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Review

Viral proteins targeting mitochondria: controlling cell death

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Abstract

Mitochondrial membrane permeabilization (MMP) is a critical step regulating apoptosis. Viruses have evolved multiple strategies to modulate apoptosis for their own benefit. Thus, many viruses code for proteins that act on mitochondria and control apoptosis of infected cells. Viral proapoptotic proteins translocate to mitochondrial membranes and induce MMP, which is often accompanied by mitochondrial swelling and fragmentation. From a structural point of view, all the viral proapoptotic proteins discovered so far contain amphipathic α-helices that are necessary for the proapoptotic effects and seem to have pore-forming properties, as it has been shown for Vpr from human immunodeficiency virus-1 (HIV-1) and HBx from hepatitis B virus (HBV). In contrast, antiapoptotic viral proteins (e.g., M11L from myxoma virus, F1L from vaccinia virus and BHRF1 from Epstein–Barr virus) contain mitochondrial targeting sequences (MTS) in their C-terminus that are homologous to tail-anchoring domains. These domains are similar to those present in many proteins of the Bcl-2 family and are responsible for inserting the protein in the outer mitochondrial membrane leaving the N-terminus of the protein facing the cytosol. The antiapoptotic proteins K7 and K15 from avian encephalomyelitis virus (AEV) and viral mitochondria inhibitor of apoptosis (vMIA) from cytomegalovirus are capable of binding host-specific apoptosis-modulatory proteins such as Bax, Bcl-2, activated caspase 3, CAML, CIDE-B and HAX. In conclusion, viruses modulate apoptosis at the mitochondrial level by multiple different strategies.

Keywords: Viral protein; Apoptosis; Mitochondrial targeting sequence; Mitochondrial membrane permeabilization; Amphipathic α-helix; C-terminal anchoring domain

1. Introduction

Apoptosis is an active death program that contributes to the elimination of damaged, mutated, aged, or virally infected cells [1]. Apoptosis may be initiated by the extrinsic pathway, in which death receptors expressed at the cell surface trigger the receptor-proximal activation of caspases and later mitochondrial membrane permeabilization (MMP) [2–4]. When cell death is triggered by the intrinsic pathway, death signals act directly on mitochondria leading to MMP before caspases are activated [2,5]. The permeabilization of mitochondrial membranes leads to the release of pro-apoptotic factors, some of which can activate caspases, a family of proteins that serve as cellular demolition experts[6], whereas others can activate caspase-

Abbreviations: $\Delta\Psi_m$, mitochondrial transmembrane potential; AEV, avian encephalomyelitis virus; ANT, adenine nucleotide translocator; CIDE, cell death-inducing DFF45-like effector; CMV, cytomegalovirus; EBV, Epstein–Barr virus; HBV, hepatits B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HPV, human papillomavirus; HTLV, human T leukemia virus; KSHV, Kaposi sarcoma herpes virus; MTS, mitochondria targeting sequence; MMP, mitochondria membrane permeabilization; PBR, peripheral benzodiazepine receptor; PT, permeability transition; VDAC, voltage-dependent anion channel; vMIA, viral mitochondria inhibitor of apoptosis; WDSV, Walleye dermal sarcoma virus

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independent death pathways [7–9]. MMP is tightly regulated by proteins from the Bcl-2 family, which inhibit or promote MMP, depending on whether they belong to the pro- or anti-apoptotic branch of the family, respectively [10–12]. MMP thus frequently marks the point-of-no-return of the apoptotic process, the point beyond which cells are condemned to die [12,13].

During the coevolution with their hosts, viruses have developed multiple strategies to manipulate all biological processes of infected cells. Viruses can regulate proliferation, differentiation, and cell death [14,15]. Thus, many viruses inhibit apoptosis, a strategy that subverts one of the most ancient (non-immune) anti-viral mechanisms, namely the apoptotic suppression of infected cells, and thereby allows the virus to replicate before its host cell dies [16,17]. In addition, viruses may induce apoptosis of either infected cells or immunologically relevant cells, with the purpose of increasing viral spread or subverting the host's immune response [14–16].

Obviously, viruses target the central parts of the pro-apoptotic signal transduction and execution machineries. Examples of proteins that subvert pro-apoptotic signals include viral proteins that block tumour necrosis factor (TNF) and its signals [18], viral proteins that inhibit ds-PKR, a protein kinase that is activated by ds-RNA (and which can initiate apoptosis in virus-infected cells) [19], viral proteins that inhibit p53 (a transcription factor that is often rate-limiting for DNA damage-induced apoptosis) [20,21], and viral proteins that inhibit caspases [22,23]. In addition, viral proteins are often acting on mitochondrial receptors and membranes to inhibit or induce MMP (Tables 1 and 2), and this is the focus of the present review.

2. Viral proteins acting on mitochondria

2.1. DNA viruses

2.1.1. Vaccinia virus: F1L

The best-characterized antiapoptotic protein from Vaccinia, an orthopoxvirus, is the general caspase inhibitor CrmA [24]. Nevertheless, Vaccinia virus strains from which the CrmA gene have been deleted are still able to protect cells against proapoptotic stimuli, indicating that this virus activates additional antiapoptotic mechanisms. This evidence led to the characterization of the F1L protein [25]. F1L is a unique Vaccinia virus protein that is localized exclusively in mitochondria [25]. Its C-terminal region contains a hydrophobic domain flanked by positively charged residues and a C-terminal hydrophobic tail, which is responsible for mitochondrial targeting as well as for the antiapoptotic function. This mitochondrial targeting sequence also has a slight homology with the C-terminal region of Bcl-2. F1L overexpression can inhibit MMP, including cytochrome *c* release, after treatment of cells with staurosporine or anti-CD95 plus cycloheximide, thereby blocking the apoptotic cascade at the mitochondrial level [25]. There are several F1L orthologs from other poxviruses, variola virus, monkeypox virus and ectromelia virus, all of which share 95% amino acid similarity in the C-terminal part of the protein. The ectromelia virus ortholog has been shown to protect cells from apoptosis initiated at the mitochondrial level, indicating that the F1L orthologs are indeed functional [25].

2.1.2. Myxoma virus: M11L

Another poxvirus, Myxoma virus, infects rabbits and is the causative agent of myxomatosis. Myxoma virus codes for

Table 1
Proapoptotic viral proteins acting at the mitochondria

Virus	Protein	Intracellular localization	Partners	Effect on mitochondrial morphology	References
HBV	X	M, N	VDAC3, Hsp60, cFLIP, p53, survivin	Yes	[33–35,102–104]
HIV	Vpr	M, N	ANT, cyclophilin A, 14-3-3 proteins, Gag, SP1, GR, TFIIB, p300/CREB-binding protein	Yes	[105–107]
IAV	PB1-F2	M, N		Yes	[92,93]
HTLV-1	P13 II	M, N	FPPS, C44, C254	Yes	[70,98,108]
BLV	G4	M, N	FPPS		[70]
AEV	VP3	M		Yes	[86]
	2C	M, N		Yes	[87]
WDSV	Orf C	M, C		Yes	[85]
HPV type 16	E1^E4	M	cytokeratin	Yes	[94]

Abbreviations: AEV, avian encephalitis virus; ANT, adenine nucleotide translocase; BLV, bovine leukemia virus; C, cytosolic; ER, endoplasmic reticulum; FPPS, farnesylpyrophosphate synthetase; HBV, hepatitis B virus; HIV-1, human immundeficiency virus-1; HPV, human papillomavirus; HSP, heat shock protein; HTLV-1, human T leukemia virus-1; IAV, influenza A virus; M, mitochondria; N, nucleus; VDAC, voltage-dependent anion channel; WDSV, Walleye dermal sarcoma virus.

Table 2
Antiapoptotic viral proteins acting at the mitochondria

Virus	Protein	Intracellular Localization	Partners	Cellular homologs	Protects cells from	References
CMV	vMIA	M	Bax, ANT		Oxidants, anti-Fas, Bax, tBid, TN, TG, STS, BFA, NFX, CPX, HCQ	[55,57,60–64]
Myxoma	M11L	M	PBR		STS, anti-Fas, PPIX	[27,28]
Vaccinia	F1L	M			STS, anti-Fas	[25]
KSHV	K7 or vIAP	M, ER, PM	CAML, Bcl-2, activated caspase 3	Survivin delta-Ex3	TG, TNF-α, anti Fas	[32,49]
	K15	M, ER	HAX-1			[52]
EBV	BHRF1	M		Bcl-2	TRAIL, t-BHP, DNA damage, virus infection	[40,43]
HCV	NS2	ER (M with CIDE-B)	CIDE-B		CIDE-B	[91]

Abbreviations: ANT, adenine nucleotide translocase; BFA, brefeldin A; CIDE-B, cell death-inducing; CMV, cytomegalovirus; CPX, ciprofloxacin; DFF45-like effector-B; EBV, Epstein–Barr virus; ER, endoplasmic reticulum; HCQ, hydroxychloroquine; HCV, hepatitis C virus; KSHV; Kaposi's sarcoma-related herpes virus; M, mitochondria; N, nucleus; NFX, norfloxacin; PBR, peripheral benzodiazepin receptor; PM, plasma membrane; PPIX, protoporphyrin IX, TG, thapsigargin; TN, tunicamycin; STS, staurosporine; *t*-BHP, *tert*-butyt-hydroperoxide.

the antiapoptotic protein M11L. M11L-deleted virus fails to induce a productive infection, indicating that this protein is important for viral pathogenesis [26]. M11L is a small protein (166 amino acids) and has no defined structural motifs except a hydrophobic stretch in the C-terminal domain that forms a putative transmembrane domain [27]. In this domain, six positively charged amino acids and a short hydrophobic tail are responsible for the mitochondrial localization of M11L [27]. In myxoma virus-infected cells, M11L inserts in the outer mitochondrial membrane, where it is exposed to the cytoplasmic face of the organelle [27]. The mitochondrial targeting sequence (MTS) of M11L is homologous to that contained in some of the Bcl-2 family members [27]. The consensus sequence of this MTS contains a hydrophobic region flanked by positively charged residues next to a short positively charged tail [27]. M11L interacts with the peripheral benzodiazepine receptor (PBR), a pro-apoptotic protein from the outer mitochondrial membrane, and this interaction is thought to participate in the M11L-mediated protection against apoptosis [28]. Moreover, M11L has been shown to interact with the pro-apoptotic protein Bak, a Bcl-2 family member that is constitutively present on mitochondria [29]. M11L prevents the release of cytochrome *c* from mitochondria, as well as the dissipation of $\Delta\Psi_m$, induced by the treatment with several apoptosis inducers including the PBR ligand protoporphyrin IX [28] and overexpression of Bak [29]. It is reasonable to speculate that M11L regulates the mitochondrial permeability transition (PT) pore by direct modulation of PBR, which can interact with the principal proteins of the PT pore, in particular the voltage-dependent anion channel (VDAC) and the adenine nucleotide translocase (ANT) [30].

2.1.3. Hepatitis B virus (HBV) X protein

HBV is one of the leading causes of chronic liver disease and infection is often associated with hepatocarcinogenesis [31,32]. The X protein of HBV (HBx) is a potent transactivator essential for virus replication and shows oncogenic properties in animal models [31,32]. HBx sensitizes hepatocytes to apoptosis induced by different stimuli such TNF-α and TRAIL [33]. HBx is a basic protein that localizes to mitochondria, and its overexpression induces a perinuclear mitochondrial distribution coupled to a $\Delta\Psi_m$ loss [34,35]. Studies with mutant proteins reveal a vast MTS in which hydrophobic residues are important for mitochondrial localization, $\Delta\Psi_m$ dissipation and cell death, independent of the transactivating function of HBx [34,36]. Moreover, PT inhibitors, antioxidants and the antiapoptotic proteins Bcl-2 and Bcl-X_L are able to protect HBx expressing cells from death. HBx reportedly interacts with at least two mitochondrial proteins, namely heat shock protein 60 (HSP60) [37] and the VDAC isoform VDAC3 [36]. It is unknown whether these interactions occur simultaneously. However, this possibility appears improbable because VDAC3 is confined to the outer mitochondrial membrane and HSP60 is mostly located in the matrix. Interestingly, VDAC3 overexpression, mitochondrial dysfunction and changes in mitochondrial morphology have been associated with chronic liver disease and carcinogenesis [38], suggesting a pathogenic role for HBx.

2.1.4. Bcl-2 homologues of Epstein–Barr virus (EBV)

EBV is a human herpesvirus that infects B-lymphocytes and promotes their survival, partly by up-regulating Bcl-2 [39]. In addition, EBV codes for BHRF1, a protein expressed early in the viral life cycle [40]. BHRF1 localizes to the mitochondrial outer membrane, where it colocalizes with Bcl-2 [40,41]. The structure of BHRF1 has been recently described; it contains two central hydrophobic α-helices that are surrounded by several amphipathic α-helices and a C-terminal hydrophobic region that targets BHRF1 to intracellular membranes [42]. Although the overall structure is similar to other anti-apoptotic multidomain Bcl-2 family members such as Bcl-2 and Bcl-X_L, there are important structural differences. Unlike Bcl-2 or Bcl-X_L, BHRF1 does not contain the prominent hydrophobic groove that mediates binding to pro-apoptotic BH3-only family members, indicating that its membrane-stabilizing mode of action may be different from those of Bcl-2 and Bcl-X_L [42]. BHRF1, like

Bcl-2, can suppress MMP and apoptosis induced by a variety of stimuli including DNA damage, viral infection and cytokines [43]. BHRF1 is highly conserved both at the sequence and functional level among different EBV isolates, indicating an important functional role for this protein [44]. In addition, EBV codes also for another Bcl-2 homolog, BALF1, which is able to interact with the proapoptotic proteins Bax and Bak [45] and which may actually antagonize the effect of BHRF1 [46]. The relative importance of BHRF1 and BALF-1 for EBV-induced cellular transformation has not been studied in a systematic fashion. However, it appears a tantalising possibility to target these viral proteins, for instance by siRNA, to facilitate apoptosis induction in EBV-expressing tumours, for instance in Burkitt lymphoma.

2.1.5. Kaposi sarcoma herpes virus (KSHV) proteins: K7 and K15

KSHV is the etiologic agent of Kaposi's sarcoma and some B-cell lymphoproliferative diseases [47]. KSHV codes for several antiapoptotic proteins such a vBcl-2 and vFLIP encoded by the ORF 16 and K13, respectively, as well as two mitochondrion-localized proteins, K7 and K15 [47–49]. K7 is a 16 kDa glycoprotein from KSHV which has no homology with proteins from related gamma-herpesviruses [48,49]. K7 contains an MTS, consisting of a single transmembrane hydrophobic region flanked by positive charged residues. This type of MTS is also found in plants and other viral proteins such as viral mitochondria inhibitor of apoptosis (vMIA) and M11L [49]. K7 blocks MMP and apoptosis induced by different stimuli such as TNF-α, ligation of the CD95 death receptor, overexpression of Bax, or addition of thapsigargin [48,49]. In addition, K7 has a BH2 domain and a half-BIR domain, allowing K7 to form a bridge between Bcl-2 and activated caspase-3 [49]. Interestingly, K7 has many similarities to a splice variant of human survivin (deltaEx3), which belongs to the inhibitor of apoptosis proteins (IAPs), a family of proteins that bind and inhibit caspase activation through BIR domains [48,50,51]. In addition, K7 is capable of binding CAML (calcium-modulating cyclophilin ligand) through the mitochondrial targeting domain, regulating the apoptotic response after calcium signalling [49].

K15 is another protein from KSHV that is localized to mitochondria and has a putative MTS in its C-terminus [52]. It is expressed during latency in infected tumours and it binds to HAX-1, a mitochondrial antiapoptotic protein with sequence similarity to Bcl-2 and the proapoptotic protein Nip3 [52,53]. Thus, KSHV codes for several antiapoptotic proteins that are capable of inhibiting apoptosis, and this could lead to the increased tumorigenesis associated with Kaposi sarcoma and other lymphoproliferative diseases [47].

2.1.6. Human papillomavirus (HPV) type 16: E1^E2 protein

HPV type 16 encodes the pro-apoptotic protein E1^E4, which can bind to mitochondria, especially in cells lacking cytokeratin, which is also binding E1^E4. The mitochondrial localization of E1^E4 has been attributed to a leucine-rich region in the N-terminus of the protein [54]. Through a yet unknown mechanism, E1^E4 induces the detachment of mitochondria from microtubules, causing the organelles to form a single large cluster adjacent to the nucleus. In addition, E1^E4 causes $\Delta\Psi_m$ dissipation, followed by apoptosis [54].

2.1.7. Cytomegalovirus (CMV): vMIA

Viral mitochondrial inhibitor of apoptosis (vMIA) is an antiapoptotic protein coded by human CMV. vMIA is a protein of 166 amino acids encoded by the first exon of the UL37 gene [55]. Its sequence is highly conserved among human and primate-derived CMV isolates. However, vMIA does not have homologies with Bcl-2 family or other known proteins [56]. vMIA is localized to mitochondria where it interacts with the adenine nucleotide translocator (ANT) [55] as well as with Bax [57,58]. vMIA has two domains important for its mitochondrial localization (in the N-terminus) and its antiapoptotic function (in the C-terminus), respectively [55]. Deletion of the central part of the molecule does not affect the anti-apoptotic function of vMIA, thus allowing for the generation of a small anti-apoptotic protein, "mini-vMIA". The vMIA homologs found in other species share similarities in the two domains important for the mitochondrial localization and the antiapoptotic function [59].

vMIA has been shown to be one of the most powerful antiapoptotic proteins. Many different cell lines overexpressing vMIA are protected against CD95 ligation [55] and oxidant stress-induced cell death [60,61], as well as staurosporine [57] or overexpression of Bid [58]. vMIA protects cells also from stress initiated at the levels of endoplasmic reticulum [62] and of lysosomes [63,64], suggesting that a vMIA-suppressible mitochondrial event is rate-limiting for cell death induced by organellar stress. How vMIA stabilize mitochondrial membranes and prevents MMP is not known in all details. However, it appears that vMIA inhibits apoptosis mediated by Bax (but not by Bak) [57,58]. Bax is normally localized in the cytosol and translocates to mitochondria only upon apoptosis induction. Once at the mitochondria, Bax inserts into the outer membrane, undergoes a conformational change and oligomerizes in a reaction that is believed to culminate in the generation of giant, protein-permeable pores [13,65]. This scenario applies to normal cells lacking vMIA expression. In cells infected by CMV or transfected with vMIA, vMIA recruits Bax to mitochondria, causes its full membrane insertion, as well as its oligomerization, and yet suppresses all manifestations of MMP [57,58]. At difference with vMIA, Bcl-2 inhibits the insertion of Bax in mitochondrial membranes (Fig. 1).

The C-terminal domain of vMIA is important for recruiting Bax to mitochondria and for its antiapoptotic effect. Indeed, this C-terminal domain is thought to participate in Bax binding [57,58] and contains a sequence that resembles the BH3 domain of Bax (Fig. 1). vMIA disrupts the reticular

morphology of mitochondria, an effect that hypothetically might be attributed to a vMIA-mediated inhibition of mitochondrial fusion or an increase in mitochondrial fission [66]. Bax translocation and inhibition of mitochondrial fusion seem to be early events in apoptosis [67]. Thus, vMIA seems to protect mitochondria even after Bax translocation and mitochondrial fragmentation, two early steps of apoptotic cell death [66]. Importantly, vMIA destroys the mitochondrial network (which passes from a "spaghetti" to a "maccaroni"-like appearance), even when overexpressed in Bax-negative cells (D.P. and N.Z. unpublished observation), indicating that Bax is not responsible for the vMIA-mediated alteration of mitochondrial morphology. The exact mode of action of vMIA remains an ongoing conundrum.

3. RNA viruses

3.1. Human T-lymphocyte virus-1 (HTLV-1): p13 II

The HTLV-1 is a retrovirus that infects T lymphocytes and causes acute T cell leukemia [14]. p13 II is an accessory protein encoded by HTLV-1. Its N-terminal domain contains a short hydrophobic leader peptide, followed by an amphipathic α-helix, which is the domain responsible for mitochondrial targeting. P13 II is associated with the inner mitochondrial membrane, as revealed by electron microscopy and digitonin fractionation [68]. p13 II overexpression induces mitochondrial alterations, swelling and fragmentation [68]. Moreover, in isolated rat mitochondria, p13 II leads to mitochondrial swelling associated with a rapid flux of K^+ and Ca^{2+} across the inner membrane and dissipation of the $\Delta\Psi_m$ [68]. In addition, p13 II overexpression enhances cell death induced by C2 ceramide [69]. Moreover, p13 II expression suppresses the growth of cells transformed with the Myc or Ras oncogenes and reduces the proliferation of Jurkat T leukemia cells [69]. Thus, p13 II has some pro-apoptotic and anti-proliferative activity.

Bovine leukemia virus (BLV) is a retrovirus with strong similarities to HTLV. It codes for the mitochondrial (and nuclear) protein G4, in which two regions are responsible for mitochondrial targeting [70]. G4 is able to alter mitochondrial morphology and colocalizes with p13 II in mitochondria [70]. BLV-infected cells are protected against

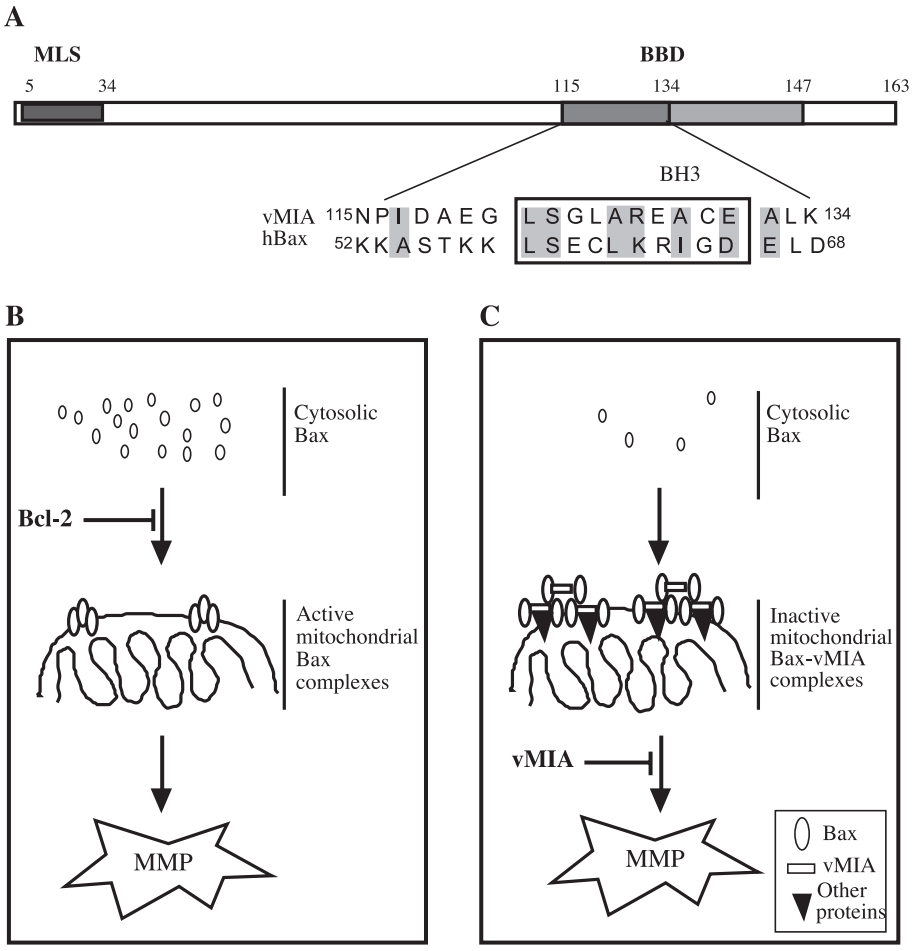

Fig. 1. Mode of action of the apoptosis-inhibitory protein vMIA. (A) Structure–function of vMIA. Residues 2–23 denote the mitochondrial localization sequence (MLS), 115–147 the Bax-binding domain (BBD) responsible for Bax sequestration/inactivation. An alignment of amino acids 116–134 from vMIA and the BH3 domain of Bax are shown. (B, C) Models of Bax inactivation by Bcl-2 like proteins (B) and the alternative mode of inhibition by vMIA (C).

apoptosis, although evidence that supports the implication of G4 in MMP regulation is still lacking [70,71]. G4 (and p13 II) are able to interact with farnesylpyrophosphate synthetase (FPPS) and this interaction seems to be important during cellular transformation [72]. Moreover, G4 is oncogenic in primary cell cultures and responsible for leukemogenesis in sheep [72]. However, it remains to be established whether these findings can be extrapolated to p13 II.

3.2. Human immunodeficiency virus-1 (HIV-1): Vpr

The presominant pro-apoptotic protein encoded by HIV-1 is the envelope glycoprotein complex [73–76]. In addition, HIV-1 codes for a small accessory protein, Vpr, which localizes to mitochondria and has pro-apoptotic properties [77]. A recombinant adenovirus coding for Vpr (adCMV-Vpr), as well as Vpr synthetic peptides derived from its C-terminus (amino acids 52–96), promotes apoptosis in many different cell types including human fibroblasts, neurons and peripheral blood T cells [78,79]. Vpr promotes MMP, cytochrome *c* release and cell death [77]. The C-terminal domain of Vpr (residues 53–96) forms an α-helix [80,81], and several arginines situated within or between the functionally important H(S/F)RIF motifs (R73, R77, R80) are crucial for the MMP-inducing effects of Vpr [77]. Mutant Vpr peptides lacking these arginine residues fail to interact with the adenine nucleotide translocase (ANT), the putative mitochondrial receptor of Vpr, and thus fail to induce MMP and cell death [77,82]. Synthetic Vpr and purified ANT can cooperate in vitro to form ion channels in synthetic lipid bilayers or in liposomes [83]. Moreover, ANT-deficient yeast strains are resistant to Vpr-mediated killing, indicating that the interaction between Vpr and ANT is crucial for cell death induction [77]. Vpr is dispensable for HIV replication in T cells but not in macrophages. Interestingly, sequence analysis of HIV long-term non-progressors (LTNP), which show detectable HIV replication but do not show immunodeficiency, presents a Vpr mutation in one crucial arginine residues (R77), yielding a mutant protein that cannot induce apoptosis. This suggests that Vpr is a virulence factor in HIV-1 infection [79,82].

3.3. Walleye dermal sarcoma virus (WDSF): Orf C

WDSV is a retrovirus associated with benign tumours in fish that regress seasonally [84]. The WDSV genome codes for a basic protein with 120 amino acids called Orf C, which localizes to mitochondria [85]. Overexpression of Orf C causes perinuclear clustering of mitochondria and $\Delta\Psi_m$ dissipation. Moreover, cytochrome *c* is partially released from mitochondria, and cells that express Orf C present signs of apoptosis such as phosphatidylserine exposure in the outer leaflet of the plasma membrane and chromatin condensation [85]. Orf C does not possess any homologies to known MTS. Tumour regression in the infected animals is often associated with apoptosis and the Orf C protein is expressed at high levels in regressing tumours, indicating that Orf C-mediated apoptosis could be a mechanism contributing to tumour involution [84,85].

3.4. Avian encephalomyelitis virus (AEV): VP3

AEV is a picornavirus that infects birds and reduces hatching frequency and life span of chicken and turkeys. AEV codes for two pro-apoptotic proteins, namely the structural protein VP3 and the non-structural protein 2C [86,87]. VP3, which localizes to mitochondria, induces apoptosis in different cell lines, activating caspase-3 leading to DNA fragmentation [86]. The 2C protein is highly conserved among picornaviruses, and can induce apoptosis accompanied by cytochrome *c* release from mitochondria. The proapoptotic domain of the protein is located in a region spanning amino acids 46 to 80, in the N-terminal domain of the protein proximal to a putative coiled-coiled domain, and has a putative α-helix structure [87]. However, 2C has been shown to localize to the endoplasmic reticulum, shedding doubts on its mitochondrion-specific mode of action [88].

3.5. Hepatitis C virus (HCV): NS2

HCV is an RNA virus that codes for a polyprotein that is cleaved into the different structural and non-structural (NS) proteins [89]. HCV-NS2 is a 23-kDa hydrophobic transmembrane protein. It is localized in the endoplasmic reticulum and its functions are not clearly defined. NS2 is able to bind and protect from CIDE-B-induced apoptosis. CIDE-B is a cell death-inducing DFF45-like effector, the overexpression of which leads to apoptosis in many different cell lines [90]. CIDE proteins are localized to mitochondria and form homodimers and heterodimers with other members of the family [90,91]. The C-terminal region of CIDE proteins is responsible for the mitochondrial localization and dimerization. NS2 from HCV interacts with CIDE-B, blocking cytochrome *c* release from mitochondria and cell death [91]. The interaction between NS2 and CIDE-B involves the C-terminus of CIDE-B, which is responsible for dimerization, as well as a four-amino acid stretch in NS2 protein. Double stainings of NS2 and CIDE-B revealed partial overlapping signals in the perinuclear region suggesting that NS2–CIDE-B complex may regulate apoptosis at the mitochondrial level [91]. Moreover, only those NS2 mutants that bind to CIDE-B are able to block cytochrome *c* release and apoptosis [91].

3.6. Influenza A virus (IAV): PBF1-F2

IAV codes for a small protein, PBF1-F2, which targets mitochondria [92,93]. Fine mapping has determined a C-terminal region which is responsible for mitochondria

localization. This region contains an amphipathic α-helix preceded by a short hydrophobic region containing several basic residues [93]. These structural features are likely responsible for the mitochondrial targeting and are conserved in many IAV strains [93]. In addition, the MTS of PBF1-F2 resembles the MTS found in p13 II, Vpr and G4. FRET techniques and electron microscopy have led to the conclusion that PBF1-F2 and fusion proteins containing this MTS are incorporated into the inner mitochondrial membrane. PBF1-F2 expression is associated with mitochondria rounding, swelling and fragmentation, accompanied by MMP. The proapoptotic effects of PBF1-F2 are cell line-dependent [93], indicating the existence of yet-to-be-defined modulators of PBF1-F2. Synthetic PBF1-F2 can decrease the stability of lipid bilayers in an electric field [94], suggesting that the protein can exert direct membrane-permeabilizing effects.

4. Mitochondrial targeting sequences of pro- and anti-apoptotic viral proteins

Mitochondrial proteins encoded by the host cell's nuclear DNA are synthesized in the cytoplasm and then imported into mitochondria. Protein sorting to mitochondria is achieved by several cytosolic factors and mitochondrial translocation machines that ensure their correct targeting to the appropriate submitochondrial compartment [95]. Mitochondrion-targeted proteins usually contain clearly defined MTS, defined as sequences sufficient to target proteins and peptides to mitochondria. Frequently, MTS are regions of 20–60 amino acids, with abundant positive charges and hydroxylated residues forming amphipathic α-helices in membranes [95]. Most matrix proteins and some inner membrane proteins possess a cleavable N-terminal presequence which functions as an MTS and can direct non-mitochondrial proteins into mitochondria when attached to their N-termini [95]. Other proteins possess an internal or C-terminal MTS [96], determining their final compartmentalization, in the matrix, the intermembrane space, and the inner mitochondrial or outer mitochondrial membrane [95–97]. Based on this knowledge, it is possible to establish rules on the structure–function relationship of virus-encoded mitochondrion-targeted proteins (Figs. 2 and 3).

The majority of mitochondria-localized viral proteins do not show the classical cleavable N-terminal presequence, however, many of them present amphipathic α-helices located in different parts of the protein. Vpr was the first viral proapoptotic protein described to act directly on mitochondria [77]. The structure of Vpr is characterized by a long [53–78] amphipathic α-helix, followed by a less defined [79–96] C-terminal domain [80, 81]. Mutational analysis of the α-helix revealed a sequence of 13 amino acids, which suffices for the MMP-inducing effect of Vpr observed in cell-free systems. This sequence forms an amphipathic α-helix, with three positive residues on one side of the helix. Substitution of these residues impairs Vpr-mediated cell killing [77], pointing to a strong correlation between mitochondrial localization and cytotoxic effect of Vpr. The p13 II protein from HTLV-1 has a 32-amino-acid stretch that contains an amphipathic α-helical MTS. In this region, 10 amino acids are sufficient for targeting the protein (or GFP in a chimeric construct) to mitochondria [98]. Four arginines are predicted to form a positively charged patch in one side of the α-helix, conferring amphipathic properties to this region [68]. Secondary structure predictions of the BLV G4 proteins also identify three α-helical regions [70], one at the hydrophobic N-terminus, one arginine-rich region between amino acids 63 and 69, and one at the C-terminus. Truncated synthetic peptides coupled to EGFP demonstrate that the two first α-helices are needed for effective mitochondrial targeting of the protein. As is true for p13 II, the arginine-rich α-helix of G4 has amphipathic properties [70].

In addition to three domains essential for transactivation, HBx has a central region important for mitochondrial

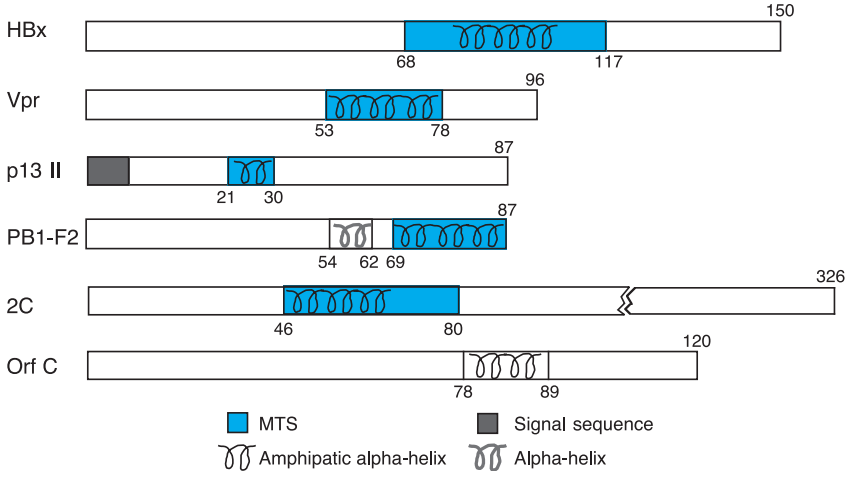

Fig. 2. Viral antiapoptotic proteins acting on mitochondria. Numbers denote the first and the last amino acid residue of each domain.

localization and pro-apoptotic effects [99]. In this region, six hydrophobic residues are important for mitochondrial localization. Interestingly, this region (amino acids 77–97) is also likely to form an amphipathic α-helix. In addition, HBx shares strong similarity (40%) with VDAC3 in the HBx MTS, indicating that HBx could be inserted into mitochondrial membranes in a fashion similar to VDAC [100].

Another viral protein that carries an amphipathic α-helix responsible for mitochondrial localization is the protein PB1-F2 from EBV. PB1-F2 has two α-helices (amino acids 54–62 and 73–82). The second helix exhibits a strong (80%) similarity with the amphipathic α-helix from HTLV-1 p13 II, and different IAV isolates show high homology in this region [93]. The minimum MTS (amino acids 69–82) is sufficient to trigger the mitochondrial import of EGFP. Interestingly a short five-amino acid stretch upstream of the amphipathic α-helix is also needed for optimal mitochondrial targeting [72].

The proapoptotic domain of 2C from AEV also forms a putative α-helix (amino acids 47 to 61) and contains an amphipathic region. However, a detailed study of the mitochondrial targeting and the apoptogenic properties of this domain is still elusive. Another proapoptotic protein from AEV, VP3 also has a putative amphipathic helix (amino acids 21–29). Yet another example of putative amphipathic α-helix that has not been characterized in detail is the protein Orf C from WDSV.

All of the aforementioned proteins containing putative amphipathic α-helices have proapoptotic properties (Fig. 2). In addition, besides their mitochondrial localization, many of these proteins are also present in the nucleus, although the significance of this dual localization remains to be established. Thus, it is tempting to speculate that viral proteins sharing these structural features promote cell death at the level of mitochondria, either by affecting membrane properties or by interacting with proteins of the PT pore such as ANT and VDAC (as this has been shown for Vpr and HBx), thus directly modulating the MMP and apoptosis.

Another class of viral proteins inserts into mitochondrial membranes by means of a tail-anchored domain [97]. Tail-anchored proteins (which include most proteins of the Bcl-2 family) are inserted into the outer mitochondrial membranes or the endoplasmic reticulum. The tail-anchored domain consists of a single hydrophobic transmembrane region located at the C-terminus of the protein and spans the membrane in an α-helical conformation. Once inserted into the membrane, the rest of the protein faces the cytosol [97,101].

The M11L protein from Myxoma virus has a C-terminal sequence responsible for mitochondrial targeting and anti-apoptotic function. This sequence consists of a hydrophobic region flanked at the N-terminus by positively charged residues and a short positively charged tail [27]. This domain is very similar to the C-terminus of Bcl-2 [27] where the tail anchor region is found [101]. Other viral proteins such as BHRF-1, KsBcl-2 and the K7 and K15 from KSHV also carry a similar MTS [27,48,49]. Several other proteins including F1L from Vaccinia virus and NS2 from HCV also possess a putative C-terminal MTS. Thus, although computer programs designed to predict the subcellular localization of proteins (e.g., PSORT, http://psort.nibb.ac.jp) fail to indicate a primordial mitochondrial localization for these viral proteins, a putative tail-anchoring domain seems to be present in all of them. Intriguingly, all of the viral proteins carrying putative tail-anchoring domains have anti- (rather than pro-) apoptotic features (Fig. 3).

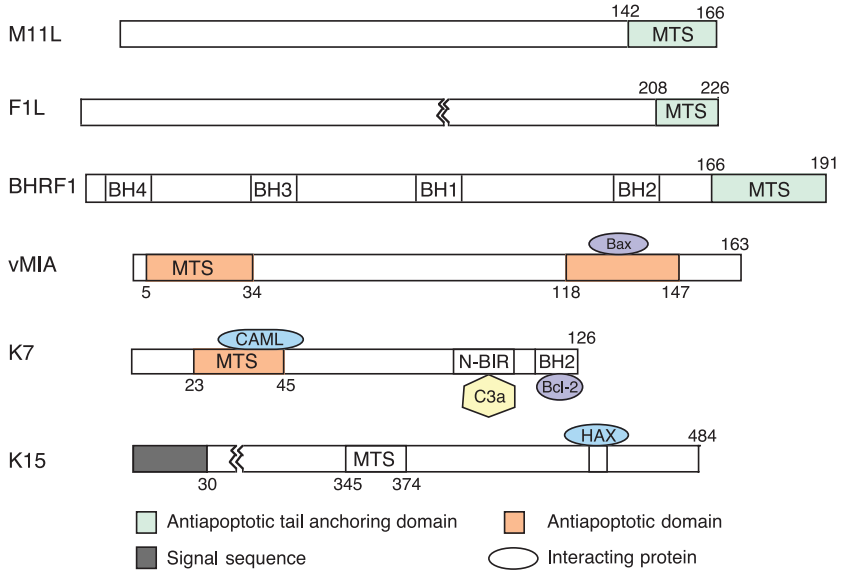

Fig. 3. Viral proapoptotic proteins acting on mitochondria. For details and comments, see main text.

5. Concluding remarks

Since mitochondria orchestrate the apoptotic response and thus function as authentic killer organelles, it is not surprising that many viral proteins directly target mitochondria for modulating apoptosis. Thus, by suppressing the cell-autonomous antiviral apoptotic response (at the beginning of the life cycle) or by promoting cell death (at the end of infectious cycle), viruses modulate apoptosis by inhibiting or inducing MMP, respectively. In an attempt to classify viral mitochondrial modulators according to the properties of their MTS, we have found that most of the proapoptotic viral proteins acting on mitochondria contain amphipathic α-helices in the N-terminus or the central portion of the protein. On the contrary, viral antiapoptotic proteins targeting mitochondria all possess a putative C-terminal anchoring domain determining a type of membrane insertion that causes the N-terminus to face the cytosol. Thus, a general pattern linking structure and function emerges. However, the exact mechanisms through which viral mitochondrial apoptosis modulators exert their local action remain to be solved for most of such proteins. It can be anticipated that a more detailed characterization of the mitochondrial targets of viral proteins may ultimately yield novel opportunities for therapeutic intervention on several viral diseases of major socioeconomic impact, including AIDS, viral hepatitis, influenza, and virus-induced cancer.

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