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Localization and Binding Characteristics of Kaposi's sarcoma-associated herpesvirus Bcl-2 Protein in the Prevention of Apoptosis

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Submitted in partial fulfillment of the requirements for the
Degree of Master of Science in Biology from the
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Abstract

Kaposi's sarcoma-associated herpesvirus (KSHV) is the causative agent of various diseases, and it encodes a Bcl-2 homolog, KS-Bcl-2. The Bcl-2 family is important in regulating cellular apoptosis. Pro-apoptotic Bcl-2 family members promote apoptosis through mitochondrial pore formation by Bak and Bax, or through BH3 only domain activators and sensitizers. Anti-apoptotic members, such as cellular Bcl-2 prevent apoptosis through interactions with pro-apoptotic proteins, for instance inhibiting pore formation by binding to Bak and/or Bax. Even though, KS-Bcl-2 has been shown to have an anti-apoptotic function like its homolog, it does not bind to either Bak or Bax, and little is known about the mechanism behind KS-Bcl-2's function. A second mitochondrial apoptotic pore is the mPTP, which is made up of ANT and VDAC. Bcl-2 and Bcl-xL have been shown to prevent apoptosis through interactions with these proteins. In order to further investigate the mechanism behind KS-Bcl-2's anti-apoptotic function the localization and binding to the mPTP needed to be determined. KS-Bcl-2 was transfected into Vero cells and then localization was observed under normal and apoptotic conditions. Under normal conditions KS-Bcl-2 localized to the mitochondria, cytoplasm, and nucleus and during apoptosis primarily the mitochondria. Furthermore, to evaluate KS-Bcl-2's interaction with mPTP proteins a GST-pull down assay was performed using isolated GST-KS-Bcl-2 and mitochondrial proteins, and then evaluated for VDAC. It was observed that VDAC and KS-Bcl-2 do interact, and that this

interaction could potentially be the mechanism behind KS-Bcl-2's anti-apoptotic function. Further studies are needed to determine the functionality of this interaction

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), is a gamma herpesvirus first detected in 1994 by Chang *et al.*, in Kaposi's sarcoma tissue of an AIDS patient (Chang *et al.*, 1994). KSHV is a large DNA virus encoding approximately ninety genes (Russo *et al.*, 1996, Taylor *et al.*, 2005) and shares sequence homology with two other gamma herpesviruses: Epstein-Barr virus (EBV) and herpes virus saimiri (HVS) (Ceaserman *et al.*, 1995, Chang *et al.*, 1994, Russo *et al.*, 1996). KSHV has been identified to be a known infectious agent important for development of Kaposi's sarcoma (KS) as well as other lymphoproliferative disorders such as primary effusion lymphomas and multicentric Castleman's disease (Boshoff *et al.*, 2001, Ceaserman *et al.*, 1995, 1996, Chang *et al.*, 1994, Fernandez *et al.*, 1999, Russo *et al.*, 1996, Sarid *et al.*, 1999, Sarid *et al.*, 2002).

Since its discovery in 1994, KSHV has been found in all forms of Kaposi's sarcoma: classical, endemic-African, iatrogenic, and epidemic-AIDS associated (Ambroziak *et al.*, 1995, Boshoff *et al.*, 1995, Huang *et al.*, 1995). The oldest and least aggressive type, classical KS, predominately affects older men of Mediterranean and Eastern European decent. Classical KS can be characterized by cutaneous lesions, or raised red/purple blotches typically found on the skin of the lower extremities. The second type, endemic-African KS, is more aggressive than classic KS and involves the lymph nodes in addition to the skin of patients (Buonaguro *et al.*, 2003). This type of KS originally predominately affected HIV-negative men, but as time went on the ratio of affected men to women has declined (Wahman *et al.*, 1991). African KS can even be

found in children (Ziegler *et al.*, 1996). African Kaposi's sarcoma will attack the lymphatic system of all ages, and becomes fatal, mainly resulting in death, after a year of onset (Wahman *et al.*, 1991, Iscovich *et al.*, 2000). Iatrogenic KS occurs in individuals who have received solid-organ transplants, and similar to classical KS, occurs at a higher frequency in men of Mediterranean decent (Harwood *et al.*, 1979, Buonaguro *et al.*, 2003). The last and most aggressive form of Kaposi's sarcoma, epidemic-AIDS associated KS, not only involves the skin and lymph nodes, but often disseminates to the lung, gastrointestinal tract, liver, and spleen. Unlike classical KS, which may take decades to present symptoms in a patient, epidemic KS can present in as little as two years (Cheng *et al.*, 1996).

In addition to Kaposi's sarcoma, KSHV can be found in other disorders, most commonly primary effusion lymphomas (PEL) and multicentric Castleman's disease (MCD). PELs are rare non-Hodgkin's lymphomas of B-cell origin, in which tumor cells contain the DNA sequence of KSHV. Generally, this disease has been found in homosexual and bisexual men with HIV infection, but in rare instances has been linked to HIV-negative individuals (Ceaserman *et al.*, 1995). Distinguishing characteristics of PELs include: proliferation in body cavities, large-cell morphology bridging immunoblastic and anaplastic lymphomas (Ablashi *et al.*, 2002), and B-cell genotype (Ablashi *et al.*, 2002, Ceaserman *et al.*, 1995). In addition to primary effusion lymphomas, KSHV sequences have been found in multicentric Castleman's disease. MCD is an atypical lymphoproliferative disorder. Castleman's disease is usually described as a polyclonal, non-neoplastic disorder, and is thought to be related to immune

deregulation. It presents in two distinct subtypes, the hyaline vascular type, which can be identified as surgically curable solitary mass, and the plasma cell type, which is associated with more generalized lymphadenopathy and immunological abnormalities. A characteristic of MCD is its close association with Kaposi's sarcoma and non-Hodgkin's lymphoma. Sequences of KSHV have been isolated from almost all cases of HIV-associated MCD. Moreover, KSHV was detected in MCD cases in HIV-negative patients (Soulier *et al.*, 1995). These results suggest that KSHV is an infectious agent responsible for the development of not only KS, but also PEL and MCD.

Latency vs. Lytic Gene Replication

Many herpesvirus genes can be classified into four distinct categories based on time of expression and whether expression occurs prior to viral protein synthesis or DNA replication. These categories include latent, immediate-early (IE), early (E), and late (L) genes. The later three gene classifications make up the lytic cycle, while the first denotes the latent cycle or latency. Latency can be defined as a persistent viral infection where virus production ceases. Gamma herpesviruses characteristically establish latent infection in lymphoid cells causing the cells to become immortalized (Miller *et al.*, 1997). Two latent viruses, EBV and HVS, immortalize lymphocytes, causing them to grow continuously *in vitro* (Miller *et al.*, 1997, Schrim *et al.*, 1984, Szomolangi *et al.*, 1987). EBV usually immortalizes B cells, and HVS immortalizes T cells (Biesinger *et al.*, 1992, Wright *et al.*, 1976). Even though KSHV codes for ninety genes, viral gene expression decreases dramatically in cells following initial infection (Renne *et al.*, 2001). This drop

in expressed genes suggests that most infected cells in a KS lesion as well as lymphoproliferative disorders are latently infected (Davis *et al.*, 1997).

Of the ninety genes KSHV encodes for, less than 10% of these genes are expressed during latency, which is quickly established after primary infection (Dupin *et al.*, 1999). Latency genes include LANA, v-FLIP, v-Cyclin, and a group of short membrane-associated proteins named Kaposin A, B, and C (Dittmer *et al.*, 1998, Sarid *et al.*, 1998). In KSHV, similar to EBV, these genes are expressed latently in B cells as well as endothelial cells (Dittmer *et al.*, 1998, Li *et al.*, 2002, Muralidhar *et al.*, 1998, Sadler *et al.*, 1999). Eventually, the virus will switch from latency to lytic gene replication. The lytic cycle can be characterized by the production of linear viral genomes and the construction of viral progeny, ultimately resulting in cell lysis.

It has yet to be determined what factors will induce lytic gene expression in KSHV *in vivo*. However, with the use of chemical inhibitors phosphonoacetic acid and cycloheximide, the life cycle of lytic infection has been characterized. This cycle can be broken down into three distinct expression groups; immediate-early (IE), early (E), and late (L). Immediate early genes are expressed soon after infection from about 2 to 4 hours and do not require *de novo* viral protein synthesis (Lacoste *et al.*, 2004). They are needed to evade cellular response to the viral infection and their proteins are transcription factors that induce of subsequent viral gene expression. Immediate early gene's mRNA will be resistant *in vitro* to the action of cycloheximide, which is an inhibitor of protein synthesis (Sun *et al.*, 1998). Early genes will be detectable at about 8 to 13 hours after induction of lytic replication and these proteins will replicate viral nucleic acids (Sun *et*

al., 1998). These genes are defined as lytic cycle transcripts whose expression will be inhibited by cycloheximide, but not blocked by phosphonoacetic acid, an inhibitor of the viral DNA polymerase (Sun *et al.*, 1998). Lastly, late genes will be expressed at 20 plus hours following induction and mainly code for proteins involved in structure as well as virus maturation. The transcription of these genes will be inhibited by phosphonoacetic acid. Once all three categories of genes have been expressed and viral proteins synthesized, they are assembled into complete viruses. The virus can now lyse the cell and the new viral progeny are free to infect other cells (Orenstein *et al.*, 1997).

Apoptosis

The proliferation of various diseases, including those previously discussed, results from the regulation, and in most cases the loss of apoptosis. The term apoptosis is often used synonymously with programmed cell death to describe a mechanism of cell destruction essential for embryonic development and maintenance of homeostasis in multi-cellular organisms (Danial *et al.*, 2004). Two classical apoptotic signaling pathways exist, extrinsic and intrinsic, which are both depicted in Figure 1. The extrinsic, or receptor mediated pathway, is activated through binding of ligands to cell surface death receptors. Upon ligand binding, adaptor proteins and pro-caspases, like caspase 8 or 10, are recruited to the receptor complex (Danial *et al.*, 2004). The caspases become activated in the complex and further amplify the apoptotic signaling cascade through activation of downstream effectors , such as caspase 3 (Danial *et al.*, 2004, Kinnally *et al.*, 2007, Pearce *et al.*, 2009, Scorrano *et al.*, 2003). Caspase 3 is an executioner of apoptosis and its activation will lead to morphological changes associated

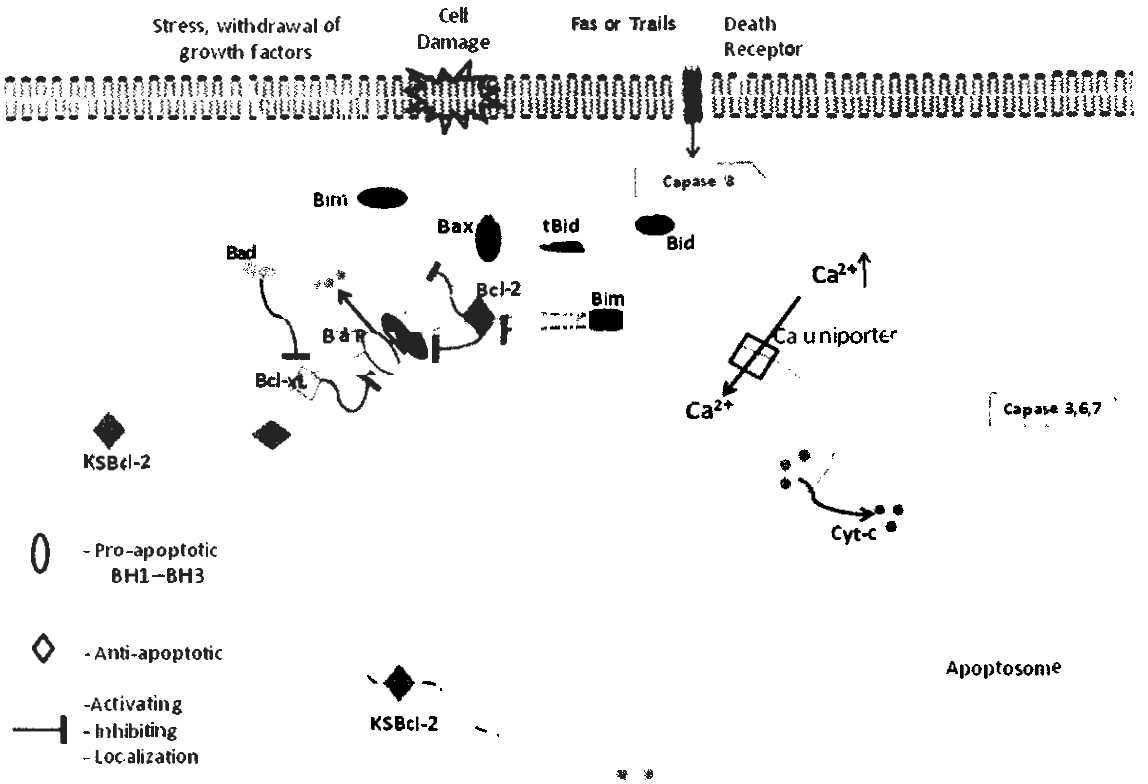

Figure 1: Apoptosis pathway. Apoptotic cell death can be initiated through two main pathways: extrinsic and intrinsic. The extrinsic pathway is activated by FAS or TRAIL binding to their receptors and stimulating receptor aggregation. This stimulates recruitment of FADD and caspase 8 cleavage and its subsequent activation. Activation of caspase 8 leads to caspase 3 cleavage, which initiates multiple pro-apoptotic processes, including DNA cleavage. The Bcl-2 family of proteins regulates the intrinsic pathway by controlling mitochondrial permeability. Anti-apoptotic proteins Bcl-2 and Bcl-xL reside on the mitochondria and inhibit cytochrome c release, while Bak, also on the mitochondria, promotes apoptosis. The pro-apoptotic Bcl-2 proteins Bad, Bid, Bax and Bim translocate to mitochondria following a death signal, where they promote the release of cytochrome c. Cytosolic Bid is cleaved by activated caspase 8 to form its active fragment, tBid, which can then translocate to the mitochondria. Bax and Bim translocate to mitochondria in response to death stimuli, including survival factor withdrawal. Bax will form a pore with Bak resulting in the release of cytochrome c. This pore can be inhibited by Bcl-2 and Bcl-xL. Bim, when sequestered to the mitochondria, can inhibit Bcl-2. Another channel involved in mitochondria permeability is the mPTP comprised of ANT and VDAC. ANT is found on the inner mitochondrial membrane and VDAC on the outer. For this pore to form and release cytochrome c VDAC and ANT must come together. Bcl-xL can inhibit VDAC from opening and Bcl-2 inhibits ANT. However, Bax can also bind to VDAC promoting the release of pro-apoptotic factors. Upon release from mitochondria, cytochrome c binds to Apaf-1 and forms an active complex with pro-caspase 9, the apoptosome, which will stimulate apoptosis through caspase 3 cleavage.

with apoptosis, including DNA degradation, chromatin condensation, and membrane blebbing (Danial *et al.*, 2004). The activation of these downstream effectors ultimately results in cell death. Also, in addition to the activation of downstream effectors of the extrinsic pathway, caspase 8 will activate the pro-apoptotic Bid and allow for activation of the mitochondrial pathway (Danial *et al.*, 2004).

The intrinsic, or mitochondrial pathway, is a complex pathway with the mitochondria at the center. Key players in mitochondrial apoptosis are members of the Bcl-2 protein family. Cellular Bcl-2 was first identified at the t (14; 18) chromosomal breakpoint of follicular B cell lymphomas (Cleary *et al.*, 1986, Tsujimoto *et al.*, 1987). It is a powerful cell death-suppressor (Reed *et al.*, 1994) and represents a proto-oncogene that extends cell survival by inhibiting apoptosis as opposed to promoting cell proliferation (Korsmeyer *et al.*, 1992). Bcl-2 family proteins function to either repress or promote apoptosis. To characterize a protein as a member of the Bcl-2 family it must contain at least, but not limited to, one of the four Bcl-2 Homology (BH) domains: BH1, BH2, BH3, and/or BH4 (Danial *et al.*, 2004). Bcl-2 family members can be divided into three groups depending on the conservation of these BH domains and also their function. The first group consists of anti-apoptotic Bcl-2 proteins, which contain domains BHI – BH4 (Cheng *et al.*, 1996). Members from this group include c-Bcl-2, Bcl-xL, Mel-I, Bcl-w (Danial *et al.*, 2004), and KS-Bcl-2 (Cheng *et al.*, 1996). Pro-apoptotic proteins make up the next two groups, multi-domain and BH3 only proteins. The multi-domain, pro-apoptotic proteins contain domains BHl – BH3, and include Bak and Bax (Danial *et*

al., 2004). Members of the last group, BH3 only proteins contain only one domain, the BH3 domain, and include Bid, Bim, Bad, Bmf, Noxa, Puma, and Bik (Akgul *et al.*, 2000, Flanagan *et al.*, 2008, Pearce *et al.*, 2009, Scorrano *et al.*, 2003 Uren *et al.*, 2007,).

The BH3 only group of proteins can be further subdivided into two separate groups, either sensitizers or activators depending on whether they can induce apoptosis on their own (Letai *et al.*, 2002). Bid, Bad, Bmf, Noxa, Puma, and Bik are considered sensitizer proteins as they cannot induce apoptosis alone, but they still exhibit a pro-death function by occupying the inhibitory pocket of anti-apoptotic BCL-2 family members, thus displacing BH3 activators (Letai *et al.*, 2002). Sensitizers therefore cause apoptosis by inhibiting the function of anti-apoptotic cellular proteins like Bcl-2 or Mcl-1 (Flanagan *et al.*, 2008, Pearce *et al.*, 2009, Scorrano *et al.*, 2003 Uren *et al.*, 2007,). Bid, once modified to truncated Bid (tBid) and also Bim, another BH3 only protein, make up the activator proteins (Flanagan *et al.*, 2008, Letai *et al.*, 2002). These proteins will induce Bax and Bak oligomerization resulting in mitochondrial outer membrane permeabilization, release of cytochrome c and commitment to programmed cell death (Flanagan *et al.*, 2008).

Within the intrinsic pathway lie several sub-pathways that can all play an individual or mutual role in apoptosis. One important pathway involves the multi-domain pro-apoptotic Bcl-2 proteins, Bax and Bak (Antignai *et al.*, 2006, Kinnally *et al.*, 2007, Uren *et al.*, 2007, Scorrano *et al.*, 2003). The activation of this pathway remains unclear, but can occur via withdrawal of growth factors, stress, cell damage and even activation of caspase 8 via the extrinsic pathway. These factors will activate upstream

signaling molecules, such as the sensitizer protein Bad, seen in Figure 1 as a green oval, the conversion of Bid to tBid, or even cause the translocation of Bax or Bim to the mitochondria. Once translocated to the mitochondria Bax, a red oval in Figure 1, will activate and heterodimerize with Bak. Once activated, Bax and Bak form a pore in the mitochondria leading to the release of pro-apoptotic factors from the mitochondria, like cytochrome c, which will lead to the activation of capase 9 and SMAC/Diablo (Kinnally *et al.*, 2007, Scorrano *et al.*, 2003, Uren *et al.*, 2007). Another sub-pathway involved in intrinsic apoptosis is the opening of the mitochondrial permeability transition pore complex (mPTPC).

Molecular composition of the mPTPC has not been fully established, but the main components consist of an inner membrane protein, adenine nucleotide translocator, ANT (two blue ovals in Figure 1), and an outer membrane protein, voltage-dependent anion channel, VDAC (two green squares in Figure 1) (Desagher *et al.*, 200, Crompton 1999, Halestrap 2003, Zoratti *et al.*, 1995). Other proteins have been proposed as regulatory components of the permeability-transition pore based primarily on their association with either the ANT or the VDAC. These proteins include the intermembrane-space protein creatine kinase, cytosolic hexokinase, and matrix cyclophilin D (Crompton *et al.*, 1999, Brdiczka *et al.*, 1998). ANT and VDAC need to associate in order to form this permeability pore. Without this association there will be no pore and homeostasis will be maintained in the mitochondria. Opening of the PTPC can be triggered by several physiological effectors such as an increase in cellular Ca+, reactive oxygen species, changes in pH, and Bax (Moriishi *et al.*, 1999). PTPC opening causes a sudden increase

in the permeability of the inner mitochondrial membrane resulting in the immediate dissipation of the proton-dependent mitochondrial membrane potential and chemical equilibration between the cytoplasm and mitochondrial matrix (Crompton *et al.*, 1999). The increase in inner membrane permeability causes osmotic swelling of the matrix and can ultimately lead to disruption of the outer membrane and release of apoptotic factors. The opening of these pores, together or alone, will result in the release of pro-apoptotic factors to amplify cell death.

Both of these pathways can be modulated by members of the Bcl-2 family. Anti-apoptotic proteins Bcl-2 and Bcl-xL are found on the mitochondrial membrane and will block both the Bax/Bak pore and mPTP, shown in Figure 1 via the red inhibitory lines (Antignani *et al.*, 2006, Brenner 2000, Jacotot *et al.*, 2001, Shimizu *et al.*, 2000, Tsujimoto *et al.*, 2000). In addition to blocking the apoptotic function of the Bax/Bak pore, c-Bcl-2 has been shown to block the translocation of Bax to the mitochondria ergo preventing the formation of this pore and inhibiting apoptosis (Antignani *et al.*, 2006). Also, several pro-apoptotic family members will inhibit these anti-apoptotic proteins. Bad, once activated, will block the anti-apoptotic effect of Bcl-xL (Downward 1999) and Bim, once tranlocated to the mitochondria, will block c-Bcl-2 (Letai et al 2002).

Viral Mechanisms to evade apoptosis

Over the past several decades numerous viral proteins have been reported to modulate, either positively or negatively, the host cell apoptotic response to viral infection. Modulation of apoptosis helps virally infected cells evade programmed cell death and ultimately propagate viral progeny. Many viral infections that effect apoptosis

act upon the intrinsic, mitochondrial pathway. Viral proteins can regulate mitochondrial membrane permeability by directly acting on the mitochondrial membrane, indirectly by regulating Bcl-2 family proteins, or possessing a function that will mimic cellular Bcl-2's functions. Some of these viruses include, but are not limited to, vesicular stomatitis virus, simian varicella virus, Epstein-Barr virus, human virus saimiri, and KSHV.

Vesicular stomatitis virus (VSV) is a well studied negative-strand RNA virus, and is an exceptionally potent inducer of apoptosis in a wide variety of cell types (Komaya *et al.*, 1995). VSV is capable of inducing apoptosis by activation of multiple apoptotic pathways. Several studies have shown that VSV induces apoptosis early via the mitochondrial pathway (Gadaleta *et al.*, 2005, 2002, Kopecky *et al.*, 2003). This activation does not require viral protein synthesis (Gadaleta *et al.*, 2005, 2002), but rather inhibition of host gene expression and modulation ofBcl-2 proteins by the VSV matrix protein M, which will lead to the induction of apoptosis (Gadaleta *et al.*, 2005, Kopecky *et al.*, 2003). In addition to the intrinsic pathway, M protein mutants deficient in the ability to inhibit host gene expression are effective inducers of the extrinsic apoptotic pathway (Gaddy *et al.*, 2007).

Simian varicella virus (SVV) causes varicella in primates, and mimics the infection ofvaricella zoster virus in humans (Gray *et al.*, 2004). SVV has been shown to be a potent activator of the intrinsic mitochondrial pathway. It causes the release of cytochrome c, a hallmark of the mitochondrial apoptotic pathway and also results in the activation of caspase 9 (Pugazhenthi *et al.*, 2009), which will result in cell death. Additionally, SVV will cause decreased expression of host Bcl-2 mRNA, and in turn

decreased expression of the Bcl-2 protein (Pugazhenthi et al., 2009), demonstrating loss of inhibition of the mitochondrial apoptotic pathway, which will lead to apoptosis.

Epstein-Barr virus (EBV) is another gamma herpesvirus, which shares sequence homology to KSHV. EBV's induction of apoptosis via the mitochondrial pathway can be either positively or negatively regulated by various viral proteins: BALF1 and BHRF1. BHRF1 is a Bcl-2 homologue, and prevents apoptosis early in infection and after exposure to apoptotic inducing events (Altmann *et al.*, 2005, Foghsgaard *et al.*, 1997, Henderson *et al.*, 1994, Tarodi *et al.*, 1993). In addition, BHRF1 can bind to Bim, a pro-apoptotic Bcl-2 protein, to prevent apoptosis (Desbien *et al.*, 2009). While BHRF1 clearly resembles cellular Bcl-2 in its anti-apoptotic function, BALF1 on the other hand, indirectly activates mitochondria membrane permeability by acting as an antagonist to BHRF1 (Bellows *et al.*, 2002). Besides BALF1 and BHRF1, EBV encodes for three latent membrane proteins, LMP– 1, 2A, and 2B (Camilleri *et al.*, 2000). An important effect of LMP-1 is the activation of the NFκB pathway, which will up-regulate the expression of several anti-apoptotic genes. Also, the MAP kinase and phosphatidylinositol-3-kinase (PI3k)/protein kinase B (Akt) pathways are activated by LMP-1, which will have important pro-survival effects on the cells (Izumi *et al.*, 1997). LMP-2A can mimic a cell surface receptor and prevent EBV from inducing apoptosis (Mancao *et al.*, 1997). Like LMP-1, LMP-2A can activate the PI3k/Akt pathway (Scholle *et al.*, 2000). The effects of LMP-2B on apoptosis remain unknown.

Herpesvirus saimiri (HVS), another gamma herpesvirus, will induce fatal lymphomas when experimentally infected in primates (Nava *et al.*, 1997). Alignment of

the HVS ORF 16 amino acid sequence with several other Bcl-2 family members revealed sequence homology and a high conservation of the Bcl-2 homology domains 1 and 2 (Nava *et al.*, 1997). ORF 16 of HVS does in fact bind to both Bak and Bax, pro-apoptotic Bcl-2 members, to inhibit virus-induced apoptosis (Nava *et al.*, 1997). While the BH1 and BH2 domains of ORF16 exhibit high conservation the BH3 domain does not, suggesting that this domain may not be essential for the anti-apoptotic functions of ORF16.

KSHV contains several cellular homologues including a homologue of cellular Bcl-2. KSHV Bcl-2 belongs to the anti-apoptotic Bcl-2 family, and is expressed early in the lytic replication cycle (Flanagan *et al.*, 2008, Sarid *et al.*, 1997). It shares functional homology to Mel-1, another anti-apoptotic protein (Flanagan *et al.*, 2008) and sequence homology to several cellular, anti-apoptotic Bcl-2 family members (Cheng *et al.*, 1996, Flanagan *et al.*, 2008). Like its cellular counter-parts, KS-Bcl-2 does in fact inhibit apoptosis; however it is not known how KS-Bcl-2 does so (Cheng *et al.*, 1996, Flanagan *et al.*, 2008).

KS-Bcl-2 contains all four BH domains; however, only BH1 and BH2 domains are highly conserved (Cheng *et al.*, 1996). BH1 and BH2 sequence motifs are important for cellular Bcl-2 to bind to the BH3 domain of other proteins and the death repressor effects of the anti-apoptotic proteins (Cheng *et al.*, 1996, Huang *et al.*, 2002). Bcl-2 homology domain 1 contains a signature "NWGR" sequence that is believed to be essential for the anti-apoptotic function of c-Bcl-2 and also for c-Bcl-2 to heterodimerize with other Bcl-2 protein family members, especially BH3 only members. KS-Bcl-2 does

contain this sequence thus explaining its anti-apoptotic function (Huang *et al.*, 2002). Both BH3 and BH4 domains of KS-Bcl-2 lack homology to the cellular BH3 and BH4 domains. Caspase cleavage of cellular Bcl-2 at Asp-34 separates the BH4 region producing a truncated protein, which explains c-Bcl-2 anti-apoptotic verses pro-apoptotic functions. KS-Bcl-2 does not contain this caspase cleavage site, and escapes this extra level of regulation (Huang *et al.*, 2002). This varying level of conservation in these domains may account for KS-Bcl-2 functional similarity to Mcl-1 as opposed to cellular Bcl-2 (Flanagan *et al.*, 2008).

Since, KS-Bcl-2 has shown conservation in the BH1 domain it was believed to interact with both pro-apoptotic groups of the Bcl-2 family via their BH3 domains. However, unlike its cellular counterpart, it does not interact with either Bak or Bax (Cheng *et al.*, 1996), but it does interact with the BH3 domain from sensitizer proteins Bim, Bid, Noxa, Bik, Puma and Bmf (Flanagan *et al.*, 2008). It is these interactions, especially with Noxa, that has shown KS-Bcl-2's functional homology to Mcl-1, since Mel-I also interacts with these BH3 proteins (Flanagan *et al.*, 2008). Also, KS-Bcl-2 has been shown to inhibit cytochrome c release from isolated mitochondria via its interactions with Bid as well as Bim (Flanagan *et al.*, 2008). This data suggests that KS-Bcl-2 does not inhibit apoptosis through direct interaction with the pro-apoptotic executioners, Bak and Bax, but rather via a secondary pathway.

Given that KS-Bel- 2 has been found localized to the mitochondria in addition to the cytoplasm and nucleoi (Kalt *et al.*, 2010) and also has been shown to block cytochrome c release, it is believed that KS-Bcl-2 acts through the intrinsic apoptotic

pathway to repress apoptosis. Within the mitochondrial pathway two separate pores exist (the mPTPC and Bax/Bak pore) to activate apoptosis in an independent fashion. Activation of either pore results in the permeability of the outer mitochondrial membrane and subsequent release of pro-apoptotic factors. Both pores are tightly regulated by cellular Bcl-2 family proteins. The Bax/Bak pore is comprised of Bcl-2 family proteins, and can be inhibited by several anti-apoptotic Bcl-2 protein members via their localization to the mitochondria and direct interaction with Bax/Bak resulting in inhibition of apoptosis (Antignani *et al.*, 2006). However, KS-Bcl-2 does not bind to either Bax or Bak, so KS-Bcl-2 is not believed to act through this pore (Cheng *et al.*, 1996). The second pore, mPTPC is also regulated by several cellular Bcl-2 proteins via direct interaction. Cellular Bcl-2 has been shown to bind directly to ANT (Brenner 2000, Jacotot *et al.*, 2001) and Bcl-xL to VDAC (Shimizu *et al.*, 2000, Tsujimoto *et al.*, 2000); each of these interactions prevent pore opening resulting in suppression of apoptosis. Bax has been shown to bind to ANT thereby enhancing mitochondrial membrane permeability and promoting apoptosis (Brenner *et al.*, 2000, Belzacq *et al.*, 2003). How KS-Bcl-2 regulates the mitochondrial apoptotic pathway remains unclear; however these previous findings can shed light on the localization and mechanism that KS-Bcl-2 utilizes to inhibit apoptosis. Based on the similarity the KS-Bcl-2 has to c-Bcl-2 and Bcl-xL and also its inability to inhibit apoptosis through direct interaction with pro-apoptotic executioners Bax and Bak, it is believed that KS-Bcl-2 localizes to primarily to the mitochondria during apoptosis ultimately preventing the formation of the mPTPC through interaction with either VDAC or ANT thus suppressing apoptosis.

Materials and Methods

Cell Lines and Reagents

Two different cell lines were used for these studies, Vero cells and BJAB cells. Vero cells are an African green monkey kidney cell (Shishihido et al., 1967), and BJAB a human B cell lymphoma (Menezes *et al.,* 1975). Both cell lines were grown at 37°C with 5% CO_2. The Vero cells were grown in DMEM + 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (p/s). Passage of Vero cells involved a rinse with phosphate buffered saline (PBS) and then cells were trypsinized for several minutes in the incubator. Following trypsinization, DMEM media was added to deactivate the trypsin and cells were split in accordance to cell numbers needed for the experiment. BJAB is a non-adherent cell line and is grown in RPMI 1640 with 10% FBS and 1% p/s. Since BJAB is a non-adherent cell line, trypsinization is not needed and the cells can be split by simple pipetting. To split cells, a cell count is performed to determine the cellular concentration using trypan blue stain, which will only stain living cells. Once the concentration of cells is determined enough cells are used for the experiment at hand, and a one to ten (v/v) dilution of cells to RPMI media is performed to maintain the cells for future experiments.

Antibodies

A mouse anti-HA antibody (Cell Signaling) was used for detection of the HA tagged protein, KSHV-Bcl-2 in western blot analysis. A rabbit anti-VDAC antibody (Cell Signaling) was used for the detection of the VDAC protein during western blot analysis. A rabbit anti-HA Tag, C29F4 (Cell Signaling) was used for detection of HA tagged proteins, KSHV-Bcl-2, in fluorescence imaging. A horse horseradish peroxidase

(HRP)-conjugated anti-mouse (Cell Signaling) antibody was used for detection of mouse antibodies used in western blots. A goat HRP-conjugated anti-rabbit (Cell Signaling) antibody was used for detection of rabbit antibodies used in western blots. An anti-rabbit conjugated to Alexa Fluor 488 (Invitrogen) was used for detection of rabbit antibodies using fluorescence imaging.

Plasmids

Two different plasmids were used in these experiments; phCMV2 from Genlantis and pGEX-5X-3 from GE Healthcare. Cloning of the KSHV-Bcl-2 gene into hCMV2 and pGEX vectors was carried out by Tiffany Chang and Maddalena Allegretta, respectively.

Transformation

Escherichia coli (*E. coli*) BL21 competent cells were thawed on ice and 50 μL was aliquoted to fresh microcentrifuge tubes. To the new tube, I μL of plasmid DNA was added. The tubes were then placed on ice for 30 minutes, heat shocked at 42°C for 90 seconds and then incubated on ice for an additional two minutes. To the cells, 450 μL of Luria broth (LB) was added and incubated at 37°C for one hour. The cells were then plated on an ampicillin or kanamycin LB-agar plates, depending on the plasmid, and allowed to incubate overnight at 37°C.

Glutathione S-Transferase Expression and Purification

Escherichia coli (*E.coli*) strain BL21 cells were transformed with pGEX-KS-Bcl-2 to express the GST-KS-Bcl-2 fusion protein and pGEX vector to express only GST. Colonies were picked and grown overnight in five mL LB and 50 ug/mL ampicillin at

37°C. After overnight growth two mL of each culture were aliquoted into 95 mL LB containing 50 ug/mL ampicillin and 0.5% glucose and cells were incubated at 37°C with shaking for several hours. In order to determine cell growth, optical densities (OD) of the cell cultures were taken at 600 nm every hour. Once the cell concentration reached an OD of roughly 0.5, the cells have reached the exponential growth phase and were induced with 0.5 mM IPTG and allowed to incubate for two hours at 37°C

After incubation, 100 mL of each culture were centrifuged for ten minutes at 10,000 rpm and 4°C and the supernatant was discarded. The pellet was resuspended in ten mL of lysis buffer (20 mM HEPES, 250 mM NaCl, 1 mM EDTA, and 0.1% NP-40) and transferred to a 15 mL conical tube for lysis. The cells were freeze-thawed by incubating on dry ice and ethanol for five minutes followed by five minutes at 37°C. The cells were then sonicated twice for fifteen seconds followed by ten seconds rest on wet ice in between and then freeze-thawed again three times with vortexing between each cycle. The lysate was centrifuged for ten minutes at 10,000 rpm and 4°C and the supernatant was transferred to a fresh 15 mL conical tube. Next, 250 μL of glutathione sepharose beads (Sigma) at a 30% (w/v) slurry were added to the supernatant and rotated for four hours at 4°C. The solution was centrifuged for five minutes at 2,000 rpm and the supernatant removed. The beads were washed three times for five minutes at 4°C in 20 mM HEPES, 250 mM NaCl, 1 mM EDTA, and 0.1% NP-40 and then once in 20 mM HEPES, 100 mM NaCl, 1 mM EDTA, and 0.1% NP-40. In between each wash the pellet was centrifuged for five minutes at 2,000 rpm, and the supernatant was discarded each

time. After the final wash, 250 μL of 20 mM HEPES, 100 mM NaCl, 1 mM EDTA, and 0.1% NP-40 was added to all samples.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

To determine if GST-KS-Bcl-2 was expressed, 30 μL of purified protein product on glutathione beads were centrifuged at 12,000 rpm for five minutes and supernatant removed. The samples were prepared for loading into the SDS-PAGE by adding 15 μL of 2x loading dye to the samples, boiled for five minutes and then centrifuged at 12,000 rpm for five minutes. The samples were resolved using a 10% (w/v) SDS-PAGE gel and then stained with Brilliant Blue stain for one hour then rinsed with a 30% methanol solution until all protein bands were visible. The full range rainbow molecular weight marker (GE Healthcare) was used for comparison of protein size.

Western Blot

Protein concentrations were determined using 1x Bradford Dye reagent. Based on the protein concentrations, samples were prepared using 50 μg of the protein mixed with an equal volume of 2x loading dye, boiled for five minutes, then quickly centrifuged. The samples were resolved using a 10% (w/v) SDS-PAGE gel using the full range rainbow molecular weight marker (GE Healthcare) for comparison. The gel was transferred onto polyvinyldifluoride (PVDF) membrane (Immobilon-P from Millipore) in 1X transfer buffer (200 mM glycine and 25 mM Tris Base pH 8.3), 20% methanol, and 0.1% SDS, either over night at 80 mA or for two hours at 30 V depending on the protein of interest. Next, the membrane was blocked (block varied for each primary antibody, see below) for an hour followed by primary antibody application. The membrane was

then rinsed with TBST four times at ten minute intervals and the appropriate secondary antibody was applied for one hour at room temperature.

For the mouse anti-HA antibody (Cell Signaling) the membrane was blocked in 1:2 (v/v) NAP (GBiosciences)/TBST (25 mM Tris pH 8.0, 125 mM NaCl, 0.1% TWEEN-20) for one hour at room temperature, and then the primary antibody applied at 1:1,000 (v/v) in a 1:4 (v/v) NAP/TBST for two to four hours also at room temperature. The membrane was then rinsed with TBST. Lastly, the secondary antibody, horse horseradish peroxidase (HRP)-conjugated anti-mouse (Cell Signaling) was used for detection. For the rabbit anti-VDAC antibody (Cell Signaling), a 5% (w/v) bovine serum albumin (BSA) in TBST block was used for one hour at room temperature, and then the primary antibody was applied at 1:1,000 (v/v) in 5% (w/v) BSA in TBST overnight at 4°C. A secondary antibody, goat HRP-conjugated anti-rabbit HRP (Cell Signaling) was used for detection. Following application of the secondary antibody the membrane was rinsed four times at ten minute intervals with TBST before detection with ECL kit (GE Healthcare) following manufacturer's instructions. The membrane was scanned using the phosphoimager Storm 860 (Molecular Dynamics) where an image was created using ImageQuant software.

Mitochondria Isolation

The mitochondrial isolation protocol was adapted from Abeam, *"Mitochondrial Purification Protocol Isolation of Mitochondria from cells."* BJAB cells, $5x10^7$ cells, were collected by centrifugation at approximately 370 g for ten minutes. The supernatant was removed and discarded and the cells re-suspended in ten packed cell volumes of

NKM buffer (1 mM Tris HCl, pH 7.4, 0.13 M NaCl, 5 mM KCl, and 7.5 mM $MgCl_2$). The cells were centrifuged again at 370 g for ten minutes, and the supernatant was removed and discarded; this wash step was repeated twice. Cells were then resuspended in six packed cell volumes of homogenization buffer (10 mM Tris-HCl, pH 6.7, 10 mM KCl, 0.15 mM $MgCl_2$, 1 mM PMSF, and 1 mM DTT). The cells were transferred into a dounce homogenizer and incubated for ten minutes on ice. Using a tight pestle (pestle A), the cells were homogenized with about 30 strokes of the pestle. The homogenate was transferred into a fresh microcentrifuge tube containing one packed cell volume of 2 M sucrose solution in water and mixed gently. Unbroken cells, nuclei, and large debris were pelleted at 1,200 g for five minutes and the supernatant was transferred to another tube. This treatment was repeated twice, transferring the supernatant to a new tube each time and discarding the pellet. The mitochondria were pelleted by centrifugation at 7,000 g for ten minutes. To the pellet, one volume of lysis buffer (50mM Tris-HCl pH 7.4, 120 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF, 0.2 mM Na_3VO_4, 1 mM DTT, and 1 mM PMSF) was added; the solution was vortexed and incubated on ice for ten minutes with vortexing every two minutes. Lysate was centrifuged for ten minutes at 12,000 rpm and the supernatant was saved.

BJAB whole cell lysate was prepared using $1x10^7$ BJAB cells. Cells were collected by centrifugation at 370 g for ten minutes. The supernatant was removed and discarded and the cells resuspended in 500 μL of lysis buffer (50mM Tris-HCl pH 7.4, 120 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF, 0.2 mM Na_3VO_4, 1 mM DTT, and 1 mM PMSF), vortexed and incubated on ice for ten minutes with vortexing every

two minutes. The lysate was centrifuged for ten minutes at 12,000 rpm and the supernatant was saved.

GST-Pull Down Assay

Protein concentrations of mitochondrial extract and BJAB whole cell lysate were determined by measuring optical density at 595 nm using the Bradford dye assay. Based on the protein concentrations, 600 ug of protein was combined with either 100 µL of GST-KS-Bcl-2 beads or 20 µL GST beads plus 80 µL of glutathione beads. Each sample was normalized to 500 µL using20 mM HEPES, 100 NaCl, 1 mM EDTA, and 1%NP-40 and were incubated with rotation at 4°C overnight. After overnight incubation, the samples were centrifuged for five minutes at 10,000 rpm. The supernatant was saved to a fresh microcentrifuge tube and the beads were washed four times for five minutes in one mL of 20 mM HEPES, 100 NaCl, 1 mM EDTA, and 1% NP-40. After the last wash, the beads were resuspended in 30 µL of SDS loading buffer and stored at -80°C or prepared for western blot.

Transfection of Vero Cells

In preparation for the transfection, Vero cells were split a day before and $1x10^6$ cells plated in a 100 mm^2 plate. The transfection reagent GeneJuice (Novagen) was used according to manufacturer's instructions. For each transfection, 600 µL of serum free media was added to tubes and a ratio of one to three µg of DNA to 1 µL of Gene Juice was used. First the media and Gene Juice were combined in a microcentrifuge tube, vortexed and allowed to incubate for five minutes. Following the incubation period the DNA was added and incubated for an additional 15 minutes. The entire mixture was then

added to the plate in a drop-wise fashion and placed in the incubator for 48 hours. After 48 hours had elapsed, the media in each well was aspirated off and protein lysis buffer (100 mM Tris-HCl pH 8.0, 100 mM EDTA, and 0.5% Triton X-100) was added. The cells were pipetted into separate microcentrifuge tubes and placed on ice for ten minutes with vortexing every two minutes. Following incubation on ice, cell lysate was centrifuged for 10 minutes at 12,000 rpm and supernatant transferred and stored at -80oC.

Hypertonic Shock

Vero cells, approximately $5x10^6$, were plated on 100 mm^2 plates in DMEM with 10% FBS and p/s and grown overnight at 37°C and 5% CO_2. A time and concentration course experiment was carried to determine optimal levels of apoptosis in Vero cells. After the 24 hours, media was removed and experimental media added in groups of three, control (no NaCl), 200 mM NaCl media or 250 mM NaCl media was added. Cells were allowed to incubate for 8, 12, 16, 20, and 24 hours at 37°C and 5% CO_2. After the incubation periods each plate was harvested according to protocol adapted from Lee *et al.* 2009 and from Hedrick Lab at UCSD Cancer Center. To harvest the cells, media along with any dead cells were removed to a labeled 15 mL conical tube, centrifuged for five minutes at 1,000 rpm, and the supernatant was discarded. To the plate, one mL of lysis buffer (100 mM Tris-HCl pH 8.0, 100 mM EDTA, and 0.5% Triton X-100), was added. The cells were then scrapped and lysis buffer plus cells were added to the respective 15 mL conical tube and incubated on ice for 20 minutes. After 20 minutes, the samples were transferred to microcentrifuge tubes and centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant was transferred to a fresh microcentrifuge tube and a one to one phenol

chloroform extraction was performed, vortexing for one minute followed by centrifugation at 12,000 rpm for five minutes. The top layer, which contains the DNA, was saved to a new microcentrifuge tube. Next, a one to two (v/v) ethanol precipitation was carried out, adding two volumes of ethanol for every one volume of DNA, for 30 minutes on dry ice, centrifuged 15 minutes at 12,000 rpm at 4°C, and the supernatant discarded. The DNA pellet was air dried, 30 μL of RNase A water was added, and DNA was resuspended by incubating for 30 minutes at 37°C. Gel electrophoresis was carried out on a 2% agarose gel to view DNA fragmentation and subsequent levels of apoptosis. Optimal apoptosis was seen at 20 hours. To determine if KS-bcl-2 inhibited apoptosis induced by hypertonic shock, $1x10^6$ Vero cells were plated on 100 mm^2 plates in DMEM media and grown overnight at 37°C and 5% CO_2. The cells were then transfected using GeneJuice, according to manufacturer's instructions, with phCMV2-KS-Bcl-2 or phCMV2 and allowed to incubate for 48 hours. The media was removed and experimental media was added in groups of two, control (no NaCl) and 250 mM NaCl media. The cells were allowed to incubate for 20 hours at 37°C and 5% CO_2. After the incubation period, each plate was harvested, DNA extracted as above and electrophoresed in a 2% agarose gel.

Fluorescence Laser Scanning Confocal Microscopy

Acid washed cover slips were placed in a 6 well plate, $2x10^5$ Vero cells were plated on the cover slips directly in the well, DMEM with 10% FBS and p/s media was added and cells were grown overnight at 37°C and 5% CO_2. Cells were transfected using GeneJuice with phCMV2-KS-Bcl-2 or just phCMV2 and allowed to incubate for 48

hours. MitoTracker Red CMXRos (Invitrogen) was used to visualize mitochondria, according to manufacturer's instructions. A stock solution of 1 mM MitoTracker Red was prepared using sterile DMSO, which was further diluted to a working concentration of 250 nm using pre-warmed DMEM/10% FBS/p/s media. Media was removed from each well; the working solution was added directly to each well, and allowed to incubate for 30 minutes at room temperature in the dark. A 3.7% paraformaldehyde solution in PBS was prepared. All subsequent steps were carried out in the dark. After the 30 minute incubation with Mitotracker Red, cells were rinsed with PBS and then fixed using the paraformaldehyde solution for 30 minutes at room temperature. Cells were again rinsed and then permeabilized with PBS/ 0.1%Triton-X-100 for 15 minutes at room temperature. The permeabilization solution was removed and 1% BSA in PBS block solution was added for 30 minutes at 37°C. After the block, primary antibody, rabbit anti-HA, was applied at a 1:1,000 (v/v) dilution in PBS/ 0.1% Triton-X/ 1% BSA for one hour at room temperature. The cells were then washed three times with PBS for five minutes. After the last wash, the secondary antibody, anti-rabbit conjugated to Alexa Fluor 488 (Invitrogen) was used for detection using fluorescence and was applied at a one to 1,000 dilution in PBS/0.1% Triton-X/1% BSA for one hour. The cells were then washed three times with PBS for five minutes. Next, the cells were counterstained for five minutes with 0.1 ug/mL 4`,6-diamidino-2-phenylindole, dilactate (DAPI) (Invitrogen) to visualize the nucleus. Cells were again washed in PBS. The cover slips were removed from the six well plate, mounted on a slide cell side down, and the edges of the cover slip were sealed. Localization of Bcl-2, mitochondria, and nucleus were visualized using an

Olympus Fluoview FV100 Confocal Microscope (Olympus, USA). MitoTracker RedCMXRos was excited using the green HeNe 588 laser since excitation peaks at 579 nm, Alex Fluor 488 was excited using the Argon lasar since excitation peaks at 495, and DAPI's excitation will peak at 358. Digital images were obtained with Olympus Flouview FV10-ASW Version 2.0 software (Olympus, USA).

Results

Hypertonic Shock of Vero cells Induces Apoptosis

Apoptosis, or programmed cell death, can be induced via two different pathways, either the extrinsic or the intrinsic pathway (Figure 1). The intrinsic pathway will have the mitochondria as its central player, and involves the release of various apoptotic factors from the mitochondrial space. There are several different mechanisms that have been shown to induce apoptosis via the mitochondrial pathway in various cells lines. Hypertonic shock can lead to apoptosis and has been shown to activate caspase 3 cleavage as well as mitochondrial fragmentation, which will cause the release of cytochrome c (Copp *et al.*, 2005), both indications of activation of the intrinsic apoptosis pathway.

In order to determine if and when hypertonic shock induced apoptosis in the Vero cell line, a time and concentration course experiment was carried out. Vero cells were plated and allowed to grow overnight, cells were harvested and DNA extracted and measured for subsequent apoptosis levels. The measure for apoptosis used was DNA fragmentation, a hallmark of apoptosis. During apoptosis, endonucleases become activated and will degrade DNA at the internucleosomal linker region creating DNA fragments of approximately 180 base pairs (Kizaki *et al.*, 1988). As seen in Figure 2, levels of DNA fragmentation, apoptosis, increased as time as well as salt concentration increased. The control (lanes 2, 5, 8, 11, and 14), which contains standard media, exhibited no apoptosis, as seen by the lack of laddering. The lanes containing 200 mM NaCl media (lanes 3, 6, 9, 12, and 15) showed a direct increase in apoptosis as time of

treatment increased, but still very little DNA laddering was observed. As with the 200 mM NaCl media group, the lanes containing 250 mM NaCl media (lanes 4, 7, 10, 13, and 16) exhibited a direct increase in apoptosis as time increased, and laddering can be seen at this concentration. At 20 hours of treatment with 250 mM NaCl media (Figure 2 lane 13) optimal DNA laddering can be observed indicating that at this time point significant apoptosis has occurred.

Figure 2: Hypertonic Shock: Vero cells were plated and allowed to grow overnight at 37°C in 5% CO_2. Media was then removed and experimental media added: control media (lanes 2, 5, 8, 11, and 14), 200 mM NaCl media (lanes 3, 6, 9, 12, and 15), or 250 mM NaCl media (lanes 4, 7, 10, 13, and 16). Cells were allowed to incubate for various time points, as indicated. DNA was extracted then separated on a 2% agarose gel and visualized by ethidium bromide staining. Lane 1 is the 1kb DNA ladder (Promega)

KSHV Bcl-2 Prevents Apoptosis Induced via Hypertonic Shock

To verify the anti-apoptotic function of KSHV Bcl-2, we examined the effect KSHV Bcl-2 had on apoptosis induced via hypertonic shock in Vero cells. Again, Vero cells were plated and allowed to grow overnight, then phCMV2-KS-Bcl-2 or just the phCMV2 vector were transfected into the cells. The cells were allowed to incubate for 48 hours and then experimental media added, this time only control containing no NaCl or 250 mM NaCl. Cells were allowed to incubate for 20 hours, since this in when optimal apoptosis was observed. The cells were then harvested, lysed, and DNA extracted. Again, levels of apoptosis were seen via DNA fragmentation. As seen in Figure 3, lane 5, which contained phCMV2-KS-Bcl-2 and treated with 250 mM NaCl, apoptosis was still observed, but to a lesser extent compared to the cells transfected with only the vector and treated with 250 mM NaCl (Figure 3, lane 3).

Figure 3: KS-Bcl2 Protects Cells from Hypertonic Shock: Vero cells were plated and allowed to grow overnight at 37°C in 5% CO_2. Cells were then transfected with only phCMV2 (lanes 2 and 3), or phCMV2-KS-Bcl-2 (lanes 4 and 5). The standard media was removed and experimental media was added: control media (lanes 2 and 4) or 250 mM NaCl media (lanes 3 and 5). Cells were allowed to incubate for 20 hours. DNA was extracted then separated on a 2% agarose gel and visualized by ethidium bromide staining. Lane 1 is the lkb DNA ladder (Promega)

KS-Bcl-2 Localization

In order to determine if the localization of KS-Bcl-2 changes under apoptotic conditions indirect immunofluorescence, in conjunction with lasar scanning confocal microscopy, was used. Vero cells were plated directly onto coverslips, and then transfected with either phCMV2-KS-Bcl-2 (Figures 4A and 5A) or just the empty vector, phCMV2 (Figures 4B and 5B). These cells were allowed to grow in normal media conditions (Figure 4) or in 250 mM NaCl media for 20 hours (Figure 5) and then viewed using the Olympus Fluoview FV100 Confocal Microscope.

Immunofluorescence was carried out using several stains to visualize location of various cellular components, nuclear DNA staining was visualized with DAPI, mitochondria were stained with MitoTracker RedCMXRos, and KS-Bcl-2 was visualized with Alex Fluor 488. Staining under normal conditions when cells were transfected with phCMV2-KS-Bcl-2 further confirmed that KS-Bcl-2 does in fact localize to the mitochondria as well as to the cytoplasm and the nucleus (Figure 4A, panel 4). Following staining the mitochondria of live cells will appear red, since MitoTracker only stains living cells, and cells containing KS-Bcl-2 will appear green. Once these images were merged, each color overlapped each other, a yellow color indicating co-localization of KS-Bcl-2 to the mitochondria (Figure 4A, panel 4). As a control, only phCMV2 was transfected into Vero cells and immunofluorescence staining was carried out under normal conditions (Figure 4B). As expected no green staining was observed indicating KS-Bcl-2 was not present and there was no back-ground staining (Figure 4B, panel 3).

To observe if the localization of KS-Bcl-2 changes under apoptotic conditions,

apoptosis via hypertonic shock was induced prior to immunofluorescence staining. Again, the cells were transfected with either phCMV2-KS-Bcl-2 or phCMV2, but allowed to grow under apoptotic conditions prior to staining. The cells that were transfected with only the vector phCMV2 (Figure 5B) did in fact undergo apoptosis as seen by the mitochondrial fragmentation, a hallmark of apoptosis indicated by the fragments of red staining (Figure 5B panels 3 and 4). As expected, there was no background staining observed with the anti-HA antibody (Figure 5B panel 2 and 4). When the cells were transfected with phCMV2-KS-Bcl-2 no mitochondrial fragmentation was observed (Figure 5B panel 3). KS-Bcl-2 localized mainly to the mitochondria as indicated by the predominant yellow color in Figure 5B panel 4. Some cytoplasmic and nuclear localization was observed, but to a much less degree, suggesting that during apoptosis KS-Bcl-2 will be primarily localized to the mitochondria. These results taken together imply that the anti-apoptotic function of KS-Bcl-2 acts on the intrinsic, mitochondrial pathway.

A **B**

Figure 4: Cellular Localization of KS-Bcl-2: Vero cells were transfected with either phCMV2-KS-Bcl-2 (A) or phCMV2 (B). Mitochondria were stained with MitoTracker Red CMXRos (panel 2) and KSHV Bcl-2 was stained with an anti-HA antibody and an anti-mouse-Alex Fluor 488 antibody (panel 3). Nuclear DNA was stained with DAPI (panel 1). Images were merged in panel 4. Magnification: 400X; 10X eyepiece, 40X objective

Figure 5: Cellular Localization of KS-Bcl-2 under Apoptotic Conditions: Vero cells were transfected with either phCMV2-KS-Bcl-2 (A) or phCMV2 (B). Apoptosis was induced for 20 hours via hypertonic shock. Mitochondria were stained with MitoTracker Red CMXRos (panel 2) and KS-Bcl-2 was stained with an anti-HA antibody and an anti-mouse-Alex Fluor 488 antibody (panel 3). Nuclear DNA was stained with DAPI (panel 1). Images were merged in panel 4. Magnification: 400X; 10X eyepiece, 40X objective

KS-Bcl-2 interacts with VDAC

Since KS-Bcl-2 inhibits apoptosis induced via the mitochondrial pathway, but does not bind to either Bak or Bax (Cheng *et al.*, 1996) other possible binding partners need to be found. To investigate possible binding partners of KS-Bcl-2 with proteins in the mPTPC, a GST-pull down assay was performed using GST bound KS-Bcl-2 with isolated mitochondrial proteins as well as whole cell lysate. pGEX-KS-Bcl-2 and pGEX was transformed into *E. coli* strain BL21 and purified using glutathione-Sepharose beads. The resulting GST fusion proteins were separated on a 10% SDS-PAGE gel, as illustrated in Figure 6. The GST protein, expressed from the pGEX-5x-3 plasmid, was shown to be approximately 26 kDa (Figure 6 lane 2) and the GST-KS-Bcl-2 fusion protein was found to be approximately 45 kDa (Figure 6 lane 3). A GST band can be seen in the GST-KS-Bcl-2 lane (lane 2), likely the result of empty vectors being expressed in *E. coli*. However, expression of both proteins was confirmed.

Mitochondrial proteins and a total protein extract were isolated from BJAB cells. Isolation of mitochondrial proteins, and specifically components of the mPTPC, was confirmed via western blot analysis for VDAC (Figure 7, lanes 4 and 7). A GST pull down assay was performed using protein extracts combined with GST-KS-Bcl-2 as well as just GST. The mixture was incubated over night at 4°C with rotation. The beads, with any bound proteins, were washed and then separated on an SDS-PAGE gel and evaluated for the presence of VDAC by western blot analysis (Figure 7). A distinct VDAC band, located at approximately 34 kDa, was visualized in the GST-KS-Bcl-2 lanes (lanes 3 and

6), thus indicating that KS-Bcl-2 does in fact partner with VDAC. In the GST only lanes (Figure 7, lanes 2 and 5), no band should be seen since no protein that will specifically interact with VDAC is present. No band was observed in lane 2 where GST was combined with mitochondrial extract; however, when combined with BJAB whole cell lysate a band was seen. This band could be a result of using too much protein extract, as 700 μg ofBJAB extract was used as compared to only 250 μg with the mitochondrial extract, and VDAC may be non-specifically interacting with GST. Even though this band is present, it is of a lesser intensity then that in the GST-KS-Bcl-2 lane, indicating a stronger, more specific interaction with KS-Bcl-2.

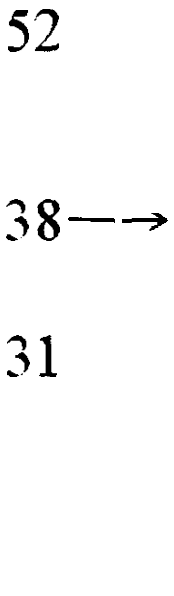

Figure 6. Expression and Purification of KS-Bcl-2. pGEX and pGEX-KS-Bcl-2 were transformed into *E. coli* strain BL21 and expression was induced with IPTG. GST (lane 2) and GST-KS-Bcl-2 (lane 3) were purified using glutathione Sepharose beads. The proteins and a rainbow molecular weight marker (lane 1) were run on a 10 % SDS-PAGE gel and stained with Brilliant blue.

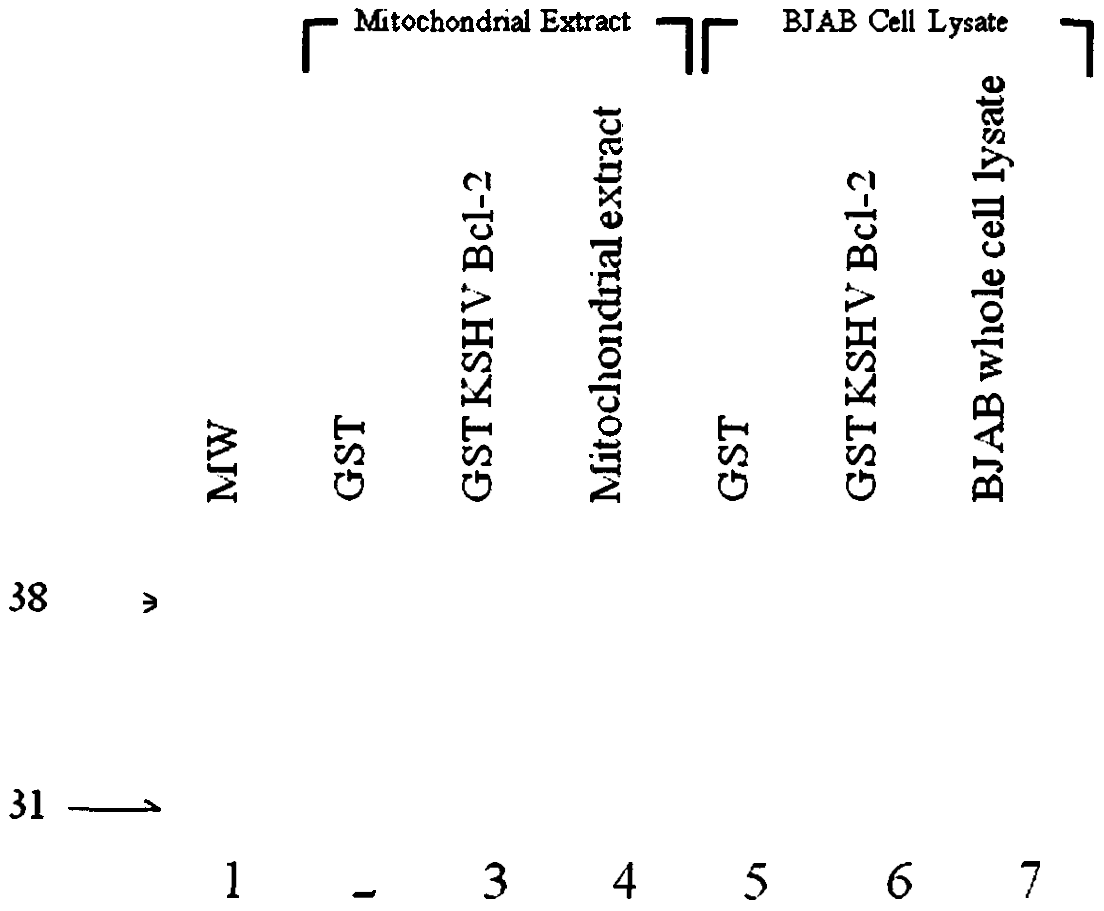

Figure 7 KS-Bcl-2 Interacts with VDAC: GST-KS-Bcl-2 and GST were individually immobilized on glutathione Sepharose beads and combined with either 250 ug mitochondrial protein extract (lanes 2 and 3) or 700 ug BJAB whole cell lysate (lanes 5 and 6). Proteins interacting with GST-KS-Bcl-2 were analyzed by western blotting for VDAC. Lane 4: mitochondrial protein extract, and lane 7: BJAB whole cell lysate, were electrophoresed as a control for western blotting and for comparison.

Discussion

Since its discovery in 1994, Kaposi's sarcoma-associated herpesvirus (KSHV) has been proven to be the causative agent important for the development of Kaposi's sarcoma (KS) as well as primary effusion lymphomas and multicentric Castleman's disease (Boshoff *et al.*, 2001, Ceaserman *et al.*, 1995, 1996, Chang *et al.*, 1994, Fernandez *et al.*, 1999, Russo *et al.*, 1996, Sarid *et al.*, 1999, Sarid *et al.*, 2002). Many cancers, including KS, along with other diseases proliferate in humans through the regulation of the cell cycle and apoptosis or programmed cell death. Apoptosis serves as a defense mechanism against the infection of disease causing agents such as KSHV. Viral proteins have been reported to modulate, either positively or negatively, the host cell apoptotic response to the infection. Modulation of apoptosis will help virally infected cells evade programmed cell death and ultimately produce viral progeny, and in the case of KSHV, cause disease proliferation. If the mechanism in which the virus evades host cell death can be determined then possible targets for new therapeutic agents will also be able to be established, and ultimately help stop the spread of virally infected cells, or even cure the diseases these viruses cause.

Apoptosis is an evolutionarily conserved cell death mechanism that plays a crucial role in multiple biological events (Danial *et al.*, 2004). There are two apoptotic pathways, the external and internal, that can be activated by various stimuli and converge toward a common death pathway. The external pathway is activated by the binding of a ligand to the death receptor and subsequent caspase activation, whereas the internal

pathway has the mitochondria at its center releasing apoptotic factors from mitochondrial pores ultimately resulting in caspase activation (Figure 1) (Danial *et al.*, 2004). A major class of proteins that regulates apoptosis is the Bcl-2 family, and many viruses will code for anti-apoptotic genes such as a Bcl-2 homolog that will promote the production of viral progeny and in some cases predispose cells to additional genetic changes in oncogenes that will enhance tumor growth (Strasser et al., 1990). Within the γ herpesvirus family, EBV, HVS, and KSHV, all which are closely associated with tumors, encode a Bcl-2 homologue (Hardwick *et al.*, 2003)

As previously mentioned, the Bcl-2 family can be divided into three classes based on the Bcl-2 homology domains present and whether it is pro or anti-apoptotic. The first group of Bcl-2 proteins, is anti-apoptotic and consists of cellular Bcl-2, Bcl-xL, Mel-1, as well as KS-Bcl-2, the second group is made up of pro-apoptotic proteins Bak and Bax (Oltvai *et al.* 1993, Wei *et al.* 2001). One mechanism by which the Bcl-2 family positively regulates apoptosis is through the formation of a mitochondrial pore by the heterodimerization of Bax and Bak to cause release of pro-apoptotic factors. To counteract this release of apoptotic factors anti-apoptotic Bcl-2 family members will bind directly to Bax and Bak thus preventing pore formation and therefore prevent apoptosis. Even though, KS-Bcl-2 contains highly conserved BH1 and BH2 domains it does not interact with either Bax or Bak (Cheng *et al.* 1996). A second mechanism of positive regulation of apoptosis is through the sensitizer Bcl-2 proteins inhibiting anti-apoptotic cellular proteins like Bcl-2 or Mel-1 or activating Bak or Bax (Flanagan *et al.* 2008, Pearce *et al.* 2009, Uren *et al.* 2007, Scorrano *et al.* 2003). However, since KS-Bcl-2

does not bind to Bax or Bak this most likely is not the main mechanism through which KS-Bcl-2 functions.

So, if KS-Bcl-2 does not act through the Bax-Bak pathway what apoptotic pathway does it act through? A second pore exists on the mitochondrial membrane, the mitochondrial permeability transition pore complex, or mPTPC. The major components of the mPTPC are ANT on the inner membrane, VDAC on the outer membrane, and cyclophorin D in the intermembranous space (Desagher *et al.*, 200, Crompton 1999, Halestrap 2003, Zoratti *et al.*, 1995). Similar to the Bax/Bak pore, once formed mPTPC will cause the release of pro-apoptotic factors from the mitochondria. Several Bcl-2 members have also been shown to regulate this pore; cellular Bcl-2 binds directly to ANT (Brenner 2000, Jacotot *et al.* 2001) and Bcl-xL directly to VDAC (Shimizu *et al.* 2000, Tsujimoto *et al.* 2000). Each of these interactions prevent pore opening and thus suppress apoptosis. Bax, on the other hand, has been shown to bind to ANT, enhancing mitochondrial membrane permeability and promoting apoptosis (Brenner *et al.* 2000, Belzacq *et al.* 2003).

While it has been known now for over a decade that KSHV expresses a Bcl-2 homologue the mechanism by which it functions remains obscure. KS-Bcl-2 was previously shown to function as an anti-apoptotic protein and that it does not interact with Bax or Bak (Cheng *et al.*, 1996). However, it does bind to BH3 only proteins Bim, Bid, Bik, and Noxa (Flanagan *et al.* 2008). Despite numerous studies observing anti-apoptotic function of KS-Bcl-2, little information is available regarding its potential apoptotic role with the mPTPC. This study looked to further investigate the potential change in the

cellular localization of KS-Bcl-2 under apoptotic conditions, as well as whether or not KS-Bcl-2 will interact with VDAC.

In order to determine if KS-Bcl-2 cellular localization changed under apoptotic conditions immunofluorescent staining for mitochondria and KS-Bcl-2 was carried out under both normal and apoptotic conditions in Vero cells either transfected with phCMV2-KS-Bcl-2 or an empty vector. Apoptosis was induced via hypertonic shock, Figures 2 and 3. In Figure 3, it can be seen that KS-Bcl-2 does in fact inhibit apoptosis, even though some level of apoptosis is still observed. One potential reason for apoptosis could be due to the fact that not only does the shock induce via the intrinsic, but also the extrinsic pathway in which Bcl-2 would not be able to prevent (Copp *et al.*, 2005, Michea *et al.*, 2000) hence still seeing apoptosis, but to a lesser extent.

The results of the staining under normal conditions (Figure 4) showed that KS-Bcl-2 can be found not only in the mitochondria, but also in the cytoplasm and nucleus. These results were consistent with that previously demonstrated by Kalt *et al.* in 2010. Under apoptotic conditions the localization of KS-Bcl-2 did in fact change. As compared to normal conditions KS-Bcl-2 primarily localized to the mitochondria (Figure 5). Even though some nuclear as well as cytoplasmic localization can still be observed the majority of KS-Bcl-2 localized to the mitochondria as compared to normal growth conditions. This localization pattern gives us significant evidence that in the prevention of apoptosis KS-Bcl-2 localizes to the mitochondria and acts through the internal apoptotic pathway, and potentially the mPTPC.

This study also investigated the potential interaction with members of the mPTPC, specifically VDAC. KS-Bcl-2 was expressed from an inducible promoter in bacterial cells and then immobilized on glutathione beads (Figure 6). GST alone was also positively expressed in bacterial cells and immobilized on beads. In addition to positive protein expression, mitochondrial proteins were isolated from BJAB cells. Following immobilization and protein isolation, a GST-pull down assay was performed using KS-Bcl-2 and isolated mitochondrial proteins as well as whole B cell lysate. A GST-pull down assay follows the bait and prey concept; the immobilized, weighted proteins, or bait, will pull down any proteins from the extract, or prey, with which it interacts. The protein interaction can then be detected by western blot analysis for the protein of interest. The immobilized KS-Bcl-2 protein serves as the "bait" and isolated mitochondrial proteins as well as whole cell lysate serves as the "prey". Once allowed to interact overnight, a western blot for VDAC was performed to test for binding. As clearly seen in Figure 7, KS-Bcl-2 does in fact interact with VDAC. Even though the functionality of this interaction has not been determined, this initial step in showing interaction between KS-Bcl-2 and VDAC is the beginning of establishing that KS-Bcl-2 functions to prevent apoptosis through the mPTPC.

Additionally, in Figure 7, a band can be seen in the GST only lane when incubated with whole cell lysate. Several reasons exist for the presence of this band, most likely being due to the amount of protein used. More whole cell lysate was incubated with the immobilized proteins as compared to mitochondrial protein extract. A band in the GST lanes is only visible with the whole cell lysate, not with the

mitochondrial extract. Also important to note is the intensity of this band compared to that in the KS-Bcl-2 lane. The GST only band is much lighter than that of the band in the KS-Bcl-2 lane, indicating a stronger, more specific interaction taking place between KS-Bcl-2 and VDAC. Therefore, this GST/VDAC band can be designated as a non-specific interaction most likely resulting from an overload of protein extract. A possible solution would be to optimize the amount of mitochondrial protein and whole cell lysate used; potentially less whole cell lysate and more mitochondrial protein could be used to observe specific interaction with KS-Bcl-2 and to minimize the non-specific interaction with GST.

Much evidence suggests that many Bcl-2 family members fulfill their apoptotic role through the mitochondrial pathway (Cheng *et al.*, 1996, Flanagan *et al.*, 2008). KS-Bcl-2 has previously been shown to inhibit several mitochondrial events that lead to apoptosis, such as the prevention of cytochrome c release (Flanagan *et al.*, 2008); however, its mechanism behind this still needs to be elucidated. This study's results, the localization change of KS-Bcl-2 under apoptotic conditions as well as KS-Bcl-2's ability to bind to VDAC, when taken together show for the first time that KS-Bcl-2 may function to inhibit apoptosis through the mPTPC.

To further investigate if this interaction between KS-Bcl-2 and VDAC is a functional one several studies still need to be carried out. One functional assay that can be carried out is to reconstitute only VDAC on liposomes and determine if KS-Bcl-2 inhibits apoptosis in this manner. If KS-Bcl-2 does in fact inhibit apoptosis then this relationship can be deemed functional since VDAC would be the only apoptotic pore on

the liposomes. Also, generation of KS-Bcl-2 mutants, each lacking a different BH domain will offer insight to which domain is needed for KS-Bcl-2's anti-apoptotic function. As demonstrated by Shimizu *et al.* in 2000, the BH4 domain of both Bcl-2 and Bcl-xL is essential for their anti-apoptotic function. Furthermore, Shimizu extended their findings and demonstrated that BH4 is the functional domain for Bcl-xL inhibition of VDAC (Shimizu *et al.*, 2000). This finding proves interesting because even though the amino acid sequence between KS-Bcl-2 and Bcl-xL is limited, structural similarity is conserved, specifically in the BH4 domain (Cuconati *et al.*, 2002)

The biological importance of KS-Bcl-2 is still not known. It may be required for inhibition of apoptosis during initial infection, or for the development and maintenance of infection, or even in the development of cancers. In addition to KS samples, KS-Bcl-2 has been detected in clinical samples of primary effusion lymphomas linking it to a potential role in cancer development (Sarid *et al.*, 1997). There is still much needed work to be done to elucidate KS-Bcl-2's role in tumor development, but these findings may provide the basis for identifying the mechanism by which KS-Bcl-2 helps evade cell death, as well to help identify therapeutic agents to combat these diseases.

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