

REVIEW

**To keep the host alive – the role
of viral Bcl-2 proteins**

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Summary. – Apoptosis, an intrinsic cellular pathway that eliminates unwanted cells from multicellular organisms, represents an important mechanism for protection against viral infections. When cells infected by viruses get recognized by immune cells, apoptosis is triggered in the infected cells. Among the many regulators of apoptosis involved in this process, a family of proteins homologous to oncogene Bcl-2 plays a central role. Their concerted activities converge to permeabilization of mitochondrial membranes and activation of apoptotic pathways in the presence of diverse apoptotic signals, including virus infection. In the genomes of many viruses, genes encoding for homologues of antiapoptotic proteins of Bcl-2 family can be found. These proteins, collectively referred to as vBcl-2 proteins, inhibit apoptosis in infected cells at the different stages of virus life cycle to enable the virus to complete its replication and to spread.

Keywords: apoptosis; vBcl-2; Bcl-2 family; autophagy; virus

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1. Introduction

When infected with viruses, host organisms employ multiple strategies to prevent the virus replication and spread. These include the recognition of infected cells by the host immune system and induction of apoptosis in these cells, eventually leading to the removal of the infection. Viruses, on the other hand, face these strategies either by 'escaping' the recognition and/or by inhibiting the apoptosis of infected cells, which provides the viruses with the opportunity to complete their life cycle and spread. Genomes of many viruses, thus, encode for multiple proteins capable of inhibition of different steps of the host apoptotic pathway. While some viruses inhibit the execution of apoptosis by inhibiting caspases – proteases involved in execution of apoptosis, many viruses are known to inhibit regulatory proteins upstream of the activation of caspases. The most prominent among these regulatory proteins are the proteins of the Bcl-2 family.

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Abbreviations: ADV = adenovirus; ASFV = African swine fever virus; BH = Bcl-2 homology motif; CMV = cytomegalovirus; EBV = Epstein-Barr virus; KSHV = Kaposi's sarcoma associated herpesvirus; MHV-68 = murine gammaherpesvirus 68; MXV = myxoma virus; VACV = vaccinia virus; vBcl-2 = viral Bcl-2

2. Cellular Bcl-2 proteins

Proteins of the Bcl-2 family are involved in the regulation of apoptosis. They integrate many distinct cell death-inducing signals and translate them into a single universal apoptosis-inducing event – permeabilization of mitochondrial outer membrane, which results in release of the cytochrome *c* and several other apoptogenic proteins from mitochondrial intermembrane space to the cytosol. Binding of cytochrome *c* to the cytosolic protein Apaf-1 then induces the formation of heptameric complexes, apoptosomes, activating procaspase 9 and downstream caspases (Fig. 1) (see e.g. Youle and Strasser, 2008 for review).

Founding member of the Bcl-2 family, Bcl-2, is an apoptosis-inhibiting protein first identified in patients with B-cell lymphoma (Bakhshi *et al.*, 1985; Cleary and Sklar, 1985;

Tsujimoto *et al.*, 1985). Other members of this protein family share structural similarity with Bcl-2 in at least one of four distinct BH (Bcl-2 homology) motifs (BH1-BH4). According to their activity, particular Bcl-2 family proteins fall into one of three subfamilies, in which the functions correlate with the presence of individual BH domains. Subfamily of multimotif proapoptotic proteins (also known as Bax subfamily) consists of Bax (Bcl-2-associated X protein), Bak (Bcl-2 homologous antagonist / killer) and several other proteins containing three BH motifs (BH1-BH3) (Youle and Strasser, 2008). Activity of these proteins results in the permeabilization of mitochondrial membranes and release of cytochrome *c* and other apoptogenic factors from mitochondria. Of these proteins, Bax and Bak are absolutely required for membrane permeabilization, as double knock-out (*Bax*^{-/-}*Bak*^{-/-}) cells are resistant to all inducers of mitochondrial apoptotic pathway

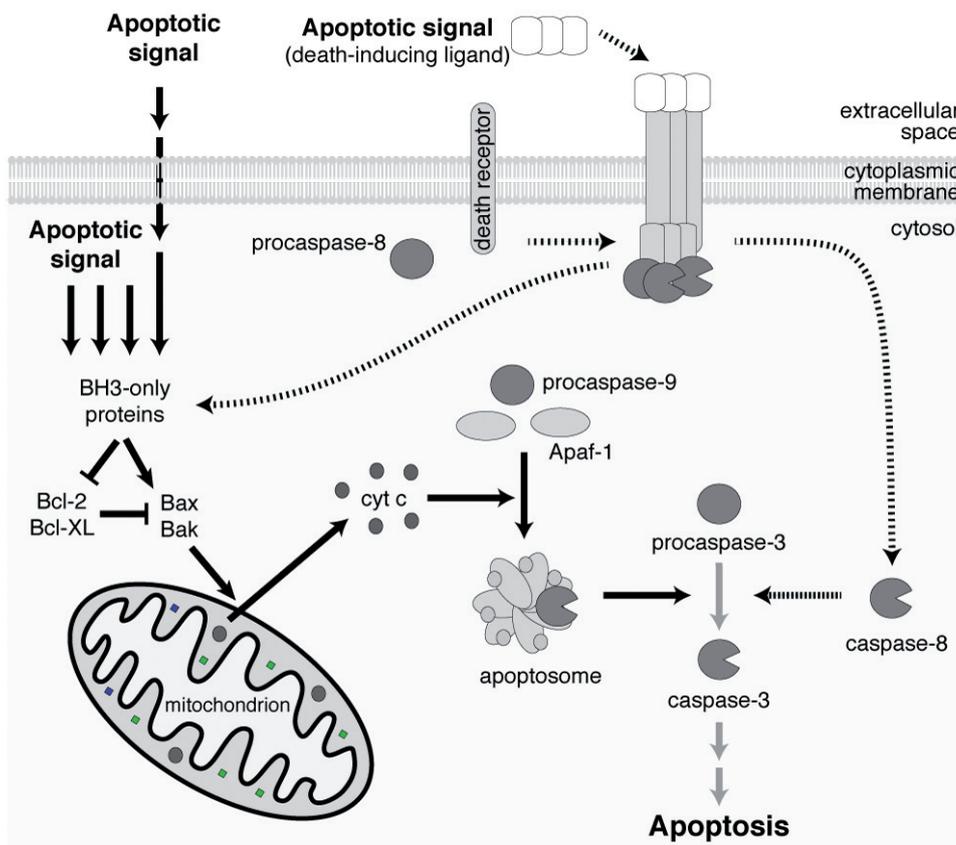


Fig. 1

Apoptosis-inducing pathways

Extrinsic (receptor) pathway of apoptosis (dotted arrows) begins with ligation of receptors of the tumor necrosis factor receptor (TNFR) superfamily that induces oligomerization and activation of initiator caspase 8, which then activates downstream executioner caspases (e.g. caspase 3). In intrinsic (mitochondrial) pathway (full arrows), distinct proapoptotic signals are sensed by proteins of the Bcl-2 family that induce permeabilization of the outer mitochondrial membrane, releasing cytochrome *c* and several other apoptogenic proteins into the cytosol. Cytochrome *c* in cytosol induces the formation of apoptosome and activation of downstream caspases.

(Lindsten *et al.*, 2000; Wei *et al.*, 2001). Subfamily of antiapoptotic proteins (Bcl-2 subfamily) consists of proteins Bcl-2, Bcl-XL, Bcl-w, MCL-1 and A1 that act to prevent the release of the cytochrome *c* from mitochondria in living cells and contain all four BH motifs (BH1-BH4). Proteins of remaining subfamily are proapoptotic proteins, e.g. Bid, Bim, Bik, Bad, which contain only BH3 motif, and are therefore collectively referred to as BH3-only proteins. These proteins regulate the activity of the two above-mentioned subfamilies in response to a wide range of signals, resulting in activation of multimotif proapoptotic proteins Bax and Bak, culminating in Bax and Bak oligomerization in outer mitochondrial membrane and membrane permeabilization.

The intracellular localization of Bcl-2 family proteins is diverse. Antiapoptotic proteins reside in intracellular membranes, in which they are anchored by C-terminal hydrophobic transmembrane domain. While Bcl-2 localizes to all intracellular membranes, Bcl-XL is specifically targeted to outer mitochondrial membrane (Kaufmann *et al.*, 2003). Multimotif proapoptotic protein Bax is localized in the cytosol in living cells and upon induction of apoptosis is translocated into mitochondrial membrane (Hsu *et al.*, 1997; Wolter *et al.*, 1997). Bak, the other multimotif proapoptotic protein, is, however, associated with mitochondrial

membrane even in the absence of apoptosis-inducing signals. Intracellular localization of BH3-only proteins reflects their function in sensing various apoptosis-inducing signals. Most of them are present in cytosol and after receiving apoptosis-inducing signal translocate to the surface of mitochondria, where they interact with other pro- or antiapoptotic proteins of the Bcl-2 family, which results in activation of Bax and Bak and ultimately in permeabilization of the membrane. Translocation of these proteins from cytosol is mostly regulated by posttranslational modifications. Bid, for example, is a cytosolic protein that is, in response to the activation of death receptors on the cell surface, activated by proteolytic cleavage by caspase 8. Cleavage of Bid produces active truncated form of Bid - tBid, in which newly generated N-terminus is myristoylated, targeting the protein to the mitochondrial membrane (Zha *et al.*, 2000). Activation and translocation of other BH3-only proteins, e.g. Bad, Bim and Bmf, may in response to the apoptotic signal be regulated by phosphorylation/dephosphorylation, and yet another BH3-only proteins may be active without posttranslational modification and be regulated at the level of transcription (e.g. p53-induced transcription of Puma and Noxa).

Interestingly, three-dimensional structure of multimotif proteins of the Bcl-2 family is similar for both proapoptotic (Bax and Bak) (Suzuki *et al.*, 2000) and antiapoptotic proteins (e.g. Bcl-2, Bcl-XL) (Muchmore *et al.*, 1996), consisting of two hydrophobic α -helices surrounded by seven amphipathic helices (Fig. 2). The overall fold of these proteins resembles the structure of pore-forming domains of bacterial toxins such as colicins or diphtheria toxin. Upon interaction with the membrane, these proteins rearrange and get integrated into membrane by hydrophobic α -helices.

Of the BH3-only proteins, structure of tBid is similar to the structure of multimotif proapoptotic and antiapoptotic proteins (Chou *et al.*, 1999; McDonnell *et al.*, 1999), while others are mostly intrinsically unstructured proteins that only undergo limited conformational change when interacting with other proteins, e.g. binding other Bcl-2 family members (Hinds *et al.*, 2007).

The mechanisms, by which BH3-only proteins activate Bax and Bak remain under dispute. All proposed models reflect the fact that the BH3 domains are capable of binding into the hydrophobic groove in multimotif proteins (Fig. 2). The 'direct activation' model proposes that activated BH3-only proteins directly bind to the inactive monomeric Bax and Bak, inducing their oligomerization and membrane insertion to form a pore in the outer mitochondrial membrane. In model of 'indirect activation', BH3-only proteins bind to antiapoptotic proteins, such as Bcl-XL and Bcl-2 to inhibit their antiapoptotic activity, activating, thus, Bax and Bak indirectly. As it appears that these two models are not mutually exclusive, other models assume that while some of the BH3-only proteins may act as 'activators' to directly

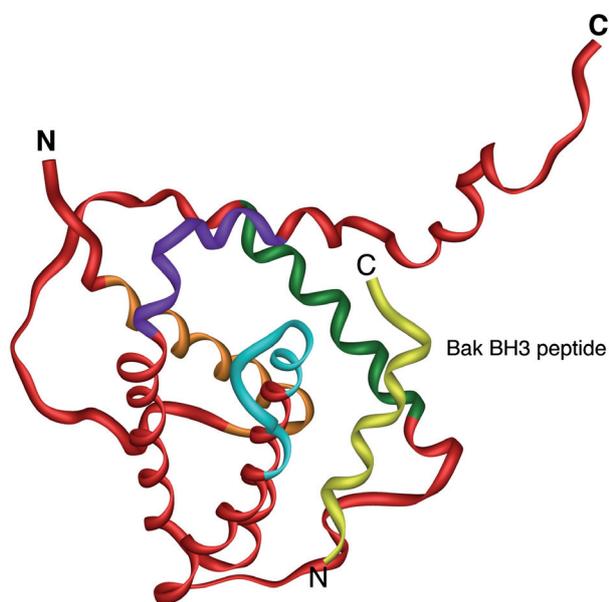


Fig. 2

Three-dimensional structure of antiapoptotic Bcl-2 family protein

Crystal structure of Bcl-XL with indicated position of individual BH motifs (BH1 blue; BH2 purple; BH3 green and BH4 orange) is shown. Yellow α -helix represents a Bak-derived BH3 peptide bound in the hydrophobic groove of Bcl-XL. C-terminal hydrophobic domain is replaced with 6xHis tag. Picture was generated by iMol 0.40 software using structural data from Sattler *et al.* (1997).

activate Bax and Bak, others, called 'sensitizers', may act by an indirect mechanism (Willis and Adams, 2005; Aouacheria *et al.*, 2013).

Among BH3-only proteins, one protein - Beclin-1 - stands out as it plays a key role in the regulation of autophagy, connecting thus the regulation of programmed cell death and autophagy.

3. Autophagy

Autophagy is an essential and evolutionarily conserved process characterized by the intracellular accumulation of double-membrane vesicles called autophagosomes that engulf cytoplasmic material including proteins, large macromolecular aggregates and even entire organelles. Autophagosomes with cargo are targeted to lysosomes for degradation and recycling. Under normal conditions, autophagy operates at a basal level to eliminate long-lived proteins and to maintain cellular homeostasis. In response to various forms of stress, including starvation, depletion of growth factors, low energy levels, hypoxia, oxidative stress or ER stress, autophagy is upregulated to provide a cell-autonomous source of energy and amino acids (Yang and Klionsky, 2010).

Several proteins coordinate and regulate autophagy in mammals. The key role in the regulation of autophagy lies with the BH3-only protein Beclin-1 due to its ability to interact with various partners, especially with class III phosphoinositide-3-kinase (PI3K), together with Vps34, Vps15 and Ambra1, to form a complex responsible for autophagosome formation (He and Levine, 2010).

Several studies have also implicated autophagy in innate and adaptive immune response against pathogens (Levine and Deretic, 2007). However, increasing evidence suggests that viruses have evolved diverse strategies not only to evade or subvert autophagy but also to take advantage of this cellular process (Liang *et al.*, 2015a).

Cellular Bcl-2, the prototype apoptosis inhibitor, has been demonstrated to be an interconnection between autophagy and apoptosis as it negatively regulates autophagy by binding to the BH3 domain of Beclin-1 autophagic protein (Pattingre *et al.*, 2005).

4. Viral Bcl-2 proteins

In many viruses homologues of antiapoptotic proteins of the Bcl-2 family, collectively referred to as vBcl-2 (viral Bcl-2) proteins, have been identified. These viral proteins differ significantly in their primary structure and include those that are closely homologous with cellular antiapoptotic proteins, proteins that contain some of BH motifs, as well as proteins, in which no sequence homology can be identified. All of

these proteins, however, contain a fold typical for antiapoptotic proteins of the Bcl-2 family. It is, thus, similarity of the three-dimensional structure, rather than sequence similarity, which is important for their antiapoptotic function.

4.1 Adenoviridae

The first viral Bcl-2 protein was identified in adenovirus (ADV). Adenoviruses are small DNA oncogenic viruses that infect mammals and birds. Human adenoviruses mainly infect epithelial cells of the respiratory and gastrointestinal tract, in which they can establish persistent infection.

In the cell lines, infection results in progressive replication. The early genes, expressed after the virus entry, involve E1A and E1B. Expression of E1A is required for the replication of viral genome but it induces the apoptosis of host cells. Mutants of adenovirus with altered gene encoding for one of the proteins encoded by E1B, E1B-19K, were described, in which the degradation of cellular DNA and cytopathy resembling the apoptosis were observed (White *et al.*, 1984). E1B-19K thus appeared to block apoptosis in infected cells, sustaining the viral replication.

The E1B-19K is a protein that displays limited sequence similarity to Bcl-2 proteins (Chiou *et al.*, 1994), containing sequences homologous to BH1 and BH3 motifs of cellular Bcl-2. Phenotypes observed in E1B-19K mutants can be suppressed by either coexpression of cellular Bcl-2 or in transgenic virus, in which native E1B coding sequence was replaced with that encoding for cellular Bcl-2 (Chiou *et al.*, 1994; Subramanian *et al.*, 1995b), indicating that E1B-19K and cellular Bcl-2 are functional homologs. Moreover, recombinant proteins constructed from E1B-19K and cellular Bcl-2 by replacing a conserved sequence motif in one by corresponding region of the other retain their antiapoptotic activity (Subramanian *et al.*, 1995a).

Similarly to cellular Bcl-2, when E1B-19K is expressed in the cell lines it is able to suppress the apoptosis induced by E1A expression or other stimuli, including treatment with death receptors ligands, such as tumor necrosis factor- α (White *et al.*, 1991, 1992).

During an adenoviral infection, E1B-19K also interacts with Beclin-1 in the host cells, resulting in modulation of the interaction of Beclin-1 with PI3K complex, and subsequent initiation (in opposite from what is described for other viral Bcl-2 proteins) of autophagy and the formation of autophagosomes. The mechanisms, by which E1B-19K promotes autophagy, involve antagonizing cellular Bcl-2 protein through direct competition to heterodimerize with Beclin-1. Functional BH3 domain of E1B-19K is required for this interaction (Piya *et al.*, 2011). Adenoviruses, thus, have evolved their own mechanism to trigger autophagy, which might be required for viral replication and/or cell lysis (Piya *et al.*, 2011; Liang *et al.*, 2015a).

4.2 Herpesviridae

Herpesviruses are a family of DNA viruses causing diseases in humans and animals. While individual herpesvirus subfamilies target different cell types, the common feature of all is their ability to establish latency in the host cell.

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), is a tumorigenic gammaherpesvirus linked to human cancers including Kaposi's sarcoma, primary effusion lymphoma and some forms of Castleman's disease (Damania and Cesarman, 2013). In KSHV genome, an open reading frame *orf16* encodes for KSBcl-2 (Kaposi's sarcoma-associated Bcl-2). The protein contains conserved BH1 and BH2 motifs and a stretch of hydrophobic amino acids followed by basic amino acids that may anchor the protein into intracellular membranes at its C-terminus (Cheng *et al.*, 1997; Sarid *et al.*, 1997).

Transgenic expression of KSBcl-2 inhibits apoptosis induced by Sindbis virus infection of BHK cells to the extent comparable to overexpression of cellular Bcl-2 or Bcl-XL (Cheng *et al.*, 1997), it inhibits apoptosis of fibroblasts induced by transfection with Bax, as well as it inhibits killing of yeast cells by ectopically expressed human Bax (Sarid *et al.*, 1997). KSBcl-2 does not form dimers, nor it forms heterodimers with Bax or Bak (Cheng *et al.*, 1997), but can bind Beclin-1 (Pattingre *et al.*, 2005; Sinha *et al.*, 2008).

Expression of KSBcl-2 was detected when cell line latently infected with KSHV was treated with phorbol ester that induces reactivation of the virus, indicating that it is expressed during lytic cycle (Cheng *et al.*, 1997). Experiments using mutant viruses with inactivated *orf16* demonstrated that while KSBcl-2 is dispensable for establishment of latent infection, it is required for virus reactivation. Mechanisms involved in this function likely include other than blocking apoptosis, because mutant with deleted *orf16* has defect in reactivation and does not express some of reactivation-specific proteins (which are therefore likely regulated by KSBcl-2) (Gelgor *et al.*, 2015).

KSBcl-2 was the first viral protein reported to be able to inhibit autophagy by directly interacting with Beclin-1 *via* the BH3-binding hydrophobic surface groove of vBcl-2, in both yeast and mammalian cells (Pattingre *et al.*, 2005) (see below for the details).

Epstein-Bar virus (EBV), also called human herpesvirus 4 (HHV-4), is another highly prevalent human gammaherpesvirus infecting B-lymphocytes. Normally the infection is either asymptomatic or leads to a benign lymphoproliferation (infectious mononucleosis) but in immuno-compromised individuals may cause several types of cancer, including Hodgkin's lymphoma, Burkitt's lymphoma and nasopharyngeal carcinoma (Longnecker *et al.*, 2013). Unlike in other gammaherpesviruses, two homologs of Bcl-2 have been identified in EBV. BHRF1 has structural homology

with cellular Bcl-2 and Bcl-XL in BH1-BH3 (Marshall *et al.*, 1999). Its expression enhances survival in transfected Burkitt's lymphoma-derived cell lines in conditions that normally trigger apoptosis, such as low serum and treatment with ionomycin (Henderson *et al.*, 1993). Cell lines expressing BHRF1 are also resistant to γ -irradiation, treatment with cytosine arabinoside (Ara-C), doxorubicin, etoposide and staurosporine, similarly to those overexpressing Bcl-2, Bcl-XL or Bcl-W (Kvansakul *et al.*, 2010).

Solution of three-dimensional structure has revealed that BHRF1 has an overall structure similar to cellular Bcl-2 proteins, but differs significantly in the structure of the hydrophobic groove, which is why BHRF1 does not bind tightly to peptides derived from Bak, Bax, Bik and Bad (Huang *et al.*, 2003). Later it was demonstrated that BHRF1 binds BH3-only protein Bim (Desbien *et al.*, 2009) and the importance of the interaction of the hydrophobic groove was further corroborated when an artificial peptide inhibitor designed to bind to BHRF1 hydrophobic groove was shown to induce apoptosis in EBV-infected cells (Procko *et al.*, 2014). It is, however, not unlikely, that mechanisms of its antiapoptotic activity also include ones that differ from those of other antiapoptotic Bcl-2 proteins, including e.g. downregulation of expression of proapoptotic protein Noxa (Yee *et al.*, 2011).

In addition to BHRF1, another Bcl-2 homologue - BALF1 - was identified in EBV genome (Marshall *et al.*, 1999). Unlike most of the other vBcl-2s, BALF1 contains all BH motifs but lacks C-terminal hydrophobic domain. When expressed in cultured cells, BALF1 protects cells from apoptosis induced by treatment with interferon γ and anti-Fas antibody (Marshall *et al.*, 1999) and renders transfected cells' growth serum independent (Cabras *et al.*, 2005).

Both EBV antiapoptotic proteins appear to be required to prevent apoptosis during initial infection of lymphocytes (Altmann and Hammerschmidt, 2005). As the expression of both, BHRF1 and BALF1, was detected in the Burkitt's lymphoma cell lines and in some other EBV-associated tumor cells, it is likely that they also play an important role in EBV-induced oncogenesis (Cabras *et al.*, 2005; Hsu *et al.*, 2012).

It has not been reported yet whether the two viral homologues of Bcl-2 encoded by EBV have influence on autophagy. However, owing to their structural and functional homology to cellular Bcl-2, it seems likely that BHRF1 and BALF1 may also have an antiautophagy activity. Nevertheless, they could be responsible for the initial autophagy inhibition that was observed early after the expression of EBV early lytic products in Burkitt's lymphoma cells (Nowag *et al.*, 2014).

Besides KSHV and EBV, homologues of Bcl-2 have been found in genomes of all gammaherpesviruses. Of these, murine gammaherpesvirus 68 (MHV-68), also referred to as murid herpesvirus 4 (MuHV-4), represents an important

model virus, because, unlike human herpesviruses, it can also be studied *in vivo* in its natural host. In MHV-68, the protein encoded by gene M11 has been identified as an homologue of Bcl-2 proteins with limited homology to cellular Bcl-2 proteins in BH1 motif and was shown to inhibit apoptosis induced by various stimuli involving FAS ligation, TNF- α treatment (Wang *et al.*, 1999), Sindbis virus infection (Bellows *et al.*, 2000), γ -ray irradiation, dexamethasone treatment or CD3 ϵ ligation (Loh *et al.*, 2005). Although sequence similarity of M11 with cellular Bcl-2 proteins is rather weak and only restricted to BH1 motif, three-dimensional structure of M11 is very similar to the structure of cellular antiapoptotic Bcl-2 family member Bcl-XL (Loh *et al.*, 2005). Cell-based binding assays as well as *in vitro* binding assays using peptides derived from BH3 domains of Bcl-2 family proteins have shown that M11 protein can bind to the regulator of autophagy Beclin-1 and to proapoptotic proteins of the Bcl-2 family, including multimotif proapoptotic proteins Bak, Bax and several BH3-only proteins, e.g. Bid, Noxa, Bim, Bmf and Puma. While the role of M11 in inhibition of autophagy by interacting with Beclin-1, which binds to M11 with the highest affinity, has been well established (Ku *et al.*, 2008; Sinha *et al.*, 2008), functional interactions of M11 with regulators of apoptosis have not been well characterized.

M11 is expressed during latency (Virgin *et al.*, 1999; Marques *et al.*, 2003), appears not to be required for replication of the virus (Gangappa *et al.*, 2002; de Lima *et al.*, 2005), but is required for *in vivo* persistence in immune-deficient mouse (Gangappa *et al.*, 2002).

Using mutants deficient in M11 it was shown that M11 may play important role in infection of immature and transitional B-cells, in which it inhibits B-cell receptor-mediated apoptosis (Coleman *et al.*, 2014).

Similarly to KSBcl-2, M11 was also shown to inhibit autophagy through its interaction with Beclin-1 (Sinha *et al.*, 2008). Whereas the interaction between cellular Bcl-2 and Beclin-1 is regulated by the phosphorylation of the three different Bcl-2 sites (at residues T69, S70, and S87 of the non-structured loop) when cells are subjected to stress, such as starvation, KSBcl-2 and M11 lack analogous phosphorylation sites (Wei *et al.*, 2008). This results in a stable interaction between these vBcl-2s and Beclin-1, leading to a constitutive repression of autophagy. Unlike its cellular counterpart, the vBcl-2-Beclin-1 complex can not be easily disrupted by other BH3-only molecules, such as Bid or Bim (Ku *et al.*, 2008). As a result, these viral homologues exhibit an enhanced capacity for autophagy inhibition as compared to cellular Bcl-2 (Liang *et al.*, 2008).

Mutations in the central hydrophobic cleft of herpesvirus genome-encoded vBcl-2s (84WGR86 of KSBcl-2 or 85SGR87 of M11) perturb the structure of the BH3-peptide-binding groove and prevent the interaction with Beclin-1, resulting in the release of the inhibition of autophagy (E *et al.*, 2009).

Furthermore, a Beclin-1-binding deficient mutant MHV-68 strain, which is impaired in autophagy inhibition but retains intact antiapoptotic activity, is compromised in the ability to maintain chronic infection, though the initial viral establishment of latency is not affected. In contrast, a mutant virus lacking the antiapoptotic activity of vBcl-2 establishes chronic infection as efficiently as the wild-type virus but is largely impaired in efficient *ex vivo* reactivation from latency (Liang *et al.*, 2015b). Thus, while the M11 protein does not appear to be indispensable for lytic replication or for acute infection *in vivo*, its antagonism of autophagy confers chronic/persistent infections (E *et al.*, 2009; Liang *et al.*, 2015b).

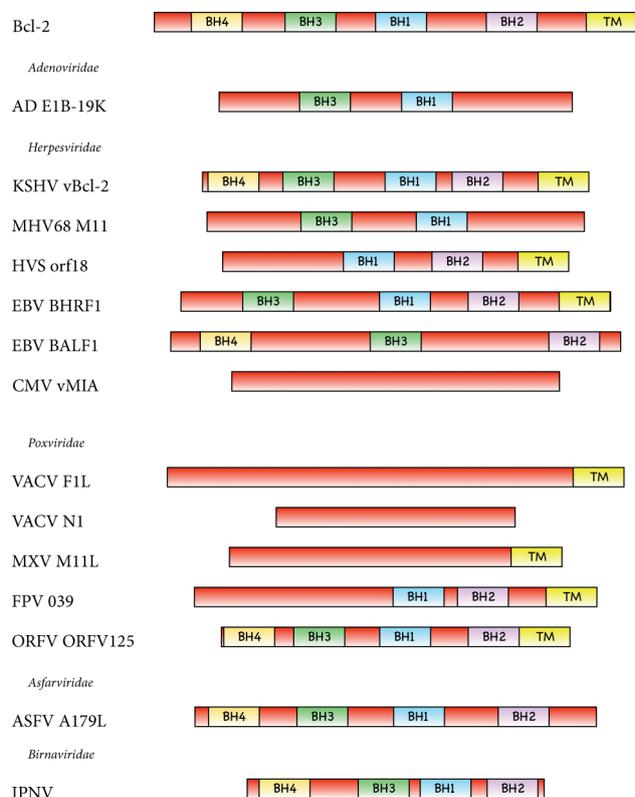


Fig. 3

Bcl-2 homology motifs within viral Bcl-2 proteins

Identified Bcl-2 homology motifs (BH1-BH4) and C-terminal transmembrane domains (TM) present in cellular Bcl-2 (Bcl-2) and its viral homologues: adenovirus E1B-19K (AD E1B-19K), Kaposi's sarcoma-associated herpesvirus vBcl-2 (KSHV vBcl-2), murine gammaherpesvirus 68 M11 (MHV-68 M11), herpesvirus saimiri orf18 (HVS orf18), Epstein-Bar virus BHRF1 (EBV BHRF1), Epstein-Bar virus BALF1 (EBV BALF1), human cytomegalovirus vMIA (CMV vMIA), vaccinia virus F1L (VACV F1L), myxoma virus M11L (MXV M11L), fowlpoxvirus 039 (FPV 039), ORF virus ORFV125 (ORFV ORFV125), African swine fever virus A179L (ASFV A179L) and infectious pancreatic necrosis virus VP5 (IPNV) are indicated. The lengths of proteins correspond to the molecular weights of individual proteins, while the lengths of BH motifs correspond to the lengths of BH motifs in human Bcl-2.

Surprisingly, KSBcl-2 differs from other vBcl-2 proteins in that it is essential for viral replication, as its knockout dramatically lowers KSHV lytic gene expression, viral DNA replication, and progeny virus production (Gelgor *et al.*, 2015; Liang *et al.*, 2015b). Most importantly, it has been found that this novel essential function of KSBcl-2 in lytic replication is separate from the apoptosis- and autophagy-inhibiting activity but correlates with an unusual localization within the cell nucleus (in addition to mitochondria) of infected cells, suggesting that this protein might execute its essential function in the nucleus (Gallo *et al.*, 2017).

Yet different antiapoptotic protein has been found in human cytomegalovirus (CMV), also known as human herpesvirus 5 (HHV-5), an important pathogen that causes significant mortality in immunocompromised individuals and a leading viral cause of congenital defects (Mocarski *et al.*, 2013). Viral mitochondria-localized inhibitor of apoptosis (vMIA) in CMV is encoded by open reading frame UL37x1 (Goldmacher *et al.*, 1999). Orthologous protein is encoded by m38.5 in murine cytomegalovirus (MCMV). vMIA has no homology to cellular Bcl-2 proteins, but likely has a fold typical for Bcl-2 proteins (Pauleau *et al.*, 2007). Unlike other antiapoptotic proteins, vMIA was shown to recruit Bax to mitochondria and bind it, but preventing it from formation of pores in mitochondrial membranes (Arnoult *et al.*, 2004). It selectively binds Bax but not Bak (Arnoult *et al.*, 2004), binding to a unique non-canonical binding site (Ma *et al.*, 2012).

Later analyses have shown that vMIA is synthesized at endoplasmic reticulum (ER), from where it traffics to mitochondria-associated subdomains of ER (mitochondria-associated membranes, MAM) at the contact sites of ER and mitochondria and to mitochondria (Mavinakere *et al.*, 2006), where it associates with lipid rafts (Williamson *et al.*, 2011). It has been shown that in cells infected with CMV, vMIA is recruiting Bax to MAM and likely targets Bax for proteosomal degradation (Zhang *et al.*, 2013).

Due to the strict host specificity of CMV, the activity of vMIA *in vivo* cannot be studied directly and the murine virus can be used as a useful model. In mice infected with MCMV, m38.5 is required for effective replication in leukocytes, while it appears to be dispensable for replication in visceral organs. As leukocytes represent a route of virus to the salivary glands, through which the virus is being disseminated, m38.5 is required to generate sufficient titer of the virus in salivary glands and efficient dissemination (Manzur *et al.*, 2009).

In addition to Bax-specific m38.5, another antiapoptotic protein localizing to mitochondria and specifically inhibiting Bak – m41.1, also known as vIBO (viral inhibitor of Bak oligomerization), was identified in MCMV (Cam *et al.*, 2010). This 57-amino-acid protein, however, does not represent a Bcl-2 homolog as it shares no homology with Bcl-2 proteins and due to its low molecular weight must

not be able to fold like Bcl-2 proteins. When expressed in cultured Bax^{-/-} cells, m41.1 protects cells from apoptosis (Fleming *et al.*, 2013). Antiapoptotic activity of m41.1 has been shown to be required for optimal virus replication in infected animals in leukocytes in blood and spleen, as well as in lungs and liver. MCMV thus expresses two antiapoptotic proteins, one for inhibition of Bax and one for inhibition of Bak with different requirement for these proteins in different tissues reflecting the different involvement of Bax and Bak in these tissues (Fleming *et al.*, 2013). Interestingly, no Bak-specific antiapoptotic protein has been identified in human CMV so far.

Although the first report suggesting a possible relationship between human CMV and autophagy appeared in 1978 (Smith and de Harven, 1978), it took 30 years to demonstrate that CMV produces a marked blockade of autophagy (Chaumorcel *et al.*, 2008). Despite the fact that other herpesviruses, such as KSHV and MHV-68, express a viral homolog of Bcl-2 able to block autophagy, CMV does not possess one – vMIA does not have that ability (Chaumorcel *et al.*, 2012).

Although CMV stimulates the expression of cellular Bcl-2 protein, which has an anti-autophagy effect via its interaction with Beclin-1, it does not use this strategy to block the autophagy. It has been shown that the TRS1 protein of human CMV blocks autophagosome biogenesis through its binding to Beclin-1 and that a TRS1 deletion mutant is defective in autophagy inhibition (Chaumorcel *et al.*, 2012).

4.3 Poxviridae

Poxviruses are large DNA viruses that infect animals and cause often fatal diseases. A prototype of poxviruses is vaccinia virus (VACV). An antiapoptotic gene F1L, protecting infected cells from apoptosis induced by intrinsic pathway inducer staurosporine, was identified by screening a set of deletion mutants of VACV strain lacking a gene encoding for a caspase 8 inhibitor CrmA (Wasilenko *et al.*, 2003). F1L encodes for a protein that bears no sequence homology with cellular Bcl-2 except for a C-terminal hydrophobic membrane anchor and localizes to mitochondria to inhibit the release of cytochrome *c* (Wasilenko *et al.*, 2003). The mitochondrial localization appears to be required for the antiapoptotic activity of F1L (Stewart *et al.*, 2005). The crystal structure of F1L revealed that protein assumes a Bcl-2 fold and forms a dimer with swapped helices (α 1) (Kvansakul *et al.*, 2008). The dimers of F1L contain two hydrophobic grooves capable of binding BH3 peptides derived from Bim or Bak (Wasilenko *et al.*, 2005; Postigo *et al.*, 2006; Campbell *et al.*, 2014). In addition to inhibiting the cell death-inducing activity of Bim and Bak, F1L was found to inhibit caspase 9 by binding it by a novel motif at the N-terminus of F1L (Zhai *et al.*, 2010; Yu *et al.*, 2011). F1L was shown to partially protect the cells from Bax-dependent cell death, while not to bind Bax. This effect,

thus, likely reflects combined inhibition of Bim and caspase 9 (Taylor *et al.*, 2006; Zhai *et al.*, 2010).

Second antiapoptotic protein in VACV is N1 encoded by gene N1L. It was described as a virulence factor, which is not required for virus replication in cell culture, but deletion mutant is attenuated in intranasal and intradermal mouse model (Bartlett *et al.*, 2002). Although it also does not contain recognizable BH motifs, its three-dimensional structure strikingly resembles that of cellular Bcl-2 proteins, and it forms homodimers (Aoyagi *et al.*, 2007; Cooray *et al.*, 2007). During viral infection, N1 binds endogenous Bid, Bak and Bax as well as ectopically overexpressed Bad and Bax (Cooray *et al.*, 2007). Inhibition of apoptosis, however, may not be the most crucial contribution of N1 to virulence, as in addition to inhibition of apoptosis, N1 also inhibits pro-inflammatory signaling by inhibiting nuclear factor- κ B (NF- κ B) activation. The latter function is independent on N1 antiapoptotic function and employs different surface of N1 protein (Maluquer de Motes *et al.*, 2011).

Myxoma virus (MXV) is a member of the *Leporipox* virus genus, which infects rabbits and causes lethal myxomatosis. One of the genes required for its virulence is M11L, as rabbits infected with M11L knock-out virus develop a mild form of the disease, from which they may fully recover (Opgenorth *et al.*, 1992). M11L encodes for a 166-amino acid protein that contains hydrophobic C-terminal domain, which targets protein to the mitochondria, but otherwise bears no homology to Bcl-2 proteins (Everett *et al.*, 2000). The solved crystal structure of M11L shows that its three-dimensional structure closely resembles the structure of cellular antiapoptotic proteins Bcl-2 or Bcl-XL (Douglas *et al.*, 2007). Either transient expression or infection with MXV protects cells from apoptosis induced by number of different stimuli (Everett *et al.*, 2002; Su *et al.*, 2006) by inhibiting activation of Bax (Su *et al.*, 2006) and Bak (Wang *et al.*, 2004).

Fowlpox virus (FPV) is a prototypical virus of the family *Avipoxviridae*, infecting poultry. Based on sequence analysis of the genome, the gene FPV039 has been identified as Bcl-2 homologue. Unlike antiapoptotic proteins of above-mentioned poxviruses, FPV039 contains highly conserved BH1 and BH2 motifs and C-terminal transmembrane domain. FPV039 has no obvious homology with BH3 and BH4 motifs (Banadyga *et al.*, 2007). When expressed in cultured human or chicken cells, FPV039 localizes to mitochondria with C-terminal hydrophobic domain being both necessary and sufficient for its mitochondrial localization. It protects cells from TNF- α -induced loss of mitochondrial membrane potential and apoptosis induced by vaccinia virus infection (is able to functionally replace vaccinia virus F1L) and from apoptosis induced by ectopically expressed Bak, Bax and BH3-only proteins (Banadyga *et al.*, 2007; Banadyga *et al.*, 2009).

When physical interaction with proapoptotic members of Bcl-2 family was assessed, binding of FPV039 with Bak,

Bax, BimL and Bik was detected (Banadyga *et al.*, 2007, 2009).

Orf virus (ORFV) of the *Parapoxvirus* genus primarily infects sheep and goats, causing contagious ecthyma, but it can also infect humans. Its genome encodes for ORFV125 (Westphal *et al.*, 2007), a 173-aminoacid protein that contains a hydrophobic sequence on C-terminus flanked by positively charged amino acids. It contains recognizable BH1 and BH3 motifs and it also has some of the conserved residues of BH4 and BH2 motifs. Expression of ORFV125 in stably transfected cells completely prevents UV-induced apoptosis as is also observed in cells infected by ORFV. ORFV125 is targeted to mitochondria by its C-terminal domain, which is both necessary and sufficient for mitochondrial targeting, and mitochondrial localization is required for its antiapoptotic activity. It inhibits activation of Bax and Bak, and all downstream apoptotic events, including caspase activation and DNA fragmentation (Westphal *et al.*, 2007).

To show that ORFV125 indeed acts as viral Bcl-2, mutant versions of the protein with mutations in conserved residues in BH motifs were tested for the antiapoptotic activity in transfected cells (Westphal *et al.*, 2009). Results indicated that mutations that affect the activity of cellular antiapoptotic proteins also affected the activity of ORFV125.

Out of the proapoptotic BH3-only proteins, ORFV125 has been shown to bind to Bik, Puma and Bim (all three isoforms) and to weakly bind to Noxa and DP5, while no binding activity was shown to Bad, Bmf, Bid or tBid. Interestingly, ORFV125 does not bind to Bak and only binds to Bax when Bax is in active conformation, inhibiting oligomerization of active Bax (Westphal *et al.*, 2009).

3.4 *Asfarviridae*

African swine fever virus (ASFV) is a virus that infects swine as well as soft ticks of the genus *Ornithodoros* that may act as a vector in virus transmission (Costa, 1990). In infected animals, it targets cells of phagocytic system, in which it induces apoptotic cell death. Apoptosis in infected cells is, however, only executed after virus replication. One of the proteins identified in ASFV that inhibit apoptosis after infection is 21 kDa protein encoded by gene A179L (5-HL) (Neilan *et al.*, 1993; Brun *et al.*, 1996). This protein shows a high degree of homology to cellular Bcl-2 in all four BH motifs but lacks the C-terminal hydrophobic domain.

A179L appears essential for ASFV replication throughout the infection cycle. A179L has been shown to inhibit apoptosis in cultured mammalian (Afonso *et al.*, 1996; Revilla *et al.*, 1997) as well as insect cells (Brun *et al.*, 1998), with this low degree of species-specificity likely enabling the protein to inhibit apoptosis both in animal (swine) and in vector (tick) cells.

Using yeast two-hybrid screen, a porcine tBid was identified as interacting Bcl-2 family protein. When other proap-

optotic proteins of the Bcl-2 family were tested, A179L was shown also to interact with Bad, Bmf, Puma, Bik, Bim, Bax and Bak (Galindo *et al.*, 2008) and very recently binding of all major proapoptotic proteins has been demonstrated (Banjara *et al.*, 2017). Solving the structure of complexes of A179L with BH3 peptides of Bid or Bax thus provided important data on how proapoptotic molecules are sequestered by an antiapoptotic protein that does not discriminate among them (Banjara *et al.*, 2017).

Similarly to herpesviral vBcl-2s, it has been shown that A179L also inhibits autophagy by interacting with Beclin-1 through its BH3 homology domain. A179L was found to colocalize with Beclin-1 at mitochondria and the endoplasmic reticulum. It was proposed that the localization of this viral protein to these two organelles is crucial to exert a dual function in the inhibition of apoptosis and autophagy. A179L expression inhibited autophagosomes formation in cells, however, autophagy induced prior to or at the time of virus infection significantly decreased the number of infected cells (Hernaiz *et al.*, 2013).

3.5 Birnaviridae

Although homologs of Bcl-2 proteins are typically found in large DNA viruses, they have also been identified in RNA viruses of the family *Birnaviridae*. Prototypic virus of the family is infectious pancreatic necrosis virus (IPNV) that infects salmonoid fish, in which it causes infectious pancreatic necrosis. The two segment RNA genome of IPNV encodes for five major proteins, among them VP5 is a 17 kDa protein that contains all four BH motifs but lacks the C-terminal transmembrane domain (Hong *et al.*, 2002). As for other above-mentioned vBcl-2s, when the VP5 is expressed in cultured cells, it is able to protect them from apoptosis (Hong *et al.*, 2002). Role of the VP5 in life cycle of IPNV, however, remains unclear as studies employing mutant virus deficient in VP5 expression revealed that VP5 is not required for virulence and persistence of the virus (Santi *et al.*, 2005b) and that it does not inhibit apoptosis induced in infected cells *in vivo* (Santi *et al.*, 2005a). Here should, however, be noted that IPNV strain used in this study contains a VP5 that differs significantly from the one characterized as functional antiapoptotic protein (Hong *et al.*, 2002) as it has substitutions in several putatively important amino acids in BH2 motif.

4. Conclusions

Viral Bcl-2 proteins provide viruses with a powerful weapon in the fight for a chance to replicate and spread. Both true homologues and proteins that lack homology in their amino acid sequences but preserve three-dimensional structure act through a shared mechanism to inhibit the per-

meabilization of outer mitochondrial membrane by cellular proteins of the Bcl-2 family. Although they may primarily target different subsets of cellular proapoptotic proteins and in some cases details in binding to the target proteins may vary, their activity ultimately translates into inhibition of activation of Bax or Bak. As detailed mechanisms of how Bax and Bak permeabilize membrane, and how antiapoptotic proteins inhibit their activity, are not understood yet, many details of the action of vBcl-2 remain to be elucidated. So does our understanding of the role of cell death inhibition by vBcl-2 proteins in life cycles of individual viruses.

In addition to inhibition of apoptosis, some of these proteins also act as either positive or negative regulators of autophagy. Both these functions of vBcl-2 appear to be key and complementary. They suggest that these viral proteins play a fundamental role in underpinning the cellular framework for viral replication. The simplicity of the system that allows a viral protein to interact with autophagy- and apoptosis-related cellular proteins is consistent with the pertinent economy of viral material and the need to execute a vast variety of functions in the course of virus-host interactions.

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