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Functional and biochemical characterization of the baculovirus caspase inhibitor MaviP35

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Many viruses express proteins which prevent the host cell death that their infection would otherwise provoke. Some insect viruses suppress host apoptosis through the expression of caspase inhibitors belonging to the P35 superfamily. Although a number of P35 relatives have been identified, *Autographa californica* (Ac) P35 and *Spodoptera littoralis* (Spli) P49 have been the most extensively characterized. AcP35 was found to inhibit caspases via a suicide substrate mechanism: the caspase cleaves AcP35 within its 'reactive site loop' then becomes trapped, irreversibly bound to the cleaved inhibitor. The *Maruca vitrata* multiple nucleopolyhedrovirus encodes a P35 family member (MaviP35) that exhibits 81% identity to AcP35. We found that this relative shared with AcP35 the ability to inhibit mammalian and insect cell death. Caspase-mediated cleavage within the MaviP35 reactive site loop occurred at a sequence distinct from that in AcP35, and the inhibitory profiles of the two P35 relatives differed. MaviP35 potently inhibited human caspases 2 and 3, DCP-1, DRICE and CED-3 *in vitro*, but (in contrast to AcP35) only weakly suppressed the proteolytic activity of the initiator human caspases 8, 9 and 10. Although MaviP35 inhibited the AcP35-resistant caspase DRONC in yeast, and was sensitive to cleavage by DRONC *in vitro*, MaviP35 failed to inhibit the proteolytic activity of bacterially produced DRONC *in vitro*.

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Cell death is essential for normal animal development and to destroy pre-cancerous and auto-immune cells, but it has been postulated that apoptosis originally evolved to defend primitive multicellular organisms against intracellular pathogens such as viruses.[1] Over evolutionary time, an 'arms race' has developed between viruses and their hosts. Cellular machineries detect infection and activate self-destruction pathways, limiting the ability of the virus to replicate and spread to other cells. Viruses, in turn, have evolved ways to suppress their hosts' apoptotic machineries during the early phase of infection. Targeting caspases is one approach adopted by viruses to block their host cells' suicidal reaction to infection.[2] The P35 family is a group of caspase inhibitors encoded by viruses that infect insects. Almost all of the viruses that possess P35 relatives are baculoviruses:[3] the sole exception known to date is the *Amsacta moorei* entomopoxvirus.[4] No cellular P35 homologs have been described as yet, although as baculoviruses usually derive their genes from their hosts,[5] it seems likely that P35 genes did evolve from a cellular ancestor.

The best-studied P35 family member is AcP35, encoded by the baculovirus *Autographa californica* multi nucleopolyhedrovirus (AcMNPV).[6] It inhibits caspases via a substrate trap mechanism.[7–9] The caspase cleaves AcP35 within the reactive site loop. This cleavage provokes a conformational change within the inhibitor, targeting its amino terminus to the caspase's active site, preventing hydrolysis of a thioester adduct between the inhibitor and the protease, and thus locking the caspase in an inactive, P35-bound form.[7] Of the many mammalian, insect and nematode caspases tested, very few were found to be insensitive to AcP35. The *Drosophila* initiator caspase DRONC was shown to be resistant to inhibition by AcP35.[10,11] Processing of downstream *Spodoptera* caspases proceeded in the presence of AcP35,[12] implying that a *Spodoptera* DRONC ortholog (denoted 'Sf-caspase-X') is also resistant to AcP35 inhibition. AcP35 could inhibit the enzymatic activity of recombinant caspase 9 (DRONC's mammalian counterpart), however extremely high concentrations of AcP35 were required to prevent apoptosome-activated caspase 9 from cleaving its physiological substrate, caspase 3.[13] This suggests that AcP35 cannot efficiently interfere with the function of naturally activated caspase 9.

Bombyx mori nucleopolyhedrovirus (BmNPV) encodes a protein (BmP35), which shares 91% of its amino-acid sequence with AcP35. BmP35 displayed only weak anti-apoptotic activity[14] and, unlike AcP35, BmP35 was dispensable for normal viral propagation.[15,16] Extracts from mammalian cells expressing BmP35 were less potent than lysates from AcP35-expressing cells at inhibiting recombinant

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Abbreviations: Mavi, *Maruca vitrata*; MNPV, multiple nucleopolyhedrovirus; Ac, *Autographa californica*; Bm, *Bombyx mori*; NPV, nucleopolyhedrovirus; Spli, *Spodoptera littoralis*; TRAIL, TNF-related apoptosis-inducing ligand; FBS, fetal bovine serum; MEF, mouse embryonic fibroblast; Xgal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; CAT, chloramphenicol acetyl transferase; RFU, relative fluorescence units
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caspase 3, although lower BmP35 expression levels may have contributed to this difference.[13] No quantitative data have been published regarding the caspase inhibitory potency or specificity of BmP35, and no other close relatives of AcP35 have been functionally or biochemically investigated to date.

Some baculoviruses encode distant relatives of AcP35, which constitute the P49 subfamily. *Spodoptera littoralis* (Spli) NPV-P49 is the best-studied member of this subfamily. Like AcP35, SpliP49 is a broad-spectrum caspase inhibitor that could suppress insect[17–20] and mammalian[21] cell death. Unlike AcP35, SpliP49 could inhibit DRONC-mediated yeast lethality,[21] but it was incapable of preventing DRICE processing in *Drosophila* cells.[19] SpliP49 could, however, prevent processing of executioner *Spodoptera* caspases,[18,20] implying that it can inhibit the proposed Sf-caspase-X. AcP35 contains the cleavage sequence DQMD'G within its reactive site loop, but SpliP49 instead possesses the sequence TVTD'G at this position. This sequence is required for SpliP49 to inhibit the distal insect caspase Sf-caspase-X, but its insertion into the AcP35 reactive site loop failed to confer this capability,[20] indicating that other regions of the SpliP49 protein, not shared by AcP35, are critical for its ability to inhibit insect initiator caspases. The caspase inhibitor AMVP33 from *Amsacta moorei* entomopoxvirus is the least homologous member of the P35 superfamily, exhibiting only 25% amino acid identity to AcP35.[4]

The baculovirus *Maruca vitrata* (*Mavi*) MNPV infects the legume pod borer *Maruca vitrata*, and may offer a biological means of controlling this important pest of legume crops.[22] The recent sequencing of the *Mavi*MNPV genome[23] revealed the presence of a P35 ortholog (MaviP35). The predicted MaviP35 protein is highly homologous to AcP35, but its predicted reactive site loop possesses a distinct caspase cleavage sequence. Here, we report our characterization of the apoptosis and caspase inhibitory properties of MaviP35.

Results

Sequencing of the *Mavi*MNPV genome[23] revealed that this virus encoded a P35 ortholog that was 81% identical to the founding member of this family, AcP35 (Figures 1a and b), and modeling suggested the two relatives may adopt similar structures (Figures 1c and d). Residues determined to be essential for the caspase inhibitory activities of AcP35, including C2[8] and D87,[24,25] were conserved in MaviP35, suggesting it too may function as a caspase inhibitor that could prevent apoptosis. We tested this hypothesis by overexpressing MaviP35 in mammalian and insect cells, and monitoring the transfectants' sensitivity to apoptosis. MaviP35 inhibited insect cell death and caspase activity triggered by infection with an AcP35-deficient baculovirus, although less efficiently than AcP35 (Figures 2a and b). MaviP35 and AcP35 inhibited insect cell death induced by actinomycin D to a similar extent (Figure 2c), but MaviP35 protected a larger proportion of insect cells than AcP35 against apoptosis induced by UV irradiation (Figure 2d). MaviP35 and AcP35 afforded similar levels of protection to mammalian cells against cisplatin-induced apoptosis (Figure 2e), but MaviP35 was less protective than AcP35 against death induced by TNF-related apoptosis-inducing ligand (TRAIL; Figure 2f).

We have previously exploited yeast-based assays to visualize caspase activity and inhibition, and these were used to provide an indication of the specificity of MaviP35 for various caspases. MaviP35 protected yeast from death induced by mammalian caspases 1, 2, 3, 5, 7 and 8, the *Drosophila* caspases DCP-1 and DRICE, and CED-3 from *Caenorhabditis elegans* (Figure 3). In this system, MaviP35 appeared to exhibit similar activity to AcP35, and protected yeast from death induced by caspases 5, 8 and CED-3 better than SpliP49 (Figure 3).

AcP35 has been shown to inhibit caspases via a pseudosubstrate mechanism, and mutation of the caspase cleavage site abolishes caspase and apoptosis inhibitory activity.[7,8] Comparison of the MaviP35 and AcP35 sequences predicted that caspases may cleave MaviP35 after residue D^{87} within the site $TQFD^{87'}G$ (Figure 1a). Consistent with this residue being critical for caspase inhibition, a putative P1 mutant failed to prevent DRICE induced yeast death (Figure 4). Lysates from yeast expressing active DRICE exhibited considerable DEVDase activity. Co-expression of untagged or carboxyl terminally FLAG-tagged wild-type MaviP35 or AcP35 abolished this activity, but their cleavage site mutants had negligible impact (Figure 4). Immunoblotting confirmed that the tagged mutants were expressed at least as abundantly as their wild-type counterparts. Interestingly, cleavage products could not be detected in lysates from yeast co-expressing DRICE with either MaviP35-FLAG or AcP35-FLAG. Because DRICE activity in this system is generated through autoactivation, we suspect that only relatively few P35 molecules would be required to block this feedback loop, and immunoblotting may not be sufficiently sensitive to detect this small number of cleaved proteins.

The yeast system is a sensitive tool for observing caspase inhibition within a naive eukaryotic environment, but only provides limited insight into the strength of inhibition. To gain a quantitative understanding of MaviP35's caspase inhibitory activity, we purified FLAG-tagged MaviP35 and AcP35 and examined their ability to prevent recombinant caspases from cleaving fluorogenic substrates *in vitro*. When present at 100–1000-fold excess, MaviP35 diminished by at least 90% the activity of caspases 2, 3, DRICE, DCP-1 and CED-3. Weaker inhibition was seen for caspases 1 and 7. MaviP35 only partially reduced the activity of caspases 6, 8 and 9, and negligible inhibition of caspase 10 was observed (Figure 5). A particularly striking difference between AcP35 and MaviP35 related to inhibition of caspase 8. As observed previously,[26] AcP35 potently suppressed the proteolytic activity of this enzyme, yet a 100-fold excess of MaviP35 only decreased its activity by about half (Figure 5).

The alignment of P35 subfamily members revealed that the P4 and P2 residues differed between AcP35 and MaviP35 (P4-DQMD-P1 *versus* P4-TQFD-P1, respectively). Mutagenesis studies of AcP35 had previously demonstrated that changing its P4 aspartate residue to either alanine or asparagine markedly impaired its ability to inhibit caspases 3 and 8,[7] highlighting the importance of the P4 amino acid for caspase inhibition. The cleavage site of MaviP35, containing a P4 threonine residue, was reminiscent of the site at which

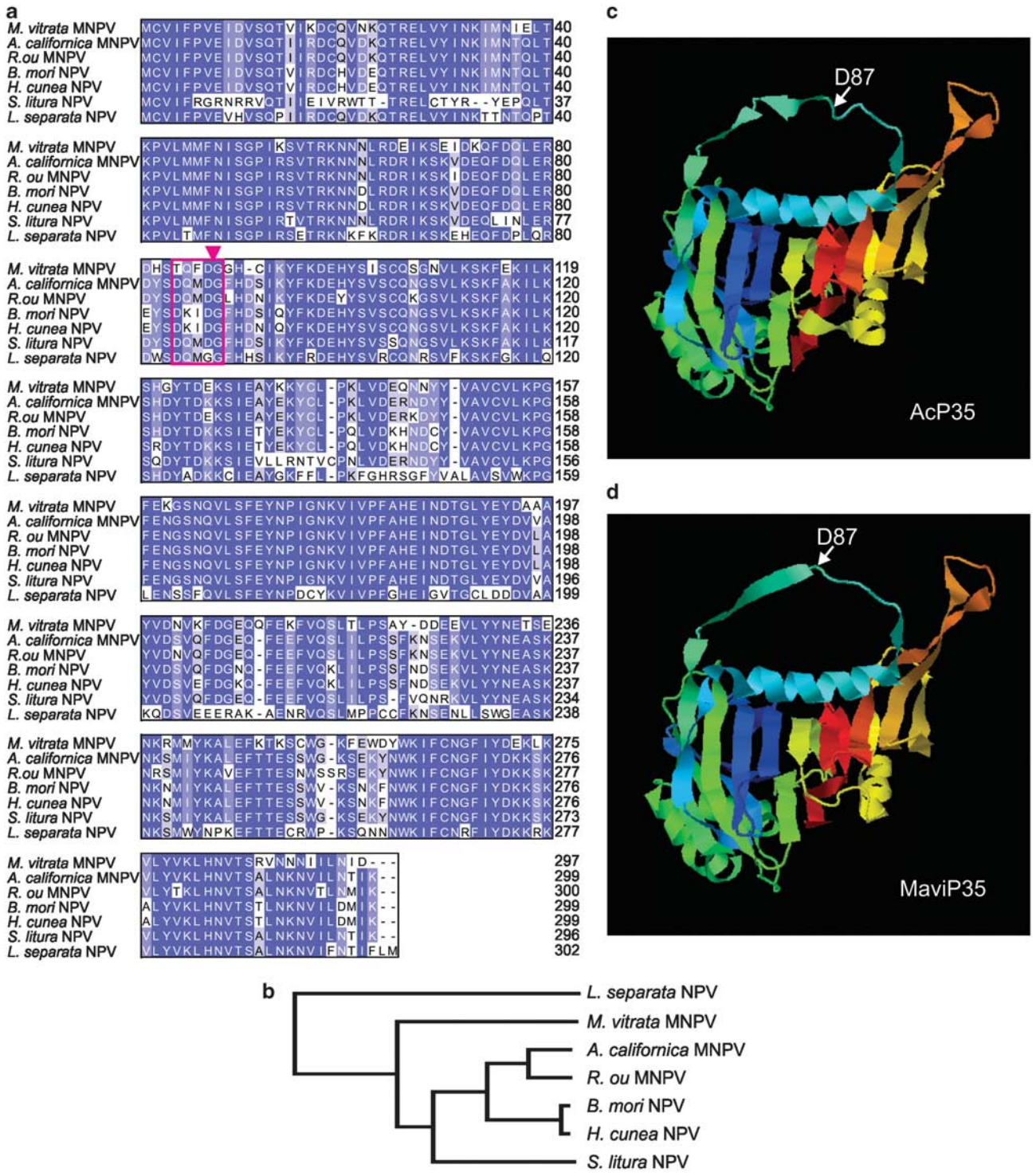


Figure 1 Relationships between MaviP35 and the other P35 subfamily members. (**a**) Alignment of baculoviral P35 subfamily proteins. Color intensity reflects the degree of conservation within the family. A pink box shows the P4-P1′ region (based on AcP35) and the caspase cleavage site is marked by an arrow. (**b**) An average distance tree was calculated using percentage identity from the multiple sequence alignment. The AcP35 structure (**c**) and predicted structure of MaviP35 (**d**) are shown. The positions of the AcP35 P1 aspartate residue and the corresponding residue in MaviP35 are indicated by arrows

DRONC auto-processes between its large and small subunit (TQTE)[11] and, to a lesser extent, the caspase cleavage site within the SpliP49 reactive site loop (TVTD).[17] This prompted us to wonder whether MaviP35 may be the first example of a P35 subfamily member that can inhibit DRONC. Consistent with this notion, expression of MaviP35 completely abolished DRONC-mediated yeast death (Figure 6a) and recombinant DRONC could cleave purified MaviP35, although not as

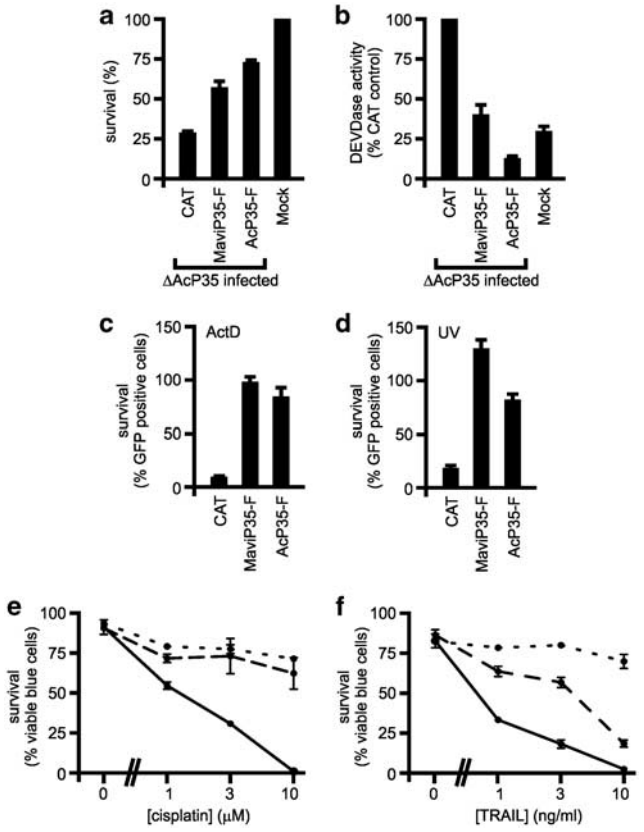


Figure 2 MaviP35 inhibits mammalian and insect cell death. (**a**, **b**) Sf9 cells were transfected with plasmids encoding MaviP35-FLAG, AcP35-FLAG or chloramphenicol acetyl transferase (CAT), then infected with an AcP35-mutant baculovirus. Untransfected cells were subjected to mock infection. (**a**) Survival was monitored relative to the mock-infected cells. (**b**) Caspase activity was detected and expressed relative to the DEVDase activity in infected cells expressing the negative control CAT protein. (**c** and **d**) Sf21 cells were co-transfected with plasmids encoding GFP and the specified proteins. After 4 h heat shock-mediated induction of transgene expression, the cells were treated with actinomycin D (**c**) or UV (**d**). Viability is expressed as the number of GFP-positive cells following exposure to apoptotic stimuli, relative to their number before treatment. (**e** and **f**) MEF (**e**) or LN18 glioblastoma cells (**f**) were co-transfected with a β-galatosidase expression plasmid and either an empty vector (solid lines), or plasmids encoding MaviP35 (dashed lines) or AcP35 (dotted lines), before treatment with the indicated doses of either cisplatin (**e**) or crosslinked TRAIL (**f**). Following 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining, the survival of blue (transfected) cells was scored using morphological criteria. Over 80 (**e**) or 150 (**f**) blue cells were scored for each condition per experiment. Error bars represent S.E.M. from three separate experiments (**a**–**f**)

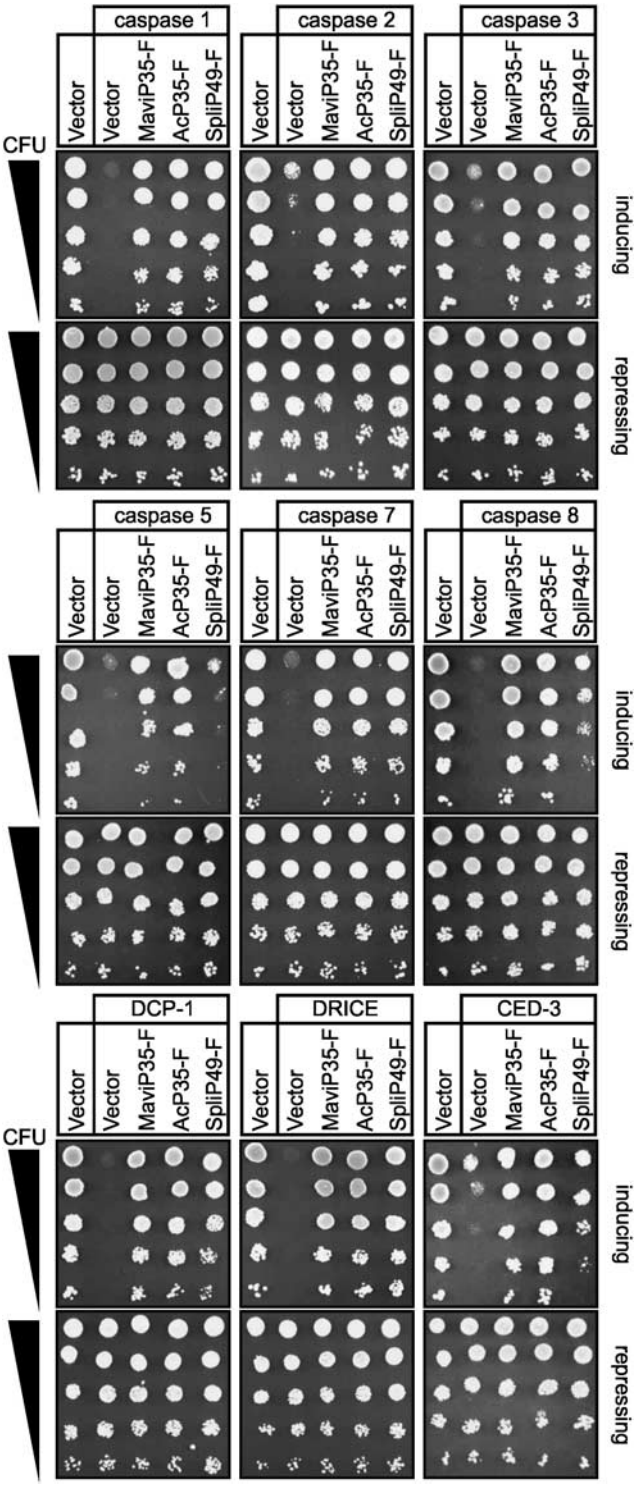


Figure 3 MaviP35 inhibits caspase-dependent yeast death. Yeast were transformed with the indicated expression plasmids. Suspensions containing equivalent concentrations of each transformant were serially diluted and 5 μl of each dilution were spotted onto plates containing galactose (to induce transgene expression) or glucose (to repress transgene expression). Growth on inducing plates indicates survival and proliferation of yeast expressing the transgenes

efficiently as DRICE (Figures 6b and c). We therefore sought to determine whether MaviP35 could suppress DRONC activity in insect cells. DRONC can cleave DRICE between its large and small subunits[10,11] and cleavage of DRICE in *Drosophila* cells has previously been used as a readout of DRONC activity.[19,27] A GFP-tagged active site mutant of DRICE expressed in *Drosophila* Kc167 cells was completely processed to yield a 39-kDa product following actinomycin D treatment (Figure 6d), as expected from DRONC cleavage between the large and small subunits of DRICE (Figure 6e). Enforced expression of DIAP1 completely inhibited cleavage of DRICE^{C211A}-eGFP in actinomycin D-treated *Drosophila* cells (Figure 6). Curiously, AcP35 partially inhibited DRICE^{C211A}-eGFP cleavage, with more than half of the protein remaining intact in cells co-expressing AcP35. MaviP35 also partially inhibited this cleavage event, although

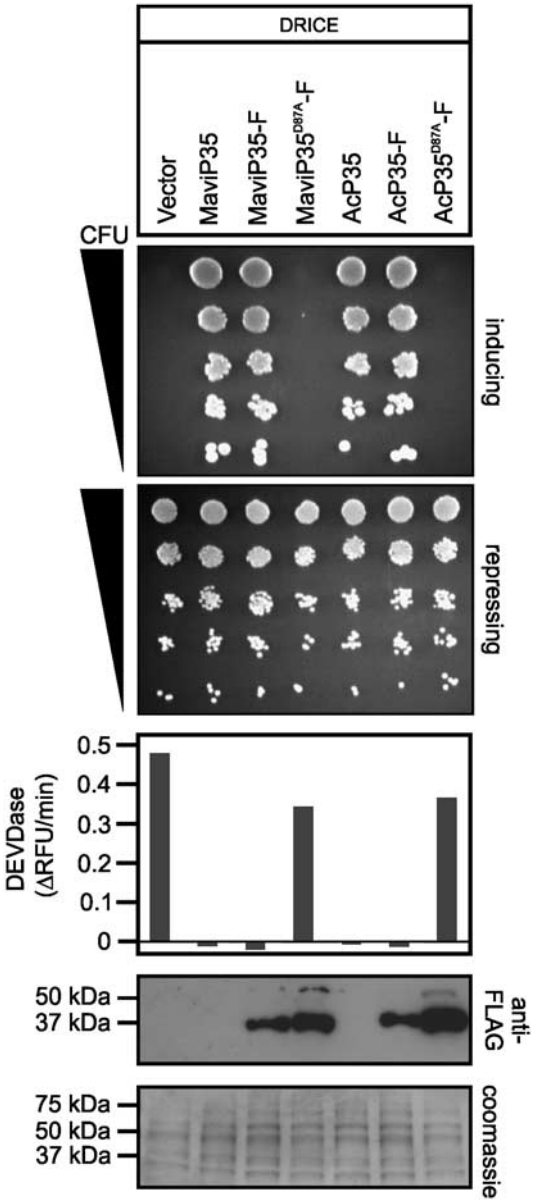


Figure 4 MaviP35 activity requires the P1 aspartate residue but is unaffected by addition of a C-terminal FLAG tag. Yeast were transformed with the indicated expression plasmids. Suspensions containing equivalent concentrations of each transformant were serially diluted, and 5 μl of each dilution were spotted onto inducing and repressing plates. The caspase activities of induced transformants' lysates were compared using the fluorogenic Ac-DEVD-AFC substrate. Anti-FLAG immunoblotting was performed and loading was visualized by coomassie staining

less potently than AcP35. Purified MaviP35 did not impair the ability of recombinant DRONC to cleave a peptide substrate (Figure 6f). Using a range of substrate and inhibitor concentrations, inhibition by bacterially produced DRONC of MaviP35 was extremely weak (Figure 7). Quantitation of MaviP35's inhibition of other caspases confirmed the data shown in Figure 5: strong inhibition of executioner caspases, but weak to negligible inhibition of initiator caspases (Figure 7c).

Discussion

This study describes the apoptosis and caspase inhibitory properties of a new P35 subfamily member: MaviP35. Like AcP35, MaviP35 could inhibit insect and mammalian cell death, was susceptible to caspase cleavage, and could inhibit the proteolytic activity of caspases. Nevertheless, AcP35 and MaviP35 differed in their specificity profile. MaviP35 inhibited executioner apoptotic caspases with similar potency to AcP35. However, MaviP35 was substantially less potent than AcP35 at inhibiting mammalian caspases 8 and 10. This may explain the weaker protection afforded by MaviP35 relative to AcP35 against TRAIL-induced cell death. MaviP35 was also a weaker inhibitor of recombinant caspase 9 than AcP35. However, it is important to note that published data suggest that the susceptibility of recombinant caspase 9 to AcP35 *in vitro* is not mirrored by apoptosome-activated caspase 9 within cell lysates,[13] so it is possible that AcP35 and MaviP35 are both incapable of interfering with the activity of naturally activated caspase 9 *in vivo*.

The P4 residue of MaviP35 (threonine) differs from that of AcP35 (aspartate). Mutation of P4 in AcP35 to asparagine reduced its ability to inhibit caspase 3 by 47-fold,[7] yet MaviP35 – which contains the slightly larger polar uncharged residue threonine at this position – inhibited caspase 3 with similar efficiency to wild-type AcP35. Presumably differences in other regions of the protein compensate, allowing MaviP35 to efficiently suppress caspase 3 activity. The MaviP35 cleavage site resembles the auto-processing site between the large and small subunits of DRONC, and MaviP35 was sensitive to DRONC proteolysis *in vitro*. MaviP35 inhibited yeast death triggered by high-level expression of DRONC, suggesting that it could function as a pseudo-substrate inhibitor of DRONC. However, two pieces of evidence argue against this possibility. First, MaviP35 was an extremely weak inhibitor of recombinant DRONC activity *in vitro*. Its K_i was indistinguishable from that of AcP35, which was incapable of inhibiting DRONC in yeast and *in vivo*[10,11] and was resistant to DRONC cleavage *in vitro*. Second, MaviP35 only partially inhibited actinomycin D-induced cleavage of the DRICE^{C211A}–eGFP fusion protein, impeding processing to a lesser extent than AcP35. This result implies that actinomycin D treatment provoked DRONC-mediated (AcP35/MaviP35 resistant) processing of the DRICE-eGFP substrate in Kc167 cells, but also suggested that other proteases, sensitive to AcP35/MaviP35 inhibition, also contributed to this proteolysis. Taken together, these data lead us to postulate that MaviP35 functions as a classical substrate for DRONC, rather than as a suicide substrate inhibitor. It is possible that the ability of MaviP35 to suppress DRONC-mediated yeast lethality reflects substrate competition: DRONC's proteolytic attention may be diverted to MaviP35 processing rather than cleavage of essential yeast proteins.

Despite the high homology between MaviP35 and AcP35, this study has revealed an important difference in their caspase specificity. Both inhibitors could suppress downstream caspases from insects and mammals (and CED-3), but only AcP35 could efficiently block the activities of mammalian caspases-8 and -10. Neither AcP35 nor MaviP35 could significantly inhibit the *Drosophila* initiator caspase DRONC.

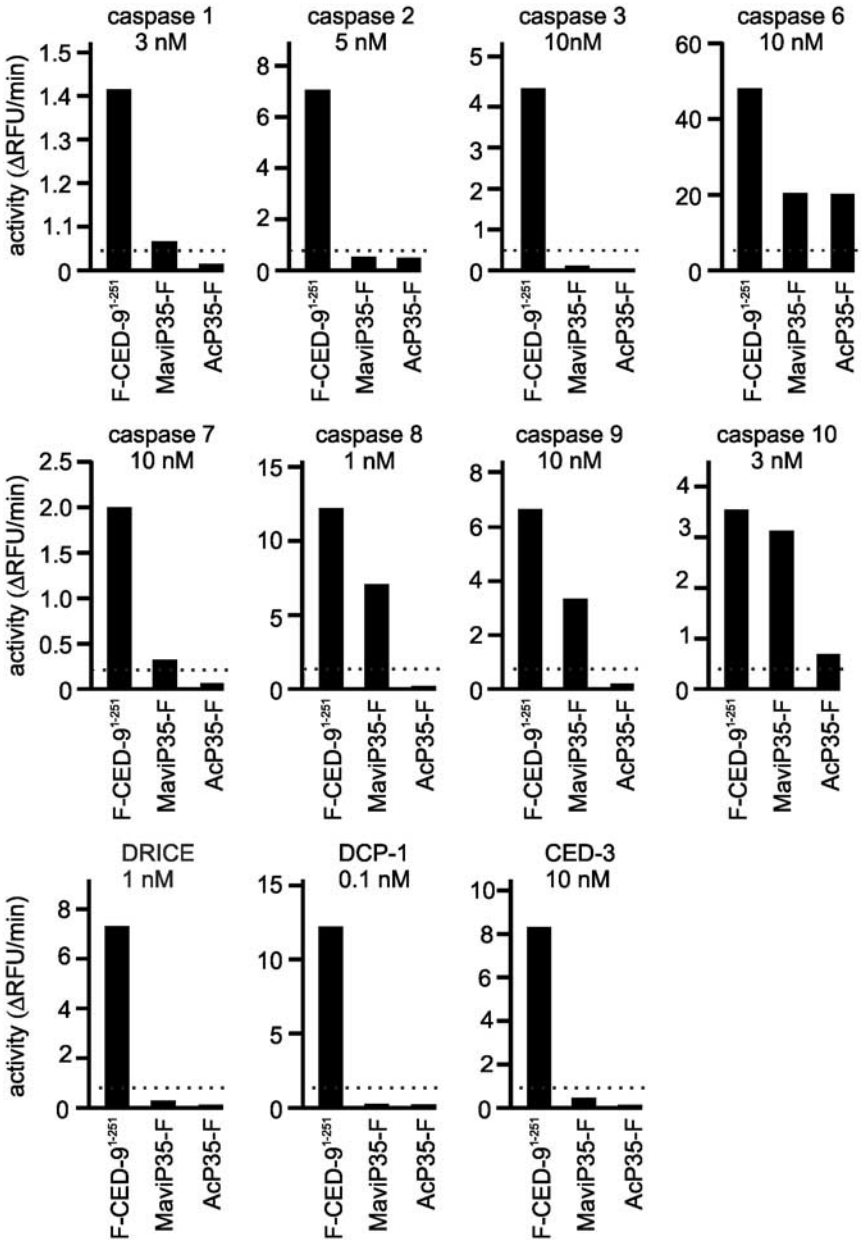


Figure 5 MaviP35 inhibits recombinant caspases *in vitro*. Cleavage of fluorogenic substrates by purified recombinant caspases (at the indicated concentrations) is plotted as the change in RFU per minute, in the presence of 1 μM of FLAG-tagged MaviP35, AcP35 or truncated CED-9 (a cytosolic protein devoid of caspase inhibitory activity). The dotted line indicates 10% activity, relative to the substrate cleavage rate in the presence of the control protein

Materials and Methods

Sequence comparisons and structural modeling. Sequences of the P35 subfamily members were aligned using the 'Multialign' program[28] and formatted using Jalview 2.0.[29] Genbank accession numbers for the P35 sequences used were: *Mavi*MNPV: YP_950833, *Ac*MNPV: NP_054165.1, *Ro*MNPV: NP_703122, *Bm*NPV: AAO12972, *Hycu*NPV: AAO17287, S*plt*NPV: CAA71304 and *Lese*NPV: AAF78504. Protein Homology/analogY Recognition Engine (Phyre) was used to predict the structure of MaviP35.[30] AcP35 and predicted MaviP35 structures were depicted using Jmol: an open-source Java viewer for chemical structures in 3D (http://www.jmol.org/).

Plasmid construction. For yeast experiments, coding DNA sequences were expressed from inducible Gal1/10 promoters.[31] Plasmids-expressing caspase 2, caspase 3-lacZ, caspase 5, caspase 7[53], caspase 8, CED-3, reverse DRICE, DCP-1, DRONC, AcP35, AcP35-F, AcP35^{D87A}-F and SpliP49-F have been described previously.[11,21,32–34] Other plasmids were generated as follows: the caspase 1 coding region was amplified using oligonucleotides 1 and 2 from pET21b-Casp-1-His (purchased from Addgene Cambridge, MA, USA). The product was cut with *Bam*HI/*Xba*I and ligated into pGALL-(*LEU2*)[32] cut with *Bam*HI/*Xba*I. The *MaviP35* coding region was amplified with oligonucleotides 3 and 4 from a plasmid kindly donated by Prof. Chung-Hsiung Wang, cut with *Bam*HI/*Xho*I and ligated into pGALL-(*HIS3*)[32] cut with *Bam*HI/*Xho*I. *MaviP35* was also amplified with oligonucleotides 3 and 5 and cloned the same way into pGALL-(*HIS3*), to incorporate a carboxyl terminal FLAG tag. Site-directed mutagenesis to generate *MaviP35^{D87A}* was performed using PCR. After excising *MaviP35* from pGALL-(*HIS3*)-MaviP35 and inserting it into Bluescript II SK + via *Bam*HI/*Xho*I, a PCR was conducted using *MaviP35-F* as a template (oligonucleotides 6 and 7) to amplify the 3′ portion of the gene incorporating a P1 mutation. This product was cut with

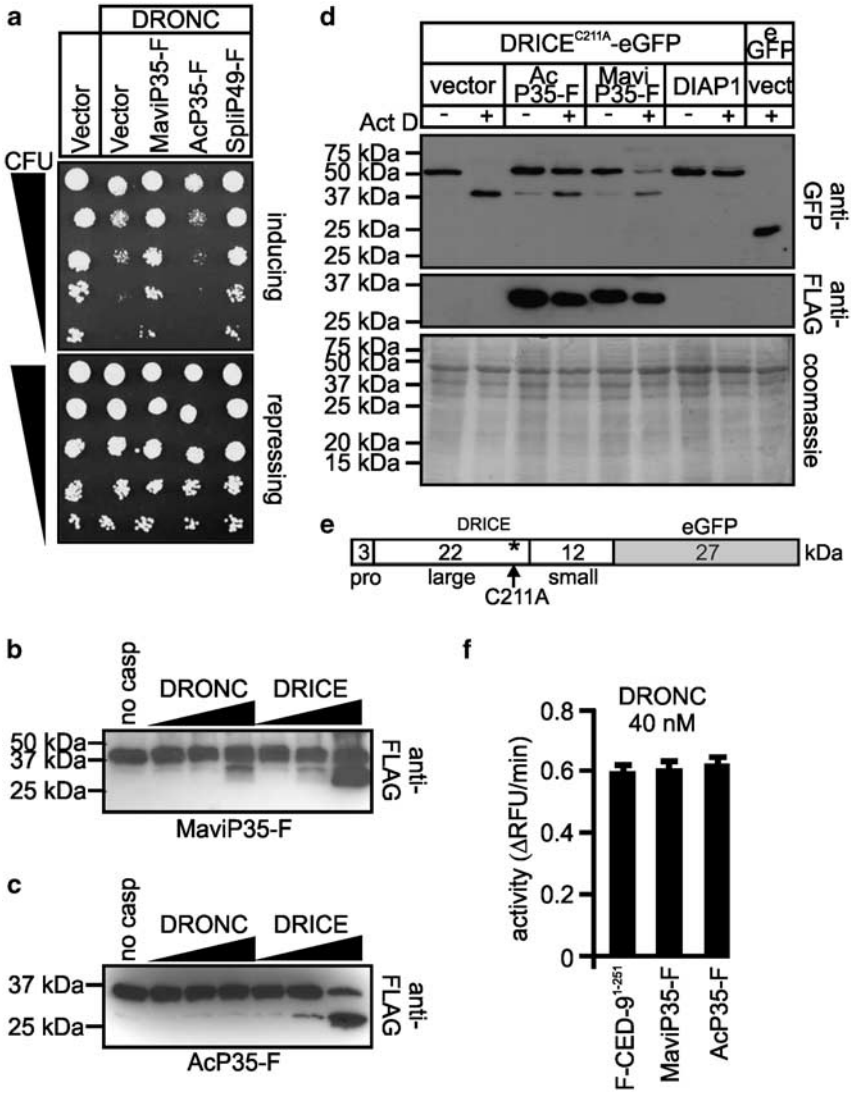
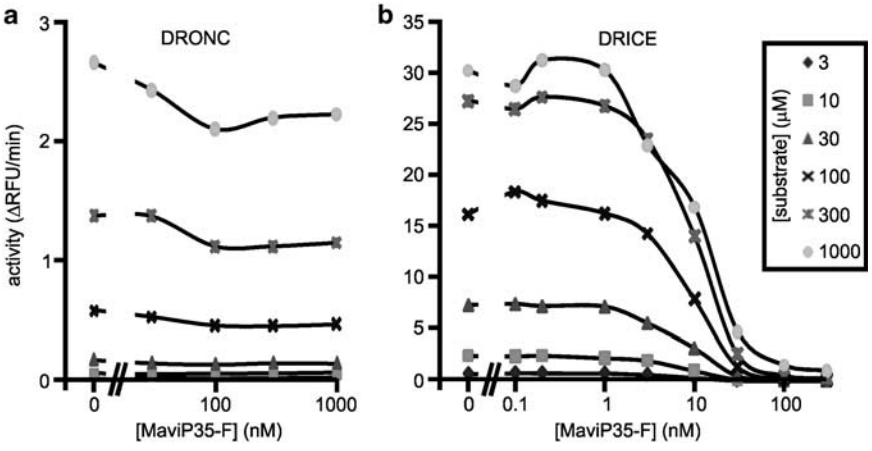


Figure 6 MaviP35 is cleaved by DRONC but does not inhibit DRONC activity *in vitro*. (**a**) Yeast were transformed with the indicated expression plasmids. Suspensions containing equivalent concentrations of each transformant were serially diluted, and 5 μl of each dilution were spotted onto inducing and repressing plates. A total of 400 ng of FLAG-tagged MaviP35 (**b**) or AcP35 (**c**) were incubated with 0, 15, 150 or 1500 nM of DRONC or DRICE, then processing was monitored by anti-FLAG immunoblotting. (**d**) *Drosophila* Kc167cells were co-transfected with a plasmid encoding either eGFP or DRICE^{C211A}-eGFP, together with either an empty vector or plasmid directing expresion of AcP35-F, MaviP35-FLAG or DIAP1. Transfectants were incubated in media containing or lacking 1 μM actinomycin D (ActD) fo 12 h. Lysates were subjected to anti-GFP or anti-FLAG immunoblotting, or coomassie staining. (**e**) The structure of the DRICE^{C211A}–eGFP fusion protein is shown. (**f**) Cleavage of Ac-TQTD-AFC by purified recombinant DRONC is plotted as the change in RFU per minute, in the presence of 1.6 μM of FLAG-tagged MaviP35, AcP35 or truncated CED-9

*Bcl*I/*Xho*I and inserted into *Bcl*I/*Xho*I cut MaviP35-Bluescript II SK + . The resulting P1-mutated *MaviP35-F* gene was then excised with *Bam*HI/*Xho*I and ligated into pGALL-(*HIS3*). For mammalian and insect cell expression, *MaviP35-F* was amplified from pGALL-(*HIS3*)-MaviP35-F with oligonucleotides 8 and 7, then cut with *Bam*HI/*Xho*I, blunted and inserted into pHSP70PLVI^{+}CAT (chloramphenicol acetyl transferase)[35] cut with *BglII*/*EcoR*I blunted or pEF[36] cut with *Bam*HI/*Xba*I blunted. *Caspase 3* was amplified using primers 9 and 10, then digested with *Nde*I/*Xho*I and ligated into *Nde*I/*Xho*I cut pET23a. All amplified fragments were sequenced to verify the absence of any unintentional mutations. The pAct5eGFP vector was kindly provided by Samuel Le Fort and David Vaux. The coding sequencing of *DRICE^{C211A}* was amplified from pGMR-DRICE^{C211A} (described below) using primers 11 and 12, cut with *BglII* and cloned into pACT5eGFP cut with *Bam*HI to generate pACT5c-DRICE^{C211A}-eGFP. Restriction analyses and sequencing were performed to determine the orientation of the insert and to check the sequence was correct. pGMR-DRICE was made by amplifying *DRICE* with primers 13 and 14, then cutting the product with *Bgl*II and *Xba*I and cloning into pGMR.[37] pGMR-DRICE^{C211A} was constructed by amplifying the 3′ portion of *DRICE* encoding the active site with a mutagenic forward primer (15) and wild-type reverse primer (16), digesting the product with *Nhe*I and *Not*I and ligating into pGMR-DRICE cut with *Nhe*I and *Not*I. The DNA encoding eGFP was removed from pAct5eGFP by cutting with *Bam*HI and *Xba*I, blunting using Klenow polymerase and religating to yield pAct5c. *Bam*HI/*Xba*I fragments encoding either DIAP1 (obtained by digesting pGALL-(*HIS3*)-DIAP1) or MaviP35-FLAG (excised from pGALL-(*HIS3*)-MaviP35-F) were ligated into *Bam*HI/*Xba*I cut pAct5c-eGFP, replacing the *eGFP* gene.

The sequences of the nucleotides referred to above were as follows:

1: 5′-CGGGATCCATGGCCGACAAGGTCCTGAAGGAG-3′
2: 5′-GCTCTAGATTAATGTCCTGGGAAGAGGTAGAAACATC-3′
3: 5′-GGGATCCCATATGTGTGTAATTTTTCCAGTAG-3′



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caspase (concentration, substrate)	AcP35-F	MaviP35-F
caspase 3 (30 nM, Ac-DEVD-AFC)	0.23 +/- 0.04 nM	0.34 +/- 0.05 nM
caspase 8 (2 nM, Ac-IETD-AFC)	0.26 +/- 0.07 nM	168 +/- 33 nM
caspase 9 (30 nM, Ac-LEHD-AFC)	14.22 +/- 7.69 nM	1.2 +/- 0.5 μM
DRICE (10 nM, Ac-DEVD-AFC)	0.89 +/- 0.28 nM	1.34 +/- 0.25 nM
DRONC (100 nM, Ac-VEID-AFC)	3.7 +/- 1.4 μM	3.8 +/- 1.6 μM

Figure 7 MaviP35 is an efficient inhibitor of executioner caspases *in vitro*. Purified FLAG-tagged MaviP35 and AcP35 were tested *in vitro* for their ability to inhibit cleavage of fluorogenic peptide substrates by human caspases 3, 8 and 9 and *Drosophila* DRICE and DRONC. Hydrolysis of the indicated substrates was monitored in the presence of a range of inhibitor concentrations. Graphs show the impact of various concentrations of MaviP35 on DRONC (**a**) and DRICE (**b**) activity. (**c**) Inhibition constants (K_i) were calculated for selected caspases using a competitive model, as described in the Materials and Methods

4: 5′-GCCTCGAGTTAATCAATGTTTAATATTATATTG-3′
5: 5′-GCCTCGAGTTACTTGTCATCGTCGTCCTTGTAGTCCATATCA ATGTTTAATATTA TATTGTTG-3′
6: 5′-CAATTTGATCAACTAGAACGCGACCACAGCACTCAATTCGCT GGAGGCC-3′
7: 5′-CTTTATTATTTTTATTTTATTGAGAGGGTGG-3′
8: 5′-GCGGATCCGCCATGTGTGTAATTTTTCCAGTAG-3′
9: 5′-GGAATTCCATATGGAGAACACTGAAAACTCAGTGG-3′
10: 5′-CCCTCGAGGTGATAAAAATAGAGTTCTTTTGTGAGC-3′
11: 5′-GTCAGATCTCAAAATGGACGCCACTAACAATGGAG-3′
12: 5′-GTCAGATCTACCCGTCCGGCTGGAGCCAAC-3′
13: 5′-CGAGATCTCCGCCATGGACGCCACTAACAATGGAGAATCC-3′
14: 5′-CGTCTAGACTAAACCCGTCCGGCTGGAGCCAACTGC-3′
15: 5′-CCTCGCTAGCCGGCAAACCCAAGTTGTTCTTCATACAGGCCG CCCAGGGC-3′
16: 5′-GCACTAGTGCGGCCGCCTAAACCCGTCCGGCTGGAGCCAAC TGC-3′

Apoptosis assays from insect cells. Sf21 cells were plated at 8×10^5 cells per well in six-well plates in TC-100 insect medium (Invitrogen, Carlsbad, CA, USA) plus 10% fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA, USA), and allowed to attach overnight at 27°C. Transfections were performed using lipofectin, which was prepared as a 1.5 : 1 mixture of DOTAP ((*N*-(1-(2,3-Dioleoyloxy)propyl)-*N,N,N*,-trimethylammonium chloride salt; Avanti Polar Lipids, Alabaster, AL, USA) and DOPE (L-α Phosphatidylethanolamine, dioleoyl; Sigma-Aldrich, St. Louis, MO, USA). For each well, 2.5 μg of the eGFP expression plasmid pHSP70GFPBsu36I[38] was mixed with 2.5 μg of either pHSP70PLVI$^+$CAT, pHSP70PLVI$^+$AcP35 or pHSP70PLVI$^+$MaviP35F. This DNA was diluted to 100 μl with TC-100 lacking FBS and incubated for 5 min at room temperature (RT). In a separate tube, 6 μl lipofectin was diluted to 100 μl with TC-100 lacking FBS and incubated for 5 min at RT. The mixtures of DNA and lipofectin were then combined and allowed to incubate for 15 min at RT. During this incubation the Sf21 cells were washed twice with 1 ml of TC-100 lacking FBS. After the last wash, 800 μl of TC-100 lacking FBS was left in each well and the DNA/lipofectin mixture (200 μl) was added to each well and allowed to incubate at 27°C for 4 h. The mixture plus the media were removed and 2 ml of TC-100 plus 10% FBS was added to each well. The cells were heat shocked 24 h post transfection at 42°C for 30 min, to drive expression from the hsp70 promoter. The cells were then induced to undergo apoptosis 4 h post heat shock, by treatment with either UV (by placing the plates on a transilluminator for 10 min) or actinomycin D (Invitrogen; 250 ng/ml). To determine cell viability, the number of GFP-expressing cells was counted in each well both immediately before and 17 h after UV or actinomycin D treatment. Three random fields of view per sample were counted per well, and three separate wells were assayed per treatment.

To assay sensitivity to infection-mediated apoptosis, Sf9 cells (10^6) were plated in six-well culture dishes for 2 h in the TC-100 medium with 10% FBS. After 2 h, the medium was replaced with Grace's insect unsupplemented medium (Invitrogen). Cells were transiently transfected with pHSP70PLVI$^+$CAT, pHSP70PLVI$^+$AcP35 or pHSP70PLVI$^+$MaviP35F using 3 μg of each plasmid and 6 μl of lipofectin. Transfection mixtures were replaced with TC-100 plus 10% FBS after 5 h incubation with cells. Cells were infected at 24 h post transfection with vAcP35KO-PG[39] at a multiplicity of infection of 1 PFU/cell, and then harvested at 48 h post infection for caspase and viability assays. Caspase assays were performed using the substrate Ac-DEVD-AFC (MP Biomedicals, Solon, OH, USA) as described previously.[40] To assess viability, three random fields of view were photographed ($\times$200 magnification), and viable cells were counted for each well. Cell viability was determined by counting the non-apoptotic cells and comparing to the number of viable cells in a mock-infected control at 0 h post infection, which was set at 100%.

DRICE cleavage assays in Kc167 cells. Two million Kc167 cells (kindly provided by Gary Hime) were transfected with 0.2 μg of either pAct5c-eGFP or pAct5c-DRICE-eGFP plus 1.8 μg of either pAct5c, pAct5c-MaviP35-F or pAct5c-DIAP1, using the Effectene transfection reagent (Qiagen, Doncaster, Victoria, Australia) according to the manufacturer's instructions. After 24 h transfection, the cells were incubated in media containing 0 or 1 μM of actinomycin D for 12 h. Cells were lysed in mammalian lysis buffer (50 mM Tris pH 7.5, 375 mM NaCl, 1 mM ethylenediamine tetra-acetic acid, 1% Triton X-100) containing protease inhibitors (protease inhibitor cocktail set 1; Calbiochem, Darmstadt, Germany) and subjected to 12% SDS-PAGE and either Coomassie stained or immunoblotted using anti-GFP (Roche Applied Science no. 11814460001; Castle Hill, New South Wales, Australia) or anti-FLAGM2 (Sigma no. F3165) and anti-mouse IgG-HRP (Sigma no. A9044).

Mammalian apoptosis assays. SV-40 transformed mouse embryonic fibroblasts (MEFs) were co-transfected with 1 μg of CMV-lacZ and either 3 μg of pEF, AcP35-pEF or MaviP35-pEF using FuGENE HD transfection reagent (Roche; Basel, Switzerland). LN18 glioblastoma cells (ATCC; Manassas, VA, USA) were co-transfected with 1 μg of CMV-lacZ and either 2 μg of pEF, AcP35-pEF or MaviP35-pEF using Lipofectamine (Invitrogen). Transfections were performed according to the manufacturers' instructions. Twenty-four hours after transfection, the medium was removed and the cells were incubated with fresh unsupplemented media or media containing cisplatin (Mayne Pharma, Mulgrave, Victoria, Australia) or Superkiller (crosslinked) TRAIL (Alexis Biochemicals, Lausen, Switzerland). After 24 h, the cells were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and the blue cells were scored for viable *versus* apoptotic morphology, as previously published.[36]

Yeast transformation and death assays. *Saccharomyces cerevisiae* yeast strain W303α was transformed[31] and analyzed in survival assays[21] as described previously.[31] Caspase 1 was expressed using the pGALL-(LEU2)-Casp1 vector described above.

Protein assays from yeast. Yeast transformants were grown and transgene expression induced as described previously.[11] The lysates were subjected to SDS-PAGE and the gels were then stained with Coomassie brilliant blue (Sigma) to visualize protein loading and immunoblotted as described previously.[11] The membranes were probed with an antibody recognizing the FLAG tag (clone M2; Sigma) and anti-mouse-HRP (Sigma). To measure the caspase activity via fluorescence analysis, the yeast were treated as follows: An overnight culture was pelleted, washed twice with 1 ml of TE (Tris HCl 10 mM pH 8, EDTA 1 mM) and induced for 6.5 h in complete media containing 2% galactose. After pelleting the yeast culture, the yeast was weighed and glass beads were added. To lyse the cells, 5 ml of CelLyticY reagent (Sigma) with 10 mM DTT was added per 1 g of yeast cells. After gently shaking the cells for 30 min at RT, the debris was removed by centrifugation at 16 100 $\times$ *g* for 10 min at 4°C. The protein concentration of the supernatant was measured using the Bicinchoninic acid protein assay kit (Sigma). In fluorescence assays, lysate (0.5 mg/ml) was mixed with Ac-DEVD-AFC (100 μM) in DRICE activity buffer (50 mM HEPES pH7.5, 10% sucrose, 0.1% CHAPS, 5 mM DTT, 100 mM NaCl, 1 mM EDTA), and the fluorescence (excitation 410 nm, emission 500 nm) was measured every 30 s for 1 h. The slope of each curve was calculated using Graphpad Prism 5.0 (La Jolla, CA, USA). The slope of each curve determines the concentration of free AFC per minute, which provides a measure of caspase activity.

Protein purification from yeast. Transformants were grown in 5 ml glucose-containing selective medium to stationary phase, then expanded by addition of 195 ml glucose-containing selective medium. After expanding the cells for 16–18 h, the pelleted yeast were washed once with 100 ml TE and resuspended in 1 l of galactose-containing complete media for 6.5 h induction. Cells were harvested at 12 100 $\times$ *g*, 4°C for 15 min then glass beads were added after weighing the pellets. To lyse the cells CelLyticY reagent (Sigma) was used as described above. The supernatant was incubated for 30 min at 4°C with 200 μl Anti-FLAG M2 affinity gel (Sigma), which had been previously washed three times with 12 $\times$ resin volume washing buffer (Tris HCl 50 mM pH 7.4, 150 mM NaCl). Incubated beads were pelleted 5 min at 3750 g, RT then washed with 32 resin volumes of washing buffer for 10 min at 4°C. Five elution fractions were collected each using one resin volume of elution buffer (1 mM HEPES pH 7.0, 0.1% PEG, 0.001% CHAPS, 0.1 mM DTT, 200 ng/μl FLAG peptide (Sigma)). For each elution step the beads were incubated for 2 min at 4°C with agitation, and supernatant was collected after a pelleting for 1 min at 16 100 g, 4°C. After subsequent SDS-PAGE analysis and Coomassie brilliant blue (Sigma) staining, the fractions containing pure FLAG-tagged proteins were pooled, and protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad, Gladesville, New South Wales, Australia).

Recombinant caspases. Caspases 1, 2, 6, 7, 8, 9 and 10 were purchased from Enzo Life Sciences (Farmingdale, NY, USA). BL21-(DE3)-pLysS (Merck, Darmstadt, Germany) bacteria were transformed with caspase 3-pET23a (described above) or the following previously published plasmids: DCP-1-pET23a,[32] DRICE-pET23a,[32] CED-3-pET23a,[34] DRONC-pET23a.[11] Caspase 3, DCP-1, DRICE and CED-3 were purified as described previously.[34] DRONC purification was carried out as follows: A transformant colony was inoculated into 1.5 ml 2YT-amp/chlor (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl, 100 μg/ml ampicillin and 35 μg/ml chloramphenicol) and grown overnight at 37°C. A volume of 1 ml of this culture was expanded into 50 ml of pre-warmed 2YT-amp/chlor and grown at 37°C for 2 h, 200 r.p.m. A total of 10 ml of this was mixed with 190 ml of pre-warmed 2YT amp/chlor in a 2-l baffled flask and shaken at 37°, 200 r.p.m. until OD_{600} reached 0.6–0.8. IPTG was added to a final concentration of 1 mM and the culture was shaken at 20°C for 18 h, then pelleted for 10 min at 3000 g, 4°C and then frozen at -80°C. The pellet was thawed then resuspended in a 10-ml Bug Buster Mastermix (Merck) by pipetting, then incubated for 20 min at RT. Insoluble cell debris was removed by centrifugation at 16 100 $\times$ *g* for 20 min at 4°C. Half a milliliter of NiNTA resin (Qiagen) was washed twice in phosphate buffer (50 mM $NaHPO_4$, 300 mM NaCl), then incubated with the induced bacterial lysate for 30 min at 4°C, gently mixing. The beads were washed twice with phosphate buffer containing 5 mM imidazole, then the caspase was eluted with phosphate buffer containing 250 mM imidazole.

***In vitro* quantitation of caspase inhibition.** Caspases 1, 2, 3, 6, 7, 8, 9, 10, DRICE, DCP-1 and CED-3 were pre-activated for 10 min at 37°C in universal caspase citrate buffer (10 mM HEPES pH 7.0, 10% sucrose, 0.1% CHAPS, 10 mM DTT, 100 mM NaCl, 1 mM EDTA, 0.65 M Na-Citrate). After the activation step, the caspase was incubated either with buffer alone, F-CED-9^{1-251}, AcP35-F or with MaviP35-F for 1 h at 37°C. The appropriate fluorescent substrate was then added (100 μM): Ac-WEHD-AFC for caspase 1; Ac-VDVAD-AFC for caspase 2; Ac-DEVD-AFC for caspases 3, 7, DRICE, DCP-1 and CED-3; Ac-VEID-AFC for caspase 6; Ac-LEHD-AFC for caspases 8, 9 and 10 and Ac-TQTD-AFC for DRONC (Enzo Life Sciences). Fluorescence (excitation 410 nm, emission 500 nm) was measured every minute for 2 h. The maximal slope of each curve was calculated using Prism 5.0 and graphed.

Determination of inhibition constants. Caspases were pre-activated for 10 min at 37°C in the following buffers: caspase 3: 100 mM HEPES pH 7.0, 10% PEG, 0.1% CHAPS, 10 mM DTT; DRICE: 50 mM HEPES pH 7.5, 10% sucrose, 0.1% CHAPS, 5 mM DTT, 100 mM NaCl, 1 mM EDTA; caspases 8 and 9: 10 mM HEPES pH 7.0, 10% sucrose, 0.1% CHAPS, 10 mM DTT, 100 mM NaCl, 0.1 mM EDTA, 0.65 M Na-Citrate; DRONC: 50 mM Tris pH 7.4, 100 mM NaCl, 0.65 M Na-Citrate. Subsequently, the caspase was incubated with either AcP35-F or MaviP35-F in the appropriate activity buffer for 1 h at 37°C. Substrates were added at concentrations ranging from 0.001 to 1000 μM. Substrates used were: Ac-DEVD-AFC for caspase 3 and DRICE; Ac-LEHD-AFC for caspases 8 and 9 and Ac-VEID-AFC for DRONC (Enzo Life Sciences). Fluorescence (excitation 410 nm, emission 500 nm) was measured every minute for 2 h. The slope of each curve was calculated using Prism 5.0. Inhibition constants were calculated by non-linear regression using Prism 5.0 software, using a competitive inhibition model as described by these equations: $K_m^{Obs} = K_m \times (1 + [I]/K_i)$ and $Y = V_{max} \times X/(K_m^{Obs} + X)$, where $[I]$ is the inhibitor concentration (μM); K_i is the inhibition constant (μM), V_{max} is the maximum enzyme velocity (relative fluorescence units (RFU)/min), K_m is the Michaelis–Menten constant (μM), X is the concentration of substrate (μM) and Y is the change in fluorescence (RFU/min).

Conflict of Interest

The authors declare no conflict of interest.

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