viruses had been obtained (data not shown).

2.4.2 Analysis of viral protein production.

To examine the role of the RSV SH protein during virus infection rPIV5, rPIV5ΔSH, rPIV5ΔSH-RSV A2 SH and rPIV5ΔSH-RSV B1 SH were compared on the basis of viral growth rate, plaque size, protein production and cytopathic effect. To determine if replacing PIV5 SH with RSV SH had an effect on viral protein production, HeLa cells were infected with an MOI of 10. One day post-infection, cells were labeled with $^{35}$S-Met and $^{35}$S-Cys, lysed, and viral proteins were immunoprecipitated using antibodies against PIV5 proteins. As shown on Figure 2-2A, synthesis of the V, P and L proteins
were similar between the recombinant viruses and PIV5. The levels HN and F1 protein were somewhat variable between viruses and varied slightly among different experiments. In general, the levels of HN and F in rPIV5ΔSH-RSV B1 SH virus infected cells were higher than or equal to those levels in PIV5 infected cells, which were similar to the HN and F levels of rPIV5ΔSH-RSV A2 SH infected cells.

In order to examine the expression of the RSV SH proteins encoded by recombinant viruses, RSV SH antibodies against the SH of strain A2 or B1 of RSV were generated using the last 17 amino acids of the C-terminal of the protein as described in Materials and Methods. HeLa cells were infected with the rPIV5ΔSH-RSV A2 SH or rPIV5ΔSH-RSV B1 SH. One day post-infection cells were labeled with $^{35}$S-Met and $^{35}$S-Cys and the SH protein was immunoprecipitated using the RSV SH antibodies. The four different forms of the RSV SH protein in infected cells were observed in PIV5ΔSH-RSV A2 SH infected cells after immunoprecipitation (Figure 2-2B). As observed previously only three forms of the protein were observed in PIV5ΔSH-RSV B1 SH infected cells (Figure 2-2C) [71].

2.4.3 Growth of PIV5 and RSV SH recombinant viruses.

Previous studies have shown that deletion of the SH gene from PIV5 does not cause significant differences in virus growth. Both rPIV5 and rPIV5ΔSH virus grow to similar titers, although rPIV5ΔSH virus grows slightly faster in the first stages of infection [62, 87]. To study the effect of replacing the PIV5 SH protein with the RSV SH protein on virus growth, the growth rate of these viruses was compared. As seen in Figure 2-3A,
the growth of the rPIV5ΔSH-RSV SH recombinant viruses was comparable to rPIV5 and rPIV5ΔSH by 1 day post-infection (dpi). Occasionally, a delay in the growth of one or both of the recombinant viruses was observed at 12 hours post infection (hpi), but by 24 or 36 hours the viruses had always reached titers comparable to the wild type virus. The plaques formed by the rPIV5, rPIV5ΔSH and rPIV5ΔSH-RSV SH viruses in BHK cells had similar size and morphology (Figure 2-3B).

2.4.4 Cytopathic effect (CPE) of virus infection.

Previous studies have demonstrated that there is no significant CPE after infection of MDBK, HeLa, A549 or L929 cells with rPIV5 [61, 62, 79]. It has also been shown that rPIV5ΔSH causes severe CPE in MDBK and L929 but not HeLa or A549 cells [61, 62, 79]. If the RSV SH protein and the PIV5 SH protein have similar functions, it is likely that the replacement of the PIV5 SH protein with the RSV SH protein from strain A2 or B1 will result in minimal CPE after infection in these cells. To determine if the RSV SH protein was able to replace the PIV5 SH protein in blocking cell death, MDBK, L929 and A549 cells were infected with rPIV5, rPIV5ΔSH, rPIV5ΔSH-RSV A2 (or B1) SH at an MOI of 5. Three to five days after infection, the cells were photographed using a Nikon Eclipse TE300 Inverted Microscope. As expected, rPIV5 had minimal CPE in all the infected cell lines (Figure 2-4A). Consistent with previous work [61, 62, 79], rPIV5ΔSH caused notable CPE in MDBK and L929 cells but not in A549 cells. The cells infected with the RSV SH recombinant viruses had a phenotype similar to those infected with rPIV5, showing no visible CPE in the time frame studied. Since the only difference between the rPIV5ΔSH virus and the RSV SH recombinant virus is the replacement of
the PIV5 SH protein with that of RSV, the survival of the cells infected with the RSV SH recombinant viruses suggests that the RSV SH protein was able to replace the PIV5 SH protein in preventing the cells from dying.

To determine if the RSV SH protein could prevent apoptosis induced by rPIV5ΔSH infection MDBK cells were infected with rPIV5ΔSH-RSV A2 (or B1) SH and one day post-infection super-infected with rPIV5ΔSH. The results are shown in Figure 2-4B. As observed previously, PIV5 infected cells super-infected with rPIV5ΔSH showed minimal CPE, especially when compared to cells that were infected with rPIV5ΔSH alone. rPIV5ΔSH-RSV A2 (or B1) SH infected cells super-infected with PIV5ΔSH had minimal CPE and a phenotype that was more similar to the rPIV5 + PIV5ΔSH infected cells. Expression of the RSV SH protein prevented the rPIV5ΔSH-infected cells from undergoing apoptosis. Afterwards, RNA was extracted from all the samples and an RT-PCR was performed with primers flanking the SH protein to confirm replication by both viruses in the super-infections (Figure 2-4C).

2.4.5 NF-κB activation by viruses.

The absence of the PIV5 SH protein during infection induces an increased production of TNF-α and activation of NF-κB, resulting in the translocation of the p65 subunit of NF-κB factors into the nucleus of rPIV5ΔSH infected L929 cells [61]. To investigate the consequence of replacing the PIV5 SH protein with RSV SH protein in relation to NF-κB activation, L929 cells were infected with rPIV5, rPIV5ΔSH or rPIV5ΔSH-RSV A2 (or B1) SH and analyzed for intracellular localization of NF-κB using antibodies against the p65 subunit of NF-κB. Activation of NF-κB results in the translocation of the protein
from the cytoplasm to the nucleus [91]. As expected, nuclear localization of p65 was observed in PIV5ΔSH infected cells and not in PIV5 infected cells (Figure 2-5A). Little if any nuclear p65 was found in PIV5ΔSH-RSV A2 (or B1) SH infected cells. While 30% of cells showed p65 in the nucleus after PIV5ΔSH infection only about 1-3 % of cells showed p65 in the nucleus after PIV5ΔSH-RSV A2 (or B1) SH infection. These results were further confirmed using an NF-κB binding enzyme-linked immunosorbent assay (ELISA) using immobilized DNA oligomers. Nuclear extracts from L929 cells infected with the recombinant viruses were used to treat 96 well plates coated with an oligonucleotide encoding an NF-κB consensus binding site. Activated NF-κB was detected by the addition of antibodies against p65. To examine the specificity of the proteins in the nuclear extract binding to the oligonucleotide coated plate, free wild type oligonucleotide or a mutated oligonucleotide were used as binding competitors. As seen in Figure 2-5B, while PIV5ΔSH virus activated NF-κB, the recombinant PIV5ΔSH-RSV SH viruses and PIV5 did not.

2.4.6 Inhibition of TNF α-induced apoptosis.

Although biologically detectable levels of TNF-α are produced after PIV5 infection, PIV5ΔSH infection produces a significantly higher amount of the cytokine [61]. Previous work from our lab indicates that the SH protein of PIV5 was able to block TNF-α signaling. To study whether the RSV SH protein has a similar function, L929F cells were transfected with a luciferase gene under the control of an NF-κB responsive promoter along with a plasmid containing the gene for RSV A2 (or B1) SH. Cells were also transfected with a plasmid containing the Renilla luciferase gene under the control of
herpes simplex virus thymidine kinase promoter as a transfection control. One day post-transfection, media was replaced with Opti-MEM or Opti-MEM and TNF-α (10 ng/mL) and cells were incubated for another 4 hours. Samples were then examined for dual luciferase activities. The RSV SH protein from both strains inhibited NF-κB activation by TNF (Figure 2-6). As observed before [62], cells transfected with the PIV5 SH also inhibited TNF from activating NF-κB. As a control, the RSV G protein did not inhibit TNF-α pathway induced NF-κB activation.

2.4.7 Induction of apoptosis by RSVΔSH.

To determine whether the RSV SH protein had a role in inhibiting apoptosis during RSV infection, a recombinant RSV without the SH gene (RSVΔSH) was generated from wild type RSV strain A2 as described in Materials and Methods. Monolayers of L929 cells were mock infected or infected with RSV or RSVΔSH at an MOI of 3 and cells were photographed 1 day post-infection (dpi) using a Nikon Eclipse TE300 Inverted Microscope. As shown in Figure 2-7A, CPE was observed in the cells infected with RSV at 1 dpi when compared to the mock-infected cells, however more severe CPE was observed in RSVΔSH infected cells at the same time point.

To quantify the number of dead cells after RSV and RSVΔSH infection, a trypan blue exclusion assay was performed (Figure 2-7B). The RSV infected sample had ~50% live cells at 1 dpi, compared to 100% for mock-infected cells and the percentage of live cells remained somewhat constant up to 7dpi. In contrast, the RSVΔSH sample had 25% live cells at 1 dpi. The number of live cells in the RSVΔSH sample decreased at a steady pace resulting in ~10% live cells in the RSVΔSH infected culture by 7 dpi. A TUNEL assay was performed to determine whether the cell death observed after RSVΔSH
infection was due to apoptosis of the cells. Monolayers of L929 cells were infected at an
MOI of 1 with RSV, RSVΔSH or mock infected and collected 1 dpi for the TUNEL
assay. As shown in Figure 2-7C, only 15% of the cells infected with RSV are apoptotic
by 1 dpi, while 95% of the RSVΔSH infected cells have started to undergo apoptosis at
this time point. Thus, RSV was capable of inducing apoptosis in L929 cells, but
RSVΔSH-infected cells caused significantly more apoptosis in this cell line.

To determine if the accelerated cell death caused by RSVΔSH was cell type specific,
A549 cells, a lung epithelial cell line, were mock infected or infected with RSV or
RSVΔSH at an MOI of 3 and cells were photographed 3 dpi. The results, shown in
Figure 2-8A, indicated that while little or no CPE was observed in the mock or RSV
infected cells, considerable CPE was observed in the RSVΔSH infected cells 3 dpi. As
before, a TUNEL assay was performed to determine whether the cell death observed in
the RSV and RSVΔSH infected A549 cells was due to apoptosis. A549 cells were
infected at an MOI of 1 and collected for the TUNEL assay 2 dpi. At this time-point
50% of the RSVΔSH infected cells were apoptotic compared to less than 1 % for RSV
(Figure 2-8B). Interestingly, A549 cells are not sensitive to cell death after treatment
with TNF-α, suggesting that RSVΔSH induces apoptosis in this cell line is through a
different pathway. To confirm that RSVΔSH-induced apoptosis in A549 cells is not
mediated through TNF-α production, the levels of TNF-α in the media were measured by
ELISA after virus infection. As seen in Figure 2-9A RSVΔSH induced detectable levels
of TNF-α production by 24 hpi and peaked at 50 pg/ml at 36 hpi. As previously
described, infection with wild type RSV also induced TNF-α production although
detectable levels were seen only after 36 hpi. To test whether 50 pg/ml is enough to
cause apoptosis in A549 cells, these cells were treated with TNF-α at 50 pg/ml, 100pg/ml and 10 ng/ml in the presence or absence of cycloheximide. Only the cells treated with 10 ng/ml of TNF-α in the presence of cycloheximide showed considerable CPE after treatment. This suggests that cell death after RSVΔSH infection of A549 cells is not due to TNF-α-induced apoptosis. To confirm this, a human TNF-α (hTNF-α) neutralizing antibody was used to block the effect of TNF-α production after RSVΔSH infection. While the h-TNF-α neutralizing antibody was able to block TNF-α + cycloheximide induced cell death (Figure 2-9C), it did not reduce apoptosis of A549 cells after RSV or RSVΔSH infection (Figure 2-9D). This suggests that RSVΔSH induced apoptosis of the human lung epithelial cell line is not mediated through TNF-α signaling. Therefore, the RSV SH protein can delay apoptosis through more than one mechanism.
2.5 Discussion

RSV encodes three glycoproteins identified as F, G and SH. Although the roles of the RSV G and F proteins have been fairly well described, the function of the small hydrophobic (SH) protein is not yet clear and somewhat controversial. Studies done by Hemingway et al. [26] found that expression of the RSV SH protein in CV-1 cells aids cell fusion due to the F and G proteins. However, another study by Techaarpornkul, et al [3] suggested that the RSV SH protein has a role in inhibiting viral fusion in the absence of the G protein when HEp-2 cells were infected with an RSVΔG virus. These contradictory results suggest that another approach may be necessary to identify a role for the RSV SH protein. To sort out the function of the RSV SH, we first compared the function of this protein with the PIV5 SH in a PIV5 genome background. Previous studies have shown that the PIV5 SH protein is necessary for inhibition of TNF-α pathway induced NF-κB activation and apoptosis [61]. PIV5, mumps virus and RSV are members of the Paramyxoviridae family. Replacement of the PIV5 SH gene with the mumps SH gene resulted in a virus that had a similar phenotype as wild type PIV5, even though these two proteins have no sequence homology. Also, the recombinant PIV5ΔSH-MuV SH virus did not cause CPE in L929 and MDBK cells, which are known undergo apoptosis after PIV5ΔSH infection. Expression of the MuV SH protein inhibited TNF-α-induced NF-κB activation. These results suggested that the MuV SH protein is a functional counterpart of the PIV5 SH protein [62].

PIV5 recombinant viruses that contained the RSV SH from either strain A2 or strain B1 in place of the PIV5 SH were generated. Comparison of the viral protein
production between the recombinant viruses and wild type PIV5 showed no deficiency in viral transcription and translation by the RSV SH-containing viruses. The RSV SH protein inhibited MDBK cells from undergoing apoptosis due to PIV5ΔSH infection. Since PIV5ΔSH infected cells die by TNF-α-mediated apoptosis, these results suggest that the RSV SH protein is capable of inhibiting this process.

Expression of the RSV SH protein in L929 cells inhibited NF-κB activation induced by TNF-α signaling. It is known that TNF-α production induces apoptosis by the activation of caspases and it activates the transcription factor NF-κB by a positive feedback mechanism that upregulates TNF-α production. NF-κB, however, also initiates the transcription of several anti-apoptotic genes [92-94]. In PIV5, it was found that although the PIV5ΔSH virus activated NF-κB, the p65 subunit was degraded by 2 dpi, allowing apoptosis of the cell. [61]. In RSV, however the mechanism may be different, since infection of cells with RSV activates NF-κB [95, 96]. In RSV, the M2-1 and NS2 genes have been found to be involved in the activation of NF-κB. It is possible that the activation of NF-κB observed during RSV infection is not induced by TNF-α signaling. A study by Fiedler et al. [97], showed that IκB is upregulated during RSV infection. Degradation of IκB is observed early during infection but normal levels are observed by 48 hours, NF-κB was activated even when normal levels of IκB were observed. Interestingly, inhibition of IκB degradation by proteasome inhibitor MG132, which was previously observed to reverse TNF-α-induced NF-κB activation [98], only partially reversed RSV-induced NF-κB activation and NF-κB-induced transcription of IL-8, while IL-8 release was not affected suggesting that TNF-α, while being produced after RSV infection, is not the major mechanism of RSV-induced NF-κB activation. Thus, although
RSV SH is capable of blocking TNF-α-mediated NF-κB activation, the actual role of the RSV SH during infection may be different.

RSV virus that lacks the SH gene has been rescued and found viable in vitro [23, 77, 78]. Studies carried out in mice and chimpanzees showed that an RSVΔSH virus from strain A2 is slightly attenuated in vivo in the lower respiratory tract of chimpanzees and upper respiratory tract of mice [4, 77]. RSV subtype B1 virus with deletions in the SH and G genes replicates efficiently in vitro but it is highly attenuated in humans [23]. The attenuation of the RSVΔSH virus during infection in vivo suggests the SH protein has a role in pathogenesis of the virus. In this study, the role of the RSV SH protein in RSV infection was analyzed using an RSV deletion mutant that did not contain the RSV SH gene (RSVΔSH). To generate the RSVΔSH virus used in this study the 3' UTR of the M gene was ligated to the SH gene end by PCR. This RSVΔSH was slightly different than the ones used in other studies [3, 4, 77]. Although RSV induced apoptosis in L929 cells, it was clear that the RSVΔSH virus accelerated apoptosis compared to the wild type. These results support the mechanism previously proposed that the paramyxovirus SH protein plays a role in blocking cell death [62]. RSVΔSH infection of A549 cells, which can produce TNF-α but are not sensitive to death caused by TNF-α-induced signaling [99, 100], also accelerated apoptosis when compared with wild type infection. Induction of apoptosis in this cell line was not due to activation of the TNF-α pathway since neutralization of TNF-α did not inhibit the apoptosis induced by RSVΔSH infection, suggesting that while the RSV SH protein plays a role in the TNF-α pathway, it may also play a role in inhibiting apoptosis in A549 cells by a different mechanism. Since Fas and TRAIL are both upregulated following RSV infection [101, 102], it is
possible that the RSV SH protein may inhibit the Fas or TRAIL extrinsic apoptotic pathways.

Studies examining whether RSV induces apoptosis have been inconsistent. On one hand it has been suggested that RSV inhibits apoptosis [103-105], with one study finding that RSV inhibits TNF-α induced apoptosis [106]. In this study by Domachowske, et al., infection of HEp-2 cells (a human laryngeal carcinoma cell line) with RSV caused upregulation of the anti-apoptosis gene IEX-1L. IEX-1L is known to inhibit TNF-α induced apoptosis. Treatment of RSV-infected HEp-2 cells with TNF-α did not cause apoptosis. However, it has been suggested that IEX-1L is a transdominant negative mutant of the pro-apoptotic gene IEX-1S and may only be expressed in some tumor cell lines [107]. A study by Takeuchi, et al. [104] suggested that RSV infection upregulates caspase 1/ICE and caspase 3/CPP32 in A549 cells but does not cause apoptosis by 48 hours post-infection (hpi). Thomas, et al [103] suggested that RSV delays apoptosis during the early course of infection through a PI3K-dependent mechanism. Infection of A549 cells with RSV induced cytotoxicity by 48 hpi. However, inhibition of PI3K, an important regulator of cell survival, induced apoptosis quickly. On the other hand, some studies suggest that RSV induces or sensitizes cells to apoptosis [102, 108, 109]. Among these studies, O’Donell, et al. found that infection of A549 cells with RSV moderately upregulated Fas expression and caused the apoptosis of about 15% of the cells [102]. Addition of a Fas crosslinking monoclonal antibody increased the percentage of apoptotic cells to about 25%. This suggests that RSV sensitizes A549 cells to apoptosis induced by Fas. A study by Kotelkin et al. [101] suggests that RSV sensitizes cells to apoptosis induced by exogenous TRAIL, but in its absence, apoptosis
does not occur until 48 hours post-RSV infection. However at this time over 60% of the cells were apoptotic. Bitko, et al. [109] suggests that RSV induces apoptosis of 95% of infected cells by 20 hpi and that the apoptosis observed is not due to a soluble product released from infected cells but rather to the activation of caspases 3 and 12. However later studies have suggested that human caspase 12 is proteolytically inactive [110] and likely does not have a role in apoptosis.

In this study, 15% of RSV infected L929 cells were apoptotic by 1 dpi. Thus, RSV was capable of inducing apoptosis in this cell line. However, in RSV-infected A549 cells less than 1% of the cells were apoptotic by 2 dpi. RSVΔSH was able to accelerate apoptosis significantly in both cell lines. Since A549 cells are not sensitive to TNF-α mediated cell death we speculate that the RSV SH protein can block more than one pathway that leads to apoptosis. Our results also suggest that the apoptotic pathway activated during RSVΔSH infection may vary depending on the cell line. It is possible that RSV inhibits TNF-α-induced apoptosis and that it also activates another pathway that leads to cell death. As mentioned above Fas and TRAIL are both upregulated following RSV infection [101, 102]. The RSV SH protein may also inhibit the Fas or TRAIL extrinsic apoptotic pathways.
Figure 2-1: Generation of PIV5ΔSH-RSV SH. (A) Sequences of the SH proteins of PIV5 and RSV strains A2 and B1. The predicted transmembrane domain of the proteins is underlined with solid lines. The amino acids sequences used to generate the RSV A2 and B1 SH antibodies are underlined with dashed lines. (B) Diagram of the insertion site of the RSV SH protein in PIV5. The RSV SH protein of strain A2 or B1 was inserted between the F and HN genes of PIV5. Primers BH 191 and BH 194 were used to amplify the region of interest for confirmation. (C) Confirmation of the generation of the PIV5ΔSH-RSV SH viruses. MDBK cells were infected at an MOI of 5 and RNA was extracted 1dpi as described in materials and methods. RT-PCR using primers BH191 and BH 194 amplified the region surrounding the SH gene in PIV5, PIV5ΔSH, PIV5ΔSH-RSV A2 SH and PIV5ΔSH–RSV B1 SH infected cells. Lane 1 is the 100bp DNA ladder.
Figure 2-2: Viral protein production in PIV5 and PIV5ΔSH-RSV SH infected cells. (A) Expression of PIV5 proteins. HeLa cells were infected at an MOI of 10. One day post-infection cells were labeled with $^{35}$S-Met and $^{35}$S-Cys and proteins immunoprecipitated using PIV5 specific antibodies. (B-C) Expression of the RSV SH protein by the PIV5ΔSH-RSV SH viruses. HeLa cells were infected as before and antibodies that recognized the C-terminal of the RSV SH protein were used to detect it. (B) anti-RSV A2 SH antibody. (C) anti-RSV B1 antibody.
Figure 2-3: Growth kinetics and plaque morphology of PIV5 and PIV5ΔSH-RSV SH. (A) Growth rate of recombinant viruses. MDBK cells were infected at high MOI and samples from the media were taken at 0, 12, 24, 36 and 48 hours post-infection and used for plaque assay. Error bars are standard error of mean. (B) Plaques from PIV5 and PIV5ΔSH-RSV SH BHK infected cells.
Figure 2-4: The PIV5ΔSH-RSV SH viruses inhibit apoptosis induced by PIV5ΔSH. (A) MDBK, L929 and A549 cells were mock infected or infected with PIV5, PIV5ΔSH, PIV5ΔSH-RSV A2 SH or PIV5ΔSH-RSV B1 SH at an MOI of 5. L929 cells were photographed three days post-infection. MDBK and A549 cells were photographed 4 days post-infection using a Nikon Eclipse TE 300 inverted microscope. (B) MDBK cells were mock infected or infected with PIV5, PIV5ΔSH-RSV A2 SH or PIV5ΔSH–RSV B1 SH at an MOI of 5. One day post-infection with these viruses cells were super-infected with PIV5ΔSH. The replication of the PIV5ΔSH virus in the experiments was confirmed by RT-PCR. Cells were photographed using a Nikon Eclipse TE 300 inverted microscope three days post-infection with the PIV5ΔSH virus. (C) Confirmation of super-infection, RT-PCRs were carried out as in Figure 1C. PCR products corresponding to SH or ΔSH as indicated.
Figure 2-5: Activation of NF-κB by recombinant PIV5. (A) L929 cells were mock infected or infected with PIV5, PIV5ΔSH, PIV5ΔSH-RSV A2 SH or PIV5ΔSH-RSV B1 SH. One day post-infection cells were fixed with 0.5% formaldehyde and permeabilized using a 0.1% saponin/ PBS solution. Cells were incubated overnight with an antibody against the p65 subunit of NF-κB. Localization of NF-κB in the cells was detected using a FITC-labeled anti-mouse IgG antibody. Fluorescence was observed using an Olympus BX-60 digital microscope with Image Pro Plus software. (B) Activation of NF-κB using ELISA. L929 cells were mock infected or infected with rPIV5, rPIV5ΔSH, rPIV5ΔSH-RSV A2 SH, or rPIV5ΔSH-RSV B1 SH at an MOI of 10. At 1 dpi, nuclear extracts were obtained as described by Lin et al. (12). One microgram of protein was used for the ELISA-based NF-κB activation assay from Active Motif (TransAM NF-κB family kit; Active Motif, Carlsbad, CA) according to the manufacturer’s instructions.
Figure 2-6: The RSV SH protein inhibits TNF-α induced NF-κB activation. L929F cells were transfected with pCAGGS-GFP, pCAGGS-PIV5 SH, pCAGGS-RSV A2 SH, pCAGGS-RSV B1 SH or pCAGGS RSV G. All samples were also transfected with pkB-TATA-Luc and phRL-TK (Promega). One day post-transfection media was replaced with Opti-MEM or Opti-MEM and TNF-α (10 ng/mL) and incubated for 4 hours at 37°C and 5% CO₂. Luciferase activity was measured using a Veritas Microplate Luminometer (Turner Biosystems) in samples treated with TNF/Opti-MEM or Opti-MEM alone. The graph represents the fold increase in luciferase activity in TNF-α treated samples over untreated samples. Error bars are the standard error of mean.
Figure 2-7: RSVΔSH virus causes apoptosis in L929 cells. L929 cells were mock-infected or infected with RSV or RSVΔSH at an MOI of 3. (A) Cells were photographed 1 day post-infection using a Nikon Eclipse TE300 inverted microscope. (B) A trypan blue exclusion assay was performed at 1, 2, 4 and 7 days post-infection. Cells were trypsinized and stained with trypan blue. Live cells were counted using a hemacytometer. Error bars represent the standard error of the mean. (C) L929 cells were mock-infected or infected with RSV or RSVΔSH at an MOI of 1. One day post-infection, cells were collected and fixed with 0.5% formaldehyde and DNA fragmentation was measured with a TUNEL assay. Error bars represent the standard error of the mean.
**Figure 2-8: RSVΔSH accelerated apoptosis in A549 cells.** A549 cells were mock infected or infected with RSV or RSVΔSH at an MOI of 3. (A) Cells were photographed 3 day post-infection using a Nikon Eclipse TE300 inverted microscope. (B) A549 cells were mock infected or infected with RSV or RSVΔSH at an MOI of 1. Two days post-infection, cells were collected and fixed with 0.5% formaldehyde and DNA fragmentation was measured with a TUNEL assay. Error bars represent the standard error of the mean.
Figure 2-9: RSVΔSH apoptosis in A549 cells is not mediated through TNF-α. (A) A549 cells were mock infected or infected with RSV or RSVΔSH at an MOI of 3. After infection media was collected at 0, 12, 24, 36 and 48 hours post-infection and used for a TNF-α ELISA. (B) A549 cells were treated only with human TNF (hTNF) at the concentrations indicated (top panel) or with hTNF-α and 20 mg/ml of cycloheximide (lower panel). Pictures were taken 1 day post-treatment. (C) A549 cells were treated as labeled with TNF-α (10 ng/ml), cycloheximide (CHX, 20 mg/ml) and neutralizing hTNF-α antibody at 1 μg/ml or 5 μg/ml concentration. Pictures were taken 1 day post-treatment. (D) A549 cells were infected at an MOI of 3. After infection media was replaced with OptiMEM containing 1μg/ml of neutralizing TNF-α antibody. Four days post-infection the cells were collected for TUNEL as described in Materials and Methods.
Chapter 3: The role of Akt phosphorylation for RSV P function
3.1 Abstract

The importance of phosphorylation of the P protein for RSV RNA synthesis remains an enigma. Recently, we have discovered that the host protein Akt1, a serine/threonine kinase, has a role in the RNA synthesis of non-segmented negative sense RNA viruses (NNSV). Inhibition of Akt reduced protein expression of parainfluenza virus 5 (PIV5), vesicular stomatitis virus (VSV) and measles virus (MeV). In this study we examined the role of Akt during RSV life cycle. Inhibition of Akt by small molecule inhibitors, siRNA and Akt dominant negative mutant results in reduced RSV protein expression. In addition, inhibition of Akt results in a significant reduction of P protein phosphorylation in RSV infected cells, suggesting that P is a target of Akt. To further investigate the role of Akt phosphorylation of P in viral RNA synthesis, we performed an in vitro kinase assay with unphosphorylated P protein purified from bacteria and active Akt1. Mass spectrometry analysis of the Akt-phosphorylated P protein revealed that the serine at position 86 was phosphorylated by Akt. This serine is well conserved among P proteins of the Pneumoviridae. Mutation of this serine resulted in a severe reduction in P protein phosphorylation by Akt in an in vitro kinase assay. Using mass spectrometry analysis we found this serine was also phosphorylated in virus infected cells. Understanding the role of phosphorylation in RSV replication will not only provide new knowledge on viral RNA synthesis but also may lead to a new strategy and rationale for vaccine design.
3.2 Introduction

RSV RNA synthesis requires the nucleocapsid (N), phosphoprotein (P), large polymerase (L) and the M2-1 protein [7]. The M2-1 protein, expressed from the first open reading frame of the M2 gene, is a transcription anti-termination factor, important for the processive transcription of long mRNAs [14]. The N protein binds and encapsidates the viral RNA, providing a stable nuclease-resistant complex. The L protein is the polymerase. The P protein can interact with L, M2-1 and N as well as itself, providing stability to the polymerase complex [8-13]. P serves as a chaperone for the N protein allowing it to stay in a soluble state and to encapsidate virus-specific RNA [1, 8]. It can also bind the L protein bringing it closer to the N-RNA complex. P is an integral member of the RNA polymerase complex and indispensable for viral RNA synthesis. As the name suggests, the RSV P protein is the most heavily phosphorylated protein of the virus [65]. The importance of the phosphorylation status of P for its role in viral transcription and replication is not completely understood.

Unphosphorylated P protein purified from E. coli is not active in transcription, suggesting that phosphorylation of the P protein is important for the function of P in this process [111]. Phosphorylated sites of the protein have been identified primarily in two clusters. The first cluster is located in the middle of the protein and is comprised of serines 116, 117 and 119. The importance of phosphorylation at these residues has been studied using a P mutant with mutations at S116L, S117R, S119L and studying the ability of these mutant P protein to drive RSV RNA synthesis. This mutant P was only slightly less
active than wild type P at replication, while there was no defect in transcription [11, 66, 112]. The second cluster is found at the C-terminal of the protein and includes serines 232 and 237 [113, 114]. Serine 232 is the major phosphorylation site of the protein containing 70% of the P protein phosphorylation [114]. Mutation of serines 232 and 237 negatively affect the ability of P to function in an *in vitro* transcription assay [11, 113-117]. Use of mutant P lacking phosphorylation at S232 and S237 residues did not affect RNA synthesis in a minigenome assay. Incorporation of this mutation to a virus does not reduce viral transcription, replication or titers in tissue culture although it does reduce titers in mice [66]. These results suggest that while S232 and S237 may modulate transcription, phosphorylation at these sites is not necessary for viral RNA synthesis. High turnover phosphorylation sites have been found at the N terminus of the protein spanning amino acids 1-90 and 104-160. Of the sites identified, only phosphorylation at serine 54 and threonine 108 seemed to be important for P function. Phosphorylation of S54 may be important for viral uncoating, while phosphorylation of T108 is important for the interaction between P and M2-1. Mutation of this threonine reduces RNA synthesis in a minigenome based assay [12, 118-120]. Phosphorylation at serine 215 has also been suggested [116]. Mutation of this serine did not reduce P protein function in transcription.

It is well known that cellular proteins are important in the RSV life cycle [45, 46, 121]. Since P does not autophosphorylate and none of the RSV proteins have been found to phosphorylate P in the cell, it is believed that host kinases phosphorylate the protein *in vivo* [111, 113, 115]. Indeed, *in vitro* kinase assays demonstrate that casein kinase II
(CKII), a cellular protein, is capable of phosphorylating P at serine residues 232 and 237 [115, 122]. Further evidence of the importance of CKII in the phosphorylation of P was provided by experiments that demonstrated that phosphorylation of P in vitro is inhibited by CKII antibody [115, 122]. In addition, casein kinase I (CKI) may be able to phosphorylate P protein at serine residue 215. However, the identity of the kinase that phosphorylates P in vivo remains unknown. Similarities between casein kinase II and the kinase that phosphorylates P during infection suggest that CKII can also phosphorylate P in vivo [113, 122]. How many kinases contribute to the phosphorylation of P in vivo is not known. We have found that Akt can phosphorylate P in an in vitro kinase assay. The role of Akt phosphorylation in the function of the P protein was explored in this study.

Akt is a serine/threonine protein kinase that has three isoforms: Akt1/α, Akt2/β, Akt3/γ. They all have a pleckstrin homology (PH) domain in their N-termini followed by a central kinase domain and a C-terminal hydrophobic domain. Akt belongs to the AGC family of kinases along with its homologs PKA and PKC. The known pathway that leads to Akt activation requires the phosphorylation of phosphatidylinositol-4,5-biphosphate (PIP₂) by phosphatidylinositol 3 kinase (PI3K) to produce phosphatidylinositol-3,4,5-triphosphate (PIP₃). PIP₃ is a high affinity ligand of Akt and recruits Akt to the plasma membrane. Once in the membrane, Akt is phosphorylated by PDK1 at threonine 308. This phosphorylation is necessary for the activation of Akt. Further phosphorylation of Akt by mammalian target of rapamycin complex 2 (mTORC2) at serine 473 increases the kinase activity of Akt. The phosphorylation motif recognized by Akt is reported to be R-X-R-X-X-S/T-B where X is any amino acid and B is a bulky hydrophobic residue. Akt
can also phosphorylate R-X-X-S/T sites. However, substrates for Akt have been found that do not contain these sequences [123, 124].

Akt is a multifunctional protein that leads to cell survival, cell growth, cell proliferation, angiogenesis, cell metabolism, and cell migration. It promotes cell survival by blocking pro-apoptotic pathways at different steps. It phosphorylates and inhibits pro-apoptotic Bcl2 family members. It phosphorylates transcription factor forkhead box O (FOXO) family members inhibiting the transcription of FOXO activated genes that promote apoptosis. Akt also phosphorylates HDM2 to inhibit p53-induced apoptosis by targeting it for degradation. It can phosphorylate procaspase 9 to inhibit its cleavage. Akt also promotes cell growth or an increase in cell mass by phosphorylating tumor sclerosis complex 2 (TSC2). As a result, TSC2 negatively regulates mTORC1, which regulates cell growth. Akt promotes cell proliferation by phosphorylating p21 and p27, among several targets. Phosphorylation of these proteins inhibits their effect on cell cycle arrest. Additionally, Akt stimulates the production of VEGF and activation of endothelial nitric oxide synthase (eNOS) to stimulate angiogenesis. Finally, Akt can change cellular metabolism by promoting glucose uptake and altering glucose metabolism within the cell. These are only some of the ways in which Akt regulates cell processes [123]. Here we propose a new role for Akt in RSV replication.

Uncontrolled regulation of the Akt pathway has been associated with many types of cancer, including gastric, pancreatic, ovarian and breast cancers [125-127]. Because of this, the Akt pathway has been targeted in anti-cancer drugs [128]. There are many Akt
inhibitors commercially available and some that are undergoing clinical trials [129, 130].

The research discussed here adds a new role for Akt in virus growth but more importantly proposes a new role for the Akt inhibitors in the control of RSV infection.
3.3 Materials and Methods

3.3.1 Viruses, cells and plasmids

A549 and BSR-T7 cells were grown in Dulbecco’s modified Eagles’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml of penicillin and 100 μg/ml of streptomycin (1% P/S) at 37°C and 5% CO₂. To maintain expression of the T7 RNA polymerase, BSR-T7 cells were also supplemented with 0.4 μg/ml of G418 (Geneticin, Invitrogen). The RSV A2 virus was a gift from Dr. Brian Murphy (National Institute of Allergy and Infectious Diseases). The RSVeGFP and RSV-Luc viruses were a gift from Dr. Michael Teng (Department of Internal Medicine, University of South Florida School of Medicine). These recombinant viruses were made by cloning eGFP or Renilla luciferase gene between the leader and NS1 gene and rescued as described before [89]. All RSV viruses were grown and titered in Vero cells. All virus infections were done in Opti-MEM I Reduced Serum Medium supplemented with 2% FBS and 1% P/S. The AKT1 dominant negative (DN) mutant construct, pMT2-AH-AKT1 (PH-AKT) contains the PH domain of Akt from amino acids 1 to 147 and a Myc tag as described in [131]. The kinase dead Akt1 dominant negative (AAA-Akt1), described previously by Srinivas, et al [132], contains three alanine mutations: K179A in the ATP binding site and T308A/S473A in the Akt activation sites and includes an HA tag for detection. The pET15b-RSV P plasmid was made by cloning the RSV P gene from strain A2 into pET15b vector (Novagen) downstream of the T7 promoter and Histidine (His) tag. The pET15b-S86A was made by PCR mutagenesis of the pET15b-RSV P plasmid. pCAGGS expression vector [83] was used to clone the coding sequence for the rA2 P (pCAGGS-
P) and the Akt1 with a Flag tag (pCAGGS-Akt Flag).

### 3.3.2 Fluorescence Microscopy

To understand the effect of the inhibition of Akt by small molecules on RSV protein expression, A549 cells were infected at a multiplicity of infection (MOI) of 3. After infection, medium was replaced with 2% FBS, 1% P/S Opti-MEM containing the Akt inhibitor IV at the concentration indicated. At 1 day post-infection the cells were photographed using a Nikon Eclipse TE300 Inverted Microscope.

### 3.3.3 Akt inhibitors, siRNA and dominant negative mutants

Akt inhibitor IV, Akt 1/2 isotype-selective inhibitor, Wortmannin and casein kinase II inhibitor from Calbiochem were dissolved in DMSO and diluted to the concentration indicated in the Figure legends with 2% FBS and 1% P/S supplemented Opti-MEM after virus absorption. DMSO was used as a negative control of the inhibitor’s effect.

Small interfering RNA (siRNA) against the three isoforms of Akt and a non-target control were purchased from Dharmacon. A549 cells in 24 well plates were transfected with 100 nM of Akt1, Akt2 or Akt3 siRNA and Oligofectamine reagent from Invitrogen. siRNA-Oligofectamine complexes were prepared and transfected according to the manufacturer’s protocol. The culture media of transfected cells was changed to 2% FBS and 1% P/S supplemented Opti-MEM after overnight incubation. Two days post-transfection, the cells were infected with RSV-Luc for the luciferase assay at an MOI of
1. For immunoprecipitation, cells were transfected in 6 cm plates with 1 μM of siRNA as mentioned above and infected 2 days post-transfection with rA2 at an MOI of 1.

Akt1 dominant negative mutants AAA-Akt1 and PH-Akt were transfected into A549 cells using Lipofectamine and Plus reagent (Invitrogen). Six μg of plasmid DNA were used per 6 cm plate for the immunoprecipitations and 400 ng of plasmid DNA per well of a 24 well plate were used for the luciferase assays. Transfection was done according to manufacturer’s recommendations.

3.3.4 RT-PCR

To quantify the expression of the different Akt isoforms in A549 cells an RT-PCR was performed. Total RNA was purified from A549 cells using Qiagen RNeasy kit. Reverse transcription of RNA was carried using oligo(dT). Equal amount of the cDNA was used for a staged-PCR using Akt1, Akt2, Akt3 and GADPH specific primers for 20, 30 or 40 cycles.

3.3.5 Luciferase assays

A549 cells in 24 well plates were transfected with Akt siRNA or Akt1 dominant negative mutant and infected with RSV-Luc as described above. At 1 day post-infection, the cells were lysed and analysed for luciferase activity using the Renilla luciferase assay system from Promega (Madison, WI) according to the manufacturer’s protocol. Following the luciferase assay, lysates were diluted 1:1 with protein lysis buffer (2% sodium dodecyl sulfate, 62.5 mM Tris-HCl [pH 6.8], 2% dithiothreitol) to confirm Akt knockdown by
siRNA. Up to 100 μl of lysate were used for the SDS-PAGE. The Akt isoforms were identified by immunoblotting with mouse monoclonal anti-Akt1 (2H10), rabbit anti-Akt2 or rabbit anti-Akt3 antibodies from Cell Signaling Technology. To detect the expression of the Akt dominant-negative mutants, lysates from the luciferase assay were collected and diluted as above. PH-Akt and AAA-Akt dominant-negative mutants were detected using anti-Myc tag or anti-HA tag antibody respectively.

3.3.6 Cell viability assays

A trypan blue exclusion assay was performed to examine the viability of cells after treatment with Akt inhibitors. For this, A549 cells in 12 well plates were treated with DMSO, Akt inhibitor IV (1 μM) and Akt 1/2 isotype-selective inhibitor (10 μM). At 1 or 2 days post-treatment cells were trypsinized and combined with floating cells in the media. Cells were spun at 1,000 rpm in a Beckman Coulter Microfuge® 18 (Fullerton, CA) centrifuge for 15 min. Pellets were resuspended in PBS. Aliquots (10 μL) of the cell suspensions were stained with 10 μL of 0.4% trypan blue (Avocado Research Chemicals Ltd, Ward Hill Massachusetts). Stained cells were loaded into a hemacytometer. Four squares of the hemacytometer were counted and averaged for each triplicate. The triplicate values were averaged and the standard error of the mean was calculated for each sample.

A Cell Titer-Glo assay, which measures cellular ATP levels, was performed to test the viability of cells after treatment with inhibitors against Akt, casein kinase II and PI3K. A549 cell in 96 well plates were treated with DMSO, Akt inhibitor IV, Casein kinase II
inhibitor 1 and Wortmannin at the concentrations indicated. One and two days post treatment a Cell Titer-Glo assay was performed according to the manufacturer’s recommendations.

### 3.3.7 Immunoprecipitation and co-immunoprecipitation

To study the effect of Akt inhibition on P protein phosphorylation, A549 cells were infected with RSV at an MOI of 3. One day post-infection, the cells were incubated in DMEM in the absence of either cysteine and methionine or phosphate for 30 minutes at 37°C. Labeling was done in media containing 35S-Met/35S-Cys or 32Porthophosphate for 4 hours at 37°C. Cells were lysed with radioimmunoprecipitation assay buffer (RIPA buffer), containing 0.3 M NaCl, 1% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 0.1% (w/v) sodium dodecyl sulfate (SDS), 0.1M Tris-HCl (pH 7.4), 1 mM phenylmethylsulphonyl fluoride (PMSF), 210 ng/ml aprotinin (0.24 trypsin inhibiting U/mL) and 10 mM iodoacetamide. Lysates were cleared by high speed centrifugation at 14,000 rpm for 30 minutes at 4°C. Proteins were immunoprecipitated by incubation with goat anti-RSV antibody RSV #PAB7133P (Maine Biotechnology) and sepharose G beads for 3 hours at 4°C. Immunoprecipitated proteins were washed with RIPA buffer containing 0.3 M and 0.15 M NaCl and separated in a 10% SDS PAGE and visualized with a PhosphorImager Storm System (Storm 860, Molecular Dynamics).

To study the effect of Akt inhibition by siRNA or Akt dominant-negative mutant in RSV protein expression, A549 cells were transfected with Akt1 siRNA or Akt dominant-negative mutants as described above. At 1 day post-infection, the cells were labeled for
three hours with $^{35}$S-Met/$^{35}$S-Cys and the proteins immunoprecipitated with monoclonal D6 antibody against P. The monoclonal antibody D6 was generated from mice injected with P purified from \textit{E. coli}. After immunoprecipitation the proteins were separated in a 15% SDS PAGE and visualized with a PhosphorImager Storm System (Storm 860, Molecular Dynamics).

To identify the sites of phosphorylation in P during virus infection, A549 cells were infected with rA2 at an MOI of 1. One day post-infection, the cells were lysed with whole cell extract buffer (WCEB; 1 M Tris [pH 8.0], 280 mM NaCl, 0.5% NP-40, 2 mM EGTA, 0.2 mM EDTA, 10% glycerol, 1X protease inhibitor cocktail, 0.1 mM phenylmethylsulfonyl fluoride) and treated with okadaic acid (0.25 uM). Lysates were immunoprecipitated with monoclonal anti-P antibody (clone D6). Proteins were separated in a 15% SDS PAGE and visualized by Coomassie Blue staining. The band corresponding to P was cut out and prepared for mass spectrometry as described below.

To determine if Akt1 and P can interact, BSR-T7 cells in 10 cm plates were transfected with pCAGGS-Akt1 Flag (2 $\mu$g), pCAGGS-P (7 $\mu$g) or both using Lipofectamine and Plus reagent (Invitrogen) according to manufacturer’s recommendations. One day post-transfection the cells were labeled with $^{35}$S-Met/$^{35}$S-Cys as described above and the protein co-immunoprecipitated with mouse anti-Akt1 clone 2H10 (Cell Signaling Technology), or rabbit anti-RSV P (produced against sequence IKGFTSPKDPKKKC from the N terminus of the protein). Proteins were separated in a 15% SDS PAGE and visualized with a PhosphorImager Storm System (Storm 860, Molecular Dynamics).
3.3.8 Purification of *Escherichia coli*-expressed RSV P protein

Purification of bacterially expressed phosphate free P protein. The pET15b-P and pET15b-S86A P plasmids were expressed in BL21(DE3)/pLysS competent cells in Luria Bertani (LB) media supplemented with ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml) for selection. Production of the 6xHis-RSV P or 6xHis-S86A P proteins was induced by treatment of the cells with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 hours to produce the T7 polymerase. Bacteria were pelleted by high speed centrifugation and lysed with 50 mM Tris-Cl pH 8, 0.05% Tween-20, 200 mM NaCl, 5 mM imidazole and sonicated in ice. Bacterial lysate was loaded into a Polyprep chromatography column (BioRad Cat No. 731-1550) containing 2 mL of the resuspended His•Bind® Ni-charged resin (Novagen Cat no. 71035). The column was washed 4-8 times with the lysis buffer and increasing concentrations of imidazole to release histidine-containing proteins. The P protein was eluted in 50 mM Tris-Cl pH 8, 0.05% Tween-20, 200 mM NaCl, 200 mM imidazole. The different fractions were separated in a 10% SDS-PAGE and visualized by staining with Coomassie Blue.

3.3.9 *In vitro* kinase assay.

Increasing concentrations of purified wt P or S86A P protein (1.65 μg, 3.3 μg and 4.95 μg) were mixed with recombinant active Akt1 (400 ng) from Upstate Biotechnology, 10 μCi of [γ-32P] ATP (Amersham Biosciences) and 1X Kinase buffer (25 mM HEPES, 25 mM β-glycerophosphate, 25 mM MgCl2, 2 mM dithiothreitol, and 0.1 mM NaVO3) and incubated for 2 hours at 30°C. The reaction was stopped by addition of protein loading buffer. Proteins were separated by 10% SDS-PAGE and phosphorylated proteins
visualized using PhosphorImager Storm System (Storm 860, Molecular Dynamics).

### 3.3.10 Mass spectrometry

To identify the site of Akt phosphorylation in the P protein, a cold *in vitro* kinase assay was performed with ATP (200 mM), 1 X kinase buffer, active recombinant Akt1 (4 μg) and RSV P (16 μg). The mixture was incubated for 2 hours at 30°C. The reaction was stopped by addition of protein loading buffer. The proteins were separated by a 10% SDS PAGE and the gel stained with Coomassie Blue. The P protein band was sliced into 1 mm² cubes, destained and dehydrated using 100 mM NH₄HCO₃ in 50% acetonitrile. Disulfide bonds were reduced and alkylated with 10 mM DTT in 25 mM NH₄HCO₃ and 55 mM iodocetamide in 25 mM NH₄HCO₃ respectively. Protein in the gel was digested with trypsin (12.5 ng/ml in NH₄HCO₃) and the resulting peptides extracted with 5% formic acid in 50% ACN. Samples were sent to the Yale Cancer Center Mass Spectrometry Resource and W.M. Keck Foundation Biotechnology Resource Laboratory, Yale University for TiO₂ enrichment and liquid chromatography-tandem mass spectrometry (LC-MS/MS). The Mascot database was used to identify the peptides [133].
3.4 Results

3.4.1 Inhibition of Akt reduces RSV protein expression

Previous studies by Sun et al [134] have shown that Akt is involved in nonsegmented negative-stranded RNA virus (NNSV) RNA synthesis. To examine the role of Akt during RSV RNA synthesis and protein expression, A549 cells were infected with RSV\textsubscript{eGFP} and treated with different Akt inhibitors after virus absorption. The results seen in Figure 3-1A show a reduction in GFP expression after treatment with Akt inhibitor IV in cells photographed at 1 and 2 dpi. The PH domain dependent, Akt 1/2 isotype-selective inhibitor has a lesser effect on RSV\textsubscript{eGFP} expression. To better quantify this reduction in RSV protein expression, A549 cells were infected with RSV-Luc and treated as above. A \textit{Renilla} luciferase assay was performed 1 dpi and the results are presented in Figure 3-1B. In the graph, luciferase activity from inhibitor-treated cells is normalized to DMSO treated cells. There is more than 50% reduction in luciferase activity in cells treated with 0.5 \(\mu\)M of the Akt inhibitor IV and over 80% reduction when the cells are treated with 1 \(\mu\)M of the inhibitor. Inhibition of PI3K with wortmannin shows only about 30% reduction in luciferase activity at the highest concentration tested. An inhibitor to casein kinase II, a kinase that can phosphorylate P \textit{in vitro} at serine 232 [115, 122], can inhibit luciferase activity only at very high concentrations. These results were confirmed by immunoblotting of lysates from infected cells treated with Akt IV inhibitor (Figure 3-1C). Cells treated with Akt inhibitor IV showed a dramatic reduction in P protein expression. Treatment of cells with Akt inhibitor IV and, to a lesser extent Akt 1/2 isotype-selective inhibitor also reduced viral titers (Figure 3-1D). As shown in
Figures 3-1E and F, the concentration of Akt inhibitors used in this study were not found to be toxic to A549 cells at the times tested using trypan blue staining and a Cell Titer-Glo Assay. Of note, A549 cells treated with Akt inhibitor IV at 1 μM concentration had 25% less live cells one day post-treatment than DMSO treated cells as measured using the Cell Titer Glo-Assay. At that concentration, there is over 80% reduction in RSV-Luc activity. Using the trypan blue assay this inhibitor was not found to be toxic at this time-point.

The effect of Akt inhibition on RSV protein expression was confirmed using small interfering RNAs (siRNA) against all three Akt isoforms, and Akt dominant negative mutants. Akt1 is the predominant isoform expressed in A549 cells, followed by Akt2, while Akt3 is barely detectable by reverse transcriptase PCR (Figure 3-2A). Knockdown of Akt1 and Akt2 expression reduced protein expression of the RSV as measured by luciferase activity (Figure 3-2B). As expected, siRNA against Akt3 did not have an effect on RSV protein expression in A549 cells. Knockdown of Akt by the siRNA was confirmed by immunoblotting. Immunoprecipitation of 35S labeled proteins from infected cells expressing Akt1 siRNA also showed a reduction in RSV protein expression in comparison to the non-target siRNA control (Figure 3-2C). To further confirm these results A549 cells were transfected with Akt dominant-negative mutants. The PH-Akt contains only the PH domain of Akt, while the AAA-Akt dominant-negative encodes Akt with a triple alanine mutation: K179A in the ATP binding site and T308A/S473A in the Akt activation sites. A549 cells were transfected with an Akt dominant-negative or empty pCAGGS vector and infected with RSV-Luc two days later. At one day post infection,
the cells were examined for luciferase activity. Luciferase activity of RSV-Luc was reduced over 40% after AAA-AKT transfection (Figure 3-2D). In contrast, the PH-Akt dominant-negative had a much lesser effect, consistent with the results observed with the PI3K inhibitor in Figure 3-1B and the PH domain dependent Akt 1/2 isotype-selective inhibitor in Figures 3-1A and B. These results suggest that Akt is important for RSV protein expression. In addition, RSV infection may be activating the Akt pathway in a PI3K-PH domain-independent manner.

3.4.2 The P protein is a target of Akt

To examine the role of Akt in RSV protein expression, the target of Akt during viral RNA synthesis was investigated. The proteins involved in RSV RNA synthesis are the N, P, L and M2-1 protein. While there is no evidence for L or N protein phosphorylation, several studies show phosphorylation of P and M2-1 [65, 135, 136]. Of these, the phosphoprotein is the most heavily phosphorylated protein in the virus [65]. The role of P protein phosphorylation during viral RNA synthesis has been the focus of many studies. To determine if Akt was involved in the phosphorylation of P, the effect of Akt inhibitor IV on P protein phosphorylation was examined. As seen in Figure 3-3, inhibition of Akt reduced P protein phosphorylation by 50%, indicating that Akt is involved in this process. In addition, an interaction between Akt1 and P was observed by co-immunoprecipitation in cells expressing both proteins (Figure 3-4). To determine whether Akt could directly phosphorylate P, unphosphorylated P protein with a hexahistidine tag was expressed in E. coli and purified by affinity chromatography to Ni²⁺. Purification of the protein was confirmed by SDS-PAGE and Coomassie blue staining.
The recombinant P protein was then incubated with recombinant active Akt from Upstate Signaling for an *in vitro* kinase assay. The results shown in Figure 3-5B, show that P is a substrate of Akt in vitro.

### 3.4.3 Mapping the Akt phosphorylation site within P

A scan of the P protein using scansite (scansite.mit.edu) revealed serine 86 as a possible phosphorylation site for Akt. No other phosphorylation sites were predicted using this software. To identify the site of Akt phosphorylation of P, a cold *in vitro* kinase assay was performed. The resulting Akt-phosphorylated P protein was digested with trypsin and analyzed by liquid chromatography, tandem mass spectrometry (LC MS/MS). The results are presented in Figure 3-6A and B. One phosphopeptide with the sequence 82-KPLVpSFKEDPTPSDNPFSK-100 was identified through the Mascot database. The phosphorylated serine was found at position 86. Mass spectrometry analysis covered 95% of the protein and no other phosphorylated serines or threonines were detected. To confirm these results, recombinant RSV P protein with a serine to alanine mutation at position 86 was expressed in *E. coli* and purified as before. An *in vitro* kinase assay with active Akt1 showed a severe reduction in phosphorylation of the S86A mutant P protein in comparison to wild type P, an effect that was maintained at increasing concentrations of the P proteins (Figure 3-6D). These results suggest that serine 86 is the major site of Akt phosphorylation. Kalign software analysis (www.ebi.ac.uk/kalign) shows that S86 is well conserved in human RSV subtype A and B and closely related bovine and ovine RSV P proteins. Conservation of this residue is also seen in the pneumonia virus of mice and the human metapneumovirus (HMPV) using the Kalign 2.0 software (Figure 3-6C).
3.4.4 P protein phosphorylation in infected cells

Studies by other labs have shown two major clusters of P protein phosphorylation. One is in the middle of the protein and includes serines 116, 117 and 119, the other is in the C-terminus and is comprised of serines at position 232 and 237. So far no phosphorylation has been detected in serine 86. To examine whether serine 86 is phosphorylated in RSV-infected cells, A549 cells were infected with RSV and the P protein immunoprecipitated one day post-infection. The P protein was digested in gel with trypsin and analyzed by LC MS/MS. The results are shown in Figure 3-7. Three phosphorylated serine residues were identified by LC MS/MS: S86, S215 and S232. Phosphorylation at serine 86 was seen in two different phosphopeptides with sequences 82 - KPLVpSFK-88 and 82 - KPLVpSFKEDPTPSDNPFSK - 100. The MS/MS spectrum for the latter phosphopeptide can be seen in Figure 3-7B. This is the first study that has detected phosphorylation at serine 86 of P from virus-infected cells. Serine 232 has been identified as the most frequently phosphorylated residue of the P protein and casein kinase II has been identified as the kinase responsible for its phosphorylation [115, 122]. Phosphorylation of this serine was seen in three different phosphopeptides with the sequences 206- MAKDTSEVLNPTSEKLNLLLEGNDpSNDLDSL - 241, 209 – DTSDEVSLNPTSEKLNLLLEGNDpSNDLDSL - 241 and 223 – LNNLLEGNDpSNDLDSL - 241. The MS/MS spectrum for the latter phosphopeptide is shown in Figure 3-7D. Dupuy, et al. identified casein kinase I as the kinase phosphorylating S215 [116]. This serine residue was phosphorylated in only one phosphopeptide of the sequence 209 - DTSDEVpSLNPTSEK - 222. Two other peptides containing both serine 215 and serine 232 did not show phosphorylation at serine 215.
3.5 Discussion

The role of phosphorylation for the function of paramyxoviruses P protein has mainly been studied in RSV and Sendai virus (SeV). During SeV infection the P protein is constitutively phosphorylated at serine 249, with phosphatase-regulated phosphorylation at multiple other sites in the protein [137-140]. Mutation of serine 249 to an alanine does not have an effect on viral RNA synthesis \textit{in vitro}, or viral titers in infected cells or in mice, suggesting that phosphorylation at this site is not important for P protein function [138]. Interestingly, mutation of the primary phosphorylation site increases phosphorylation in the other sites of the protein. In addition, mutation of proline 250 to alanine abolishes phosphorylation at S249 suggesting that phosphorylation at S249 is by a proline-directed kinase. However, this P250A mutant is not deficient in driving SeV minigenome expression indicating that phosphorylation of P by the proline-directed kinase is not important for viral RNA synthesis [138, 139]. Mutation of the other known phosphorylation sites in the protein did not reduce RNA synthesis [141]. All of this suggests that specific phosphorylation sites of the P protein are not important for the role of P during transcription and replication. However, studies by Huntley, et al have shown that inhibition of SeV P protein phosphorylation by PKC-ζ reduced viral RNA synthesis and titers [142]. Thus, suggesting that phosphorylation by PKC-ζ at a yet to be discovered site in the protein is important for P protein function.

In the case of the RSV phosphoprotein, phosphorylation in the middle terminal cluster does not seem to be important \textit{in vitro} or in virus infected cells [66, 117]. Phosphorylation at the C-terminus of the protein, specifically at serine 232, is important
for transcription in vitro [111]. Incorporation of this mutation into a recombinant virus does not affect virus growth in HEp-2 cells, suggesting that phosphorylation of serine 232 is not necessary for RNA synthesis, but may modulate this process [66]. Phosphorylation of T108 has been shown to modulate the interaction between M2-1 and P protein. Phosphorylation at this site disrupts the interaction between P and M2-1. A mutant P with a mutation at T108 has less viral RNA synthesis in a minigenome based assay [118]. Since the interaction between P and M2-1 is important during transcription, phosphorylation and dephosphorylation of this site may be a way to regulate the switch between transcription and replication of the viral genome. In addition, high turnover phosphorylated residues have been found in the N-terminus of the protein. Mutation of all of these residues to alanine did not have an effect on viral RNA synthesis, although S54 may be involved in viral uncoating [119, 143].

So far, casein kinase II and protein kinase C (PKC) have been identified as RSV and SeV P protein kinases, respectively [115, 122, 142]. Casein kinase II purified from rabbit reticulocyte lysates can phosphorylate the RSV P in an in vitro kinase assay [115]. The PIV5 P protein was also found to be phosphorylated by Akt in an in vitro kinase assay using bacterially purified P and enzymatically active Akt1 [134]. Inhibition of Akt by small molecule inhibitors leads to a significant reduction in P protein phosphorylation. In addition, inhibition of Akt by small molecule inhibitors and Akt1 siRNA lead to a reduction in viral protein expression [134]. Viral titers for PIV5, measles and vesicular stomatitis virus were also found to be reduced after treatment of infected cells with small molecule inhibitors against Akt. This suggests that phosphorylation of P by Akt is
important for viral gene expression of non-segmented negative sense single stranded RNA viruses [134]. In contrast, phosphorylation of the PIV5 P by PLK1 is a mechanism of negative regulation of viral gene expression. A naturally occurring PIV5 mutant with a mutation in the PLK1 phosphorylation site grows faster than wild type and causes considerably more cytopathic damage [144]. All of this suggests that phosphorylation of the P protein is important for modulating this protein’s function during viral transcription and replication.

Here we report that phosphorylation of P by host kinase Akt is important for RSV RNA synthesis. Inhibition of Akt with small molecule inhibitors results in an inhibition of RSV protein expression and titers. While inhibition of Akt can be cytotoxic, the concentrations of inhibitors used were not excessively toxic for the cells at the time-points used in our experiments. Two Akt inhibitors were used in this study. The PH domain dependent Akt 1/2 iso-selective inhibitor, described in [145], had a small effect on RSV gene expression and titers. The Akt inhibitor IV [146] inhibits Akt activation by an unidentified upstream kinase and does not act directly on Akt. This inhibitor had a dramatic effect on RSV gene expression and titers. Recently the target of the Akt inhibitor IV has been called in to question. A study by Dunn, et al suggests that the Akt IV inhibitor does not reduce phosphorylation of the two Akt activation sites T308 and S473. Despite this 4EBP-1 a downstream target of Akt has reduced phosphorylation after Akt inhibitor IV treatment [147]. In a study by Kumar et al, HT-29 cells treated with insulin show oscillatory Akt kinase activity while showing no differences in Akt phosphorylation at the same time points [148]. This suggests that while T308 and S473
have to be phosphorylated in order for Akt to be active, phosphorylation of these sites is not necessarily an indication of Akt kinase activity. In general, specificity of small molecule inhibitors to a single target can be an issue; because of this, siRNA and Akt dominant negative mutants where also used to confirm our results. The reduction in RSV protein expression observed after siRNA inhibition of Akt1 and Akt2 expression confirms the involvement of this host kinase in RSV RNA synthesis. These results were further confirmed using a dominant-negative kinase-dead Akt1. We speculate that the lack of significant inhibition using the PH dominant-negative and the PH domain dependent Akt 1/2 isotype-selective inhibitor is due to activation of Akt through a virus-induced pathway. In PIV5, the L protein has been found to increase Akt activation [149]. It is possible that a similar mechanism is seen during RSV infection and that activation of Akt after virus infection is mediated through two different pathways: a PH domain-dependent PI3K-Akt pathway [103] and another mediated by one or more viral proteins that may not require binding of Akt to the membrane.

The P protein was considered as a target of Akt since inhibition of Akt reduced P protein phosphorylation and Akt was found to directly phosphorylate P \textit{in vitro}. So far, only casein kinase II and casein kinase I have been suggested as RSV P protein kinases [115, 116, 122]. Akt can also phosphorylate the P protein of PIV5 and its inhibition can reduce the titers of VSV, MeV and PIV5. Serine 86 of the RSV P protein was phosphorylated by Akt \textit{in vitro}. Mutation of this serine to alanine resulted in over 80% reduction in P protein phosphorylation by Akt, suggesting that serine 86 is the main site of P protein phosphorylation. No other sites of Akt phosphorylation were found by mass
spectrometry analysis. The protein coverage was 95% and only one threonine at position 18 was not covered. These results are in agreement with a kinase motif scan of the P protein from strain A2 that shows only serine 86 as a predicted site of Akt phosphorylation (scansite.mit.edu). It will be interesting to examine the effect of this mutation on virus growth. For this reason, we are making viruses containing a mutation in the putative Akt phosphorylation site. The growth kinetics, mRNA and protein expression, and P phosphorylation status of this virus will be studied in comparison to wild type. We speculate that mutation of the Akt phosphorylation site will be detrimental for virus growth. Serine 86 was found to be phosphorylated in P protein immunoprecipitated from virus infected cells. Further analysis still needs to be done to confirm serine 86 as the site of Akt phosphorylation during RSV infection.

In the past, RSV infected patients were treated with the nucleoside analog ribavirin with limited success. Patients at high risk for developing severe disease, such as premature babies, are now treated by immunoprophylaxis with monoclonal RSV F protein antibodies. While prevention of lower respiratory tract infection by immunoprophylaxis has been successful, the cost-effectiveness of this method has been questioned [49, 51, 56]. Respiratory syncytial virus is the most common cause of lower respiratory tract infection in infants. Thus, it is imperative to find alternate methods for treating RSV. Studies are already on their way to test the safety of using Akt inhibitors as a treatment for different cancers on humans. Our studies show that targeting Akt can be a new strategy for RSV therapeutics. In addition identifying the site of Akt phosphorylation may aid in the design of RSV vaccines. Many vaccine candidates have been made with
varying degrees of success in attenuating the virus while providing protection against subsequent RSV infections. If mutation of the putative Akt phosphorylation site results in reduced viral RNA synthesis, the mutation could be incorporated into a vaccine candidate.
A

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>Akt inhibitor IV</th>
<th>Akt 1/2 inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 dpi</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>2 dpi</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
</tbody>
</table>
### B

<table>
<thead>
<tr>
<th></th>
<th>Relative luciferase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus only</td>
<td>100%</td>
</tr>
<tr>
<td>DMSO</td>
<td>100%</td>
</tr>
<tr>
<td>0.5 μM Akt</td>
<td>100%</td>
</tr>
<tr>
<td>1.0 μM Akt</td>
<td>100%</td>
</tr>
<tr>
<td>0.5 μM Wortmannin</td>
<td>100%</td>
</tr>
<tr>
<td>1 μM Wortmannin</td>
<td>100%</td>
</tr>
<tr>
<td>1 μM Akt</td>
<td>100%</td>
</tr>
<tr>
<td>10 μM Akt</td>
<td>100%</td>
</tr>
<tr>
<td>20 μM Akt</td>
<td>100%</td>
</tr>
<tr>
<td>30 μM Akt</td>
<td>100%</td>
</tr>
</tbody>
</table>

### C

- UN
- DMSO
- Akt (1 μM)
- CKII (10 μM)

### Actin

### P

### D

#### Log 10 (cfu/mL)

<table>
<thead>
<tr>
<th>Hours post-infection</th>
<th>rA2</th>
<th>rA2 + DMSO</th>
<th>rA2 + Akt inhibitor IV</th>
<th>rA2 + Akt 1/2 inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>48</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### E

#### Viable cells (%)

- DMSO
- Akt inhibitor IV
- Akt 1/2 inhibitor

<table>
<thead>
<tr>
<th>1 dpt</th>
<th>2 dpt</th>
<th>3 dpt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Figure 3-1: Inhibition of RSV by Akt small molecule inhibitors.** (A) A549 cells were infected at an MOI of 3 and treated with DMSO, Akt inhibitor IV (1 μM) or Akt 1/2 isotype-selective inhibitor (10 μM). Expression of GFP was determined by fluorescence microscopy. Cells were photographed 1 (upper panels) and 2 (lower panels) days post-infection. (B) A549 cells were infected with RSV-Luc virus at an MOI=1 and treated with DMSO, Akt inhibitor IV, wortmannin or CKII inhibitor 1 after absorption at the concentration indicated. Cells were lysed 1 dpi and used for Renilla luciferase assay. Graph shown is an average of three independent experiments. Levels of Renilla luciferase activity for Akt inhibitor IV, wortmannin and CKII inhibitor 1 treated cells are expressed as a ratio of the DMSO control treated sample. Error bars correspond to the standard error of the mean (SEM). (C) A549 cells were infected at an MOI of 3 with rA2 and treated as follows: media alone (UN), treated with DMSO (DMSO), treated with 1 μM of Akt inhibitor IV (AKT) or treated with 10 μM of casein kinase inhibitor (CKII). One day post-infection the cells were lysed and lysate used for immunoblotting with polyclonal RSV P antibody. (D) A549 cells were infected with RSV from strain A2 at an MOI of 3 and treated with DMSO, Akt inhibitor IV (1 μM) or Akt isotype-selective 1/2 inhibitor (10 μM). Media was collected at the timepoints indicated. RSV titers were determined using plaque assay. Error bars represent the SEM. (E) Toxicity of Akt inhibitors in A549 cells. A549 cells were treated with Akt inhibitor IV (1 μM), Akt -1/2 isotype-selective inhibitor (10 μM) or DMSO. 1, 2 and 3 days post-treatment (dpt) cell viability was examined with a trypan blue exclusion assay. Error bars are the SEM of triplicate samples. (F) A549 cells were treated with small molecule inhibitors at the concentrations indicated and viability of A549 cells was assessed with a Cell Titer-Glo assay at 1 or 2 days post-treatment (dpt).
**A**

<table>
<thead>
<tr>
<th></th>
<th>Akt1</th>
<th>Akt 2</th>
<th>Akt 3</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycle #</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>20</td>
</tr>
</tbody>
</table>

**B**

![Bar graph showing relative luciferase activity](image)

- Non-target siRNA: Non-target Akt-1 siRNA: Akt-1 siRNA
- Akt-2 siRNA: Akt-2 siRNA
- Akt-3 siRNA: Akt-3 siRNA

- P = 0.004
- P = 0.006
- P = 0.3

**C**

- **siRNA:** Non-target Akt1
- **Antibody:** Anti-Akt1
- **Antibody:** Anti-Akt2
- **Antibody:** Anti-Akt3

- Mock,
- RSV,
- Mock
- RSV

![Western blot images](image)

- Akt1
- Akt2
- Akt3
Figure 3-2: Inhibition of RSV protein expression by Akt siRNA and Akt dominant negative. (A) Expression of Akt isoforms in A549 cells. Total RNA was purified from A549 cells using Qiagen RNeasy kit. Reverse transcription of RNA was carried using oligo(dT). Equal amount of the cDNA was used for a staged-PCR using Akt1, Akt2, Akt3 and GADPH specific primers. Cycle numbers are indicated at the top. Expected PCR fragments are about 380 bp, 280 bp, 330 bp and 200 bp for Akt1, Akt2, Akt3 and GADPH, respectively. (B) A549 cells are transfected with non-target, Akt1, Akt2 or Akt3 siRNA and two days post-transfection cells were infected with RSV-Luc at an MOI of 1. Cells were lysed and used for Renilla luciferase assay 1 dpi. Levels of Renilla luciferase activity for Akt siRNA treated cells are expressed as a ratio of the non-target siRNA treated sample. Graph is an average of three independent experiments. Error bars correspond to the SEM. To confirm the knockdown of Akt by the siRNA western blot was performed using the lysate from the luciferase assay and immunoblotting with the specified antibodies. (C) A549 cells were transfected with Akt1 or non-target siRNA and infected as above. One day post-infection the cells were labeled with media containing $^{35}$S-Met/$^{35}$S-Cys for 3 hours. After lysis the cells were incubated with anti-P (D6 clone), anti-Myc tag and anti-HA tag. Immunoprecipitated proteins were separated with a 15% SDS PAGE and proteins visualized with a PhosphorImager Storm system. (D) A549 cells were transfected with Akt dominant negative mutants and infected with RSV-Luc as described in Material and Methods. Renilla luciferase assay was done 1 dpi. Graph is an average of four independent experiments. Levels of Renilla luciferase activity for dominant negative transfected samples are expressed as a ratio of pCAGGS transfected cells. Error bars represent the SEM. Right panel, proteins in the lysate were separated in a 15% SDS-PAGE and proteins identified by immunoblotting using anti-HA, anti-myc and anti-actin antibodies.
Figure 3-3: Reduction of Akt phosphorylation by Akt inhibitor IV. (A) A549 cells were infected with RSV at an MOI of 3, and 1 day post-infection they were labeled with $^{35}$S-Met/$^{35}$S-Cys or $^{33}$Porthophosphate for 4 hours. The cells were lysed with RIPA buffer and proteins immunoprecipitated with anti-RSV antibody. Immunoprecipitated proteins were run in a 10% SDS-PAGE and visualized with a PhosphoImager Storm System. (B) Phosphorylation of P was quantified using Image Quant. The bars refer to the $^{33}$P signal normalized by the $^{35}$S signal. Level of phosphorylation of Akt inhibitor IV treated samples was calculated as a ratio of the DMSO treated sample. Mean ± SEM of three separate experiments.
Figure 3-4: Interaction between P and Akt1. BSR-T7 cells were transfected with, pCAGGS, pCAGGS-P, pCAGGS-Akt1 Flag or both pCAGGS-P and pCAGGS-Akt1 Flag. One day post-transfection cells were labeled with $^{35}$S and proteins immunoprecipitated with anti-Flag or anti-P antibodies. Proteins were separated in a 15% SDS PAGE and visualized using a PhosphorImager Storm system.
Figure 3-5: Purification of Escherichia coli expressed RSV P protein and in vitro kinase assay.  (A) Purification of bacterially expressed phosphate free P protein. The RSV P gene from strain A2 was cloned into pET-15b vector and expressed in BL21(DE3) pLysS competent cells by induction with IPTG. Bacterial lysate was loaded into a chromatography column containing resuspended Ni-charged His•Bind® resin. After 4 washes with increasing concentrations of imidazole the P protein was eluted. The different fractions were separated in a 10% SDS-PAGE and visualized by staining with Coomassie Blue. M, Marker; U, uninduced bacterial lysate; I, IPTG induced bacterial lysate; F, flow through from column; E, elution (B) In vitro kinase assay. The purified, bacterially expressed P protein was incubated with recombinant active Akt1 in the presence of [γ-32P] ATP for 2 hours at 30°C. Proteins were separated in a 10% SDS-PAGE and phosphorylated proteins visualized using Storm PhosphorImager (Molecular Dynamics)
Figure 3-6: Identification of the Akt phosphorylation site within P. (A) Cold kinase assay. Phosphate free P protein purified from bacteria was incubated by itself or in the presence of recombinant active Akt1 (Upstate Signaling) and 5’-ATP in 1X kinase buffer for 2 hours at 30°C. Proteins were visualized by SDS-PAGE stained with Coomassie blue. The band corresponding to P from the in vitro kinase assay was excised and prepared for LC MS/MS as described in the Materials and Methods section. MS/MS spectra from all peptides generated were analyzed and phosphopeptides identified using Mascot. MS/MS spectra shown is for the only phosphopeptide found, KPLVpSFKEDPTPSDNPFSK. Based on intact mass (+80 amu) the peptide contains a site of phosphorylation. y-ions and b-ions, some with neutral loss of -98 (H3PO4), covering all possible phosphosites verify S86 (position 5 in the peptide sequence) as a phosphosite. (B) Schematic showing the amino acids covered in the LCMS/MS analysis. The serine found to be phosphorylated is highlighted in red. Gray highlighted residues were not covered. (C) Sequence alignment of P proteins from the Pneumoviridae showing the conservation of serine 86. (D) The serine at position 86 was mutated to alanine and the protein expressed in E. coli. The protein was purified by affinity chromatography. An in vitro kinase assay with the recombinant active Akt1 and increasing concentration of wild type P or S86A P (1.65 μg, 3.3 μg and 4.95 μg) was performed in the presence of [γ-32P] ATP or 5’-ATP for 2 hours at 30°C.
MEKFAPEFHG EDANNRATKF LESIKGKFTS PKDPKKDSI ISVNSIDIEV

TKESPITSNS TIINPTNETD DTAGNKPNYQ RKPLVSFKED PTSPDNPFSK

LYKETIETFDDNVEEESYYSEIONDQDN ITARLDRIE KLSEILGMLH

TLVVASAGPT SARDGIRDAM VGILREEMIEK IRTEALMTND RLEAMARLRN

ESEKMAKDT SDEVSLNPTS EKLNNLLEGN D8DNDL5LED F

---

### Relative Abundance vs. Mass (m/z)

- **C**
  - Graph showing relative abundance and mass (m/z) with peaks labeled as y10++, y17++, b9++, b10++ etc.
  - M/z values and peak intensities are indicated.

---

**Legend**

82 - KPLVSFKEDPTSPDNPFSK - 100

---

**Note:**

- **A** and **B** sections of the diagram include protein sequences and an image of a gel or blot, respectively.
Figure 3-7: Identification of phosphosites in P from infected cells. (A) A549 cells were infected with RSV at an MOI of 1. One day post-infection cells were lysed with WCEB and proteins immunoprecipitated with monoclonal anti-P clone D6. Immunoprecipitated proteins were separated with a 15% SDS-PAGE and stained with Coomassie blue. The band for the P protein was excised and processed for LC MS/MS analysis as described in Materials and Methods. (B) Schematic showing the amino acids covered in the LCMS/MS analysis. The serines found to be phosphorylated are highlighted in red. Gray highlighted residues were not covered. (C) MS/MS spectra of phosphopeptide KPLV$_8$FKEDPTPSDNPF$_5$K, covering serine 86. Based on intact mass (+80 amu) the peptide contains a site of phosphorylation. Good series of b- and y-ions covering all possible phosphosites, as well as neutral loss (-98 amu [H$_3$PO$_4$]) from multiple ions, verify S86 (position 5 in the peptide sequence) as a phosphosite. (D) MS/MS spectra of phosphopeptide DTSDEV$_5$LNPTSEK, covering serine 215. Based on intact mass (+80 amu) the peptide contains a site of phosphorylation. Very good series of b- and y-ions covering all possible phosphosites, as well as neutral loss (-98 amu [H$_3$PO$_4$]) from multiple ions, verify S215 (position 7 in the peptide sequence) as a phosphosite. (E) MS/MS spectra of phosphopeptide LNNLLEGND$_9$DNDSL$_2$LEDF covering serine 232. Based on intact mass (+80 amu) the peptide contains a site of phosphorylation. Good series of b- and y-ions covering the two possible phosphosites, as well as neutral loss (-98 amu [H$_3$PO$_4$]) from several ions, verify S232 (position 10 in the peptide sequence) as a phosphosite. (F) Summary of all the peptides found phosphorylated in the MS/MS analysis.
Chapter 4: Summary and Conclusions
4.1 Inhibition of apoptosis by the RSV small hydrophobic protein

Viruses have different strategies to prolong their survival in the cell. One such mechanism is the inhibition of apoptosis or programmed cell death. Previous studies have shown that RSV can inhibit apoptosis early during infection [103, 104]. In Chapter 2, we studied the role of the RSV SH in the inhibition of apoptosis. The small hydrophobic proteins, present in some paramyxoviruses, share no sequence homology with each other. Despite this, so far the SH protein of PIV5 and mumps virus have been found to inhibit TNF-α induced apoptosis [61, 62]. A recombinant PIV5ΔSH virus causes apoptosis by producing TNF-α. The PIV5 or mumps SH protein expressed in cells in the absence of other viral proteins can inhibit the TNF-α pathway that leads to apoptosis. In this study we used two approaches to analyze the role of the RSV SH protein in apoptosis. First, we replaced the PIV5 SH gene with the RSV SH gene from subtype A or subtype B. This recombinant virus did not induce apoptosis in any of the cell lines tested, suggesting that the RSV SH protein is a functional counterpart of the PIV5 and mumps SH protein. Expression of the RSV SH protein in cells in the absence of other viral proteins was able to inhibit the TNF-α pathway that leads to NF-κB activation, suggesting that like its mumps and PIV5 counterparts the RSV SH protein can inhibit TNF-α induced apoptosis. In the second approach we used an RSVΔSH virus to study the function of the protein during RSV infection. This recombinant RSVΔSH virus induced apoptosis in L929 and A549 cells, confirming that the SH protein has a role in the inhibition of apoptosis. We know from previous work that a PIV5ΔSH virus causes increased TNF-α production and apoptosis in L929 cells [61]. We hypothesize that
RSVΔSH also induces apoptosis in L929 cells through TNF-α production. However the same cannot be said for A549 cells since these cells are not sensitive to apoptosis triggered by TNF-α production. To confirm that RSVΔSH induced apoptosis was not due to TNF-α, the levels of TNF-α in the media of RSV or RSVΔSH infected cells was measured by ELISA at different time-points. RSVΔSH infected cells produced TNF-α faster than RSV infected cells, however the highest concentration of TNF-α produced in A549 cells after RSV and RSVΔSH infection was not enough to induce apoptosis in this cell line when the same concentration of exogenous TNF-α was added to the cell media. All this suggests that RSVΔSH induced apoptosis in A549 cells is not induced by TNF-α and that the RSV SH can inhibit more than one pathway that leads to apoptosis. It also suggests that RSVΔSH induces apoptosis by different mechanisms in L929 and A549 cells. Previous studies have shown that Fas and TRAIL are both upregulated during RSV infection. It is possible that the SH protein can inhibit one or both of these pathways.

The mechanism of TNF-α pathway inhibition by the RSV SH is unknown. Apoptosis induced by TNF requires binding of TNF-α to TNFR1 in the surface of the cells. Once activated this receptor can lead to apoptosis and/or NF-κB activation. TNFR1 contains dead domain that interact with downstream proteins TNF-receptor associated via DD (TRADD) and receptor interacting protein serine-threonine kinase 1 (RIP1). These proteins mediate signaling downstream of the TNFR1. The SH is a transmembrane protein and may be interfering with TNFR homotrimerization or interaction of TNFR1 with RIP1 or TRADD. More studies are necessary to elucidate the mechanism of action of the RSV SH protein.
4.2 Role of Akt phosphorylation of P in RSV RNA synthesis

Efficient RSV RNA synthesis requires the nucleocapsid protein (N), the large polymerase protein (L), the phosphoprotein (P), and the M2-1 protein. The P protein is essential for RNA synthesis. It serves as a co-factor for the viral polymerase and as a chaperone for the nucleocapsid protein allowing it to stay in a soluble form. P can also bind the M2-1 protein. This interaction may help bring the M2-1 protein into the polymerase complex to aid in the elongation of the transcript. The interaction between P and the other members of the polymerase brings stability to the complex. The P protein of RSV the major phosphorylated species of the virus and several studies suggest that phosphorylation of the P protein is an important step in making the P active in the transcription process. There are two major cluster of serine phosphorylation in P with slow to moderate turn over rate: serines 232 and 237 in the C-terminal and serines 116, 117 and 119 in the middle of the protein. Several high turnover phosphorylation sites have been identified in the N-terminal and the middle of the protein. Of these, only the phosphorylation at T108 has been found to have a role in viral RNA synthesis in vitro. Casein kinase II can phosphorylate the C-terminal of the protein in vitro and may phosphorylate the protein in infected cells. The kinases responsible for phosphorylation at other sites have not been identified.

In Chapter 4, we identify Akt as a P phosphorylating kinase and examined the role of Akt phosphorylation in RSV RNA synthesis. Inhibiting Akt with the use of small molecule inhibitors, siRNA or Akt dominant negatives reduces RSV gene expression,
suggesting that Akt is important for RSV RNA synthesis. The target of Akt in the virus is the P protein since inhibition of Akt activation reduces P protein phosphorylation by an average of 50%. Akt and P can interact in transfected cells. In addition, Akt was found to phosphorylate P directly in an \textit{in vitro} kinase assay with active Akt1 and unphosphorylated P purified from bacteria. The working mechanism for role of Akt in RSV RNA synthesis is shown in Figure 4-1.

Mass spectrometry analysis identified the serine at position 86 of P as being phosphorylated by Akt \textit{in vitro}. Coverage of the protein was 95% and only the threonine at position 18 was not covered in this analysis. A mutant S86A P had considerably reduced phosphorylation in comparison to wild type P when used in an \textit{in vitro} kinase assay with active Akt1. Still there was some phosphorylation by Akt1 in this assay. This suggests that while S86 may be the major site of Akt phosphorylation, other sites of the protein may also be phosphorylated by Akt. The site of Akt phosphorylation during RSV infection is still unknown; of note, S86 was found to be phosphorylated in virus infected cells, suggesting that this serine may also be phosphorylated by Akt during infection. However, further studies need to be performed to confirm this. Analysis of a S86A P mutant virus may help us understand the role of Akt phosphorylation in RSV RNA synthesis. We speculate that mutation of the Akt phosphorylation site/s within P will result in an attenuated virus. The lack of an RSV vaccine makes research on the control of RSV replication imperative. Our studies suggest that Akt may be a plausible target for RSV treatment and prevention.
Figure 4-1: Working model for the role of Akt in RSV RNA synthesis. RSV replication involves the N, P, L and M2-1 proteins. P protein phosphorylation is important for this process since unphosphorylated P is not active in transcription. Phosphorylation of P by Akt leads to the activation of P. While activation of Akt1 is often through the PI3K pathway activation after RSV infection may also occur in a PI3K-PH domain-independent pathway.
References


77. Bukreyev, A., et al., *Recombinant respiratory syncytial virus from which the entire SH gene has been deleted grows efficiently in cell culture and exhibits site-


Vita
Sandra M Fuentes

EDUCATION
Ph.D., Pathobiology Program, Pennsylvania State University (2004-2010)
BS in General Biology, University of Puerto Rico (1999-2004)

PUBLICATIONS

*co-authors

ACADEMIC HONORS AND AWARDS

• Bunton Waller Award for graduate students (2004-2008)
• NIH Research Supplements for Underrepresented Minorities (2005-2007)
• Alfred P. Sloan Scholarship (2007 - 2010)
• Travel Award for the American Society for Virology Annual Meeting (2007-2010)
• CAS Graduate Student Competitive Grant (Spring 2008)
• Mr. and Mrs. E. Bosworth Grier Scholarship (2009)