Published in final edited form as:

Annu Rev Microbiol. 2008 ; 62: 171–192. doi:10.1146/annurev.micro.62.081307.163009.

Viral Subversion of Apoptotic Enzymes: Escape from Death Row*

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Abstract

To prolong cell viability and facilitate replication, viruses have evolved multiple mechanisms to inhibit the host apoptotic response. Cellular proteases such as caspases and serine proteases are instrumental in promoting apoptosis. Thus, these enzymes are logical targets for virus-mediated modulation to suppress cell death. Four major classes of viral inhibitors antagonize caspase function: serpins, p35 family members, inhibitor of apoptosis proteins, and viral FLICE-inhibitory proteins. Viruses also subvert activity of the serine proteases, granzyme B and HtrA2/Omi, to avoid cell death. The combined efforts of viruses to suppress apoptosis suggest that this response should be avoided at all costs. However, some viruses utilize caspases during replication to aid virus protein maturation, progeny release, or both. Hence, a multifaceted relationship exists between viruses and the apoptotic response they induce. Examination of these interactions contributes to our understanding of both virus pathogenesis and the regulation of apoptotic enzymes in normal cellular functions.

Keywords

caspase inhibition; serine protease; CrmA; p35; IAP; vFLIP

INTRODUCTION

The successful replication of a virus within an individual cell requires a remarkable cascade of interactions between virus and host, beginning at the first engagement of the cell receptor to the final release of progeny virions. Viruses utilize everything from cellular enzymes and transcription factors to membranes and organelles to facilitate their replication (11). However, hijacking of the cell by a virus is not without a potent antiviral response. One such response is apoptosis, the genetically and biochemically controlled process of cell death that functions in development and homeostasis of multicellular organisms through selective removal of unwanted or damaged cells (120). Apoptosis can also serve as an innate cellular response to infection that limits both the time and cellular machinery available for virus replication (120). Many of the key biochemical events that occur during apoptosis are mediated by proteases (62), the most important of which are caspases (cysteine-dependent aspartate-specific proteases). Not surprisingly, viruses from diverse families have evolved mechanisms to evade or delay cell death by suppressing the activity of caspases and other enzymes with central roles in the implementation of apoptosis. Examination of interactions between viruses and the host apoptotic machinery has contributed extensively to our understanding of virus replication and pathogenesis. Furthermore, the elucidation of how viruses modulate these responses has

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DISCLOSURE STATEMENT

The author is not aware of any biases that might be perceived as affecting the objectivity of this review.

furthered our knowledge of apoptotic pathways and how they contribute to both normal and disease states. This review enumerates the specific mechanisms underlying virus-induced suppression of enzyme activity during apoptosis.

In its simplest form, apoptosis can be considered a two-step proteolytic pathway (92). The first is an initiating phase resulting in activation of initiator caspases. These caspases are responsible for the second execution phase by cleaving and activating executioner or effector caspases. During this second phase, effector caspases cleave target host proteins, culminating in the step-wise demise of the cell (66). Apoptosis is initiated through two general mechanisms: from outside the cell (extrinsic) or from within (intrinsic) (Figure 1). Extrinsic triggering of apoptosis occurs following ligation of death receptors by the tumor necrosis factor (TNF) superfamily including TNF, Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL) (Figure 1). Ligation of the TNF receptor family results in the formation of the death-inducing signaling complex (DISC) (63) required for activation of initiator caspases, particularly caspase-8 and caspase-10.

Apoptosis: refers to morphological changes occurring in a controlled process of cell death, resulting in membrane-bound cell fragments usually eliminated by phagocytosis

Caspase: cysteine-dependent aspartate-specific protease

Death induced signaling complex (DISC): multimeric assembly platform required for caspase activation induced after death receptor ligation

Intrinsic activation of apoptosis is triggered following translocation of a proapoptotic Bcl-2 family member, Bid or Bax, to the mitochondria (133,135) (Figure 1). In particular, cytosolic Bid is cleaved by caspases or other proapoptotic proteases to form truncated Bid (tBid), which then localizes to the mitochondria. Here, tBid interacts with other proapoptotic Bcl-2 member proteins such as Bax and/or Bak, resulting in pore formation and permeabilization of the outer mitochondrial membrane (45,133). This initiates the release of proteins from the inner membrane into the cytosol. One such protein, cytochrome *c*, is central to this apoptosis program (45) because it binds to apoptosis protease-activating factor (Apaf-1) to induce the formation of an oligomeric assembly platform, termed the apoptosome (7,92). The initiator caspase-9 is subsequently recruited to the apoptosome, resulting in its activation (92). A wide variety of cellular insults result in loss of mitochondrial membrane integrity, including aberrant calcium signaling, growth factor withdrawal, treatment with various cytotoxic agents, and endoplasmic reticulum stress. However, receptor-mediated cell death can also result in caspase-8-mediated cleavage of Bid, mitochondrial membrane permeabilization, and activation of caspase-9, thus amplifying apoptotic signaling pathways (133) (Figure 1). Active caspase-8, -10, and -9 then target effector caspases for cleavage. The individual pathways involved in initiating and regulating apoptosis are expanded upon in the following sections.

Apoptosome: multimeric assembly platform required for caspase-9 activation induced following disruption of mitochondrial membrane potential

CASPASES

As highlighted above, the key effector proteins activated during apoptosis and targeted by viruses for inhibition are caspases (1). There are currently 13 members of the mammalian caspase family, characterized by a near-absolute specificity for substrates containing an Asp in the P₁ cleavage position. In addition, these enzymes contain both a Cys and a His in the active site that assist in peptide bond hydrolysis (24,115). Caspases that function in apoptosis

include caspase-2, -8, -9, -10, and -12 (the initiator caspases) as well as caspase-3, -6, -7, and -14 (the effector caspases). Caspase-1, -4, -5, and -11 function in inflammation.

Caspases exist in the cell as inactive procaspases, or zymogens, and based on the length of their prodomains, they can be classified in two main categories (66). The first category is the large-prodomain caspases (caspase-1, -2, -4, -5, -8, -9, -10, -11, and -12), which have a long prodomain characterized by death domain (DD) motifs including death effector domains (DEDs) and caspase activation and recruitment domains (CARDs) (90). These caspases are present in the cell as monomers and, following recruitment into multimeric protein platforms such as the DISC (63) or apoptosome (7,92), dimerize and undergo a conformational change resulting in activation. The second category is the short-prodomain caspases (the initiator caspase-3, -6, -7, and -14). These caspases exist as preformed dimers and, in contrast to the large-prodomain enzymes, require proteolysis to be activated (99). Active caspases are obligate homodimers with each monomer composed of a large (17- to 20-kDa) subunit and a small (10- to 12-kDa) subunit (42).

VIRUS-MEDIATED INHIBITION OF CASPASES

Because of their central role in controlling cell fate decisions, caspases are subject to regulation at multiple levels, through prevention of activation as well as inhibition of activity following maturation (28). This property renders caspases a logical target for inhibition by viruses. Inhibition can be achieved through indirect mechanisms such as the virus-induced downregulation of death receptor expression (116) or the expression of secreted viral TNF receptor homologs (9), both of which prevent signaling at the cell surface. An important general mechanism viruses use to suppress caspase activation is inhibition of mitochondrial membrane permeabilization, thus preventing or delaying the release of cytochrome *c*. These approaches utilized by viruses to prevent apoptosis have been reviewed in detail elsewhere (2,14,17,39, 47,61,107,113).

Viruses can also directly antagonize enzyme function. This is achieved either by interacting with the caspase active site or by acting as competitive inhibitors of signaling molecules required for caspase activation. Four major classes of virus-encoded inhibitors directly regulate caspases: (a) the serine protease inhibitor (serpin) family, (b) the p35 family (for which no cellular homologs are known), (c) viral inhibitor of apoptosis proteins (vIAPs), and (d) viral FLICE (Fas-associated death domain-like interleukin-1 β -converting enzyme) inhibitory proteins, or viral FLICE inhibitory proteins (vFLIPs) (Table 1). Each class of inhibitor is discussed below.

DD: death domain

DED: death effector domain

Serpin: serine protease inhibitor

IAP: inhibitor of apoptosis protein

vFLIP: viral FLICE inhibitory protein

CrmA: cytokine response modifier A

CrmA: Building a Better Mousetrap

The first caspase inhibitor discovered was cytokine response modifier A (CrmA) encoded by cowpox virus, an orthopoxvirus. CrmA is a member of the serpin superfamily. It was originally identified as an inhibitor of interleukin-1 β (IL-1 β)-converting enzyme (ICE), now known as

caspase-1, by preventing its ability to process the precursor of IL-1 β into its active, secreted form (89). This discovery was made before it was understood that caspases were the central proteases of cell death (16). However, when the critical nature of caspases in apoptosis was determined following the discovery of CED-3 (a caspase that functions as both an initiator and an executioner) from *Caenorhabditis elegans* (84,136), the ability of CrmA to inhibit apoptosis was tested. CrmA can inhibit apoptosis triggered by overexpression of caspase-1 (84) as well as by treatment of cells with TNF or ligation of Fas receptors. In addition to direct inhibition of caspase-1 in the low picomolar range, CrmA can also suppress caspase-8 and caspase-10 activity (44,44,139,140), thus explaining its protective properties when apoptosis is initiated through death receptors (Table 1). CrmA can inhibit a wide range of caspases including caspase-3, -6, -7, and -9 (140). However, the efficiency of inhibition is generally lower than that demonstrated for caspase-1 and caspase-8, and caspase-9 is not thought to be an important target in vivo (27).

CrmA is unique among the serpins, as this family normally functions to suppress the activity of serine proteases rather than that of cysteine proteases. Serpins have a conserved core globular domain consisting of three β -sheets and eight to nine α -helices. CrmA maintains the serpin architecture and fold even though it lacks conserved features of the serpin superfamily including the D-helix, half of the A-helix, and part of the E-helix (91,101). In addition to these differences, CrmA contains a pseudosubstrate of the caspases (Leu-Val-Ala-Asp³⁰³) within the serpin flexible reactive site loop (RSL), with Asp³⁰³ in the P₁ position an unusual residue for a serpin.

RSL: reactive site loop

CrmA inhibits caspase-1 by a mechanism of conformational trapping similar to that determined for serpin inhibition of serine proteases (106,110). The exposed RSL of CrmA is inserted into the caspase active site where the caspase catalytic cysteine is covalently linked to the serpin P₁ residue. The RSL is then cleaved at the P₁-P₁' bond, leading to a radical conformational change whereby the N terminus of the newly cleaved loop inserts into the center of the major β -sheet of CrmA (sheet A) (Figure 2). This causes the caspase tethered to the P₁ residue to be translocated to the opposite end of the serpin. The caspase is inactivated as its active site becomes conformationally distorted and trapped (106). In addition, the small subunit of caspase-1 and caspase-8 can disassociate from the large subunit during the trapping process, providing an additional irreversible inhibition of the protease (32). The structure of the serpin in complex with its target is less energy rich than its native structure (101), thus providing the energetics for this virus-encoded mousetrap.

Other members of the poxviruses encode serpins homologous to CrmA, including serine protease inhibitor (SPI)-1 and SPI-2 [in orthopoxviruses including vaccinia (31), ectromelia (118), and rabbitpox (76) viruses] or serpin-2 (SERP-2) from myxoma virus, a *Leporipoxvirus* (87). SERP-2 shares 34.9% identity at the amino acid level, and 57.3% similarity (87), with CrmA and can inhibit caspase-1, -8, and -10 in vitro (77) (Table 1). Both CrmA and SERP-2 are required for full virus virulence in their models of infection, chicken chorioallantoic membranes (CAMs) and rabbits, respectively. This property is dependent on the P₁ Asp residue present in both proteins (77). Despite these similarities, SERP-2 and CrmA are not functionally interchangeable. For example, replacement of CrmA by SERP-2 enabled the resulting recombinant cowpox virus to inhibit apoptosis and reach wild-type virus yields in infected CAMs. However, SERP-2 did not block inflammation in this context (86), which is the original hallmark of CrmA function (89). Similarly, the reciprocal recombinant myxoma virus containing CrmA in place of SERP-2 was not fully virulent in its European rabbit host and did not cause the fulminant skin lesions characteristic of infection with wild-type virus

(86). Both recombinant viruses could inhibit apoptosis of infected cells, suggesting that poxvirus serpins may have host-specific roles in control of inflammation.

DRONC: *Drosophila* Nedd2-like caspase

p35 Inhibition of Caspases: A Ménage à Trois

p35 is a broad-spectrum caspase inhibitor identified from the baculovirus, *Autographa californica* multiple *Nucleopolyhedrovirus* (AcMNPV) (23). This protein inhibits caspases from *C. elegans* (CED-3), *Drosophila melanogaster* (the effector caspase DrICE), *Spodoptera frugiperda* (the effector Sf-caspase-1), and mammals (caspase-1, -3, -6, -8, -7, and -10) (67, 130,139) (Table 1). p35 is least effective against caspase-2 and caspase-9. The related p49 protein from *Spodoptera littoralis* *Nucleopolyhedrovirus* (SINPV) (34) inhibits a wider range of caspases compared with p35, including caspase-2 and caspase-9 as well as *Drosophila* Nedd2-like caspase (DRONC), a caspase-9-like initiator caspase (56,142) (Table 1). However, unlike p35, p49 cannot suppress DrICE activity, suggesting host-specific roles for this class of inhibitors (70). Recently, a homolog of the p35 protein, called p33, was discovered in *Amsacta moorei* entomopoxvirus (AmEPV), and it is the first homolog found outside of the baculoviruses (78). p33 is only 25% identical to p35 at the amino acid level but has strikingly similar predicted secondary structure and can inhibit similar effector caspases in vitro (78).

Similar to CrmA, p35 is a suicide inhibitor of caspases, acting as bait for the active caspase to attack. In addition, the exposed RSL of p35 contains P₄-P₁ residues, Asp-Gln-Met-Asp⁸⁷, with the critical Asp residue in the P₁ position. However, the molecular mechanism of inhibition differs between p35 and CrmA, as demonstrated for the p35-mediated inhibition of caspase-8 (74). Following insertion of the RSL into the caspase active site and cleavage of p35, the cleavage residue Asp⁸⁷ forms a thioester intermediate with the caspase active site Cys (Cys^{casp8}). This enables the portion of the RSL distal to Asp⁸⁷ to dissociate from the caspase, leading to dramatic conformational changes in p35 (Figure 3). This conformational change liberates the N terminus of p35 from where it is buried in the core of the molecule and enables it to insert into the caspase active site (74). During normal substrate cleavage by caspase-8, a water molecule is activated by the active site His to quickly hydrolyze the thioester intermediate. However, the p35 N-terminal cysteine residue (Cys²) attacks the thioester bond of Asp⁸⁷ and excludes solvent, thereby trapping Asp⁸⁷ at the thioester intermediate step. In addition, once attacked by the thiol of Cys², the carbonyl carbon of Asp⁸⁷ is no longer accessible to the free amino group of Cys² much in the same way that water is excluded from attacking the Asp⁸⁷-Cys^{casp8} bond (74). In the absence of this second step, chemical ligation of the two ends is not completed and p35 does not become circularized. Instead, an equilibrium of reversible *trans*-thioesterification between Asp⁸⁷-Cys^{casp8} and Asp⁸⁷-Cys² is established (74), trapping the caspase active site in a futile ménage à trois (Figure 3). It is currently unknown if this mechanism of inhibition is limited to p35/caspase-8 interactions or if this is a general mechanism that contributes to the promiscuity of the p35 class of caspase inhibitors (103).

Suicide inhibitor: a protein that inhibits by irreversibly binding to its target

Viral Inhibitor of Apoptosis Proteins: The Ol' Bait and Switch

The first IAP was discovered in a genetic screen of baculoviruses searching for genes that could complement the deletion of p35 from AcMNPV (26). In addition to the baculoviruses, homologous IAPs are encoded by entomopoxviruses, iridoviruses, and African swine fever virus (21,22,29). Cellular homologs of IAPs also exist in diverse organisms, from yeast to humans, with eight identified in the human genome. However, all IAPs do not function in

modulating apoptotic responses, as they have roles in diverse cellular functions including innate immunity to bacterial infection and the regulation of chromosome segregation during mitosis (reviewed in References 95 and 121). The IAP family is characterized by the presence of baculovirus IAP repeat domains (BIRs), a novel zinc binding fold of approximately 70 amino acids (52,83). Cellular IAPs (cIAPs) contain up to three BIRs while viral IAPs (vIAPs) only contain one or two. In addition, the IAPs contain a really interesting new gene (RING) domain that possesses E3 ubiquitin ligase activity and is required for IAP-mediated suppression of apoptosis (121,122).

To understand how vIAPs function to suppress caspase activity, it is important to understand the function and regulation of cIAPs during apoptosis. Of the human IAPs, X-linked IAP (XIAP), cellular IAP1 (c-IAP1), and c-IAP2 can directly bind specific caspases, including caspase-3, -7, and -9 in vitro (35,98,109). However, only XIAP can directly inhibit caspase activity, and therefore it is thought to be the primary IAP responsible for caspase inhibition in vivo (36). XIAP has two distinct domains that bind either to caspase-9 (via its BIR3) or to caspase-3 and caspase-7 (via the region immediately N-terminal to BIR2) (30), resulting in caspase inhibition (Figure 4a). Similar to XIAP, *Drosophila* IAP1 (DIAP1) is essential for negative regulation of the initiator caspase DRONC and the effector caspases DrICE and *Drosophila* caspase-1 (DCP-1). However, in this case, binding of caspases by the BIR1 domain of DIAP1 alone is not sufficient for inhibition. Following binding, DIAP1 is cleaved by *Drosophila* effector caspases at its N terminus, resulting in the targeting of DIAP1 for ubiquitin-dependent proteasome-mediated degradation. Presumably, the active caspase tethered to DIAP1 is also degraded (108,109,131).

Activity of the cellular antiapoptotic IAPs is modulated by IAP-interacting proteins often termed IAP antagonists. In humans and mice, the major IAP antagonist is termed SMAC (second mitochondrial activator of caspases) or DIABLO (direct IAP binding protein with low pI), respectively, and resides in the intermembrane space of the mitochondria (33,123). The release of SMAC/DIABLO occurs in response to apoptotic stimuli along with cytochrome *c*, at which time it can bind to XIAP (72,129) and displace the active caspase(s) to promote apoptosis. *Drosophila* has multiple IAP antagonists, including Reaper, HID (head-involution defective), Grim, and Sickie, that can bind to DIAP1 and antagonize its ability to bind to DRONC. These IAP antagonists share a common IAP binding motif [IBM (98), also known as the Reaper-HID-Grim (RHG) motif] that enables them to bind to and suppress the function of DIAP1 (128) and XIAP (Figure 4b), suggesting a mechanism of binding conserved between flies and humans. One function of Reaper, HID, and Grim, as well as the *Drosophila* ubiquitin conjugase-related protein termed Morgue, is to promote degradation of *Drosophila* IAPs (54, 95,134). Degradation is dependent on a functional RING domain in the IAP itself (93,127), suggesting that binding by the antagonist induces IAP autoubiquitination (95).

BIR: baculovirus IAP repeat

RING domain: specialized type of zinc-finger domain involved in protein-protein interactions that possesses E3-ubiquitin ligase activity

Given the complex roles of cIAPs in apoptosis suppression, how do vIAPs with anti-apoptotic activity function? vIAPs such as the baculovirus Op-IAP (from *Orgyia pseudotsugata* NPV) do not bind to insect or mammalian caspases (127), nor do they undergo caspase-mediated cleavage (108). As these two events are essential for XIAP- and DIAP1-mediated inhibition of caspases, respectively, it is unlikely that vIAPs function as direct caspase inhibitors. Instead, it is thought that vIAPs act as decoys for the IAP antagonists. Op-IAP contains two BIR domains and one RING domain. The BIR2 domain of Op-IAP can bind to Reaper, HID, and Grim (124–126). Op-IAP can also efficiently bind SMAC/DIABLO via its BIR2 domain, a

property required for its ability to prevent apoptosis when expressed in human cells (127). Furthermore, the RING domain of Op-IAP also contributes to IAP function, facilitating ubiquitination of itself and of HID (22,49) or SMAC/DIABLO (127) and presumably degradation. Thus, Op-IAP and probably vIAPs in general function in an old-fashioned bait-and-switch tactic. They deceive the IAP antagonists and then disable them, permitting endogenous IAPs such as DIAP1 to inhibit caspases (Figure 4c).

The observations that cIAPs are conserved in diverse genomes, including flies and humans, and function in apoptosis by conserved mechanisms suggest that vIAPs might be encoded by a wide variety of viruses. However, such homologs have only been found in viruses that infect arthropods (22). This restricted presence of viral homologs is consistent with the relative role of IAPs in apoptotic programs of insects versus humans. The prevailing view is that induction of apoptosis in humans is regulated by the activation of inactive zymogens, whereas apoptosis in *Drosophila* is initiated by the liberation of constitutively active caspases from their complex with IAPs (94). Hence, vIAPs may impart a greater degree of caspase control if the virus's host is an arthropod rather than a higher vertebrate, thus creating the selective pressure required to retain IAPs as principal modulators of apoptosis. This suggestion is supported by the observation that known genomes of two entomopoxviruses do not encode serpins like their mammalian counterparts, although at least one of these, *Amsacta moorei* (AmEPV), has a functional IAP (71).

FADD: Fas-associated via death domain

Viruses FLIP Out Over the DISC

Signaling through death receptors, including TNF and Fas, generates oligomeric signaling assemblies such as the DISC (reviewed in References 7,15, and 92) (Figure 5). The intracellular region of death receptors contains a DD, which in the case of Fas recruits the Fas-associated via death domain (FADD) adaptor protein via a homotypic interaction with the DD of FADD. FADD contains an additional DED that interacts with DEDs in the prodomains of caspase-8 and caspase-10. The recruitment of these initiator caspases into the DISC results in their dimerization and maturation. During this activation process, caspase N-terminal DEDs are removed by intermolecular processing, resulting in the release of caspases into the cytosol where they activate effector caspases (24).

DISC assembly and caspase activation are negatively regulated by cellular FLIP (cFLIP) that directly competes with caspase-8 for recruitment to FADD (65) (Figure 5). Two major isoforms of cFLIP exist, the 26-kDa short form (cFLIP_S) and the 55-kDa long form (cFLIP_L) (112). cFLIP_S contains two DEDs and completely inhibits proteolytic processing of caspase-8 (65). In contrast, in addition to two DEDs, cFLIP_L contains a longer C terminus than does cFLIP_S that closely resembles the overall structure of caspase-8 and caspase-10 (65). cFLIP_L can form a heterodimer with caspase-8 through interactions between the DEDs and caspase-like domains. This association causes a conformational change in caspase-8, exposing the caspase active site (19,81). The result is a partial autoprocessing of caspase-8 that in turn cleaves cFLIP_L (19,82). Cleavage of cFLIP_L augments its ability to recruit TNF-receptor-associated factor 2 (TRAF2) and receptor-interacting protein 1 (RIP1) (60). These events facilitate NF-κB activation, which is the quintessential role of TNF signaling. A similar partial activation of caspase-8 may also be important in T-cell activation (reviewed in Reference 15).

vFLIPs (111) are encoded by several γ-herpesviruses and by the molluscum contagiosum virus (MCV) (10,10,55,112). vFLIPs contain two DED domains and, similar to their cellular counterparts, interfere with recruitment, processing, and release of caspase-8 and caspase-10 following ligation of death receptors. However, the precise mechanism utilized by various

viruses differs, as illustrated by their interactions with Fas receptor-induced DISC formation. vFLIP encoded by γ -herpesviruses is analogous to cFLIP_s outcompeting caspases for recruitment to FADD (132) (Figure 5). In contrast, the MCV genome contains two vFLIPs, termed MC159 and MC160. MC159 interacts with both FADD and Fas (100) but does not compete with caspase-8 for recruitment. Instead, it binds at least two FADD molecules and disrupts FADD oligomerization (132). Hence, caspase inhibition results from the absence of FADD self-association and DISC formation (132) (Figure 5). In addition, MCV FLIPs are unique in that they have C-terminal extensions responsible for binding TRAF2 and TRAF3 and recruiting them to the Fas DISC in a FasL-dependent manner. The binding of TRAF3 in particular contributes to inhibition of Fas receptor internalization (114), a step that occurs after DISC formation and caspase-8 activation and is required for optimal induction of apoptosis. MCV-encoded vFLIPs also interact with proteins that regulate NF- κ B activation including TRAFs, RIP1, NF- κ B-inducing kinase (NIK), and inhibitor of κ B-kinase 2 (IKK2) (20). Thus, MCV vFLIPs may impart multiple blocks following death receptor ligation, resulting in the inhibition of initiator caspase activation as well as the promotion of cellular survival signals such as NF- κ B activation.

Although not a vFLIP, the E6 protein of human papillomavirus type 16 (HPV-16) also targets DISC assembly at multiple levels to suppress death receptor-induced apoptosis. The long isoform of E6 can bind to the DD of TNF receptor 1 and interfere with the recruitment of TNF receptor-associated via death domain (TRADD) and therefore DISC formation (41). In addition, E6 can bind to the DEDs of both FADD and procaspase-8. The consequences of this binding are twofold. First, E6 prevents the normal association of caspase-8 with FADD, and second, it accelerates the degradation of both molecules (40,41) and prevents DISC formation. Furthermore, the large isoform of E6 accelerates degradation of the tumor suppressor p53 (40,97), thus rendering cells less sensitive to apoptosis initiated by genotoxic stress. E6 is therefore a multifunctional protein (with functions additional to those described here) that blocks numerous initiating signals of apoptosis. Finally, the human cytomegalovirus UL36 gene product termed vICA also associates with procaspase-8 and blocks its activation, although it possesses no sequence homology with vFLIPs (102).

EVADING SERINE PROTEASES: THRUST AND PARRY

Effective antiviral immune responses involve the generation of virus-specific cytotoxic T lymphocytes (CTLs), whose function is to kill virus-infected cells. The two main weapons utilized by CTLs are perforin-/granzyme- and Fas-mediated apoptosis. Following recognition of virus-infected cells, perforin and granzymes are released by CTLs and are endocytosed by the target cell (5,73). Perforin then polymerizes and inserts into the membrane of the endocytic vesicle, forming a pore through which granzymes including granzyme B are transported into the cytosol (73). Granzyme B is a unique serine protease owing to its strict requirement for Asp in the substrate P₁ position. Apoptosis is initiated following direct cleavage and activation of caspase-3 by granzyme B, or following cleavage and activation of Bid (8,51,73). Truncated Bid translocates to the mitochondria and recruits Bax to facilitate alterations in membrane permeabilization, release of cytochrome *c*, and activation of downstream caspases (8,51).

In addition to potentially inhibiting caspases, CrmA can antagonize the proteolytic activity of granzyme B (88). Granzyme B is structurally and mechanistically distinct from the caspases, demonstrating a remarkable cross-class ability of CrmA to inhibit proteolysis (64). The 100,000-kDa (100 K) assembly protein of human adenovirus type 5 (Ad5-100K) is also a strong inhibitor of granzyme B (3,5). The Ad5-100K protein has several essential functions in the adenovirus life cycle, including virus assembly, activation of late viral protein synthesis, and inhibition of cellular protein synthesis (53). Inhibition of granzyme B is absolutely dependent on Asp⁴⁸ within a granzyme B consensus recognition sequence in the Ad5-100K

protein (3). Although granzyme B from multiple species (human, rat, and mouse) can cleave Ad5–100K, these proteins cleave at different sites within the viral protein and only activity of human granzyme B is inhibited (4). The precise molecular mechanisms by which Ad5–100K prevents granzyme B activity are not known, although at least two intermolecular interactions are required. These are between the viral protein RSL and the protease active site, resulting in the cleavage of the former, as well as an additional interaction involving the extreme C-terminal residues of Ad5–100K (residues 688–781) that may stabilize interactions between protease and inhibitor (4).

The fencing match between host and virus does not end following the initial thrust with granzyme B and the parry of the adenovirus inhibitor. The human host has a riposte in the form of granzyme H that is also delivered to cells by activated CTLs. Granzyme H cleaves and inactivates Ad5–100K, as well as the adenoviral DNA binding protein (6). Cleavage of these proteins not only significantly restricts virus replication, but also relieves virus-mediated inhibition of granzyme B and restores its ability to process caspase-3 (6). Granzyme H does not have orthologs in species other than humans, and the inhibitory activity of Ad5–100K is specifically directed toward human granzyme B. Thus, despite generally well-conserved processes of apoptosis, these findings suggest a remarkably complex evolutionary relationship between the induction and suppression of host cell apoptosis and virus replication.

Granzyme: serine esterases present within granules of cytotoxic T cells and natural killer cells

Viruses target proapoptotic serine proteases other than granzymes to suppress the cell death response. The high-temperature requirement protein A2 (HtrA2/Omi) is a serine pro-tease released from the mitochondrial inner membrane space along with cytochrome *c* and SMAC/DIABLO following membrane permeabilization (50). Once released, HtrA2/Omi can degrade XIAP and thereby relieve its inhibition of caspases (105). When used in combination as clinical therapy for some tumors, interferon (IFN) and retinoic acid promote apoptosis in part through the upregulation of genes associated with retinoid-interferon-induced mortality (GRIM) protein expression (59). In particular, GRIM-19 associates with HtrA2/Omi and augments XIAP degradation (75). The Kaposi's sarcoma-associated herpesvirus oncoprotein termed viral IFN-regulatory factor 1 (vIRF1) binds to GRIM-19 and prevents its interaction with HtrA2/Omi (75). This preserves cellular XIAP levels in response to IFN and retinoic acid and ablates caspase-9 activation.

The original function identified for vIRF1 was a suppressor of IFN signaling (141), a potent antiviral response that viruses devote large amounts of their genomes to evade. It is well known that IFN stimulation can eventually lead to apoptosis through the increased expression of proapoptotic genes. However, the targeting of GRIM-19 and HtrA2/Omi by a viral homolog of the IFN signal transduction pathway suggests that there may be direct regulation, or cross-talk, between the two cellular pathways. The recent identification of HtrA2/Omi as a mitochondria-resident protein with roles in apoptosis in *Drosophila* (18) suggests a level of conservation that facilitates virus coevolution. Therefore, it is of interest to determine if additional oncogenic viruses, which may persist in the presence of chronic expression of proapoptotic cytokines such as IFN, also target HtrA2/Omi to suppress apoptosis.

WHEN RESISTANCE IS FUTILE: UTILIZATION OF CASPASES FOR VIRUS REPLICATION

The combined efforts of viruses to suppress apoptosis in general and caspase activity in particular suggest that this cellular response is to be avoided at all costs. However, caspases

also have roles in cell proliferation, differentiation, and NF- κ B activation (69). These roles for caspase activation raise the question of whether viruses can positively modulate or utilize caspase activity to facilitate replication. The role of the HPV-16 E6 long isoform in caspase-8 degradation has already been highlighted. The E6 short isoform, which is only produced by so-called high-risk HPV types, can also bind to caspase-8. However, this molecular interaction results in caspase-8 stabilization (40). The implications from these findings are that, like inhibition of caspase activity, stabilization of limited caspase-8 activity may be important for cell survival or maintaining a cell at a particular stage of differentiation. Thus, caspases may be targeted by certain viruses to prolong cell viability or aid in cellular transformation.

Examples of viruses that directly utilize caspase activity to facilitate replication also exist. Permissive replication of Aleutian mink disease parvovirus (ADV) in cell culture is associated with an apoptotic response that can be blocked by treatment with caspase inhibitors. There are numerous examples of virus infection in which blockage of apoptosis increases the virus titer recovered from treated cells, consistent with the antiviral role of apoptosis (23,38,137). However, in the case of ADV, inhibition of specific caspases results in decreased virus yield, suggesting that caspase activity facilitates virus replication (13). The major nonstructural protein of ADV, NS1, is required for many replication functions including control of viral and cellular gene transcription, viral DNA replication, and capsid assembly. Caspases mediate cleavage of NS1 early in virus replication, particularly by effector caspase-3, caspase-7, or both (12; S.M. Best, unpublished data). In the absence of cleavage, translocation of NS1 to the site of virus replication in the nucleus is impaired. It appears that the role of caspases is to generate a C-terminal cleavage product that contains the NS1 nuclear localization sequence. This product forms oligomers with full-length NS1 and facilitates transport of the latter to the nucleus (M.E. Bloom & S.M. Best, unpublished data).

Although a clear requirement exists for caspases in ADV replication, it is currently unknown how this contributes to virus pathogenesis. In infected mink kits, permissive replication of ADV occurs in pulmonary type II pneumocytes, resulting in high levels of cytopathology and mortality. In contrast, infection of adult mink results in restricted replication in lymph node macrophages and persistent infection. It is possible that cell-type-specific regulation of caspase activity modulates the degree of NS1 nuclear translocation and hence its function in DNA replication and viral gene expression. In adult mink, for example, tight regulation of caspase activity in macrophages may restrict nuclear translocation of NS1 and virus replication, contributing to persistent infection. However, apoptosis may be triggered during virus infection of pneumocytes, resulting in elevated caspase activation and permissive replication in mink kits.

Adenoviral proteins are also cleaved during virus replication. The early transcription units E1A (which encodes two proteins, 12S and 13S) and E1B encode proteins involved in transactivation of both cellular and viral transcription as well as cellular transformation. Following transient expression of the E1A proteins from Ad2 or Ad12, caspases cleave both 12S and 13S at multiple sites, resulting in progressive truncation from the N termini (48). This cleavage disrupts interactions of E1A with cellular transcription-regulating proteins that bind to the N terminus but not to the C terminus (48). Thus, caspases have a potential regulatory role in E1A-mediated gene expression. E1A is also proteolytically cleaved by caspases during Ad5 replication. However, cleavage of E1A proteins, as well as cellular proteins normally cleaved during apoptosis, is limited and dependent on cell type (48). The degree of cleavage is likely influenced by temporal expression of adenovirus-encoded inhibitors of apoptosis such as E3, the protein responsible for the downregulation of cell surface death receptors (116). Thus, an intricate interplay between caspase activation and inhibition in a particular cell type may be required for optimal virus replication.

Additional examples of viruses that utilize caspase activity include human astroviruses that cause viral gastroenteritis. In this case, caspase-mediated cleavage of the capsid precursor protein facilitates the release of viral particles from the cell (80). Other examples of virus-encoded proteins cleaved by caspases include NS5A of hepatitis C virus (46,58,96), the nucleocapsid protein of influenza A virus (138), and ICP22 of herpes simplex virus (85), although the role of protein cleavage in replication of these viruses is unclear (11). Cleavage may facilitate protein degradation and negatively impact virus replication as described for the granzyme H-mediated cleavage of the Ad5-100K protein. However, because mutation of the crucial Asp residue would eliminate the enzyme recognition site, conservation of these sequences suggests a selection for caspase-mediated cleavage in virus replication (11). Hence, in addition to facilitating virus release, caspases may have roles in regulation of virus protein maturation and function as suggested by studies using ADV and adenoviruses.

CLOSING REMARKS

To prolong cell viability after infection, the function of enzymes essential for the promotion of host cell death, such as caspases and serine proteases, are obvious targets for suppression by viruses. Although not discussed here, additional proteases including cathepsins, calpains (104,117,119), phosphatidylinositol 3-kinase (PI3K) (25), mitogen-activated protein kinases (MAPK) (57), and protein kinase R (PKR) (43) have important roles in regulating apoptosis. PI3K, MAPK and PKR are all targeted by various viruses to modulate cell death. It is possible that other proteases like the cathepsins or calpains are also targeted by viruses for this purpose.

The discovery of virus-encoded inhibitors of apoptotic proteases has been invaluable, both in our understanding of virus requirements for replication and for tools used to study apoptosis. In this arena, CrmA, p35, vIAPs, and vFLIPs have yielded enormous insight into apoptotic signaling pathways stimulated by a plethora of insults. These proteins also have potential use as therapeutics to treat conditions in which apoptosis is a major contributor to disease. Owing to the observation that many viruses encode several inhibitors of apoptosis and that individual proteins have multiple functions, the full spectrum of viral protein activity in modulating cell death is probably not fully realized. Furthermore, caspases have roles in nonapoptotic cellular processes (68,69) as well as in the facilitation of virus replication (11). Thus, it is likely that further knowledge of both cell death and cell survival programs will be gained from understanding specific mechanisms by which virus proteins modulate these enzymes.

Taken together, the studies highlighted in this review suggest that complex relationships exist between enzyme activity, virus replication, and cell survival and transformation. Furthermore, although general mechanisms of apoptosis are conserved from *Drosophila* to humans, many of the viral proteins that modulate enzyme activity during apoptosis do so in a host-specific context, including CrmA, p35 and p49, vIAPs, and Ad5-100K. Combined with the fact that apoptosis is a strong antiviral response, these studies suggest that virus-encoded proteins are important contributors to virus cell tropism and host range specificity. Thus, challenges remain in understanding how these multifaceted virus-host interactions within the individual cell contribute to the outcome of virus infection at the level of the organism.

SUMMARY POINTS

1. Viruses suppress the function of proteases with central roles in promoting apoptosis to prolong cell viability and facilitate replication. The functions of caspases and serine proteases are most commonly modulated by viruses to suppress apoptosis.
2. Four classes of viral proteins directly suppress caspase function. Two of these, serpins and p35 family members, have in common a RSL that is inserted into the caspase active

site. However, serpins function by conformational distortion of the caspase active site, whereas p35 engages the active site in a process of chemical ligation.

3. Two other classes of virus inhibitors suppress caspase activity by competing with signaling molecules involved in caspase activation. vIAPs act as decoys for cellular IAP antagonists, thus enabling cellular IAPs to inhibit caspase activity. vFLIPs prevent DISC assembly in response to ligation of death receptors, resulting in suppression of caspase-8 and caspase-10 activation.

4. Viral gene products also antagonize the proapoptotic function of the serine proteases, granzyme B and Htr2A/Omi.

5. Despite the antiviral role of apoptosis, some viruses appear to exploit this response by utilizing caspase activity to cleave viral proteins and facilitate replication. Thus, complex relationships exist between caspase activity, virus replication, and cell survival.

FUTURE ISSUES

1. Elucidation of protein structures in complex with their targets has provided enormous insight into distinct mechanisms utilized by p35 to suppress apoptosis. Additional structural studies, for example, of Ad5-100K in complex with granzyme B, are required to fully elucidate the mechanisms underlying virus protein function in apoptosis suppression.

2. The diverse functions of cIAPs in the regulation of apoptosis are beginning to be revealed. Hence, functions of vIAPs in addition to their ability to subvert cellular IAP antagonists may exist and remain to be identified.

3. In examples in which viruses utilize caspases for replication, little is known regarding how caspase activity is positively regulated while limited to complete replication. Thus, the temporal relationships between viral pro- and antiapoptotic signals are poorly understood. Furthermore, it is unknown how caspase-dependent replication contributes to virus pathogenesis.

4. A major challenge continues to be converting knowledge of viral protease inhibitors into viable therapeutics to treat conditions involving aberrant apoptosis or inflammation.

ACKNOWLEDGMENTS

Thank you to Marshall Bloom, Dana Mitzel, Shelly Robertson, Travis Taylor, and Dan Voth for critique of the manuscript and to Anita Mora for graphical expertise. This work was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

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Figure 1.

Apoptosis signaling pathways. Apoptosis is initiated extrinsically following ligation of death receptors including the Fas receptor. This results in the recruitment of the adaptor protein FADD and the formation of the death-inducing signaling complex (DISC). Procaspase-8 is also recruited to the DISC, where it is activated, enabling it to cleave and activate caspase-3 and caspase-7. Intrinsic initiation of apoptosis is triggered following cleavage and translocation of a proapoptotic Bcl-2 family member such as Bid to the mitochondria, where it is involved in altering membrane permeabilization and pore formation, enabling the release of cytochrome *c*. Cytosolic cytochrome *c* binds Apaf-1 to induce formation of the apoptosome. This complex recruits and activates caspase-9, enabling it to cleave and activate caspase-3 and caspase-7. Caspase-8 can also activate intrinsic signaling through the cleavage of Bid. Abbreviations: Apaf-1, apoptosis protease-activating factor; DD, death domain; DED, death effector domain; FADD, Fas-associated death domain; tBID, truncated Bid.

Figure 2.

Model of CrmA-mediated caspase inhibition. (a) Ribbon diagram of a serpin in its native state demonstrating the position of the reactive site loop (RSL). The crystal structure of native CrmA is not known so the structure of the related α_1 -antitrypsin (PDB# 1psi) is depicted to illustrate the mechanism of inhibition by CrmA (32,37). (b) Diagram of CrmA following cleavage and insertion of RSL into the center of the molecule (PDB# 1c8o) (91,101). A caspase covalently linked to the P₁ residue (*labeled*) within the RSL would be translocated to the opposite end of CrmA during this conformational rearrangement. Protein structures were generated using PyMOL.

Figure 3.

Mechanism of p35-mediated inhibition of caspase-8. (a) Worm diagram of p35 in its native state. The extreme N terminus (*red*) is buried in the core of the protein. (b) Diagram of p35 after complex formation with caspase-8. Cleavage of the reactive site loop (RSL) enables Asp⁸⁷ to form a thioester intermediate with the caspase active site Cys residue. The liberated Cys² of the p35 N terminus inserts into the caspase active site, trapping Asp⁸⁷ at the thioester intermediate step and establishing equilibrium of reversible *trans*-thioesterification between Asp⁸⁷-Cys^{casp8} and Asp⁸⁷-Cys². In panels *a* and *b*, the C terminus of p35 is depicted in light blue and the remaining N terminus is dark blue. This figure was reproduced with modifications from Reference 74 with permission from the publisher.

Figure 4. Mechanism of vIAP inhibition of caspase activity. (a) Direct inhibition of caspases by cellular XIAP. The BIR3 domain of XIAP binds to caspase-9, and the region proximal to the XIAP BIR2 binds to caspase-3 and caspase-7 and suppresses their activity. (b) IAP antagonists (such as SMAC/DIABLO) are released from the mitochondria during apoptosis and competitively bind the IAP, resulting in displacement of active caspases. (c) vIAPs function by binding to IAP antagonists, permitting endogenous IAPs such as XIAP to continue to inhibit caspases. Abbreviations: BIR, baculovirus IAP repeat domain; IAP, inhibitor of apoptosis protein; vIAP, viral IAP; XIAP, X-linked IAP.

Figure 5.

Mechanism of vFLIP-mediated inhibition of caspase activation. Death-receptor induced apoptosis occurs following ligation of the receptor, recruitment of the adaptor protein FADD via its DD and higher-order DISC assembly. Procaspase-8 or procaspase-10 are subsequently recruited and activated. DISC assembly and caspase activation are negatively regulated by cFLIP_s or by γ -herpesvirus vFLIP that also contains DEDs and directly compete with caspase-8 for recruitment to FADD. The molluscum contagiosum virus MC159 protein binds at least two FADD molecules to disrupt FADD oligomerization and higher-order DISC assembly. Abbreviations: vFLIP, viral FLICE (Fas-associated death domain-like interleukin-1 β -converting enzyme) inhibitory protein; cFLIP_s, short isoform of cellular FLIP; DD, death domain; DED, death effector domain; DISC, death induced signaling complex; FADD, Fas-associated death domain.

Table 1

Virus-encoded caspase inhibitors

	Protein family	Specific protein	Virus	Caspases targeted	Reference(s)
Direct caspase inhibitor	Serpin	CrmA/SPI-1 SERP2	Cowpox virus orthopoxvirus Leporipoxvirus	Caspase-1, -8, and -10 Caspase-1, -8, and -10	(44,64,140) (77)
	p35	p35	Baculoviruses	Broad spectrum including caspase-1, -3, -6, -7, -8, and -10	(130,139)
		p49	Baculoviruses	Similar to p35 plus caspase-9 and DRONC	(56,142)
		p33	<i>Amsacta moorei</i> entomopoxvirus	Similar to p35 (as far as tested)	(78)
Competitive caspase inhibitor	IAP	Op-IAP	Baculoviruses	Indirect inhibition of caspase-3, -7, -9, DRONC, and DrICE	(79,131)
		AMV-IAP A224L	Entomopoxvirus African swine fever virus		
	FLIP	vFLIP	γ -herpesviruses	Indirect inhibition of caspase-8 and caspase-10 activation	(10,55)
		MCI59	Molluscum contagiosum virus		

Abbreviations: CrmA, cytokine response modifier A; DrICE, *Drosophila* interleukin-1 β converting enzyme; DRONC, *Drosophila* Nedd-2-like caspase; FLIP, FLICE (Fas-associated death domain-like interleukin-1 β converting enzyme) inhibitory protein; IAP, inhibitor of apoptosis protein; SERP-2, serine protease inhibitor-2; SPI-1, serine protease inhibitor-1; vFLIP, viral FLIP.