

Modified vaccinia virus Ankara protein F1L is a novel BH3-domain-binding protein and acts together with the early viral protein E3L to block virus-associated apoptosis

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Abstract

Infection with viruses often protects the infected cell against external stimuli to apoptosis. Here we explore the balance of apoptosis induction and inhibition for infection with the modified vaccinia virus Ankara (MVA), using two MVA mutants with experimentally introduced deletions. Deletion of the E3L-gene from MVA transformed the virus from an inhibitor to an inducer of apoptosis. Noxa-deficient mouse embryonic fibroblasts (MEF) were resistant to MVA- Δ E3L-induced apoptosis. When the gene encoding F1L was deleted from MVA, apoptosis resulted that required Bak or Bax. MVA- Δ F1L-induced apoptosis was blocked by Bcl-2. When expressed in HeLa cells, F1L blocked apoptosis induced by forced expression of the BH3-only proteins, Bim, Puma and Noxa. Finally, biosensor analysis confirmed direct binding of F1L to BH3 domains. These data describe a molecular framework of how a cell responds to MVA infection by undergoing apoptosis, and how the virus blocks apoptosis by interfering with critical steps of its signal transduction.

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Abbreviations: MVA, modified vaccinia virus Ankara; BH3 domain, Bcl-2 homology domain 3; MEF, mouse embryonic fibroblasts; CEF, chicken embryonic fibroblasts

Introduction

Cell death by apoptosis is a common response of human cells to extrinsic stimuli. It is commonly thought that one of the purposes of apoptosis is the defence against noxious stimuli, such as genotoxic influences or infectious agents. In this context, it has long been assumed that apoptosis can serve as

a defence of human tissues against infecting viruses, and the hypothesis has even been put forward that apoptosis evolved to combat viral infections.^{1,2}

Apoptosis occurs upon triggering of a specialised signal transduction pathway. A key event in most forms of apoptosis is the release of cytochrome *c* from the mitochondria into the cytosol, where it initiates the activation of caspase proteases. Active caspases then are instrumental in the appearance of the typical morphological changes of apoptosis, such as nuclear condensation (for a review of the mechanisms of apoptosis see Hengartner³).

The idea that apoptosis serves as a defence mechanism against viruses has received strong support by the fact that many viruses carry genes whose products can directly interfere with the cell's apoptotic apparatus. An early and clear example of this situation was the demonstration that a baculovirus has the capacity to induce apoptosis in infected insect cells and that, at the same time, this capacity is countered by the expression of the viral gene p35.⁴ Since then, apoptosis inhibitors have been found in viruses that infect diverse species, such as Epstein Barr virus,⁵ African swine fever virus⁶ and various baculoviruses.⁷ In most of these cases, viral inhibitors of apoptosis are known cellular homologues, such as the antiapoptotic protein Bcl-2.

Poxviruses have been found to encode for a multitude of proteins that regulate virus–host interactions.⁸ Within this virus family, especially vaccinia virus, the closely related cowpox virus and the rabbit pathogen myxoma virus have been studied and found to possess genes that can interfere with cellular activation pathways, such as interferon- and TNF signalling. It is noteworthy that important host regulatory genes are even conserved in the genomes of highly attenuated vaccinia virus strains, such as modified vaccinia virus Ankara (MVA) or NYVAC, candidate strains for future vaccination protocols.^{9,10} One of these regulatory proteins, E3L, can bind double-stranded (ds) RNA and inhibit the dsRNA-stimulated enzymes, protein kinase R and RNA-specific adenosine deaminase.^{11,12} A vaccinia virus deficient in E3L is highly sensitive to the activity of type I interferons and restricted in growth in some cell lines such as HeLa but not in others such as chicken embryonic fibroblasts (CEF).^{13,14}

We have previously generated the mutant virus MVA- Δ E3L deficient in E3L coding sequences (open reading frame 050, transcribed leftward through nucleotides 43 269 to 42 697 in the MVA genome).^{15,16} Intriguingly, unlike MVA, MVA- Δ E3L caused apoptosis in infected CEF cells.¹⁵ Furthermore, a vaccinia virus mutant lacking E3L has been reported to cause apoptosis in HeLa cells.¹⁷ Therefore, the viral early protein E3L can be said to have an antiapoptotic function in some situations.

Besides such viral proteins that may be involved in signal transduction and indirectly in apoptosis inhibition, a vaccinia virus protein was recently described that appears to have broad antiapoptotic activity against experimental apoptotic triggers.¹⁸ This protein, F1L, can block apoptosis induced by Fas and by staurosporine, two agents that use different upstream pathways to apoptosis. This potent activity together with its mitochondrial localisation^{18,19} suggests that F1L directly targets the cellular apoptotic pathway and is a specialised inhibitor of apoptosis. Interestingly, the open reading frame encoding F1L is highly conserved between the MVA genome (ORF 029, leftward transcription through nucleotides 26 046 to 25 378, 98% amino-acid identity and vaccinia virus strain Copenhagen).¹⁶

Thus, many viruses can protect infected cells against experimental insults. It has, however, not been well explored what the physiological function of viral antiapoptotic proteins is on a molecular level. Antiapoptotic proteins are only required if there is apoptosis to be inhibited. One hypothesis is that a host cell is able to detect the replicating virus and responds by undergoing apoptosis. This would mean that viruses in fact induce apoptosis that needs to be inhibited to allow viral replication. In this study we investigate this idea for the infection with MVA and two mutant viruses each lacking one of the genes encoding E3L or F1L.

Results

Apoptosis induction by MVA-ΔE3L

The mutant virus MVA-ΔE3L was originally generated to investigate the cellular response to MVA-based vaccine vectors. When analysing this response, we noticed that

MVA-ΔE3L had lost its ability to grow in CEF, and that this failure correlated with the induction of apoptosis in CEF cells.¹⁵ To find out whether this apoptotic response was confined to CEF, we first infected HeLa human epithelial cells with MVA or MVA-ΔE3L. Cells infected with MVA did not undergo apoptosis and showed a strong resistance to apoptosis induced by treatment with staurosporine (Figure 1a). This activity was lost in MVA-ΔE3L. Instead, this mutant virus actively induced killing of HeLa cells, as detected by assessment of nuclear morphology and by measurement of effector caspase activity (Figure 1b and c). The apoptosis-inducing activity of MVA-ΔE3L is thus not restricted to CEF cells.

MVA-ΔE3L activates the mitochondrial pathway and utilises Noxa

We next investigated whether MVA-ΔE3L-induced apoptosis proceeded via the release of mitochondrial cytochrome *c*. As shown in Figure 2a, infection of HeLa cells with MVA-ΔE3L but not MVA caused the release of mitochondrial cytochrome *c*, as shown by microscopic analysis and by flow cytometry.²⁰ Cytochrome *c* release is the result of the activation of the proapoptotic Bcl-2-family members Bax/Bak. Staining for conformationally activated Bax showed that infection with MVA-ΔE3L led to Bax activation (Figure 2b). Thus, apoptosis induction by MVA-ΔE3L activates the mitochondrial pathway to apoptosis.

Bax and Bak are the most downstream molecules of the cytochrome *c*-release machinery known.²¹ Although they seem to be redundant in some circumstances,^{22,23} there is a clear preference for one over the other in some situations.^{24,25}

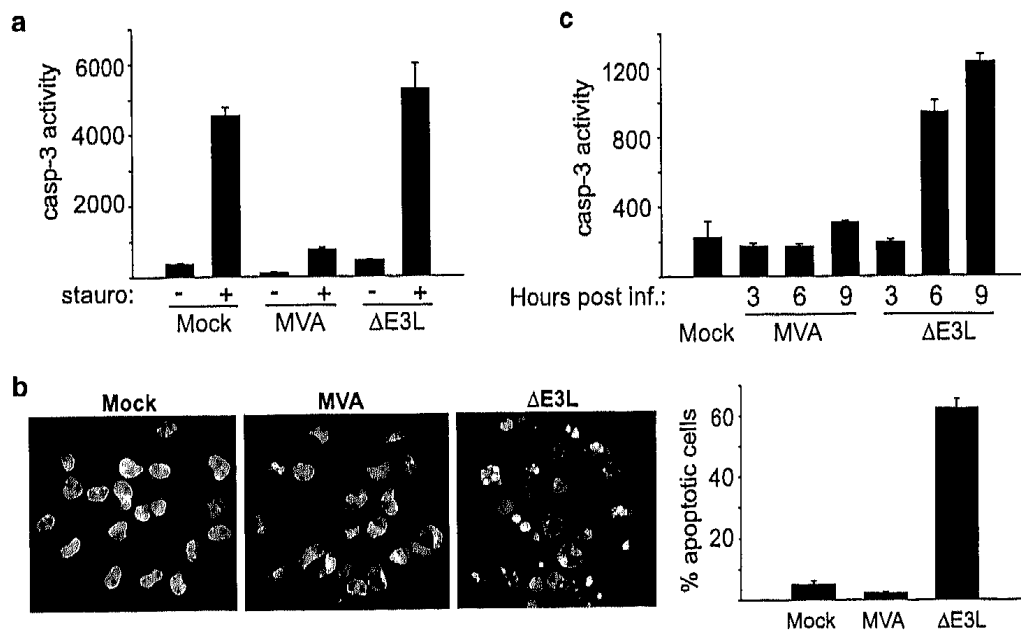


Figure 1 Apoptosis induction by MVA-ΔE3L. (a) HeLa cells were infected with an MOI of 10–15 of either MVA or MVA-ΔE3L. At 8 h p.i., some wells were treated with staurosporine (1 μM). After 3 h, cells were extracted and caspase activity was measured by enzyme assay. (b) HeLa cells were infected as under (a). After 15 h, nuclei were stained with Hoechst. Left, representative areas of the plate were photographed. Right, quantification of nuclear apoptosis (mean/S.D. of triplicate wells). Similar results were obtained in three separate experiments. (c) HeLa cells were infected at various times with either MVA or MVA-ΔE3L (MOI = 10–15). All cells were harvested at the same time. Data are mean/S.D. from triplicate measurements of one infected well. Similar results were obtained in three separate experiments. Note the difference in scaling between a and c

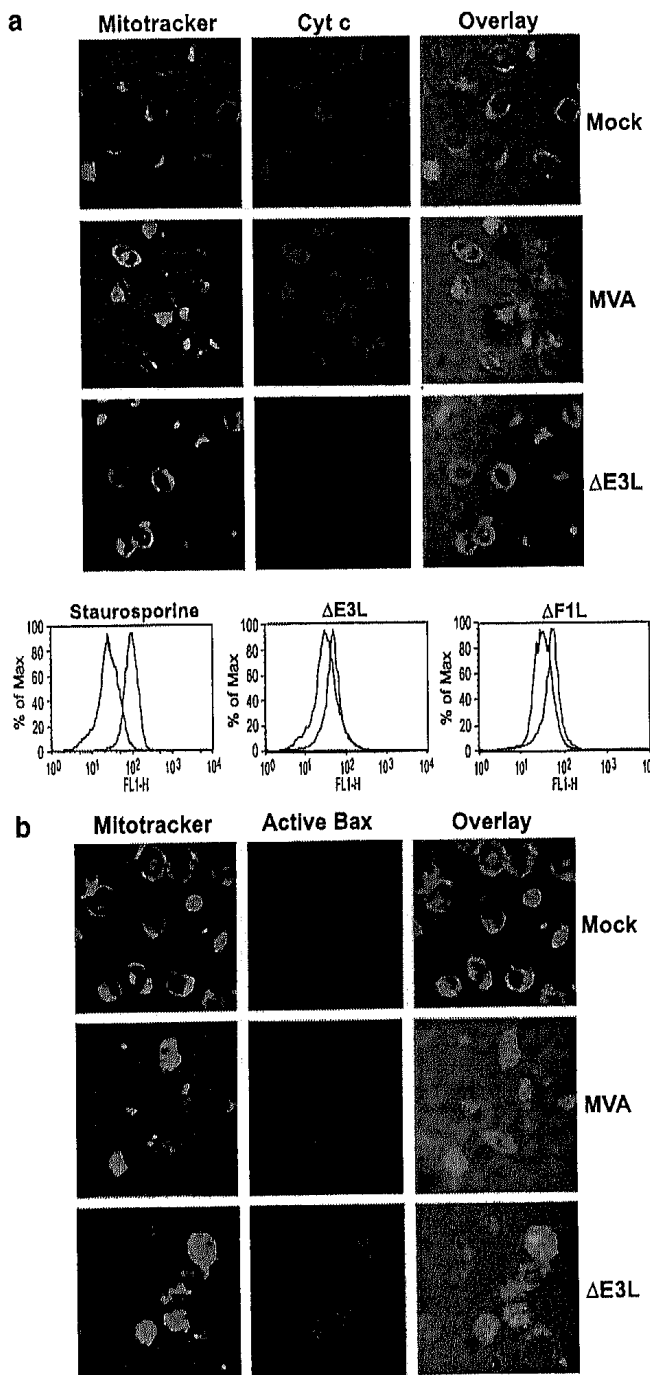


Figure 2 MVA- Δ E3L induces the release of cytochrome c and causes the activation of Bax. HeLa cells grown on coverslips were infected with either MVA or MVA- Δ E3L (MOI = 10–15). At 10 h p.i., cells were fixed and stained with MitoTracker Green FM and with antibodies either directed against cytochrome c (a) or against conformationally altered Bax (b). The bottom panel in (a) shows a flow cytometric analysis of cytochrome c content during treatment (blue lines) with staurosporine (control of apoptosis induction, 500 nM, 8 h) and infection with MVA- Δ E3L or MVA- Δ F1L as above (12 h; see below on the MVA- Δ F1L virus). Red lines represent controls (for staurosporine, no treatment; for infection with mutant viruses, controls represent infection with MVA). Similar results were obtained in four separate experiments. Note loss of cytochrome c and presence of conformationally altered Bax during apoptosis

The activation of Bax/Bak is the consequence of the activation of members of the BH3-only group of Bcl-2 protein family members.^{21,26} We therefore sought to identify the BH3-only protein required for apoptosis induction by MVA- Δ E3L. Since

E3L binds and probably sequesters dsRNA that is generated during viral replication, we hypothesised that apoptosis induction by MVA- Δ E3L was caused by the appearance of dsRNA and could be mimicked by treatment of the cells with poly-I:C (a synthetic dsRNA-mimetic). The human keratinocyte cell line HaCaT was found to be sensitive to poly-I:C, with about 50–60% of cells undergoing apoptosis when treated with 50 μ g/ml poly-I:C for 24 h (data not shown). The expression levels of the BH3-only proteins, Bim and Puma, did not change during poly-I:C-treatment, but Noxa expression was clearly induced (Figure 3a and data not shown). Noxa induction by dsRNA was recently also reported by others.²⁷ Intriguingly, Noxa was induced by infection of HeLa cells with either MVA or MVA- Δ E3L to a similar extent (Figure 3b). This suggests that the transcriptional induction of Noxa may not be the sole result of free dsRNA, which should be present more abundantly in infections with MVA- Δ E3L than in infections with MVA, but may be induced through some other mechanism.

To assess the importance of Noxa for apoptosis induction by MVA- Δ E3L, we infected mouse embryonic fibroblasts (MEF) with either MVA or MVA- Δ E3L. As in CEF and HeLa cells, MVA- Δ E3L caused a strong apoptotic response in MEF. When MEF from mice deficient in the Noxa gene were used, this response was abrogated demonstrating that Noxa crucially contributes to apoptosis induction by MVA- Δ E3L (Figure 3c and d). Furthermore, transient expression of Bcl-2 by transfection blocked the induction of apoptosis through MVA- Δ E3L in HeLa cells (not shown), consistent with a model where Noxa is activated and induces apoptosis by MVA in the absence of E3L.

This raises the question why MVA, which induces Noxa to a similar extent, does not induce apoptosis. We considered the possibility that MVA- Δ E3L is defective in the expression of the antiapoptotic protein F1L. Northern blot analysis showed that the mRNA for F1L is made at early times, but apparently less abundantly during infection with MVA- Δ E3L, as compared to MVA infection (Figure 3e). The most likely model is therefore that MVA and MVA- Δ E3L both induce the expression of Noxa but only MVA is able to block the apoptosis-inducing activity of Noxa. It should further be added that mere Noxa expression is a poor apoptosis stimulus at least in HeLa cells. When Noxa was induced in a tetracycline-inducible system to levels much higher than the ones achieved during infection, only approximately 10–20% of cells underwent apoptosis (Figure 3f and data not shown). Therefore, although Noxa is required, other unknown events must contribute to apoptosis induction by MVA- Δ E3L.

Loss of E3L causes the induction of apoptosis in MVA-infected cells, and E3L may therefore be considered an antiapoptotic protein. However, unlike other viral proteins, E3L does not appear to directly target the apoptotic pathway, as do for instance viral Bcl-2 homologues or the baculoviral inhibitor of apoptosis proteins (IAP). As mentioned above, the vaccinia F1L protein has been found to have strong antiapoptotic activity.²⁸ This broad inhibition together with its mitochondrial localisation suggested that F1L was a specialised inhibitor of apoptosis that is expressed during viral infection in order to counter apoptosis induction that may be induced by the virus.

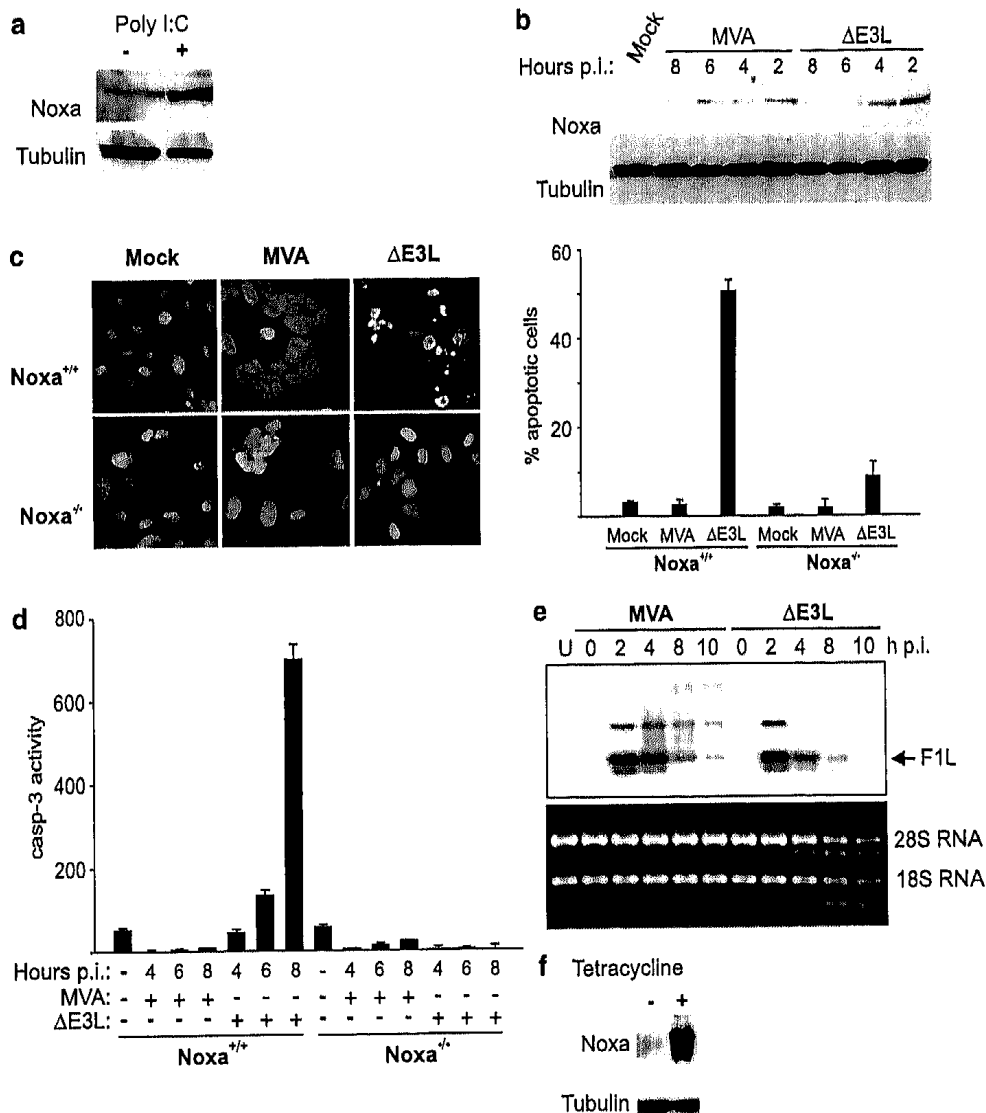


Figure 3 Noxa is induced during infection and is necessary for apoptosis induction by MVA-ΔE3L. (a) HaCaT human keratinocytes were treated with poly-I:C for 24 h and analysed for the expression of Noxa and tubulin (loading control) by Western blotting. Similar results were obtained in five experiments. (b) HeLa cells were infected at various periods with either MVA or MVA-ΔE3L (MOI = 10–15). At the time points shown, expression of Noxa was analysed by Western blotting. In a series of five experiments, the levels of expression were similar between the two viruses, with slight variations between individual experiments. (c) MEF generated from WT or from Noxa-deficient mice were grown on coverslips and infected with either MVA or MVA-ΔE3L. At 15 h p.i., representative areas of the coverslip were photographed (left). Right, 300 nuclei were scored visually for apoptosis. Data are mean/S.D. of triplicate wells. (d) MEF from WT or Noxa-deficient mice were infected with either MVA or MVA-ΔE3L. At the time points indicated, caspase-3 activity was measured in cell extracts by enzyme assay. Similar results were obtained in four separate experiments. (e) HeLa cells were mock infected (U) or infected with either MVA or MVA-ΔE3L at a multiplicity of infection (MOI) of 10. Total RNA was isolated at 0, 2, 4, 8 and 10 h postinfection (h p.i.) and electrophoretically separated in 1% agarose formaldehyde gels applying 1 μg of total RNA per lane. For loading control ribosomal RNAs were stained with ethidium bromide. In MVA-ΔE3L-infected cells, degradation of ribosomal RNA was detectable at 4 h p.i. and later during infection. Subsequently, RNA was transferred onto a positively charged nylon membrane (Roche Diagnostics) via vacuum blot and hybridised to riboprobes specific for F1L. (f) HeLa cells stably carrying a tetracycline-responsive repressor and Noxa under the control of this repressor were incubated for 24 h in the presence or absence of tetracycline and subjected to Western blotting for Noxa

Induction of apoptosis by MVA-ΔF1L

If the role of F1L is to block apoptosis caused by the cell's response to MVA infection, a mutant virus lacking F1L would be expected to induce apoptosis. MVA has an F1L-gene that is very closely related to vaccinia F1L. We generated an F1L-deficient MVA mutant (MVA-ΔF1L) and tested it for apoptosis induction in HeLa cells. As shown in Figure 4a and b, infection with MVA-ΔF1L caused significant apoptosis, as detected as nuclear morphology changes and caspase activity. (The detected caspase activity was somewhat smaller than during apoptosis induced by MVA-ΔE3L (see

above). This could mean that F1L also blocks caspase-independent apoptotic events or that the time course of apoptosis induction was different in the individual cell (comparing the two mutant viruses), leading to a different total caspase activity at a given time in the population.) Apoptosis induction was associated with the activation of Bax and the release of mitochondrial cytochrome *c* (Figure 5a and b; see Figure 2a (bottom panel) for flow cytometric analysis of cytochrome *c* release). These results indicate that MVA has an intrinsic apoptosis-inducing capacity, which is normally blocked by F1L.

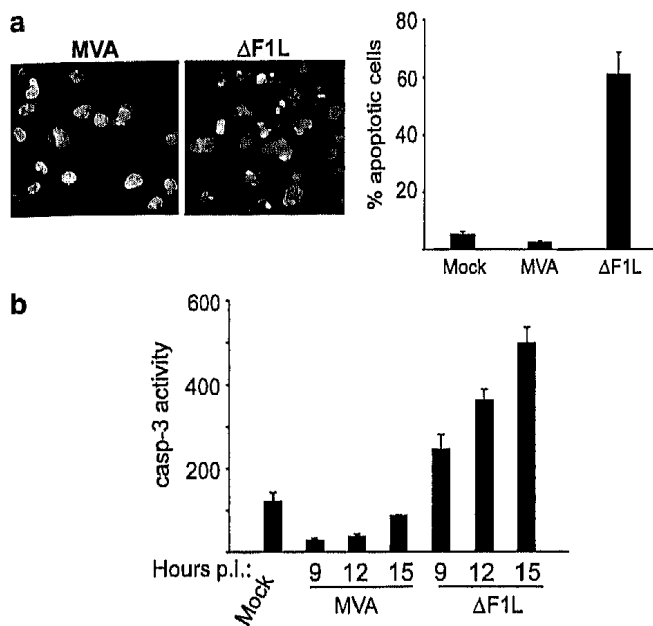


Figure 4 Apoptosis induction by MVA-ΔF1L. (a) HeLa cells grown on coverslips were infected with either MVA or with MVA-ΔF1L (MOI = 10–15). At 15 h p.i., nuclei were stained with Hoechst. Left, a representative area of the coverslip is shown. Right, 300 nuclei per coverslip were scored visually for apoptosis. Data are mean/S.D. of triplicate wells. Data are taken from the same experiment shown in Figure 1b. (b) HeLa cells were infected with MVA or with MVA-ΔF1L for the time periods shown. Caspase-3 activity was measured in cell extracts by enzyme assay

Analysis of apoptosis induction by MVA-ΔF1L

We used MVA-ΔF1L to analyse the pathway to apoptosis that is triggered by MVA infection. First, we tested whether the cellular anti apoptosis protein Bcl-2 was able to inhibit MVA-ΔF1L-induced apoptosis. HeLa cells were transiently transfected with a vector driving Bcl-2 expression and infected with MVA-ΔF1L. As shown in Figure 6a, expression of Bcl-2 inhibited apoptosis induction by MVA-ΔF1L, indicating that MVA activated apoptosis in a way that could be blocked by Bcl-2.

This suggested that F1L has a molecular function similar to that of Bcl-2. How Bcl-2 works on a molecular level is still not completely clear, but its function is embedded in the wider Bcl-2 family of proteins. Within this family, three subfamilies are commonly recognised. Bcl-2 itself and its close relatives (such as Bcl-x_L, Bcl-w, A1) are inhibitors of apoptosis. Bax and Bak are the most downstream molecules known in the pathway, and activation of these proteins is linked to the release of cytochrome *c*. Upstream of Bax and Bak lies the family of proapoptotic Bcl-2 Homology Domain-3-only (BH3-only) proteins (such as Bim, Puma, Noxa; 8 are known at present). An apoptotic stimulus activates one or several BH3-only proteins, which then cause activation of Bax and/or Bak. Bcl-2 and Bcl-2-like molecules can bind active BH3-only proteins. Bcl-2-like proteins may thus function by sequestering active BH3-only proteins. Alternatively, BH3-only proteins may act by displacing Bcl-2-like molecules and thereby allowing autoactivation of Bax/Bak.

High-level expression of BH3-only proteins induces apoptosis, which can be blocked by coexpression of Bcl-2. F1L was found to have the same capacity: like Bcl-2, F1L inhibited

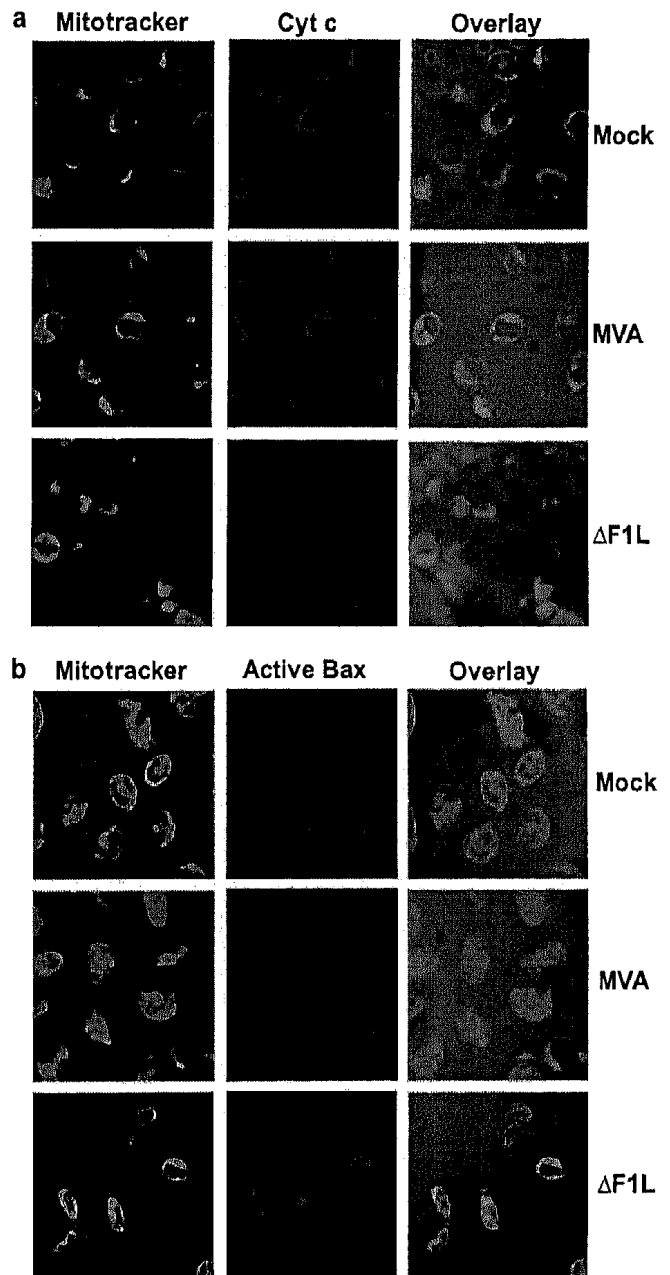


Figure 5 MVA-ΔF1L induces cytochrome *c* release and activation of Bax. HeLa cells grown on coverslips were infected with either MVA or MVA-ΔF1L (MOI = 10–15). At 10 h p.i., cells were fixed and stained with MitoTracker Green FM and with antibodies either directed against cytochrome *c* (a) or against active Bax (b). Similar results were obtained in four separate experiments. Data in a are taken from the same experiment as the one shown in Figure 2

apoptosis induced by the BH3-only proteins Bim, Puma and Noxa when coexpressed in HeLa cells (Figure 6b). Like Bcl-2, F1L must therefore act by blocking a process downstream of BH3-only protein activation. Note that the induction of apoptosis by transient transfection of Noxa is much higher than during tetracycline-regulated expression (Figure 3f), probably either because of higher expression levels or because of stress associated with the transient transfection.

We next analysed the contribution of Bax and Bak to apoptosis induced by MVA-ΔF1L. MEF lacking either or both of these proteins were infected with MVA or MVA-ΔF1L and

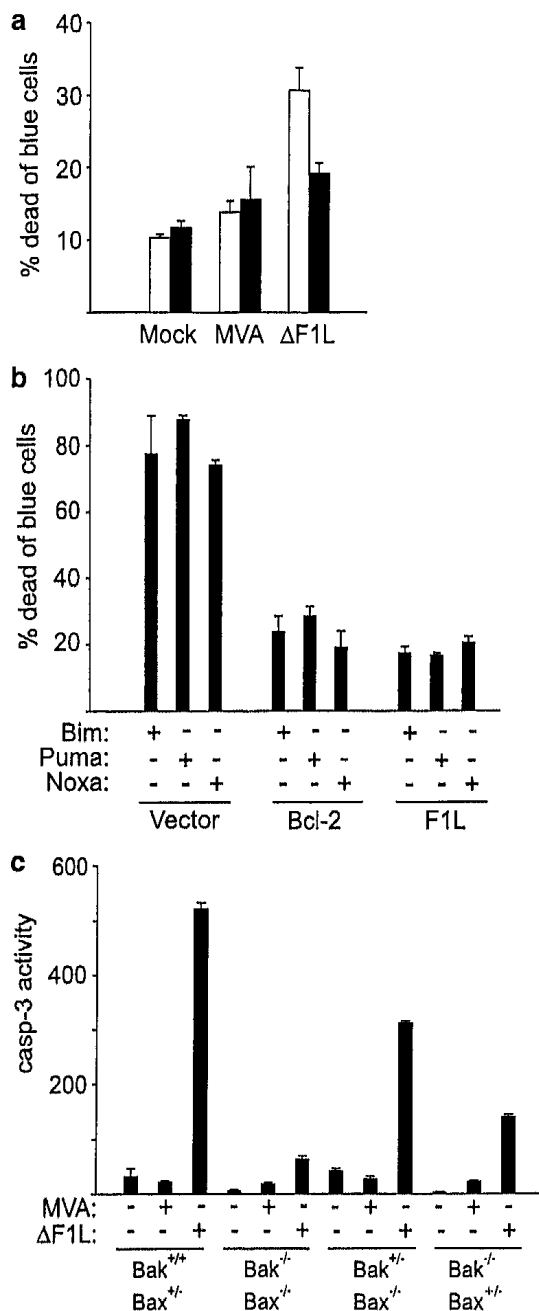


Figure 6 F1L functions in a Bax/Bak-dependent manner downstream of BH3-only protein activation. (a) HeLa cells were transiently cotransfected with an expression vector for β -galactosidase and either the empty vector as a control (white bars) or the same vector driving the expression of human Bcl-2 (black bars). At 24 h after transfection, cells were infected either with MVA or with MVA- Δ F1L as indicated. After 15 h, cultures were fixed, stained for β -galactosidase expression, and blue cells were scored for cell death.^{48,49} In all, 900 cells per well were counted, and data are represented as mean/S.D. of results from three independent transfection/infections. (b) HeLa cells were transiently cotransfected with a β -galactosidase expression vector and expression vectors for Bim, Puma or Noxa together with expression vectors for Bcl-2, F1L or the empty vector as indicated. After 15 h, cells were fixed and blue cells were scored for cell death. One out of three experiments with very similar results is shown. (c) MEF generated from mice deficient for Bak and/or Bax as indicated were infected with either MVA or MVA- Δ F1L. After 12 h, caspase-3 activity in cell extracts was measured by enzyme assay. Data are mean/S.D. of triplicate measurements from one extract. Similar results were obtained in four experiments

apoptosis was monitored by measuring caspase activity. MVA- Δ F1L-induced apoptosis was abrogated in cells lacking both Bax and Bak. Cells lacking only Bak showed a strong reduction in their apoptotic response, while cells that lacked only Bax were protected only to a moderate degree (Figure 6c). This indicates that the apoptotic pathway activated by MVA preferentially activates Bak, but can also, to a lesser degree, work via the activation of Bax.

MEF deficient in the BH3-only proteins, Bim, Bid, Puma, Noxa, Bik or Bmf, were also tested for their ability to respond with apoptosis to infection with MVA- Δ F1L. All of them underwent apoptosis like the wild-type MEF (data not shown). The most likely explanation for this finding is that MVA- Δ F1L-infection triggers more than one BH3-only protein, and that the absence of one of them is insufficient to block apoptosis.

Our results with the MVA mutants suggest that they trigger apoptosis by a Bcl-2-dependent mechanism. Since the killing is abrogated by the absence of the essential apoptosis mediators Bax/Bak (Figure 6c) and infection by the cytotoxic mutants drive Bax activation (Figures 2b and 5b), an attractive model for F1L function is direct sequestration of these molecules. However, unlike certain other antiapoptotic viral proteins, such as EBV BHRF-1, which share sequence and structural homology²⁹ with mammalian Bcl-2, F1L does not have any obvious sequence homology to the wider Bcl-2 family, or indeed any other protein. When F1L was analysed using FUGUE, a programme for recognizing distant homologues by sequence-structure comparison and incorporating BLAST searches,³⁰ only F1L homologues from other orthopoxviruses were identified. The next best match fell below statistical significance.

However, the antiapoptotic proteins expressed by human cytomegalovirus vMIA and that expressed by myxoma virus M11L sequester Bax³¹ and Bak,³² respectively. To test this hypothesis for F1L-function, we initially assessed the capacity of purified recombinant F1L expressed in *Escherichia coli* to bind Bcl-2 homology domain (BH3 domain) peptides since these regions mediate the killing activity of the proapoptotic BH3-only proteins as well as Bax/Bak.^{33,34} These *in vitro* binding assays were performed using the Biacore optical biosensor, which can sensitively and accurately determine binding affinities as we have recently undertaken for mammalian Bcl-2 family proteins.³⁵ Strikingly, we found that F1L Δ C20, a form that lacks its hydrophobic C-terminus, binds BimBH3 avidly with a dissociation equilibrium constant (K_D) of 80 nM (Figure 7a and b). This significant binding was confirmed in solution competition assays (Figure 7c) as the IC50, the concentration of free Bim peptide required to reduce F1L Δ C20 binding to the immobilised BimBH3 peptide by half, was 75 nM, which approximates the K_D . F1L Δ C20 could also bind peptides spanning the BH3 regions of Bax and Bak, but not a subtle point mutant of this, although at lower affinities ($\sim 1 \mu$ M) consistent with results obtained using isothermal calorimetry (data not shown).

In agreement with our observation that F1L functions in a manner akin to mammalian prosurvival Bcl-2, F1L can bind BH3 regions. Thus, F1L despite having no significant primary sequence similarity to mammalian prosurvival Bcl-2 proteins, probably possesses a groove targeted for BH3 binding.^{36,37} By implication, F1L may act to sequester BH3-containing

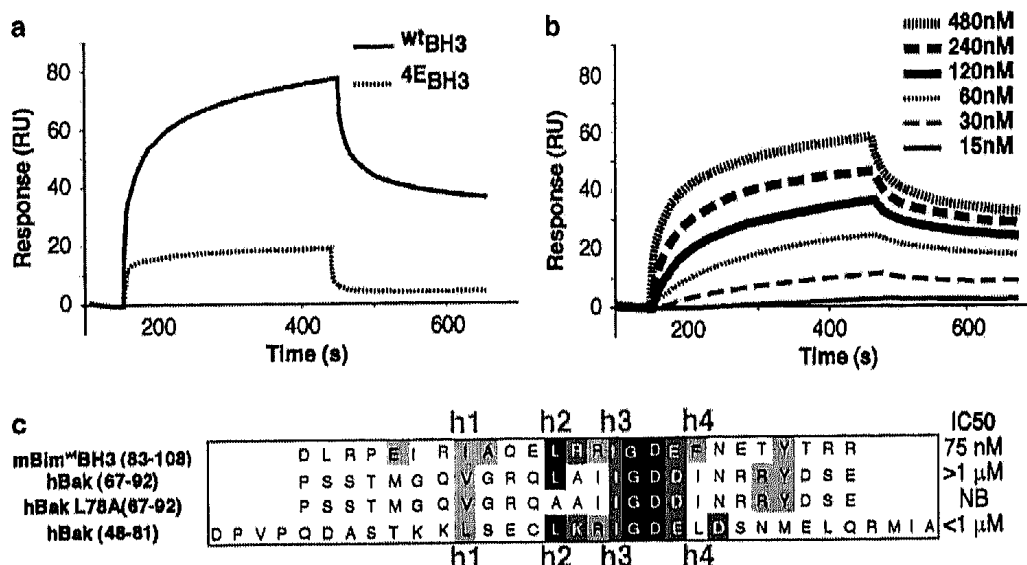


Figure 7 F1L binding to selected BH3 domain peptides. (a) Recombinant F1L Δ C20 was injected onto sensorchips with Bim^{wt}BH3 (solid line) or mutant Bim^{4E}BH3 (dotted line) peptides immobilised. To obtain the specific binding, the baseline response with ^{4E}BH3 was subtracted from that with ^{wt}BH3. (b) Biosensor responses when 15–480 nM was injected over the BimBH3 sensorchip. (c) BH3 peptides used in the solution competition assays. The BH3 region contains four hydrophobic residues (h1–h4) required for interacting with the prosurvival proteins. The competitor peptides were derived from human proteins except for mouse Bim. Sequences were aligned as described.⁵¹ The IC50 (nM) for the indicated interactions are shown on the right and are from representative experiments (NB, no binding)

proapoptotic proteins, but precise molecular mechanism(s) by which it promotes cell survival await further studies.

Discussion

In this study, we investigated two MVA mutants with regard to their potential to activate the apoptotic pathway. It is commonly accepted that viruses carry antiapoptotic genes (some of which have been well characterised), and it has been demonstrated on many occasions that viral infection can protect the infected cell against experimental apoptotic insults. The role of viral antiapoptotic proteins for the viral infection, however, is far less well documented. The fact that a virus can block apoptosis suggests that there is a concomitant proapoptotic activity that needs to be blocked by the virus. This most likely means that the viral infection in fact induces apoptosis or, from the cell's perspective, that the cell notices the replicating virus and undergoes apoptosis. Our data provide strong support for this model.

In the absence of E3L, MVA-infection induces apoptosis that critically involves Noxa. Expression of E3L by cotransfection failed to block apoptosis upon forced expression of Noxa (not shown). It is therefore more likely that E3L acts upstream of the activation of Noxa. However, the molecular pathway is not as straightforward as appears to be at first glance. Since E3L is known to bind dsRNA we tested induction of BH3-only proteins through dsRNA and found that expression of Noxa was induced (this was very recently also reported by others²⁷ although another group recently reported findings suggesting that dsRNA induces apoptosis not through Noxa but through the formation of a caspase-8-activating protein complex^{38,39}). Indeed, Noxa expression was induced upon infection with MVA- Δ E3L. However, the expression was not very high and, surprisingly, was very similar in cells infected with MVA (where no apoptosis was induced). Based on results in

Noxa^{-/-} cells, Noxa clearly contributed to apoptosis induction through MVA- Δ E3L. Why, then, did MVA not also induce apoptosis?

One factor that is of likely relevance is the finding that F1L expression may be reduced in infection with MVA- Δ E3L as compared to MVA. As shown above, strong F1L expression protects against apoptosis induced by overexpression of Noxa, suggesting that F1L, if present at high enough levels, would prevent apoptosis through MVA- Δ E3L. The observed reduction in F1L is therefore probably relevant. A second possibility is suggested by data indicating that Noxa may be required, but on its own would not be enough for apoptosis. Noxa expression was found to be insufficient to induce apoptosis on its own but able to cooperate in apoptosis induction with concomitant expression of Bad, probably because of its limited binding capacity to some but not all antiapoptotic Bcl-2-like proteins.³⁵ It has further been suggested that the majority of known BH3-only proteins (and Noxa amongst them) fail to activate Bax directly but act to sensitise the system to the proapoptotic activities of the BH3-only proteins Bim and tBid.⁴⁰ In addition, our own data presented here show that experimentally induced high-level expression of Noxa only weakly causes apoptosis. Therefore, it appears likely that loss of E3L leads to the induction of Noxa and also provides a second trigger (perhaps the activation of another BH3-only protein), which then collaborate to induce apoptosis during infection with MVA- Δ E3L.

Our data suggest that apoptosis induced by MVA- Δ E3L is indirect, possibly via the reduction in F1L expression. F1L, on the other hand, appears to be a true antiapoptotic protein in that it localises to mitochondria, the site of cytochrome c release^{18,19}, and directly targets components of the core apoptosis machinery of this study. Apoptosis induction by MVA- Δ F1L required Bak and/or Bax. The only known way to activate these molecules is via the activation of BH3-only

proteins. The finding that loss of any one of the six investigated BH3-only proteins failed to protect the cell against MVA- Δ F1L therefore suggests that more than one BH3-only protein is activated (or that other, perhaps unidentified BH3-only proteins contribute). Bcl-2-expression was able to cover for the loss of F1L, further indicating that both proteins at least act in the same pathway. Bax/Bak are the most downstream proteins known prior to cytochrome *c* release, and active Bax is sufficient to induce the release of molecules from synthetic liposomes.⁴¹ BH3-only proteins are required to activate Bax probably in most if not all instances of Bcl-2-controlled apoptosis. F1L was able to block apoptosis induced by overexpression of the BH3-only proteins Bim, Puma and Noxa. Furthermore, evidence for direct binding of F1L to BH3-containing proapoptotic proteins was found. Therefore, F1L either binds and sequesters BH3-only proteins or binds to Bak/Bax precluding their activation. Future work will have to clarify what the direct targets of F1L *in vivo* are.

Taken together, this study shows that an infected cell is able to detect the presence of infecting MVA and to react to this infection by activating the apoptotic pathway. Understanding this relationship will allow us to appreciate an important aspect of the infectious biology of viruses.

Materials and Methods

Cells and viruses

HeLa cells (human cervical adenocarcinoma cell line) were obtained from the American Type Culture Collection (ATCC) and HaCat cells (human keratinocyte cell line) were obtained from the German Cancer Research Centre (DKFZ, Heidelberg, Germany). Mouse embryonic fibroblasts deficient in Noxa, Puma, Bim or Bik (kindly provided by A Strasser, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, immortalised by C. Borner, Albert-Ludwigs-University Freiburg, Germany), Bid (S Korsmeyer, Harvard Medical School, Boston, USA) were used. Bax^{+/-} Bak^{-/-}, Bax^{-/-} Bak^{+/-}, Bax^{+/+} Bak^{+/-} and Bax^{-/-} Bak^{-/-} MEF were derived from E14 C57Bl/6 mice and immortalised (at passage 2–4) with SV40 large T antigen.

Culture of all cells was carried out in DMEM supplemented with 10% FCS.

Vaccinia virus MVA (cloned isolate F6 at 582nd CEF passage),⁴² and MVA- Δ E3L¹⁵ were routinely propagated and titrated by vaccinia virus-specific immunostaining on BHK-21 cells to determine the numbers of infectious units (IU) per milliliter. Vaccinia virus MVA (cloned isolate II_{new})^{42,43} and MVA- Δ F1L were amplified and titrated on CEF. In experiments using MVA- Δ E3L, the MVA isolate F6 was used as a control, in experiments with MVA- Δ F1L, the control was MVA isolate II_{new}.

MVA DNA sequences flanking the F1L gene (MVA029L nucleotides 25 378–26 046, GenBank accession No. U94848) were amplified by polymerase chain reaction (PCR) using genomic MVA (isolate F6) DNA as template. The resulting PCR fragments were inserted into the plasmid p Δ K1L⁴⁴ to obtain the F1L deletion plasmid p Δ F1L.

For generation of F1L deletion mutant virus, monolayers of 1×10^6 confluent CEF cells grown in six-well tissue-culture plates were infected with MVA isolate II_{new} at a multiplicity of infection (MOI) of 0.01 IU per cell. At 90 min after infection cells from one well were transfected with 2 μ g plasmid DNA of p Δ F1L using FUGENE (Roche Diagnostics) as recommended by the manufacturer. At 48 h after infection the cells and medium were harvested, freeze thawed three times and homogenized in a

cup sonicator (Sonopuls HD 200, Bandelin, Germany). From this material MVA mutant virus was isolated following previously described methodology.⁴⁴ Briefly, 10-fold serial dilutions (10^{-1} to 10^{-4}) of the harvested material in medium were used to infect subconfluent monolayers of RK-13 cells grown in six-well tissue-culture plates. After 3 days incubation at 37°C, single foci of infected RK-13 cells were picked and processed by freeze thawing and sonication for another infection of RK-13 cell monolayers. After elimination of parental MVA during passage on RK-13 cells, 10-fold serial dilutions (10^{-1} to 10^{-6}) of the recombinant viruses were used for infection of subconfluent CEF cells grown in six-well tissue-culture plates. Well-separated foci of infected CEF cells were harvested to isolate K1L-negative mutant viruses. Viral DNA from cloned MVA isolates was routinely analysed by PCR as described previously.⁴⁴ Details of PCR and the cloning procedure are available from the authors upon request.

Induction and detection of apoptosis

Host cells (2×10^5 /well in 12-well plates seeded the day before) were either infected or not, treated with staurosporine (1 μ M, Sigma) and apoptosis was detected as described previously.⁴⁵ Briefly, for detection of nuclear apoptosis, cells were stained with 20 μ M Hoechst 33258 (Sigma) for 30 min and nuclear morphology was assessed under a fluorescence microscope. At least 300 nuclei per sample were scored. For detection of caspase-3-like activity, cells were lysed in NP-40 lysis buffer and⁴⁵ triplicates of aliquots were added to a fluorogenic peptide containing a caspase-3-recognition sequence (DEVD-AMC, 10 μ M, Bachem, Heidelberg, Germany) in assay buffer containing BSA and HEPES. Free AMC was measured after 1 h of incubation at 37°C and values are presented as arbitrary relative fluorescence units (mean \pm S.D. of triplicate reactions). After treatment with poly-I:C (50 μ g/ml) for 24 h, probes were harvested and subjected to Western blot analysis for detection of Noxa as described below.

Microscopy, immunocytochemistry and flow cytometry

HeLa cells were grown on glass coverslips, infected or left uninfected. Cells were fixed with 2% formalin for 30 min, stained with mouse anticytochrome *c* mAb (Becton Dickinson) and Cy3-labelled anti-mouse antiserum in PBS-containing 1% FCS and 1% saponin. For the detection of active Bax, cells were stained with antiactive Bax mAb (6A7, Upstate Biotechnology,⁴⁶) and Cy3-labelled anti-rabbit antiserum (Dianova). Then, cells were stained with MitoTracker Green FM (Molecular Probes). Pictures were obtained with a Zeiss laser scanning microscope.

For detection of cytochrome *c* release by flow cytometry, cells were stained and analysed as described.²⁰ Briefly, cells were fixed in 2% formaldehyde, washed consecutively in PBS, BPS/BSA 0.5% and PBS/BSA/Saponin 0.5%. Cells were stained with anticytochrome *c* mAb in PBS/BSA/Saponin, followed by staining with FITC-labelled goat anti-mouse Ab (Dianova). Cells were analysed in a Becton Dickinson FACSCalibur.

Western blot analysis

Cells were harvested and detergent extracts were prepared by lysis of 2×10^5 cells in 50 μ l Triton buffer (1% Triton X-100, 0.05 M Pipes-NaOH, 0.05 M HEPES pH 7.0, 2 mM MgCl₂, 1 mM EDTA, 10 mM DTT and protease inhibitors (Roche)) for 30 min on ice. After centrifugation at $2000 \times g$ at 4°C for 10 min, loading buffer was added and lysates were run

on a 12% polyacrylamide gel. Proteins were transferred to nitrocellulose membranes, which were consecutively probed with antibodies specific for Noxa (Alexis) or tubulin (Sigma). Proteins were visualized by a chemiluminescence detection system (Perkin-Elmer Lifescience, Boston, MA, USA).

Transfection of cells

Cells were transfected using ethylene imine polymer solution (Fluka⁴⁷) as described previously.⁴⁸ Briefly, HeLa cells were transfected with 1.5 μ g of control vector pEF, pEF-Bcl-2-expression vector or F1L-expression vector together with 1.5 μ g Bim-, Puma- or Noxa-expression vector (kindly provided by Andreas Villunger, Innsbruck) and 0.5 μ g of a vector driving the expression of *E. coli* β -galactosidase (CMV-LacZ).⁴⁸ The F1L-expression vector was generated by PCR amplification of the F1L ORF from genomic MVA DNA, followed by sub-cloning into the pEGFP-C2-expression vector (Clontech). For transfection of cells by electroporation (5×10^6 cells at 240 V, 960 μ F), 15 μ g of control vector (pEF) or pEF-Bcl-2-expression vector together with 5 μ g CMV-LacZ was used. At 24 h after transfection, cells were infected or left uninfected for 12 h. Cells were then stained for β -galactosidase activity and blue cells were viewed under a microscope and scored alive or dead using morphological criteria.^{48,49}

Northern blot analysis

HeLa cells cultured in 35 mm-diameter dishes were mock infected or infected with MVA or MVA- Δ E3L using an MOI of 10. Following 30 min adsorption at 37°C, virus inocula were replaced by prewarmed tissue culture medium (DMEM with 2% FCS). After 0, 2, 4, 8 and 10 h, total RNA of mock infected, MVA and MVA- Δ E3L-infected cells was isolated with TRIzol reagent (Invitrogen) following the manufacturer's instructions. Quality of RNA was examined by electrophoresis in 1% agarose formaldehyde gels and ethidiumbromide staining. Subsequently, RNA was transferred onto a positively charged nylon membrane (Roche Diagnostics) via vacuum blot. For synthesis of riboprobes for detection of MVA-F1L transcript, PCR using viral DNA as template and primer pair HL68 (5'-ATG TAG ATG GTA TAG TAC AGG-3')/HL69 (5'-CTA ATA CGA CTC ACT ATA GGG AGA ATT ATC TGG TGG TGA AAT GTC-3') was employed. Reverse primer contained a T7 RNA polymerase promoter recognition sequence (underlined). Digoxigenin (DIG)-labelled riboprobes were obtained by *in vitro* transcription with T7 RNA polymerase (Roche Diagnostics) using the PCR-generated DNA fragment as template. *In vitro* RNA labelling, hybridisation and signal detection were carried out according to the manufacturer's instructions (DIG RNA labelling kit and detection chemicals, Roche Diagnostics), applying 68°C for prehybridisation, hybridisation and high stringency wash.

Recombinant protein production

HexaHIS-tagged F1L Δ C20 was expressed using the pET Duet vector (Novagen) in *E. coli* BL21 DE3 pLysS cells. Following induction of protein expression, the cell lysate was homogenised, cleared and the protein purified on a HiTrap (Amersham)-chelating column charged with nickel. The protein was eluted in 50 mM Tris pH 8.0, 150 mM NaCl and 5 mM 2-mercaptoethanol, 250 mM imidazole and subjected to gel-filtration chromatography in 20 mM Tris pH 8.0, 150 mM NaCl and 5 mM 2-mercaptoethanol using a Superdex 200 column; it eluted as a single peak.

Affinity measurements and solution competition assays

Affinity measurements were performed at room temperature on a Biacore 3000 biosensor as previously described.⁵⁰ All the peptides used were supplied by Mimotopes, Australia, and their sequences are indicated in Figure 7c. Isothermal calorimetry data were collected on a VP-ITC (MicroCal) with F1L Δ C20 diluted in 20 mM Tris pH 8.0, 150 mM NaCl and 5 mM 2-mercaptoethanol to a final concentration of 10 μ M. Data were obtained at 25°C and analysed using the MicroCal Origin software.

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