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Cell death suppression by cytomegaloviruses

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Cytomegaloviruses (CMVs), a subset of betaherpesviruses, employ multiple strategies to suppress apoptosis in infected cells and thus to delay their death. Human cytomegalovirus (HCMV) encodes at least two proteins that directly interfere with the apoptotic signaling pathways, viral inhibitor of caspase-8-induced apoptosis vICA (pUL36), and mitochondria-localized inhibitor of apoptosis vMIA (pUL37 × 1). vICA associates with pro-caspase-8 and appears to block its recruitment to the death-inducing signaling complex (DISC), a step preceding caspase-8 activation. vMIA binds and sequesters Bax at mitochondria, and interferes with BH3-only-death-factor/Bax-complex-mediated permeabilization of mitochondria. vMIA does not seem to either interact with Bak, a close structural and functional homologue of Bax, or to suppress Bak-mediated permeabilization of mitochondria and Bak-mediated apoptosis. All sequenced betaherpesviruses, including CMVs, encode close homologues of vICA, and those vICA homologues that have been tested, were found to be functional cell death suppressors. Overt sequence homologues of vMIA were found only in the genomes of primate CMVs, but recent observations made with murine CMV (MCMV) indicate that non-primate CMVs may also encode a cell death suppressor functionally resembling vMIA. The exact physiological roles and relative contributions of vMIA and vICA in suppressing death of CMV-infected cells *in vivo* have not been elucidated. There is strong evidence that the cell death suppressing function of vMIA is indispensable, and that vICA is dispensable for replication of HCMV. In addition to suppressed caspase-8 activation and sequestered Bax, CMV-infected cells display several other phenomena, less well characterized, that may diminish, directly or indirectly the extent of cell death.

Keywords: alphaherpesvirus; apoptosis; Bak; Bax; Bcl-2; betaherpesvirus; caspase-8; cell death suppressor; cytomegalovirus; gammaherpesvirus; herpesvirus; inhibitor of apoptosis; programmed cell death; vICA; vMIA.

The notion that organisms use apoptosis as an anti-viral defense has became widely accepted.[1,2] Infected cells undergo apoptosis and thus are eliminated, limiting viral propagation. In addition to triggering of what is loosely called "intrinsic" or "innate" apoptosis, viral infections activate cytotoxic effector cells of the immune system, which then induce cell death "extrinsically" *via* ligation of Fas[3] and/or injection of granzymes.[4] To prevent premature death of infected cells, viruses have evolved with encoding various cell death suppressor proteins that block apoptotic signaling pathways.[1,2] Two other phenomena associated with cell death suppression during viral replication were also reported: up-regulation of the expression of cellular cell death suppressors, and expression of viral genes products that promote the survival of the infected cells, but do not directly interfere with the apoptotic machinery.

Several recently published reviews discuss various aspects of cell death suppression by herpesviruses.[5–15] The field is advancing rapidly, and these reviews are already not up to date on the subject. Here I will discuss cell death suppression by human and animal CMVs.

vICA, a cell death suppressor that blocks caspase-8 activation

Anti-apoptotic function of vICA

vICA is a product of the immediate early *UL36* gene of HCMV. vICA suppresses apoptosis triggered by ligation of death receptors Fas, tumor necrosis factor receptor-I (TNFR-I), and Apo-2,[16] but appears to only marginally protect cells against death induced by cytotoxic drugs, or by infection with adenovirus lacking the *E1B19k* cell death suppressor gene.[16]

vICA associates with pro-caspase-8 and appears to prevent its recruitment to DISC

Ligation of Fas leads to recruitment of pro-caspase-8 to the cytoplasmic portion of Fas through FADD adaptor protein, and subsequent proteolytic processing and activation of pro-caspase-8.[17] vICA blocks apoptosis by interfering with caspase-8 activation. It constitutively associates with pro-caspase-8 *via* the caspase-8-pro-domain region that contains two non-identical death effector

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domains (DED).[16] Unlike pro-caspase-8, FADD does not co-immunoprecipitate with vICA,[16] and thus appears to not associate with it. This result favors a model in which vICA blocks the recruitment of pro-caspase-8 to FADD (Figure 1). It still needs to be determined whether vICA associates with pro-caspase-8 directly or through an intermediary.

Homologues of vICA encoded by other betaherpesviruses

Betaherpesviruses are the only viruses or organisms found so far that encode vICA homologues. All sequenced genomes of betaherpesviruses (except for an incompletely sequenced guinea pig CMV genome) encode highly conserved vICA homologues (Table 1). Two of these proteins, the *M36* protein product (M-vICA) of MCMV, and the *Rh61* (In our study we denoted this gene as *Rh36*,[18] because we were not yet using the new nomenclature of Hansen *et al*.[20]) protein product (Rh-vICA) of rhesus macaque CMV (RhCMV) were tested and found to be functional cell death suppressors, and M-vICA was found to associate with pro-caspase-8 in transfected cells.[18,19] Because of a high degree of sequence similarity among vICA homologues encoded by betaherpesviruses, it is likely that the cell death suppressing activity is a general property of these vICA homologues. A short segment of guinea pig CMV genome was sequenced and found to be corresponding to the human CMV *UL32-UL37* region. This segment did not contain a vICA homologue ORF,[21] an unexpected result in light of the findings that other betaherpesviruses, more distantly related to HCMV encode a homologue of vICA. It remains to be determined if guinea pig CMV encodes a vICA homologue in another part of its genome.

Figure 1. The cell death suppressing activity of vICA. Ligation of a cell death receptor results in recruitment of pro-caspase-8 to DISC through its association with the adaptor protein FADD *via* a homotopic interaction of their DED regions. Pro-caspase-8 then self-activates into active caspase-8, which mediates apoptosis by cleaving its substrates such as BID and (in type I cells) pro-caspase-3. vICA binds to the DED region of pro-caspase-8 and thus prevents its association with FADD.

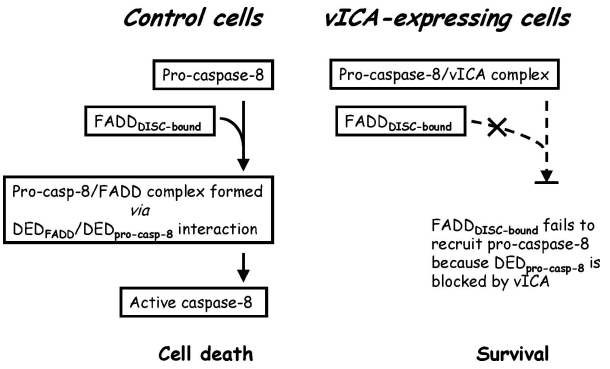


Table 1. vICA and vMIA homologues of betaherpesviruses[18]

	vICA homologue			vMIA homologue		
Virus	**Gene**	**Protein**	**Cell death suppressing activity**	**Gene**	**Protein**	**Cell death suppressing activity**
Rat cytomegalovirus	*R36*	R-vICA	Not tested	Not found		
Murine cytomegalovirus	*M36*	M-vICA	Yes	Not found	A hypothetical functional analogue of vMIA (protein X)[64]	
Herpesvirus tupaia	*T36*	T-vICA	Not tested	Not found, except for a short homologous segment in *T40*		Not tested
Rhesus macaque cytomegalovirus	*Rh61*	Rh-vICA	Yes	*Rh62*	Rh-vMIA	Yes
African green monkey cytomegalovirus	Not assigned	Agm-vICA	Not tested	Not assigned	Agm-vMIA	Not tested
Chimpanzee cytomegalovirus	*UL36*	Ch-vICA	Not tested	*UL37*	Ch-vMIA	Not tested
Human herpesvirus-6	*U17-U16*	HHV6-pU17-16	Not tested	Not found		
Human herpesvirus-7	*U17-U16*	HHV7-pU17-16	Not tested	Not found		

In our study we denoted *Rh61* and *Rh62* as *Rh36* and *Rh37*, respectively[18], because we were not yet using the new nomenclature of Hansen *et al*.[20]

Structure-function analysis of vICA

The ability of vICA to bind to the pro-caspase-8 DED region seems to be necessary for its cell death suppressing activity.[16] At present, we do not know which part of vICA is responsible for this function, and if there is any other function of vICA required for its anti-apoptotic activity. Alignment of the amino acid sequences of vICA homologues of CMVs reveals the conserved area in their ORFs,[18] which may help to design deletion mutants for the future structure-function analysis.

Cellular and viral non-homologous functional analogues of vICA

Gammaherpesviruses encode viral FLIPs (vFLIPs), which, like the cellular FLIP (cFLIP), contain two non-identical death-effector domains, and are highly homologous to the similarly organized pro-domain of pro-caspase-8. Several FLIPs were tested on their functional activities and found to associate with either pro-caspase-8 and/or with the pro-caspase-8 adaptor protein FADD, and to interfere with activation of caspase-8.[2,22–24] These associations appear to be effected through the homotypic interactions of the death-effector domains of vFLIPs and their binding partners, pro-caspase-8 or FADD.[17] One can speculate that depending on the binding specificity of a particular vFLIP (a preference to pro-caspase-8 or to FADD) it may block either the recruitment of pro-caspase-8 to DISC, or the next step, caspase-8 activation. The mechanism of action of vICA seems to be similar but not identical to that of cFLIP. One difference between vICA and cFLIP is that vICA does not associate with FADD,[16] while cFLIP is capable of forming a complex with either pro-caspase-8 or FADD,[24] although it has not been established whether cFLIP interacts with each of these two proteins directly, or whether one of them serves as an adaptor for interaction with the other. Another difference between vICA and cFLIP is that the former associates with pro-caspase-8 constitutively in the absence of death-receptor ligation of pro-caspase-8, while the latter, under physiologically-relevant conditions, forms a complex with FADD and pro-caspase-8 only at the DISC following ligation of a death receptor.[25] vICA and vFLIPs may also differ in that the latter may contribute to the suppression of apoptosis by inducing NF-kappa B.[25a]

Adenovirus encodes a 14.7-kDa protein that also seems to target pro-caspase-8, but shares no homology with either FLIPs or vICA.[26]

vICA is active as a cell death suppressor in both type I cells, and type II cells,[16] and is similar to cFLIP in this property.[25,27,28] The ability of vICA and cFLIP to suppress apoptosis in both type I and type II cells is consistent with their mode of action: interruption of the apoptotic signaling pathway upstream of its branching into the type I and type II pathways.

vMIA, a cell death suppressor targeting the mitochondrial apoptotic pathway

Cell-death suppressing activity of vMIA and its splice variants

UL37 gene of HCMV encodes at least eleven differentially spliced protein products[29–31] eight of which contain vMIA-encoding region, *UL37 exon 1*. vMIA suppresses apoptosis triggered by a variety of cytotoxic effectors such as infection with HCMV, ligands of death receptors, various cytotoxic compounds, infection with an *E1B19k*-deficient mutant of adenovirus, HIV-encoded Vpr and drugs that interfere with functioning of the secretory pathways.[5,29,32,33,119,119a] Two longer splice variants of vMIA, gpUL37 and $pUL37_M$, both containing the full-length exon 1-encoded sequences, are also capable of suppressing apoptosis.[29]

Intracellular localization and processing of vMIA and its splice variants

In HCMV-infected cells, as well as in human cells transiently or stably transfected with *vMIA*, most of the newly synthesized vMIA molecules relocate to mitochondria,[29,34,35] where it appears to associate with the outer mitochondrial membrane.[29,36] In addition, a fraction of vMIA in transfected or HCMV-infected cells is localized to the endoplasmic reticulum.[35–37] vMIA retrovirally transduced into the murine NIH-3T3 fibroblasts also localize preferentially at mitochondria[38] indicating that the mitochondria-targeting signal of vMIA is not species-specific.

gpUL37, a predominantly expressed splice variant of vMIA, was predicted from its sequence to be an integral membrane protein,[39] and is *N*-glycosylated.[37] Consistent with this prediction, gpUL37 was observed at mitochondria, endoplasmic reticulum, and the Golgi apparatus of CMV-infected cells, and on the plasma membrane of transiently transfected human cells.[36] vMIA and $pUL37_M$, another splice variant of vMIA, are not glycosylated.[40]

The *N*-terminal 22 amino acid-long hydrophobic segment of vMIA resembles a signal peptide,[39] and, as such, in principle, it could be cleaved in the process of protein maturation. We examined the *N*-terminal sequence of a vMIA protein sample that was immunoprecipitated through its *C*-terminal myc-tag from a lysate of a stably transfected HeLa clone by protein microsequencing without prior partial tryptic digestion. The *N*-terminal peptide was not cleaved (Goldmacher and Bartle, unpublished results), consistent with its essential role in the

mitochondrial targeting and cell death suppression activity of vMIA.[29,41]

Recently, Mavinakere and Colberg-Poley reported[40] that at the endoplasmic reticulum gpUL37 is cleaved onto an *N*-terminal polypeptide containing the vMIA amino acid sequence, and a *C*-terminal polypeptide containing a *UL37 exon 3*-encoded sequence. The *N*-terminal cleavage product largely accumulates at mitochondria, while the *C*-terminal product accumulates at the endoplasmic reticulum where it gets *N*-glycosylated. $pUL37_M$ lacks the peptidase-cleavage-site present in gpUL37, and is neither cleaved, nor *N*-glycosylated, and traffics to both the endoplasmic reticulum and mitochondria.

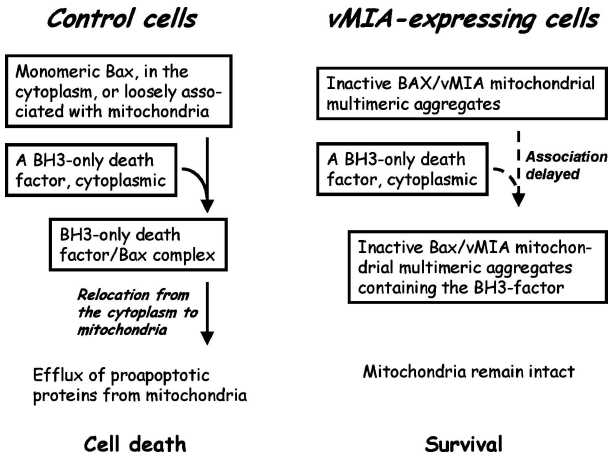
The mechanism of association of vMIA with the mitochondrial outer membrane remains unknown. The hydrophobic *N*-terminal leader-like sequence of vMIA is likely to serve as an anchor in the membrane. vMIA forms mixed aggregates with Bax (see below), but this association with Bax is not required for its relocation and binding to mitochondria, and the mitochondria-targeting function of vMIA is retained in vMIA deletion mutants that are incapable of binding Bax.[29,34,41,42] We cannot rule out a possibility, however, that the Bax mitochondrial targeting function may still contribute to this process, in addition to that of vMIA.

Two mitochondrial proteins have been implicated in being involved in the mitochondrial apoptotic signaling pathway, voltage-dependent anion channel (VDAC)[43] and adenine nucleotide translocator (ANT).[44] vMIA does not appear to physically interact with VDAC,[45] and it does pull down ANT in co-immunoprecipitation experiments.[5,29,45] Our recent experiments indicated, however, that the cell-death-suppressing activity of vMIA mutants did not correlate with their ability to associate with ANT in co-precipitation experiments, and that there appeared to be no defined segment of vMIA responsible for its affinity towards ANT.[42] These data suggested to us that the interaction of ANT with vMIA might be of a non-specific nature.

vMIA associates with Bax and blocks Bax-mediated permeabilization of mitochondria

Investigations independently performed by Guido Kroemer's group in France and by us in collaboration with Richard Youle's group at NIH revealed the molecular mechanism of action of vMIA: it binds and sequesters Bax at mitochondria[34,42] in the form of high-molecular-weight mixed aggregates,[34] thus depleting the intracellular pool of Bax and preventing Bax-mediated permeabilization of mitochondria (Figure 2). Several lines of evidence either support, or are at least consistent with this mechanism: (i) Bax co-precipitates with vMIA from lysates of vMIA-expressing cells[34,42] (Previously we had reported[29] that vMIA does not bind Bax. This conclusion

Figure 2. The cell-death-suppressing activity of vMIA. Bax associates with a BH3-only death factor induced or up-regulated during apoptosis. The complex then relocates to mitochondria and triggers efflux of mitochondrial proapoptotic proteins. vMIA depletes the pool of monomeric Bax by sequestering it into inactive multimeric aggregates. The association of the BH3-only death factor with these Bax/vMIA aggregates is delayed, and the resulting mixed aggregates remain inactive.



was based on co-immunoprecipitation experiments done with 293T cells transiently transfected with vMIA. We did not know at the time (it was published later) that E1B19k protein, which is expressed in 293T cells, inhibits Bax-mediated apoptosis and prevents relocation of Bax from the cytoplasm to mitochondria.[9] These data suggest that 293T cells may have been a poor choice to examine vMIA/Bax interaction: the presence of E1B19k might interfere (directly or indirectly) with the interaction between Bax and vMIA. Cos-7 cells are similar to 293T cells in that they also express the large T antigen and are capable of replicating plasmids containing SV40 origin. These cells, however, do not express E1B19k, and we easily co-precipitated Bax with vMIA from Cos-7 lysates.[42]); (ii) a vMIA fragment that consists of the Bax-binding domain (a synthetic peptide), binds recombinant bacterially produced Bax, indicating that vMIA-Bax interaction is direct and does not require an intermediary;[42] (iii) during Fas-mediated apoptosis vMIA blocks tBID-mediated permeabilization of mitochondria and the downstream events, such as caspase-9 activation and PARP cleavage, but not the upstream events, such as Fas-ligation-mediated proteolytic processing of caspase-8, or the proteolytic processing of BID;[29] (iv) in *vMIA*-transfected cell lines or HCMV-infected normal human fibroblasts the majority of Bax molecules are constitutively associated with mitochondria and co-localized with vMIA, while in the control vMIA-negative cells, most of the intracellular Bax pool is dispersed throughout the cytoplasm;[34,42] (v) mitochondria isolated from vMIA-expressing cells are refractory to the permeabilizing effect of tBid (because of the absence of available Bax), but are permeabilized by an excess of

activated recombinant Bax;[42] (vi) mutations within the Bax-binding domain that inactivate the Bax-binding activity of vMIA, abolish the cell-death-suppressing activity of vMIA as well.[41,42] vMIA does not compete with tBid for association with Bax[42] indicating that tBid has affinity for Bax irrespective of whether Bax is or is not associated with vMIA. On the other hand, vMIA delays relocation of ectopically introduced GFP-tBid from the cytoplasm to mitochondria,[34] probably because in the absence of vMIA, GFP-tBid relocates to mitochondria after it forms a complex with cytoplasmic Bax, while in the presence of vMIA, there is no cytoplasmic Bax present.

vMIA does not prevent mitochondrial damage in intact cells or isolated mitochondria exposed to peptides containing either a Bax-BH3 or Bcl-2-BH3 sequence.[46] Thus, in this property, these peptides are similar to recombinant activated Bax, and dissimilar to recombinant tBid which does not permeabilize mitochondria isolated from vMIA-expressing cells. It is not clear whether Bax and the peptides act *via* a similar molecular mechanism.[47]

It has been recently reported that Bax operates as a pro-apoptotic factor not only at mitochondria, but also at the endoplasmic reticulum.[48,49] vMIA and its splice variants have been detected at the endoplasmic reticulum,[36] but it has not been determined if these proteins are located on the luminal or the cytoplasmic side of the endoplasmic reticulum. In principle, it is possible that these proteins neutralize endoplasmic reticulum-localized Bax, and thus suppress the endoplasmic reticulum-localized-Bax-mediated apoptosis, but this hypothesis has not been tested experimentally.

Richard Youle and his collaborators reported that Bax may be involved in fission of mitochondria during apoptosis,[50] and, in a separate study, McCormick *et al.* found that vMIA induces mitochondrial fission in infected and transfected cells.[51] Outcomes of these possibly related phenomena are opposite: death, or survival of the cells. The mechanisms and the functional significance of the Bax- and vMIA-mediated mitochondrial fission are at present unclear. Both full-length vMIA and a functionally active mini-vMIA consisting only of the mitochondria-targeting domain and the Bax-binding domain, induce the mitochondrial fission, while a vMIA mutant lacking Bax-binding activity does not,[51] suggesting that the fission is associated with the presence of vMIA-Bax complex at mitochondria. It still needs to be determined if vMIA-induced fission has any effect (beneficial or adverse) on the mitochondrial physiological function.

Cell-type specificity of the anti-apoptotic activity of vMIA

vMIA suppresses death receptor ligation-mediated apoptosis in type II cells, but not in type I cells,[16] and in this property vMIA is similar to Bcl-2.[52,53] These observations are consistent with the mechanisms of action of vMIA and Bcl-2 which target the mitochondrial apoptotic signaling pathway. The difference in apoptotic pathways in type I and type II cells have been described only for Fas-mediated apoptosis. There is some evidence that apoptosis induced by other stimuli, such as ionizing radiation, cytotoxic drugs, viral infections, DNA damage, or immune effector cells, proceeds not through caspase-8 activation and the subsequent Bid activation,[54,55] but through up-regulation of another BH3-only death factor such as BBC-3/Puma[56,57] or Noxa,[58] and that, in accord, vMIA,[45,59] and Bcl-2[54,55,60] suppress apoptosis induced by at least some of these stimuli in type I cells.

The cell death suppressing activity of vMIA is more restricted than that of Bcl-2, and there are several lines of evidence that vMIA, unlike Bcl-2, interacts physically and functionally with Bax but not with Bak, and can only suppress Bax- but not Bak-mediated mitochondrial damage during apoptosis:[34,42] (i) vMIA does not seem to associate with Bak; (ii) vMIA does not suppress mitochondrial damage in transformed murine embryonic fibroblasts (MEF) during staurosporine-induced apoptosis, unless *Bak* is knocked out; (iii) vMIA suppresses apoptosis in HCT116 cells in which Bax is functional and Bak is not.

Considerable evidence has accumulated that at least in some types of cells tBid and other BH3-only death factors mediate apoptosis exclusively through activation of either Bax or its close structural and functional homologue Bak,[42,61–63] and that cells can be divided into several phenotypes distinct in the relative contributions of Bax and Bak in this process. In Bax-dominant (Bax^D) cells, apoptosis is mainly effected by Bax, while Bak is inactive. In Bax/Bak co-dominant cells (Bax/Bak^{coD}), both Bax and Bak are capable of mediating this apoptotic pathway. In Bak^D cells Bak is the main mediator of apoptosis (Table 2). The recently proposed[42] classification of cells into Bax^D, Bax/Bak^{coD}, and Bak^D phenotypes is based on experiments with only a few types of cells, and more work will be needed to characterize the relative contributions of Bax and Bak into the apoptotic process in other types of cells. In these experiments, vMIA can be used as a tool to reveal Bax^D cells as those protected by vMIA.[42]

Structure-function analysis of vMIA

vMIA, gpUL37, and $pUL37_M$ share an identical 162 *N*-terminal amino acid sequence, the entire length of *UL37 exon 1*, which constitutes essentially the full length of vMIA (Figure 3), indicating that this polypeptide segment is responsible for the anti-apoptotic activities of these proteins, and, by extension, that these proteins suppress cell death by the same mechanism. It is very likely that the other seven vMIA splice variants that also

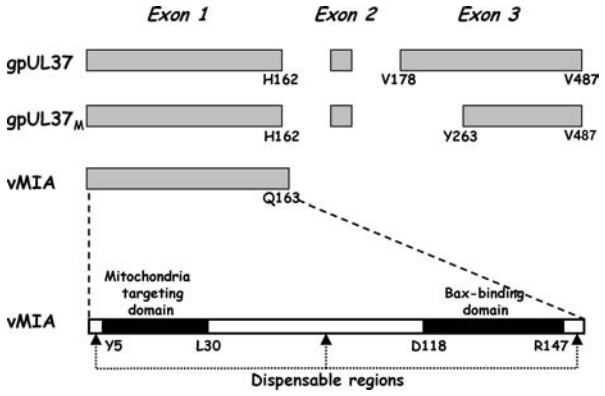
Table 2. Relative contributions of Bax and Bak into the mitochondrial apoptotic signaling pathway

Cell type	Species	Stimulus of apoptosis	Tentative phenotype[a]	Reference
HCT116 cell line	Human	Ligation of death receptors; tBid-mediated permeabilization of cell-free mitochondria; exposure to thapsigargin	Bax^{D}	[42] and references therein;[140]
HeLa cell line	Human	ligation of death receptors; exposure to various cytotoxic drugs; infection with E1B19k-deficient adenovirus	Bax^{D}	[42] and references therein
Normal fibroblasts	Human	Ligation of death receprtors; infection with vMIA-deficient human cytomegalovirus	Bax^{D}	[42] and references therein;[38,119a]
Glioblastoma	Human	Exposure to UV irradiation, staurosporine, or doxorubicin	Bax/Bak^{coD}	[61]
Rat-1 fibroblasts	Rat	c-myc overexpression	Bax^{D}	[141]
Embryonic fibroblasts	Mouse	Ligation of death receptors, or transfection with tBid; exposure to various cytotoxic drugs; UV irradiation; serum withdrawal	Bax/Bak^{coD}	[42,62]
Embryonic fibroblasts	Mouse	c-myc overexpression	Bax^{D}	[141]
COS-7 cells	African green monkey	Exposure to staurosporin	Bax^{D}	L. M. Bartle and V. S. Goldmacher, unpublished
pre-B cells	Mouse	IL-7 deprivation; deregulation of c-myc expression	Bak^{D}	[142]
Hepatocytes	Mouse	tBid-mediated permeabilization of cell-free mitochondria	Bak^{D}	[63]
Non-synaptosomal brain	Rat	tBid-mediated permeabilization of cell-free mitochondria	Bak^{D}	[143]
Baby mouse kidney (BMK) E1A-transformed dominant-negative p53 cells	Mouse	adenovirus TNF-α	Bax/Bak^{coD}	[144,145]
Jurkat T cell line	Human	Exposure to UV irradiation, staurosporin, VP-16, bleomycin, cisplatin, or transduction with an expression adenoviral vector for granzyme B	Bak^{D}	[146]
LoVo, DU145, and LS180 cell lines	Human	Bid-mediated permeabilization of cell-free mitochondria	Bax^{D}	[80]

[a]This classification is my interpretation of the published data.

contain *UL37 exon 1*-encoded sequence[31] have a similar cell death suppressing activity. Characterization of a panel of vMIA mutants with deletions across vMIA coding region revealed[41] that vMIA contains two functional domains, one confined within the Tyr^{5}-Leu^{30} segment, and the other confined within the Asp^{118}-Arg^{147} segment (Figure 3). These two domains are necessary and, together, sufficient for the cell death suppressing function of vMIA.[41] Tyr^{5}-Leu^{30} segment contains a previously unreported mitochondria-targeting motif and an endoplasmic reticulum targeting motif.[35,41] Asp^{118}-Arg^{147} segment contains a Bax-binding motif.[34,42] The two domains are functionally autonomous i.e. retain their functions as isolated polypeptides.[41,42]

Figure 3. Amino acid sequences of the three known functional cell death suppressors encoded by UL37 of HCMV.



Sequence homologues of vMIA

To date, sequence homologues of vMIA have been found only in genomes of non-human primate CMV (Table 1). Both the mitochondria-targeting domain and the Bax-binding domain of vMIA are highly conserved in Ch-vMIA, Rh-vMIA, and Agm-vMIA, while the rest of the amino acid sequence of vMIA is not conserved, suggesting that the main function of these proteins is cell death suppression. We tested Rh-vMIA and confirmed that this protein has an anti-apoptotic activity.[18]

MCMV may encode a cell death suppressor similar to vMIA in its mechanism of action

Only primate CMVs encode vMIA. Other CMVs and non-CMV betaherpesviruses do not encode any overt homologues of vMIA (and neither do any other viruses or organisms). Andoniou *et al*. recently reported[64] that MCMV-infected dendric cells become refractory to apoptosis induced by growth factor deprivation, and that in these cells Bax is associated with mitochondria where it forms mixed high-molecular-weight mixed aggregates with Bak. These data suggest that MCMV may encode a yet unidentified cell death suppressor (protein X) which functionally resembles vMIA, and thus that not only primate CMVs, but other CMVs as well target the mitochondrial apoptotic signaling pathway by sequestering Bax. One property in which this hypothetical cell death suppressor may differ from vMIA is that it appears to sequester not only Bax, but Bak as well.[64]

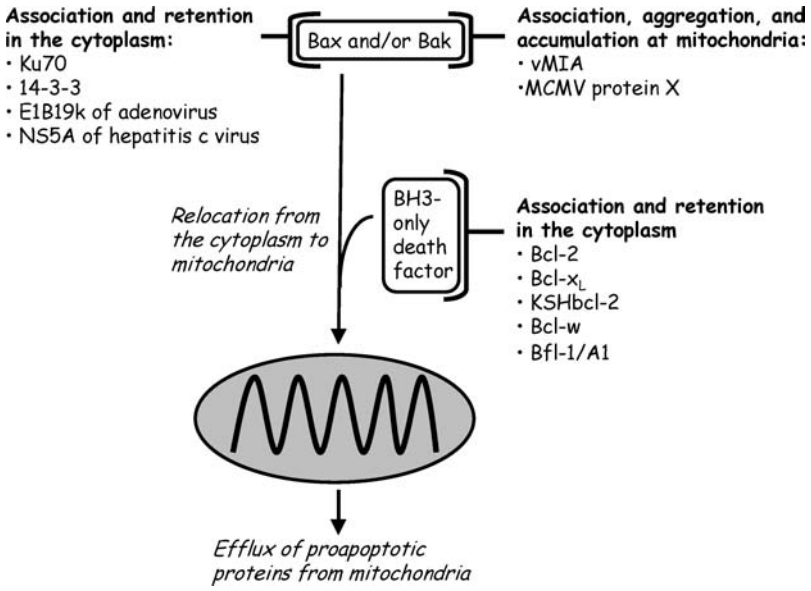
Possible functional roles of the longer splice variants of vMIA and of their homologues in CMV-infected cells

The two longer splice variants of vMIA, gpUL37 and $pUL37_M$ are dispensable for replication of HCMV in cultured fibroblasts, as long as vMIA is expressed.[29,65–67] On the other hand, genomes of various CMVs and, more broadly, betaherpesviruses (MCMV, rat CMV, tupaia herpesvirus, HHV-6 and HHV-7), contain sequence homologues (which are positional homologues as well) of *UL37 exon 3*, but are not homologous to vMIA (*UL37 exon 1*) region. Proteins encoded by these genes are thus unlikely to be cell death suppressors (based on their sequence alone). One of them, m37 of MCMV was characterized and it was found that m37 lacks anti-apoptotic activity.[18,64] m37 is dispensable for *in vitro* replication of MCMV, but essential for *in vivo* replication of MCMV.[68] gpUL37 and $pUL37_M$ of HCMV and their homologues of other betaherpesviruses may be important for replication of these viruses *in vivo* for a reason unrelated to cell death suppression.[5,69] Recently, it was reported[40] that gpUL37 of HCMV undergoes an internal cleavage that separates a vMIA-containing *N*-terminus polypeptide, which accumulates at mitochondria, and a pUL37exon-3-containing *C*-polypeptide, which accumulates at the endoplasmic reticulum, likely to perform separate functions.

Functional analogues of vMIA that are non-homologous to vMIA

Cell death suppressors that target the mitochondrial apoptotic signaling pathway upstream of the permeabilization of mitochondrial membrane can be tentatively divided into three classes (Figure 4). The first class is comprised of Bcl-2, Bcl-x_L, and, probably, KSHbcl-2, Bcl-w, and Bfl-1/A1, which appear to block apoptosis primarily through binding and sequestration of BH3-only death factors, and not through direct binding to Bax or Bak. The following observations support this notion. (i) Bcl-2 and Bcl-x_L bind and sequester BH3-only death factors during apoptosis.[47,70] (ii) Bcl-2 does not associate with Bax in the absence of Triton X-100.[71,72] (iii) KSbcl-2 does not associate with Bax or Bak.[73] (iv) Cell death suppressing activity of Bcl-w and A1 does not correlate well with their affinity for Bax or Bak.[74] (v) Bcl-2 and Bcl-x_L mutants that lack affinity to Bax retain their cell-death-suppressing activity.[75–78] (vi) Bcl-2 and Bcl-x_L localize at mitochondria of cells transfected with these genes, but this does not affect the cytoplasmic distribution of GFP–Bax in these cells.[79] (vii) The Bcl-2- and Bcl-x_L-induced resistance of cell-free mitochondria to tBid-induced permeabilization induced can be overcome by an excess of tBid.[42] (viii) A Bid mutant with a diminished affinity for Bax but intact affinity for Bcl-2 and Bcl-x_L has a decreased ability to permeabilize mitochondria, while a Bid mutant with intact affinity for Bax but a diminished affinity for Bcl-2 and Bcl-x_L has intact ability to permeabilize mitochondria.[80] The second tentative class of proteins which appear to target the mitochondrial apoptosis pathway includes the cellular proteins humanin,[81] Ku70,[82] and isoforms of 14-3-3,[83,84] and the viral proteins E1B19k, encoded by adenovirus,[85]

Figure 4. Three tentative classes of cell death suppressors, according to their mode of action, that target the mitochondrial apoptotic signaling pathway upstream of mitochondrial permeabilization.



and NS5A, encoded by hepatitis c virus.[86] These proteins bind to Bax, either in its non-activated, or activated form (E1B19k), and appear to prevent relocation of Bax to mitochondria during apoptosis. The third class of cell death suppressors targeting mitochondria includes vMIA and its homologues. Unlike Bcl-2, vMIA does not seem to interact with tBid, and unlike humanin, Ku70, or E1B19k, vMIA does not prevent relocation of Bax to mitochondria, but, on the contrary, induces spontaneous (in the absence of apoptosis) relocation of Bax to mitochondria and formation of mixed high-molecular-weight aggregates. vMIA does not share any significant amino acid sequence homology with any mitochondria-targeting cell-death suppressors except for a weak homology with humanin.

A number of other cellular and viral sequence homologues of Bcl-2[5,11] have been identified, which have not yet been well characterized, and it is not clear if they act through sequestering a BH3-only death factor, or through an interaction with Bax and/or Bak, or both. Also, a sequence-unrelated *Neisseria meningitides* PorB have been reported to interact with mitochondria of infected cells and protect cells from apoptosis by a not yet characterized mechanism.[87]

Other modes of cell death suppression/ survival promotion in CMV-infected cells

M41

Brune *et al*.[38] found that infection of murine fibroblast, endothelial, or bone marrow stromal cell lines with a *M*41-deficient MCMV mutant, triggers massive apoptosis of these cells, unlike the wild-type virus. The molecular function of *M*41 protein products is not known. One of these proteins, m41, is localized at the Golgi. This protein did not suppress Fas-mediated apoptosis in transiently transfected HeLa cells (Goldmacher and Brune, unpublished observations). In principle, m41 may be a cell death suppressor that either targets a pathway distinct from the Fas-mediated apoptotic pathway, or suppresses the death of murine but not of human cells. As an alternative, m41 may prevent cell death by supporting a vital physiological function in MCMV-infected cells.

NF-κB, a transcription factor that may function as a cell death suppressor, is induced in CMV-infected cells

Activation of NF-κB suppresses apoptosis induced by diverse stimuli,[88–91] in particular, apoptosis induced by infection with a virus distantly related to CMV, herpes simplex virus.[92] HCMV,[93,94] and MCMV[94] also induce activation of NF-κB, suggesting a possible role of NF-κB in suppression of apoptosis in CMV-infected cells. On the other hand, activation of NF-κB appears to not affect the replication kinetics of HCMV and MCMV in human and mouse permissive cells,[95] respectively, and it was recently reported[96] that NF-κB induced by certain cytotoxic stimuli represses cellular anti-apoptotic genes. More data will be needed to determine if NF-κB induced during CMV infections affects the survival/apoptosis of the infected cells.

Suppression of TNFR-1-mediated apoptosis in CMV-infected cells

Cells infected with HCMV become refractory towards TNFR-I-mediated apoptosis[29,97] by several mechanisms: vMIA activity,[29] vICA activity,[16] down-regulation of cell surface TNFR-I,[98] and, possibly, *via* an involvement of a potential decoy receptor, a product of *UL144*,[12] although there is no direct experimental evidence supporting the latter. It is not clear if CMV infection *in vitro* or *in vivo* triggers TNFR-I ligation and induces the TNFR-I-mediated apoptotic pathway.

IE1 and IE2 transcription factors of HCMV: do these proteins suppress cell death?

It was reported that HeLa cells transfected with either *IE1* or *IE2* were protected against apoptosis induced by either TNF-α, or by infection with an *E1B19k*-deficient adenovirus.[97] In our experiments, transient transfection with either *IE1* or *IE2* did not protect HeLa cells against apoptosis induced by TNF-α- or anti-Fas.[29] Another group reported that IE2 (but not IE1) protected coronary artery smooth muscle cells against cell death induced by doxorubicin and by ectopic p53.[99] In still another study it was found that a human astrocytoma clone constitutively expressing HCMV IE1 was more resistant to etoposide-induced apoptosis than the parental cell line.[100] More work will be needed to reconcile these data and to understand these phenomena better.

p53 and p73 may be inactivated or sequestered during CMV infection

The intracellular levels of p53 are greatly up-regulated several hours after the start of HCMV infection.[101,102] While elevation of the intracellular concentration of p53 under other circumstances has been implicated in induction of apoptosis *via* either its activity as a transcription factor (reviewed in[103]), and/or direct targeting of the mitochondrial apoptotic pathway,[104] it is not clear if p53 induces apoptosis during infections by cytomegaloviruses. Some observations suggest that p53 may be inactivated in CMV-infected cells, but the available data are inconclusive and, to a degree, contradictory. It was reported that in CMV-infected cells p53 was excluded from the nucleus,[105] apparently because of suppression of its nuclear localization signal,[106] and that the CMV-encoded immediate early protein IE2 bound to and inactivated p53.[99,101,107] Other researchers found that transfection of cells with *IE2* did not change p53 localization: p53 was still located in the nucleus.[108] Still another group observed p53 sequestered within the nuclei rather than in the cytoplasm of CMV-infected human fibroblasts.[109] Recently it was reported that HCMV infection up-regulates the expression of ΔN-p73, a dominant-negative isoform of p73, which inhibits activities of p53 and p73, and that the elevated expression of ΔN-p73 may contribute to the cell death suppressing activity of HCMV infection in some types of cells.[110]

M45 of MCMV

Infection with *M45*-deficient MCMV induces cell death of cultured murine endothelial cells, but not fibroblasts, bone marrow stromal cells, or hepatocytes.[111] The mutant virus is not virulent in SCID mice.[112] *M45* failed to protect HeLa cells against Fas-mediated apoptosis in transient transfection assays (Skaletskaya, and Goldmacher, and Lembo, unpublished). It is not clear yet if the m45 pro-survival activity stems from a direct blocking of an apoptotic pathway, or if this protein protects cells by another mechanism. m45 shares homology with the R1 subunit of HSV-2 ribonucleotide reductase which was recently reported to protect cells against apoptosis at, or upstream of, caspase-8 activation by an unknown mechanism.[113] The HCMV homologue of *M45*, *UL45*, is dispensable for replication of HCMV in endothelial and fibroblast cells in culture, and its deletion moderately sensitizes cells to Fas-mediated apoptosis but, unlike *M45*, does not induce spontaneous apoptosis during infection[66,67,114,115].

Upregulation of Bcl-2 and its homologues

Elevated expression of Bcl-x_L and Bcl-2 was reported in HCMV-infected endothelial cells,[116] and colon tumor[117] and neuroblastoma cells,[118] respectively. It is not clear how much these phenomena contribute to cell death suppression in infected cells since Bcl-2 and its homologues act mainly by sequestering BH3-only death factors and thus suppressing both relocation of monomeric Bax from the cytoplasm to mitochondria, and its activation (discussed above), but in HCMV-infected cells and MCMV-infected cells cytoplasmic Bax is depleted.[34,42,64]

Anti-apoptotic functions and replication of cytomegaloviruses

Apoptotic pathways induced during CMV infections

Until recently, it was not clear if CMV infections trigger apoptosis. The main difficulty in detecting this phenomenon was the ability of these viruses to suppress the apoptotic process (presumably after it has been initiated) in the infected cells.[29,64,97] The first direct evidence

that CMV infections induce intrinsic apoptosis came from studies with double vMIA-negative/vICA-negative HCMV mutants. These mutant viruses caused massive apoptosis of infected fibroblasts, a phenomenon reversed by introducing into the cells an anti-apoptotic gene targeting the mitochondrial apoptotic pathway, vMIA, Bcl-2, Bcl-x_L, or E1B19k.[38,119,119a]

In addition to intrinsic apoptosis, HCMV-infected cells appear to undergo apoptosis induced by the immune system as well. Cytotoxic T-cells (CTL) and natural killer cells (NK) constitute a key part of the immune response against CMV infections *in vivo*.[120–122] CTL and NK cells express Fas ligand on their surface, and Fas ligation appears to be a major mechanism of killing of Fas-expressing target cells by CTL and NK cells,[123,124] making it likely that Fas-ligand-mediated killing of CMV-infected cells is a major part of the immune response. Unlike TNFR-1,[98] Fas is not down-regulated during in HCMV-infected cultured normal human fibroblasts (Dionne and Goldmacher, unpublished).

vMIA is indispensable for replication of HCMV

There is strong evidence that the anti-apoptotic activity of vMIA is required for replication of HCMV. Neither $HCMV^{vICA-/vMIA-}$, nor $HCMV^{vICA+/vMIA-}$ are able to replicate in cultured fibroblasts; $HCMV^{vICA-/vMIA-}$ causes massive apoptosis in cultured fibroblasts;[38,66,67,119,119a] most of the *UL37x1* ORF sequence including the two functional domains of vMIA does not diverge in primary and in laboratory strains of HCMV.[41] The two domains are highly conserved in ChCMV, RhCMV, and AgmCMV,[18] indicating that that the anti-apoptotic activity of vMIA plays a major role in replication of these viruses as well.

The involvement of vICA in replication of CMVs

The anti-apoptotic function of vICA is dispensable for replication of HCMV and RhCMV in cultured fibroblasts,[16,18,125,66] and appears to be dispensable for *in vivo* replication and pathogenesis of RhCMV in rhesus macaques.[18] These results, and a close resemblance of RhCMV to HCMV (a high degree of sequence homology, co-linearity in their genome organization,[20] and similarity in the patterns of their replication and pathogenesis in their respective hosts[126,127]), suggests that vICA may also be dispensable for replication of HCMV *in vivo*, and it will be of interest to sequence *UL36* regions in clinical HCMV isolates in order to determine if any of the isolates contain inactivating mutations in *UL36*. In contrast to Rh-vICA, which is dispensable for *in vitro* and *in vivo* replication of RhCMV,[18] M-vICA is essential for *in vivo* replication of MCMV (Koszinowski, personal communication). The amino acid sequence of vICA is conserved throughout the betaherpesvirus sub-family, suggesting that its cell death suppressing function, while dispensable for viral replication of some CMVs, still provides an advantage for replication of betaherpesviruses in their hosts. One possible role for vICA during *in vivo* HCMV infections may be suppression of CTL or NK-induced apoptosis triggered by Fas ligation in infected type I cells of the host, where vMIA is not functional.

The kinetics of acquired resistance of HCMV-infected cells to apoptosis, and the expression of vICA and vMIA

vICA, a component of the HCMV virion, comes into contact with infected cells immediately following viral adsorption, but at a low level[125] not detectable by tandem mass spectrometry.[127a] Similarly, M-vICA was not detected in MCMV virions[127b], and vMIA in HCMV virions.[127a] The transcription of *UL36* and *UL37* in infected cells starts within several hours following viral absorption and continues throughout infection.[29,39,128,129] HCMV-infected fibroblasts start expressing detectable amounts of newly synthesized vICA and mitochondria-associated vMIA proteins by 4 h and 8 h after infection, respectively; vICA reaches its maximal expression by 8 h post-infection, while the mitochondrial expression of vMIA reaches its maximum later, probably by 48 h.[29,34,35,125] Expression of the *UL36* and *UL37* homologues of RhCMV and of the*UL36* homologue of MCMV was examined only at the mRNA level, and was found to be similar to those of *UL36* and *UL37*.[18]

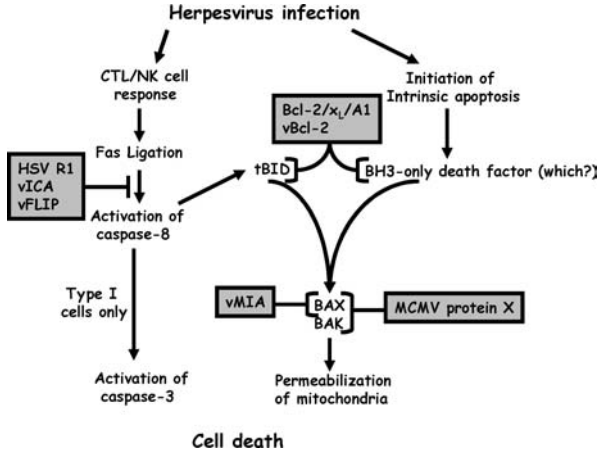
Fibroblasts infected with $HCMV^{vICA+/vMIA+}$ acquire resistance towards Fas-mediated apoptosis at about 24 h post-infection, while those infected with vICA-deficient strains of HCMV remain sensitive towards Fas-mediated apoptosis for an additional 12 to 24 h period.[16,29] This sequence of events correlates with the order of appearance of vICA and vMIA in infected cells. The delay between the onset of massive expression of vICA and the onset of resistance to apoptosis in HCMV-infected cells may reflect the time needed for the complex formation between vICA and pro-caspase-8. Similarly, the delay between the onset of massive mitochondrial expression of vMIA and the onset of resistance to apoptosis of cells infected with vICA-deficient HCMV may reflect the time needed for the depletion of cellular Bax. These coincidences between the kinetics of expression of vICA and vMIA, and the kinetics of acquired resistance of cells to apoptosis, supports the notion that infected cells are protected first by vICA, and later by both vICA and vMIA, against Fas-mediated apoptosis induced by effector cells of the immune system.

A comparison of the cell death suppression strategies of betaherpesviruses with those of alpha- and gammaherpesviruses

Mechanisms of cell death suppression used by betaherpesviruses bear similarities with those employed by other herpesviruses. Most gammaherpesviruses encode homologues of cFLIP, some of them proven functional (ref.[22,130] and Entrez database), and homologues of Bcl-2, some of them functional.[5,11] At least some alphaherpesviruses appear to interrupt Fas-mediated apoptosis at, or upstream of, caspase-8 activation,[113,131] and either increase the expression of cellular cell death suppressors targeting mitochondrial permeabilization, or decrease expression of cellular pro-apoptotic factors targeting mitochondrial permeabilization, or both.[132–34] In addition, some alphaherpesviruses encode several genes with less-well characterized anti-apoptotic activities.[13,135–139] Alphaherpesviruses may not require as stringent suppression of apoptosis as beta- and gammaherpesviruses because they may possibly evade apoptosis prior to its completion due to their faster replication cycle (suggested by J. Blaho).

A hypothetical model of cell death suppression during CMV infections, in comparison with cell death suppression induced by other herpesviruses, is shown in Figure 5. All three families of herpesviruses, alpha-, beta-, and gammaherpesviruses, appear capable of suppressing both the caspase-8 activation step of Fas-mediated apoptosis induced by the cytotoxic cells of the immune system, and the Bax/Bak-activation step of the intrinsic apoptosis induced by the infection. It remains to be determined which of the BH3-only death factors is the mediator of the intrinsic CMV-triggered apoptosis. In the case of HCMV, tBID is an unlikely candidate, otherwise the expression of vICA would be sufficient to suppress HCMV-induced apoptosis in the absence of vMIA, and it is not. It is also unclear if any herpesviruses suppress cell death induced by CTL granzyme-induced activation of caspases.

Figure 5. A comparison of hypothetical strategies of cell death suppression by alpha- beta- and gammaherpesviruses. Alphaherpesvirus HSV-2 R1, vICAs of betaherpesviruses, and vFLIPs of gammaherpesviruses block caspase-8 activation during Fas-mediated apoptosis triggered by the immune effector cells. HCMV vMIA and the MCMV hypothetical protein X sequester Bax, and Bax and Bak, respectively, interrupting intrinsic apoptosis induced by these viral infections. Cellular Bcl-2 and its homologues induced by some alpha- and betaherpesviruses, and vBcl-2 encoded by gammaherpesviruses sequester BH3-only death factors that emerge during Fas-mediated apoptosis (tBid), and during intrinsic apoptosis (a factor not yet identified) and thus suppress activation of Bax and Bak.



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