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Review

Enter the kill zone: Initiation of death signaling during virus entry

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ABSTRACT

Infection of host cells by a variety of viruses results in programmed cell death or apoptosis. In many cases, early events in virus replication that occur prior to synthesis of viral proteins and replication of viral genomes directly or indirectly activate signaling pathways that culminate in cell death. Using examples of viruses for which prodeath signaling is better defined, this review will describe how cell entry steps including virus attachment to receptors, virus uncoating in endosomes, and events that occur following membrane penetration lead to apoptosis. The relevance and physiologic consequences of early induction of prodeath signaling to viral pathogenesis also will be discussed.

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Introduction

Apoptosis is the programmed destruction of cells characterized by plasma membrane blebbing, cell shrinkage, nuclear condensation, and ultimately, degradation of genomic DNA. Apoptosis is induced by the activation of caspases (a family of cysteine proteases) through two different pathways (Shi, 2004; Sprick and Walczak, 2004) (Fig. 1). The intrinsic pathway is initiated when cellular stress is sensed by BH3-only proteins of the Bcl-2 family such as Bim, Bad, Puma and Noxa and leads to insertion of proapoptotic Bcl-2 family members such as Bax and Bak into the mitochondrial membrane (Breckenridge and Xue,

2004). The subsequent release of cytochrome c into the cytosol activates caspase-9 and results in formation of the apoptosome, a large protein complex comprised of cytochrome c along with Apaf-1 and caspase-9 (Liu et al., 1996; Zou et al., 1997; Narita et al., 1998; Srinivasula et al., 1998; Finucane et al., 1999). The apoptosome mediates the activation of caspase-3 and caspase-7 (Li et al., 1997), which execute the final stages of the apoptotic response, through degradation of many cellular proteins involved in DNA repair and maintenance of cellular architecture (Green, 2000). Optimal activation of this pathway also requires release of Smac/Diablo from the mitochondria along with cytochrome c. Smac/Diablo promotes apoptosis by relieving inhibition of caspase-3 and caspase-7 by inhibitors of apoptosis (IAPs) (Du et al., 2000; Srinivasula et al., 2000). The extrinsic pathway is activated by ligation of death domain-

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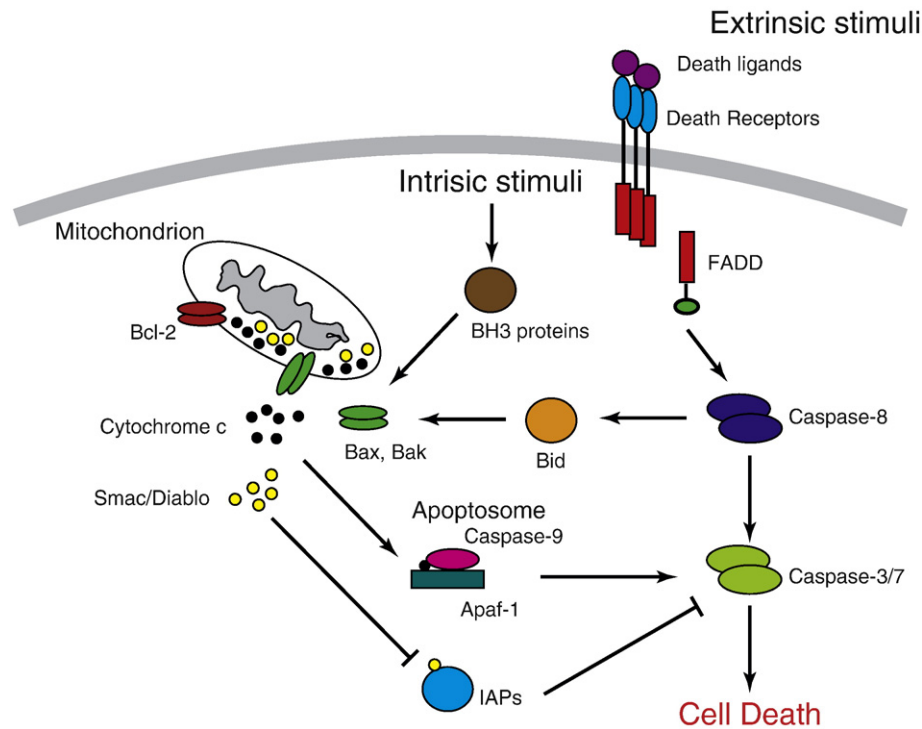


Fig. 1. Classical apoptotic pathways. Apoptotic death of host cells is dependent on cleavage of important cellular factors by effector caspases such as caspase-3 and 7. Two major pathways govern the activation of effector caspases. In the intrinsic pathway, intracellular stresses or insults sensed by the BH3-only members of the bcl-2 family promote the formation of the apoptosome through release of proapoptotic molecules such as cytochrome c and Smac/Diablo from the mitochondria. The apoptosome directly activates effector caspases. In the extrinsic pathway, ligation of death receptors such as Fas and TNF-R to death ligands such as FasL and TNF α results in formation of a death inducing signaling complex (DISC) which contains adaptor proteins including FADD. Signaling through DISC results in activation of initiator caspase, caspase-8, which directly mediates effector caspase activation.

containing receptors such as Fas, TNF-R, and TRAIL-R, which leads to formation of the death-induced signaling complex (DISC). The DISC contains adaptor proteins such as Fas-associated death domain (FADD) and TNF-R-associated death domain (TRADD) and mediates the activation of the initiator caspase, caspase-8 (Juo et al., 1998). Caspase-8 activation has two different consequences depending on the cell type (Scaffidi et al., 1998). In some cells, caspase-8 activation results in direct activation of the apoptosis effectors, caspase-3 and caspase-7 (Scaffidi et al., 1998). In other cells, usually under conditions when extrinsic apoptotic signaling results in insufficient levels of caspase-8 activation, apoptosis requires amplification of death signals through stimulation of the mitochondrial pathway via the function of another BH3-only member of the Bcl-2 family, Bid (Wei et al., 2000; Zha et al., 2000).

Apoptosis plays an important regulatory role in many biological processes such as organ development, cellular homeostasis, and immunity. A critical feature of apoptosis is that it does not produce inflammation. Thus cellular turnover and removal during the biological processes described above can occur with minimal activation of the host immune response (Opferman and Korsmeyer, 2003; Zhivotovsky, 2003). This prevents immune-mediated damage to healthy tissues and limits exposure of self-antigens. In addition to its importance in development and physiology, apoptosis is also commonly observed following viral infection of host cells.

As obligate intracellular parasites, viruses are dependent on the host for each stage of replication and therefore constantly interface with multiple components of the host cell machinery, including cellular receptors and uptake pathways, gene expression mechanisms and cell division apparatus. Viral appropriation of these systems may cause cell stress and activate death-signaling pathways or alter expression of genes that control cell survival. Therefore, virtually every stage in the viral replication cycle can evoke cell death. Thus, it is not surprising to find evidence for the proapoptotic effect of

multiple proteins from the same virus, each activating death signaling at a different stage of replication.

One of the earliest stages at which virus infection results in apoptosis is virus entry. Three distinct stages of virus entry into cells are known to activate cell death pathways (Fig. 2 and Table 1). First, apoptosis signaling can be initiated by binding of viruses to cell surface receptors. Second, apoptosis may be activated as a result of viral uncoating, either as a consequence of fusion between viral and host membranes, or due to membrane breach by nonenveloped viruses. Apoptosis also can occur as a consequence of exposure of viral components following disassembly. This review will focus on how these viral replication events that occur prior to de novo synthesis of viral genomes, mRNAs, or proteins evoke apoptotic cell death. Because the mechanisms that govern apoptosis following infection with each virus are not completely elucidated, the organization of the review is based on the most likely cell entry stage at which prodeath signaling is activated.

Initiation of apoptosis during virus attachment

Viruses use a variety of cell surface molecules including carbohydrates, lipids and proteins as receptors for attachment to cells, efficient internalization, or uncoating (Helenius, 2007). The physiologic functions of protein receptors utilized by viruses to enter cells include sensing soluble molecules such as growth factors and cytokines, binding to components of the extracellular matrix, and facilitating interactions between cells. Engagement of these cell surface proteins with their respective ligands often results in activation of signaling pathways, which can alter the properties of target cells. Because viruses often engage receptors in a manner analogous to their native ligands, they elicit a similar signaling

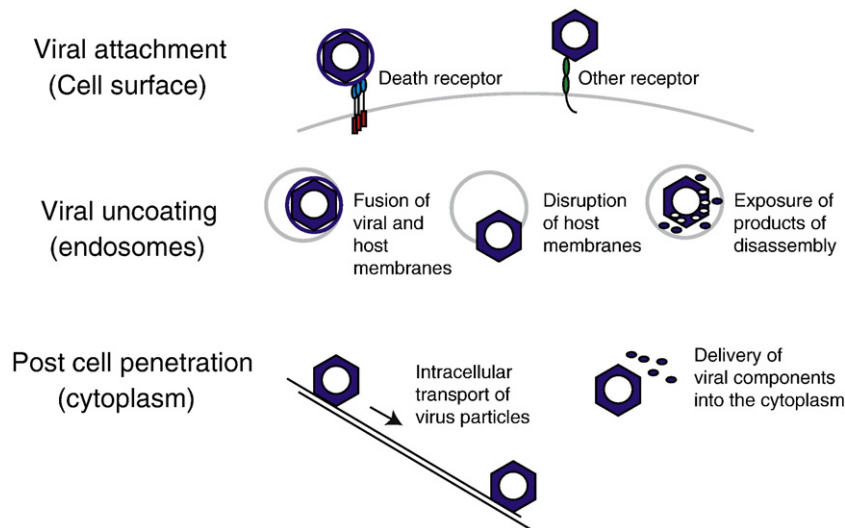


Fig. 2. Initiation of proapoptotic pathways during cell entry. Three different stages of virus infection that occur early in infection prior to de novo synthesis of viral mRNA and proteins can initiate death signaling. Virus-receptor interactions at the cell surface can trigger death signaling either directly by engagement of death receptors by virus or indirectly through activation of signaling pathways that regulate the function of classical apoptotic pathways. Virus uncoating within the endosome may also result in activation of prodeath signaling by perturbing the properties of host membranes through fusion of host and viral membranes by enveloped viruses or by disruption of membrane integrity by nonenveloped viruses. In addition, products of viral disassembly generated within endosomes may trigger signaling pathways that culminate in cell death. The delivery of subviral particles or disassembled viral components into the host cytosol subsequent to successful membrane penetration also can mediate proapoptotic signaling.

cascade. Viruses such as avian leukosis and sarcoma virus (ALV) and bovine herpesvirus-1 (BHV-1) are thought to induce death signaling by directly binding a cell surface receptor involved in death signaling. For other viruses, such as poliovirus, virus-receptor interaction has been implicated in initiation of prodeath signaling, although mechanisms by which proapoptotic signaling cascades are initiated as a consequence of receptor engagement are not known.

Apoptosis induction as a result of virus attachment to death receptor

The envelope (Env) protein of many retroviruses, including ALV, determines the capacity of the virus to produce cytopathic effect (CPE) (Dorner and Coffin, 1986; Riedel et al., 1988; Siliciano, 1996). This suggests that the envelope protein which functions in cell attachment and fusion-mediated entry, may directly contribute to cell killing. Clues about mechanisms by which the ALV Env protein contributes to cell death were obtained following identification of the cellular receptor for cytopathic ALV strains ALV-B and ALV-D by expression cloning methods (Brojatsch et al., 1996). Mouse cells that usually do not support infection by ALV were rendered permissive by

expression of a cDNA encoding CAR1, a member of the tumor necrosis factor receptor (TNF-R) family (Brojatsch et al., 1996). These findings, along with evidence for direct interaction of CAR1 with the surface (Su) subunit of ALV Env, indicate that CAR1 serves as a receptor for ALV (Brojatsch et al., 1996). Based on the evidence for the function of TNF-R family members in apoptosis, the capacity of ALV to induce cell death following CAR1 engagement was assessed. Soluble ALV-B-derived Su was capable of inducing apoptosis in a CAR-1 dependent manner (Brojatsch et al., 1996). These findings indicate that attachment of cytopathic ALV strains to CAR-1 results in apoptosis. Although the precise pathway to cell death evoked following CAR-1 ligation remains undefined, it is likely similar to proapoptotic signaling following attachment of death ligands with members of the TNFR family (Lavrik et al., 2005).

Apoptosis induction following BHV-1 infection of peripheral blood mononuclear cells or bovine B lymphoma cells also may be dependent on receptor binding. Initial investigation into the mechanism by which BHV-1 induces apoptosis indicated that BHV-1 virions inactivated by photochemical treatment with trioxsalen and UV irradiation remained capable of inducing apoptosis (Hanon et al.,

Table 1

Viruses that initiate prodeath signaling during cell entry.

Mechanism of apoptosis induction	Virus	Reference
Initiation of apoptosis during virus attachment		
Engagement of viruses to death receptors	Avian sarcoma and leukemia virus (ASLV) Bovine herpesvirus (BHV-1)	(Brojatsch et al., 1996) (Hanon et al., 1999)
Attachment of viruses to nondeath receptors	Poliovirus	(Gosselin et al., 2003)
Initiation of prodeath signaling during uncoating		
Endosomal uncoating of enveloped viruses	Iridoviruses Murine coronavirus Sindbis virus Vaccinia virus	(Chinchar et al., 2003; Chitnis et al., 2008) (Liu et al., 2003) (Jan et al., 2000) (Ramsey-Ewing and Moss, 1998)
Endosomal disassembly of nonenveloped viruses	Avian reovirus (ARV) Bluetongue virus (BTV)	(Labrada et al., 2002) (Mortola et al., 2004)
Initiation of prodeath signaling subsequent to membrane penetration		
Intracellular transport of viral particles	African swine fever virus (ASFV)	(Hernaiz et al., 2004)
Delivery of viral components into the postendosomal compartment	Mammalian reovirus (MRV)	(Connolly and Dermody, 2002; Danthi et al., 2006, 2008a)

1996). These findings suggested that early steps in viral infection, namely attachment to cells, fusion of viral and host membranes, or viral uncoating were sufficient to drive the death response. A mutant BHV-1, deficient in gH, an envelope glycoprotein dispensable for cell attachment but required for cell penetration, was found to be fully capable of eliciting apoptosis (Hanon et al., 1998). These findings suggest that death signaling following BHV-1 infection requires virus attachment but not penetration. Moreover, these findings indicated that BHV-1 glycoproteins other than gH are required for induction of cell death. To identify the viral trigger for apoptosis, the apoptosis-inducing capacity of a gD-deficient mutant was analyzed (Hanon et al., 1999). Although this virus was capable of efficiently attaching to cells, it failed to induce apoptosis, suggesting a function of gD in BHV-1-induced apoptosis (Hanon et al., 1999). These findings indicate that engagement of one or more cell surface molecules by gD is required for BHV-1-induced apoptosis. Purified gD, was incapable of inducing apoptosis although it could prevent BHV-1-induced apoptosis likely by competing for virus attachment (Hanon et al., 1999). These findings suggest that gD alone is not sufficient for apoptosis. However, it is also possible that gD fails to induce apoptosis in its purified form due to its inability to crosslink receptors in a manner analogous to binding by intact BHV-1 virions. The precise mechanism by which attachment of BHV-1 evokes death signaling remains unknown. Based on evidence from related herpes simplex virus (HSV-1), whose gD protein binds the TNF-R family member HVEM (Montgomery et al., 1996), it is hypothesized that death signaling pathways may be directly activated by virus-receptor interactions. However, experimental evidence to support this idea is lacking.

Apoptosis following virus attachment to receptors other than death receptors

Infection of cell such as enterocytes, dendritic cells, monocytes, and neuronal cells with poliovirus results in apoptosis (Ammendolia et al., 1999; Girard et al., 1999; Lopez-Guerrero et al., 2000; Wahid et al., 2005). Although the precise mechanism by which early steps in poliovirus infection lead to apoptosis remains unknown, analysis of neuroblastoma cells persistently infected with poliovirus suggests that virus-receptor interaction contributes to poliovirus-induced cell death.

Persistent infection of neuroblastoma cells with poliovirus results in mutation in the membrane distal domain of poliovirus receptor (PVR/CD155), which is important for virus attachment (Freistadt and Racaniello, 1991; Morrison et al., 1994; Gosselin et al., 2003). To determine the physiologic consequence of the observed PVR mutation, the capacity of the mutant receptor to mediate poliovirus infection in non-permissive cells was assessed. Although the mutant receptor supported poliovirus attachment and infection to the same extent as the wild-type receptor, cells expressing the mutant receptor displayed a markedly lower level of apoptosis (Gosselin et al., 2003). These findings suggest a function of PVR in poliovirus-induced apoptosis. It is not known how PVR contributes to poliovirus-induced cell death. One possibility is that poliovirus-PVR interaction triggers death signaling through PVR (Gosselin et al., 2003). However, since virus attachment appears to be unaffected in cells expressing mutant receptors, it is not clear how a mutation in the PVR membrane-distal domain can influence proapoptotic signaling. Another possibility is that PVR interacts with host molecules involved in prodeath signaling. Determining the physiologic function of PVR and studies using cytoplasmic tail-deleted PVR molecules, which are known to support poliovirus infection (Freistadt and Racaniello, 1991), or analysis of PVR-associated proteins such as CD44 (Shepley and Racaniello, 1994; Freistadt and Eberle, 1997) may help address these questions. Regardless of the mechanism, c-Jun N terminal kinase (JNK)-mediated activation of the mitochondrial apoptotic pathway is required for poliovirus-induced apoptosis (Autret et al., 2007). JNK activation

promotes release of cytochrome c and results in loss of mitochondrial membrane potential in a Bid- and Bim-independent but Bax-dependent manner (Autret et al., 2007). Interestingly, although UV-inactivated poliovirus can activate JNK, it fails to induce apoptosis (Autret et al., 2008). These findings suggest that poliovirus-PVR interaction is sufficient to activate JNK signaling. In addition, these results indicate that steps in the poliovirus replication cycle that occur subsequent to receptor engagement work in concert with signaling pathways initiated by virus attachment to induce apoptosis. These steps could include expression of poliovirus proteases 2A and 3C following translation of viral RNA. Ectopic expression of these viral proteases induces apoptosis, likely by cleaving cellular molecules involved in host transcription and translation (Barco et al., 2000; Goldstaub et al., 2000; Calandria et al., 2004).

The combined requirement for receptor engagement and de novo synthesis of viral RNA and proteins for apoptosis induction by poliovirus observed in cells of the neuronal origin contradicts findings from studies of poliovirus-induced apoptosis of HeLa cells. Although productive poliovirus infection of HeLa cells does not evoke apoptosis, blockade of poliovirus infection prior to viral protein synthesis results in apoptosis (Tolskaya et al., 1995). Based on these findings, it has been proposed that the initial stages of poliovirus infection, which occur prior to translation of the incoming viral genome evoke prodeath signaling, whereas synthesis of viral proteins blocks apoptosis (Agol et al., 2000). While it remains unclear why different research groups observed incongruent requirements for apoptosis, a simple explanation for this finding could be that poliovirus utilizes distinct mechanisms to induce apoptosis in different cell types. Thus it is possible that for each cell type, the outcome of poliovirus infection is dependent on the balance between the strength of pro- and antiapoptotic signaling.

Initiation of prodeath signaling during uncoating

Following attachment to cell surface receptors, many viruses are internalized by cellular uptake pathways and delivered into the host endocytic compartments. Virus particles are disassembled or uncoated in the endosomes to facilitate the release of their genomes into host cells. For enveloped viruses, viral uncoating requires conformational changes in the envelope glycoproteins that drive fusion between the viral and host membranes (Harrison, 2008; White et al., 2008). Some viruses use endosomal proteases to cleave their envelope glycoproteins, thereby priming them for fusion (Harrison, 2008; White et al., 2008). Others use the low pH environment of the endosome to drive fusion-inducing conformational changes in the glycoproteins (Harrison, 2008; White et al., 2008). The endosomal environment also can affect the uncoating of nonenveloped viruses. In some cases, endosomal proteases remove a protective outer coat from the virus to expose the membrane penetration machinery. Alternatively, the low pH environment induces conformational changes in viral capsid proteins to expose the membrane penetration apparatus. The newly exposed regions facilitate delivery of the viral genomes into cells by forming pores or channels in membranes, or by rupturing them (Tsai, 2007; Banerjee and Johnson, 2008). Perturbation of membranes as a consequence of fusion or disruption can initiate signaling pathways that evoke cell death.

Apoptosis induced by endosomal uncoating of enveloped viruses

For a variety of enveloped viruses – Iridoviridae (Chinchar et al., 2003; Chitnis et al., 2008), murine coronavirus (Liu et al., 2003), Sindbis virus (Jan and Griffin, 1999), and vaccinia virus (Ramsey-Ewing and Moss, 1998), entry steps that occur within endosomes are thought to initiate prodeath signaling. In each case, lysosomotropic agents that block infection by inhibiting fusion between host and viral membranes block apoptosis. Studies using UV-inactivated virus or agents that block post-

entry steps in viral replication suggest that endosomal entry events are sufficient to induce apoptotic cell death.

Although it is not clear how these otherwise unrelated enveloped viruses initiate apoptosis at a common step in infection, studies on Sindbis virus offer some clues about how uncoating of an enveloped virus can induce death signaling. Following delivery into endosomes, Sindbis virus envelope glycoproteins undergo low-pH-dependent conformational changes (Bron et al., 1993). Fusion of host and viral membranes by these conformationally altered glycoproteins requires the presence of sphingomyelin in host cell membranes (Nieva et al., 1994). During entry into cells, Sindbis virus rapidly activates acid sphingomyelinase leading to the generation of ceramide (Jan et al., 2000). The initial increase in ceramide is sustained by activation of neutral sphingomyelinase at later times following infection (Jan et al., 2000). Decreasing intracellular ceramide levels by overexpression of ceramidase results in diminishment of apoptosis (Jan et al., 2000). Based on these findings, and the function of ceramide in inducing apoptosis following exposure to other stimuli (Pettus et al., 2002), it is thought that ceramide generation by activation of sphingomyelinases during entry stimulates prodeath signaling in Sindbis virus-infected cells (Jan et al., 2000).

Although it is not clear how sphingomyelinases are activated during entry of Sindbis virus into host cells, the above findings suggest a role for viral structural components in initiating this response. Consistent with this idea, native Sindbis virus particles elicit greater cytopathology than Sindbis replicons that lack viral structural proteins (Frolov and Schlesinger, 1994). Furthermore, ectopic expression of envelope glycoproteins, E1 or E2, or their transmembrane domains alone induces apoptosis (Joe et al., 1998), suggesting that the transmembrane domains of E1 and E2 may directly interact with acid sphingomyelinases to induce their activation during fusion (Joe et al., 1998). Further studies are needed to determine if E1 or E2 contribute to death signaling during cell entry and to delineate molecular mechanisms underlying the activation sphingomyelinases. It is unknown how ceramide accumulation leads to cell death following infection with Sindbis virus. Pharmacologic inhibitors that block the function of ceramide targets such as protein kinases and phosphatases diminish apoptosis suggesting the importance of protein phosphorylation in Sindbis virus-induced cell death (Jan et al., 2000). However, since these inhibitors target a wide variety of molecules, the precise signaling cascade stimulated by ceramide is not known. Other studies also have indicated a function for reactive oxygen species and transcription factor NF- κ B in cell death signaling following Sindbis virus infection (Lin et al., 1995). However, the relationship between these pathways and ceramide-induced death signaling remains unknown.

Apoptosis induction during endosomal disassembly of nonenveloped viruses

A characteristic feature of the reoviridae family of viruses is the presence of concentric protein shells, the outer capsid and the core (Roy, 2007; Schiff et al., 2007). The outer capsid is partially disassembled within the endocytic compartment (Roy, 2005; Danthi et al., 2010a), thereby revealing the membrane-penetration protein which functions to allow the delivery of viral core across membranes into the cytoplasm for replication. Apoptosis induction by at least two different members of the reoviridae family, avian reoviruses (ARV) and bluetongue virus (BTV) is thought to occur during virus disassembly in the endosome.

Treatment of infected cells with ribavirin, which blocks de novo synthesis of ARV or BTV mRNA does not affect virus-induced cell death (Labrada et al., 2002; Mortola et al., 2004). These findings suggest that de novo synthesis of viral RNA and proteins are not required for apoptosis induction by ARV and BTV. Consistent with this idea, UV-inactivated virions of ARV and BTV retain the capacity to

induce apoptosis (Labrada et al., 2002; Mortola et al., 2004). In each case, inhibitors of endosomal acidification, such as ammonium chloride or chloroquine, which block virus disassembly, prevent apoptosis induction (Mortola et al., 2004). These findings suggest that viral products generated during endosomal disassembly of both ARV and BTV trigger apoptosis.

Despite this similarity, it is not entirely clear if these viruses trigger apoptosis by a shared mechanism. For ARV, initial studies suggested that the apoptosis inducing-factor could be viral attachment protein σ C, which unlike its mammalian reovirus (MRV) counterpart σ 1, induces apoptosis following ectopic expression (Shih et al., 2004; Coffey et al., 2006). However, subsequent studies demonstrated that initiation of signaling pathways that contribute to σ C-induced apoptosis require viral replication (Ping-Yuan et al., 2006), suggesting that σ C may induce apoptosis at a later stage of infection. Thus, the viral intermediary that connects ARV entry steps to the cell death machinery remains undefined. How BTV infection results in apoptosis is much better defined. Ectopic expression of either viral attachment protein VP2, or the viral entry effector VP5 does not result in apoptosis (Mortola et al., 2004; Roy, 2005; Forzan et al., 2007). Interestingly, extracellular co-administration of VP2 and VP5, which likely results in uptake of VP2-VP5 complexes into the endosomes, induces apoptosis (Mortola et al., 2004). These findings indicate the function of these capsid components in the endosome triggers apoptosis. Apoptosis induction by bluetongue virus requires signaling via JNK and the activation of transcription factor NF- κ B (Mortola et al., 2004; Mortola and Larsen, 2010). The similarity in the requirement for the function of the membrane penetration protein and for signaling via JNK and NF- κ B for both bluetongue virus and MRV (as described below) suggests that these two viruses share a common strategy for apoptosis induction. However, further work is needed to determine if this is indeed the case.

Initiation of prodeath signaling subsequent to membrane penetration

Different types of payloads are delivered into cells following successful penetration of membranes by viruses. Consequently these payloads have different fates. For viruses such as poliovirus, the cargo delivered into the cells is free genomic positive sense RNA, which is directly subjected to translation. For other viruses such as Sindbis virus, Adenovirus and African swine fever virus (ASFV), the viral nucleocapsid may be delivered into the cell for further uncoating within the cytosol or at the nuclear membrane. Members of the reoviridae family, which contain concentric protein shells, deliver the inner capsid or core into the cytoplasm to initiate transcription of viral RNA by the core-associated viral polymerase. It is possible that delivery of these components directly or indirectly activates proapoptotic signaling pathways.

Apoptosis induction during intracellular transport of viral capsids

ASFV infection leads to apoptosis of infected cells (Oura et al., 1998). Analogous to viruses that appear to initiate proapoptotic signaling during uncoating steps within cellular endosomes, ASFV-induced apoptosis is blocked by lysomotrophic agents that block virus entry but unaffected by agents that interfere with DNA or protein synthesis (Carrascosa et al., 2002). These data implicate steps associated with ASFV entry in the induction of apoptosis. Following low pH-driven fusion of viral and host membranes, the viral nucleocapsid deposited into the cytoplasm is transported along microtubules to the perinuclear region for replication. Microtubule-dependent transport of the nucleocapsid to the perinuclear region requires viral protein p54, which binds directly to the light chain of cytoplasmic dynein (Alonso et al., 2001). Because virus transport to the perinuclear region also would be blocked by lysomotrophic agents,

but not affected by pharmacologic inhibitors that block viral DNA and protein synthesis, it is possible that these cytosolic events contribute to initiation of death signaling following ASFV infection.

Consistent with this idea, plasmid-driven p54 expression activates effector caspases and evokes apoptosis (Hernaiz et al., 2004). Since the apoptosis-inducing capacity of p54 appears to be dependent on a 13 amino acid motif that mediates interaction with dynein light chain, it has been suggested that p54-dynein interaction may play a role in induction of prodeath signaling (Hernaiz et al., 2004). It is not clear if a similar interaction between p54 and dynein early during ASFV infection contributes to apoptosis. How p54-dynein interaction leads to apoptosis also remains unknown. Bim, a proapoptotic BH3-only member of the Bcl-2 family (O'Connor et al., 1998), is translocated to the mitochondria following ASFV infection (Hernaiz et al., 2004). Interestingly, in its inactive state, Bim is associated with microtubules via its interaction with dynein light chain using a binding site very similar to ASFV p54 (Puthalakath et al., 1999; Hernaiz et al., 2004). These findings raise the possibility that p54 induces apoptosis by displacing Bim from the microtubules, which results in its relocalization to mitochondria leading to activation of the intrinsic apoptotic pathway. Although Bim is relocalized from microtubules to mitochondria during ASFV infection (Hernaiz et al., 2004), whether this relocalization is a consequence of p54-dynein interaction remains to be determined.

Apoptosis induction by delivery of viral components into the postendosomal compartment

In cultured cells, MRV-induced apoptosis is not dependent on *de novo* synthesis of viral RNA and protein (Connolly and Dermody, 2002; Danthi et al., 2006), indicating that components of the incoming viral capsid are sufficient for initiation of prodeath signaling. Consistent with these findings, differences in the capacity of prototype reovirus strains to induce apoptosis segregate genetically with the S1 and M2 gene segments (Tyler et al., 1995, 1996; Connolly et al., 2001), which encode the viral attachment protein $\sigma 1$ and the viral membrane penetration protein $\mu 1$, respectively (McCrae and Joklik, 1978; Mustoe et al., 1978). Although initial studies suggested that attachment of MRV $\sigma 1$ with JAM-A or sialic acid on the host cells is important for apoptosis (Barton et al., 2001; Connolly et al., 2001), antibody-dependent uptake of MRV virions into host cells in a JAM-A- and sialic acid-independent manner also leads to apoptotic cell death, indicating that signaling pathways triggered by $\sigma 1$ -receptor interactions are dispensable for MRV-induced apoptosis (Danthi et al., 2006). These findings also suggest that the previously suspected functions of $\sigma 1$, JAM-A, and sialic acid in MRV-induced apoptosis are related to the requirement of high affinity binding of MRV to the host cell surface in order to efficiently initiate infection of host cells.

Regardless of the receptors used to mediate attachment, initiation of prodeath signaling following MRV infection requires viral disassembly in cellular endosomes (Connolly and Dermody, 2002; Danthi et al., 2006), suggesting an essential function for the $\mu 1$ protein in apoptosis induction. Introduction of single amino acid substitutions into the central δ region of $\mu 1$ decreases the capacity of the resultant mutant viruses to effect membrane penetration, mobilize NF- κ B, and evoke apoptosis (Danthi et al., 2008b). These findings suggest that the membrane-penetration and apoptosis-induction functions of $\mu 1$ are linked and that the δ region of $\mu 1$ is an essential modulator of both processes (Danthi et al., 2008b). It is possible that membrane penetration directly initiates proapoptotic signals. Alternatively, membrane penetration might allow delivery of the $\mu 1$ cleavage fragments into the cytoplasm where prodeath signaling is elicited. Two lines of evidence support the latter possibility. First, plasmid-driven expression of the $\mu 1$ C-terminal ϕ domain in the cytoplasm is sufficient to induce apoptosis (Coffey et al., 2006). Second, recombinant viruses with engineered substitutions in ϕ are diminished in NF-

κ B activation and apoptosis induction (Danthi et al., 2008a). Importantly, a membrane-penetration-proficient ϕ mutant is impaired in the capacity to activate prodeath signaling, indicating that ϕ modulates apoptosis independent of an effect on membrane penetration (Danthi et al., 2008a). Based on these findings, delivery of ϕ to the cytoplasm subsequent to membrane penetration may initiate prodeath signaling following MRV infection (Danthi et al., 2008a).

There are two possible models to explain how delivery of ϕ into the cytosol leads to cell death. First, ϕ localizes to the mitochondria and endoplasmic reticulum following expression from plasmids, suggesting that ϕ triggers apoptosis by directly altering mitochondrial integrity and promoting release of prodeath molecules such as cytochrome c (Coffey et al., 2006; Wisniewski et al., 2010). Second, since transcriptional activity of NF- κ B is required for prodeath signaling following MRV infection (Danthi et al., 2010b), ϕ may induce apoptosis by stimulating expression of proapoptotic targets of NF- κ B.

A unique combination of upstream regulators, I κ B kinase (IKK) α and the NEMO adaptor are required for MRV-induced apoptosis (Hansberger et al., 2007), but how their signaling function is co-opted by MRV ϕ remains unknown. In addition to NF- κ B signaling, MEKK1-mediated activation of JNK signaling also is required for activating the mitochondrial apoptotic pathway (Yujiri et al., 2000; Clarke et al., 2001, 2004). While the $\mu 1$ -encoding M2 gene segment also has been implicated in regulating JNK signaling (Clarke et al., 2001), the mechanism by which viral proteins initiate this signaling cascade also remains undefined. The innate immune transcription factor IRF-3 also contributes to proapoptotic signaling following MRV infection (Holm et al., 2007). Activation of IRF-3 requires the detection of genomic dsRNA by RIG-I and MDA-5 (Holm et al., 2007; Loo et al., 2008), and also occurs during virus entry independent of *de novo* synthesis of viral RNA (Holm et al., 2007). Since MRV genomic dsRNA is thought to remain encapsidated in viral cores throughout the viral infectious cycle, how it is exposed to cellular sensors such as RIG-I and MDA-5 to initiate signaling pathways that contribute to prodeath signaling via IRF-3 remains unknown. Although a function for IRF-3 in apoptosis induction has been defined for other systems (Chattopadhyay et al., 2010), how IRF-3 modulates the cellular death machinery following MRV infection is not clear.

Conclusions and future directions

Viruses from wide variety of families induce apoptosis during three different cell entry steps – attachment, uncoating and post-invasion. Although unrelated viruses appear to share mechanisms that govern the execution of apoptosis, much remains unknown about how proapoptotic signaling is initiated during virus entry into cells and what its effects are on the pathogenesis of viral disease.

Mechanisms of virus entry-induced death signaling

For a complete understanding of virus host interactions that lead to apoptosis, it is important to know the identity and nature of viral factors that initiate the death response and its cellular binding partners. Other than apoptosis induced by virus-receptor interaction, knowledge about a viral trigger for apoptosis and its host target is either incomplete or unknown. For apoptosis initiated as a consequence of virus-receptor engagement, the viral attachment protein (Env for ASLV) or a structural feature (the canyon region for poliovirus) is important for binding to its cell surface receptor (Brojatsch et al., 1996; Belnap et al., 2000). When apoptosis is initiated during uncoating within endosomes, it is possible that a specific viral factor initiates a death response by binding to an as yet unknown endosomal host factor. Alternatively, proapoptotic signaling may be initiated by cell stress produced due to changes in membrane properties as a consequence of membrane fusion or membrane

rupture. Apoptosis induction subsequent to membrane invasion is dependent on a specific viral factor (ASFV p54 and MRV ϕ) (Hernaiz et al., 2004; Danthi et al., 2008a). However, it remains unknown if these factors evoke prodeath signaling by directly binding a specific host factor resident in the cytoplasm (such as dynein for ASFV p54) or by injuring cellular organelles such as mitochondria (as has been suggested for MRV ϕ) (Coffey et al., 2006; Danthi et al., 2008a; Wisniewski et al., 2010).

For most viruses, how virus host interactions that occur during cell entry leads to activation of intrinsic or extrinsic apoptotic pathways also remains unknown. In many cases, viral components initiate cell death signaling by binding to cellular factors not known to directly play a role in death signaling (such as poliovirus attachment to PVR). In others, viruses appear to activate death signaling from within endosomes, which are not usually known to contain components of the death machinery. In many of these cases, initiation of death signaling appears to be indirect. For example, acid sphingomylinase activity stimulated as a result of Sindbis virus uncoating in endosomes initiates a signaling cascade requiring kinases and phosphatases (Jan et al., 2000). Although the nature of these signaling pathways is unclear, it is possible that such kinases and phosphatases activate death signaling by directly interfacing with a critical component regulating initiator or effector caspase activity (Kurokawa and Kornbluth, 2009). For some viruses, indirect activation of death signaling may require de novo expression of host genes. For example, MRV-induced death signaling is dependent on activity of NF- κ B (Connolly et al., 2000; Hansberger et al., 2007; Danthi et al., 2010b). Further studies are needed to define how host proteins that detect viral components initiate a signaling cascade that culminates in cell death.

Relevance and consequences of virus entry-induced death signaling

Apoptosis induction by many of the viruses described above is only evident when infection is initiated at high multiplicity (Joe et al., 1998; Connolly and Dermody, 2002; Danthi et al., 2006). Because of this requirement, apoptosis evoked by this mechanism is often considered to be an artifact of the experimental conditions and questions are raised about its relevance (Urban et al., 2008). Two lines of evidence support the idea that this mechanism of apoptosis also may be relevant in vivo. First, histological analyses of organs of experimentally infected animals indicate only a small subset of cells within a particular organ is antigen positive even though the viral titer in the organ homogenate is relatively high. For example, peak titer of MRV in infected mouse brains is greater than 10^8 PFU per brain even though a very small fraction (<5%) of the total cells are positive for reovirus antigen (Danthi et al., 2008a,b, 2010b). Similar observations also have been made in the central nervous system (CNS) of mice infected with Sindbis virus (Levine et al., 1996). Thus, it is possible that the effective MOI in local foci within the organs is indeed high, and that apoptosis may be initiated during entry of virus into cells adjacent to those initially infected. Second, evidence supporting the in vivo function of viral and cellular factors previously identified to be important for entry-induced apoptosis in cell culture suggests a shared mechanism. For example, for MRV, ϕ mutant viruses are apoptosis defective both in cell culture and in vivo (Danthi et al., 2008a). Similarly, reovirus-induced apoptosis in cell culture and in the CNS is diminished in the absence of the NF- κ B p50 subunit (Connolly et al., 2000; O'Donnell et al., 2005).

The physiological consequence of apoptosis induced by virus entry into cells varies with each viral system, and is likely dependent on the rate of viral replication and the speed with which the host cell succumbs to death signaling initiated during entry. In some cases, initiation of death signaling during virus entry may be an intrinsic host response to invasion by a virus (Roulston et al., 1999; Hay and Kannourakis, 2002). For this response to be successful, the cell must undergo apoptotic cell death prior to completion of virus replication.

Premature death of cells is detrimental to the virus since it prevents spread of progeny viruses to neighboring cells. In order to overcome this host response, some viruses, especially those that replicate slowly and have genomes with larger coding capacity encode proteins that delay or prevent apoptotic cell death until viral replication is completed (Benedict et al., 2002; Hay and Kannourakis, 2002). Among viruses discussed in this review, a late event in poliovirus replication blocks apoptosis (Tolskaya et al., 1995) and ASFV encodes antiapoptotic proteins (Nogal et al., 2001; Galindo et al., 2008). In other cases, induction of apoptosis by a virus early in its replication cycle may be a viral immune evasion strategy. Induction of prodeath signaling by the virus may prevent or attenuate the development of immune response, thereby allowing the virus to replicate its genome and generate progeny (Roulston et al., 1999; Hay and Kannourakis, 2002). It is possible that even for the same virus, the consequence of initiation of apoptosis during cell entry differs between cell types or between hosts. Thus, the true outcome of death signaling initiated during entry may only be evident during examination of viral pathogenesis. For example, apoptosis induction by MRV does affect viral replication in cell culture but appears to enhance viral replication within the murine CNS (Danthi et al., 2010b).

Regardless of whether the virus or the host benefits from apoptosis initiated during virus entry, death of infected cells has a profound impact on the pathogenesis of viral disease. In cases where virus infects and induces apoptosis in terminally differentiated, non-renewable cell population such as neurons or cells that are critical to the physiology of the host, such as myocytes or liver cells, apoptosis of infected cells often correlates with and contributes to the severity of disease. Among viruses that induce apoptosis during cell entry, pathogenesis of ASFV, BHV-1, Sindbis virus and MRV is associated with apoptosis (Lewis et al., 1996; Oberhaus et al., 1997; Oura et al., 1998; Jones and Chowdhury, 2007). Pharmacologic blockade of apoptosis alleviates viral disease in experimental animals (DeBiasi et al., 2001, 2004; Beckham et al., 2007). These findings indicate that virus-induced death signaling may be a viable antiviral target. Ongoing studies to enhance an understanding of the virus-host interface that initiates death signaling and the signaling cascade that connects these initial events to activation of classical apoptosis pathways will highlight novel therapeutic targets to ameliorate the deleterious effects of apoptosis on viral disease.

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