University of Alberta

The Vaccinia Virus F1L Protein: Characterization of Mitochondrial Localization and Inhibition of Apoptosis

by

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ABSTRACT

Poxviruses encode multiple immune evasion proteins including proteins that regulate the apoptotic cascade. We have recently identified one such protein, F1L, which localizes to mitochondria and inhibits apoptosis. Close analysis of the C-terminal tail of F1L revealed a short transmembrane domain flanked by positively charged lysines. By generating a series of F1L constructs, we show that these positively charged amino acids are necessary for targeting F1L to mitochondria. In addition, we concluded that F1L displays classical tail-anchored topology where the N-terminus faces the cytoplasm. Utilizing the various F1L mutant constructs we found that F1L localization to the mitochondria is necessary to inhibit apoptosis since mutant constructs that fail to localize to the mitochondria have reduced anti-apoptotic ability. Our studies show that F1L is a new member of the tail-anchored protein family which localizes to mitochondria during virus infection and inhibits apoptosis as a means of guaranteed vaccinia virus survival.

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LIST OF ABBREVIATIONS

AIF	apoptosis inducing factor
ANT	adenine nucleotide translocator
Apaf-1	apoptotic protease activating factor-1
Bak	Bcl-2 homologous antagonist- killer
Bax	Bcl-2 associated X-protein
Bcl-2	B-cell lymphoma protein 2
Bcl-xL	B-cell lymphoma protein xL
BH	Bcl-2 homology domain
Bid	Bcl-2 like inhibitor of death
CAD	caspase activated DNAase
CPV	cowpox virus
CrmA	cytokine response modulator A
C-terminus	carboxy terminus of peptide
CTL	cytotoxic T lymphocyte
Cyb5	cytochrome b5
DED	death effector domain
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
EEV	extracellular enveloped virion
EGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
EV	ectromelia virus

FADD	Fas associated death domain
ICAD	inhibitor of caspase activated DNAase
ICE	interleukin-1β-converting enzyme also known as caspase-1
IMV	intracellular mature virion
LMP-1	latent membrane protein-1
MCV	molluscum contagiosum virus
MOI	multiplicity of infection
NF-κB	nuclear factor kappa-B
NK	natural killer
NP-40	nonionic detergent P-40
N-terminus	amino terminus of peptide
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PT	permeability transition
RNA	ribonucleic acid
RPV	rabbitpox virus
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Serp-2	Serine protease-2
Smac	Second mitochondria-derived activator of caspase
Spi2	Serine protease inhibitor-2
SRP	signal recognition particle

TA	tail-anchored
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TNT	transcription and translation
TRADD	TNF receptor associated death domain
Vamp	vesicle-associated membrane protein
VDAC	voltage dependent anion channel
vFLIPS	viral Fas-associated death domain like interleukin-1β converting enzyme inhibiting protein
vMIA	viral mitochondrial inhibitor of apoptosis
VV	vaccinia virus
VV811	a deletion vaccinia virus missing 55 open reading frames including F1L

CHAPTER 1 – GENERAL INTRODUCTION

1.1 Introduction to *Poxviridae*

The Poxviridae family is composed of two subfamilies, the *Chordopoxvirinae* which infect vertebrates and the *Entomopoxvirinae* which infect invertebrates (49). The *Chordopoxvirinae* subfamily is further comprised of eight genera including *Orthopoxviruses, Parapoxvirus, Capripoxivirus, Suipoxvirus, Leporipoxvirus, Aviopoxvirus, Yatapoxvirus, Molluscipoxivirus* and possibly a ninth genera which is currently reserved for those poxviruses that are unclassified (49). The host range of all eight genera is uniquely large ranging from humans to monkeys to crocodiles and skunks (Table 1-1). Two species of poxviruses that commonly cause disease in humans, include the well-known variola virus, the causative agent of smallpox, and molluscum contagiosum virus, which causes benign lesions on the skin of infected individuals (43, 192). However, monkeypox, cowpox, and vaccinia virus have also been linked to human infections (49, 59, 79, 98).

1.2 Introduction to *Orthopoxviruses*

Members of the *Orthopoxviruses* genus are the best characterized poxviruses and have numerous implications on disease and immune modulation (49). Variola virus, the causative agent of smallpox, is a species found within the *Orthopoxvirus* genus and has been responsible for causing more deaths world-wide than any other known virus (49, 192). Vaccinia virus and cowpox virus, the vaccine agents used for vacciniation against variola virus, are also two members of the *Orthopoxvirus* genus. Vaccinia virus has

Table 1-1: The *Poxviridae* Family. Adapted from Fenner 1996

Subfamily:	Genus:	Members:	Reservoir Host:	Other Infected Hosts:
Chordopoxvirinae	Orthopoxvirus	Variola Virus Vaccinia Virus Ectromelia Virus Cowpox Virus Monkeypox Virus	Humans Unknown Rodents Rodents Squirrels	None Humans, Cows, Buffaloes Silver fox, blue fox, mink Cats, cows, humans Monkeys, humans
	Avipoxvirus	Fowlpox Virus Canarypox Virus	Birds	Humans
	Capripoxvirus	Sheepox Virus Goatpox Virus	Sheep Goats	None
	Leporipoxvirus	Myxoma Virus	*Sylvilagus brasiliensis* *Sylvilagus bachmani*	*Oryctolagus*, other leporids *Oryctolagus*, other leporids
	Molluscipoxvirus	Molluscum contagiosum	Humans	None
	Parapoxvirus	Orf Virus	Sheep	Other ruminants, Humans
	Suipoxvirus	Swinepox virus	Swine	None
	Yatapoxvirus	Yaba Monkey Tumor Virus	Monkeys	Humans (rare)
	Unclassified	Macropod poxvirus Crocodilian poxvirus	Kangaroos, Quokkas Crocodiles	None None
Entomopoxvirinae	Entomopoxvirus A	Melolontha melolontha Virus		
	Entomopoxvirus B	Amsacta moorei Virus		
	Entomopoxvirus C	Chironomus luridus Virus		

particularly contributed to our current understanding of poxviral biology due to several of its proteins being involved in immune evasion (49, 116, 122). Together, the *Orthopoxviruses* have contributed to major discoveries in science ranging from the elucidation of cellular pathways to the global lessons learned during the eradication program of smallpox in 1977 (49).

1.2.1 Vaccinia Virus

The best characterized member of the poxvirus family is vaccinia virus which shares >90% homology to variola virus but rarely causes disease in humans (45, 52, 60, 122, 184). Vaccinia virus is considered to be the prototypic family member and is an important research tool. The origins of vaccinia virus however are obscure and its reservoir host is unknown (49). Vaccinia virus became widely known when it was used as the vaccine of choice against smallpox in the 1950s and replaced the preceding vaccine, cowpox virus (49). A stable freeze dried form of vaccinia virus is inoculated onto the upper arm resulting in a localized skin lesion or "pox" resulting in a lifetime visible scar only seen on individuals born before 1977, the year vaccination was discontinued. Although smallpox is no longer a threat, continued research on vaccinia virus is still extremely important for understanding poxviral biology and unraveling host immune pathways.

1.2.2 Ectromelia Virus

Ectromelia virus, also referred to as mousepox, was discovered in 1930 after causing a smallpox "like" disease in laboratory mice (49, 50). Ectromelia virus shares

significant sequence homology to vaccinia virus and is extensively studied since it is an ideal model system to study poxviral pathogenesis (50). Most importantly, it is of current interest in the field of apoptosis for it has been shown to express three gene products which potentially inhibit apoptosis (J.Taylor and M.Barry unpublished data) (19, 211). Two of these gene products, Spi2/CrmA and F1L, have homologous counterparts in vaccinia virus (J.Taylor and M.Barry unpublished data) (211).

1.3 Myxoma Virus

Myxoma virus a member of the *Leporipoxvirus* genus causes benign fibromas in wild rabbits in America (*Sylvilagus brasiliensis*) and fatal myxomatosis in European rabbits (*Oryctolagus cuniculus*) (49). Myxoma virus provides one of the first examples of viral biological pest control: in this case the European rabbit in Australia and Europe. Like Ectromelia virus, myxoma virus is also useful for studying poxvirus pathogenesis and has played an important role in understanding regulation of apoptosis by poxviruses (130). Myxoma virus expresses four anti-apoptotic inhibitors, one of these, M11L, is of particular interest (10, 46, 47, 54, 111, 123, 219). M11L shares no homology to any vaccinia virus protein but shares functional homology to the vaccinia virus protein F1L (46, 47, 219).

1.4 The Poxvirus Genome

The genome of poxviruses is composed of a linear double-stranded DNA molecule and is one of the largest mammalian viral genomes ranging in length from approximately 130 to 300 kilobases (122). The molecular structure of the poxvirus

genome is unique in that it contains hairpin loops at both ends which join complementary inverted terminal repeats (Figure 1-1). The central core of the genome is highly conserved between all members of the family and encodes essential housekeeping genes involved in transcription, translation, and DNA replication (52). The inverted terminal repeats vary in length and sequence between the different poxviruses and contain open reading frames typically involved in immune evasion (115, 146).

1.5 The Poxvirus Virion

Unlike all other animal viruses, poxvirus virions may be observed with the use of a light microscope (20). A more detailed view by electron microscopy reveals a 300nm virion containing a homogenous core surrounded by lateral bodies and an external phospholipid bilayer (Figure 1-2) (40, 128). F17R, L4R, A3L, and A10L are the most abundant structural vaccinia virus proteins accounting for ~70% of the virion weight (83, 122, 213, 214, 227). Two forms of poxviral virions are produced during infection, intracellular mature virions (IMV) and extracellular enveloped virions (EEV) (122, 143).

1.6 Vaccinia Virus Lifecycle

The replication cycle of vaccinia virus, and other poxviruses, is very complex. Distinctively, poxviruses are the only mammalian DNA viruses that replicate in the cytoplasm of infected cells (122). Their large genomes encode enough genetic material to successfully replicate without relying on host cellular machinery. Virus infection begins with attachment and fusion to the host cell membrane releasing the DNA containing core into the cytoplasm. Interestingly, very little is known regarding the entry of vaccinia virus

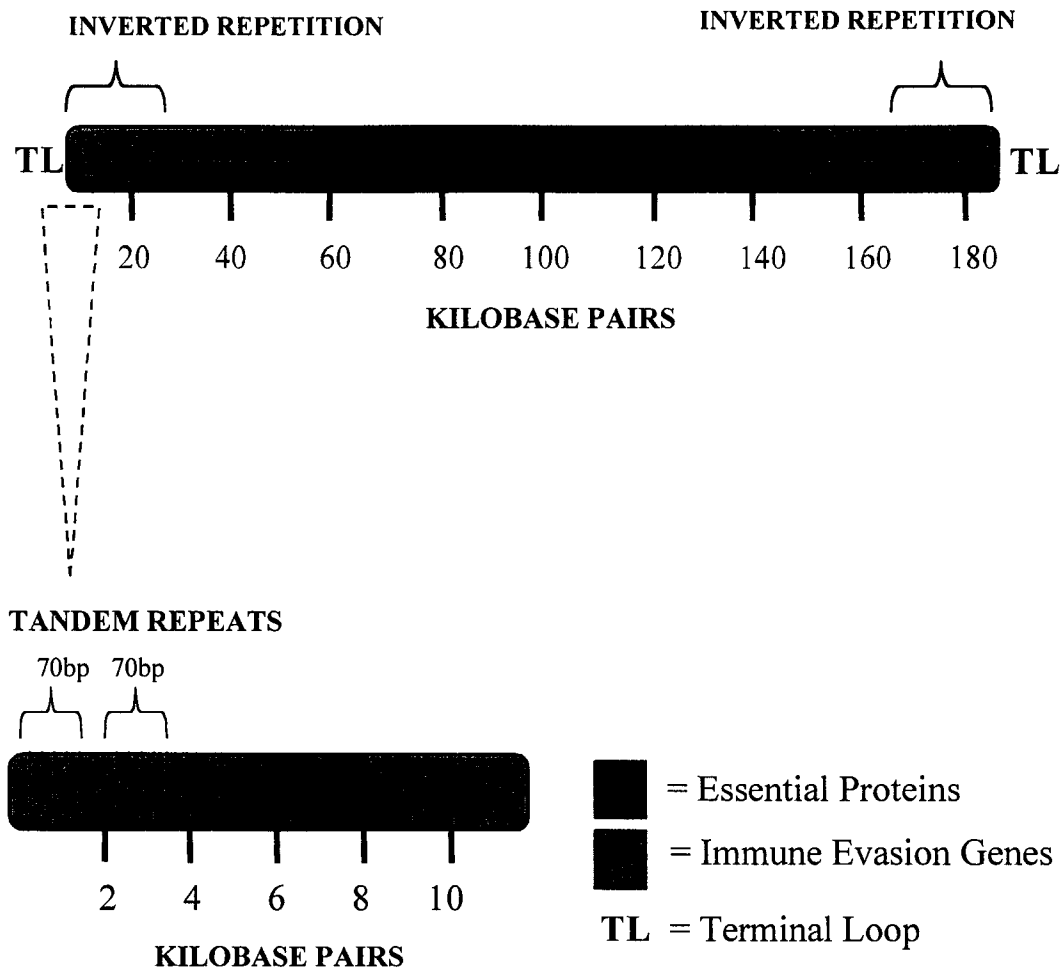
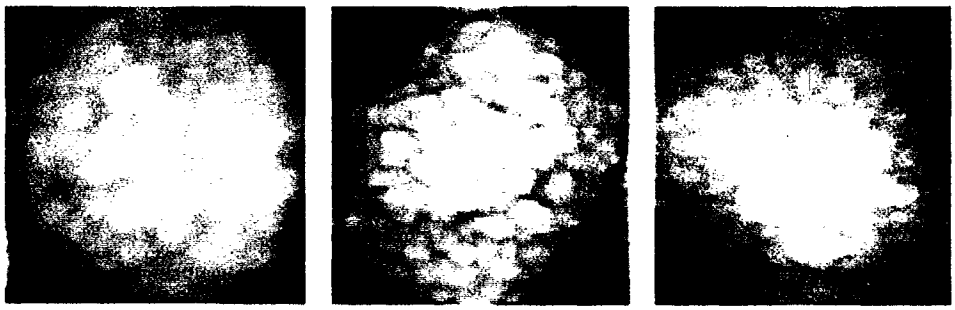


Figure 1-1: The vaccinia virus genome. A representation of the entire poxvirus genome using vaccinia virus as a model. Poxvirus genomes range in size from 130-300kb in length and contain terminal loops (TL) at both ends which join inverted repeats. The central component (purple) encodes essential proteins, while immune evasion genes (blue) are found at the ends of the genome. Adapted from Moss 1996.

A)



Malkin, A.J. *et al.* J.Virol 77(11)6332-40

B)

M. Nermut, National Institute for Biological Standards and Control, U.K.

Figure 1-2: The poxviral virion. **(A)** High-resolution, in situ atomic force microscopy are 385 by 385 nm (a), 425 by 425 nm (b), and 365 by 365 nm (c). **(B)** Electron microscopy of vaccinia virus virion

into cells nor has a cell surface receptor been identified (122). The possible receptor candidate is thought to be highly conserved based on the wide host range of vaccinia virus (122). Proceeding viral entry, early mRNAs are synthesized immediately from the core, producing proteins important for immune evasion and proteins involved in replication and transcription (125). Subsequently to uncoating, the genomic DNA is replicated into concatemers (122). Following replication, intermediate and late gene expression occurs producing various proteins involved in assembly and maturation. After cleavage of the concatamer DNA, the genomic DNA is packaged in discrete membrane structures referred to as "virus factories" forming immature virions, which eventually develop into IMV. IMVs are further wrapped with Golgi membranes and fuse with the plasma membrane resulting in the release of EEV (Figure 1-3) (122).

1.7 General Overview of Poxviruses and Immune Evasion

In response to the selective pressures initiated by the host immune response, poxviruses rely on their large coding capacity to encode numerous proteins for immune evasion (191). Covering all facets of the immune response, poxviruses encode proteins involved in modulating innate and adaptive immune responses (122, 191). However, poxviruses are notorious for inhibiting several key points in the apoptotic cascade by a variety of mechanisms such as encoding TNF receptor decoys, vFLIPS, caspase inhibitors, and recently the discovery of a novel inhibitor that functions at the mitochondria, F1L (11). The following description of the various apoptotic pathways and

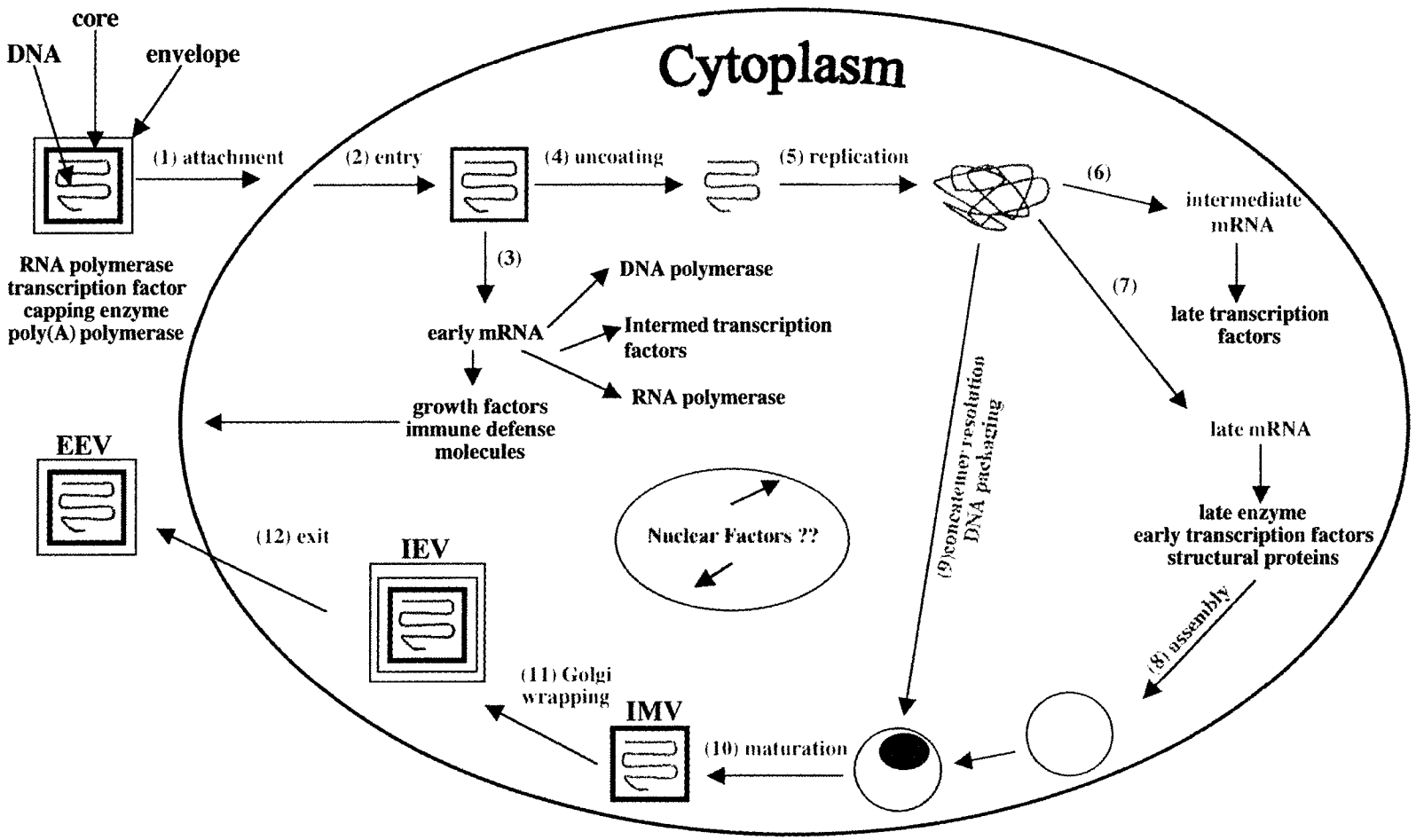


Figure 1-3: The Vaccinia Virus Lifecycle. (1) virions attach to cells and fuse to membrane (2) core component of virions are release into the cytoplasm (3) cores produce early mRNAs (4) uncoating of double stranded DNA occurs (5) replication occurs to produce concatemeric DNA (6,7) intermediate and late genes are transcribed from concatemeric DNA (8) assembly of virions begins (9) concatemeric DNA is cleaved and packaged into individual virions (10) maturation into infectious intracellular mature virions (IMV) (11) virions are wrapped with Golgi membranes to produce intracellular enveloped virions (IEV) (12) IEV fused with the cell surface releasing extracellular enveloped virions (EEV). Adapted from Moss 1996.

the poxviral proteins that inhibit them demonstrates how research on poxviruses has increased our knowledge of apoptotic cascades and viral-host interactions.

1.8 Apoptosis

Apoptosis or cell suicide, is a highly controlled pathway tightly regulated by a family of cysteine proteases referred to as caspases (133, 203). Caspases are found as inactive zymogens within the cell. After the onset of an apoptotic stimulus, caspases become cleaved at aspartic acid residues and activated. Cleavage and activation of caspases results in the dismantling of the cell through various pathways. Unlike other posttranslational modifications, cleavage of caspases is irreversible which is why they are involved in several irreversible cellular pathways such as the cell cycle, development, and cell death (203).

The term apoptosis, which originated from the Greek word describing leaves falling from trees, was adopted in 1972 to describe an uncharacterized form of cell death (87). Interestingly, although apoptotic death is characterized by the activation of caspases, its relationship to caspases was not revealed until 18 years later when Robert Horvitz and colleagues discovered *ced-3* and *ced-4* genes in *Caenorbabditis elegans* (41, 87, 242). Ced-3 and Ced-4 protein products were implicated in nematode cell death and later found to be homologous to interleukin-1β-converting enzyme (ICE), also referred to as caspase 1, and apoptotic protease-activating factor-1 (Apaf-1) in mammalian cells, respectively (232, 241, 242, 249). This discovery eventually led to Robert Horvitz winning the Nobel prize in 2002 and the genesis of apoptotic biology. Since the discovery of *ced-3* and *ced-4*, new members of the caspase family have gradually been

added to the list which is now currently composed of 13 members (Figure 1-4) (203). The caspase family is divided into three functional categories: (1) initiator caspases, involved in instigating apoptosis in response to apoptotic stimuli (2) effector caspases, involved in carrying out cell death, and (3) caspases involved in cytokine maturation. Capsases activation requires specific protein-protein interactions between pro-caspases and adapter molecules such as Apaf-1. Two domains found in members of the caspase family that are important for carrying out these interactions include death effector domains (DED) and caspase recruitment domains (CARD) (55). Caspases and their adapter molecules contain either a DED or CARD domain and their subsequent interaction leads to activation.

Members of the caspase family share a number of unifying characteristics. First, all caspases contain a conserved QACXG pentapeptide domain within the active site (32, 33). Second, caspases are constitutively expressed but have little or no activity until apoptosis is instigated (155, 203, 233). Third, caspases are structurally composed of a prodomain, large subunit, and small subunit, and it is only after proteolytic processing that these three domains are liberated and the heterodimer active form of the enzyme can assemble (Figure 1-5) (33, 165, 217, 229). Fourth, caspases are unique in their strict requirement for cleavage at an aspartic acid residue and lastly, due to their distinct substrate specificity, caspases are involved in the cleavage of themselves and other caspases resulting in a caspase cascade (33, 155, 203, 233).

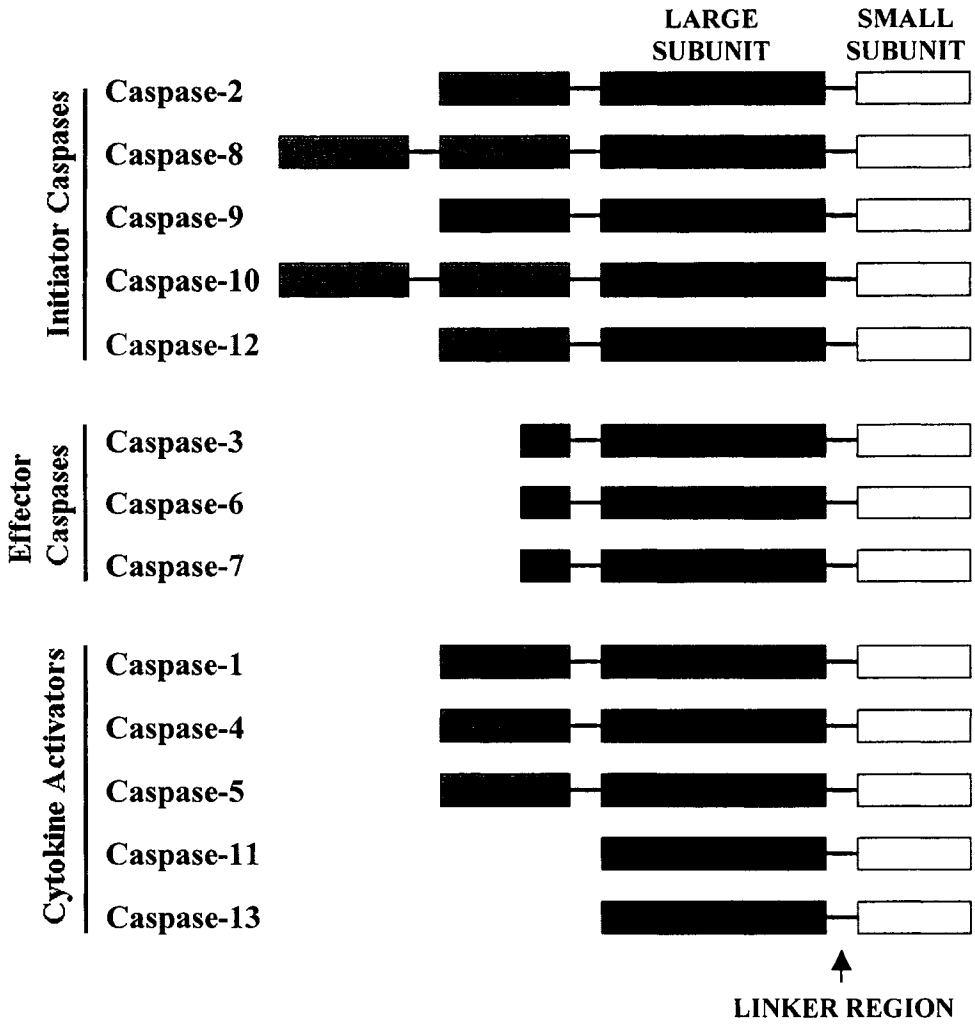


Figure 1-4: Members of the caspase family. Caspases are grouped according to their *in vivo* role: initiator, effector, and cytokine activating caspases. Caspase recruitment domains (CARD) (green) and death effector domains (DED) (blue) are involved in recruitment and activation. The N-peptide (N) found on effector caspases is removed during apoptosis. The large subunit (purple) and small subunit (yellow) of each caspase is separated by a linker region. Adapted from Degterev, A. et al. 2003; Denault & Salvesen 2002.

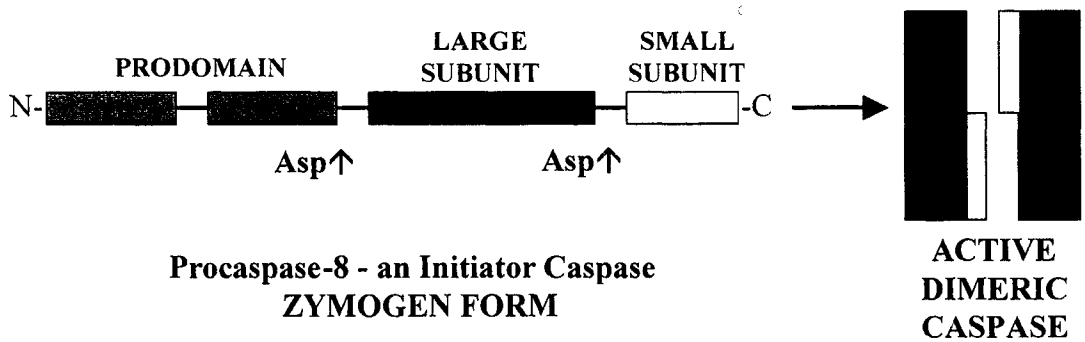


Figure 1-5: Processing of Caspases. Caspases are synthesized as inactive zymogens that undergo proteolytic processing. Activation involves cleavage at conserved aspartic acid residues between domains, followed by the association of the large and small subunits to form a active heterodimer. Processing is mediated by either caspases or other proteases or by autocatalysis. The amino terminal end (N) of each procaspase contains either a caspase recruitment domain (CARD) or death effector domain (DED) domain or N-peptide, all used for recruitment and activation. In this example the prodomain of caspase-8 contains two DED domains. Adapted from Earnshaw, W.C. *et al.* 1999; Thornberry and Lazebnik 1998.

1.8.1 Pathways of Apoptosis

Caspase activation eventually leads to the hallmark features of apoptosis such as loss of the mitochondrial membrane potential, release of cytochrome c, chromatin condensation, DNA fragmentation, and membrane blebbing (67, 231). As a result, apoptosis leads to a "silent" death avoiding inflammation and is undetected by the immune system (203). Although numerous apoptotic pathways exist, the pathways eventually converse leading to a highly efficient dismantling of the cell (56). The initial trigger of apoptosis can be stimulated either by extra-or intracellular signals (162). Common extracellular pathways of apoptosis can be induced by members of the tumor necrosis family (TNF) receptors such as tumor necrosis family receptor-1 (TNFR1) and Fas, often referred to as the death-receptor pathways (8, 127, 168, 206, 240). A second extracellular pathway of apoptosis is the granule secretion from cytotoxic T lymphocytes (CTL) (8, 66, 68, 237). Stimulation of intracellular pathways is often caused by physiological changes such as DNA damage, organelle malfunction, or virus infection (89, 134, 157, 158)

1.8.1.1 TNF and Fas Apoptotic Pathways

Activation of apoptosis via TNFR-1 or Fas is often triggered as an active process by cytotoxic T lymphocytes (CTL) and natural killer cells (NK) to defend against virus infection (8, 82, 168). This extracelluar apoptotic pathway is a strategy developed by the host to prevent the replication and dissemination of viral particles. Additionally, TNFR-1 and Fas pathways are also involved in maintenance of cellular homeostasis, embryogenesis, and tissue atrophy (127). While TNF is a soluble protein commonly

secreted by NK cells, macrophages, and CTLs and the Fas ligand, is a cell surface protein found on CTLs, both ligands initiate a common apoptotic pathway (Figure 1-6 A and B) (127, 205). Upon binding of TNFα or Fas ligand with TNFR1 or Fas receptors respectively, they trimerize the receptors leading to the recruitment of the adapter molecule Fas-associated-death-domain (FADD). FADD is recruited directly to the Fas receptor while indirectly to the TNFR1 via a second adapter called TNF-receptor-associated-death-domain (TRADD). Once FADD is recruited to the cytoplasmic domain of either TNFR1 or Fas, pro-caspase-8, the chief initiator caspase, is autocatalytically cleaved and once activated, inititates the cleavage of downstream caspases (33, 96, 126, 203, 236). Interestingly, the pathway downstream of caspase-8 depends on the cell type (175). In type I cells such as SKW6.4 and H9 cells, a strong activation of caspase-8 occurs leading to direct activation of the chief effector caspase, caspase-3 (175). In type II cells such as Jurkat and CEM cells, lower levels of caspase-8 are activated resulting in a apoptotic pathway that passes through the mitochondria via cleavage and activation of the pro-apoptotic Bcl-2 member Bid, resulting in the loss of the inner mitochondrial membrane potential and release of cytochrome c (100, 175). Once released from mitochondria, cytochrome c associates with the adapter molecule Apaf-1. This association leads to the recruitment of pro-caspase-9 resulting in the formation of the apoptosome (21, 250). The formation of the apoptosome allows pro-caspase-9 to become activated eventually leading to activation of caspase-3 linking the type I and type II pathways together and subsequently resulting in death of the cell (56, 162, 175) (Figure 1-6 A and B).

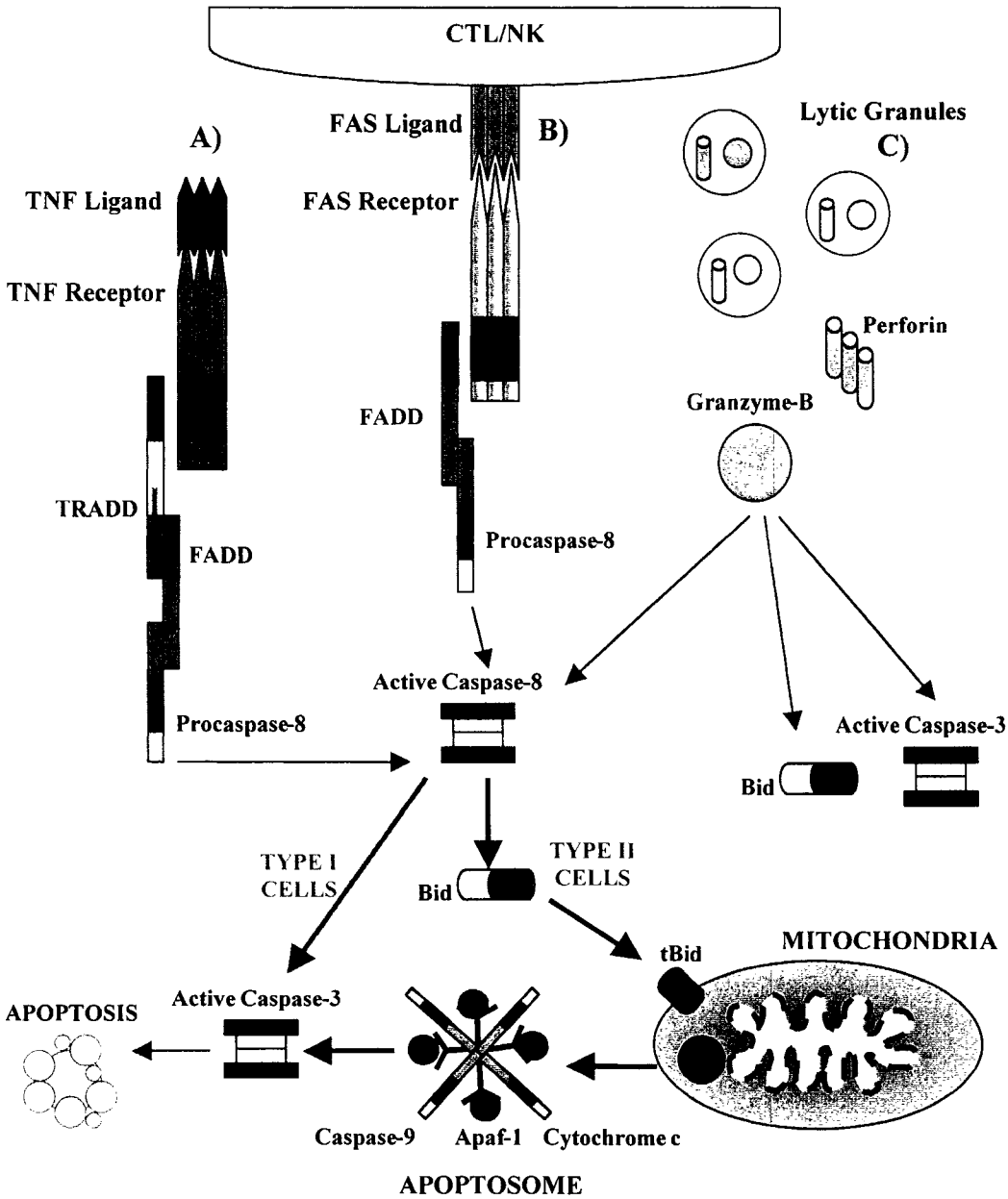


Figure 1-6: Extrinsic apoptotic pathways. **(A)** Tumor necrosis factor (TNF) death receptor pathway **(B)** Fas death receptor pathway **(C)** Granule secretion.

1.8.1.2 Granule Secretion and Granzyme B

A second method CTLs and NK cells use to destroy virally infected cells is by delivery of cytotoxic granules directly into target cells (Figure 1-6 C) (8, 105). The contents of cytotoxic granules includes perforin and granulysin, two pore forming proteins, and granyzmes A, B, C, D, E, F, G, H, K, and M all of which are serine proteases (114). While eleven granzymes have been discovered, the majority of studies have focused on granzyme B. Like caspases, granzyme B is a protease with specificity for aspartic acid residues (151). Once granzyme B enters into the target cell, it induces death through the direct activation of caspase-3 and Bid (8). Although all members of the caspase family become activated upon granzyme B delivery, granzyme B predominately initiates apoptosis through direct cleavage of caspase-3 (7, 118). Additionally, granzyme B has also been widely demonstrated to cleave the pro-apoptotic Bcl-2 family member Bid (3, 9, 118). Granzyme B cleavage of Bid results in a unique cleavage product of Bid which differs that of caspase-8. Cleavage of Bid by granzyme B results in translocation of Bid to the mitochondria resulting in loss of the inner mitochondrial membrane potential and release of cytochrome c (3, 65).

1.8.2 Mitochondria and Apoptosis

Although apoptotic pathways connect several organelles within a cell, most research has been focused on the major regulator of the apoptotic caspase, the mitochondria. Since the discovery of apoptosis as a unique form of cell death nearly 30 years ago, research has quickly unraveled the existence of multiple apoptotic pathways within cells. It is now clear that all of these pathways converge at a central and ultimate

checkpoint within the cell, the mitochondria (55, 56, 132). Once, only thought to maintain life via energy production, mitochondria are now known to be the key organelles for controlling death based on harboring numerous toxin proteins within the intermembrane space (55, 56, 132).

Mitochondria have a double membrane system. The inner mitochondrial membrane is responsible for electron transport and oxidative phosphorlyation (132). The outer mitochondrial membrane contains proteins that reside in the intermembrane space (132). During electron transport, proton translocation occurs generating a net positive charge within the inter-membrane space. Consequently, the low proton concentration in the matrix produces a net negative charge and generates a charge differential across the inner mitochondrial membrane referred to as the inner mitochondrial membrane potential. To maintain the charge gradient generated by electron transport, the outer membrane inhibits diffusion of intermembrane contents except via pores formed by the voltage-dependent anion channel (VDAC) (132). The major mitochondrial events that occur in an apoptotic cell include (1) loss of the inner mitochondrial membrane potential, (2) release of pro-apoptotic proteins from the intermembrane space and (3) opening of the mitochondrial permeability transition (PT) pore (55, 56, 132).

Maintenance of the inner mitochondrial membrane potential is essential for ATP production and the release of soluble proteins from the intermembrane space is necessary for death. Important pro-apoptotic proteins that are released from the mitochondria upon induction of apoptosis include apoptosis-inducing factor (AIF), second mitochondria-derived activator of caspase (Smac), endonuclease-G, and most importantly cytochrome c (Figure 1-7) (39, 101, 142, 170, 245). Of all the pro-apoptotic proteins released from the

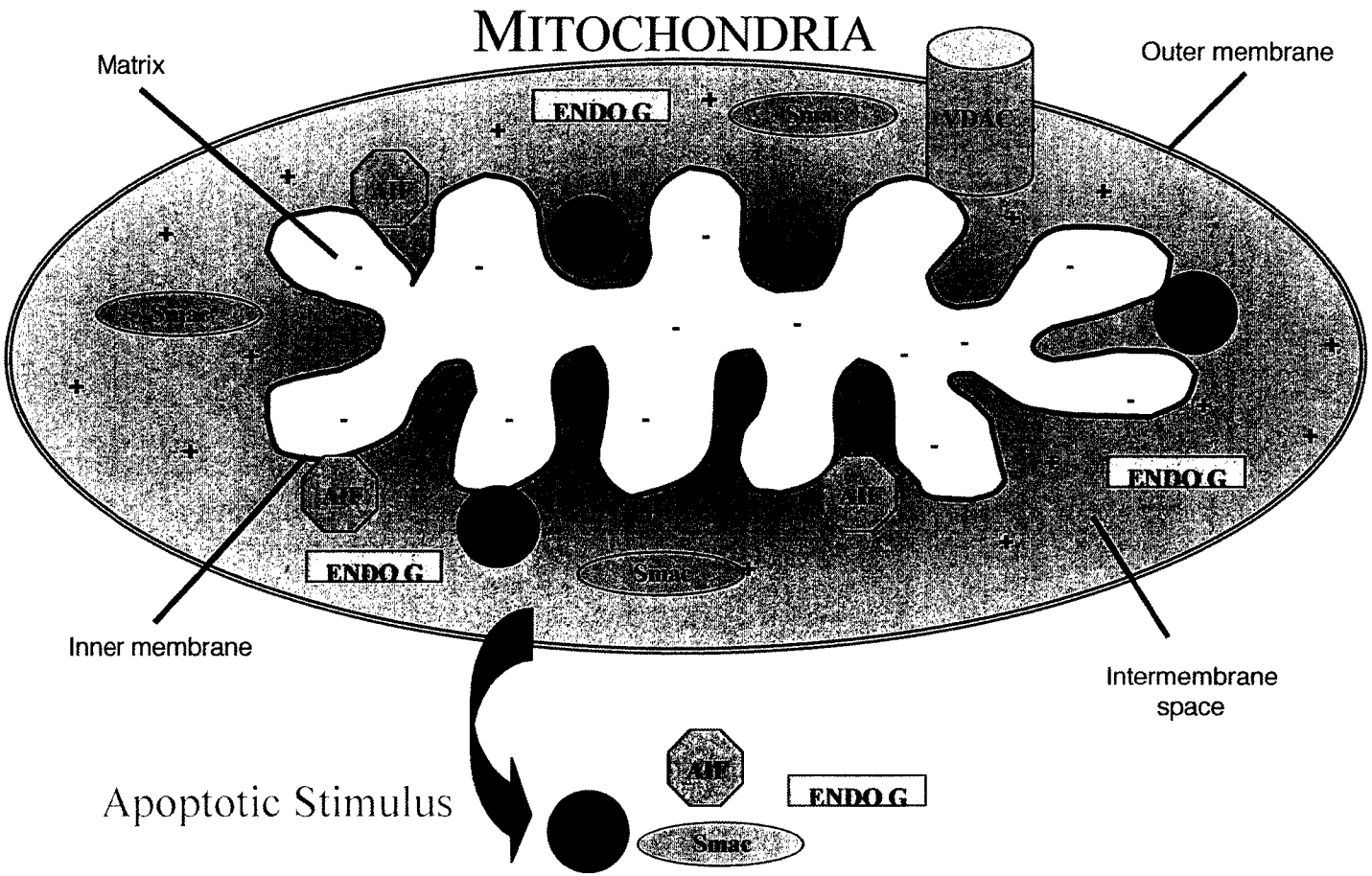


Figure 1-7: Mitochondria and Apoptosis. In healthy cells mitochondria maintain an inner mitochondrial membrane potential and toxic proteins are held within the intermembrane space. During an apoptotic stimulus the charge gradient between the matrix and and intermembrane space is lost resulting in the release of toxic proteins such as cytochrome c (Cyto c) apoptosis-inducing factor (AIF), second mitochondria-derived activator of caspase (Smac) and endonuclease-G (Endo G).

mitochondria, the role of cytochrome c is the best characterized. Cytochrome c is an essential component of the electron transport chain but once released from the mitochondria it binds to Apaf-1 exposing the CARD domain of Apaf-1 allowing docking of caspase-9 via its CARD domain. This three-protein complex, referred to as the apoptosome, is essential for activation of caspase-9 (102, 170, 250).

1.8.3 Control of Apoptosis by Bcl-2 Family Members

Members of the Bcl-2 family tightly control the mitochondrial pathway of apoptosis (58) There are currently 21 members of the Bcl-2 family which are classified as either pro-or anti-apoptotic proteins (Figure 1-8) (139). Several members localize to various subcellular compartments where they await an apoptotic stimulus while other members are cytosolic and undergo a conformational change that exposes targeting motifs allowing them to translocate to membranes to regulate death (58). The first member discovered was Bcl-2 which was originally shown to be the cause of transformation and stimulation of cellular proliferation in follicular lymphomas (70, 207).

Members of the Bcl-2 family possess one or more specific domains referred to as Bcl-2 homology (BH) domains (1, 86). There are four BH domains referred to as BH1, BH2, BH3, and BH4 and all four BH domains contribute to either the pro or anti-apoptotic function of these proteins. BH1 and BH2 are thought to be essential for the anti-apoptotic function of Bcl-2 and Bcl-xL by dimerizing and forming a channel allowing for the appropriate interaction with pro-apoptotic proteins such as Bax and Bak (181, 239). Although all Bcl-2 family members possess a BH3 domain, it is this

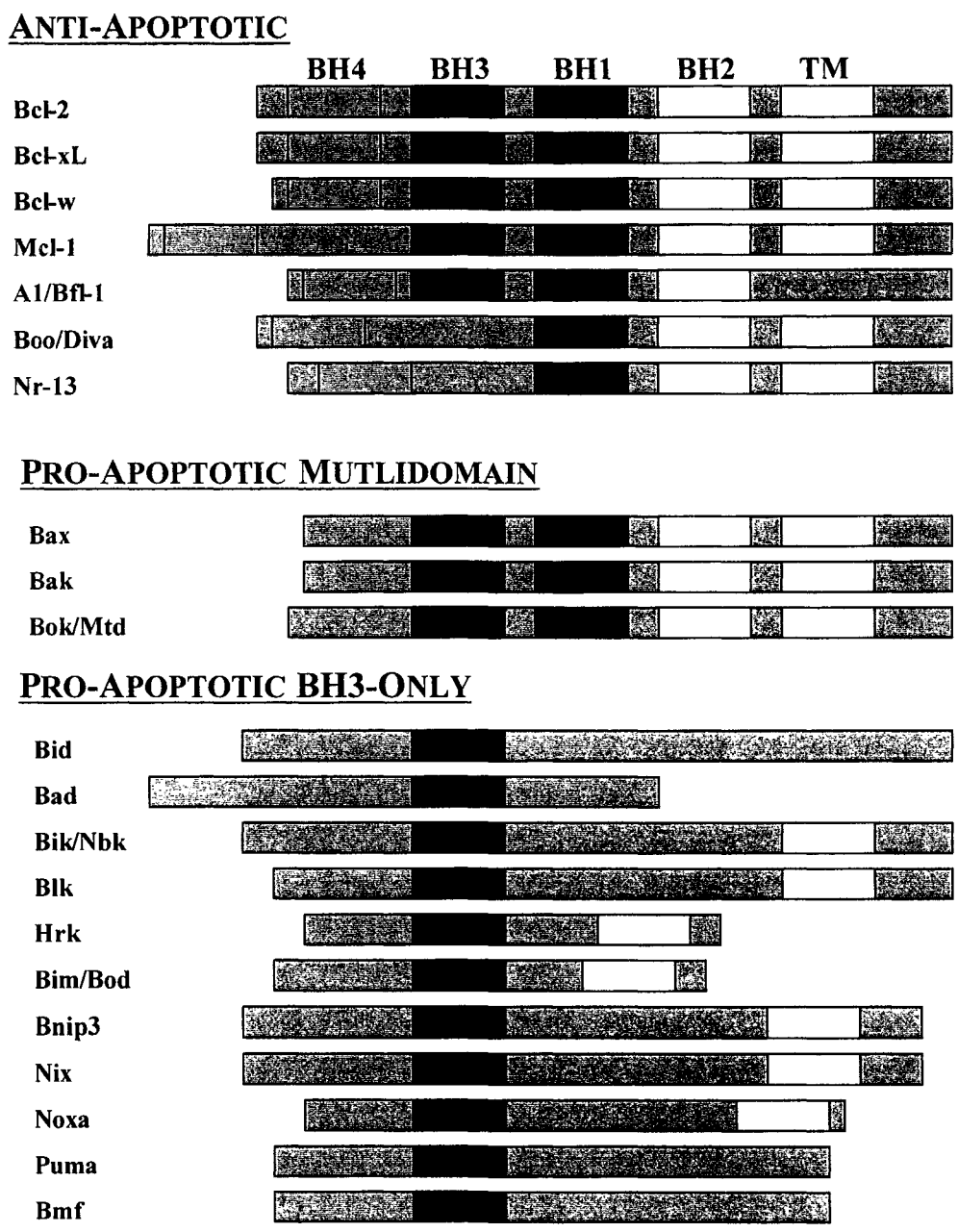


Figure 1-8: Members of the Bcl-2 family. Anti-apoptotic members contain four Bcl-2 homology (BH) domains, while pro-apoptotic members are subdivided into either multidomain family members or BH3-only members. TM = transmembrane domain.

particular domain that is important for promoting cell death by interacting with anti-apoptotic proteins (30, 174). The exact function of the BH4 domain is currently unknown but BH4 domains are only present in anti-apoptotic family members and is thought to be involved in protein-protein interactions (16, 221). Based on the characterization of BH domains, Bcl-2 family members have been further subdivided based on the number of BH domains each possesses (1, 86). The anti-apoptotic proteins Bcl-2 and Bcl-xL contain four BH domains (BH1-BH4) while certain pro-apoptotic members such as Bax and Bak contain only three BH domains (BH1-BH3) and are referred to as multidomain members. In addition, there are "BH3-only" pro-apoptotic proteins such as Bid, Bik, and Bim which contain the minimal amount of sequence necessary to induce cellular suicide. Although pro-and anti-apoptotic members are present within a cell at all times it is the delicate balance between these proteins that decides the outcome of the cell.

1.8.3.1 Pro-Apoptotic Bcl-2 Family Members

There are currently two groups of pro-apoptotic Bcl-2 members: BH3-only proteins such as Bid, and multi-domain BH proteins including Bak and Bax (58). In both classes, the BH3 domain plays a significant role in enabling pro-apoptotic proteins to instigate apoptosis (1, 86). It is currently believed that inhibitors of apoptosis such as Bcl-2 and Bcl-xL sequester BH3-only proteins, preventing them from activating Bax and Bak (28, 99). Therefore, following an apoptotic stimulus, BH3-only proteins are activated first followed by activation of Bak and Bax.

1.8.3.1.1 BH3-only Bcl-2 family members

BH3-only pro-apoptotic proteins are kept in check by both transcriptional and post-translational events (75). The pro-apoptotic protein Noxa is transcriptionaly upregulated by p53 or interferon regulatory factor-1 (138). Post-translational control has been demonstrated for both the pro-apoptotic proteins Bad and Bim (29, 154). Bad and Bim are normally dormant but phosphorylation of Bad causes its release from Bcl-2 whereas phosphorylation of Bim causes its release from microtubules (29, 154, 234).

Bid is an important BH3-only protein that plays a significant role in upstream mitochondrial events. In healthy cells, full-length Bid is found in the cytoplasm. In cells undergoing apoptosis however, Bid becomes cleaved and translocates to the mitochondria (100). Regardless of the apoptotic stimulus, activation of Bid via cleavage is universal and suggests that inhibition of apoptosis by Bcl-2 family members is downstream of Bid cleavage (23, 28, 58). As previously mentioned, Bid becomes activated by either caspase-8 in the death receptor pathway or by granzyme-B following CTL granule exocytosis (3, 9, 65). Caspase-8 cleaved Bid generates a truncated 15-kDa fragment (p15-tBid) that becomes N-myristolylated due to the exposure of a glycine residue (100, 109, 246). In contrast, granzyme B activated Bid produces a 13-kDa fragment (p13-tBid) that is not N-myristolyated (23, 153, 166, 226). Although the specific downstream events proceeding Bid activation are not clear, Bid plays an extensive role in apoptosis and has been documented repeatedly to be essential for the activation of Bax and Bak and the cause of mitochondrial disfunction and release of cytochrome c (23, 100, 109, 153, 166, 225, 246).

1.8.3.1.2 Multidomain Bcl-2 family members

The importance of Bax and Bak in apoptosis was demonstrated when cells lacking both Bax and Bak were found to be resistant to Bid-induced cytochrome c release and apoptosis (226). Although Bax and Bak are both part of the multidomain-BH subfamily of Bcl-2 proteins, they are activated by unique mechanisms. Bax exists primarily as a monomer in the cytosol of healthy cells but translocates to the mitochondria and oligomerizes during apoptosis (5, 71, 199). It is thought that Bax translocates to the mitochondria following a conformational change (197). In contrast, Bak, a member of the tail-anchored protein family, is constitutively found at the mitochondria where it also undergoes a conformational change and oligomerizes in response to an apoptotic stimulus (57).

There are currently two models regarding the release of cytochrome c from mitochondria. Firstly, opening of the permeability transition (PT) pore is thought to cause rupturing of the outer mitochondrial membrane and release of cytochrome c (243, 244). Sustained opening of the PT pore allows for ion equilibrium between the intermembrane space and the cytoplasm. This eventually leads to loss of the inner mitochondrial membrane potential, osmotic swelling, and rupturing of the outer membrane releasing cytochrome c (243, 244). Alternatively, it is thought that pro-apoptotic Bcl-2 family members may form ion channels in the outer membrane allowing ion equilibrium thus causing the release of cytochrome c (55, 56, 81, 230).

Although Bax and Bak are initially found at different locations within a healthy cell and are possibly activated by diverse stimuli, it is suggested they induce similar pro-apoptotic changes at the mitochondria resulting in the release of cytochrome c (23, 153,

226, 246). The oligomerization of Bax and Bak are key events that indicate the "point of no return" in apoptosis. Oligomerization of Bax and Bak is thought to form pores in the outer mitochondrial membrane ultimately resulting in the release of cytochrome c (44, 92, 225). It is thought that oligomerization of Bax and Bak are not independent of each other as indicated when a stringent requirement of Bax was necessary for Bak oligomerization (119). It has also been documented that Bax and Bak can interact with the preexisting PT pore and trigger its opening also causing the release of cytochrome c (129, 185, 186)

In addition to functioning at the mitochondria, traces of Bax and Bak have both been found at the ER (136). It is unclear whether Bak and Bax use the same mechanisms at the ER but early work has demonstrated that both proteins cause the release of Ca^{2+} from the ER (135, 180, 202). Whether this is linked to the pore forming capabilities of Bax and Bak is unknown. It is hypothesized that a distinct apoptotic stimulus holds the key to activating Bax and Bak at the ER or mitochondria (137, 180, 198). For example, BH3-only proteins are thought to be the prerequisite for triggering the mitochondrial pathway whereas lipid secondary messengers are thought to trigger the ER pathway. More importantly however, the promiscuous localization of Bax and Bak to the ER provides evidence that communication between the ER and mitochondria is essential for apoptosis to occur and the ER's role in apoptosis is ever increasing.

1.8.3.2 Anti-Apoptotic Bcl-2 Members

Like a controlled road system, apoptosis is not only composed of "green" lights but inhibitors of the apoptotic cascade exist enforcing "red" lights and preventing death

of the cell. Two major inhibitors of apoptosis in the Bcl-2 family include Bcl-2 and Bcl-xL. Both Bcl-2 and Bcl-xL are integral membrane proteins and members of the tail-anchored (TA) protein family (25, 84, 91). While Bcl-2 localizes to the endoplasmic reticulum, outer mitochondrial membrane, and nuclear membrane, Bcl-xL only resides at the outer mitochondrial membrane (84, 93). Inhibition by Bcl-2 and Bcl-xL commonly prevents the release of cytochocrome c and loss of the inner mitochondrial membrane potential and three mechanisms for this inhibition have been proposed: (1) neutralization of BH3-only proteins (2) neutralization of multidomain proteins or (3) competition with pro-apoptotic proteins for effector molecules (80, 132, 235).

Evidence suggesting that Bcl-2 neutralizes BH3-only proteins was demonstrated when Bcl-2 was shown to inhibit insertion of tBid into mitochondria. Bcl-2 has also been shown to sequester BH3-only molecules thus preventing Bax and Bak oligomerization as well as antagonize the pore-forming effects of Bax (4, 28, 238). Evidence supporting the direct inhibition of multidomain proteins has been demonstrated when oligomerization of Bak was inhibited by competitive binding between Bak and Bcl-2 (169). Bcl-2 has also been shown to inhibit the pore-forming activity of Bax therefore preventing the release of inter-membrane proteins (4). Evidence that Bcl-2 may inhibit apoptosis by competing for effector molecules has been demonstrated in studies on the PT pore. As previously mentioned, opening of the PT pore can result in rupturing of the outer mitochondrial membrane causing release of cytochrome c. Preliminary results have shown that Bax interacts with the ANT and VDAC components of the PT pore and that Bcl-2 may inhibit this interaction (113, 129, 185, 186).

Bcl-2 was originally thought to function mainly at the mitochondria but it is now documented that Bcl-2 also functions at the ER (93, 164). How Bcl-2 regulates apoptosis at the ER is under debate but several studies have shown that Bcl-2 inhibits apoptosis by causing the passive release of Ca^{2+} from the ER and thus causes a partial emptying of the agonistic Ca^{2+} stores (97, 150, 164). Contrary to this, another group has shown that Bcl-2 inhibits apoptosis by preserving high levels of ER luminal Ca^{2+} and preventing the "set-off" of the PT pore (135). Interestingly, Bcl-2Cyb5, a chimera form of Bcl-2 that localizes exclusively to the ER, has been shown to inhibit a constitutive mitochondrial mutant of Bax suggesting a possible "third-party" that shuttles between the ER and mitochondria linking Bcl-2Cyb5 and Bax together (201, 202). Whether this mysterious "third party" is Ca^{2+} or BH3-only proteins has not been determined.

The close relative of Bcl-2, Bcl-xL, is also a potent inhibitor of apoptosis at the mitochondria. Similar to Bcl-2, Bcl-xL interacts with a wide variety of proteins such as BH3-only proteins. However, none of these interactions have narrowed down the possible mechanism of Bcl-xL (28, 99, 107). Evidence supporting the model in which Bcl-xL functions by sequestering active pro-apoptotic proteins has been provided in a crystallography study revealing the close and intimate interaction between Bcl-xL and Bak (124, 174). It was this first initial crystallography study that revealed the importance of the BH3 domains in binding. It also predicted that the BH1, BH2, and BH3 domains of anti-apoptotic proteins form a hydrophobic pocket with the BH3 domain of pro-apoptotic proteins (124, 174). As an alternative mechanism of inhibition, evidence that Bcl-xL might be competing with pro-apoptotic proteins for effecter molecules has been demonstrated in a study where Bcl-xL interacted with Apaf-1 and displaced the

apoptosome thereby inhibiting activation of caspase-9 (58, 74). In addition, specific mutations that disrupt Bcl-xL binding to Bax and Bak still preserve the ability of Bcl-xL to inhibit apoptosis thus suggesting a possible mechanism of action not involving a direct interaction with Bax and Bak (26).

1.8.4 Tail-Anchored Proteins

Several members of the Bcl-2 family are also members of a growing group of proteins referred to as tail-anchored (TA) proteins (17, 18, 25, 91, 176, 224). TA proteins are characterized by: (1) a single transmembrane domain which is presumably helical located at the C-terminus, (2) a hydrophobic transmembrane sequence flanked on both sides by positively charged amino acids, (3) lack of an N-terminal signal sequence, (4) being integral membrane proteins that insert post-translationally, and (5) adopting a topology where the N-terminus is facing into the cytoplasm (18, 224). Members of the Bcl-2 family that possess all five of these characteristics include Bcl-2, Bcl-xL, and Bak (61, 84).

Originally it was thought that TA proteins were only found at the ER or mitochondria. With the discovery of additional TA proteins it has now been concluded that TA proteins are also found at the Golgi apparatus, nuclear membrane, and plasma membrane (95). Research on TA proteins is currently focused on determining how these proteins reach their final destination within a cell. The conventional targeting of type II integral membrane proteins is normally via the signal recognition particle (SRP) pathway (159, 218). Type II integral membrane proteins are classified as having the same orientation as TA proteins but upon initiation of translation an internal hydrophobic

signal sequence is exposed in which a SRP complex binds and transports the polypeptide to the target membrane (69). After type II proteins are targeted to their specific membrane they are anchored by either their signal sequence or by other hydrophobic segments. In contrast, TA proteins do not follow these conventional rules and instead rely on their C-terminal hydrophobic domain for insertion. Since the C-terminal transmembrane anchor is not exposed until completion of translation, it is predicted that TA proteins insert into membranes post-translationally (18).

The C-terminal tail of TA proteins holds the key to understanding which membrane a TA protein will target to. While each TA protein has its own distinct characteristic signal hidden within its C-terminus, a standard claim made is that the length of the hydrophobic domain as well as the presence of positively charged amino acids play a role in deciding the destination of the protein (17, 18, 224). For proteins that target to the outer mitochondrial membrane, the transmembrane domain is usually comprised of no more than 20 amino acids. In contrast, TA proteins that target to the ER have longer transmembrane domains to compensate for the thicker ER membrane (18, 144). The importance of positively charged amino acids of TA proteins was determined by tampering with the C-terminal tail and mutating positively charged amino acids to neutral residues (17, 18, 224). Numerous labs have shown that decreasing the number of positively charged amino acids flanking the hydrophobic domain can cause a severe alteration in localization of mitochondrial targeted proteins and send them to the ER (78, 84, 94).

Tail-anchored proteins perform a wide variety of functions at several different locations within a cell (Table 1-2). A considerable portion are members of the Bcl-2

Table 1-2: Function and localization of various tail-anchored proteins. MOM = mitochondrial outer membrane ER = endoplasmic reticulum C = cytoplasm N = nucleus. Adapted from Wattenberg & Lithgow 2001.

Tail-anchored Protein:	Localization:	Function:
Bcl-2 Family Members		
Bcl-2	MOM,ER,N	Anti-apoptotic
Bcl-xL	MOM	Anti-apoptotic
Bak	MOM,ER	Pro-apoptotic
Bcl-w	MOM	Anti-apoptotic
Viral Proteins		
Myxoma virus MIIL	MOM	Anti-apoptotic
Vaccinia virus H3L	VF	
HSV-2 UL34	ER	
HSV-2 UL56	G	
EBV BHRF-1	MOM	
Protein Localization		
Vamp-1A	ER	Vesicle targeting
Vamp-1B	MOM	Unknown
OMP25	MOM	Binds of inositol 5'phosphatase
Sec12	ER	Sar1p binding
Tom5,6,7,22	MOM	Binding/translocation of mitochondrial precursor proteins
Enzymes/Cofactors		
Cytochrome b5	ER	Electron transfer
OM cytochrome b5	MOM	Electron transfer
Ubc6	ER	Conjugation of Ubiquitin to substrates
Monoamine oxidases	MOM	Oxidation of metabolites

family including Bcl-2 and Bcl-xL (25, 84, 90, 91, 176). While Bcl-xL resides exclusively at the mitochondia to inhibit apoptosis, Bcl-2 is found to localize to the mitochondria, ER, and nuclear membrane (84, 93). For Bcl-2 to localize to three different membranes further emphasizes the complexity involved in cracking the localization code of TA proteins.

1.9 Viral Inhibitors of Apoptosis

Removal of virally infected cells occurs through the activation of CTL and NK cells which stimulate an apoptotic response (8, 168). In response, viruses have evolved counter-strategies to inhibit apoptosis in order to ensure virus replication and survival. Strategically, viruses have evolved mechanisms to target key control points within the apoptotic cascade (Figure 1-9).

1.9.1 Receptor Dismantling

The extrinsic apoptotic pathway starts with engagement of ligand to TNF and Fas receptors followed by recruitment of adaptor molecules (168, 228). Not surprisingly, several viruses have selectively targeted this stage of apoptosis as an early maneuver to prevent cell death. Adenoviruses and poxviruses, for example, are two families of viruses that successfully inhibit apoptosis by causing down-regulation of death receptors or by encoding soluble receptor decoys (Figure 1-9) (14).

E3-10.4K and E3-14.5K are expressed early during adenovirus replication and are responsible for internalization and degradation of Fas in infected cells thereby protecting cells from Fas mediated death (37, 42, 103, 187, 204). In addition, E3-10.4K and E3-

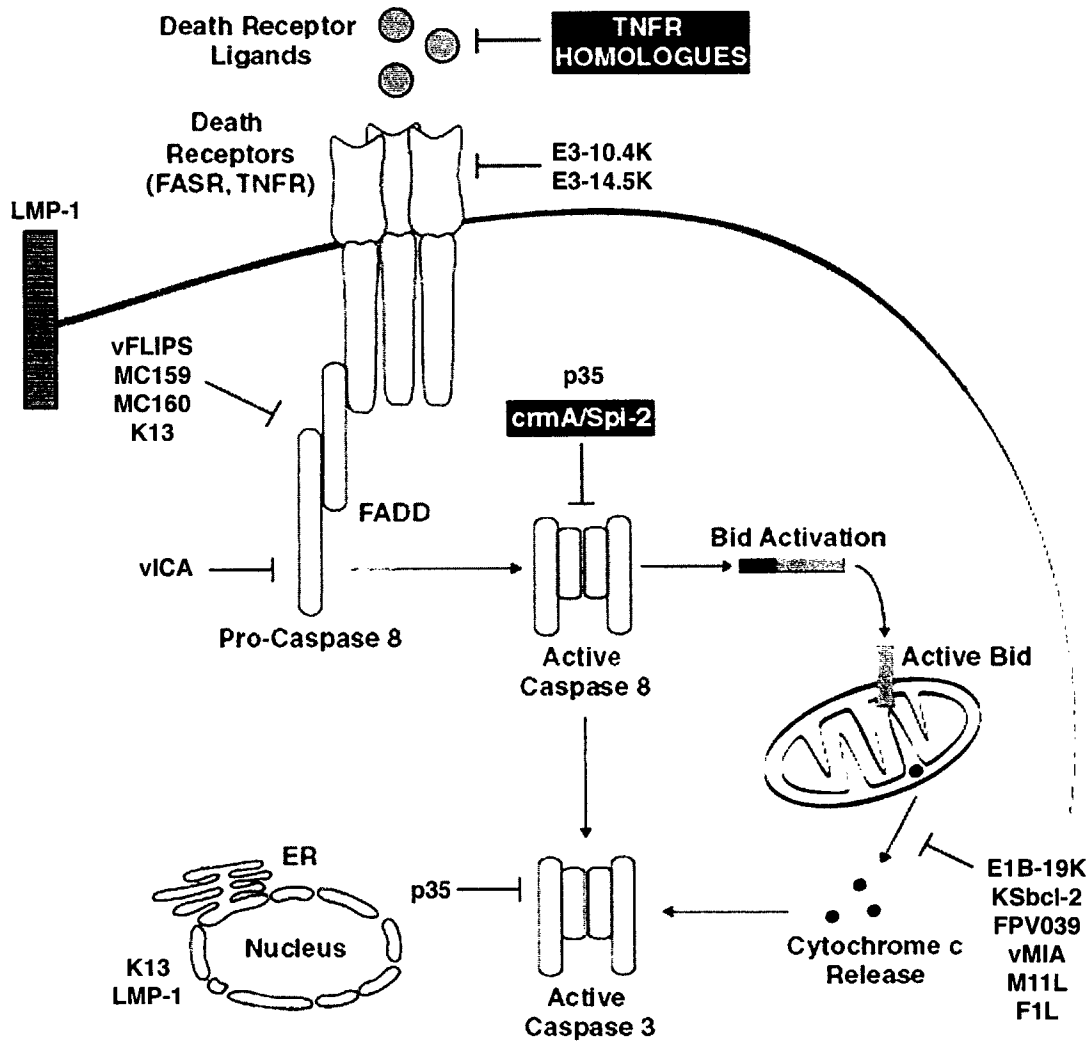


Figure 1-9: Viral inhibitors of apoptosis. Viruses have developed multiple strategies that target distinct components of the apoptotic cascade. In order to inhibit apoptosis viruses can down regulate death receptors, encode soluble receptor decoys, encode vFLIPS, encode caspase inhibitors, or encode novel or Bcl-2 homologous proteins that inhibit apoptosis at the mitochondria. Adapted from Barry, M. *et al.* 2004.

14.5K have also been shown to protect against TNF induced apoptosis although, the levels of TNF receptors on the cell surface remain unchanged. This suggests that E3-10.4K and E3-14.5K inhibit apoptosis by two mechanisms (37, 104, 187).

Several members of the poxvirus family encode soluble TNF receptor (TNFR) proteins as a means to ensure virus survival (167). One of the first described TNF receptor decoys was M-T2, which was discovered in myxoma virus (177-179). M-T2, shares homology to the TNFR ligand binding domain, is secreted as a dimer and sequesters TNFα in a species specific manner (177, 178). Deletion of the M-T2 gene significantly impairs viral infection (212). Cowpox virus encodes CrmB, CrmC, CrmD, and CrmE all of which have been shown to evade the immune system by preventing initial TNF receptor stimulation. (72, 108, 161, 172, 190). Characterizational studies have shown that CrmB, C, D, and E all have sequence similarity to the N-terminal binding domain of TNFRs but are missing the C-terminal transmembrane anchor, except for CrmE which also has a membrane bound form (72, 108, 161, 172, 190). Interestingly CrmB, C, D, and E have different binding affinities and expression profiles, which might provide insight into the infection tactics of cowpox virus (72, 108, 161, 172, 190).

Homologs to CD30 have also been discovered in cowpox virus and ectromelia virus (141, 173). CD30, a member of the tumor necrosis factor receptor family, is normally upregulated during virus infection and acts as a co-stimulatory molecule to sustain the T-cell response against antigen (31). The CD30 homolog expressed by ectromelia virus not only binds and sequesters CD30 ligand but also inhibits intracellular signaling in cells expressing CD30 ligand (173). The role of cowpox virus CD30 homolog has not been characterized.

1.9.2 Signaling Interference

While some viruses have evolved strategies to block the initial stimulation of death receptors, others encode proteins that block downstream signaling events prior to the activation of initiator caspases. Both herpesviruses and poxviruses encode proteins that impede the intracellular signaling of the extrinsic apoptotic pathway. Members of the herpesviruses and poxviruses families encode Fas-associated death-domain like interleukin-1β converting enzyme inhibitory proteins (vFLIPS) that contain DED which are responsible for linking TRADD and FADD to procaspase 8 (Figure 1-9) (15, 73, 200).

vFLIPS encoded by bovine herpesvirus-4, equine herpesvirus, herpesvirus samari, and human herpesvirus-8 all block the interaction between caspase-8 and the death receptor-adaptor complex (15, 73, 200, 220). Interestingly, K13 encoded by human herpesvirus-8 is able to transform fibroblast cells by activating the NFκB pathway, a quality not found in other vFLIPS (194). The suggestion that vFLIPS, which originally were thought to only interfere with upstream death receptor events also influence NFκB signaling provides an interesting twist on the role of vFLIPS during virus infection since the NFκB pathway plays such an important role in the immune response (24, 194).

MC159 and MC160 are two vFLIPS encoded by the poxvirus molluscum contagiosum virus (15, 73, 200). MC159 and MC160 are well characterized and like other vFLIPS have been shown to disrupt recruitment and activation of caspase 8 (15, 73, 200). Molluscum contgiosum virus is the only poxvirus that encodes vFLIPS however, this correlates with the fact that molluscum contagiosum virus is missing the caspase 8 inhibitor CrmA, a common orthopox inhibitor of apoptosis (182, 183). While MC159 has

been shown to inhibit apoptosis by interaction with FADD and caspase 8, MC160 is unable to inhibit apoptosis without the presence of MC159 but is able to interact with FADD and caspase-8 (15, 51, 73, 188, 200, 208). It is not fully understood why MC160 is dependent on MC159 but it is thought that the two proteins help stabilize each other during virus infection (188)

In addition to vFLIPS, viruses have evolved other strategies to block apoptotic cell signaling. A unique apoptotic signaling inhibitor encoded by Epstein Barr virus, a member of the herpesevirus family, is LMP-1 (latent membrane protein-1) (36, 85, 121, 216). LMP-1, an oncogene product, is the major transforming protein in EBV and acts as a constitutively active CD40 receptor by interacting with TRAFs and TRADD in the TNF extrinsic pathway. This interaction up regulates interleukin-10 and NFκB which in turn up regulates anti-apoptotic proteins (36, 85, 121, 216). Although LMP-1 induces transformation via multiple signaling pathways it is still unclear which downstream event leads to the inhibition of apoptosis. However, recently it has been shown that LMP-1 suppresses DNA repair and therefore contributes to genomic instability, a key event leading to transformation and apoptosis (106).

1.9.3 Caspase Inhibitors

Several viruses encode proteins that directly inhibit caspases to prevent the initiation of apoptosis. The best known inhibitor of caspase 8 is the poxviral protein CrmA encoded by cowpox virus. CrmA is a member of the serpin protein family, which consists of proteins that inhibit serine proteinases by forming stable protein complexes (148, 160, 248). CrmA inhibits Fas and TNF induced apoptosis by binding to caspase 8.

CrmA is also capable of inhibiting caspase-1, also referred to as interleukin-1β-converting enzyme (ICE) (Figure 1-9) (148, 160, 248).

CrmA related proteins are found throughout the poxvirus family. Spi-2 found in rabbitpox, VV, and ectromelia virus, and Serp-2 found in myxoma virus all share similar properties to Crm A (38, 88, 110). Spi-2 from rabbitpox virus and VV have high homology to CrmA. While Spi-2 from VV plays a role in resistance to TNF and Fas induced apoptosis and inhibits caspase 1, Spi-2 from rabbitpox virus has only been implicated in inhibiting Fas induced apoptosis although TNF studies have not been performed (38, 88, 110). The ectromelia virus encoded Spi-2, like CrmA, protects cells from TNF induced apoptosis and is able to inhibit caspase 8 and 1 (211). Serp-2 encoded by myxoma virus inhibits caspase 1 activity however, no substantial evidence has been found in regards to whether it can inhibit apoptosis (22, 88, 147, 210). However, a strain of myxoma virus lacking Serp-2 causes an increase in apoptosis and inflammation in infected rabbits suggesting its possible role as an apoptotic inhibitor (117). Interestingly, although there is a common functional theme between the different CrmA homologs expressed by the various poxviruses, an elegant study has shown that CrmA and Serp-2 are not interchangeable during infection of rabbits (131). This suggests CrmA and Serp-2 inhibit apoptosis by mechanisms that have not been supported by previous biochemical studies (Figure 1-9).

Two other caspase inhibitors are p35 encoded by baculovirus and vICA encoded by human cytomegalovirus. p35 has been shown to inhibit Fas and TNF induced apoptosis by its unique ability to inhibit a vast array of caspases including caspase 1, 2, 3, 4, 6 ,7, 8, and 10 (12, 35, 120, 247). Although p35 lacks sequence homology to other

serpins, it appears to use a similar mechanism of inhibiting caspases by trapping caspases in its active site (163). In addition to p35 being as able of inhibiting a wide variety of caspases it also has a wide variety of other functions. Its ability to inhibit oxidant-induced apoptosis was demonstrated when p35 was able to quench free radicals thus preventing release of cytochcrome c and activation of caspase 3 (171). vICA has been shown to prevent the activation of caspase-8 by binding to its prodomain in cells induced with TNFα and anti-Fas (189). vICA does not show sequence homology to any known suppressors of apoptosis suggesting that vICA represents a new class of viral inhibitors of apoptosis (Figure 1-9) (189).

1.9.4 Bcl-2 Homologs

Based on the already described importance of Bcl-2 at inhibiting apoptosis, it is not surprising that viruses would mimic this inhibitory effect by encoding Bcl-2 homologous proteins (14, 34, 63). Virus encoded Bcl-2 homologs have been described in several viruses such as adenovirus, herpesviruses, and poxviruses. Although the sequence homology between cellular Bcl-2 (cBcl-2) and viral Bcl-2 homoglues (vBcl-2) is generally low (~20-30%) they do have conserved structural homology (34, 63). Whether the limited sequence homology is relative to differences in protein function has yet to be determined. However, studies on vBcl-2 crystal structures have revealed a similar overall fold with cBcl-2 and a striking similarity to a typical pore forming protein (34, 76, 77, 124).

The first identified functional Bcl-2 homolog was E1B-19K found in adenovirus (149, 193). E1B-19K, a protein expressed early during adenovirus replication, inhibits

both Fas and TNF induced apoptosis and interestingly inhibits its own internal apoptotic inducer E1A (48, 112, 145, 156). E1B-19K displays functional homology to Bcl-2 by inhibiting Bax and Bak interactions, blocking Bax oligomerization, and preventing the loss of the inner mitochondrial membrane potential and release of cytochcrome c (Figure 1-9) (62, 156, 195, 196).

The Bcl-2 homolog encoded by the herpesvirus Karposi sarcoma virus, KSbcl-2, has also been shown to inhibit apoptosis (27). However, unlike Bcl-2, KSbcl-2 does not interact with Bax or Bak suggesting it may have evolved to avoid any negative interactions between cellular Bax and Bak proteins (27). In addition, KSbcl-2 also contains a poorly conserved BH3 domain (27). This suggests that although KSbcl-2, as well as other vBcl-2s may be homologous to cBcl-2 in sequence or in structure they may possess novel mechanisms of inhibiting apoptosis (Figure 1-9).

It is perhaps surprising that although poxviruses encode numerous anti-apoptotic proteins, only fowlpox virus encodes an obvious Bcl-2 homolog called FPV039 (2, 209). Although minimal experiments have been performed with FPV039 it contains both BH1 and BH2 domains and closely resembles the cellular Bcl-2 family member MCL-1 (2, 209). It is currently unknown whether FPV039 is capable at inhibiting apoptosis.

1.9.5 Novel Proteins with Bcl-2 Function

Viral proteins that have no sequence or structural homology to Bcl-2 may display similar functioning mechanisms such as inhibition of apoptosis at the mitochondria. Three well-characterized novel proteins that inhibit apoptosis at the mitochondria are vMIA, M11L, and F1L (Figure 1-9).

1.9.5.1 vMIA

vMIA (mitochondrial-localized inhibitor of apoptosis), encoded by human cytomegalovirus (CMV), was first identified by Goldmacher and colleges when CMV infected cells were found to be resistant to apoptosis downstream of caspase 8 activation (53). Although vMIA lacks sequence homology to Bcl-2, it does share some functional homology as indicated by the ability of vMIA to localize to the mitochondria and prevent the loss of the inner mitochondrial membrane potential (13, 53, 215). Two domains within vMIA that are necessary to inhibit apoptosis, (1) its N-terminus (amino acids 5-34) which contains a mitochondrial localizing signal and (2) amino acids 117-147 which are necessary for vMIA to inhibit apoptosis (6, 64, 152).

The anit-apoptotic ability of vMIA is based on its ability to associated with Bax (6, 64, 152). Evidence supporting the association of vMIA with Bax has been shown by confocal microscopy where expression of vMIA alone was responsible for triggering Bax translocation to the mitochondria (6, 152). Although it may seem ironic that an inhibitor of apoptosis would promote the translocation of Bax to the mitochondria, an event that leads to apoptosis, it is hypothesized that vMIA sequesters Bax at the mitochondria in an "unusable" form therefore preventing Bax activity (152). Further evidence of the association of vMIA with Bax has been demonstrated by immunoprecipitation studies in which vMIA interacted with Bax and amino acids 117-147 were necessary and sufficient for this interaction (6). vMIA was also shown to interact with Bak albeit at reduced levels compared to Bax (6). To clarify that vMIA was actually inhibiting the effects of Bax and not Bid, immunoprecipitation and apoptotic studies were performed. Results indicated that vMIA did not associate with tBid during immunoprecipitation assays. Furthermore,

vMIA prevented the release of HtrA2/Omi, Smac/Diablo, and cytochrome c from mitochondria treated with an excess of recombinant tBid but not mitochondria treated with excess oligomeric Bax (6). These results indicate that vMIA has no direct relationship with tBid but rather inhibits apoptosis by a Bax depend mechanism and therefore functions by a novel anti-apoptotic mechanism.

1.9.5.2 M11L

M11L was discovered when a deletion virus was generated resulting in a severe abolishment of symptoms associated with myxomatosis (140). Initial characterizational studies on M11L revealed a 166 amino acid protein that prevented myxoma virus-induced apoptosis in rabbit T lymphocytes (140). M11L contains a C-terminal transmembrane domain flanked by positively charged amino acids which are necessary and sufficient to target M11L to the mitochondria (46). Apoptotic studies on M11L demonstrated that M11L inhibits apoptosis by preventing the loss of the inner mitochondrial membrane potential and release of cytchrome c (46). It was discovered more recently that M11L specifically interacts with a component of the permeability transition (PT) pore called the peripheral benzodiazepine receptor (PBR) and it was this interaction that allowed M11L to control apoptosis at the mitochondria (47). Even more recently, M11L has been shown to inhibit apoptosis by interacting with Bak (219). Sequence analysis has shown that M11L shares no homology to any known cellular protein except for a pseudo-BH3 domain and was therefore the first novel viral protein with no homology to any known cellular protein discovered to localize to the mitochondria and associate with the PBR component of the PT pore.

1.9.5.3 F1L

For years it was believed CrmA/Spi-2 was the major inhibitor of apoptosis expressed by VV (148, 160). The first hint of F1L came from studies performed in our laboratory showing that VV strain Copenhagen, (VV65), which is devoid of CrmA/Spi-2 still retained the ability to inhibit Fas-induce apoptosis downstream of caspase-8 (222). Using purified mitochondria from Jurkat cells infected with VV65 in which the apoptotic pathway was reconstituted with recombinant Bid and granzyme B, it was further confirmed that VV65 expressed a second inhibitor of apoptosis that functioned directly at the mitochondria (222). With the use of deletion viruses it was further confirmed that the deletion virus VV811 which is missing open reading frames B13R-B29R was unable to inhibit apoptsis (222). With the goal of trying to find possible candidates, sequence homology searches were performed on VV strain Copenhagen genome to determine proteins that contained a putative mitochondrial targeting sequence and that were absent from VV811. F1L was a positive match and its discovery has resulted in an entire branch of research based on understanding how F1L is able to inhibit apoptosis at the mitochondria.

F1L consists of 226 amino acids and localizes to the mitochondria in the absence or presence of virus infection where it inhibits loss of the inner mitochondrial membrane potential and prevents the release of cytochrome c; two hallmark events that occur at the mitochondria during apoptosis (Figure 1-10 A) (223). F1L displays no obvious sequence homology to any known cellular protein. F1L orthologs are only found in members of the *orthopoxvirus* genus (Figure 1-11). Each F1L ortholog is approximately the same size with exception of ectromelia virus, variola virus, and camelpox. F1L of these

orthopoxivirus species contain a variable N-terminus with ectromelia virus diverging the greatest compared to all other F1L orthologs. The ectromelia F1L ortholog contains an extra 240 acids consisting of an 8 amino acid repeat. Preliminary data suggests these extra 240 amino acids are not required for inhibition of apoptosis since a truncated version of F1L from ectromelia virus lacking this N-terminal repeat still inhibits apoptosis (J. Taylor and M. Barry unpublished data). Whether F1L is functional in any of the other orthopoxvirus species has yet to be determined.

1.10 Experimental Rational and Hypothesis

Activation of apoptosis is commonly seen in virally infected cells due to the cell mediated immune response of CTLs and NK cells (8, 168). This cell-mediated immune response is a strategy evolved by the host to prevent propagation and dissemination of viral particles. In response, viruses have evolved their own counter-strategies by encoding proteins that target and inhibit key points in apoptosis. Vaccinia virus, the prototypic member of the poxvirus family, was recently demonstrated to control apoptosis directly at the mitochondria by encoding a previously unidentified protein, F1L (223). F1L has no obvious homology to Bcl-2 or any other cellular protein and is the first identified vaccinia virus protein that has been identified that localizes to mitochondria to inhibit apoptosis.

Due to the recent identification of F1L, little is known about its mechanism of action. However, sequence analysis of F1L revealed a putative C-terminal mitochondrial localizing sequence consisting of a 12 amino acid hydrophobic domain flanked by positively charged lysines followed by an eight amino acid hydrophilic tail, similar to that

of Bcl-2 and other TA proteins (Figure 1-10 B). Based on F1L possessing several qualities that make it an excellent candidate for studying viral-host interactions and for understanding TA proteins the objectives of my thesis were as follows: (1) to identify the mitochondrial targeting sequence of F1L (2) to determine if F1L is a member of the tail-anchored protein family, and (3) to determine if localization of F1L to the mitochondria is necessary for inhibition of apoptosis.

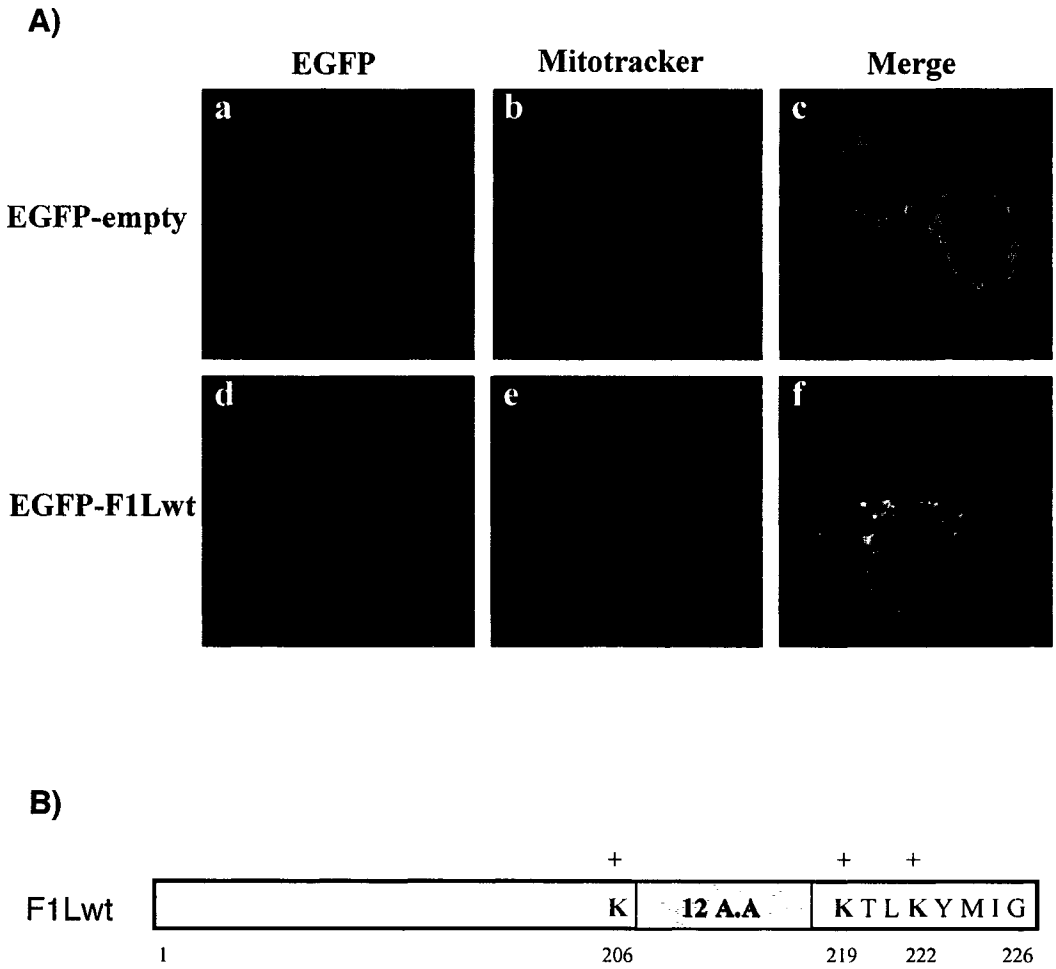


Figure 1-10: Vaccinia virus protein F1L **(A)** Intracellular localization of vaccinia virus protein F1L. HeLa cells were transiently transfected with pEGFP (a) or pEGFP-F1Lwt (d) and visualized by confocal microscopy. Mitochondria were labeled with Mitotracker Red (b and c). Merged images (c and f) indicated that EGFP-F1Lwt localized to the mitochondria. **(B)** Schematic representation of F1L and its C-terminal domain.

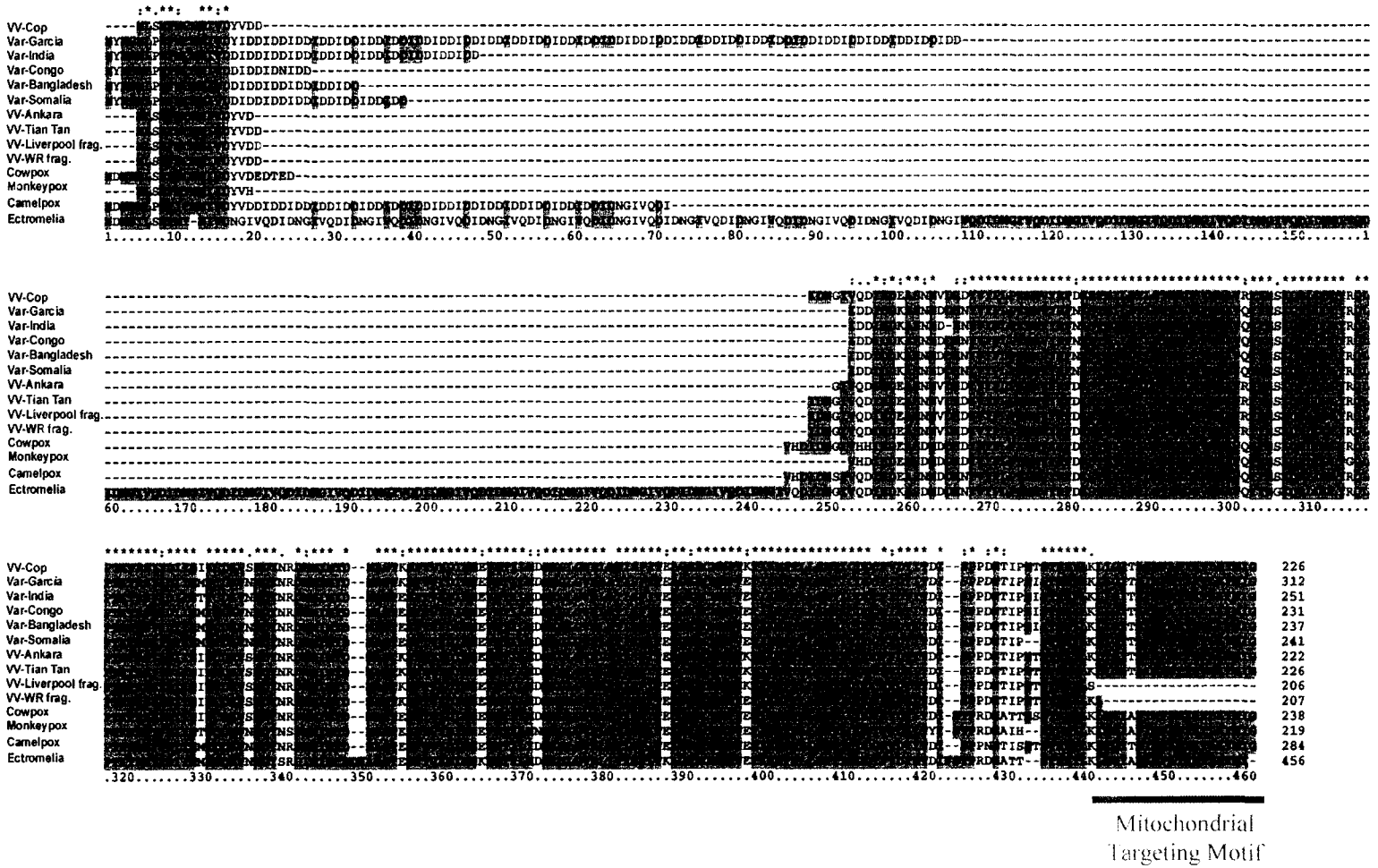


Figure 1-11: Sequence alignment of F1L and its orthologs generated using the ClustalX alignment program. Conserved residues are highlighted in green. Complete length in amino acids is denoted on the right.

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CHAPTER 2 – THE VACCINIA VIRUS F1L PROTEIN IS A TAIL-ANCHORED PROTEIN THAT FUNCTIONS AT THE MITOCHONDRIA TO INHIBIT APOPTOSIS

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Preface

The data presented in this chapter has been accepted by the Journal of Virology and has been formatted to comply with the paper thesis format. The data presented in all figures was generated by experiments carried out by myself and I wrote the first draft of the manuscript. A major editorial contribution from my supervisor, Dr. Michele Barry, led to the final version of the paper.

The Vaccinia Virus F1L Protein is a Tail-Anchored Protein that Functions at the Mitochondria to Inhibit Apoptosis

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2.1 Introduction

Apoptosis, or programmed cell death, is a naturally occurring process that specifically eliminates unwanted cells through a tightly controlled pathway (27). The key players are a family of intracellular proteases referred to as caspases (45, 59). Caspases are cysteine proteases that are activated by proteolytic cleavage following an apoptotic stimulus. Activated caspases are responsible for the proteolysis of cellular proteins resulting in the characteristic morphological and physical changes associated with apoptotic death. Some of these characteristics include chromatin condensation, DNA fragmentation, cell shrinkage and membrane blebbing (27). Recent evidence indicates that mitochondria function as key organelles and act as the ultimate checkpoint responsible for regulating apoptosis (15, 34). Apoptosis is associated with significant mitochondrial changes including the loss of the inner mitochondrial membrane potential and the release of cytochrome c (15, 34).

Both pro- and anti-apopotic members of the Bcl-2 family tightly regulate the mitochondrial checkpoint and the balance between pro- and anti-apoptotic members of the family decide the fate of the cell (12, 24). Anti-apoptotic family members, such as Bcl-2 and Bcl-xL, localize to the mitochondria where they function to inhibit the release of cytochrome c (12, 24). In contrast, pro-apoptotic members, such as Bax and Bak, disrupt the inner mitochondrial membrane potential resulting in the release of cytochrome c and death of the cell (12, 24). Although it is generally accepted that the loss of the inner mitochondrial membrane potential and release of cytochrome c lead to the demise of the cell, the exact mechanism of cytochrome c release is unresolved and controversial.

The detection and elimination of virus infected cells can occur through the action of cytotoxic T lymphocytes and natural killer cells resulting in apoptosis and the subsequent destruction of virus-infected cells (6, 52). In response, viruses have developed counter strategies to inhibit apoptosis. For example, many viruses such as members of the γ-herpesvirus family, Epstein-barr virus, adenovirus, African swine fever virus and fowlpox virus encode obvious Bcl-2 homologues that mimic the activity of Bcl-2 thereby maintaining the integrity of the mitochondria and blocking release of cytochrome c (13, 25). Additionally, recent evidence indicates that a subset of viruses, which lack obvious Bcl-2 homologues, encode unique proteins that directly inhibit mitochondrial events leading to apoptosis (18, 23, 64). For example, myxoma virus, a member of the poxvirus family, encodes a protein referred to as M11L that localizes to the mitochondria and prevents apoptosis (18, 19). Human cytomegalovirus encodes a novel mitochondrial-localized inhibitor of apoptotsis denoted vMIA which inhibits the release of cytochrome c (5, 23, 50). More recently, we have identified an additional mitochondrial-localized inhibitor of apoptosis, F1L, encoded by vaccinia virus (VV), the prototypic member of the poxvirus family (64). The F1L open reading frame in VV strain Copenhagen encodes a protein of 226 amino acids that localizes to the mitochondria where it inhibits the loss of the inner mitochondrial membrane potential and release of cytochrome c (64).

A common trait shared by several members of the Bcl-2 family is that they belong to a growing family of proteins referred to as tail-anchored (TA) proteins and are specifically targeted to intracellular membranes by virtue of a C-terminal transmembrane domain (8, 9, 53, 65). F1L possesses a putative C-terminal membrane anchoring domain consisting of a short hydrophobic domain flanked by positively charged amino acids and

a short C-terminal hydrophilic tail similar to that of Bcl-2 (32, 53). This observation led us to speculate that F1L might be a new member of the TA family of proteins. TA proteins are characterized by containing a membrane anchor located at their C-terminus (8, 9, 65). This membrane anchor is composed of a helical hydrophobic domain generally 12-24 amino acids flanked by positively charged amino acids (65). TA proteins do not possess N-terminal signal sequences and are therefore inserted into membranes post-translationally since the C-terminal membrane anchor is only exposed upon completion of translation (36, 65). The C-terminal membrane anchor encodes all the necessary information that allows each unique TA protein to be targeted to its specific destination. TA proteins are found in a wide variety of cellular membranes including the endoplasmic reticulum (ER), mitochondria, nuclear membrane, Golgi apparatus, and plasma membrane where they carry out a wide range of biological functions (8, 9, 36).

Based on the presence of a putative C-terminal membrane anchoring domain in F1L that exhibits similarity to domains in TA proteins we hypothesized that F1L would be a new member of the TA family and that localization of F1L to the mitochondria would be necessary for efficient apoptosis inhibition. In support of this hypothesis, our studies revealed that the C-terminal domain of F1L is necessary and sufficient for localization to the mitochondria. By constructing a series of F1L mutants containing deletions and point mutations we further show that the positively charged residues within the C-terminal domain are necessary for mitochondrial localization of F1L. Using *in vitro* transcription/translation studies we show that the mitochondrial targeting information is present in the C-terminal domain of F1L and we provide evidence that F1L demonstrates the characteristics and membrane topology of a TA protein. Importantly, we now report

that localization of F1L to the mitochondria is necessary for efficient inhibition of apoptosis. Our studies indicate that the VV-encoded F1L protein is a tail-anchored protein that localizes to the mitochondria during infection where it retains classical TA topology in order for it to inhibit apoptosis.

2.2 Materials and Methods

Cell culture and viruses. HeLa, CV-1, and TK$^-$H143 cells were obtained from the American Type Culture Collection and maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (GIBCO Invitrogen Corp.), 50 units/mL penicillin (GIBCO Invitrogen Corp.), 50μg/mL streptomycin (GIBCO Invitrogen Corp.) and 200μM glutamine (GIBCO Invitrogen Corp.). Jurkat cells were maintained in RPMI 1640 medium (GIBCO Invitrogen Corp.) supplemented with 10% FBS, 100μM 2-mercaptoethanol, 50 units/mL penicillin and 50μg/mL streptomycin at 37°C and 5% CO_2. Rabbitpox virus strain Utrecht and cowpox virus strain Brighton Red were a kind gift from Dr. Richard Moyer, University of Florida, Gainsville Florida. Ectromelia virus strain Moscow containing *lacZ* was a generous gift from Dr. Mark Buller, St. Louis University, St. Louis Missouri. VV811 and VV65 strain Copenhagen were generated as previously described (48). All viruses were propagated in baby green monkey kidney (BGMK) cells and grown in DMEM supplemented with 10% newborn calf serum (GIBCO Invitrogen Corp.), 50 units/mL penicillin, 50μg/mL streptomycin and 200μM glutamine at 37°C and 5% CO_2.

Generation of F1L antisera. The F1L open reading frame (ORF) was amplified by polymerase chain reaction (PCR) from pEGFP-F1Lwt using the forward oligonucleotide F1LBamHI 5'-GGATCCATGTTGTCGATGTTTATG-3' containing a Bam HI restriction site and the reverse oligonucleotide F1LNotI-5'-AGCGGCAATAGA TGCCATATCATA-3' to construct pGEX4T-3:F1L(1-120) (Amersham Biosciences) containing amino acids 1-120 of F1L appended to Glutathione-S-transferase (GST). PGEX4T-3:F1L(1-120) was transformed into BL31(DE3) and protein expression was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Rose Scientific Ltd.). GST-F1L(1-120) was purified using glutathione Sepharose 4B according to the manufacturer's instructions (Amersham Biosciences). Rabbits were immunized by injection of 500 μg of purified GST-F1L(1-120) in Freund's complete adjuvant. At biweekly intervals the animals were boosted with 500 μg of GST-F1L(1-120) in Freund's incomplete adjuvant.

Generation of recombinant vaccinia virus. The construction of pSC66-FLAG-F1Lwt, which was used to generate vaccinia virus strain Western Reserve containing a FLAG-tagged copy of F1L has been described elsewhere (64). Vaccinia virus strain Western Reserve was used to generate VV-WR-FLAG-F1Lwt as previously described (43). Recombinant virus was selected on TK-H143 cells in the presence of 25 μg/ml BUdR and plaque purified three times using X-gal to visualize recombinant viruses. The presence of FLAG-F1Lwt in VV-WR-Flag-F1L was confirmed by PCR and by anti-FLAG M2 antibody (Sigma Aldrich).

Plasmid construction. The F1Lwt ORF was amplified from virus DNA as described (64). Plasmids pEGFP-F1LHTR(1-218), pEGFP-F1LTR(1-206), pEGFP-F1Ltail(+)(206-226), pEGFP-F1Ltail(-)(207-226), pEGFP-F1L-K219A, pEGFP-F1L-K222A, pEGFP-F1L-K219/222A, and pEGFP-F1LCyb5 were generated by PCR. The forward primer used for F1LHTR(1-218), F1LTR(1-206), and F1L-K219/222A was *EcoRI-*(GAATTCTCATGTTGTCGATGTTTATG). The forward primers used for FILtail(+)(206-226) and FILtail(-)(207-226) were *Eco*R1(GGATTCTCAAGATTAT TGGCATCACA) and *Eco*R I-(GAATTCTCCTTATTGGCATCACAGCT) respectively. The reverse primers for F1LHTR(1-218) and F1LTR(1-206), were *Bam*H I-(GGATCCTTAATATGTAGCAAACATGATAGC) and *Bam*H I-(GGATCCTTA CTTTAGATATTCACGCGTGCT) respectively. The reverse primer for F1L-tail(+)(206-226) and F1Ltail(-)(207-226), was *Bam*H I-(GGATCCTTATCCTAT CATGTATTTGAG). The forward primer used for F1L-K222A, F1L-K219A was *Xho* I-(CTCGAGATGTTTATG) and for F1LLL-K219/22A was *EcoR* I-(GAATTCTCATGTTGTCGATGTTTATG). Lysine to alanine mutations in F1L-K219A, F1L-K222A, and F1L-K219/222A, were produced using the reverse primers *Bam*H I-(GGATCCTTATCCTATCATGTATTTGAGAGTTGCATATGTAGCAAAC AT), *Bam*H I-(G GATCCTTATCCTATCATGTATGCGAGAGTTTTATA) and *Bam*H I-(GGATCCTTATCCT ATCATGTATGCGAGAGTTGCATATGTAGCAAACAT).

The pEGFP-F1LCyb5 chimera was generated using a combination of three PCR reactions. In the first PCR reaction, the primers used in conjunction with pEGFP-F1Lwt template were *Eco*R I-(GAATTCTCATGTTGTCGATGTTTATG) and (CGATTCAAC GGTAGTGATCTTTAGATATTCACGCGT) resulting in a PCR product encoding the

first 206 amino acids of F1L with an overhang corresponding to amino acids 100-105 of cytochrome b5. The C-terminal domain of cytochrome b5 was amplified by PCR from pSPUTK-Bcl2Cyb5 using (ATCACTACCGTTGAATCGAAC) and *Bam*H I-(GGATCCTTAATCTTCAGCCATGTCAG) resulting in a PCR product encoding amino acids 100-134 of cytochrome b5. The products from the first two PCR reactions were combined with primers *Xho* I-(CTCGAGATGTTTATG) and *Bam*H I-(GGATCCTTAATCTTCAGCCATGTACAG) resulting in a PCR product that encoded the N-terminal portion of F1L (amino acids 1-206) and C-terminal tail of cytochrome b5 (amino acids 100-134). All amplified F1L constructs were verified by DNA sequence analysis, and subcloned into pEGFP-C3 (Clonetech). pSPUTK-Bcl2Cyb5 and pSPUTK-Bcl2ActA were a generous gift from Dr. David Andrews McMaster University, Hamilton Ontario (67).

For construction of pSPUTK-FLAG-F1Lwt, the F1L ORF was amplified using primers *Bam*H I-(GGATCCATGGACTACAAAGACGATGACGACAAGTTGTCGAT GTTTATGTGT) which contains an N-terminal FLAG epitope and *Xho* I-(CTCGAGTTATCCTATCATGTATTT). The resulting amplified products were subcloned into pSPUTK (20) using the restriction sites *Bam*H I and *Sac* I. Construction of pSPUTK-F1LTR was performed by subcloning F1LTR(1-206) into the restriction sites *Bam*H I and *Nco*I of pSPUTK.

Confocal microscopy. To determine localization of F1L orthologs in ectromelia, rabbitpox and cowpox virus infected cells, HeLa cells were infected with an multiplicity of infection (MOI) of 5 for 8-24 hours. Cells were fixed in 4% paraformaldehyde in PBS

for 10 minutes at room temperature and permeabilized using 0.02% NP-40 in PBS. For analysis of F1L localization, cells were stained with anti-F1L at a dilution of 1:2000 followed by the addition of 10μg/mL Alexa Fluor 546 goat anti-rabbit (Molecular Probes). Mitochondrial localization was determined by staining cells with 10μg/mL mouse anti-cytochrome c (clone 6H2.B4) (Pharmingen) followed by the addition of 10μg/mL Alexa Fluor 546 goat anti-mouse (Molecular Probes). For topology studies, HeLa cells were infected with VVWR-FLAG-F1Lwt with and MOI of 5 for 8 hours and either fixed with 4% paraformaldehyde and permeabilized with 25μg/mL of digitonin (Sigma Aldrich) for 2 minutes at room temperature or permeabilized with 1U/1x10^5 cells of streptolysin O (SLO) (Sigma Aldrich) for 5 minutes at 37°C and then fixed (16, 46).

To determine the localization of EGFP-F1L constructs in live cells, HeLa cells were seeded onto 18mm coverslips (Fisher Scientific) in modified 3.5cm diameter cell culture dishes. Cells were transfected with 2μg of plasmid DNA using Lipofectamine 2000 (GIBCO Invitrogen Corp.) according to the manufacturers specifications. For analysis of mitochondrial localization, the mitochondria of transfected cells were stained with 15ng/mL of Mitotracker Red CXMRos (Molecular Probes). To visualize the ER, transfected cells were stained with 1μM of ER-Tracker Blue-White DPX (Molecular Probes). Live cells were examined using a LSM510 laser scanning confocal microscopy at 543nm to assess Mitotracker fluorescence or at 350nm to assess ER-Tracker or at 489nm to assess EGFP fluorescence.

For fixed cell confocal microscopy, HeLa cells were transfected as described above. Cells were fixed using 4% paraformaldehyde in PBS for 10 minutes at room temperature and permeabilized using 0.02% NP-40 in PBS. For analysis of

mitochondrial localization, cells were stained with 10μg/mL mouse anti-cytochrome c (clone 6H2.B4) (Pharmingen) followed by the addition of 10μg/mL Alexa Fluor 546 goat anti-mouse (Molecular Probes). For analysis of ER localization, cells were stained with rabbit anti-calnexin (residues 575-593) (Stressgen Biotehnologies) at dilution of 1:400 followed by the addition of 10μg/mL Alexa Fluor 546 goat anti-rabbit (Molecular Probes). Coverslips were mounted using 50% PBS/50% glycerol containing 4mg/mL n-propyl gallate (Sigma Aldrich). Fixed cells were examined using a LSM510 laser scanning confocal microscopy at 543nm to assess cytochrome c and calnexin staining. Staining intensities were profiled with the use of the Zeiss LSM 510 image software.

Apoptotic killing assays. For apoptotic killing assays, HeLa cells were transfected as described above and induced to undergo apoptosis by the addition of 10ng/mL of tumor necrosis factor α (TNFα) (Roche Diagnostics) and 5μg/mL cycloheximide (ICN Biomedicals Inc.) for 5 hours. Following treatment with TNFα, cells were stained with 0.2μM tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes), a dye that preferentially stains healthy respiring mitochondria (17, 21, 39). Loss of mitochondrial membrane potential in EGFP positive cells was measured as a decrease in TMRE fluorescence by two-color flow cytometery (FACScan Becton Dickinson). The level of TMRE fluorescence was measured through the FL-2 channel equipped with a 585nm filter (42nm band pass). The level of EGFP fluorescence was measured through the FL-1 channel equipped with a 488nm filter (42nm band pass). Data was acquired on 10,000 cell per sample with fluorescent signals at logarithmic gain. Data was analyzed with CellQuest software. Loss of the inner mitochondrial membrane potential in positively

transfected cells was calculated using the following equation: (# $EGFP^+TMRE^-$ cells / total # of $EGFP^+$ cells) x 100.

Purification of mitochondria. Mitochondria used for membrane-insertion assays were freshly purified from Jurkat cells as previously described (26, 63). Briefly, 1 x 10^8 Jurkat cells were washed once with buffer "A" containing 20mM MOPS pH 7.4, 100mM sucrose and 1.0mM EGTA. The cells were centrifuged for 10 minutes at 2000xg and resuspended in buffer "B" containing 5% percoll (Sigma Aldrich) and 191μg/mL digitonin (Sigma Aldrich) in buffer "A" at a final concentration of 2 x 10^7 cells/mL. The cells were incubated on ice for 15 minutes with occasional inversion. The nuclei were pelleted by centrifugation at 2500xg for 10 minutes at 4°C. The supernatant was collected and further fractionated by centrifugation at 15,000xg for 15 minutes at 4°C. The remaining mitochondrial pellet was washed 3 times with buffer "A" and resuspended in buffer "C" containing 20mM MOPS pH 7.4, 300mM sucrose and 1mM EGTA. Protein concentration was determined using a bicinchoninic acid kit (Pierce Chemical Co.).

To determine the topology of mitochondrial localized F1L during virus infection, Jurkat cells were infected with VVWR-FLAG-F1Lwt at an MOI of 5 PFU per cell. Five hours after infection mitochondria were purified as described previously (26, 63). Protein concentration was determined using a bicinchoninic acid kit (Pierce Chemical Co.). Mitochondria derived from infected cells were treated with either enterokinase, proteinase K or trypsin as described below.

***In vitro* transcription-translation.** The TNT SP6-coupled reticulocyte lysate system (Promega Corp.) was used as described by the manufacturer. A 50μL reaction contained 25μL rabbit reticulocyte lysate, 20μM amino acid mixture (minus methionine), 2.5% TNT reaction buffer, 40 U RNASin (Promega Corp.), 10μCi [^{35}S] methionine (Perkin-Elmer Life Sciences), and 1μg of either pSPUTK, pSPUTK-FLAG-F1Lwt, pSPUTK-F1LTR(1-206), or pSPUTK-Bcl2ActA. Reactions were incubated at 30°C for 90 minutes and translation was stopped by the addition of 2μg/mL cycloheximide (Sigma Aldrich).

For membrane-insertion assays, 10μg of purified mitochondria were added to 10μL of TNT generated protein (L) and incubated at 30°C for 30-60 minutes to allow for membrane association. The lysate/mitochondria mixture was centrifuged for 20 minutes at 20,000xg at 4°C. Supernatants were saved and pellets were washed with PBS followed by centrifugation at 20,000xg for 20 minutes at 4°C. The resulting pellet was resuspended in 50μL of PBS. The supernatant (S) and pellet (P) samples were analyzed by SDS-PAGE and detection by autoradiography. To determine if TNT generated proteins were integral membrane proteins the mitochondrial pellet was washed with 200μL of 0.5M Na_2CO_3 (pH 11.5) to disrupt non-specific binding to membranes (4, 22). To determine protein topology in mitochondrial membranes, samples were treated with either 2.5μg/mL of trypsin (Grade III, Sigma Aldrich) or 2.5μg/mL of proteinase K (Sigma Aldrich) for 1 hour at 30°C or 1-2 U of enterokinase (Novagen) for 2 hours at room temperature.

Immunoblotting. Purified mitochondria were analyzed on SDS polyacrylamide gels and proteins were transferred to nitrocellulose membranes. FLAG-F1Lwt was detected using

rabbit anti-F1L (1:5,000) followed by goat anti-rabbit horseradish peroxidase-conjugated (Bio-Rad) at 1:10,000 or anti-FLAG conjugated to horseradish peroxidase (Clone M2) at 1:2,500 (Sigma Aldrich). Proteins were visualized with a chemiluminescent detection system according to the manufacturers' protocol (Amersham Biosciences).

Detection of protein expression levels for the various EGFP-F1L constructs was determined by transfecting HeLa cells. Cell lysates were analyzed by SDS polyacrylamide gels and proteins were transferred to nitocellulose membranes. The EGFP-F1L constructs were detected using rabbit anti-EGFP (provided by Dr. L. Berthiaume, University of Alberta, Edmonton, Alberta, Canada) at 1:20,000 followed by goat anti-rabbit horseradish peroxidase-conjugated at 1:10,000.

2.3 Results

F1L Orthologs Localizes To Mitochondria

Recently we identified a VV-encoded protein, F1L, that localizes to mitochondria and inhibits apoptosis (Wasilenko et al 2003). Sequence analysis of F1L failed to reveal any obvious similarity to cellular proteins including members of the Bcl-2 family which regulate apoptosis at the mitochondria. The poxvirus family, of which VV is the prototypical member, consists of a large family of viruses that infect both vertebrates and invertebrates (42). VV-related F1L open reading frames are only found in members of the *Orthopoxvirus* genus and are highly conserved with greater than 95% sequence identity over the last 220 amino acids. The greatest sequence diversity among the various F1L orthologs is located within the N-terminal regions, with strains of variola virus, camelpox virus and ectromelia virus displaying a series of unique repeats, the function of which is

currently unknown. To determine if F1L orthologs in other members of the *Orthopoxvirus* genus also localize to mitochondria we infected HeLa cells with either the deletion virus VV811, VV strain Copenhagen, a recombinant VV expressing a FLAG-tagged version of F1L (VVWR-FLAG-F1Lwt), ectromelia virus, rabbitpox virus, or cowpox virus. F1L was visualized by confocal microscopy using an anti-F1L antibody. When cells were infected with the deletion virus VV811, which is devoid of the F1L open reading frame, no F1L was detected within the cells (Fig. 2-1A d-f). Upon infection of cells with the F1L expressing vaccinia virus, VV65, F1L demonstrated a similar localization pattern to that of cytochrome c indicating F1L localizes to mitochondria during virus infection (Fig. 2-1A g-i). A similar pattern was detected for cells infected with the recombinant VV expressing a FLAG-tagged version of F1L (Fig. 2-1A j-l). HeLa cells infected with either ectromelia virus (Fig 2-1A m-o), rabbitpox virus (Fig. 2-1A p-r), or cowpox virus (Fig. 2-1A s-u) and stained with anti-F1L all showed colocalization with cytochrome c indicating that F1L orthologs in other *Orthopoxvirus* genus members also localize to mitochondria during infection.

The C-terminal Tail of F1L Is Critical for Mitochondria Localization

Sequence analysis of all the F1L orthologs revealed the presence of a conserved C-terminal transmembrane domain flanked by positively charged amino acids and a short hydrophilic tail C-terminal to the transmembrane domain (Fig. 2-2B a). Tail anchored proteins, including several members of the Bcl-2 family, localize to membranes through a

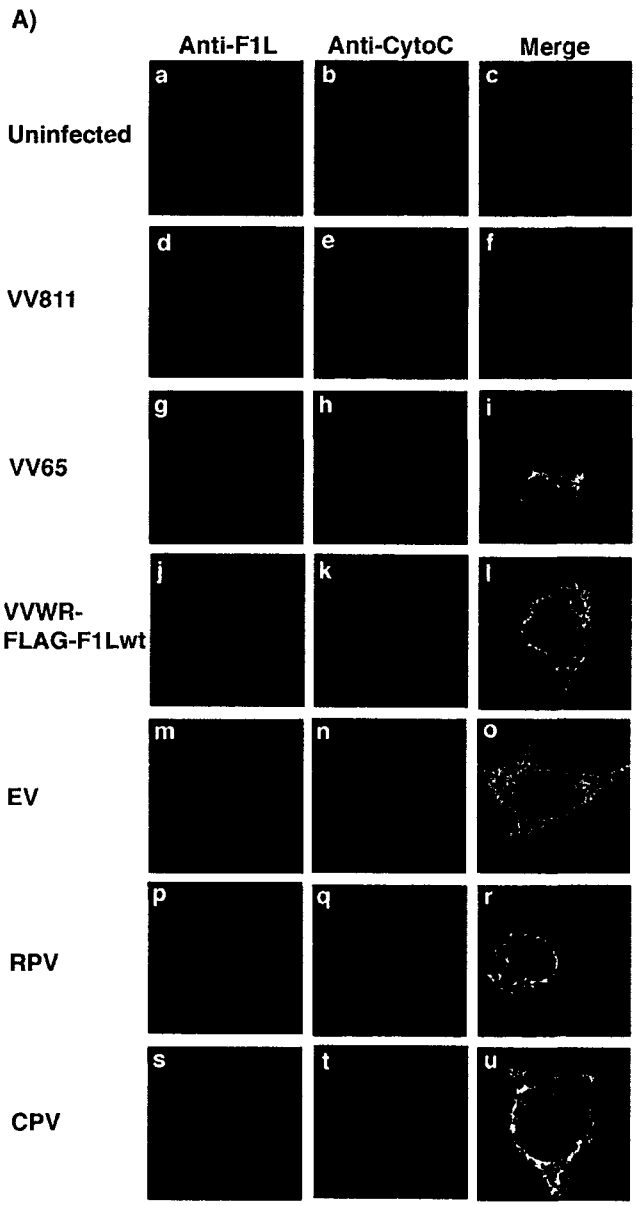
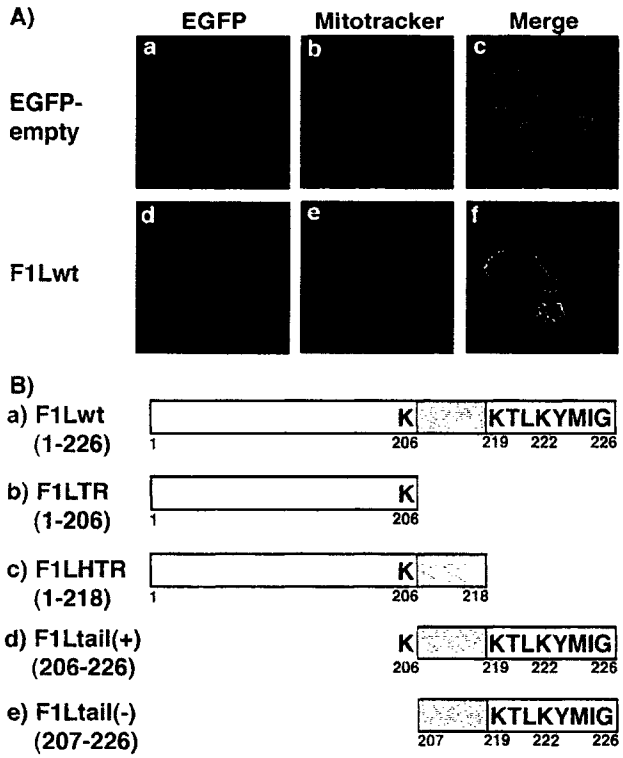


Figure 2-1



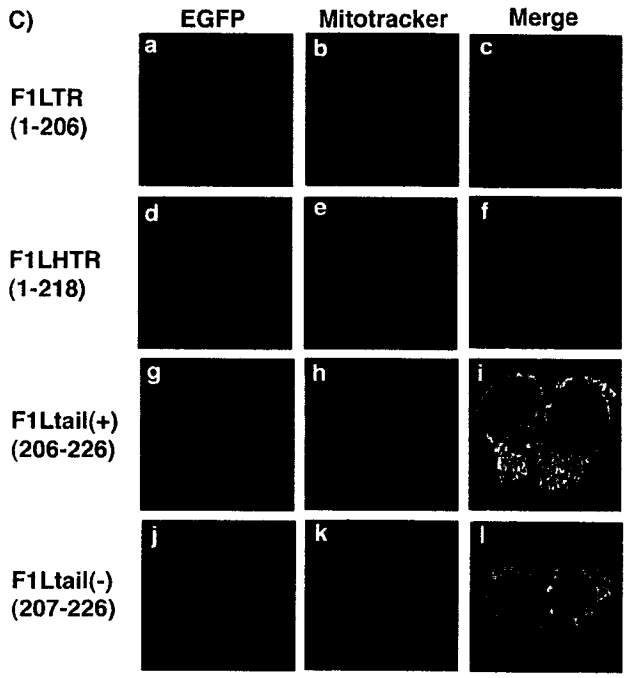


Figure 2-2

C-terminal domain containing a transmembrane region (8, 9, 53). The presence of this conserved C-terminal domain within F1L suggested that the domain might serve as a mitochondrial targeting sequence (30, 44). In order to determine if F1L localizes to mitochondria in the absence of virus infection, we generated an EGFP-tagged version of F1L and determined localization by confocal microscopy. HeLa cells were transiently transfected with pEGFP or pEGFP-F1Lwt, stained with the mitochondrial-specific dye, Mitotracker, and visualized by confocal microscopy. The fluorescent signal of EGFP alone was found throughout the cell and did not overlap with Mitotracker (Fig. 2-2A a-c). In contrast, cells transfected with pEGFP-F1Lwt showed a similar staining pattern to Mitotracker (Fig. 2-2A d and e). When the two images were superimposed, a uniform yellow image was produced indicating that F1L localized to mitochondria (Fig. 2-2A f).

To determine if the C-terminal transmembrane domain of F1L served as a mitochondrial localization signal, four EGFP-F1L deletion constructs were created (Fig. 2-2B). When HeLa cells were transfected with either pEGFP-F1LTR(1-206), which no longer contains the C-terminal domain or pEGFP-F1LHTR(1-218), which is missing the last 8 amino acids, a diffuse signal was found throughout the cells which did not colocalize with Mitotracker indicating that these amino acids were necessary for localization to mitochondria (Fig. 2-2C a-f). To determine if the C-terminal tail of F1L was sufficient for mitochondrial localization we fused the C-terminal tail of F1L to EGFP and visualized localization by confocal microscopy. Uncertain as to whether the positively charged lysine(206) upstream of the hydrophobic domain was important for localization, two F1L-tail constructs were created: F1L-tail(+)(206-226) which contains

lysine 206 and F1L-tail(-)(207-226) which is missing lysine 206. When HeLa cells were transfected with either pEGFP-F1Ltail(+)(206-226) or pEGFP-F1Ltail(-)(207-226) both constructs showed a similar staining pattern to Mitotracker and when the signals were superimposed, a uniform yellow image was produced indicating that the C-terminal tail of F1L is necessary and sufficient to target F1L to the mitochondria (Figure 2-2C g-l).

F1L Inserts Into Mitochondria *In vitro* as a Tail-Anchored Protein

Since our evidence demonstrated that the C-terminal tail of F1L was necessary and sufficient for mitochondrial localization, this suggested that F1L might be a member of the TA family of proteins. Due to the presence of the targeting sequence at the C-terminus, TA proteins are inserted post-translationally into cellular membranes (8, 9). To investigate whether F1L was a representative TA protein and therefore post-translationally inserted into mitochondrial membranes, we utilized an *in vitro* transcription-translation (TNT) assay. FLAG-tagged F1Lwt was cloned into the vector pSPUTK to allow for efficient transcription and translation of F1L in the presence of [^{35}S]-methionine. Following translation, cycloheximide was added to the [^{35}S]-labeled FLAG-F1Lwt to inhibit further translation. The generated [^{35}S]-FLAG-F1Lwt protein was added to purified mitochondria and translated protein was visualized by autoradiography. Results showed that approximately half of the translated FLAG-F1Lwt was recovered in the mitochondrial pellet (Fig.2-3A compare lanes 1-3). This observation suggested that F1L was capable of post-translational insertion into mitochondria similar to other members of the TA family (Fig. 2-3A compare lanes 1-3). We routinely found

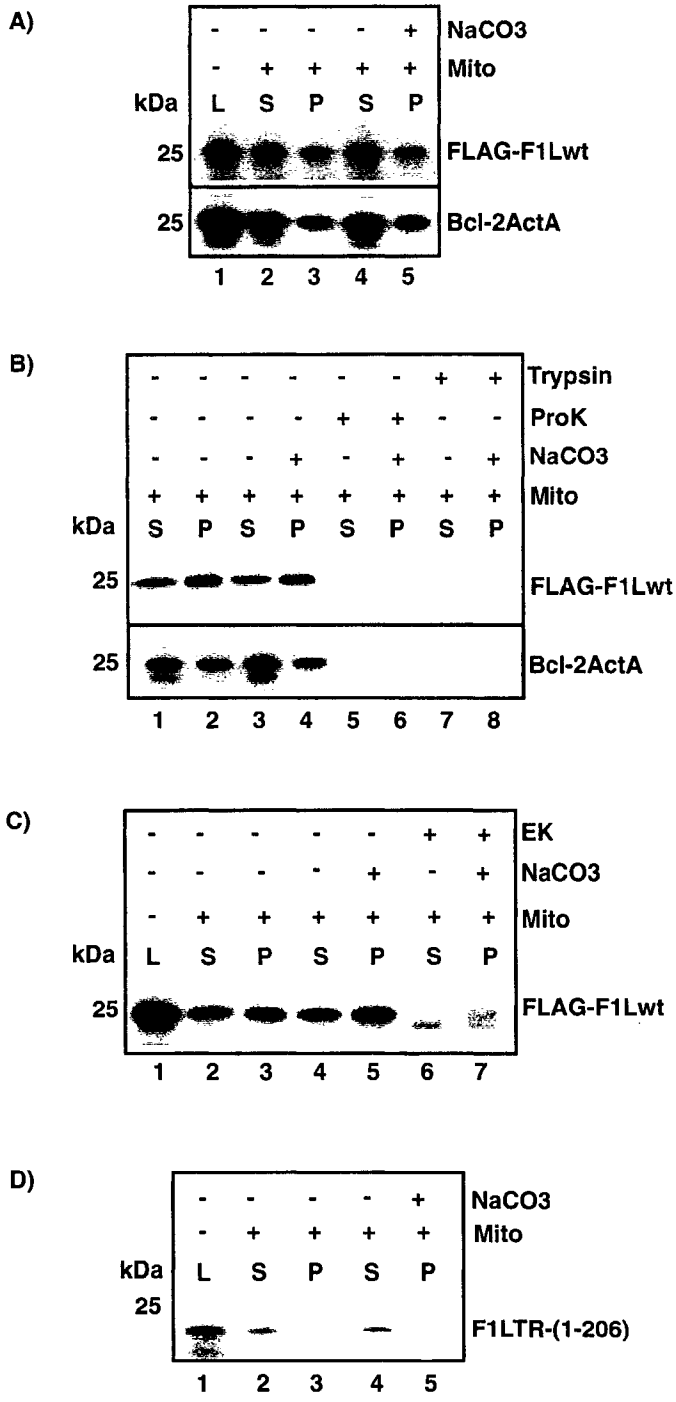


Figure 2-3

that after the addition of mitochondria approximately half of the FLAG-F1Lwt protein remained in the supernatant (Figure 2-3A lane 2), however the addition of increasing amounts of mitochondria supported an increased insertion into the mitochondrial pellet (data not shown). As a control, we used a TNT [^{35}S]-labeled Bcl-2ActA chimera, containing the mitochondrial targeting polypeptide from *Listeria monocytogenes* ActA protein which has been shown to localize Bcl-2 exclusively to the mitochondria (49, 67). Similar to FLAG-F1Lwt, Bcl-2ActA was also recovered in the mitochondrial pellet with approximately half of the protein remaining in the supernatant (Fig. 2-3A lanes 2 and 3).

To determine if F1L was in fact inserted into the mitochondrial membrane and not just peripherally associated with the mitochondria, mitochondria were washed with an alkaline solution of Na_2CO_3 pH 11.5 following post-translational insertion of FLAG-F1Lwt. The addition of Na_2CO3 pH 11.5 removes peripheral proteins but not integral membrane proteins (4, 22). No reduction of FLAG-F1Lwt or Bcl-2ActA in mitochondria was seen between the untreated and treated mitochondrial pellet fractions following Na_2CO_3 pH 11.5 washing (Fig. 2-3A compare lanes 3 and 5) suggesting that like Bcl-2ActA, F1L is anchored into mitochondrial membranes and is not a peripheral protein.

Topological studies on TA proteins reveals that they are anchored into the phospholipid bilayer by the C-terminus with the N-terminus exposed to the cytosol (8, 9). To determine the topology of F1L in mitochondria we utilized proteinase K, trypsin and enterokinase which will readily degrade exposed portions of F1L. Following the addition of proteases to the mitochondria, we found that the membrane-associated FLAG-F1Lwt was sensitive to both proteinase K and trypsin as indicated by the complete loss of radiolabeled protein suggesting that the majority of F1L was in fact oriented towards the

cytoplasm (Fig. 2-3B lanes 5-8). Similarly, the previously characterized TA protein Bcl-2ActA was also sensitive to proteinase K and trypsin (30, 67) (Fig. 2-3B lanes 5-8). Unfortunately, we were unable to detect the presence of the protected C-terminal fragment of F1L in these assays. This may be due to the presence of only one methionine in the short C-terminal tail of F1L and the small size of the fragment (~3KDa). Therefore, to further confirm the topology of F1L we used enterokinase which specifically recognizes and cleaves the N-terminal FLAG sequence (AspAspAspAspLys↓) from F1L. Enterokinase was added to either the supernantant fraction containing soluble FLAG-F1Lwt (Fig. 2-3C lane 6) or FLAG-F1Lwt associated with mitochondria (Fig. 2-3C lane 7) followed by analysis by autoradiography. Using this approach, a noticeable shift in F1L mobility resulted due to the loss of the FLAG tag from the N-terminus of F1L indicating that FLAG-F1Lwt was inserted into the mitochondria with the N-terminus exposed (Fig. 2-3C lanes 6 and 7). This cumulative data suggests that FLAG-F1Lwt displays typical TA orientation with the N-terminus facing the cytoplasm.

Since our confocal results demonstrated that expression of EGFP-F1LTR(1-206), which lacks the C-terminal 20 amino acids, was unable to localize to the mitochondria (Fig. 2-2C), we assessed the role of the F1L C-terminus in membrane anchoring using the *in vitro* TNT assay. TNT reactions were performed using the DNA template pSPUTK-F1LTR(1-206) which transcribes and translates a truncated [^{35}S]-labeled F1L lacking the C-terminal transmembrane domain and the short hydrophilic tail (Fig. 2-2B). Results showed only a minimal association of F1LTR(1-206) with mitochondria while most of the protein was found in the supernatant (Fig. 2-3D lanes 1-3). To determine if the small

amount of F1LTR found in the mitochondrial pellet was anchored into the membrane, the mitochondrial fraction was washed with Na_2CO_3 pH 11.5. Alkaline extraction resulted in a complete loss of radiolabeled FILTR(1-206) thereby confirming that the C-terminal domain of F1L was necessary for membrane insertion (Fig. 2-3D lanes 4 and 5).

F1L Inserts into Mitochondria During Virus Infection

To determine the topology of F1L during virus infection, we performed confocal microscopy utilizing selective permeabilization procedures and antibodies directed specifically to the N-terminus of F1L. HeLa cells were infected with VVWR-FLAG-F1Lwt and F1L localization was visualized using an anti-FLAG or anti-F1L antibody. When HeLa cells were treated with NP-40, which permeabilizes both the plasma and mitochondrial membranes, anti-FLAG and anti-F1L antibodies demonstrated colocalization of F1L with cytochrome c (Fig. 2-4A a-c and Fig. 2-4B a-c). In contrast, when HeLa cells were permeabilized with either digitionin or streptolysin O (SLO), which selectively permeabilize the plasma membrane but not the mitochondrial membrane, anti-FLAG antibody and an anti-F1L antibody generated to the N-terminal 120 amino acids of F1L still gained access to F1L indicating that the N-terminus of F1L was exposed to the cyotoplasm (Fig. 2-4A d to I and Fig.2-4B d to i) (16, 46). Notably, however, anti-cytochocrome c was unable to gain access to the inner mitochondrial membrane space indicating that the digitonin and SLO treatment selectively permeabilized the plasma membrane but not the mitochondrial membrane as indicated by a loss of cytochrome c staining (Fig 2-4A d-i and Fig. 2-4B d-i).

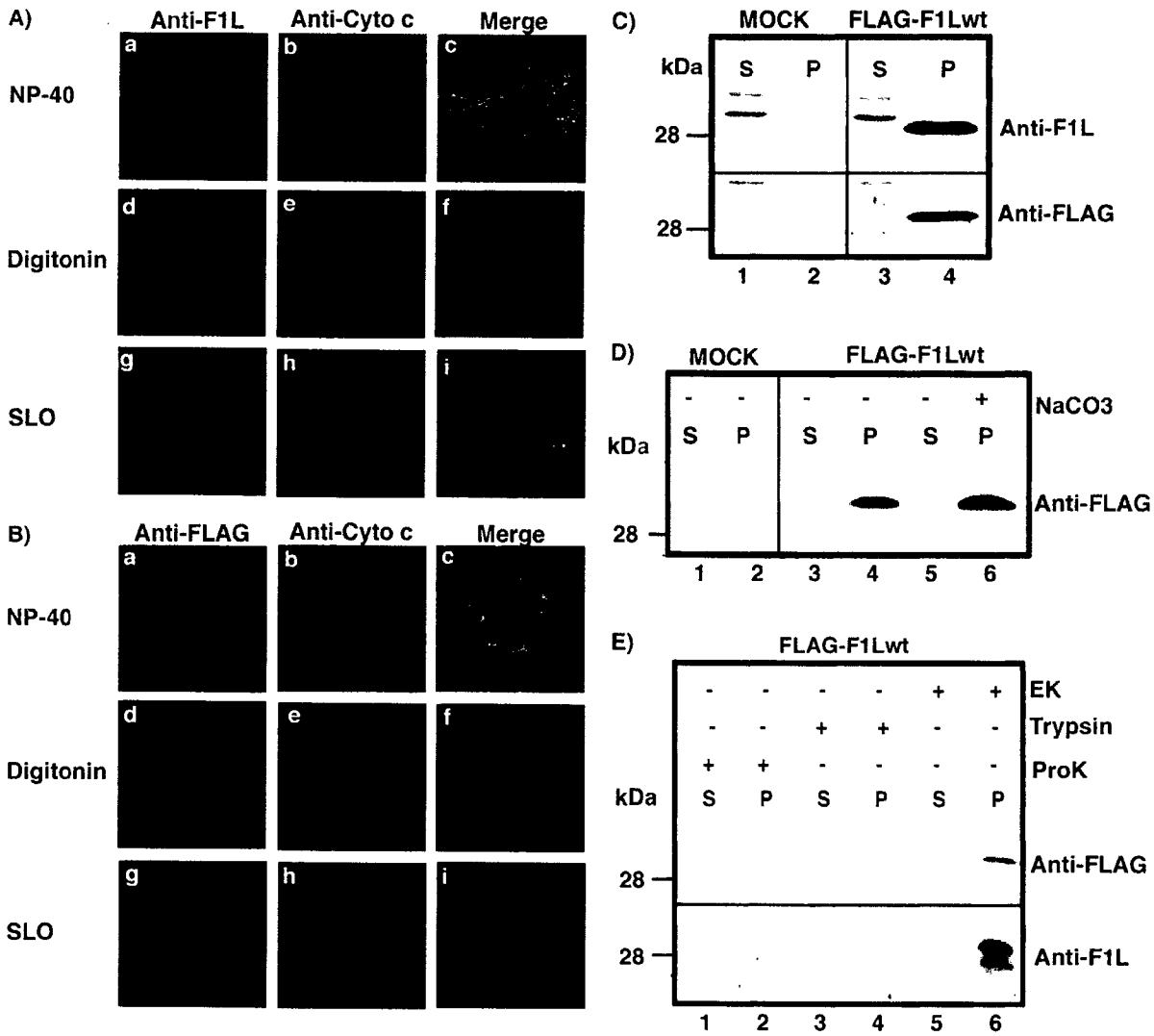


Figure 2-4

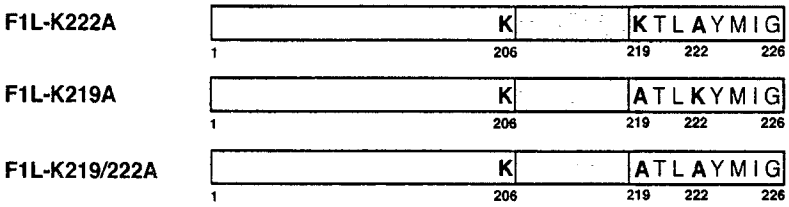
To further confirm the topology of F1L during virus infection, Jurkat cells were infected with the recombinant virus VVWR-FLAG-F1Lwt, mitochondria were purified, and the presence of F1L in mitochondria was determined by immunoblot using both anti-F1L and anti-FLAG antibodies. Mitochondria purified from mock-infected cells showed the absence of F1L in both the supernatant and pellet fractions as expected (Fig. 2-4C). In contrast, Jurkat cells infected with VVWR-FLAG-F1Lwt revealed the presence of F1L only in the mitochondrial pellet fraction with undetectable levels in the supernatant (Fig. 2-4C lanes 3 and 4). To determine if F1L was an integral membrane protein, mitochondria were washed with Na_2CO_3 pH 11.5 and F1L was detected using an anti-FLAG antibody. Similar to our *in vitro* results, F1L was only detected in the mitochondrial pellet fraction and Na_2CO_3 washing did not result in a reduction of F1L levels (Fig. 2-4D compare lanes 4 and 6). The topology of F1Lwt during virus infection was determined by isolating mitochondria from virus-infected cells followed by treatment with proteinase K and trypsin. Under these conditions no F1L was detected using either anti-FLAG or anti-F1L both of which are directed to the N-terminal portion of FLAG-F1L suggesting that the N-terminus of F1L was digested by the proteases (Fig. 2-4E lanes 1 to 4). When purified mitochondria were treated with enterokinase and blotted with anti-FLAG antibody, an overall reduction in F1L was observed indicating that the majority of the FLAG epitope was no longer accessible to the antibody due to cleavage by enterokinase (Fig. 2-4E lane 6 compared to 2-4D lane 6). When the same samples were blotted with the anti-F1L antibody which recognizes amino acids 1-120 a doublet was produced confirming that FLAG-F1L was cleaved by enterokinase and further demonstrating that the N-terminus of F1L is facing the cytoplasm (Fig. 2-3E lane 5).

Together these data indicate that F1L is inserted into mitochondria during virus infection with the N-terminus of the protein exposed to the cytoplasm.

Basic Amino Acids in the C-terminal tail of F1L are Critical For Mitochondria Targeting.

F1L shares several characteristics with other TA proteins including a transmembrane domain flanked by positively charged lysines (Fig.2-2B) (28, 32). Mutagenesis of the positively charged amino acids surrounding the transmembrane domain in other TA proteins has revealed a significant role for these amino acids in directing them to the mitochondria (28, 32). We found that deletion of the last eight amino acids, including lysine 219 and 222, resulted in the inability of F1L to localize to the mitochondria suggesting that these eight residues are critical for mitochondrial localization (Fig, 2-2C d-e). Therefore, to investigate if the positively charged amino acids in the C-terminal tail of F1L play a role in localization of F1L to the mitochondria, we generated three F1L mutants by PCR directed mutagenesis. EGFP-F1L-K219A, EGFP-F1L-K222A, and EGFP-F1L-K219/222A were created by mutating either lysine 219, lysine 222, or both lysines 219 and 222 to neutral alanine residues (Fig. 2-5A). The localization of the three mutant constructs was determined by transfecting HeLa cells and visualizing protein localization by confocal microscopy. Expression of EGFP-F1L-K222A demonstrated a characteristic mitochondrial staining pattern that colocalized with Mitotracker indicating that mutagenesis of lysine 222 did not influence mitochondrial localization (Fig. 2-5B a-c). As an indicator of co-localization we plotted the merge

A)



B)

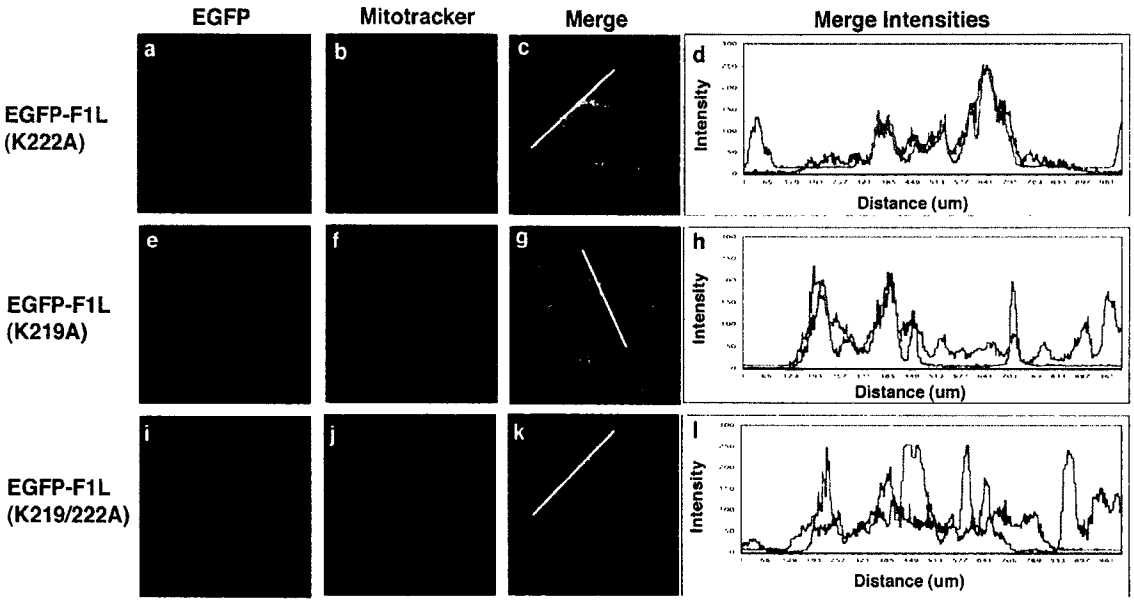


Figure 2-5

intensities for both EGFP-F1L-K222A with Mitotracker using a profile option within the Zeiss LSM 510 image software program that accurately compares the intensities of various fluorescent signals along a cellular distance (represented by white lines in the confocal images). This approach strongly confirmed co-localization of EGFP-F1L-K222A with Mitotracker as shown by the almost perfect overlap of the EGFP and Mitotracker merge intensities (Fig. 2-5B d). When HeLa cells were transfected with pEGFP-F1L-K219A, we routinely observed only partial localization with Mitotracker (Fig. 2-5B e-g). The remainder of EGFP-F1L-K219A localized to an unidentified membrane-like structure throughout the cytoplasm. The merge intensity profile of EGFP-F1L-K219A demonstrated some regions of co-localization with Mitotracker and other regions where co-localization was not evident (Fig. 2-5B h). When HeLa cells were transfected with the double lysine to alanine mutant, pEGFP-F1L-K219/222A, the mitochondrial targeting of F1L was completely abolished and the entire F1L signal was found localized to a membrane-like structure throughout the cytoplasm (Fig. 2-5B i-k). This observation was supported by the complete lack of co-localization when the merge intensities for EGFP-F1L-K219/22A and Mitotracker were plotted (Fig. 2-5B i). These data clearly indicated that mutation of the positively charged lysines in the tail of F1L dramatically affected localization.

To determine if EGFP-F1L-K219A and EGFP-F1L-K219/222A, were rerouted to the ER, a common default membrane when positive charges are eliminated from mitochondrial-localized TA proteins, HeLa cells were transfected with the various F1L mutant constructs and stained with ER-Tracker (8, 9, 28, 32). Cells transfected with either pEGFP, pEGFP-F1Lwt, or pEGFP-F1L-K222A demonstrated no colocalization

with ER-Tracker and this observation was supported by plotting the merge intensities of EGFP and ER-Tracker (Fig 2-6 a to l). In contrast, cells transfected with pEGFP-F1L-K219A again showed both a mitochondrial and membrane-like staining pattern throughout the cell (Fig. 2-6 m to p). When the fluorescent signals of pEGFP-F1L-K219A and ER-Tracker were superimposed, co-localization was evident demonstrating that in addition to localizing to the mitochondria F1L-K219A was also localized to the ER (Fig. 2-6 m-o). The merge intensity profile demonstrated some regions of co-localization as well as regions where the merge intensities were very different (Fig. 2-6 p). When cells were transfected with pEGFP-F1L-K219/222A a uniform image was produced with the ER-Tracker with no indication of mitochondrial staining demonstrating that mutating lysines 219 and 222 completely shifted the localization of F1L from the mitochondria to the ER and the merge intensity profile supported this observation (Fig. 2-6 q-t). The ER localization of EGFP-F1L-K219A and EGFP-F1L-K219/222A was also confirmed by staining cells with an antibody that recognizes calnexin, an ER localized protein (Fig. 2-7). These observations demonstrated that lysine 219 and 222 played a critical role in localization of F1L to the mitochondria.

Mitochondrial Localization of F1L is Required for Apoptosis Inhibition

Originally we found that VV strain Copenhagen, which is naturally devoid of the caspase 8 inhibitor CrmA/Spi2, inhibits apoptosis by maintaining mitochondrial integrity (63). Our recent findings show that this inhibition is due to expression of F1L which localizes to the mitochondria and prevents the release of cytochrome c and loss of the inner mitochondrial membrane potential (64). Unlike Bcl-2, which localizes to both the

A)

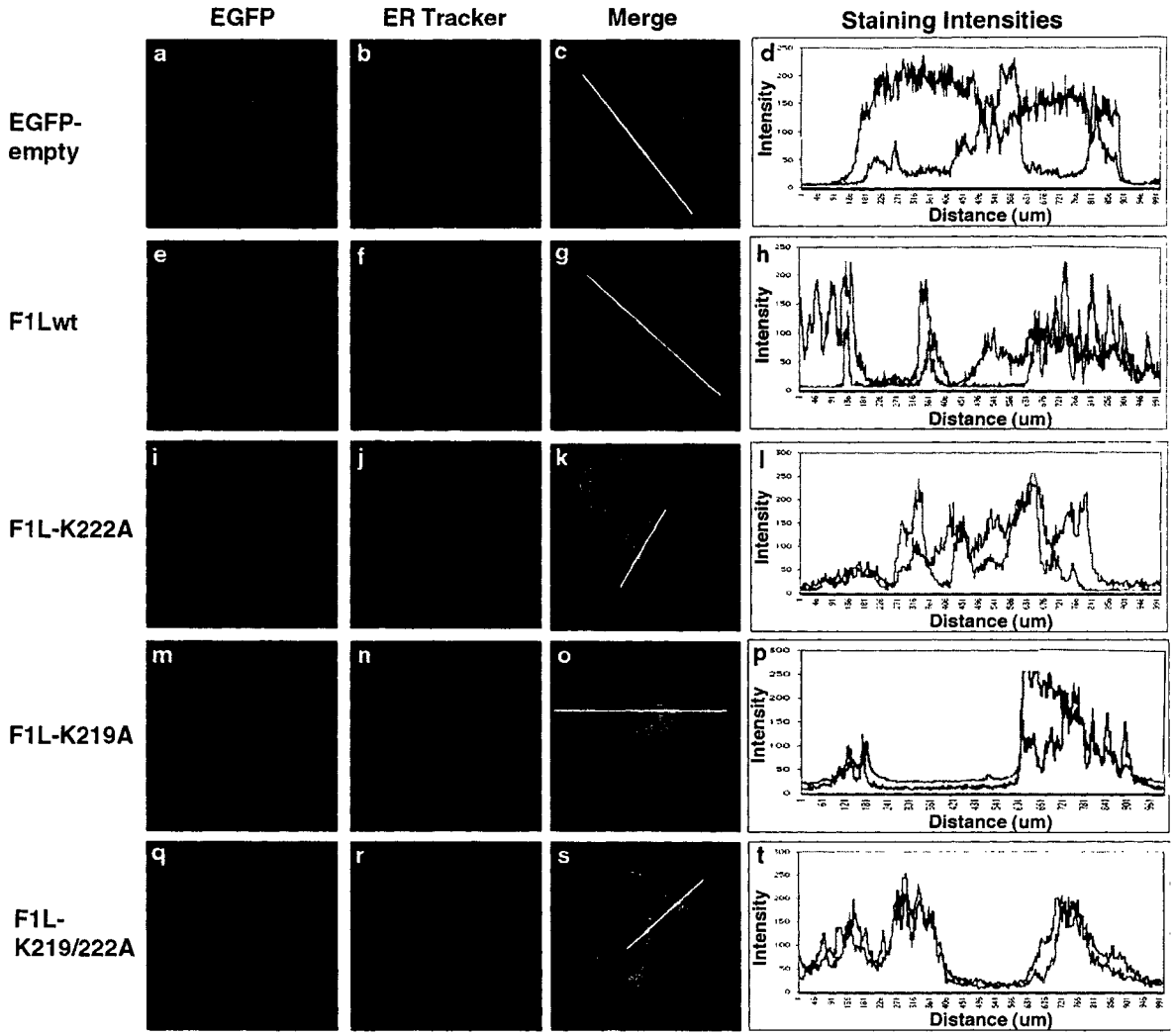


Figure 2-6

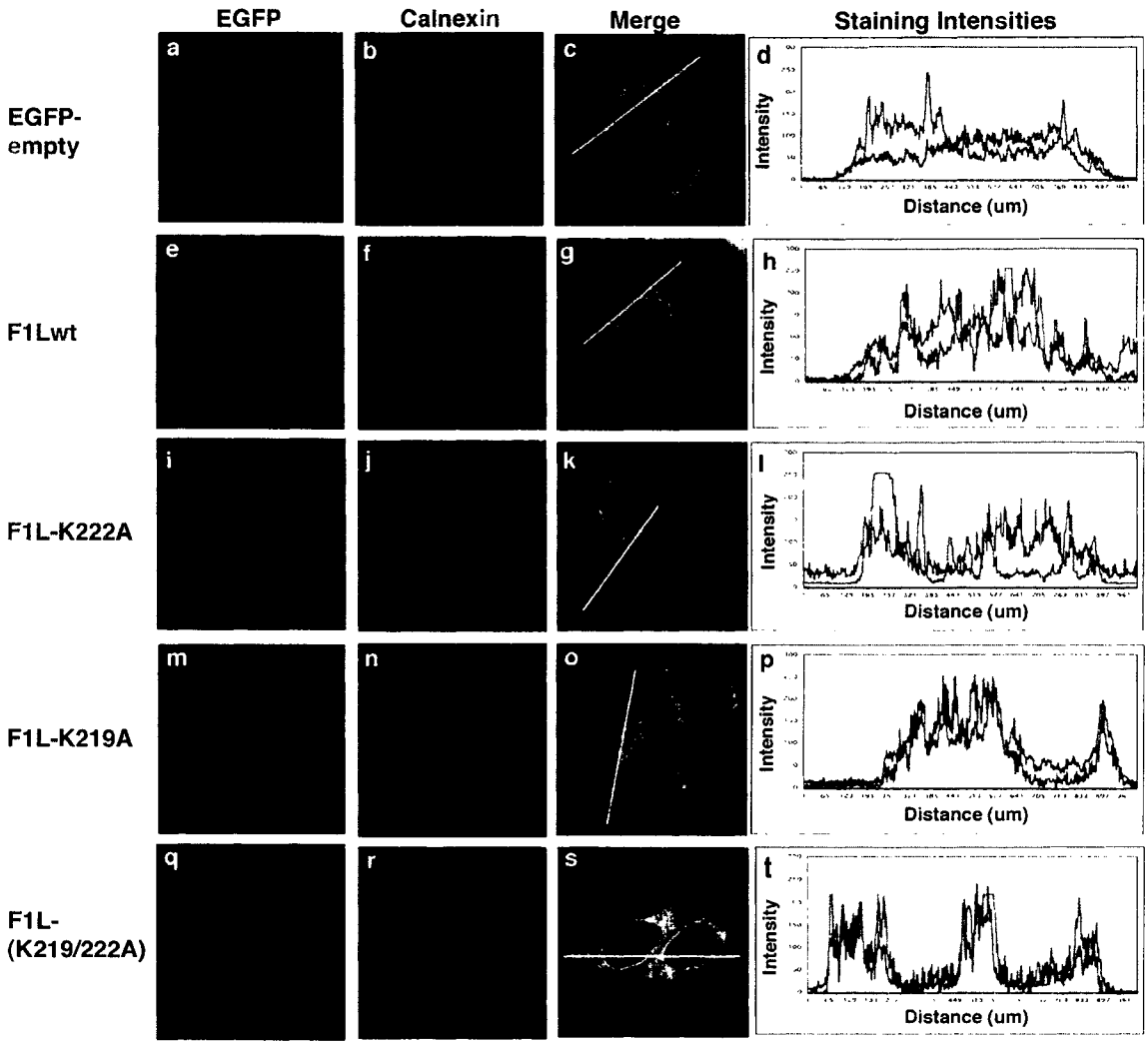


Figure 2-7

ER and mitochondrial membranes and functions to inhibit apoptosis at both locations, F1L localizes exclusively to the mitochondria suggesting that localization to the mitochondria during virus infection might be critical for apoptosis inhibition (33, 57, 58, 64).

During our characterization of F1L as a TA protein, we showed that localization of F1L was shifted from the mitochondria to the ER as a consequence of a series of point mutations made within the F1L gene (Fig. 2-5A a-c). Therefore, to determine if the presence of F1L at the mitochondria was necessary for inhibition of apoptosis, we transfected HeLa cells with pEGFP, pEGFP-F1Lwt, pEGFP-F1LK222A, pEGFP-F1LK219A, and pEGFP-F1LK219/222A, treated with TNFα, and measured the loss of the inner mitochondrial membrane potential ($\Delta\Psi_m$), a hallmark feature of apoptosis. Loss of the inner mitochondrial membrane potential was monitored by staining mitochondria with TMRE, a mitochondrial specific fluorescent dye which is incorporated into healthy respiring mitochondria, and apoptosis was analyzed by two color flow cytometry (17, 21, 39). HeLa cells transfected with empty vector pEGFP and stained with TMRE showed a population of cells that were both positive for EGFP and TMRE, representing cells that were transfected and containing mitochondria that retained their inner mitochondrial membrane potential (Fig. 2-8A a upper right quadrant). After treatment with TNFα, approximately 40% of the cells transfected with empty pEGFP showed a loss of TMRE fluorescence verified by the drop in cell population from the upper right quadrant to lower right quadrant, indicating that expression of EGFP alone was not sufficient to inhibit apoptosis (Fig. 2-8A a-b). Additionally, TMRE fluorescence was also lost in the TMRE positive EGFP negative population of cells as shown by the drop in this

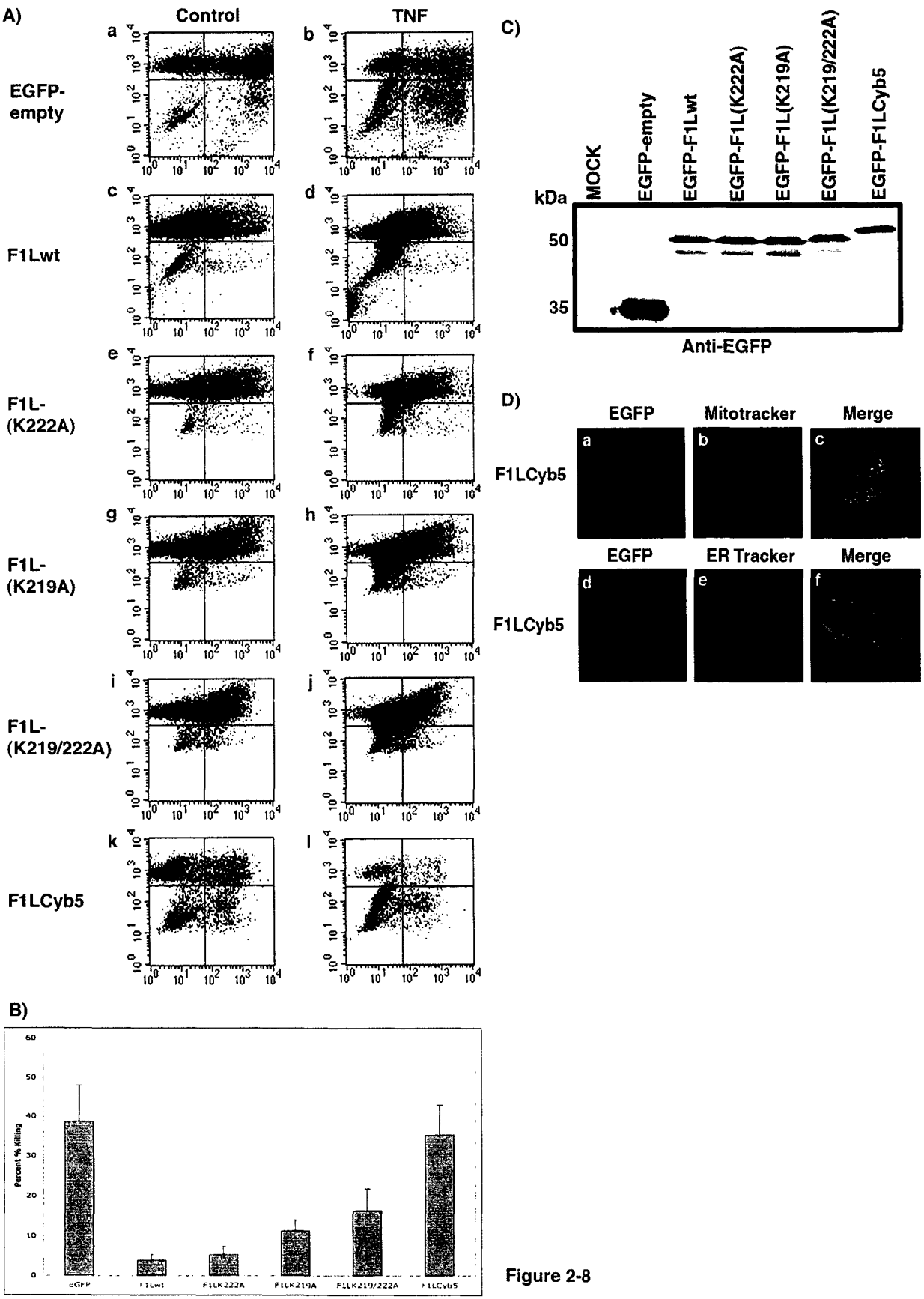


Figure 2-8

population from the upper left to the lower left quadrant (Fig 2-8A. a-l). In contrast, HeLa cells transfected with pEGFP-F1Lwt and treated with TNFα exhibited only a 3% reduction in TMRE fluorescence indicating that expression of F1L protected cells from loss of the inner mitochondrial membrane potential following an apoptotic trigger (Fig. 2-8A c-d). Similarly, upon treatment with TNFα, HeLa cells transfected with pEGFP-F1LK222A, which retains localization at the mitochondria, also resulted in inhibition of apoptosis (Fig. 2-8A e-f). However, HeLa cells transfected with pEGFP-F1LK219A, which localized to both the mitochondria and ER, resulted in a slight reduction in the ability of this construct to inhibit apoptosis with approximately 11% of cells showing a loss of the inner mitochondrial membrane potential (Fig.2-8A g-h). Interestingly when, HeLa cells were transfected with the ER localizing construct EGFP-F1LK219/222A we observed an even greater loss in TMRE fluorescence (15%) (Fig. 2-8A i-j). To ensure that a loss in apoptosis protective ability was not simply due to differences in protein expression profiles of these constructs, we analyzed protein expression levels by western blotting. Figure 2-8C demonstrates that EGFP-F1Lwt and the mutant constructs were all expressed at equivalent levels (Fig. 2-8C).

Our results indicated that localization of F1L to the ER resulted in a moderate decrease in apoptosis inhibition following TNFα treatment, but not to the degree seen in cells transfected with EGFP alone. Unsure if this was due to the ability of F1L to function at the ER or if inhibition was the result of minute amounts of EGFP-F1L-K219/222A still localizing to the mitochondria which was undetected by confocal microscopy, we used an alternative approach to further investigate this result. Due to the difficulty of performing subcellular fractionation and purifying mitochondria free of ER, we instead generated an

F1L chimera that would exclusively target F1L to the ER. To create this chimera, we swapped the C-terminal tail of F1L (amino acids 207-226) with the C-terminal tail of cytochocrome b5, a TA protein that has been shown to localize exclusively to the ER (40). To confirm the localization of F1LCyb5, we transfected HeLa cells with pEGFP-F1LCyb5, stained the cells with Mitotracker and ER-Tracker, and visualized localization by confocal microscopy. The fluorescent signal of EGFP-F1LCyb5 demonstrated a web-like staining pattern throughout the cell that colocalized with ER-Tracker but not Mitotracker indicating EGPF-F1LCyb5 localized exclusively to the ER (Fig. 2-8D).

To determine if EGFP-F1LCyb5 expression could inhibit apoptosis, HeLa cells were transfected with pEGFP-F1LCyb5 and treated with TNFα. Our data demonstrated that approximately 36% of cells underwent apoptosis, which was comparable to cells expressing EGFP indicating that EGFP-F1LCyb5 was unable to inhibit apoptosis when targeted to the ER (Fig. 2-8A k-l). To ensure that lack of inhibition was not a result of differential expression levels, expression of EGFP-F1LCyb5 was determined by western blot using an anti-EGFP antibody. As demonstrated in Figure 2-8C, expression of EGFP-F1Lwt and EGFP-F1LCyb5 was equivalent. The results for three or more separate experiments are graphically represented in Figure 2-8B. This suggests that any inhibition demonstrated by F1L-K219/222A might be due to small amounts of protein still localizing to the mitochondria or that the specific C-terminal tail of F1L plays a role in apoptosis inhibition.

To further determine if mitochondrial localization of F1L is essential for the anti-apoptotic function of F1L, we used the panel of EGFP-F1L deletion constructs generated in Figure 2. HeLa cells transfected with either pEGFP-F1LHTR(1-218), pEGFP-

F1LTR(1-206), pEGFP-F1Ltail(+)(206-226), or pEGFP-F1Ltail(-)(207-226) were treated with TNFα and cell death was determined by loss of the inner mitochondrial membrane potential ($\Delta\Psi_m$), a hallmark feature of apoptosis, in the EGFP positive population (38). HeLa cells transfected with either pEGFP-F1LHTR(1-218) or pEGFP-F1LTR(1-206) showed no protection from apoptosis following treatment with TNFα as demonstrated by a loss of TMRE fluouresence of approximately 29 and 32%, respectively (Fig. 2-9A e-h). This result suggested that expression of the N-terminal region of F1L, which is no longer capable of localizing to mitochondria, is unable to inhibit apoptosis. Similar results were found upon expression of a cytoplasmic form of Bcl-2 and M11L (18, 19, 44). Additionally, we found that expression of the two C-terminal tail constructs EGFP-F1Ltail(+)(206-226) and EGFP-F1Ltail(-)(207-226), both of which localize to the mitochondria, were also unable to provide protection from TNFα-induced apoptosis (Fig. 2-9A i-l). The results from three or more separate experiments were graphically represented in figure 2-9B. These observations indicated that expression of the F1L tail alone was not sufficient to inhibit apoptosis. The results indicate that expression of either the N-terminus alone or the mitochondrial-localized tail of F1L is not sufficient to inhibit apoptosis.

To ensure that differences in protein expression levels were not influencing the level of inhibition, the expression levels of various EGFP-F1L deletion constructs were analyzed by western blotting. HeLa cells transfected with EGFP-F1Ltail(+)(206-226) and EGFP-F1Ltail(-)(207-226) showed higher expression levels compared to wildtype F1L. Even at higher expression levels, both of these constructs were clearly unable to inhibit apoptosis (Fig. 2-9C). Western blot analysis demonstrated that transfection of EGFP-

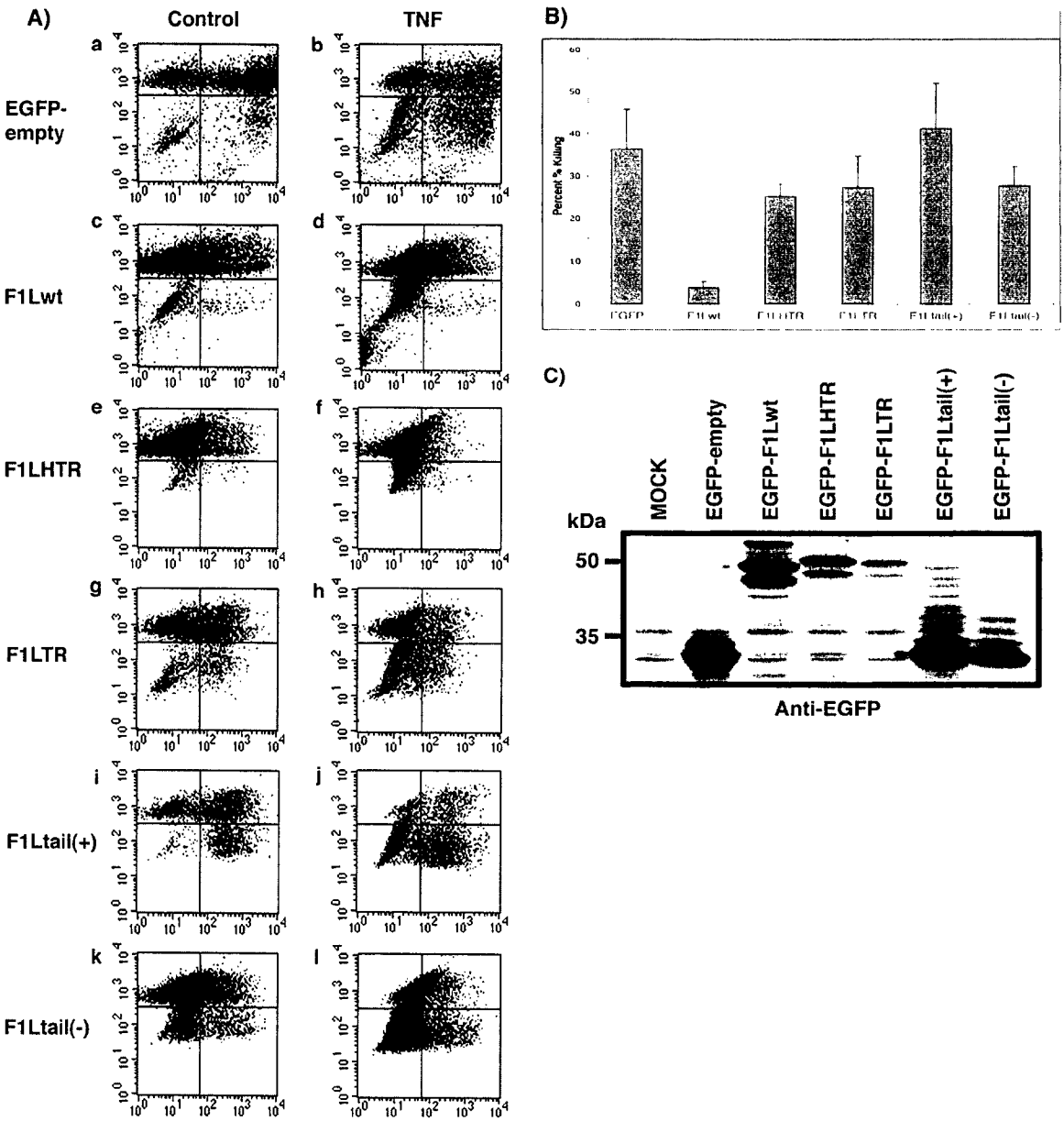


Figure 2-9

F1LHTR(1-218) had a slightly lower expression level than EGFP-F1Lwt. Unfortunately, the expression level of EGFP-F1LTR(1-206) was significantly lower than EGFP-F1Lwt. All of our attempts to increase the expression level of this truncated version of F1L were unsuccessful (data not shown). Concerned that a lack of inhibition by F1LTR(1-206) was due to lower levels of protein following transfection we reanalyzed our flow cytometry data for all of the transfected constructs by specifically gating on the population of lower EGFP expressing cells at 10^2 and determined the percent of TMRE loss following the addition of TNFα. Using this approach to reanalyze only the lower EGFP positive population the data again showed the same trend in that expression of the truncated and tail only versions of F1L were unable to inhibit apoptosis (data not shown). These results indicated that truncated F1L found in the cytoplasm is unable to inhibit apoptosis while the C-terminal tail of F1L is sufficient to localize F1L to the mitochondria but on its own is not sufficient to inhibit apoptosis.

2.4 Discussion

Viruses are under constant pressure from the host immune system, therefore, to avoid detection and elimination, many viruses resist by adopting strategies to counteract various facets of the immune response including strategies that interfere with apoptosis (13, 25, 51). Poxviruses, of which vaccinia virus is a member, express numerous proteins which mediate immune evasion including proteins aimed at interference with apoptosis (7, 31, 55). Historically, the best characterized apoptosis modulator encoded by members of the poxvirus family is CrmA/Spi-2, which inhibits apoptosis by preventing the activity of caspsase 8 (56, 66). Until recently, it was assumed that CrmA/Spi-2 was the major

intracellular anti-apoptotic protein encoded by vaccinia virus. We have recently demonstrated however, that vaccinia virus encodes an additional anti-apoptotic protein F1L. F1L localizes to mitochondria and inhibits both the loss of the inner mitochondrial membrane potential, a hallmark feature of apoptotic cells, and prevents the release of cytochrome c, the pivotal commitment step (64).

Currently, within the poxvirus family, only members of the *Orthopoxvirus* genus, which includes vaccinia virus, variola virus, cowpox virus, monkeypox virus, and ectromelia virus encode F1L orthologs, suggesting an important role for F1L during *Orthopoxvirus* infection. All known orthologs share >95% sequence identify over the last 220 amino acids. The greatest sequence diversity among the various F1L orthologs is located within the N-terminal regions, with strains of variola virus, camelpox virus and ectromelia virus displaying a series of unique repeats, the function of which is currently unknown. Although it is currently unknown if all F1L orthologs are functional, we have shown in this study that F1L orthologs present in ectromelia, rabbitpox, and cowpox virus localized to mitochondria (Fig. 2-1).

One obvious feature of F1L is a C-terminal transmembrane domain flanked by positive charged lysine residues followed by a short eight amino acid hydrophilic tail (Fig. 2-2B). Similar domains are important for membrane localization with both the length of the hydrophobic domain and the presence of flanking positive charges playing a role (8, 9, 65). Therefore, to investigate the sequence requirements necessary for F1L mitochondrial localization we generated two C-terminal tail F1L constructs; F1Ltail(+)(206-226), which includes amino acids 206-226, and F1Ltail(-)(207-226), which includes amino acids 207-226 minus lysine 206 (Fig. 2-2B). Although we were

unsure of the role of lysine 206 in mitochondrial localization, both EGFP-F1Ltail constructs localized to the mitochondria indicating that amino acids 207-226 were sufficient for mitochondrial localization and that the presence of lysine 206 was not required. Data from our previous publication revealed that the last 27 amino acids of F1L (F1L 199-226) was necessary and sufficient for localization to the mitochondria (64). This construct contained eight additional amino acids upstream of the transmembrane domain and our current data reveals that these eight amino acids are dispensable for mitochondrial localization. Additional analysis further revealed that transfection of pEGFP-F1LHTR(1-218), which contains the hydrophobic transmembrane domain, but lacks the last eight amino acids of the hydrophilic tail, no longer localized to the mitochondria and was in fact cytosolic suggesting that the short hydrophilic tail of F1L was playing a role in mitochondrial localization (Fig. 2-2B). Initially we predicted that F1LHTR(1-218) would be rerouted to the ER which is a common default membrane for TA proteins (9, 29, 32, 35). However, due to the presence of a short 12 amino acid hydrophobic transmembrane domain in F1L it is unlikely that this domain would be of sufficient length to span the ER membrane thereby resulting in cytoplasmic distribution (8, 47). The tail-anchored proteins Tom5 and outer mitochondrial membrane isoform of cytochcrome b5 (OMb), both normally localize to the mitochondria but also demonstrate cytoplasmic staining when the C-terminal hydrophilic tail is deleted (28, 35). These observations indicate that the membrane targeting signal in F1L is not solely dependent upon the presence and length of a transmembrane domain but rather additional amino acids downstream of the transmembrane domain are also required.

The presence of a C-terminal mitochondrial targeting motif in F1L suggests that F1L is a member of a growing group of proteins referred to as TA proteins. This family of proteins has received considerable attention due to their unique, but still poorly understood, membrane targeting mechanisms (8, 9, 65). TA proteins constitute a family of proteins that lack N-terminal signal sequences and insert into membranes using a C-terminal transmembrane domain. TA proteins have been discovered in a vast array of organisms, performing a diverse array of functions, and are therefore found in numerous membranes including the ER, Golgi aparatus and mitochondria (8, 9, 65). Due to the ordered steps involved in the secretory pathway leading from ER to Golgi to plasma membrane, TA proteins need only discriminate between the ER and mitochondria (8, 36). The mitochondrial outer membrane contains numerous TA proteins including Tom proteins involved in protein translocation and members of the Bcl-2 family such as Bcl-2, Bcl-xL, and Bak which function to regulate the release of mitochondrial pro-apoptotic proteins (8, 9, 65). Tail-anchored proteins have also been identified in viruses such as the vaccinia virus H3L protein, herpes simplex type II protein UL34, adenovirus E3-6.7K protein, and myxoma virus encoded anti-apoptotic protein M11L (14, 18, 41, 54). Although M11L and F1L display no sequence similarity they share similar functional features including localization to mitochondria and the ability to inhibit the loss of the inner mitochondrial membrane potential and release of cytochrome c (18, 19, 62).

TA proteins demonstrate a specific membrane topology with the C-terminus spanning and anchoring the protein into the membrane and the N-terminus facing the cytoplasm (36). To determine the topology of F1L we performed *in vitro* TnT assays. Using this *in vitro* assay we confirmed that the last 20 amino acids of F1L were necessary

for insertion of F1L into mitochondria. We also demonstrated using proteinase digestion that the N-terminus of F1L faces the cytoplasm (Fig. 2-3). This topology for F1L was also present during virus infection as determined by using purified mitochondria from VVWR-FLAG-F1Lwt infected Jurkat cells and by confocal microscopy coupled with selective permeabilization procedures (Fig. 2-4). This specific topology for TA proteins has led to a common theme where the C-terminal transmembrane domain serves as a membrane anchor while the cytosolic facing N-terminus carries out specific functions (8, 9). At present the precise anti-apoptotic mechanism of action of F1L is undefined but the specific topology of F1L suggests that the N-terminal cytosolic domain may be interacting with cellular proteins to inhibit apoptosis. We are currently pursuing experiments to identify potential F1L interacting proteins that may elucidate the mechanism of action of this newly identified anti-apoptotic protein.

A common trend seen among mitochondrial TA proteins is that in addition to the transmembrane domain, flanking positively charged amino acids also play a role in localization (28, 29, 32, 35). F1L contains two positively charged lysines downstream of the transmembrane domain; lysines 219 and 222 (Fig. 2-2B). Mutation of lysine 222 to generate F1L-K222A, did not affect the localization of F1L. In contrast, when lysine 219 was mutated to alanine to generate F1L-K219A, F1L localized to both the mitochondria and ER, and when both lysines were mutated to generate the construct F1L-K219/222A, only ER localization was observed. These data clearly indicated a role of lysine 219 and 222 in mitochondrial localization of F1L. Mutation of lysines 219 and 222 to alanine in F1L essentially extended the length of the hydrophobic domain from 12 to 20 amino acids resulting in a transmembrane domain predicted to be sufficient for ER localization

(9, 47). In a similar fashion, mutation of lysine residues downstream of the hydrophobic domain of Tom5, also results in altered localization to the ER (28). In contrast, a single mutation of lysine 219 in F1L to generate F1L-K219A lengthened the hydrophobic domain to 15 amino acids resulting in localization of F1L to both the mitochondria and ER.

To determine if the altered localization of F1L from mitochndria to ER impeded the anti-apoptotic function of F1L, HeLa cells were transfected with the various F1L point mutation consttucts. HeLa cells transfected with the two mitochondrial localizing constructs, EGFP-F1Lwt and EGFP-F1LK222A, both inhibited apoptosis. In contrast, cells transfected with EGFP-F1L-K219A demonstrated a slight decrease in apoptosis inhibition and cells transfected with EGFP-F1L-K219/222A demonstrated an even greater decrease in apoptosis inhibition. Initially this result led us to speculate that F1L could inhibit apoptosis at the ER as well as the mitochondria, in a similar fashion to Bcl-2 (33, 57, 58). However, it was possible that minute amounts of F1L-K219/222A still localized to the mitochondria which was undetected in our images. Due to the difficulty in performing subcellular fractionations and obtaining purified mitochondria free of ER, we instead swapped the C-terminal tail of F1L with the tail of cytochrome b5 which is necessary for ER localization allowing us to determine if F1L could inhibit apoptosis at the ER (40). Our results showed however, that F1L-Cyb5 was completely unable to inhibit apoptosis suggesting that F1L functions only at the mitochondria or its specific C-terminal tail is important for inhibition of apoptosis.

Unlike Bcl-2, which localizes to the mitochondria, ER and nuclear membrane, our data indicates that F1L localizes exclusively to the mitochondria (3, 33, 64). Confocal

analysis of F1L expression, even during virus infection, clearly suggests that the presence of F1L at the mitochondria is important during vaccinia virus infection. HeLa cells transfected with the two F1L truncated constructs, F1LHTR(1-218) and F1LTR(1-206), both of which demonstrate cytoplasmic distribution were unable to inhibit apoptosis. Similar observations were made with truncated versions of Bcl-2 and M11L which no longer localize to the mitochondria and are also unable to inhibit apoptosis (18, 44). Additionally, expression of F1Ltail(+)(206-226) and F1Ltail(-)(207-226) were unable to inhibit apoptosis induced by TNFα. Collectively our data indicates that the localization of F1L to the mitochondria is necessary for apoptosis inhibition.

Our studies have now shown that the newly identified anti-apoptotic protein F1L, is a member of the TA family of proteins that localizes to the mitochondria where it functions to inhibit apoptosis. Our data supports the idea that F1L localization at the mitochondria is important during vaccinia virus infection since truncated and chimeric versions of F1L are no longer able to inhibit apopotosis. Although, F1L-related open reading frames are currently only present in members of the *Orthopoxvirus* genus, various members of the *Leporipoxvirus*, *Capripoxvirus*, *Yatapoxvirus* and *Suipoxvirus* genera encode M11L-like proteins (2, 10, 11, 37, 61). Strikingly, members of the *Avipoxvirus* genus are the only poxviruses to encode obvious Bcl-2 homologues (1, 60). Recent evidence strongly demonstrates that the M11L protein from myxoma virus functions to inhibit apoptosis by interacting with the peripheral benzodiazepin receptor, a component of the mitochondrial permeability transition pore that has been linked to apoptosis (19). In addition, M11L interacts with Bak, and under some conditions, Bax, both are pro-apoptotic members of the Bcl-2 family (62). It is currently unknown if the

Avipoxvirus-encoded Bcl-2 proteins or the *Orthopoxvirus*-encoded F1L proteins function through similar interactions but the presence of the various poxvirus-encoded anti-apoptotic proteins that function to inhibit the mitochondria apoptotic pathway clearly indicates the importance of interfering with this pathway for members of the poxvirus family.

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CHAPTER 3 – DISCUSSION AND FUTURE DIRECTIONS

3.1 Discussion

At the beginning of this study, an accumulation of results suggested that F1L was a tail-anchored protein that localized to mitochondria to inhibit apoptosis (41). Originally we were interested in understanding how information within the C-terminal tail of F1L determined its localization to the mitochondria. However, tampering with the F1L gene to create various F1L mutant constructs led to an interesting observation regarding the mechanism of action for F1L. Now at the end of my studies I have successfully identified the targeting information within the C-terminal tail of F1L and have concluded that localization of F1L to mitochondria is necessary for inhibition of apoptosis.

Performing a blast search using the F1L sequence as a query led to the discovery of orthologs only found within the *Orthopoxvirus* genus (Figure 1-10). Initial studies on F1L showed that F1L localizes to mitochondria through a C-terminal hydrophobic domain where it prevented the loss of the inner mitochondrial membrane potential and release of cytochrome c (Figure 2-2) (41). Sequence alignment of the various F1L orthologs demonstrates that all of the F1L orthologs contain a similar C-terminal mitochondrial targeting motif and we have now shown that the F1L orthologs in vaccinia virus, ectromelia virus, rabbitpox virus, and cowpox virus, all localize to mitochondria during virus infection (Figure 2-1). While the sequence homology between the various F1L orthologs is greater than 95%, a region that intrigued us was the N-terminus of the ectromelia virus F1L protein. Unlike the other F1L orthologs, F1L expressed from ectromelia virus contains an 8 amino acid repeat that extends 240 amino acids. Due to

difficulties in cloning this particular F1L ortholog, perhaps due to the large N-terminal repeat, we deleted the first 240 amino acids and interestingly this particular F1L construct was still able to inhibit apoptosis at the mitochondria (J.Taylor and M.Barry unpublished data).

Tail-anchored proteins have received considerable attention due to their ability to localize to various cellular membranes post-translationally solely by the use of a C-terminal targeting motif (5, 6, 21, 42). Although the majority of studies on TA proteins have focused on trying to crack the localization code found within the C-terminal targeting motif, each TA protein is unique and contains a distinctive targeting motif (5, 6, 42). Unfortunately comparing one TA protein to another is often inconclusive. However, a common trend that researchers do agree on is that the length of the hydrophobic domain as well as the presence of positively charged amino acids within the C-terminal tail play a role in determining the final destination of TA proteins (5, 6, 21, 42).

Eight TA proteins that have been researched extensively include, cytochrome b5, Vamp1-A, Vamp1-B, Bcl-2, Bcl-xL, M11L, Tom5, and now F1L (12, 15, 16, 18, 20, 41). While Vamp1-A and cytochrome b5 use their C-terminal tails to localize to the ER, Bcl-xL, M11L, Vamp1-B, Tom5, and F1L localize to the mitochondria (Figure 3-1). Interestingly, the C-terminal tail of Bcl-2 carries information within it to allow Bcl-2 to localize to the ER, mitochondria, and nucleus (19). For Bcl-2 to localize to three different membranes further emphasizes the complexity involved in cracking the localization code of TA proteins.

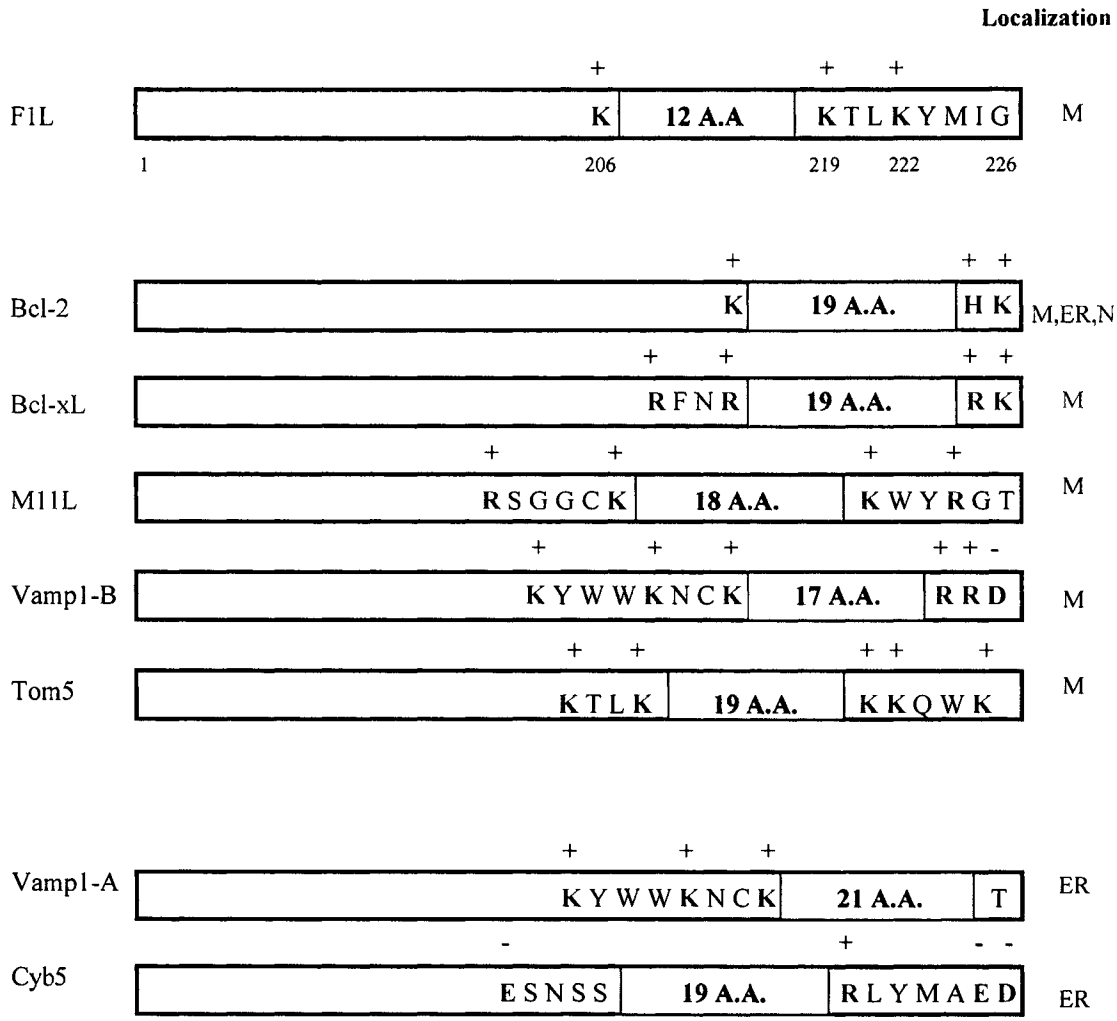

Figure 3-1: Schematic representation of the C-terminal tail of F1L compared to other tail-anchored proteins. Acidic and basic amino acids are shown in red and blue respectively.Transmembrane domains are represented by the shaded region. M = Mitochondria, ER = Endoplasmic Reticulum, A.A = amino acids

A debatable claim made for TA proteins that localize to the mitochondria is that the hydrophobic transmembrane domain should not exceed 20 amino acids (5, 6, 42). In contrast, TA proteins that localize to the ER contain transmembrane domains that consist of 21 or more amino acids (5, 6, 42). The logic behind this claim is based on the relative thickness of the mitochondria vs ER membranes (26). While the outer and inner mitochondrial membranes are made up of the thinnest membranes of the cell, the ER is composed of thicker membranes. A close analysis of the hydrophobic domains of tail-anchored proteins that localize to the mitochondria reveals an amino acids length ranging from 12 to 19 amino acids (Figure 3-1). Interestingly, the hydrophobic domains of the ER proteins Vamp1-A and cytochrome b5 contain 21 and 19 amino acids respectively. Based on these numbers there appears to be a "fine line" with regards to the length of the hydrophobic domain determining localization suggesting something else within the C-terminal tail is contributing to the localization patterns.

The deletion of positively charged amino acids in the C-terminal tail of TA proteins was the first piece of evidence supporting their importance in subcellular localization (5, 6, 42). Mutating positively charged amino acids to neutral residues had a drastic effect that resulted in TA proteins that normally localize to the mitochondria to change course and localize to the ER (15, 16, 20). To determine if F1L would show a similar trend we mutated positively charged lysines 219 and 222 to neutral alanines in the C-terminal tail of F1L and this too resulted in F1L localizing to the ER (Figure 2-5, 2-6 and 2-7). To explain this altered localization, we hypothesized that mutating positive charges in the C-terminal tail of F1L essentially extended the length the hydrophobic domain from 12 to 20 amino acids allowing for sufficient insertion into the ER (26).

The contribution that both the positively charged amino acids and the hydrophobic transmembrane domain play in targeting TA proteins is vague. Researchers often look to Bcl-2 and Bcl-xL as a means to crack the TA code. Bcl-2 and Bcl-xL both have a 19 amino acid hydrophobic domain with a similar degree of hydrophobicity (Figure 3-1) (16). However, as previously mentioned, Bcl-2 localizes to the mitochondria, ER, and nucleus while Bcl-xL exclusively localizes to the mitochondria. So what is the reason for the difference in localization? Kaufmann et al. (2003) and colleagues claim that Bcl-xL possesses an additional positively charged amino acid on each side of the transmembrane domain which allows Bcl-xL to localize to mitochondria (see Figure 3-1). In other words, these studies suggest that at least two basic amino acids flanking each end of the hydrophobic domain is what truly characterizes a mitochondrial TA protein (16). Anything less, and a TA-protein will be found in the midst of a foreign organelle. However, a contradiction to this has been demonstrated in work done on two similar proteins, Vamp1-A and Vamp-1B (15). Like Bcl-2 and Bcl-xL, Vamp1-A and B have very similar C-terminal tails with the exception that Vamp1-A lacks basic amino acids downstream of its hydrophobic domain and as a result Vamp1-A localizes to the ER (Figure 3-1) (15). However, adding positively charged residues downstream of this hydrophobic domain does not create a mitochondrial sequence and is not sufficient for localization to the mitochondria (15). Our studies on F1L further dispute this claim since creating two C-terminal tail constructs F1Ltail(+)(206-226) and F1Ltail(-)(207-226), with the latter lacking lysine 206, had no effect on localization and both constructs targeted to mitochondria (Figure 2-2).

To determine if F1L was a TA protein, several mutant F1L constructs were created containing deletions or point mutations. By generating these various F1L constructs we have essentially created a database of F1L constructs that localized to various subcellular locations including the mitochondria, cytoplasm and ER (Figure 3-2). Based on our previous evidence showing that F1L normally localizes to mitochondria to inhibit apoptosis we immediately saw the opportunity to utilize these constructs to further our understanding regarding the mechanism of F1L inhibition. While most studies on TA proteins are focused on understanding localization signals we decided to make full use of our studies and determine whether F1L needed to be at the mitochondria to inhibit apoptosis.

Bcl-2 has been shown to have the unique ability to inhibit apoptosis at both the ER and mitochondria (2, 9, 25, 38, 39). To determine if F1L could function at both organelles, we transfected HeLa cells with various F1L mutant constructs that localized to the ER, treated transfected cells with TNFα, and measured the loss of the inner mitochondrial membrane potential as an indication of apoptosis. Interestingly, as F1L moved from the mitochondria to the ER it was less efficient at inhibiting apoptosis as demonstrated by the construct F1L-K219/222A (Figure 2-8). Even more interesting was the complete loss of inhibition seen by F1LCyb5, which also localizes to the ER (Figure 2-8). This may suggest that F1L-K219/222A inhibits apoptosis at the ER, albeit at a reduced level, and that the C-terminal tail of F1L is essential for inhibition as demonstrated by F1LCyb5. Alternatively, the partial inhibition displayed by EGFP-F1LK219/22A could be due to trace amounts of EGFP-F1L-K219/222A localizing to

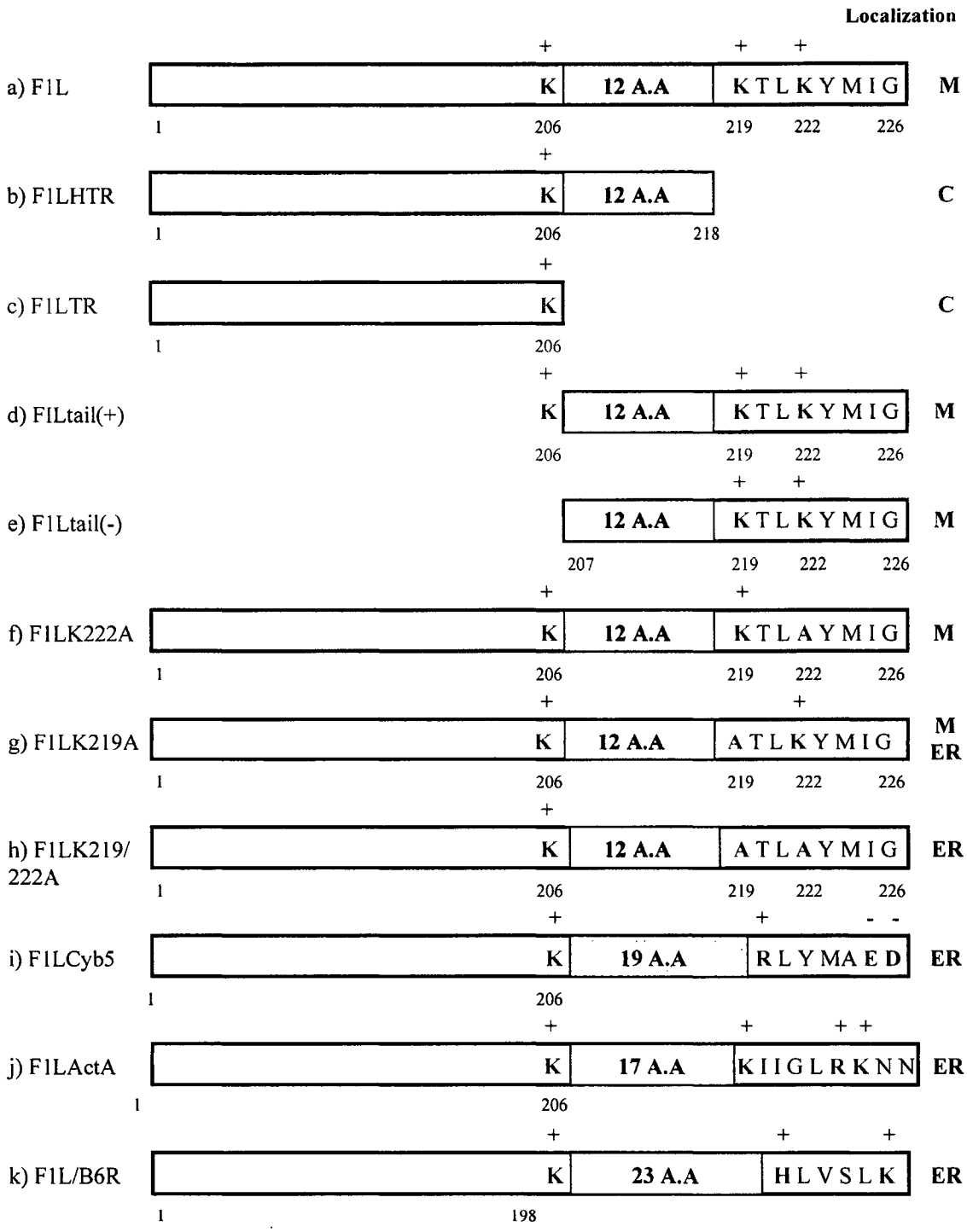

Figure 3-2: Various F1L constructs and their localization. M = Mitochondria, ER = Endoplasmic Reticulum, C = Cytoplasm, A.A = amino acids

mitochondria undetected by confocal microscopy. Contrary to this latter hypothesis, the F1L chimera EGFP-F1L/B6R, which also localizes to the ER, showed anti-apoptotic properties similar to F1L-K219/222A (Figure A-1). Although this further suggests that F1L is able to partially inhibit apoptosis at the ER, we hypothesize that EGFP-F1L/B6R and EGFP-F1LK219/222A are still able to inhibit apoptosis due to the close proximity of the ER and mitochondrial membranes (30, 31). The reason for the differences between F1L/B6R and F1LCyb5 remain unclear.

Although EGFP-F1LK219/222A localized the ER in the absence of viral infection, trace amounts were detected at the mitochondria during virus infection (Figure A-4). This was most likely due to the overexpression of protein due to constructs being under the control of a highly efficient synthetic poxviral promotor (8). Yet this result indicates that EGFP-F1LK219/222A is capable of localizing to mitochondria which explains the possible inhibitory effect of this construct. Secondly, this clearly demonstrates that vaccinia virus has recognized the importance of controlling apoptosis at the mitochondria and localization of F1L to mitochondria is essential for successful viral replication.

F1Ltail(+)(206-226) and F1Ltail(-)(207-226) were unable to inhibit apoptosis induced by TNFα suggesting that although the C-terminal tail is sufficient for mitochondrial localization it is insufficient for inhibition of apoptosis (Figure 2-9). To further study the role of the C-terminal tail at inhibiting apoptosis, a third F1L chimera was generated which contained that N-terminus of F1L and the C-terminal tail of ActA, a TA protein that has been shown to localize exclusively to mitochondria. (28,44). Interestingly, although F1LActA was within the realm of mitochondria, it did not inhibit

apoptosis efficiently suggesting a role for the C-terminal tail of F1L (Figure A-3). In addition, this suggests that the C-terminal tail of F1L does not localize F1L to mitochondria randomly. Perhaps the C-terminal tail localizes F1L to discrete locations.

It has been proposed that Bcl-2 and Bcl-xL inhibit apoptosis by inhibiting the pro-apoptotic effects of Bax (33, 37, 38). The pro-apoptotic protein Bax, normally resides in the cytoplasm of cells and it is not until the onset of an apoptotic stimulus that Bax undergoes a conformational change allowing the protein to localize to the mitochondria (4, 36). The translocation of Bax to the mitochondria is a significant apoptotic event and usually means death of the cell due to the suspected pore-forming capabilities of oligomerized Bax at the mitochondria (3, 37, 43). Although truncated forms of Bcl-2 and M11L, which localize to the cytoplasm are unable to inhibit apoptosis, we examined whether F1L had the capability to inhibit apoptosis while cytoplasmic and perhaps prevent the translocation of Bax (12, 24). To test this theory, we utilized two F1L constructs: EGFP-F1LHTR(1-218) and EGFP-F1LTR(1-206), both of which localize to the cytoplasm. However cells transfected with EGFP-F1LHTR(1-218) and EGFP-F1LTR(1-206), and treated with TNFα, were unable to inhibit apoptosis. These results suggest that for F1L to effectively inhibit apoptosis it localize to the mitochondria.

In addition to the caspase-8 inhibitor, CrmA/Spi2, we have now found that VV expresses a second anti-apoptotic inhibitor, F1L (41). A sequence comparison between members of the *Orthopoxvirus* genus has revealed that all members contain both CrmA/Spi2 and F1L with the exception of ectromelia virus that expresses a third anti-apoptotic protein called p28 (7). Interestingly, preliminary studies on ectromelia virus suggests that ectromelia virus is able to inhibit apoptosis more effectively than VV

(J.Taylor and M.Barry unpublished data). If this is a consequence of ectromelia virus expressing p28 than this explains why several viruses express multiple anti-apoptotic inhibitors since multiple proteins means an additive effect. Taking into account the ordered steps of the apoptotic pathway it is not surprising that members of the *Orthopoxvirus* genus would encode CrmA/Spi2 to inhibit the initial onset of apoptosis as well as express F1L to inhibit downstream events at the mitochondria. Encoding multiple anti-apoptotic proteins is a strategic move to ensure survival. This strategy is also used by several other viruses such as adenoviruses Epstein Barr virus, herpes simplex virus, human cytomegalovirus, and myxoma virus (14).

Analysis of viruses that express multiple anti-apoptotic proteins reveals protein functions that are specialized for specific components of the apoptotic cascade (14). Interestingly, the function of E1B-19K, an anti-apoptotic protein expressed from adenovirus, is to inhibit the virus induced induction of apoptosis (13, 22, 27, 29). During the course of infection, adenoviruses express E1A which stimulates cell cycle progression allowing the viruses to gain access to cellular machinery for DNA replication (32). The stimulation of cell cycle progression by adenoviruses is via induction of p53 which unfortunately leads to infected cells being over sensitive to TNF and FasL and therefore could cripple viral replication (10, 11, 35). However, upon adenoviruses infection, E1B-19K is also expressed to inhibit the adverse effects of E1A therefore allowing the viruses to successfully replicate and spread (13, 22, 27, 29). Although no pro-apoptotic proteins have been discovered in vaccinia virus, preliminary evidence suggests vaccinia virus may use similar strategies as adenoviruses. Experimental data using the deletion virus VV811, which is devoid of both CrmA/Spi2 and F1L, is not only unable to inhibit

apoptosis but also causes the induction of apoptosis (M.Barry unpublished data). Are CrmA/Spi2 and F1L expressed to counteract virus-induced apoptosis? This would make sense since early onset of apoptosis would significantly interfere with virus replication in the cytoplasm of infected cells, a dangerous territory in which to replicate. However, apoptosis induced later during infection could act as a Trojan horse resulting in the release of viral particles undetected by the immune system. We are currently investigating whether any pro-apoptotic genes exist within the vaccinia virus genome.

Since F1L displays no homology to any known cellular protein, the sequence of F1L has told us little about its actual mechanism of action. Originally, we searched for BH domains within the F1L gene, which are a characteristic of Bcl-2 family members (1, 17). However, analysis of the F1L sequence failed to reveal any such domains. One result that has provided insight to the mechanism of F1L is a web based software program, pro-site, that predicts tertiary structures (data not shown). Interestingly, F1L was predicted to adopt a hydrophobic pocket that resembles the fold of the anti-apoptotic protein Bcl-xL (S.Wasilenko and M.Barry unpublished data) (23, 34). This hydrophobic pocket is thought to sequester Bax and Bak allowing Bcl-xL to inhibit apoptosis (23, 34). Although the crystal structure of F1L has not been solved, immunoprecipitation studies and affinity chromatography in our lab have demonstrated that F1L interacts with Bak (S.Wasilenko and M.Barry unpublished data). Furthermore, the anti-apoptotic protein expressed by myxoma virus, M11L, which was thought to function in a similar manner to F1L, also interacts with Bak but contains a pseudo-BH3 domain similar to members of the Bcl-2 family (40). As previously mentioned, no domain was found in F1L therefore, it appears that F1L maybe functioning differently than M11L.

3.2 Future Directions

When this study began little was known about F1L. Initial studies showed that F1L localized to mitochondria where it inhibited apoptosis by preventing the loss of the inner mitochondria membrane potential and release of cytochrome c (41). We have now shown that F1L localizes to the mitochondria solely by a C-terminal targeting motif and positively charged amino acids within this motif are important for localization. In addition we have shown that F1L inserts into membranes post-translationally and adopts a topology with the N-terminus exposed to the cytoplasm. Based on these results we have concluded that F1L is a member of the tail-anchored protein family. In addition, we have shown that localization of F1L to mitochondria is necessary to efficiently inhibit apoptosis.

Our understanding on F1L has advanced considerably and we have learned much about a protein that was not discovered until four years ago. However many questions regarding the mechanism of F1L and the mystery behind its subtle function at the ER remain unanswered. Future experiments that would further shed light on the function of F1L include performing apoptotic assays that directly stimulate death at the ER, creating additional mutations within the F1L gene, solving the crystal structure of F1L and identifying any cellular proteins that F1L may interact with. To test whether F1L can truly inhibit apoptosis at the ER we need to trigger cells with various ER-inducing apoptotic stimulants such as thapsigargin or overexpression of ER proteins and utilize type I cell lines that bypass the mitochondria during apoptosis. To further determine the functional domains of F1L we need to generate additional F1L constructs such as truncated mutants lacking various portions of the N-terminus and determine whether the

N-terminus is responsible for inhibiting apoptosis. Solving the crystal structure of F1L, and most importantly, identifying any cellular proteins that interact with F1L will help to unravel the anti-apoptotic mechanism of F1L. In addition, identification of any binding partners such as possible chaperon proteins may also help to understand the targeting mechanisms of TA proteins. Cleary there is still a great deal to be done and I am looking forward to future discoveries on F1L.

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APPENDIX A – SUPPORTING METHODS AND RESULTS

A.1 F1L/B6R Chimera

Other organelles besides the mitochondria have also been shown to regulate apoptosis individually or by means of communicating with the mitochondria (1, 3, 4, 6, 7). One such organelle is the endoplasmic reticulum. To further determine if F1L needed to be at the mitochondria to inhibit apoptosis, I created an additional chimeric F1L protein, F1L/B6R, where the C-terminal tail of F1L was replaced with the C-terminal tail of the vaccinia virus protein B6R which is necessary and sufficient for localization of B6R to the ER (Figure 3-2 k).

A.1.1 Method for creating F1L/B6R

Amplification of the coding sequence for pEGFP-F1L/B6R was performed using a combination of three PCR reactions. In the first PCR reaction, pEGFP-F1Lwt was used as the template with the forward primer *Xho* I-(CTCGAGATGTTTATG) and the reverse primer 5'-(TGCGTATCGTACAACTTGTTAGGGATGGTATTATC) resulting in a PCR product that encodes the first 198 amino acids of F1L with an overhang of amino acids that corresponded to amino acids 143-149 of B6R. In the second PCR reaction, pEGFP-B6Rwt was used as the template with forward primer 5'-(AAGTTGTACG GATACGCATCT) and the reverse primer *Bam* HI-(GGATCCTTATTTATACAAAC TAACTAG) resulting in a PCR product encoding for amino acids 143-174 of B6R. In the third PCR reaction, the products of the first two PCR reactions were ligated together along with primers *Xho* I-(CTCGAGATGTTTATG) and *Bam* HI-

(GGATCCTTATTTATACAAACTAACTAG) resulting in a PCR product that encodes for the N-terminal portion of F1L (amino acids 1-198) and the C-terminal tail of B6R (amino acids 143-174). PCR amplified F1L/B6R was cloned into the TA cloning vector pGEMT (Promega), verified by DNA sequence analysis, and subcloned into pEGFP-C3 (Clonetech).

A.1.2 F1L/B6R localizes to the ER and demonstrates partial inhibition of apoptosis

Our data indicated that EGFP-F1LK219/222A, which localized to the ER, partially inhibited apoptosis (Figure 2-8). Unsure if this was due to EGFP-F1LK219/222A function at the ER or to minute amounts of protein localizing to the mitochondria, we originally created an F1L chimera, F1L/B6R, to address this issue. F1L/B6R was generated by swapping the C-terminal tail of F1L (amino acids 199-226) with the C-terminal tail of B6R (amino acids 143-174), a vaccinia virus protein that has been shown to localize exclusively to the ER (M.Barry unpublished data). Results with F1L/B6R were originally intended for publication but because B6R is an uncharacterized protein we alternatively generated F1LCyb5.

To confirm that the C-terminal tail of B6R is sufficient for ER localization, HeLa cells were transfected with pEGFP-B6Rtail, stained with Mitotracker and ER-Tracker, and visualized by confocal microscopy. The fluorescent signal for EGFP-B6Rtail displayed a web-like staining pattern throughout the cell that colocalized with ER-Tracker but not Mitotracker indicating the C-terminal tail of B6R is sufficient for ER localization (Figure A-1 A). Similarly, the fluorescent signal for EGFP-F1L/B6R

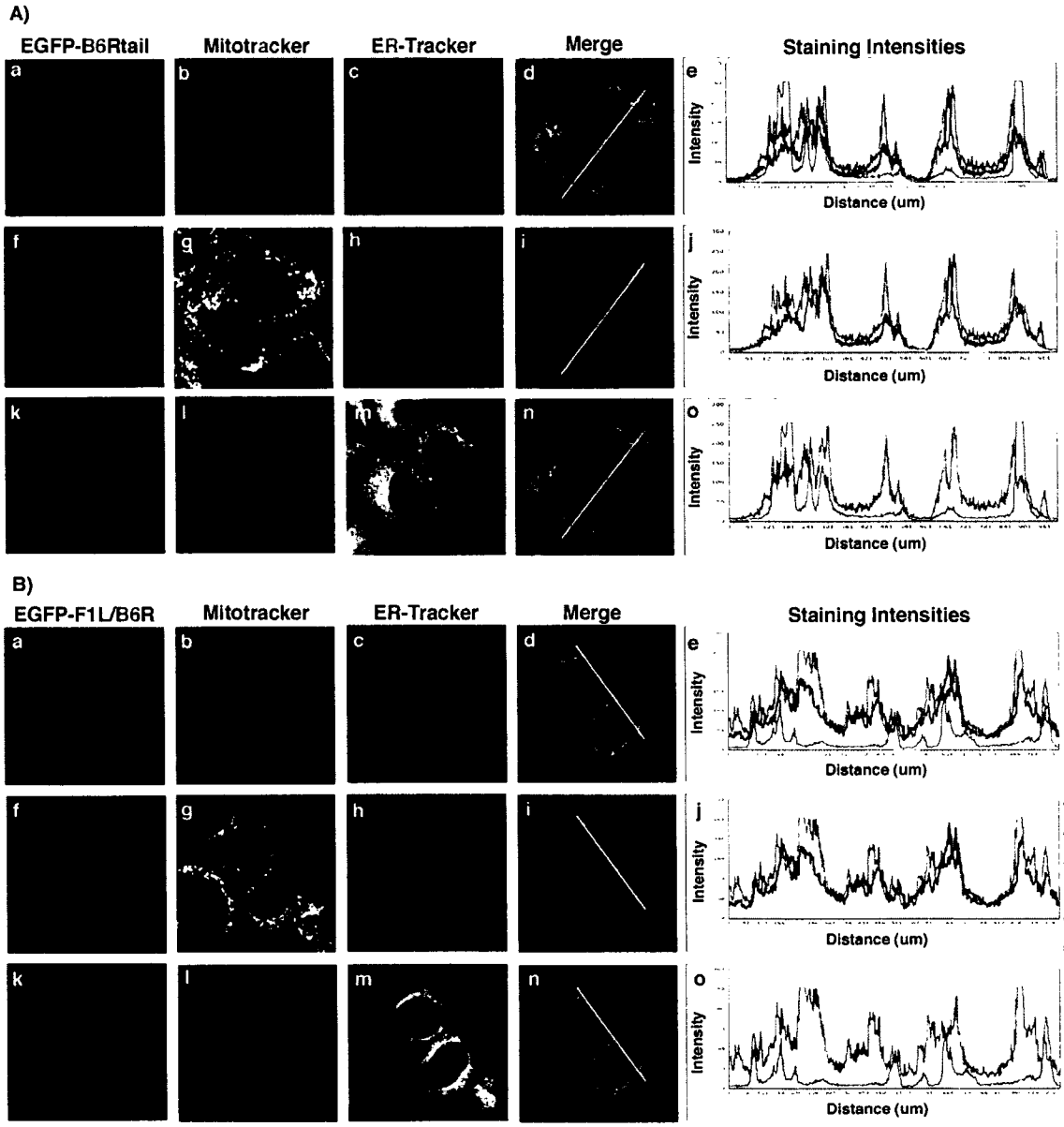

Figure A-1: EGFP-F1L/B6R localizes to the ER. HeLa cells were transfected with either pEGFP-B6Rtail (A-a,f,k) or pEGFP-F1L/B6R (B-a,f,k) and stained with Mitotracker Red (A-b,g,l and B-b,g,l) and ER-Tracker (A-c.h,m and B-c,h,m). The merge image of EGFP-B6Rtail and Mitotracker red (A-n) shows no colocalization. In contrast, the merge image of EGFP-B6Rtail and ER-Tracker (A-i) indicate colocalization, which is further supported by the merge intensity plots (A-j). Similarly, the merge image of EGFP-F1L/B6R and Mitotracker Red (B-n) shows no colocalization however, the merge image with ER-Tracker (B-i) indicates colocalization, which is further supported by the merge intensity plot (B-j). White images represent color channels that have been turned off.

displayed a web-like staining pattern throughout the cell that colocalized with ER-Tracker but not Mitotracker indicating EGFP-F1L/B6R localized exclusively to the ER (Figure A-1 B). This observation was supported by the complete lack of co-localization when the merge intensities for EGFP-F1L/B6R and Mitotracker were plotted (Figure A-1 B o). The ER localization of EGFP-F1L/B6R was also confirmed by staining HeLa cells with an antibody that recognizes calnexin (Figure A-2 A)

To determine if EGFP-F1L/B6R expression inhibited apoptosis, HeLa cells were transfected with pEGFP-F1L/B6R and treated with TNFα. Our data demonstrated a loss in the ability of this construct to inhibit apoptosis with approximately 17% of cells showing a loss of the inner mitochondrial membrane potential (Figure A-2 B). This data suggests that localization of F1L to mitochondria is necessary for efficient inhibition of apoptosis

A.2 F1LActA Chimera

To map the location of the anti-apoptotic domain of F1L, HeLa cells were transfected with the two truncated versions of F1L, EGFP-F1LHTR(1-218) and EGFP-F1LTR(1-206) and with the two C-terminal tail constructs of F1L, EGFP-F1Ltail(+) and EGFP-F1Ltail(-) and treated with TNFα. However, neither the truncated or tail constructs of F1L were able to inhibit apoptosis suggesting F1L needs to be at the mitochondria to inhibit apoptosis and that the expression of the C-terminal tail was insufficient for this function. To further determine the role that the C-terminal tail of F1L played at inhibiting apoptosis, an F1L chimera was generated that had the C-terminal tail of F1L

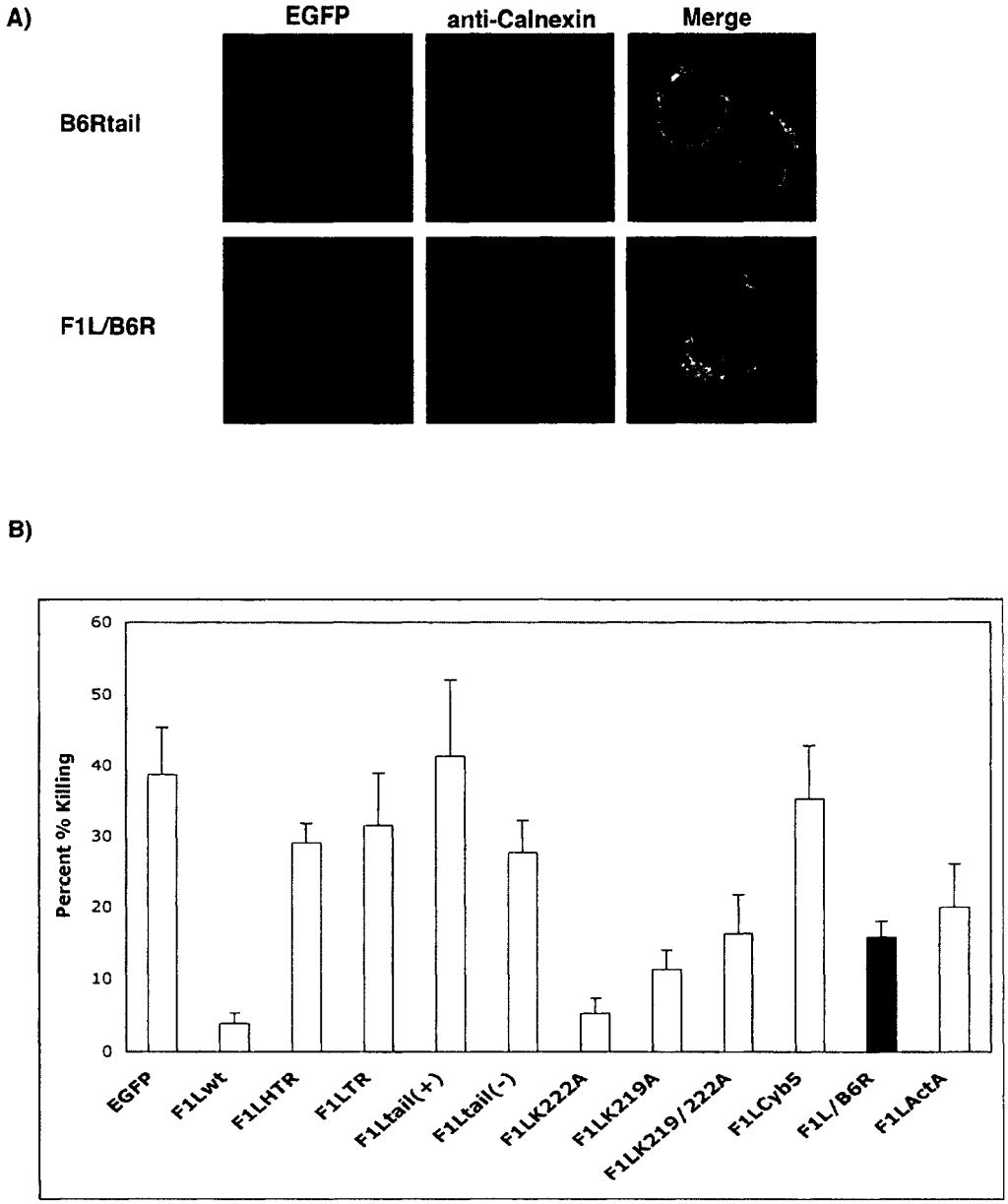

Figure A-2: EGFP-F1L/B6R colocalizes with calnexin and demonstrates partial inhibiton of apoptosis. (A) HeLa cells were transfected with either pEGFP-B6Rtail or pEGFP-F1L/B6R and stained with anti-calnexin, an ER resident protein, to detect the ER. Merge images (c and f) indicate that EGFP-B6Rtail and EGFP-F1L/B6R colocalize with calnexin. **(B)** HeLa cell were transiently transfected with pEGFP-F1L/B6R for 16 hours and treated with 10ng/mL of TNFa for 8 hour. Loss of the inner mitochondrial membrane potential was assessed by TMRE flourescence in EGFP positive cells by two-color flow cytometry. Standard deviations are calculated from three or more independent experiments. Grey bars are from Stewart *et al.* 2004.

(amino acids 207-226) swapped with the mitochondrial targeting polypeptide from *Listeria monocytogenes* ActA protein (5).

A.2.1 Methods for creating F1LActA

Amplification of the coding sequence for pEGFP-F1LActA was performed using a combination of three PCR reactions. In the first PCR reaction, pEGFP-F1Lwt was used as the template with the forward primer *Eco* RI-(GGATCCTCATGTTGTCGATG TTTATG) and the reverse primer 5'-(CAGCATGGCGAGAATCAGCTTTAGATA TTCACGCGT) resulting in a PCR product encoding for the first 206 amino acids of F1L with an overhang of amino acids that corresponded to the beginning of the C-terminal targeting motif of ActA. In the second PCR reaction, pSPUTK-Bcl-2ActA was used as the template with forward primer 5'-(CTGAATCTCGCCATGCTGGCA) and the reverse primer *Bam* HI-(GGATCCTTAATTGTTCTTCCGGAGCTG) resulting in a PCR product that encodes the C-terminal mitochondrial targeting motif of ActA. In the third PCR reaction, the products from the first two PCR reactions were ligated together with primers *Eco* RI-(GGATCCTCATGTTGTCGATGTTTATG) and *Bam* HI-(GGATCCTTAA TTG TTCTTCCGGAGCTG) resulting in a PCR product that encoded for the N-terminal portion of F1L (amino acids 1-206) and the C-terminal tail ActA PCR amplified F1LActA was cloned into the TA cloning vector pGEMT (Promega), verified by DNA sequence analysis, and subcloned into pEGFP-C3 (Clonetech).

A.2.2 F1LActA localizes to mitochondria and demonstrates partial inhibition of apoptosis

To test the localization of F1LActA, we transfected HeLa cells with pEGFP-F1LActA, stained the cells with Mitotracker Red or ER-Tracker, and visualized localization by confocal microscopy. The fluorescent signal of EGFP-F1LActA demonstrated a mitochondrial-like staining pattern throughout the cell that colocalized with Mitotracker but not ER-Tracker indicating that EGFP-F1LActA localizes to mitochondria (Figure A-3 A). This observation was supported by the lack of co-localization with the merge intensities for EGFP-F1LActA and ER-Tracker but with a perfect mirroring of the EGFP-ActA and Mitotracker merge intensity (Figure A-3 d and h).

To determine if EGFP-F1LActA expression could inhibit apoptosis, HeLa cells were transfected with pEGFP-F1LActA and treated with TNFα. Our results demonstrated that approximately 20% of transfected cells underwent apoptosis (Figure A-3 B). However, the percent of apoptosis demonstrated by EGFP-F1LActA was never as high as cells transfected with EGFP alone. This could be due to lower protein expression levels as demonstrated by western blot analysis or that the C-terminal tail of F1L plays a role in inhibition of apoptosis (Figure A-3 C).

A.3 Localization of various F1L constructs during virus infection

One concern regarding intracellular localization studies of viral proteins in the absence of virus infection is they may localize differently during virus infection. I therefore, subcloned all EGFP-F1L constructs into the vaccinia virus expression vector

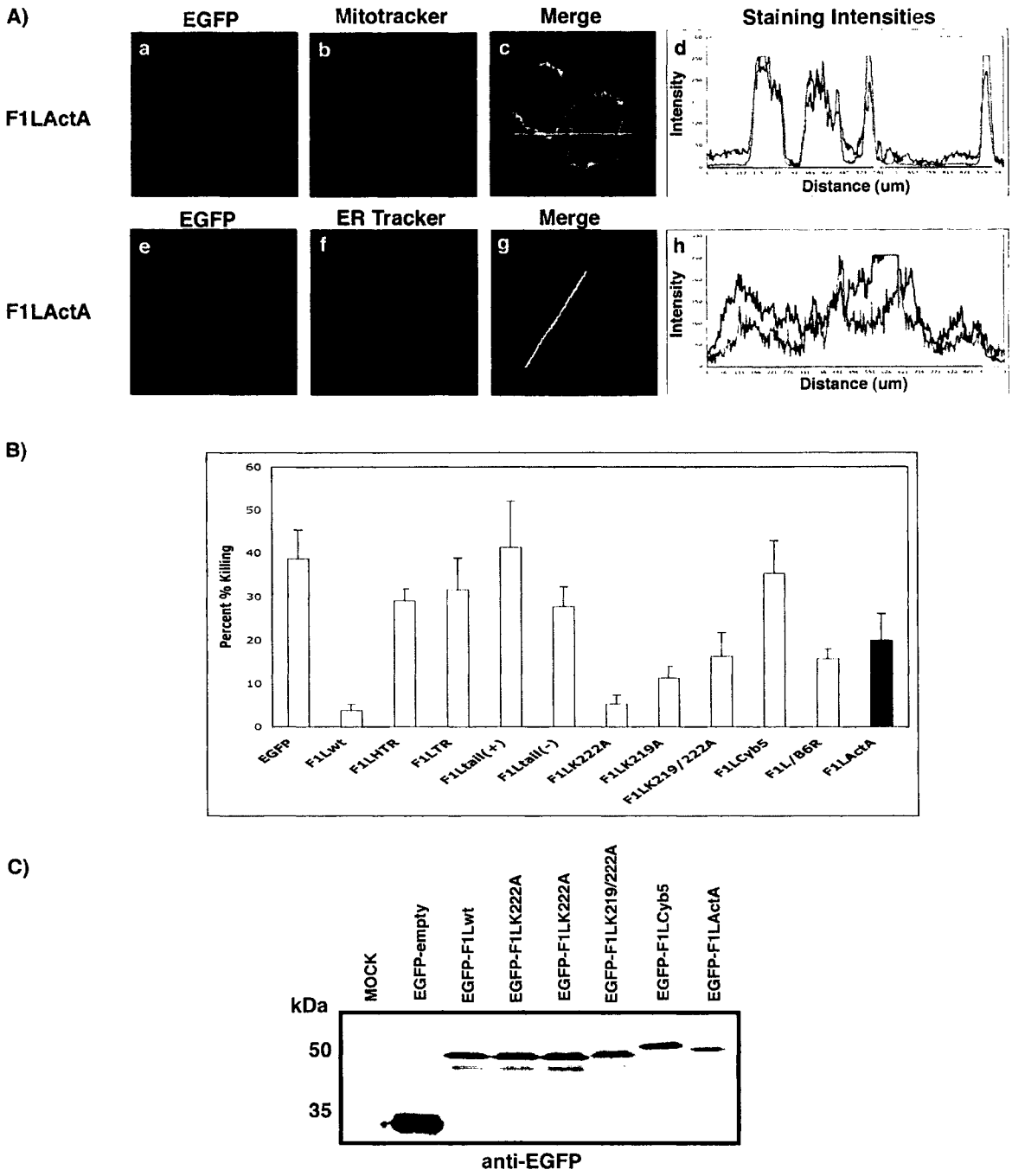

Figure A-3: F1LActA localizes to mitochondria and shows reduced inhibition of apoptosis. (A) HeLa cells were transfected with pEGFP-F1LActA (a and e) and stained Mitotracker Red (b) or ER-Tracker (f). Merge images and intensity plots (c,d,g,h) indicate that EGFP-F1LActA localizes to mitochondria but not ER. **(B)** HeLa cells were transfected with pEGFP-F1LActA for 16 hours and treated with 10ng/mL of TNFa for 8 hours. Loss of the inner mitochondrial membrane potential was assessed by TMRE flourescence in EGFP postive cells by two-color flow cytometry. Standard deviations are calculated from three or more independent experiments. Grey bars are from Stewart *et al.* 2004. **(C)** Expression levels of various F1L constructs. HeLa cells were transfected and protein levels were determined by western blotting using anti-EGFP.

pSC65, which is under a synthetic poxvirus early/late promotor, and repeated all confocal studies performing infection/transfection studies.

A.3.1 Method for Infection/Transfection

To determine the subcellular localization of the various EGFP-F1L constructs during virus infection, each EGFP-F1L construct was cloned into pSC65 where it is placed under the control of a synthetic poxvirus early/late promotor (2). EGFP-F1Lwt, EGFP-F1LHTR(1-218), EGFP-F1LTR(1-206), EGFP-F1Ltail(+)(206-226), EGFP-F1Ltail(-)(207-226), EGFP-F1LK219A, EGFP-F1LK222A, and EGFP-F1LK219/222A were generated by PCR using the forward primer 5'-(ATGGTGAGCAAGGGCG AGGAGCTG) specific for the N-terminus of EGFP. The reverse primers for each F1L construct were 5'*Bam* HI-(GGATCCTTATCCTATCATGTATTT), 5'-*Bam* HI-(GGATCCTTAATATGTAGCAAACATGATAGC), 5'*BamH1*-(GGATCCTTACTTTA GATATTCACGCGTGCT), 5'*Bam* HI-(GGATCCTTATCCTATCATGTATTTGAG), 5'*Bam* HI-(GGATCCTTATCCTATCATGTATTTGAGAGTTGCATATGTAGCAAAC AT), 5'*Bam* HI-(GGATCCTTATCCTATCATGTATGCGAGAGTTTTATA), and 5'*Bam* HI-(GGATCCTTATCCTATCATGTATGCGAGAGTTGCATATGTAGCAAAC AT), respectively. Each PCR reaction was performed using PWO-*Taq* DNA polymerase (Roche) to produce blunt end PCR products, verified by DNA sequencing, and blunt-end PCR products were cloned into the *Sal* I sites of pSC65 (Roche).

To determine the localization of EGFP-F1L constructs during virus infection, HeLa cells were seeded onto 18mm coverslips (Fisher Scientific) in a 12 well dish to achieve 80% confluency. Cells were infected with vaccinia virus strain Copenhagen

VV65 at an MOI of 3 and simultaneously transfected with 2μg of DNA using Lipofectamine 2000 (GIBCO Invitrogen Corp.) according to the manufactures specifications. Infection/transfections went for 8 hours after which cells were fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature and permeabilized with 0.02% NP-40 in PBS. For analysis of mitochondrial localization, cells were stained with 10μg/mL mouse anti-cytochrome c (clone 6H2.B4) (Pharmigen) followed by the addition of 10μg/mL Alexa Fluor 546 goat anti-mouse (Molecular Probes). For analysis of ER localization, cells were stained with rabbit anti-calnexin (residues 575-593) (Stressgen Biotechnologies) at a dilution of 1:400 followed by the addition of 10μg/mL Alexa Fluor 546 goat anti-rabbit (Molecular Probes). Coverslips were mounted using 50% PBS/50% glycerol containing 4mg/mL n-propyl gallate (Sigma Aldrich). Fixed cells were examined using LSM510 laser scanning confocal microscopy at 543nm to assess cytochrome c and calnexin staining and 489nm to assess EGFP fluorescence.

A.3.2 Various F1L constructs retain their specific subcellular distribution during virus infection

The generation of various F1L constructs that localize to a diverse array of subcellular compartments has allowed us to elucidate the mechanism of inhibition of apoptosis by F1L. However, since the mechanism of F1L is only relevant during virus infection, the localization of each F1L construct was confirmed during virus infection. HeLa cells were simultaneously infected with VV65 and transfected with either pSC65-EGFP, pSC65-EGFP-F1Lwt, pSC65-F1LHTR(1-218), pSC65-F1LTR(1-206), pSC65-F1Ltail(+), or pSC65-F1Ltail(-). Mitochondria were stained with anti-cytochrome c and

localization was visualized by confocal microscopy. HeLa cells infected and transfected with pSC65-EGFP demonstrated a fluorescent signal throughout the cell and did not colocalize with cytochrome c (Figure A-4 A e-h). In contrast, HeLa cells infected and transfected with pSC65-F1Lwt showed a similar staining pattern to cytochrome c. When the two images were superimposed, a yellow image was produced indicating that F1L localized to mitochondria during virus infection (Figure A-4 A i-l). Similar to the transient transfection of HeLa cells, both truncated versions of F1L, EGFP-F1LHTR(1-218) and EGFP-F1LTR(1-206) were cytoplasmic during infection and the two F1L-tail constructs, F1Ltail(+)(206-226) and F1L(-)(207-226) localized to the mitochondria (Figure A-4 A m-bb).

An observation made during the transient transfection of HeLa cells was that mutating positively charged amino acids in the C-terminal tail of F1L resulted in an altered localization pattern from mitochondria to ER. To determine if a similar localization pattern would occur during virus infection, HeLa cells were infected and transfected with pSC65-EGFP-F1LK222A, pSC65-EGFP-F1LK219A, and pSC65-EGFP-F1LK219/222A and subcellular localiztion was visualized by confocal microscopy. Similar to the transient expression of the various F1L point mutants, EGFP-F1LK222A localized to mitochondria and EGFP-F1LK219A localized to both the ER and mitochondria (Figure A-4 B a-h). Interestingly, EGFP-F1LK219/222A demonstrated its usual ER localization with subtle amounts of protein localizing to mitochondria which was never seen in the absence of virus infection (Figure A-4 B i-l). The altered localization of EGFP-F1LK219A and EGFP-F1LK219/222A to the ER was confirmed by anti-calnexin staining (Figure A-5 a-f). To determine the localization of the chimera

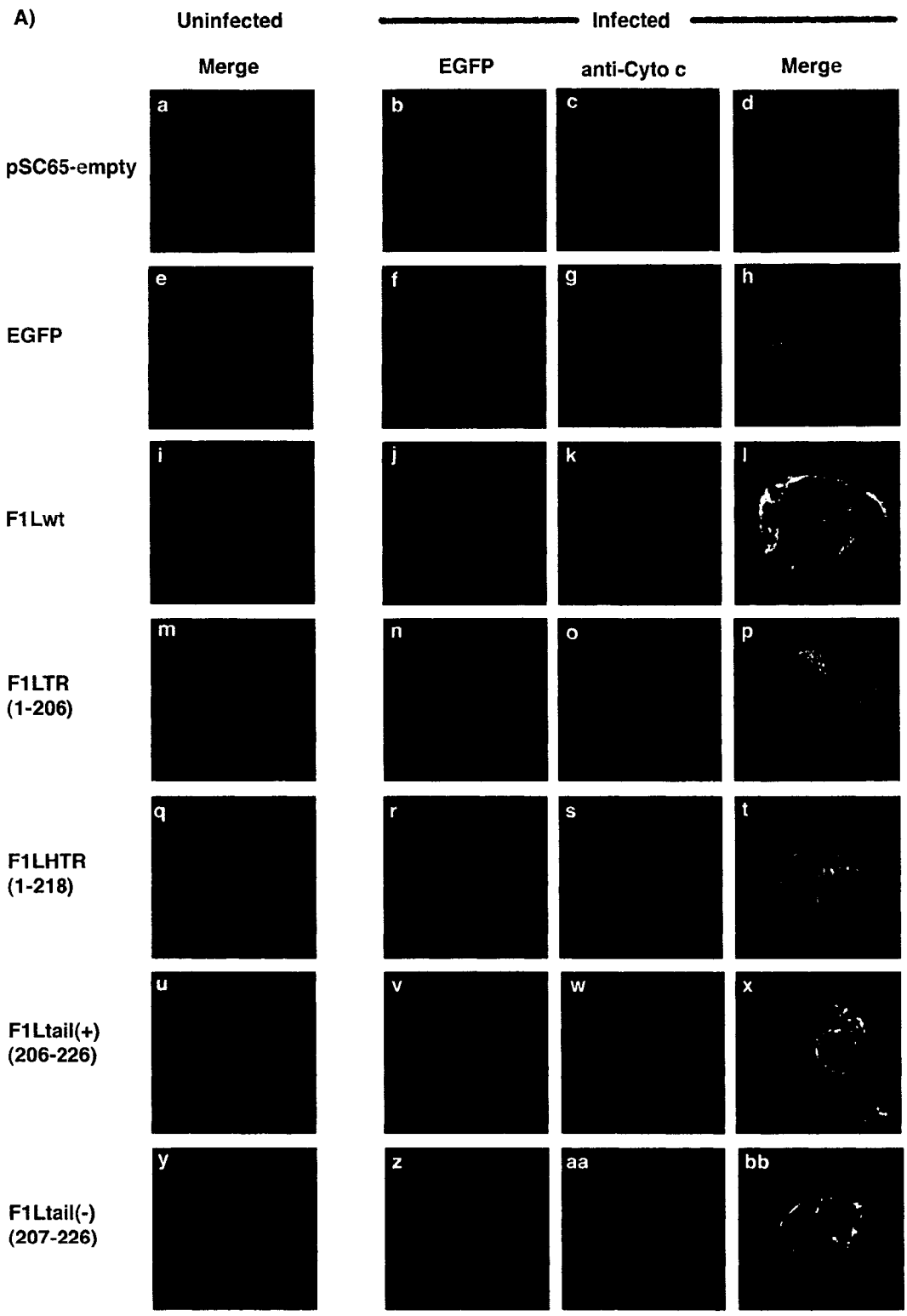

Figure A-4 (A): F1L constructs retain their specific subcellular localization during virus infection.

F1L/B6R, HeLa cells were infected with VV65 and transfected with pSC65-EGFP-F1L/B6R and visualized by confocal microscopy. Similar in the absence of virus infection, EGFP-F1L/B6R demonstrated no localization to mitochondria but colocalized with anti-calnexin (Figure A-4 B m-p, Figure A-5 g-i).

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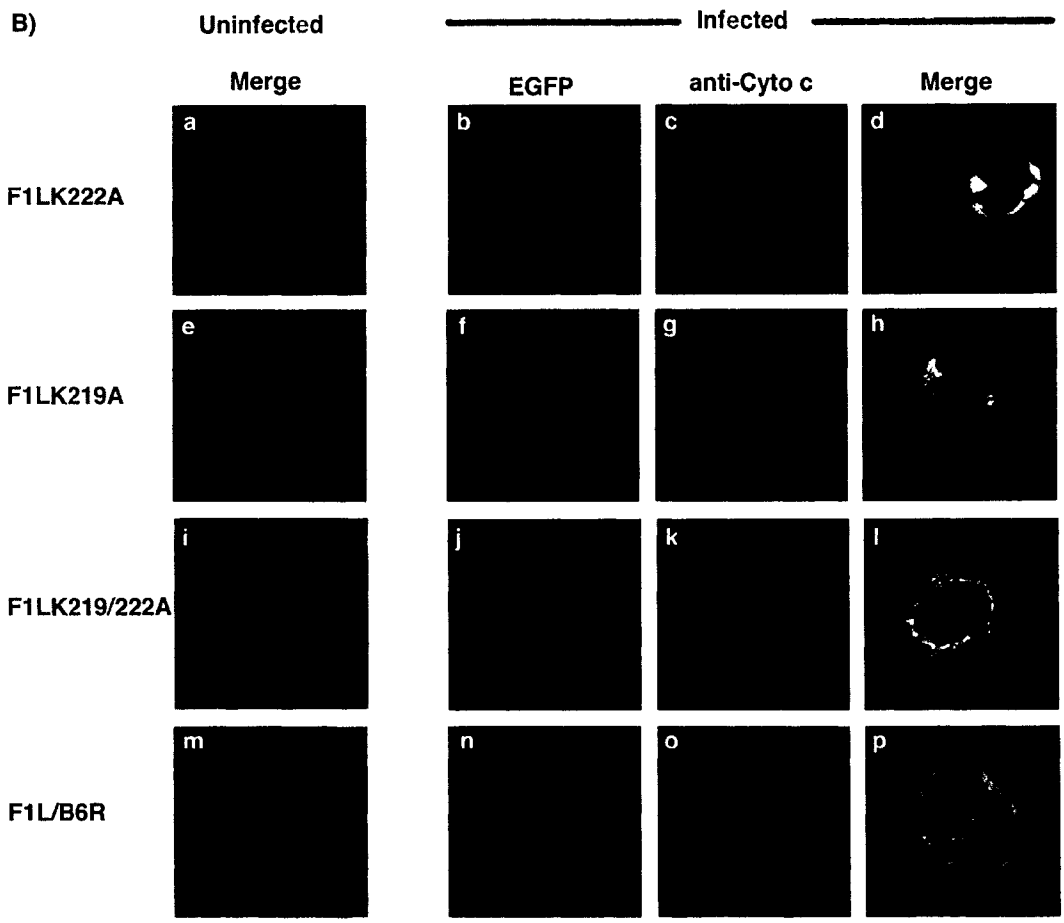

Figure A-4: F1L constructs retain their specific subscellular localization during virus infection. (B) HeLa cells were transiently transfected with pSC65-EGFP-F1LK222A, pSC65-EGFP-F1L K219A, pSC65-EGFP-F1LK219/222A, and pSC65-EGFP-F1L/B6R and were either uninfected (a,e,i,m) or infected with VV65. Mitochondria were detected using anti-cytochrome c (red) (c,g,k,o) and localization was determined by confocal microscopy. The merge image of EGFP-F1LK222A and cytochrome c (d) shows a uniform yellow image indicating mitochondrial localization during virus infection. The merge image of EGFP-F1LK219A (h) demonstrates mitochondrial localization but in additon demonstrates a web-like staining distribution throughout the cell. The merge image of EGFP-F1LK219/ 222A demonstrates a staining pattern characterisitic of ER and displays partial localization to mitochondria. The merge image of EGFP-F1L/B6R (p) also demonstrates a staining pattern similar to ER and displays no colocalization with cytochrome c.

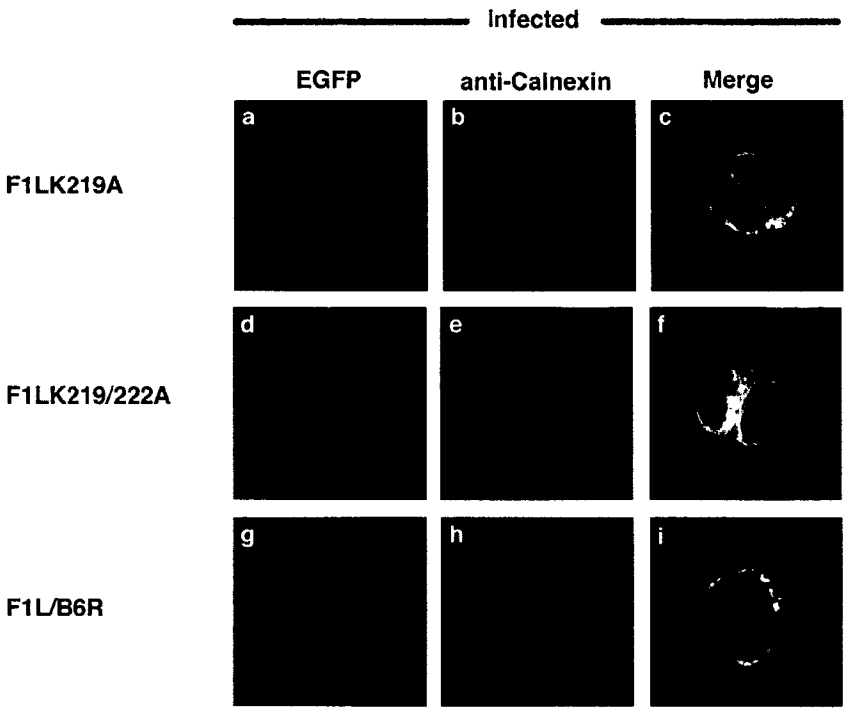

Figure A-5: EGFP-F1LK219A, EGFP-F1LK219/222A, and EGFP-F1L/B6R colocalize with calnexin during virus infection. Hela cells were transfected with either pSC65-EGFP-F1L-K219A, pSC65-EGFP-F1LK219/222A, or pSC65-EGFP-F1L/B6R and infected with VV65. ER was detected using anti-calnexin (red), and ER resident protein (b,e,h). The Merge image (c) indicates that EGFP-F1LK219A only partially localizes to ER. The merge image of EGFP-F1L-K219/222A and calnexin (f) indicates EGFP-F1L-K219/222A colocalizes with calnexin during virus infection. The merge image of EGFP-F1L/B6R and calnexin (i) demonstrates colocalization indicating that EGFP-F1L/B6R localizes to ER during virus infection.

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