VESICULAR STOMATITIS VIRUS INDUCED APOPTOSIS OCCURS BY A MECHANISM INVOLVING THE ACTIVATION OF PRO-APOPTOTIC BCL-2 PROTEINS BAK AND BID AND THE INACTIVATION OF ANTI-APOPTOTIC BCL-2 PROTEINS BCL-\textsubscript{XL} AND MCL-1

BY

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fan, always there to cheer me on and to open my beer—elephant shoe. To my sons, Alexander and Benjamin, thank you for showing me how much fun latex gloves and pipettes can be. I love you both, you have brought great joy to my life.
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<td>Apoptotic peptidase activating factor-1</td>
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<td>BH3</td>
<td>Bcl-2 homology domain 3</td>
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<td>BHK cells</td>
<td>Baby hamster kidney cells</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>DISC</td>
<td>Death-inducing signaling complex</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s Medium</td>
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<tr>
<td>dsRNA</td>
<td>Double-stranded RNA</td>
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<tr>
<td>EM</td>
<td>Electron Microscopy</td>
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<tr>
<td>ERKs</td>
<td>Extracellular signal-related kinases</td>
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<tr>
<td>FADD</td>
<td>Fas-associated protein with death domain</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>G protein</td>
<td>Glycoprotein</td>
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<td>HeLa-EV</td>
<td>HeLa cells stably transfected with pcDNA3.1(+) empty vector control plasmid</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>IFNR-/</td>
<td>Interferon receptor knockout</td>
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<tr>
<td>hpi</td>
<td>Hours postinfection</td>
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<td>JNK</td>
<td>Jun N-terminal kinase</td>
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<tr>
<td>L protein</td>
<td>Large protein</td>
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<td>M protein</td>
<td>Matrix protein</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MAPKs</td>
<td>Mitogen activated protein kinases</td>
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<td>MEFs</td>
<td>Mouse embryonic fibroblasts</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
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<tr>
<td>N protein</td>
<td>Nucleocapsid protein</td>
</tr>
<tr>
<td>P protein</td>
<td>Phosphoprotein</td>
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<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
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<tr>
<td>PARP</td>
<td>Poly(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque forming units</td>
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<tr>
<td>p-JNK</td>
<td>Phosphorylated Jun N-terminal kinase</td>
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<tr>
<td>p-p38</td>
<td>Phosphorylated p38</td>
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<tr>
<td>PPRs</td>
<td>Pathogen-recognition receptors</td>
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<tr>
<td>RIG-I</td>
<td>Retinoic acid-inducible gene</td>
</tr>
<tr>
<td>RLHs</td>
<td>Retinoic acid-inducible gene-like RNA helicases</td>
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<tr>
<td>rM51R virus</td>
<td>Recombinant virus containing M protein that has a methionine to arginine substitution at position 51 of the 229 amino acid sequence</td>
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<tr>
<td>rWT virus</td>
<td>Recombinant virus containing wild-type M protein</td>
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<tr>
<td>SAPKs</td>
<td>Stress-activated protein kinases</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>ssRNA</td>
<td>Single-stranded RNA</td>
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<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
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<td>Vesicular stomatitis virus</td>
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<tr>
<td>WT</td>
<td>Wild-type</td>
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<tr>
<td>WTO</td>
<td>Wild-type Orsay</td>
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ABSTRACT

Pearce, Alicia F.


Dissertation under the direction of Douglas S. Lyles, Ph.D., Professor and Chairman of Biochemistry

In the first chapter of my thesis I address the question of what role Bcl-2 family proteins play in apoptosis induced by vesicular stomatitis virus (VSV) with wild-type (wt) M protein (rWT virus). My results demonstrate that of the two major proapoptotic multidomain proteins Bak and Bax, Bak is more important for the induction of apoptosis by rWT virus. Additionally activation of proapoptotic BH3-only protein Bid through activation of caspase-8 also plays an important role. Finally, inactivation of both antiapoptotic proteins Mcl-1 and Bcl-X_L is required. The inactivation of Mcl-1 involves loss of the protein due to inhibition of new host gene expression, while inactivation of Bcl-X_L involves the activation of a BH3-only protein, presumably Bid. In the second part of my thesis I address the question of the dependence on the mitochondrial pathway of apoptosis induced by M protein mutant VSV. My results demonstrate that in HeLa cells, the mitochondrial pathway plays an important role in apoptosis and that once again Bak, Mcl-1 and Bcl-X_L are involved. In the third part of my thesis I address the question
of whether the stress-activated protein kinases (SAPKs) p38 and JNK play an important role in the induction of apoptosis by rWT virus. My results demonstrate that both SAPKs were activated during the time of induction of apoptosis, but inactivation of p38 or JNK did not affect the rate of the induction of apoptosis by rWT virus. Collectively, these results have shown that VSV activates multiple pathways, some of which are shared by both wildtype and M protein mutant viruses and others which are different.
INTRODUCTION

Many viruses induce cytopathic effects in the cells they infect and this is often due to the induction of apoptosis. However, viral survival is dependent upon effective use of existing host cellular machinery, so it has been debated whether apoptosis is part of the antiviral response or a mechanism for viral dissemination (reviewed in [1-3]). Like many things in nature, the answer here is both. The induction of cell death is a common host immune defense against viral infection and often apoptosis of a few infected cells can protect the whole host [4]. Therefore, many viruses encode inhibitors of apoptosis to avoid this host response [5, 6]. For other viruses, the induction of apoptosis is advantageous and allows viral dissemination [7]. Regardless of the outcome, apoptosis plays a major role in the virus-host interaction. In addition, the cytolytic activity of viruses has the potential for therapeutic applications such as the development of oncolytic viruses for the treatment of cancer [8]. Vesicular stomatitis virus (VSV) is well studied as a prototype for negative-strand RNA viruses, and is an exceptionally potent inducer of apoptosis in a wide variety of cell types [9, 10]. Due to its particularly rapid cytopathic effects, VSV is one of the major viruses being developed as an oncolytic agent [8]. VSV is capable of inducing apoptosis by activation of multiple apoptotic pathways. It is important to determine how these pathways are activated and the role they play in apoptosis induced by VSV in order to understand the virulence and oncolytic activity of the virus as well as to provide a model by which other viruses can be compared.
Replication of VSV

VSV is widely studied as a prototype for negative strand RNA viruses due to its relatively simple structure, high replication capacity, and rapid disease course [11]. VSV is an enveloped, bullet-shaped virus with a single-stranded non-segmented RNA genome that codes for 5 proteins. A complex of the phosphoprotein (P) and the large (L) protein form the viral polymerase. The nucleocapsid (N) protein and the viral polymerase surround the ~11kb RNA genome comprising the virion nucleocapsid. The glycoprotein (G) is located on the outer surface of the envelope and is responsible for interacting with the host cell surface and initiates virus entry into the cell. The matrix (M) protein binds the nucleocapsid to the envelope lipid bilayer. The VSV replication cycle takes place in the cytoplasm of susceptible host cells. Initially the G protein attaches to the host cell membrane initiating penetration via endocytosis. Low-pH-induced membrane fusion in the endocytic compartment releases the parental genome into the cytoplasm where it undergoes primary transcription to initiate production of viral genes. The parental genome is also replicated to make progeny genomes that can either be packaged into the virion or can undergo secondary transcription to enhance the expression of viral proteins. Finally the virus is assembled at the inner surface of the host plasma membrane from which it buds. The early events of attachment, penetration, and primary transcription occur within the first two hours of exposure to virus. The processes of viral replication, secondary transcription and virus assembly occur over the next 12-18 hours and during this time the host cell is responding to the virus infection. By 24 hours post-infection production of virus has finished.
**Antiviral response**

This process of replication occurs in cells susceptible to VSV infection, but not all cells are susceptible. While the virus is entering the cell and replicating, the cell is responding to the viral infection. Initiation of the antiviral response occurs when the pathogen-associated molecular patterns (PAMPs) present in viral proteins and nucleic acids activate host pathogen-recognition receptors (PRRs) (reviewed in [12]). Two families of PRRs known to function in virus recognition are the Toll-like receptors (TLRs) and the retinoic acid-inducible gene (RIG-I)-like RNA helicases (RLHs). Recognition may occur at many different stages of the viral life cycle. For instance, TLR3 and TLR7 are located in the endosome where they recognize dsRNA and ssRNA respectively. Additionally, RLHs reside in the cytoplasm where they recognize various forms of viral RNA including ssRNA and dsRNA. Upon ligand recognition, both TLRs and RLHs initiate the activation of multiple signal transduction pathways, including the IKK and IKKe/TBK1 kinase complexes and stress activated mitogen-activated protein kinase (MAPK) pathways, which are required to activate a wide range of immunoregulatory genes and proteins that initiate the antiviral response [13]. The antiviral response involves the synthesis of cytokines such as type I interferons (IFN-a/ß), interleukins, and chemokines. When cells with a functional antiviral response are infected with virus they produce and secrete interferon. This interferon then binds to neighboring uninfected cells, prompting these cells to enter an antiviral state rendering these cells resistant to virus infection. When cells without a functional antiviral response are infected with virus, they may produce interferon, but the neighboring cells are unable to respond to interferon making these cells susceptible to virus infection. The importance of the interferon response was
shown with mice lacking the receptor for IFN-a/ß. These mice are highly susceptible to infection with VSV leading to ubiquitous and nearly unrestricted viral replication and death within 3 to 6 days. The lethal dose for 50% of infected animals (LD50) is less than 50 PFU in IFN-a/ß receptor knockout mice, as compared to about $10^8$ PFU of VSV in control animals [14]. Additionally, VSV is neurotropic in wt mice, but is able to replicate in every tissue examined in IFNR-/- mice indicating that the IFN response is responsible for tissue tropism by limiting replication of virus in tissues outside the central nervous system. Therefore, the ability to mount an antiviral response often determines the susceptibility or resistance of cells to VSV infection and this is what governs the pathogenesis of the virus.

In addition to governing pathogenesis, the antiviral response forms the premise of oncolytic viral therapy. The basis of oncolytic viral therapy is selectivity of the virus for tumor cells such that the virus kills tumor cells preferentially over normal cells. The basis of selectivity of VSV for tumor cells is that many tumor cells have defective antiviral responses making them susceptible to virus infection [15, 16]. In contrast, most normal cells have intact antiviral responses making them resistant to virus infection. The problem is that infection by wildtype VSV does not induce a robust antiviral response, allowing replication in some normal cells (e.g. respiratory epithelial cells, olfactory epithelial cells, neuronal cells[17]) as well as tumor cells. The reason for this is that wildtype VSV suppresses interferon production. The way that wildtype VSV suppresses interferon production is by producing an inhibitor of new host gene expression as a means of counteracting the host antiviral response. For VSV the inhibitor of host gene expression is
the M protein [18]. Therefore, it is the M protein that suppresses the production of interferon and other antiviral proteins.

Functions of M protein

M protein has two main functions, one relating to structure and virus assembly, and the other to the suppression of new host gene expression. As a structural component, M protein binds the nucleocapsid to the virus envelope and plays an important role in virus assembly. Independent of its role as a structural component, M protein is able to inhibit new host gene expression. The purpose of this inhibition is to suppress the host cell antiviral response [19]. This can be done at the level of transcription, transport and translation. M protein can inhibit transcription by inhibiting initiation of all three host RNA polymerases [20]. More specifically, for RNA polymerase II, M protein is responsible for inactivating the TATA-binding protein of TFIID, thereby inhibiting transcription initiation [21]. M protein also blocks transport of host mRNAs from the nucleus to the cytoplasm by complexing with Nup98 and Rae1 disrupting their function in mRNA export [22, 23]. Additionally, M protein can inhibit translation by altering the eIF4F translation initiation complex [24]. The M protein’s ability to inhibit new host gene expression also enables the M protein to play a role in the regulation of apoptosis.

In previous experiments to determine whether M protein was involved in apoptosis, HeLa and BHK cells were transfected with M mRNA in the absence of other viral components [10]. Additionally, mRNA from an M protein mutant that is defective in the ability to inhibit host gene expression was also transfected. The results from these experiments indicated that wildtype M protein induces apoptosis in the absence of other
viral components, whereas the mutant M protein does not [10]. To compare the effects of these two M proteins in the context of a virus infection, recombinant viruses were engineered. The recombinant viruses were derived from cDNA clones from multiple strains of VSV. These viruses have an identical genetic backbone with the exception of a single point mutation in the M protein DNA sequence. The first virus retains the wildtype M protein sequence and is referred to as the rWT virus. The rWT virus with the wildtype M protein has the ability to inhibit new host gene expression. The second virus is identical to the rWT virus except for a methionine to arginine substitution at position 51 of the M protein sequence. This virus is referred to as the rM51R virus. Studies have shown that this mutation does not effect viral growth, but does render the virus defective at inhibiting new host gene expression. The inability of the rM51R virus to inhibit new host gene expression also renders the virus defective in the ability to suppress the antiviral response [19].

Previous experiments using these two recombinant viruses have shown that unlike the results in M mRNA transfected cells, in the context of a virus infection both the wildtype and mutant M protein viruses induce apoptosis, but the rate of apoptosis differs in different cell types [10]. For instance, in HeLa cells the rWT virus induces apoptosis faster than the rM51R virus, while for BHK and L929 cells the rM51R virus induces apoptosis faster than the rWT virus [10, 25]. These differences are due to the M protein. In HeLa cells, the wildtype M protein accelerates VSV induced apoptosis while in BHK and L929 cells the wildtype M protein delays apoptosis with respect to apoptosis induced by rM51R virus. The different effects of the M protein in different cell types can be attributed to the inhibition of new host gene expression. This was demonstrated by the
infection of HeLa cells with rM51R virus in the presence of actinomycin D which induces apoptosis at a rate similar to that of infection with rWT virus or the addition of actinomycin D alone [26]. The similarity between drugs like actinomycin D and M protein indicate that the inhibition of host gene expression is sufficient to induce apoptosis in some cell types. In contrast to HeLa cells, experiments in BHK cells demonstrated that new host gene expression is required to enter apoptosis quickly following infection with rM51R virus. Again this was demonstrated by treating cells infected with rM51R virus with actinomycin D, which delayed apoptosis to a rate similar to that observed in BHK cells infected with rWT virus [26]. Taken together these results indicate that, for some cell types, the inhibition of new host gene expression is sufficient to induce apoptosis rapidly, whereas for other cell types rapid apoptosis requires the production of new host cellular products. This observation led to the hypothesis that there are two mechanisms for virus induced apoptosis.

**Mechanisms for virus induced apoptosis**

Two types of mechanisms for virus-induced apoptosis were elucidated utilizing the rWT and rM51R recombinant viruses, as well as expression of M protein in transfected cells in the absence of other viral gene products. One mechanism of induction of apoptosis occurs when the M protein inhibits the expression of new host genes to prevent the antiviral response. This inhibition of host RNA and protein synthesis is a signal to the cell that it has been infected causing the induction of apoptosis via the intrinsic (mitochondrial) apoptotic pathway thereby activating the initiator caspase, caspase-9, [26]. This mechanism was thought to be the primary mechanism induced by virus with
wildtype M protein. A second mechanism for virus-induced apoptosis involves the activation of the host antiviral response which activates the initiator caspase, caspase-8, thereby causing the induction of apoptosis via the extrinsic (death receptor) apoptotic pathway [25]. This mechanism was thought to be the primary mechanism induced by M protein mutant viruses. Combined, these results demonstrated that M protein has both pro-apoptotic and anti-apoptotic activity. The pro-apoptotic activity occurs by the inhibition of new host gene expression which activates the mitochondrial pathway. The anti-apoptotic activity also occurs by the inhibition of new host gene expression, which inhibits the host antiviral response, thereby blocking induction of expression of genes involved in the death receptor pathway. These two mechanisms are differentially active in different cell types. For this reason, multiple cell types have been used to explore these different mechanisms.

**Activation of the intrinsic pathway**

Studies in HeLa cells have demonstrated that the wildtype M protein present in the rWT virus induces activation of both caspase-8 and caspase-9-like enzymes [26]. However, in M mRNA transfected cells, inhibition of the caspase-9 pathway inhibited the rate of apoptosis, while inhibition of the caspase-8 pathway did not [26]. Taken together, these data indicate that expression of M protein induces apoptosis via the intrinsic apoptotic pathway in HeLa cells and that caspase-8 is activated via crosstalk with the caspase-9 pathway [26]. In the intrinsic pathway, mitochondria play a central role in the integration and propagation of death signals originating from inside the cell such as DNA damage, oxidative stress, starvation, as well as those induced by chemotherapeutic drugs.
The signals generated by these cellular stresses lead to the disruption of the mitochondrial inner transmembrane potential. This induces the rupture of the outer mitochondrial membrane, resulting in the release of pro-apoptotic proteins from the mitochondrial intermembrane space into the cytoplasm. Released proteins include factors such as the apoptosis-inducing factor AIF, Smac/Diablo, and, most notably, cytochrome c. Upon release into the cytosol, cytochrome c activates the apoptosome, a complex of APAF1 bound to procaspase-9. This activation leads to autocatalytic processing and activation of procaspase-9 initiating a caspase cascade which ultimately activates the effector caspase, caspase-3.

The goal of this project is to determine which signal transduction pathways VSV activates to induce apoptosis via the mitochondrial pathway. My original model for activation of the mitochondrial apoptotic pathway was that M protein is seen as a form of cell damage, thereby activating signaling pathways which in turn activate Bcl-2 family members leading to the permeabilization of the mitochondrial membrane. Permeabilization of the mitochondrial membrane results in the release of pro-apoptotic proteins, including cytochrome c, leading to activation of the apoptosome. The activation of the apoptosome leads to activation of caspase-9 and subsequent activation of caspase-3 leading to apoptosis. Mitochondrial membrane permeabilization is regulated by the Bcl-2 family of proteins. Identification of the Bcl-2 proteins that are important for VSV induced apoptosis will provide insight into the upstream pathways that are responsible for their activation. Contrary to my original model in which rWT virus induces apoptosis primarily via the mitochondrial pathway, my results in Chapter 1 show that rWT virus also depends on elements of the death receptor pathway. Additionally, in Chapter 2 I
show that the rM51R virus depends not just on the death receptor pathway, but also relies on elements of the mitochondrial pathway to induce apoptosis in some cell types. Finally, I found no evidence for a role of stress activated protein kinase (SAPK) signaling in rWT virus induced apoptosis (Chapter 3).

**Bcl-2 Family Proteins**

Bcl-2 family proteins function to either suppress or promote mitochondrial outer membrane permeabilization, thereby regulating the release of pro-apoptotic factors into the cytosol, such as cytochrome c, apoptosis inducing factor (AIF) and Smac/Diablo [31]. Bcl-2 family proteins are subdivided into three groups depending on conservation of Bcl-2 homology (BH) domains and function, reviewed in [32, 33]. The multidomain antiapoptotic Bcl-2 proteins contain BH domains BH1-BH4 and function to inhibit apoptosis by binding to pro-apoptotic Bcl-2 family members. Members of this group include Bcl-2, Bcl-XL, Mcl-1, Bcl-w, and BFL-1/A1. The pro-apoptotic Bcl-2 proteins are comprised of two groups, the multidomain proteins and the BH3-only proteins. Bax and Bak are the two main members of the multidomain group, containing BH domains BH1-BH3. These proteins are primarily responsible for the permeabilization of the mitochondrial outer membrane, if their activity is not suppressed by anti-apoptotic Bcl-2 family members. The BH3-only proteins contain only one Bcl-2 homology domain (BH3) and include Bid, Bad, Bim, Puma, Noxa and Bik, among others. These proteins function as upstream sensors of signaling pathways and convey to other Bcl-2 family proteins the signals to initiate apoptosis. These death signals can be transmitted from the
BH3-only proteins by either binding to anti-apoptotic proteins causing the release of Bak and Bax, or they may bind to Bak and Bax directly, thereby causing their activation [34].

The pathways leading to activation of Bak differ from those that activate Bax. Interestingly, only two anti-apoptotic Bcl-2 proteins, Mcl-1 and Bcl-X\textsubscript{L}, have been shown to interact with Bak, while Bax appears able to interact with all of the anti-apoptotic proteins with the exception of Mcl-1 [35, 36]. BH3-only proteins have strong binding affinities to the anti-apoptotic proteins, indicating a major role may be to derepress Bak and Bax by binding and inhibiting the anti-apoptotic proteins [37]. However, some BH3-only proteins, such as Bid and Bim, can bind to not only anti-apoptotic Bcl-2 proteins but also to Bak and/or Bax leading to speculation that some BH3-only proteins are direct initiators of apoptosis [38, 39]. For some stimuli, such as the protein kinase inhibitor staurosporine, the topoisomerase II inhibitor etoposide, and ultraviolet radiation, Bak and Bax appear to be redundant [40]. In contrast, Bak and Bax were non-redundant in the induction of apoptosis by Neisseria gonorrhoeae and cisplatin such that both were required for apoptosis to occur [41]. In Chapter 1 I determined the relative importance of Bak and Bax in VSV induced apoptosis by transfecting HeLa cells with Bak and/or Bax siRNA and subsequently infecting with rWT virus. My results showed that Bak is more important than Bax in this system. Bak is regulated by Mcl-1, which is rapidly turned over, and Bcl-X\textsubscript{L}, which is relatively stable. M protein-mediated inhibition of host gene expression resulted in the degradation of Mcl-1, but not Bcl-X\textsubscript{L}. However, inactivation of both Mcl-1 and Bcl-X\textsubscript{L} was required for cells to undergo apoptosis. While inactivation of Mcl-1 was due to inhibition of its expression, inactivation of Bcl-X\textsubscript{L} indicates a role for one of more BH3-only Bcl-2 family members.
BH3-only proteins act as sensors that initiate apoptosis in response to developmental cues or stress signals and they may be regulated by either transcriptional control or posttranslational modification. For instance, both Noxa and Puma are regulated at the level of gene expression by the tumor suppressor p53 [42, 43]. While Bad, Bim and Bid have all been shown to be regulated post-translationally. [44-46]. In Chapter 1, I found that Bid and caspase-8 play a role in the induction of apoptosis by rWT virus in HeLa cells, indicating a role for the extrinsic pathway in the induction of apoptosis by virus with wildtype M protein.

**Activation of the extrinsic pathway by M protein mutant virus.**

Previous experiments have demonstrated that despite the inability of the M protein mutant viruses to inhibit new host gene expression, these viruses still effectively induce apoptosis in most cell types [10, 16, 25, 47, 48]. Additionally, the rM51R virus and other M protein mutant viruses show greater selectivity for many types of tumor cells, including prostate, ovarian, and renal carcinoma, over normal cells as compared to the rWT virus as evidenced by decreased pathogenicity in tumor-bearing mice treated with the rM51R versus rWT viruses [16, 49]. This observation makes M protein mutant viruses superior candidates for oncolytic viral therapy [50, 51]. Interestingly, studies using murine L929 cells have demonstrated that rM51R virus induces activation of both caspase-8 and caspase-9 similar to HeLa cells infected with rWT virus [25]. However, unlike rWT virus-infected HeLa cells, in rM51R virus-infected L929 cells, inhibition of the mitochondrial pathway by overexpression of Bcl-2 does not affect the rate of apoptosis, while inhibition of caspase-8 reduces the rate of apoptosis. These data indicate apoptosis
induced by the rM51R virus occurs via the death receptor pathway in L929 cells and that caspase-9 is activated through crosstalk with the caspase-8 pathway [25].

In the death receptor pathway, death ligands (e.g. FasL, TNFa) induce apoptosis through binding and activation of specific death receptors belonging to the superfamily of tumor necrosis factor/nerve growth factor (TNF/NGR) receptors [52]. These death receptors are characterized by a unique intracellular death domain, which recruits adaptor proteins (e.g. FADD- Fas-associated protein with death domain) important for death ligand-induced apoptosis [53]. Recruitment of FADD leads to the recruitment of procaspase-8. The binding of death ligand to its receptor leads to trimerization of the receptors. This trimerization of death receptors and subsequent association of three death domains lead to the formation of a death-inducing signaling complex (DISC). The increased proximity of procaspase-8 to itself in the DISC allows for its autocatalytic cleavage and activation [54]. Active caspase-8 leads to the activation of effector caspase, caspase-3. Recently, dominant-negative mutants of Fas, FADD, an alternative Fas adaptor protein, Daxx, and dsRNA activated protein kinase PKR have been employed to determine the importance of these gene products in rM51R virus induced apoptosis. Additionally, microarray data showed infection with rM51R virus increased the expression levels of Daxx and PKR which explains in part the requirement for new host gene expression to undergo apoptosis via the death receptor pathway. This work indicates that Fas, PKR, and Daxx are more important than FADD in rM51R virus induced apoptosis in L929 cells [55].

In L929 cells, the death receptor pathway appears to be all that is required for the cells to undergo apoptosis as there is no delay in rM51R virus induced apoptosis in L929
cells that overexpress Bcl-2 [25]. However, in HeLa cells that overexpress Bcl-2 there is a reduction in the rate of apoptosis induced by rM51R virus [26]. Additionally, in caspase-9 knockout MEFS and caspase 2/9 KO MEFS there is little if any apoptosis induced by an M protein mutant virus [47]. These results indicate that the cell type may dictate what role the mitochondrial and death receptor pathways play in rM51R virus induced apoptosis. In Chapter 2, I show that the mitochondrial pathway is important for apoptosis induced by rM51R virus in HeLa cells, and that this is likely to be due to cross-talk with the death receptor pathway. The main result from Chapters 1 and 2 is that apoptosis induced by the rWT and rM51R viruses is very similar. The difference in HeLa cells is the turnover of Mcl-1 which occurs rapidly in rWT virus infected cells but does not occur in rM51R virus infected cells leading to faster cell killing by rWT virus.

**Role of stress-activated protein kinases in VSV-induced apoptosis**

Previous experiments in transfected HeLa cells that express wildtype M protein in the absence of other viral components showed that wt M protein induced apoptosis independent of caspase-8 [10]. This result suggests there is an additional signal to activate pro-apoptotic Bcl-2 family members other than Bid. I hypothesized that the stress of the inhibition of new host gene expression would activate one or more stress-activated protein kinases. Mitogen activated protein kinase (MAPK) pathways constitute a large kinase network that regulates a variety of physiological processes, such as cell growth, differentiation, and apoptotic cell death. Thus far, three MAPK pathways have been characterized in detail- the ERK pathway and the SAPK pathways c-Jun NH₂-terminal kinase (JNK) and p38 [56]. The ERK pathway is activated by a large variety of mitogens
and by phorbol esters, whereas the JNK and p38 pathways are stimulated mainly by environmental stress and inflammatory cytokines [57, 58]. The ERK pathway is typically involved in cell proliferation and differentiation while the JNK and p38 pathways have been shown to play a role in apoptosis [59]. Additionally, SAPKs have been shown to activate proapoptotic BH3-only proteins [60, 61]. Additionally, JNK has been shown to be involved in Daxx-dependent apoptosis in response to rM51R virus infection in L929 cells [55]. Therefore, I focused on the JNK and p38 pathways in Chapter 3. In these experiments I show that neither of these pathways are important for rWT virus-induced apoptosis in HeLa cells. This leaves open the question of how M activates the mitochondrial pathway in the absence of other viral components.
MATERIALS AND METHODS

Cell lines. HeLa cells were cultured in Dulbecco’s modified Eagle medium supplemented with 7% fetal bovine serum (FBS). Stably transfected cell lines were generated by transfecting HeLa cells with the h-Mcl-1 pcDNA3 plasmid or the h-Mcl-1-S159A pc3DNA plasmid (gifts of Ulrich Maurer), h-Bcl-XL pcDNA3 (gift of John Wilkinson) or pcDNA3 plasmid for empty vector (EV) cells as previously described [10]. Stably transfected cells were cultured in Dulbecco’s modified Eagle medium supplemented with 7% FBS and 200 µg/ml of G418.

Viruses Wild-type Orsay (wtO) is a naturally occurring, laboratory-adapted strain of VSV. The recombinant viruses rWT and rM51R were isolated from cDNA clones and grown as previously described [10]. Infections were carried out at a multiplicity of infection of 10 PFU per cell.

RNA Interference For silencing experiments, HeLa cells were transiently transfected with Bak siRNA (h), Bax siRNA (h), Bcl-XS/L (h), Bid (h), Control siRNA-A (Santa Cruz Biotechnology) or On-Targetplus Duplex J-004501-17 Human Mcl-1(Dharmacon) using TransIT-siQuest transfection reagent (Mirus Bio Corporation). Briefly, HeLa cells were grown to approximately 75% confluence in 6-well plates and just before transfection, 1250 µL of fresh growth media was added to each well. For each well, a mixture of 250 µL Optimem media, 2 µL TransIT-siQuest transfection reagent and 25nM siRNA was incubated for 20min before adding to the plate. After 24hrs, cells were split into 96-well plates at a density of 8 X 10^3 for cell viability and caspase-3-like activity assays, 6-well plates for cell lysates to confirm silencing and 6- or
24-well plates for time-lapse microscopy experiments. At 48hrs posttransfection, cells were infected with rWT virus.

**Western blot analysis** HeLa cells or siRNA transfected HeLa cells were grown to approximately 75% confluence in 6-well dishes and infected with recombinant viruses. At the indicated times postinfection, cell lysates were generated and analyzed by SDS-PAGE and Western blotting as previously described [55]. The antibodies used in this study were obtained as follows: Bak and Mcl-1 were from Santa Cruz Biotechnology, Bax, Bcl-XL, Bid, JNK, MAPKAPK-2, PARP and p38 were from Cell Signaling Technologies and β-actin was from Sigma-Aldrich Incorporated. Protein band intensities were quantitated by scanning and analysis with Quantity One software (Bio-Rad).

**Time-lapse microscopy** siRNA transfected HeLa cells (described above) were grown to approximately 50% confluence in 6- or 24-well dishes. Cells were infected and incubated for 1hr in 500ul (6-well) or 200ul (24-well) Dulbecco’s modified Eagle medium supplemented with 3% FBS to allow attachment of virus to cells, then transferred to an environmental chamber attached to a Zeiss Axiovert S200. Images were captured at 15-min intervals using a Hamamatsu charge-coupled device camera run by Openlab software (Improvision) and were saved in QuickTime Movie format. Cells were monitored for 16h or 36hr postinfection and cells entering apoptosis as determined by membrane blebbing were quantified.

**Cell viability assays** Cell viability was assessed by MTT assay, according to manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN, USA). SiRNA transfected cells were seeded in 96-well plates as described above. Cell viability
was evaluated as described previously and survival estimated relative to untreated controls [26].

**Caspase-3 activity assay** SiRNA transfected and control cells were grown to approximately 75% confluence as described above. Cells were either infected with rWT virus or treated with staurosporine (SSP) (1 µg/ml; Cell Signaling Technologies) as a positive control for the activation of the mitochondrial pathway. Duplicate wells were lysed and caspase-3 activation was determined with a fluorogenic substrate for caspase-3 (DVED-AFC; R&D Systems, Inc.). Each sample was incubated for 2h with the peptide substrate and fluorescence intensities were measured at excitation and emission wavelengths of 400nm and 505nm, respectively, using a Safire II fluorescent microplate reader (Tecan). Prior to addition of the fluorogenic substrate, 10 µL were removed and used to determine the protein concentration by Bio-Rad DC Protein Assay (Bio-Rad Laboratories).

**Caspase-8 inhibitor** For MTT assays, cells were pretreated for 2hr with a synthetic inhibitor of caspase-8 activity (Z-IETD-CHO; R&D Systems) at a final concentration of 100µM. Cells were infected with recombinant viruses at an MOI of 10. MTT analyses were carried out as described above.

**Inhibition of p38 and JNK activities** Where indicated, cells were pretreated for 2hr with 30, 50 or 100µM of SB203580 (Cell Signaling Technologies) to inhibit p38 activity, or SP600125 (Calbiochem) to inhibit JNK activity.

**Statistical analysis** A paired Student’s t-test was used to compare significance of individual time points. $P<0.05$ was considered statistically significant.
CHAPTER ONE

APOPTOSIS INDUCED BY RWT VIRUS OCCURS BY A MECHANISM INVOLVING THE ACTIVATION OF BAK AND BID AND INACTIVATION OF BCL- XL AND MCL-1

rWT virus induces apoptosis more slowly in HeLa cells lacking Bak than those lacking Bax.

Wild-type strains of VSV induce apoptosis primarily via the mitochondrial pathway [9, 26]. Since the mitochondrial pathway is regulated by the Bcl-2 family of proteins, I determined which Bcl-2 family members play a role in VSV-induced apoptosis. Pro-apoptotic proteins Bax and Bak in their active form are the main proteins responsible for the destabilization of the mitochondrial membrane. Bak and Bax have been shown to be redundant in some systems [40], while in other systems one is more important than the other [41]. To determine the relative importance of Bak and Bax in VSV-induced apoptosis, HeLa cells were transfected with siRNA against either Bak, Bax, or both, or with non-targeting siRNA as a control. At 24, 48 and 72hrs post-transfection, cell lysates were prepared and analyzed by SDS-PAGE and Western blotting. Figure 1A shows a representative western blot for the silencing of Bak or Bax with analysis of actin as a loading control. Levels of Bak and Bax were lowest at 48hrs posttransfection, therefore cells were infected with virus at 48hrs posttransfection for all
Figure 1. rWT virus induces apoptosis more slowly in HeLa cells lacking Bak than those lacking Bax. (A) HeLa cells were transfected with 25nM Bak, Bax or non-targeting siRNA as a control. At the indicated times posttransfection, cell lysates were analyzed by western blotting with antibodies for Bak, Bax and actin as a loading control. (B) HeLa cells were transfected with 25nM Bak siRNA (closed circles), Bax siRNA (closed squares), Bak and Bax siRNA (closed triangles), 25nM non-targeting siRNA (open circle) as a control for single transfection, 50nM non-targeting siRNA (open triangle) as a control for double transfection. At 48hrs post-transfection, cells were infected with rWT virus and analyzed by time-lapse microscopy. The cumulative percentage of cells entering apoptosis was determined as the number of cells that underwent apoptotic membrane blebbing and is plotted as a function of time postinfection. The data represent the average of three experiments. In subsequent assays only one control is shown as the data are almost identical. (C) Caspase-3-like activity was assayed in HeLa cells transfected for 48hrs with the same siRNAs as above, then infected with rWT virus for the times indicated. Caspase-3 activity in cell lysates were measured with a fluorogenic substrate (DEVD-AFC) and is expressed in arbitrary fluorescence units per microgram of total protein, and these data are normalized to the maximum value expressed by HeLa cells incubated with staurosporine (as a positive control) for 12hrs. The data represent the average +/- standard deviation of three experiments. (D) Cell viability was analyzed with an MTT assay. HeLa cells were transfected and infected as above. At the indicated times, MTT was added to each sample for 4hrs at which point a solubilization solution was added. Samples were analyzed with an ELISA plate reader. Cell viability was determined as a percentage of a transfected, uninfected control. The data represent the average +/- standard deviation of three experiments ( *, p< 0.05 compared to control).
subsequent experiments. In the experiments shown in Fig. 1B, cells were infected with a recombinant wild-type (rWT) strain of VSV and imaged using time-lapse microscopy. The graph shows the average of 3 experiments. The percentages of cells entering apoptosis were determined as a function of time postinfection by the time of onset of apoptotic membrane blebbing. rWT virus rapidly induced apoptosis in the control non-targeting siRNA-transfected cells (open circles, open triangles) with over 75% of cells undergoing apoptosis by 16hrs postinfection. A similar timecourse was observed for the Bax siRNA cells (closed squares). However, the rate of apoptosis was delayed in both the Bak siRNA cells (closed circles) and the Bak plus Bax siRNA cells (closed triangles) with less than 30% of cells undergoing apoptosis by 16hrs postinfection.

The delay in the induction of apoptosis in Bak siRNA cells was confirmed by an analysis of caspase-3-like activity in cell lysates using a fluorogenic substrate (Fig. 1C) and by analysis of cell viability using the MTT assay (Fig. 1D) which measures metabolically active cells. For the caspase-3 assay, cells treated with staurosporin (SSP) were used as a positive control. Bax and Bak siRNA cells both had capase-3-like activity when treated with SSP compared to control non-targeting siRNA cells (data not shown). Caspase-3-like activity is presented in Fig. 1C as a percent of maximal SSP activation. For the MTT assay, data are expressed as a percent of mock infected cells (Fig. 1 cells). Control siRNA cells as well as Bax siRNA had high levels of caspase-3-like activity and reduced viability by 16hrs postinfection while the Bak siRNA cells and Bak plus Bax siRNA cells had significantly less active caspase-3 and higher cell viability than control siRNA cells at 10, 12, and 16hrs postinfection (p<.05)(Figs. 1C,D). Collectively, the
data in Figure 1 (B, C, D) show that silencing of Bak expression slows the rate of apoptosis, while silencing of Bax expression does not.

**Mcl-1 levels are reduced in rWT virus infected HeLa cells while Bcl-X<sub>L</sub> levels remain unchanged.**

In healthy cells, Bak is kept in an inactive state by interaction with anti-apoptotic Bcl-2 family members. Only two anti-apoptotic family members, Bcl-X<sub>L</sub> and Mcl-1, have been found to interact directly with Bak. Bcl-X<sub>L</sub> is a relatively stable protein with a turnover rate of over 24hrs [62]. However, Mcl-1 protein and mRNA have been shown to have a rapid turnover rate in many cell types [63]. For example, the half-life of Mcl-1 in cycloheximide-treated HeLa cells was <1hr (data not shown). VSV inhibits new host gene expression due to the ability of M protein to inhibit host transcription, nuclear-cytoplasmic transport and translation (1). I hypothesized that Mcl-1 levels would decay rapidly in VSV-infected cells allowing for the activation of Bak and subsequent apoptosis. To test this hypothesis, HeLa cells were infected with rWT virus and at different times postinfection cell lysates were prepared and analyzed for Mcl-1 by SDS-PAGE and Western blotting (Fig. 2A). There was a visible reduction in the levels of Mcl-1 in rWT virus-infected HeLa cells by 8hrs postinfection with levels below the level of detection by 24hrs postinfection. The timing of reduction in Mcl-1 levels was consistent with the induction of apoptosis (Fig. 1) and with the inhibition of host gene expression [64]. To confirm that the loss of Mcl-1 was due to the shut-off of new host gene expression, HeLa cells were infected with a recombinant M protein mutant virus that is isogenic with rWT virus except for a point mutation in the M protein, which renders the virus defective in the ability to inhibit host gene expression (rM51R virus).
Figure 2. McI-1 levels are reduced in rWT virus infected HeLa cells while Bcl-XL levels remain unchanged. (A) At the indicated times postinfection with rWT virus, cell lysates were analyzed by western blotting with antibodies for McI-1. The graph shows quantitation of McI-1 expression normalized to actin expression as percentages of the average ratio in mock infected cells. The data represent the averages +/- standard deviation from three experiments for cells infected with rWT virus (closed squares) or rM51R virus (open squares) or mock infected cells (open circles). (B) Cells were analyzed for Bcl-XL expression as in (A).
The graph in Figure 2A depicts the quantitation of Mcl-1, expressed as a ratio to actin and normalized to mock infected controls. There was a significant reduction in the levels of Mcl-1 in rWT virus-infected cells (closed squares) by 8hrs postinfection with levels continuing to decline throughout the 24hr timecourse. In contrast, Mcl-1 levels were increased by 4hrs postinfection and were similar to mock-infected cells (open circles) for the remainder of the timecourse in rM51R virus-infected cells (open squares). A similar western blot analysis of Bcl-XL levels showed that, in contrast to Mcl-1, there were no significant changes in the level of Bcl-XL in rWT virus-infected cells at any time postinfection when compared to the level in mock-infected cells (fig 2B). These data support the hypothesis that loss of Mcl-1 is due to its rapid turnover combined with the inhibition of host gene expression by wt VSV.

**Bak levels in rWT infected HeLa cells are not significantly different from mock infected samples, while Bax levels decrease at late times postinfection.**

Apoptosis is regulated by levels of both pro-apoptotic and anti-apoptotic Bcl-2 proteins, therefore I analyzed the pro-apoptotic protein levels as well. Figure 3 shows western blot analysis of pro-apoptotic Bak and Bax following rWT virus infection. There were no significant changes in the level of Bak at any time postinfection (Fig. 3A). Levels of Bax began to decline at 12hrs postinfection and were significantly decreased by 24hrs postinfection (Fig. 3B). The observation that levels of Bak did not decline during infection is consistent with the idea that loss of Mcl-1 contributes to the activation of Bak. Since Bax levels do not have an effect on the rate of apoptosis in rWT virus infected cells (Fig. 1), the decrease in the level of Bax at late times postinfection is not
Figure 3. Bak levels in rWT infected HeLa cells are not significantly different from mock infected samples, while Bax levels decrease at late times postinfection. At the indicated times postinfection with rWT virus, cell lysates were generated and analyzed by western blotting with antibodies for Bak (A) and Bax (B). Representative gels are shown at the top of panels A and B and the graphs show quantitation of Bak (A) or Bax (B) protein expression normalized to actin protein expression and are shown as percentages of mock infected samples. The data represent the averages +/- stand deviation from three experiments for cells infected with rWT virus (closed squares) or mock-infected cells (open circles).
likely to be important in VSV-induced apoptosis in HeLa cells.

**Apoptosis is delayed in Mcl-1 overexpressing cells infected with rWT virus.**

To determine whether the rapid decrease in the level of Mcl-1 contributes to the induction of apoptosis in rWT virus infected cells, HeLa cells that stably overexpressed Mcl-1 were generated. Due to the short half-life of Mcl-1, I also generated cell lines that express a mutant Mcl-1, S159A. This is a phosphorylation site mutant which exhibits enhanced stability and confers increased protection from apoptosis compared to wt Mcl-1 [65]. Two clonal cell lines were generated for both the wt Mcl-1 and the mutant Mcl-1 and are both shown to indicate differences are not due to clonal variation. The cell lines overexpress Mcl-1 compared to an empty vector (EV) control cell line as determined by western blots (Fig 4A). To determine if the increase in Mcl-1 levels delayed the induction of apoptosis by rWT virus, HeLa-wt Mcl-1, HeLa mutant Mcl-1 and HeLa-EV cells were infected with rWT virus, and cells entering apoptosis were quantitated using time-lapse microscopy (Fig. 4C). HeLa-EV cells entered apoptosis with a timecourse similar to untransfected HeLa cells (Fig. 1). Compared to control cells, there was approximately a 1hr delay in the time it took for 50% of cells to enter apoptosis for the two wt Mcl-1-overexpressing cell lines, and a 2hr delay for the two mutant Mcl-1 overexpressing cell lines. The effects of overexpression of Mcl-1 on virus-induced apoptosis were also determined by assaying caspase-3-like activity. In contrast to the relatively modest change in the timecourse of onset of membrane blebbing, the level of caspase-3 activated in HeLa-Mcl-1 cells was dramatically reduced compared to that
Figure 4. Apoptosis is delayed in Mcl-1 overexpressing cells infected with rWT virus. Clonal HeLa cell lines that overexpress wildtype Mcl-1 (Mcl-1-13, Mcl-1-16), a mutant Mcl-1 (S159A-7, S159A-8) or an empty vector control were generated. (A) Cell lysates were analyzed by western blotting using an antibody against Mcl-1. (B) The graph shows quantitation of Mcl-1 expression normalized to actin expression as percentages of the average ratio in mock infected cells. The data represent the averages +/- standard deviation from three experiments. Mcl-1-13 (open squares), Mcl-1-16 (closed squares), S159A-7 (open triangles), S159A-8 (closed triangles) and empty vector cells (closed circles) were infected with the rWT virus. Empty vector mock cells (open circles) were not infected with virus. (C) Mcl-1-13 (open squares), Mcl-1-16 (closed squares), S159A-7 (open triangles), S159A-8 (closed triangles) and empty vector cells (open circles) were infected with the rWT virus. Cells entering apoptosis were analyzed by time-lapse microscopy as described in the legend to Fig. 1. The data represent the averages from three experiments. (D) Mcl-1 overexpressing cells and empty vector cells were infected with rWT virus for the times indicated and caspase-3-like activity was analyzed using a fluorogenic substrate as described in the legend to Fig. 1. The data represent the averages +/- standard deviation from three experiments.
activated in HeLa-EV cells (Fig. 4C). This difference in magnitude of the effects of VSV is likely due to how little caspase-3 activation is required to cause HeLa cells to undergo morphological changes associated with apoptosis. Despite the overexpression of Mcl-1, the protein still decays during VSV infection which accounts for the fact that overexpression of Mcl-1 only marginally delays apoptosis (Fig. 4B). From these data I concluded that overexpression of Mcl-1 was able to marginally inhibit the onset of the morphological changes accompanying apoptosis in rWT virus-infected HeLa cells, but there was a significant reduction in activation of caspase-3-like activity.

**Apoptosis is significantly delayed in Bcl-X\textsubscript{L} overexpressing cells infected with rWT virus.**

Since there was a decrease in the rate of apoptosis in cells transfected with Mcl-1 despite the rapid turnover, I hypothesized that overexpression of a more stable antiapoptotic Bcl-2 family member, i.e., Bcl-X\textsubscript{L}, would have a more dramatic effect on the induction of apoptosis compared to overexpression of Mcl-1. To determine the effects of Bcl-X\textsubscript{L} overexpression, a HeLa cell line that stably overexpressed Bcl-X\textsubscript{L} was generated. A western blot demonstrating the overexpression of Bcl-X\textsubscript{L} is shown in Fig. 5A. The induction of apoptosis in HeLa-Bcl- X\textsubscript{L} cells and HeLa-EV cells infected with rWT virus was quantified by time-lapse microscopy (Fig. 5B) and by assay of caspase-3-like activity (Fig. 5C). rWT virus rapidly induced apoptosis in the control, empty vector cells (open circles) with approximately 90% of cells undergoing apoptosis by 16hrs postinfection, while only 20% of Bcl-X\textsubscript{L} transfected cells (closed squares) underwent apoptosis by the same time (Fig. 5B). Similar results were obtained for the caspase-3
Figure 5. Apoptosis is significantly delayed in Bcl-X<sub>L</sub>-overexpressing cells infected with rWT virus. HeLa cells that overexpress Bcl- X<sub>L</sub> (Bcl- X<sub>L</sub>-3) or an empty vector control were generated. (A) Cell lysates were generated and analyzed by western blotting using an antibody against Bcl- X<sub>L</sub>. (B) Bcl- X<sub>L</sub>-3 (closed squares) and empty vector cells (open circles) were infected with the rWT virus. Cells entering apoptosis were analyzed by time-lapse microscopy as described in the legend to Fig. 1. The data represent the averages from three experiments. (C) Bcl- X<sub>L</sub>-3 and empty vector cells were infected with rWT virus for the times indicated and caspase-3-like activity was analyzed using a fluorogenic substrate as described in the legend to Fig. 1. The data represent the averages +/- standard deviation from three experiments (*, p< 0.05 compared to empty vector control).
assay in which caspase-3-like activity was completely abrogated in the Bcl-X\(_L\) transfected cells (Fig. 5C). Both assays showed that overexpression of the more stable anti-apoptotic protein, Bcl-X\(_L\) caused a dramatic reduction in the rate of induction of apoptosis in rWT virus-infected cells compared to overexpression of Mcl-1.

**Simultaneous silencing of Bcl-X\(_L\) and Mcl-1 by siRNA transfection induces cell death.**

The data in Figs. 2-5 indicate that the rapid induction of apoptosis by rWT virus in HeLa cells is due in part to the depletion of Mcl-1. SiRNA silencing experiments were used to determine whether loss of Mcl-1 or Bcl-X\(_L\) or both was sufficient to cause cells to undergo apoptosis. Fig. 6A is a representative western blot showing the siRNA silencing of Mcl-1 and Bcl-X\(_L\). There was no significant reduction in cell viability of Mcl-1 or Bcl-X\(_L\) siRNA cells at either 24 or 48hrs post-transfection determined by MTT assay (Fig. 6B). However, the combined silencing of Mcl-1 and Bcl-X\(_L\) decreased cell viability to approximately 40% of control at both timepoints. Thus, silencing of either Mcl-1 or Bcl-X\(_L\) alone was not sufficient to induce apoptosis, while silencing of both was sufficient to induce cell death in the absence of a viral infection. These results suggest that the loss of Mcl-1 alone in VSV-infected cells is not sufficient to induce apoptosis, and suggest that inactivation of Bcl-X\(_L\) is also required.
Figure 6. Simultaneous silencing of Bcl- X<sub>L</sub> and Mcl-1 by siRNA transfection induces cell death. (A) HeLa cells were transfected with 25nM Mcl-1, Mcl-1 and Bcl-X<sub>L</sub> or non-targeting siRNA as a control. At 24hrs posttransfection, cell lysates were analyzed by western blotting using antibodies against Mcl-1 and Bcl-X<sub>L</sub>. (B) HeLa cells were transfected with Mcl-1, Bcl-X<sub>L</sub>, Mcl-1 and Bcl-X<sub>L</sub> or non-targeting siRNA as a control. Cell viability was analyzed at 24 and 48hrs posttransfection by MTT assay as described in the legend to fig.1. The data represent the average and standard deviation from three experiments. (*, p< 0.05 compared to single silenced cells).
A

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<th>Mcl-1</th>
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B

![Graph showing viable cells (% of control) over time for Mcl-1, Bcl-XL, and Mcl-1 Bcl-XL+ control conditions.](image)
Apoptosis is induced significantly faster by rWT virus in Bcl-X<sub>L</sub> silenced cells.

To test whether silencing of Mcl-1 or Bcl-X<sub>L</sub> would accelerate virus-induced apoptosis, HeLa cells were transfected with either Bcl-X<sub>L</sub>, Mcl-1, or non-targeting siRNA for 48hrs and then infected with rWT virus. Silencing of Mcl-1 led to a slightly faster induction of apoptosis than in control siRNA cells as measured by time-lapse microscopy (open circles versus closed triangles, Fig. 7A). However, silencing of Bcl-X<sub>L</sub> (closed squares) dramatically increased the rate of virus-induced apoptosis. Similar results were obtained by assaying caspase-3-like activity and cell viability (Fig. 7B and 7C). There was no significant difference in the amount of virus-induced caspase-3-like activity or reduction in cell viability in Mcl-1 siRNA cells as compared to control siRNA cells throughout the time course (Fig. 7B). In contrast, virus infection activated significantly more caspase-3 and reduced cell viability to a significantly greater extent in Bcl-X<sub>L</sub> siRNA cells than in control siRNA cells. These data are consistent with the idea that Mcl-1 is degraded quickly upon infection with VSV, therefore removing Mcl-1 prior to infection does not increase the rate of apoptosis. However, inhibition of Bcl-X<sub>L</sub> requires the activation of one or more BH3 only proteins, so removing Bcl-X<sub>L</sub> prior to infection significantly increases the rate of apoptosis, presumably by reducing the time required for inactivation of anti-apoptotic proteins by activated BH-3 only proteins.

Apoptosis is significantly delayed in Bid silenced cells infected with rWT virus.

The experiments in Fig. 7 raise the question of which BH3-only protein(s) were involved in the deactivation of Bcl-X<sub>L</sub>. There are many BH3-only proteins present in HeLa cells, but one of the requirements was for the BH3-only protein to be post-
Figure 7. Apoptosis is induced significantly faster by rWT virus infection in Bcl- X\textsubscript{L} silenced cells. (A) HeLa cells were transfected with Mcl-1 (closed triangles), Bcl- X\textsubscript{L} (closed squares) or non-targeting siRNA (open circles) for 48hrs and then infected with rWT virus. Cells entering apoptosis were analyzed by timelapse microscopy as described in the legend to Fig. 1. The data represent the averages from three experiments. (B) HeLa cells were transfected with Mcl-1, Bcl- X\textsubscript{L} or non-targeting siRNA for 48hrs and then infected with rWT virus for the times indicated and caspase-3-like activity was analyzed using a fluorogenic substrate as described in the legend to Fig. 1. The data represent the averages +/- standard deviation from three experiments. (C) HeLa cells were transfected with Mcl-1, Bcl- X\textsubscript{L}, or non-targeting siRNA for 48hrs and then infected with rWT virus. Cell viability was analyzed at the indicated times by MTT assay as described in the legend to fig.1. The data represent the average and standard deviation from three experiments (*, p<0.05 compared to control).
translationally activated due to the inhibition of new host gene expression by the M protein of VSV. This requirement resulted in three main BH3-only protein candidates—Bad, Bim and Bid. Bim is released from dynein motor complex upon c-Jun N-terminal kinase (JNK)-mediated phosphorylation [60, 66]. Bad is activated by loss of phosphorylation in response to growth-factor deprivation [67]. Bid is proteolytically processed by caspase-8 following the activation of the extrinsic pathway of apoptosis [68, 69]. However, studies involving the inhibition of the JNK pathway during VSV infection made Bim an unlikely candidate (see Chapter 3). Additionally, studies involving VSV infection of LNCaP cells found no loss of phosphorylation and therefore no activation of Bad during infection (J. H. Connor and G. Kulik personal communication). Consequently Bid became the primary candidate BH3-only protein involved in Bcl-\(X_L\) inactivation and Bak activation. To determine whether silencing of Bid would decrease the rate of VSV induced apoptosis, HeLa cells were transfected with either Bid or non-targeting siRNA for 48hrs and then infected with rWT virus. Silencing of Bid led to a slower induction of apoptosis than in control siRNA cells as measured by time-lapse microscopy (open circles versus closed squares, Fig. 8A). Similar results were obtained by assaying caspase-3-like activity and cell viability (Fig. 8B and 8C). Virus infection activated significantly less caspase-3 and reduced cell viability to a significantly lesser extent in Bid siRNA cells than in control siRNA cells. These data are consistent with the idea that activation of Bid during VSV infection leads to the inactivation of Bcl-\(X_L\) and/or the activation of Bak.
Figure 8. Apoptosis is significantly delayed in Bid silenced cells infected with rWT virus. (A) HeLa cells were transfected with Bid (closed squares) or non-targeting siRNA (open circles) for 48hrs and then infected with rWT virus. Cells entering apoptosis were analyzed by timelapse microscopy as described in the legend to Fig. 1. The data represent the averages from three experiments. (B) HeLa cells were transfected with Bid or non-targeting siRNA for 48hrs and then infected with rWT virus for the times indicated and caspase-3-like activity was analyzed using a fluorogenic substrate as described in the legend to Fig. 1. The data represent the averages +/- standard deviation from three experiments. (C) HeLa cells were transfected with Bid or non-targeting siRNA for 48hrs and then infected with rWT virus. Cell viability was analyzed at the indicated times by MTT assay as described in the legend to Fig. 1. The data represent the average and standard deviation from three experiments. (*, p<.05 compared to control).
Apoptosis is significantly delayed in HeLa cells infected with rWT virus in the presence of caspase-8 inhibitor.

Since caspase-8 is known to cleave Bid into tBid, I next determined whether a caspase-8 inhibitor would reduce the rate of VSV induced apoptosis. To test this, HeLa cells were preincubated with either 100uM caspase-8 inhibitor, 100uM pan-caspase inhibitor or no inhibitor for 2hrs prior to infection with VSV. The media contained 100uM caspase-8 or pan-caspase inhibitor for the duration of the infection. Cell viability was analyzed at 12, 16, and 24hrs postinfection by MTT assay. HeLa cells infected in the presence of either caspase-8 inhibitor or pan-caspase inhibitor showed significantly greater cell viability at all three timepoints (Fig. 9). These results suggest that both the intrinsic and extrinsic pathways are important for VSV induced apoptosis and indicates that crosstalk between the two pathways occurs via caspase-8 cleavage of Bid. Interestingly, even in the presence of a pan-caspase inhibitor VSV is able to induce apoptosis in HeLa cells suggesting that a caspase independent pathway may also be activated.
Figure 9. Apoptosis is significantly delayed in HeLa cells infected with rWT virus in the presence of caspase-8 inhibitor. (A) HeLa cells or (B) HeLa cells that stably overexpress Bcl-X_L were incubated in 100uM caspase-8 inhibitor, 100uM pan caspase inhibitor or no inhibitor for 2 hrs. Cells were then infected with rWT virus for the indicated times. The inhibitors were present in the media at the same 100uM concentration throughout the timecourse. Cell viability was analyzed at the indicated times by MTT assay as described in the legend to Fig. 1. The data represent the average and standard deviation from three experiments. (*, p<.05 compared to control).
Apoptosis is significantly delayed in Bcl-X\textsubscript{L} overexpressing cells infected with rM51R virus.

The induction of apoptosis by rM51R virus in murine L929 cells is due primarily to activation of the death receptor pathway. Inhibition of the mitochondrial pathway by overexpression of Bcl-2 had no effect on the rate of apoptosis [25]. On the other hand, the mitochondrial pathway appears to be the main pathway responsible for apoptosis induced by M protein mutant VSV in Jurkat cells and MEFs [47]. Likewise, overexpression of Bcl-2 in HeLa cells reduced the rate of rM51R induced cell death [26]. Thus in some cells, rM51R virus-induced apoptosis is independent and in others, it is dependent on the mitochondrial pathway. I hypothesized that this apparent difference is due to the role of crosstalk in apoptosis induced by the rM51R virus in different cell types. The experiments presented here were designed to further test the importance of the mitochondrial pathway in rM51R virus-induced apoptosis. To determine the effect of inhibiting the mitochondrial pathway on apoptosis, HeLa cell lines that stably overexpresses Bcl- X\textsubscript{L} or empty vector (EV) (as shown in Fig. 5A) were infected rM51R virus. The effect on cell viability in Bcl- X\textsubscript{L} overexpressing cells and HeLa-EV cells infected with rM51R virus was determined by MTT assay (Fig 10).
Figure 10. Apoptosis is significantly delayed in Bcl-X<sub>L</sub> overexpressing cells infected with rM51R virus. HeLa cells that stably overexpress Bcl-X<sub>L</sub> or empty vector cells were incubated in 100uM caspase-8 inhibitor, 100uM pan caspase inhibitor or no inhibitor for 2 hrs. Cells were then infected with rWT virus for the indicated times. The inhibitors were present in the media at the same 100uM concentration throughout the timecourse. Cell viability was analyzed at the indicated times by MTT assay as described in the legend to Fig. 1. The data represent the average and standard deviation from three experiments. (*, p<.05 compared to control).
Cell viability was significantly increased in Bcl-X<sub>L</sub> overexpressing cells as compared to empty vector cells at 24 and 36hrs postinfection (p<.05). This result indicates that the mitochondrial pathway plays a role in the induction of apoptosis in rM51R virus infected HeLa cells and confirms results obtained previously in a HeLa cell line that stably overexpresses Bcl-2 [26].

**Apoptosis induced by rM51R virus is significantly faster in Bcl-X<sub>L</sub> and Mcl-1 silenced cells and significantly slower in Bak silenced cells.**

In Chapter One, it was demonstrated that in the case of infection with rWT virus, anti-apoptotic proteins Bcl-X<sub>L</sub> and Mcl-1 and pro-apoptotic Bak all play an important role in the induction of apoptosis. siRNA silencing experiments were used to determine whether these same proteins are important in the context of infection with rM51R virus. To test whether silencing of Bcl-X<sub>L</sub> or Mcl-1 would accelerate virus induced apoptosis, HeLa cells were transfected with siRNA against either Bcl-X<sub>L</sub>, Mcl-1, or with non-targeting siRNA as a control for 48hrs and then infected with rM51R virus. Similar to results in cells infected with rWT virus, Bcl-X<sub>L</sub> siRNA cells entered apoptosis significantly faster than control siRNA cells as determined by both caspase-3-like activity and cell viability assays throughout the timecourse (p<.05)(Fig. 11A,B). Silencing of Mcl-1 led to a significant increase in the rate of apoptosis similar to that induced by silencing Bcl-X<sub>L</sub> (p<.05)(Fig. 11A,B). This differs from the results involving rWT virus-infected cells, in which silencing Bcl-X<sub>L</sub> had a greater effect on the rate of apoptosis than silencing Mcl-1 (Fig. 7). These results indicate that both Mcl-1 and Bcl-X<sub>L</sub> are important for regulating the induction of apoptosis by rM51R virus.
Figure 11. Apoptosis induced by rM51R virus is significantly faster in Bcl-X<sub>L</sub> and Mcl-1 silenced cells and significantly slower in Bak-silenced cells. HeLa cells were transfected with 25nM Bcl-X<sub>L</sub>, Mcl-1 or non-targeting siRNA for 48 hrs and then infected with rM51R virus. (A) At the indicated times postinfection, caspase-3-like activity was analyzed using a fluorogenic substrate as described in the legend to Fig.1. The data represent the averages +/- standard deviation for three experiments. (B) Cell viability was analyzed at the indicated times by MTT assay as described in the legend to Fig. 1. The data represent the average and standard deviation from three experiments. (*, p<.05 compared to control). (C) HeLa cells were transfected with Bak, Bax or non-targeting siRNA for 48hrs and then infected with rM51R virus. At the indicated times postinfection, caspase-3-like activity was analyzed using a fluorogenic substrate as described in the legend to Fig.1. The data represent the averages +/- standard deviation for three experiments. (*, p<.05 compared to control).
The importance of Bcl-X<sub>L</sub> and Mcl-1 in this system indicates a role for Bak and/or Bax, the main multidomain pro-apoptotic Bcl-2 family members. To test whether silencing of Bak or Bax would decrease the rate of rM51R virus induced apoptosis, HeLa cells were transfected with siRNA against either Bak, Bax or non-targeting siRNA as a control for 48hrs and then infected with rM51R virus. Similar to the results in rWT infected cells, silencing of Bax had no significant effect on the rate of virus induced cell death (Fig. 11C). Additionally, silencing of Bak led to a significant decrease in the amount of virus-induced caspase 3-like activity as compared to control siRNA cells at 20, 24 and 28hrs postinfection (p<.05)(Fig. 11C). These results show that silencing of Bak, but not Bax, expression significantly slows the rate of apoptosis in rM51R virus-infected cells.

**Apoptosis is significantly delayed in HeLa cells and Bcl-X<sub>L</sub> overexpressing HeLa cells infected with rM51R virus in the presence of caspase 8 inhibitor.**

The results in Figures 10 and 11 show that the mitochondrial pathway is also important for rM51R virus induced apoptosis. I hypothesized that this pathway is activated by crosstalk with the death receptor pathway. To test this hypothesis, HeLa cells were preincubated with either 100uM caspase-8 inhibitor, 100uM pan-caspase inhibitor or no inhibitor for 2 hours prior to infection with virus. Additionally, the media contained 100uM caspase inhibitor for the duration of the infection. Cell viability was analyzed at 16, 24 and 30hrs postinfection by MTT assay. HeLa cells infected in the presence of either the caspase-8 inhibitor or the pan-caspase inhibitor showed
Figure 12. Apoptosis is significantly delayed in HeLa cells and Bcl-X\textsubscript{L} overexpressing cells infected with rM51R virus in the presence of caspase-8 inhibitor. (A) HeLa cells or (B) HeLa cells that stably overexpress Bcl-X\textsubscript{L} were incubated in 100uM caspase-8 inhibitor, 100uM pan caspase inhibitor or no inhibitor for 2 hrs. Cells were then infected with rM51R virus for the indicated times. The inhibitors were present in the media at the same 100uM concentration throughout the timecourse. Cell viability was analyzed at the indicated times by MTT assay as described in the legend to Fig. 1. The data represent the average and standard deviation from three experiments. (*, p<.05 compared to control).
significantly increased cell viability at the 24 and 30hr timepoints (Fig. 12A). To determine whether combined inhibition of both the mitochondrial and death receptor pathways would have an additive effect on the rate of apoptosis, HeLa cells that overexpress Bcl-X<sub>L</sub> were infected with rM51R virus in the presence or absence of capse-8 or pan caspase inhibitor as described above. Bcl-X<sub>L</sub> overexpressing HeLa cells were analyzed at 24, 36 and 48hrs postinfection by MTT assay. Similar to the results in HeLa cells, HeLa-Bcl-X<sub>L</sub> cells infected in the presence of either caspase-8 or pan-caspase inhibitors showed significantly increased cell viability at 36 and 48hrs postinfection. These results suggest that activation of the death receptor pathway initiator caspase, caspase-8, plays a role in the activation of the mitochondrial pathway via crosstalk, and that inhibition of both pathways causes a greater delay in apoptosis than inhibiting either pathway alone.

Experiments presented here for HeLa cells along with previous experiments done in other cell types suggest that the differences in requirement for activation of the mitochondrial pathway by M protein mutant VSV is similar to the classification of cell types into type I (mitochondria-independent) or type II (mitochondria-dependent) based on their response to death ligands such as Fas [68]. I propose a model which outlines how type I vs type II cells respond to infection by VSV (Fig. 13). For type I cells, infection with rM51R virus results in the induction of an antiviral response which stimulates new host gene expression and activates the interferon inducible dsRNA-dependent protein kinase PKR. PKR activates the death receptor Fas/CD95 which signals through the adaptor protein Daxx resulting in the activation of the stress kinase JNK. This is followed by subsequent activation of caspase-8 and caspase-3 leading to
Figure 13. Response of type I and type II cells to infection with VSV. Infection of type I cells with rM51R virus results in the stimulation of new host gene expression. This causes the activation of the interferon-inducible dsRNA-dependent protein kinase PKR. PKR is involved in activating the death receptor Fas/CD95. In response to rM51R-M virus infection, Fas signals through the adaptor protein Daxx, resulting in the activation the stress kinase JNK. Unidentified downstream components of this pathway activate caspase-8 and subsequently caspase-3, leading to cell death. I propose that infection of type II cells with either rWT or M51R virus have similar effects. Infection of Type II cells with rM51R virus may follow a similar pattern to that of type I cells infected with rM51R in which the death receptor pathway is activated. This leads to activation of caspase-8, cleavage of Bid into its active form which can inhibit Mcl-1 and Bcl-XL resulting in the activation of Bak. Active Bak permeabilizes the outer mitochondrial membrane leading to activation of caspase-9 and subsequently caspase-3, leading to cell death. I propose that type II cells infected with rWT virus follow that same pathway just described for rM51R virus, but the rWT virus inhibition of new host gene expression also results in the degradation of Mcl-1. This turnover of Mcl-1 is quicker than that activation of Bid in some cell types leading to type II cells that are killed more rapidly by rWT virus than by rM51R virus.
Apoptosis

Death Receptors?

M51R virus

Stimulation of new host gene expression

Activation of PKR, Fas, Daxx and JNK

Caspase-8

Caspase-3

Apoptosis

rWT virus

Degradation of Mcl-1

Activation of Bak

Inactivation of Bcl-XL

Cleavage of Bid

Caspase-8

Caspase-9

Caspase-3

Apoptosis

M51R virus

Activation of PKR, Fas, Daxx and JNK

Caspase-8

Caspase-3

Apoptosis

rWT virus

Degradation of Mcl-1

Activation of Bak

Inactivation of Bcl-XL

Cleavage of Bid

Caspase-8

Caspase-9

Caspase-3

Apoptosis
cell death [55]. In type II cells, infection with rM51R virus may lead activation of a death receptor similar to what occurs in type I cells, this is still under investigation. It is known that caspase-8 is activated, which leads to the activation of Bid. Bid can then lead to the activation of Bak due to inactivation of Mcl-1 and Bcl-XL causing activation of caspase-9, subsequent activation of caspase-3 resulting in cell death. For type II cells infected with rWT virus, the activation of the death receptor pathway occurs and contributes to activation of the mitochondrial pathway, but the inhibition of new host gene expression leading to the turnover of Mcl-1 accelerates the rate of activation.
INHIBITION OF SAPKS P38 AND JNK DOES NOT AFFECT THE RATE OF
APOPTOSIS INDUCED BY RWT VIRUS

Stress-activated protein kinases are activated during infection with VSV.

It has been shown that rWT virus induces apoptosis in part by the activation of caspase-8 and subsequent cleavage of BH3-only protein Bid. However, previously it had been shown that transfection of wildtype M protein in the absence of other viral proteins induces apoptosis independently of caspase-8 [26]. This indicates that there may be an additional signal as a result of M protein-induced shut-off of new host gene expression. The cell may perceive this shut-off as a type of intracellular stress leading to the activation of stress activated protein kinases (SAPKs). I hypothesized that shut-off of new host gene expression induces SAPKs p38 and/or JNK and that this is an additional apoptotic signal. To test this, I first identified whether these SAPKs are activated during infection with VSV. HeLa cells were infected with wildtype Orsay virus (WTO, a naturally occurring strain of VSV used as an experimental control) at an MOI of 10 or mock infected and at various times post-infection cell lysates were prepared and analyzed by SDS-PAGE and Western blotting. Figure 14 A shows a representative blot of phosphorylated p38 (p-p38) with total p38 as a control. No p-p38 or total p38 was detected at the 24hr time point which may be due to low protein yield at this point because most of the cells have undergone apoptosis (Fig. 14A). There was maximal
Figure 14. p38 and JNK are phosphorylated during infection with VSV. HeLa cells were mock-infected or infected with WTO for 4, 8, 12, 16 and 24hrs. (A) Western blot detections of phosphorylated p38 (top panel) and total p38 (bottom panel) are shown. Maximal p38 phosphorylation occurs at 8 and 12hrs post-infection. (B) Western blot detections of phosphorylated JNK (top panel) and total JNK (bottom panel) are shown. Maximal JNK phosphorylation occurs at 8 and 12hrs post-infection.
activation of p38 at 8 and 12hrs post infection (Fig. 14A). To determine whether JNK was also activated during VSV infection, blots were stripped and probed for phosphorylated JNK (p-JNK). Similar to the results for p-p38, there was maximal phosphorylation of JNK at 8 and 12hrs post infection (Fig. 14B).

**PARP cleavage correlates with the time of maximal activation of p38 and JNK.**

The determination that both JNK and p38 were activated during VSV infection led to the question of the timing of this activation as compared to the induction of apoptosis in these cells. To determine whether activation of the SAPKs coincided with the induction of apoptosis, the lysates from above were analyzed by western blotting for PARP cleavage. PARP (poly(ADP-ribose) polymerase) is an enzyme implicated in DNA damage and repair mechanisms. During apoptosis, PARP is cleaved by caspase-3 and cleavage of PARP is considered a hallmark of apoptosis. Figure 15 shows a representative blot of PARP cleavage in VSV infected cells. The upper band is uncleaved PARP and the lower band is cleaved PARP. PARP cleavage begins around 8 hours post infection which correlates with maximal activation of p38 and JNK. PARP cleavage is complete by 24hrs post-infection (Fig 15).

**PARP cleavage is unchanged in the presence of a p38 inhibitor.**

p38 is activated by infection with WTO virus. To determine whether this activation plays an important role in VSV induced apoptosis, a synthetic inhibitor of the p38 pathway, SB203580 was used. HeLa cells were infected with WTO virus or mock infected in the presence of three different concentrations (30, 50,100uM) of the p38
Figure 15. PARP cleavage is complete by 24hrs postinfection with VSV.

HeLa cells were infected with WTO. At the given times postinfection, cell lysates were prepared and probed for PARP. The upper band represents uncleaved and the lower band represents cleaved PARP. PARP cleavage is detectable as a faint band in the cleaved PARP lane by 4hrs postinfection and all PARP has been cleaved by 24hrs postinfection with WTO virus.
Uncleaved PARP
Cleaved PARP

m 4 8 12 16 24 hours postinfection
inhibitor, while HeLa cells infected with WTO virus or mock infected without the inhibitor were used as controls. Cell lysates were prepared 12hrs post-infection and analyzed by Western blotting. As a control for the inhibition of the p38 pathway, I probed for phosphorylated MAPKAPK-2, a downstream target of p38, because the p38 inhibitor allows phosphorylation of p38, but prevents p38 from phosphorylating its downstream targets. In the absence of any inhibitor, there is phosphorylation of MAPKAPK-2 by 12hrs post-infection with WTO virus (Fig. 16, upper panel, lane 2). There was no phosphorylation of MAPKAPK-2 in cells infected with WTO virus in the presence of the p38 inhibitor at any of the inhibitor concentrations (Fig. 16, upper panel, lanes 6-8). These data indicate that there is inhibition of the p38 pathway at all three inhibitor concentrations. These blots were then stripped and reprobed for PARP to determine whether this inhibition of the p38 pathway inhibits the rate of apoptosis. The upper band is uncleaved PARP and the lower band is cleaved PARP. There is nearly complete PARP cleavage by 12hrs post-infection in WTO virus infected cells without inhibitor (Fig. 16, lower panel, lane 2). Cells infected with WTO virus in the presence of the p38 inhibitor also had nearly complete cleavage of PARP by 12hrs post-infection (Fig. 16, lower panel, lanes 6-8). This indicates that inhibition of the p38 pathway does not delay PARP cleavage in WTO infected HeLa cells.
Figure 16. p38 inhibitor does not delay the rate of PARP cleavage in WTO infected HeLa cells. HeLa cells were mock-infected or infected with WTO for 12 hrs in the presence or absence of p38 inhibitor at 30, 50, or 100uM concentrations. Western blot detection of phosphorylated, total MAPKAPK-2 and PARP are shown. Phosphorylation of MAPKAPK-2 is blocked by all three concentrations of inhibitor (upper panel, lanes 6-8). PARP cleavage is unchanged at all p38 inhibitor concentrations (lower panel, lanes 6-8).
A

Lane 1 2 3 4 5 6 7 8
m wt 100 50 30 100 50 30
mock wildtype virus

p-MAPKAK-2
Total MAPKAPK-2
[uM] p38 inhibitor

B

Lane 1 2 3 4 5 6 7 8
m wt 100 50 30 100 50 30
mock wildtype virus

Uncleaved PARP
Cleaved PARP
[uM] p38 inhibitor
**PARP cleavage is reduced in the presence of a JNK inhibitor.**

Similar to p-38, JNK is activated during VSV infection. To determine whether this activation plays role in the induction of apoptosis a synthetic inhibitor of the JNK pathway, JNKII was used. HeLa cells were infected with WTO virus or mock infected in the presence of three different concentrations (30, 50, 100uM) of the JNK inhibitor. Additionally, HeLa cells infected with WTO virus or mock infected without the inhibitor were used as controls. Cell lysates were prepared 12hrs post-infection and analyzed by Western blotting. The control for inhibition of the JNK pathway was to probe for phosphorylated c-Jun. However, the Western blots probed for both phosphorylated and total c-Jun were consistently blank. Therefore, these blots were analyzed for PARP cleavage without a positive indication the JNK inhibitor had successfully inhibited the JNK pathway indicating that a negative result would be not be interpretable while a positive result would indicate successful inhibition of JNK. Figure 17 shows a representative blot of PARP cleavage in the presence of the JNK inhibitor. The upper band represents uncleaved PARP and the lower band represents cleaved PARP. As before, the cells infected with WTO virus without JNK inhibitor underwent nearly complete PARP cleavage by 12hrs post-infection (Fig. 17, lane 2). However, in cells infected with WTO virus in the presence of JNK inhibitor, only about 50% of the PARP was cleaved by 12hrs post-infection (Fig. 17, lanes 6-8). These results suggest that JNK is involved in the induction of apoptosis in VSV infected HeLa cells. However, the JNK inhibitor only resulted in a partial inhibition of PARP cleavage. I therefore tested the effect of JNK inhibition using additional approaches.
Figure 17. PARP cleavage is delayed in HeLa cells infected with WTO virus in the presence of JNK inhibitor. HeLa cells were mock-infected or infected with WTO virus for 12hrs in the presence or absence of a JNK inhibitor at 30, 50, or 100uM concentrations. Western blot detections of PARP are shown. PARP cleavage was reduced in cells infected with WTO in the presence of all three concentrations of JNK inhibitor (lanes 6-8 as compared to lane 2).
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Uncleaved PARP
Cleaved PARP
The percent of cells entering apoptosis is unchanged (or increased) in the presence of JNK inhibitor.

In an attempt to confirm the PARP cleavage data indicating a role for JNK in the induction of apoptosis in HeLa cells, time lapse microscopy was used to determine the timing of morphological changes indicative of apoptosis. HeLa cells were infected with VSV or mock infected in the presence of 30uM JNK inhibitor or 3% EtOH (solvent for JNK inhibitor). In addition, cells were pretreated for 2hr with 30uM JNK inhibitor or 3% EtOH as a control, prior to infection with VSV. As a final control, cells were infected with VSV or mock infected without inhibitor or 3% EtOH. Cells entering apoptosis were recorded and quantitated for 24hrs post-infection using a time lapse digital microscope. Figure 18A illustrates the time lapse microscopy results. The data are expressed as the cumulative percentage of cells entering apoptosis as a function of time post-infection. The criterion used to distinguish cells entering apoptosis was membrane blebbing. Cells treated with inhibitor at the time of infection with VSV (pink) entered apoptosis as rapidly as VSV infected controls without inhibitor. However, incubation with the JNK inhibitor for 2hrs prior to infection with VSV (green) increased the rate apoptosis with ~55% of cells undergoing apoptosis by 8hrs post-infection. None of the mock infected cells underwent appreciable apoptosis, regardless of treatment (orange, red, teal). The data indicate that inhibition of the JNK pathway does not inhibit apoptosis by this assay and that activation of
Figure 18. Apoptosis is not delayed in HeLa cells infected with WTO virus in the presence of JNK inhibitor as measured by timelapse microscopy. HeLa cells were infected in the presence of 30uM JNK inhibitor with rWT virus (pink square) or mock infected (orange X). In addition, cells were pretreated for 2hr with 30uM JNK inhibitor (green triangle) prior to infection with rWT virus or pretreated for 2hrs with 3% EtOH (solvent for JNK inhibitor) as a control, prior to infection with rWT virus (black X) or mock infection (red circle). As a final control, cells were infected with rWT virus (dark blue diamond) or mock infected (teal +) without inhibitor or 3% EtOH. Cells entering apoptosis were analyzed by timelapse microscopy as described in the legend to Fig. 1. The data represent the averages from three experiments.
the JNK pathway may be required to delay apoptosis. These results are not consistent with the PARP cleavage data, therefore I analyzed caspase-3-like activity in cell lysates using a fluorogenic substrate. For the caspase-3 assay, cells treated with staurosporin (SSP) were used as a positive control. Similar to the results obtained by timelapse microscopy, HeLa cells incubated with 30uM JNK inhibitor exhibited higher amounts of caspase-3-like activity after VSV infection as compared to HeLa infected with VSV without the JNK inhibitor (Fig. 19).
Figure 19. Caspase-3-like activity is increased in HeLa cells infected with rWT virus in the presence of JNK inhibitor. HeLa cells were incubated in 30uM JNK inhibitor or no inhibitor for 2 hours and then mock infected, infected with rWT virus or incubated with SSP for the indicated times. Caspase-3-like activity was analyzed using a fluorogenic substrate as described in the legend to Fig.1. The data represent the averages +/- standard deviation for three experiments.
**JNK inhibitor reduces cell division in mock infected cells.**

During the course of these experiments, I noted that inhibition of JNK slowed the rate of proliferation of mock-infected cells. In Fig. 20, mock infected cells were analyzed by timelapse microscopy in the presence or absence of the JNK inhibitor. Since the addition of the JNK inhibitor did not induce apoptosis in mock infected cells, the total number of cells at 2hrs post-infection was compared to the total number of cells at 24hrs post-infection. The data are expressed as the fold increase over the initial cell number. HeLa cells mock-infected in the absence of JNK inhibitor increased two-fold by the 24hr time point (Fig. 20). However, cells mock-infected in the presence of the JNK inhibitor only increased 1.2 fold by the 24hr time point (Fig. 20). These results indicate that the JNK inhibitor does have an effect in that it reduces the ability of the cells to divide. These data indicate that the JNK pathway is important for cellular proliferation in uninfected HeLa cells. Collectively, these data suggest that the SAPKs do not play a major role in VSV induced apoptosis in HeLa cells.
Figure 20. **JNK pathway is required for cellular proliferation.** HeLa cells were mock infected in the presence or absence of 30uM JNK inhibitor and the total number of cells at 2hrs post-infection was compared to the total number of cells at 24hrs post-infection as measured by timelapse microscopy. The data represent the averages from three experiments. (*, p<.05 as compared to control).
DISCUSSION AND CONCLUSIONS

Initially this work was begun with the idea that VSV with wt M protein and M protein mutant VSV activated different apoptotic pathways. Previous studies involving HeLa cells indicated that rWT virus induced apoptosis primarily through the mitochondrial pathway [10, 26]. HeLa cells transfected with wt M protein in the absence of other viral components also induced apoptosis mainly through caspase-9 and the mitochondrial pathway [26]. In contrast, L929 cells infected with rM51R virus require the death receptor pathway to undergo apoptosis [25]. Another difference between HeLa cells and L929 cells is that in HeLa cells rWT virus induces apoptosis faster that rM51R virus, while in L929 cells rM51R virus induces apoptosis faster than rWT virus [25, 26]. Together these observations led to the hypothesis that rWT and rM51R viruses induce apoptosis by activating different apoptotic pathways.

To understand how rWT virus activates the mitochondrial pathway, I began investigating how the Bcl-2 family of proteins is affected by infection of HeLa cells with rWT virus. The idea was that once the Bcl-2 proteins involved in rWT virus induced apoptosis were known, this would lead to the upstream pathways by which these proteins are activated. In Chapter one, I showed an unexpected result that Bak was more important than Bax in this system. This was surprising due to numerous publications indicating the redundancy of these two proteins [40, 70, 71]. It was also shown that the turnover of Mcl-1 plays a role in accelerating apoptosis induced by rWT virus, but that inactivation of Bcl-X_L is also required for apoptosis to occur. Another unexpected result was that Bid and caspase-8 were also shown to play a role, suggesting the death receptor pathway is also involved.
In Chapter 2, I revisited the role of the mitochondrial pathway in M protein mutant VSV-induced apoptosis. This was due to previous results that showed a decrease in the rate of apoptosis in rM51R virus induced apoptosis in Bcl-2 overexpressing HeLa cells, as well as work done in other laboratories using Jurkat cells and MEFs, which indicated a role for the mitochondrial pathway in the induction of apoptosis by M protein mutant VSV [26, 47]. In agreement with these studies, I showed that the same factors involved with rWT virus induced apoptosis in HeLa cells were also involved with rM51R virus induced apoptosis, namely Bcl-X<sub>L</sub>, Mcl-1, Bak and caspase-8. The main difference between these two viruses was that infection with rM51R virus did not lead to the degradation of Mcl-1, which explains why rWT virus induces apoptosis faster than rM51R virus in HeLa cells. Otherwise, these two viruses are very similar in their effects on HeLa cell.

Finally, in Chapter 3 I revisited the fact that in cells transfected with wt M protein in the absence of other viral components, caspase-8 is not important for the induction of apoptosis. I hypothesized that SAPKs were activated by wt M protein due to the inhibition of host gene expression. However, inhibitors of both p38 and JNK disproved this hypothesis. This leaves open the question of other pathways that are involved in apoptosis due to inhibition of new host gene expression. Collectively, the results in this thesis provide new insights into the role of Bcl-2 family members in virus-induced apoptosis, and provide a framework for understanding differences between cell types in the induction of apoptosis by virus with wt versus mutant M protein.
Bak is more important than Bax for induction of apoptosis induced by VSV with wt M protein.

Cell death via the mitochondrial pathway depends on the multidomain proapoptotic proteins Bak and Bax [72] which function to permeabilize the outer mitochondrial membrane. In some systems, Bak and Bax have been shown to be functionally redundant. Studies utilizing knockout MEFs showed that MEFs deficient in either Bak or Bax alone underwent apoptosis induced by multiple apoptotic stimuli. Conversely, deficiency in both Bak and Bax conferred resistance to these apoptotic stimuli [40]. These results indicate that there is functional redundancy for Bak and Bax in MEFs. Subsequently, other groups have reported similar functional overlap in different cell types [40, 70, 71] giving rise to the idea that Bak and Bax are redundant regulators of intrinsic mitochondrial apoptosis and that inactivation of both is necessary to abrogate cell death. The results presented in Chapter One contrast with this view by showing that Bak plays a more significant role than Bax in the induction of apoptosis in VSV infected cells (Fig. 1).

Lack of functional redundancy of Bak and Bax is not surprising given the differences between them. Inactive Bax is a latent monomer in the cytosol [73]. Once activated by apoptotic stimuli, Bax undergoes conformational changes causing altered epitope availability, the formation of homodimers and oligomers, and translocation to the mitochondrial membrane [32]. In contrast, Bak resides in a membrane-bound protein complex [74] which suggests that Bak and Bax are activated by different mechanisms. In fact, recent evidence has shown that some apoptotic stimuli, such as staurosporine, actinomycin D, TRAIL and overexpression of Puma activate Bak preferentially, and that
Bak can lead to the release of cytochrome c in the absence of Bax activation [75]. Our observation that, in the context of a VSV infection, Bak appears to play a greater role in the induction of apoptosis than Bax is consistent with these more recent results.

Bak and Bax are usually constitutively expressed at relatively constant levels and are mainly regulated post-translationally by other Bcl-2 proteins. Antiapoptotic proteins, such as Bcl-2, Mcl-1 and Bcl-X\textsubscript{L}, keep Bax and Bak from permeabilizing the outer mitochondrial membrane. Since Bax exists as a monomer in the cytosol, there is no repressor bound to Bax in healthy cells [73]. However, once apoptotic signals from BH3 only proteins have induced a conformational change in Bax, it translocates to the mitochondrial membrane where Bax can be held in check by Bcl-2, Bcl-B and Bcl-X\textsubscript{L} [76]. Bak, constitutively present in membranes, has been shown to be kept inactive by interaction with multidomain antiapoptotic Bcl-2 family members Mcl-1 and Bcl-X\textsubscript{L} in organelle membranes [35, 36]. In addition, some studies have indicated that Bak requires activation by interaction with a BH3-only family member, similar to the activation of Bax [27, 44, 76] Of the two anti-apoptotic proteins that interact most strongly with Bak, Mcl-1 normally has a much more rapid turnover rate than Bcl-X\textsubscript{L} due to degradation by the ubiquitin-proteosome pathway [62, 63]. Therefore, I hypothesized that inhibition of new host gene expression by the VSV M protein induces apoptosis due to the loss of Mcl-1, since the protein cannot be replaced in VSV-infected cells once it has been degraded. This hypothesis was supported by the timing of the reduction of Mcl-1 protein levels during VSV infection which coincide with the induction of apoptosis (Fig. 2A). However, depletion of Mcl-1 alone is not sufficient to induce apoptosis (Fig. 6B) indicating that inactivation of Bcl-X\textsubscript{L} is also required for cells to enter apoptosis.
Inactivation of Bcl-X<sub>L</sub> in VSV infected cells is likely due to activation of one or more BH3-only proteins.

**Role of the death receptor pathway in the induction of apoptosis by VSV with wt M protein.**

The question of which BH3-only protein(s) were involved in the deactivation of Bcl-X<sub>L</sub> first appeared daunting due to the number of BH3-only proteins that have been described. However, this number was reduced to three likely candidates, Bad, Bim and Bid. M protein mediated shut-off of new host gene expression would likely inhibit the expression of BH3-only proteins that must be newly synthesized, which suggested that BH3-only proteins that are post-translationally regulated were the most likely candidates. Additionally, previous studies involving Bad and JNK (an upstream activator of Bid) suggested that Bad and Bid are not involved in VSV induced apoptosis (J.H. Connor and G. Kulik personal comm., Figs. 19, 20). This led to our hypothesis that BH3-only protein Bid, which is activated by proteolytic cleavage by caspase-8, was involved in Bcl-X<sub>L</sub> inactivation and Bak activation. This hypothesis was supported by the significant delay in VSV induced apoptosis in both Bid siRNA cells (Fig. 8) as well as in the presence of caspase-8 inhibitor (Fig. 9). These results suggest that both the intrinsic and extrinsic pathways are important for apoptosis induced by VSV with wt M protein, and that crosstalk between the two pathways occurs via caspase-8 cleavage of Bid.

**Role of the mitochondrial pathway in the induction of apoptosis by M protein mutant VSV.**
The idea that both the mitochondrial and death receptor pathways play a role in VSV-induced apoptosis is interesting in light of previous studies with M protein mutant virus. Conflicting data exists for which pathway is important for mutant M protein VSV induced apoptosis. Data from our laboratory has shown that in L929 cells inhibition of the mitochondrial pathway has no effect on the rate of apoptosis, while inhibition of the extrinsic pathway significantly reduces the rate of apoptosis [25]. Additionally, loss of functional PKR, Daxx or Fas can actually lead to clearance of the virus [55]. On the other hand, in caspase-8 -/- Jurkat cells and Fadd -/- Jurkat cells there is no reduction in the rate of apoptosis as compared to wt Jurkat cells induced by an M protein mutant VSV, while there is significantly less, if any, apoptosis in caspase-9/- MEFS and caspase-2,9 -/- MEFS infected with mutant M protein VSV [47]. These results indicate that in some cell types the death receptor pathway is all that is required for mutant M protein virus induced apoptosis, while in other cell types the mitochondrial pathway is required. This observation led to the hypothesis that the intrinsic pathway is activated via crosstalk with the extrinsic pathway and that this crosstalk is important for the induction of apoptosis in type II cells (mitochondrial dependent) as defined by Scaffidi et al 1998. In type II cells activation of the intrinsic pathway via crosstalk is required for death receptor Fas/CD95 induced apoptosis, while in type I cells the intrinsic pathway is not required for apoptosis to occur [68].

In the experiments in Chapter 2, the mitochondrial pathway was found to be important for induction of apoptosis in M protein mutant VSV infected HeLa cells (Figs. 10, 11). The same Bcl-2 family proteins that were important for VSV with wt M protein induced apoptosis –Bak, Mcl-1 and Bcl-XL were found to be important for mutant M
protein induced apoptosis in HeLa cells. Additionally, the experiments with caspase-8 inhibitor indicated that the extrinsic pathway was also important for mutant M protein induced apoptosis in HeLa cells, which is consistent with previous results in L929 cells (Fig. 12) [25]. Overall, the results in Chapter 2 support the idea that in some cell types activation of the death receptor pathway is enough to induce apoptosis analogous to type I cells, while in other cell types activation of the mitochondrial pathway in addition to the death receptor pathway is required for apoptosis analogous to type II cells.

The main difference between the results shown in Chapter 1 for rWT virus and those shown in Chapter 2 for rM51R virus is the effect of Mcl-1 silencing. Silencing of Mcl-1 does not have a significant effect on the rate of apoptosis induced by VSV with wt M protein. This is due to the fact that in the context of infection with wt M protein, Mcl-1 is lost quickly due to the normal rapid turnover rate of this protein coupled with the inhibition of new host gene expression. However, in the case of rM51R virus infection, silencing of Mcl-1 significantly increases the rate of apoptosis. This suggests that in the context of infection with M protein mutant VSV, an additional signal or BH3-only protein is required to remove Mcl-1 from the equation.

Additional pathways that contribute to the induction of apoptosis by VSV.

Chapter 3 addressed the hypothesis that the SAPKs were involved in the induction of apoptosis by wildtype M protein. Previous work has shown that wt M protein expressed in transfected cells in the absence of other viral components induces apoptosis, while mutant M protein does not [26]. Furthermore, inhibition of caspase-8 did not affect wt M protein-induced apoptosis, suggesting that apoptosis induced by the shut-off of host
gene expression relies on capase-9 activation but not caspase-8 [26]. I hypothesized that the inhibition of new host gene expression induced by wt M protein was perceived by the cell as a type of stress, leading to the activation of the one or both of the SAPKs, JNK and p38. Despite the activation of both JNK and p38 during VSV infection (Fig. 14), there was no reduction in the rate of apoptosis due to the inactivation of either JNK or p38 (Figs. 16, 18, 19). These results indicate that the SAPKs are not the mechanism by which wt M protein induces apoptosis. However, it is likely that JNK plays a role in inducing the expression of pro-apoptotic genes in cells infected with M protein mutant virus. L929 cells infected with rM51R virus in the presence of JNK inhibitor had significantly reduced percentage of apoptotic cells and significantly increased percentage of viable cells as compared to cells infected in the absence of JNK inhibitor [55]. It is possible that JNK may play a role in making L929 cells independent of the mitochondrial pathway, which could be tested by further experiments on the role of JNK in L929 cells.

My experiments leave open the question of how apoptosis is induced by wt M protein in a caspase-8 independent manner. Other elements of the death receptor pathway such as caspase-10 may be involved, or perhaps other regulators of the mitochondrial pathway aside from Bid may provide the crosstalk between the pathways. This may be the major pathway by which some wt VSV-infected cells die since there are several examples of cells in which inhibition of caspase-8 had no effect on apoptosis [47, 55].

Cell-type differences in the induction of apoptosis by VSV.
The results presented here provide new insight into why different cell types vary in their sensitivity to induction of apoptosis by VSV with wt versus mutant M protein [25, 26]. Cells that depend on Mcl-1 to keep Bak inactive will be particularly sensitive to viruses with wt M protein, which inhibits host gene expression leading to Mcl-1 degradation. As shown here, VSV with wt M protein induces apoptosis faster than M protein mutant VSV in HeLa cells. Silencing of Mcl-1 in HeLa cells infected with rM51R virus causes apoptosis to occur close to the rate of that induced by rWT virus. In contrast, some cells are more sensitive to viruses encoding the rM51R mutant M protein [25, 26]. In these cells, the expression of new proapoptotic gene products is required for the rapid induction of apoptosis, and apoptosis occurs primarily through the death receptor pathway [25]. Even though the mitochondrial pathway is activated, induction of apoptosis in these cells is independent of the mitochondrial pathway [68]. rM51R virus infection activates caspases-8, 9, and 12 in L929 cells, but inhibition of caspases 9 and 12 has no effect on apoptosis [25]. Another example is mouse prostate epithelial cells in which rM51R virus kills cells faster than rWT virus (D.F.Gaddy and D.S.Lyles, personal communication).

Finally, there are many cell types in which viruses with wt versus mutant M protein induce apoptosis at similar rates. These are likely to be cells in which the induction of apoptosis by both wt and M protein mutant virus is dependent on the mitochondrial pathway, presumably through cross-talk with the death receptor pathway, and not dependent on regulation of Bak by Mcl-1 [47]. Examples of such cells include human prostate epithelial cells and prostate cancer cells [16], as well as many other cancer cell types in which the rM51R and rWT viruses have similar apoptotic rates.
(Maryam Ahmed, personal communication). This is important for use of VSV as an oncolytic virus. VSV with wt M protein is not likely to be used as oncolytic virus due to its neurotropism. However, M protein mutant virus is unable to inhibit host gene expression, induces IFNs and other antiviral gene products, and is therefore unable to infect normal cells with intact IFN signaling pathways, such as those in the CNS [49]. The fact that these two viruses induce apoptosis to similar extents in different cancer cells supports the use of M protein mutant virus in oncolytic virus therapy. Furthermore, the role of the mitochondrial pathway in apoptosis induced by M protein mutant virus supports the use of small molecule inhibitors of Bcl-2 to enhance viral oncolysis, as reported recently in chronic lymphocytic leukemia [77].

A comparison of all the cell types that have been studied shows that VSV activates many different pathways that contribute to apoptosis to varying degrees in different cell types. These pathways include both of the major apoptosis pathways, the mitochondrial pathway and the death receptor pathway. In contrast to previous work, my data bring a new view to VSV-induced apoptosis which emphasizes the similarity of the rWT and rM51R viruses. These viruses likely share a common pathway involving Bak, Bcl-XL, Mcl-1 and caspase-8. This common pathway may then be modified by activation of other pathways depending on whether wt or mutant M protein is involved as well as by what proteins are present in the host cell prior to infection, and what proteins are synthesized during infection. These principles are likely to be true of other viruses in which common pathways are activated, but are modified based on the nature of the virus-host interaction.


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CONFERENCE ABSTRACTS
