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Interaction of F1L with the BH3 domain of Bak is responsible for inhibiting vaccinia-induced apoptosis

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Abstract

Apoptosis represents an important cellular defence mechanism against viral pathogens by virtue of its ability to remove infected cells. Consequently, many viruses have developed numerous strategies to prevent or delay host cell apoptosis in order to achieve productive replication. Here we report that deletion of the *F1L* gene from the vaccinia genome results in increased apoptosis during infection. We demonstrate that F1L, which has no sequence homology to Bcl-2 family members, inhibits apoptosis at the level of mitochondria by binding to Bak. As a consequence, F1L prevents Bak activation, oligomerization and interaction with active Bax, all critical steps in the induction of apoptosis. We demonstrate that residues 64–84 of F1L interact directly with the Bcl-2 homology domain 3 (BH3) domain of Bak. This region of F1L has limited sequence similarity to known Bak-interacting BH3 domains. We also find that such additional BH3-like domains exist in the vaccinia genome. We conclude that F1L uses this specific, BH3-like domain to bind and inhibit Bak at the mitochondria.

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Abbreviations: BH3 domain, Bcl-2 homology domain 3; STS, staurosporine; TM, transmembrane; WR, vaccinia virus Western Reserve

Introduction

Apoptosis is a highly regulated process by which damaged or unwanted cells are eliminated from multicellular organisms. In most cells, a key regulatory step during the progression of apoptosis occurs at the level of the mitochondria, where pro- and antiapoptotic signals are integrated. The Bcl-2 family of proteins are important regulators of these signals at the mitochondria.[1,2] Members of this family include both pro- and antiapoptotic proteins, which share up to four conserved regions, named BH1–4 domains.[1] Proapoptotic members, such as Bak and Bax, contain BH1–3 domains.[2] Following an apoptotic trigger, Bak and Bax become activated, leading to their oligomerization in the outer mitochondrial membrane (OMM), membrane permeabilization and release of factors such as cytochrome *c*.[1] Antiapoptotic proteins such as Bcl-2 and Bcl-XL share all four BH domains and act to oppose the actions of Bak and Bax.[2] Bcl-2 homology domain 3 (BH3)-only family members, such as Bid, Bad, Noxa and Bim, only have homology in their α-helical BH3 region, which is required for their proapoptotic activity.[2] These BH3-only proteins are thought to neutralize antiapoptotic members and/or activate Bak and Bax directly.[3–5] The specificity of the interactions is determined by the wide range of binding affinities between members of the Bcl-2 family, mediated largely through limited sequence variation in their BH3 domains.[3,4]

Since apoptosis provides a potent cellular defence mechanism against viral pathogens, it is not surprising that viruses have developed many strategies to prevent or delay host cell apoptosis.[6] Large DNA viruses such as adenovirus, baculovirus, herpes viruses and poxviruses, whose life cycles can be relatively long, encode proteins that interfere with apoptotic signals at multiple levels, including at the mitochondria.[6,7] For example, all gamma herpesviruses contain sequence homologs of cellular Bcl-2 that have been shown to prevent apoptosis.[8]

In contrast to herpes viruses, sequence analysis of the vaccinia genome failed to identify Bcl-2 family homologues, although it was known that the virus encodes a protein that inhibits the intrinsic mitochondrial pathway during infection.[9] Recently, Wasilenko *et al*.[10] have shown that expression of the vaccinia F1L protein was able to restore the ability of a vaccinia virus strain lacking 44 genes to inhibit apoptosis. Transient expression of GFP-tagged F1L also prevented drug-induced disruption of the mitochondrial potential and cytochrome *c* release in noninfected cells.[10] In this paper, we set out to determine how F1L prevents apoptosis during vaccinia infection. We found that F1L interacts with the proapoptotic Bcl-2 family member Bak, preventing its activation. This interaction is mediated by a specific, BH3-like domain in F1L, which, we find, may also be present in other uncharacterized vaccinia proteins.

Results

Recent observations have demonstrated that overexpression of GFP-F1L can protect noninfected cells against staurosporine (STS)-induced apoptosis.[10] However, the question still remains whether F1L actually protects cells from apoptosis during vaccinia infection. To address this issue, we generated a virus strain lacking only the F1L coding sequence (ΔF1L) using a guanine phosphoribosyl transferase (gpt) cassette replacement strategy. Immunofluorescence analysis reveals that F1L, which is first expressed between 2.5 and 3 h post-infection (h.p.i.), is readily observed on mitochondria

in cells infected with the wild-type Western Reserve (WR) vaccinia strain, but is absent in ΔF1L-infected cells (Figure 1a–c). Immunofluorescence analysis with a number of viral markers demonstrates that an absence of F1L does not result in any appreciable defect in viral assembly and overall life cycle (Figure 1c; data not shown). Deletion of F1L also had no effect on the perinuclear collapse of the mitochondrial network, which is often observed during infection (Figure 1c).

To examine whether F1L is indeed required to protect from cell death during vaccinia infection, we first analysed the survival rate of cells infected with WR or ΔF1L virus at 12 and 24 h.p.i. Whereas uninfected HeLa cells increased 1.6-fold over this time period, there was a dramatic loss of WR- and ΔF1L-infected cells from the dish. However, significantly less ΔF1L-infected cells remained than WR-infected cells at 12 and 24 h.p.i., respectively (Figure 2a), consistent with a role for F1L in prevention of cell death. Next, we examined whether WR- or ΔF1L-infected cells die by apoptosis and at which point after infection (Figure 2b). We performed FACS analysis on infected cells stained with propidium iodide, to quantify the percentage of cells with less than 2*n* of DNA (i.e. fragmented DNA). Over a period of 36 h, an increasing percentage of cells infected with WR or ΔF1L contain fragmented DNA (Figure 2b). However, from 12 h.p.i., the percentage of ΔF1L-infected cells with fragmented DNA was significantly higher than those infected with WR (Figure 2b).

To further investigate the antiapoptotic properties of F1L, we examined whether vaccinia infection would protect HeLa cells from STS-induced apoptosis in an F1L-dependent fashion. We found that treatment of noninfected cells with STS for 2 or 4 h resulted in increasing numbers of cells undergoing apoptosis (Figure 2c). The same STS treatment of cells previously infected with WR for 6 h reveals that vaccinia virus significantly protects cells from undergoing apoptosis (Figure 2c). In contrast, infection of cells with the ΔF1L virus did not result in any protective effect against the STS treatment. Activation of the proapoptotic Bcl-2 family members Bak and Bax upstream of cytochrome *c* release is a hallmark of mitochondrial dependent apoptosis. To assess whether F1L blocks Bax activation, we immunoprecipitated Bax from lysates from cells infected for 12 h with ΔF1L or WR virus using an antibody that specifically recognizes an active conformation of Bax. In ΔF1L-, but not WR-infected lysates, Bax is precipitated using this antibody (Figure 2d). In addition, in these cells active Bax coimmunoprecipitates Bak, indicative of hetero-oligomer formation, which is a hallmark of apoptosis.[11,12] Taken together, our data demonstrate that F1L can inhibit STS-induced apoptosis and significantly delays the onset of apoptosis during vaccinia infection.

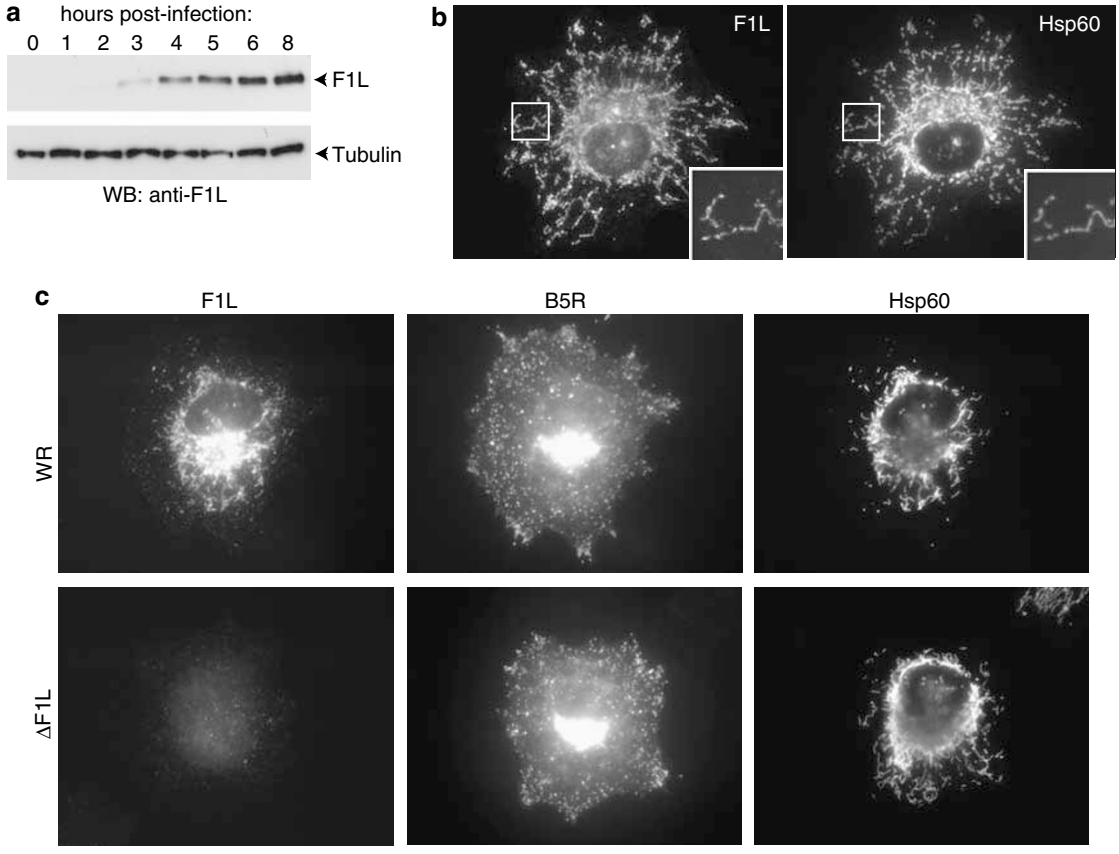

Figure 1 F1L is expressed early during vaccinia infection. (**a**) Western blot analysis of whole cell lysates from WR-infected cells with anti-F1L antibodies over a time course of 0–8 h.p.i. (**b**) Immunofluorescence analysis at 6 h.p.i. reveals that F1L is associated with mitochondria, as judged by its colocalization with Hsp60. (**c**) Immunofluorescence analysis of infected cells (8 h.p.i.) confirms the specificity of anti-F1L antibody and reveals that deletion of F1L (ΔF1L) does not inhibit virus particle assembly as the judged presence of B5R-positive virus particles in the cytoplasm

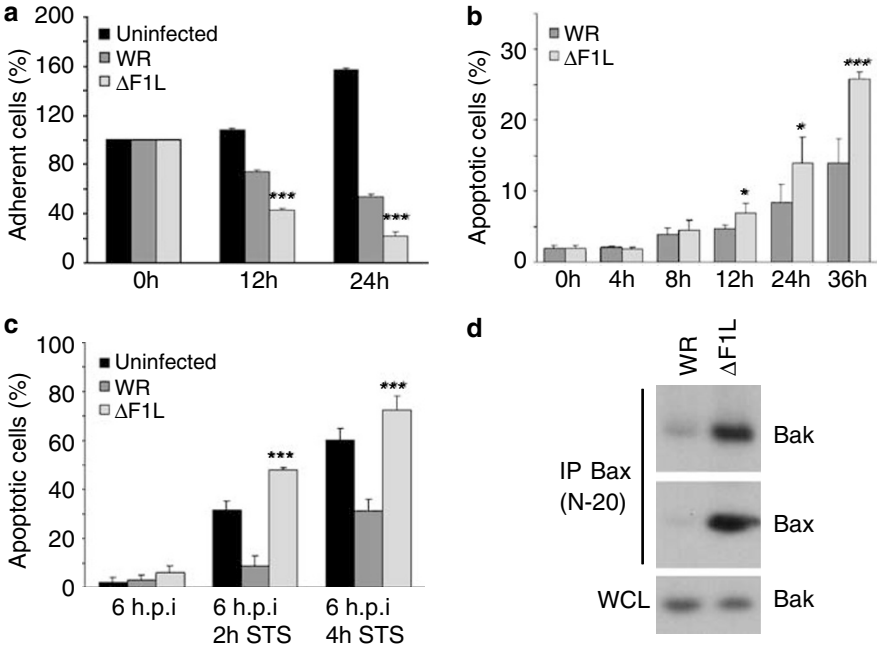

Figure 2 Deletion of F1L results in increased apoptosis during vaccinia infection. (**a**) Quantification of the number of adherent HeLa cells remaining at 12 and 24 h.p.i. after mock, WR or ΔF1L virus infection. Cell numbers are expressed as a percentage of their numbers at $t = 0$ h. (**b**) Quantification of FACS analysis of WR- or ΔF1L-infected cells containing fragmented DNA was determined after propidium iodide staining. (**c**) HeLa cells were infected with mock, WR or ΔF1L virus for 6 h before treatment with 1 μM STS for 2 and 4 h. The percentage of apoptotic nuclei was determined using DAPI counterstaining. Differences between WR- and ΔF1L-infected cells in (**a–c**) are depicted as $^{*}P<0.05$, $^{**}P<0.01$ or $^{***}P<0.001$, Student's *T*-test. All data were from at least three independent experiments. (**d**) Western blot analysis reveals that Bak can be coimmunoprecipitated with active Bax using N-20 antibodies in ΔF1L-, but not WR-infected cells

Residues 60–76 F1L are required for its antiapoptotic effects

We next examined the ability of a series of GFP-tagged F1L deletion constructs to protect cells from STS-induced apoptosis to facilitate identification of the region of the molecule responsible for its antiapoptotic activity (Figure 3a). All deletion constructs localize to the mitochondrial network, with the exception of the mutant lacking the transmembrane (TM) domain (Figure 3b).

Quantitative immunofluorescence analysis with an antiactive caspase 3 antibody reveals that cells expressing GFP-F1L, but not GFP alone, inhibited STS-induced caspase 3 activation (Figure 3c). In agreement with Stewart *et al*.,[13] we found that the carboxy-hydrophobic terminus of F1L, which targets the protein to the OMM, is critically required for its antiapoptotic effect. Specific amino-acid residues within the TM domain of F1L do not appear to be important for the antiapoptotic effect of F1L beyond their mitochondrial targeting role, as replacing this region of the molecule with the equivalent sequence from the OMM-resident Tom5 also potently prevents activation of caspase 3 (Figure 3c). Deletion analysis of F1L reveals that the sequence between amino-acid residues 60 and 76 is critical to prevent STS-induced apoptosis (Figure 3c). To extend these findings, we analysed whether further mutants in this region are able to prevent the release of cytochrome *c* after UV treatment (Figure 3d). The 44C and 60C mutants prevent UV-induced cytochrome *c* release, but the 76C and Δ57–78 mutants do not. Secondary structure prediction analysis reveals that F1L has nine potential α-helices (data not shown). Examination of the amino-acid sequence between residues 60 and 76 of F1L reveals that it overlaps with a predicted α-helix running from residue 64 to 84. To determine the relevance of this predicted helix for the antiapoptotic properties of F1L, we inserted proline or acidic residues into this region. We found that introduction of a helix-breaking proline residue (M67P) abrogated the ability of F1L to inhibit cytochrome *c* release in UV-treated cells (Figure 3d). Assuming further that this predicted helix may share characteristics with Bcl-2-binding BH3 domains, we made Val to Glu mutations that would disrupt Bcl-2 protein/BH3 peptide-like interactions.[14] We found that, when valine 66 and 70 were substituted for glutamic acid (V66E/V70E), F1L cannot prevent UV-induced cytochrome *c* release (Figure 3d). Overall, our deletion and point mutant analyses indicates that amino-acid residues 60–76, which are part of a predicted α-helix, play a critical role in the antiapoptotic properties of F1L.

F1L interacts with Bak

To investigate whether F1L might exert its antiapoptotic effect by binding to a Bcl-2 family member, we examined whether His-F1L produced in *Escherichia coli* would interact with GST-tagged Bcl-2, Bcl-XL, Bax and Bak. After stringent washing of the glutathione resin, we found that His-F1L binds only to Bak-ΔTM (Figure 4a). In contrast, His-F1L containing the M67P mutation, which disrupts the antiapoptotic activity of the protein, was unable to bind Bak-ΔTM (Figure 4a).

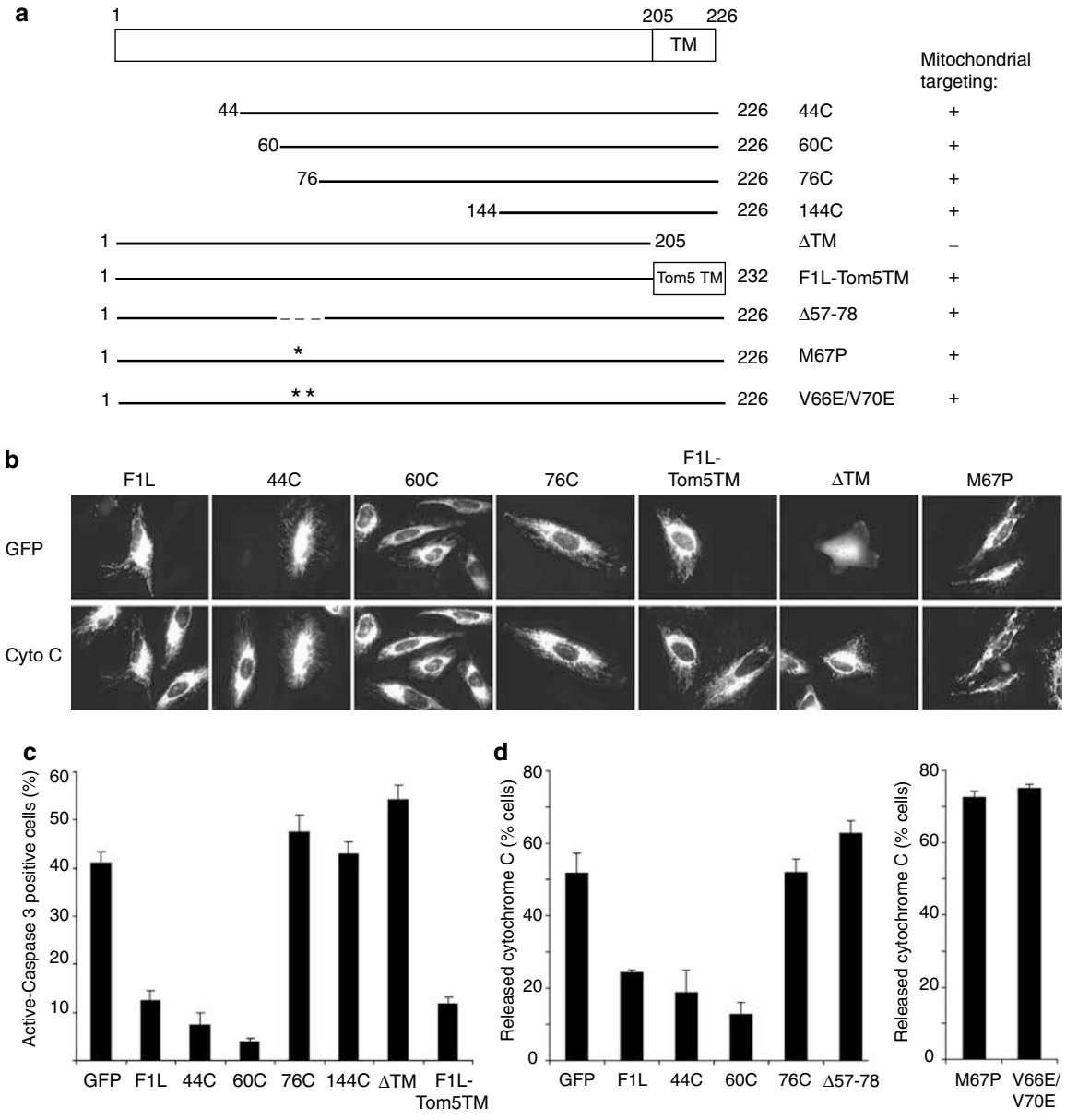

Figure 3 The antiapoptotic properties of F1L require residues 60–76. (**a**) Schematic representation of GFP-tagged F1L deletion and point mutants together with their ability to be recruited to mitochondria. (**b**) HeLa cells transfected with N-terminal GFP-tagged constructs of F1L reveal colocalizations of constructs with the mitochondrial network, as revealed by immunofluorescence stainings with an anti-cytochrome *c* antibody. Images were collected at identical microscope settings and cells with very low or high levels of GFP expression were excluded from subsequent quantification experiments. (**c**) Quantification of the ability of GFP-F1L mutants to protect HeLa cells from STS-induced apoptosis. The percent of GFP-positive cells undergoing apoptosis was determined by immunofluorescence with an anti-active caspase 3 antibody. (**d**) HeLa cells transfected for 24 h with GFP-F1L deletion or point mutants were irradiated with UV and subsequently fixed for immunofluorescence analysis 4 h later. The percentage of GFP-positive cells that had released their cytochrome *c* from the mitochondria was determined by immunofluorescence. The data represent the mean ($\pm$S.E.M.) of a minimum of three independent experiments

Immunoblot analysis of pulldown assays also demonstrated that glutathione beads with bound GST-F1L-ΔTM, but not GST, were able to retain Bak from HeLa cell lysates (Figure 4b). To test whether the region encompassing residues 60–75 is sufficient to retain Bak, we coupled a peptide corresponding to residues 57–78 to agarose beads. This peptide, but not the corresponding peptide containing the M67P substitution, is able to bind Bak from HeLa lysates (Figure 4c). Endogenous Bak was also found to coimmunoprecipitate with GFP-F1L, but not GFP-F1L-M67P or GFP, from extracts prepared from HeLa cells expressing these GFP-tagged proteins (Figure 4d). Lastly, immunoblot analysis of immunoprecipitation (IP) assays confirmed that endogenous Bak does indeed interact with F1L during vaccinia infection (Figure 4e).

F1L binds to the BH3 domain of Bak

To further investigate the interaction between F1L and Bak, we performed far Western analysis using GST-F1L or

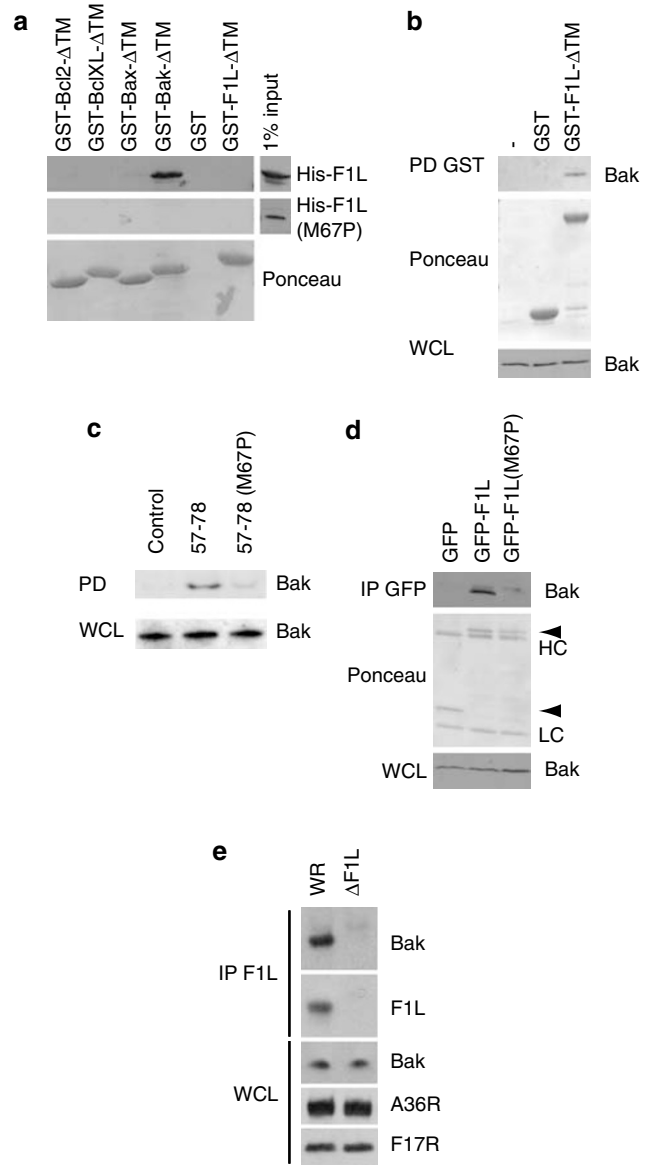

Figure 4 F1L interacts specifically with Bak. (**a**) Western blot analysis of pulldown experiments using GST-tagged Bcl-2, Bcl-XL, Bax and Bak lacking their TM domains bound to glutathione sepharose beads reveals that Bak interacts with His-F1L, but not His-F1L-M67P from soluble *E. coli* extracts. Western blot of 1% of the input bacterial lysate and ponceau of GST-tagged BcL-2 family members is shown. (**b**) HeLa cell lysates were incubated with glutathione sepharose beads alone or beads with bound GST or GST-F1L-ΔTM. Immunoblots of the pulldown (PD) and the whole-cell lysate (WCL) were probed with anti-Bak. (**c**) Peptide corresponding to residues 57–78, 57–78(M67P) and a random peptide were covalently coupled to beads before incubation with HeLa lysates. Immunoblots of the PD and the WCL were probed with anti-Bak. (**d**) Lysates of HeLa cells transfected with GFP, GFP-F1L or GFP-F1L-M67P were coimmunoprecipitated with anti-GFP antibodies. Western blots of the IP and the WCL were probed using anti-Bak antibodies. Arrowheads indicate GFP-tagged proteins, while HC and LC represent antibody heavy and light chains, respectively. (**e**) Western blot analysis reveals that Bak coimmunoprecipitates with F1L from extracts prepared from WR- but not ΔF1L-infected cells. Input cell extracts were probed with anti-Bak, anti-A36R and anti-F17R antibodies to confirm equivalent loadings and infection levels

GST-Bak as probes on a series of overlapping 18-mer peptides of Bak or F1L, respectively. Western analysis with anti-GST antibodies of the peptide spot scan reveals that GST-F1L-ΔTM strongly bound to residues 71–90 of Bak, which corresponds to its BH3 domain (Figure 5a). Although the structure of Bak has not been determined, based on sequence similarity to Bcl-XL and Bcl-2, the Bak BH3 domain is predicted to form one side of a hydrophobic groove, which forms several hydrophobic and hydrophilic interactions with an incoming BH3-containing α-helix.[14] In addition to the BH3 domain, residues from the α4/α5 BH1 region further contribute to binding such α-helices.[14] Interestingly, we find that GST-F1L-ΔTM also binds to peptides corresponding to residues 110–130 of Bak, which contains the BH1 domain, albeit with lower affinity (Figure 5b). In the reciprocal experiment, GST-Bak-ΔTM was found to interact with peptides covering amino acids 64–85 of F1L (Figure 5c). These residues overlap with the region required for the antiapoptotic activity of F1L identified during our deletion analysis (Figure 3). This raises the possibility that this region of F1L has BH3-like properties, allowing it to bind Bak.

Alignment of F1L with Bcl-2 proteins identifies other potential BH3-like domains in vaccinia genome

We aligned amino-acid residues 64–84 of F1L with the sequences containing the BH3 domains of Bcl-XL, Mcl-1, Bcl-2, Bak and Bax. In addition, we included the predicted BH3 domain of M11L, an antiapoptotic myxoma virus protein shown to bind Bak.[15] This alignment shows that the selected region of F1L has only limited sequence homology to BH3 domains (Figure 5d). The domain consensus for BH3 domains described in Prosite contains (LIVAT)-(3x)-L, which is similar to the L-(3x)-V found in F1L. On the other hand, F1L, like M11L, lacks the conserved Gly residue, while F1L further lacks the conserved Asp residue in the C-terminal half of the domain. This Asp plays a critical role in binding of BH3 domains in Bcl-2 family members.[14] Furthermore, the F1L region contains an insertion of two tyrosine residues absent in Bcl-2 family members. These two features set F1L apart from the BH3 consensus; yet, our studies reveal that this region of F1L is critically required to interact with Bak. Therefore, we refer to the sequence in F1L as BH3-like. Next, we asked if combining the features found in our BH3-like domain with conventional BH3 domains could aid in uncovering other BH3-like sequences. Particularly, we were interested if the vaccinia genome might contain additional BH3-like domains. We devised a variation of the Prosite BH3 domain consensus, incorporating charged residue features at the C-terminal half of the domain, as well as freedom to incorporate 4–6 residues between the N-terminal (LIV)-(3x)-(LV) and the C-terminal charged residues (Figure 5d). In devising our search, we further gave preference to residues that have been shown to participate in intermolecular interactions.[16–18] We searched the vaccinia genome using this variant consensus sequence and found two additional proteins (A6L and N1L) that contain a peptide stretch that fits our new search criteria (Figure 5d). A6L is an unknown protein that can cosediment with microtubules.[19] N1L was described to promote virulence independent of a role in virus replication, consistent with a potential antiapoptotic role.[20]

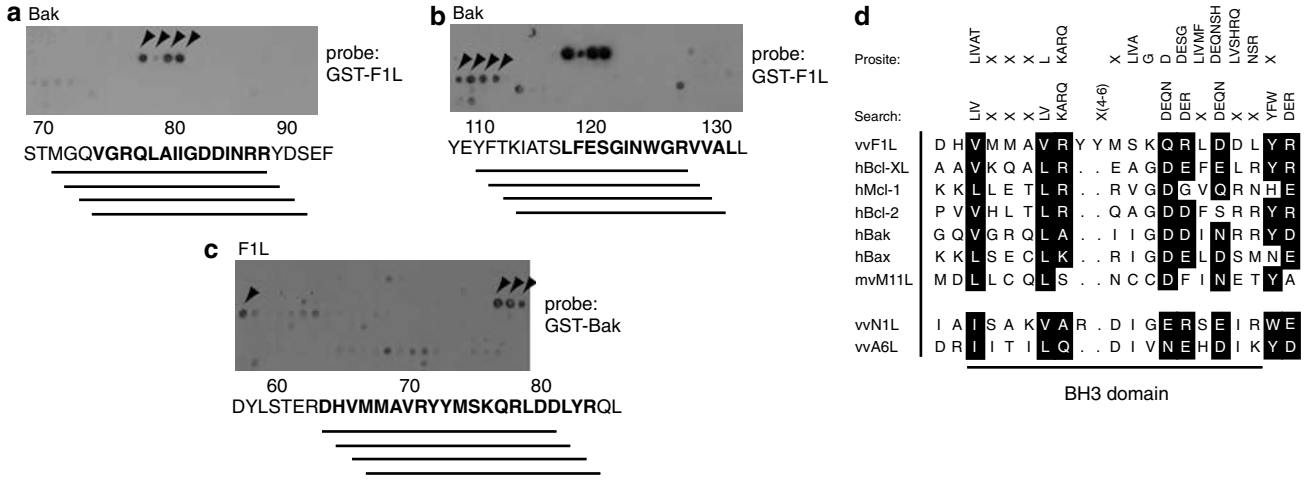

Figure 5 Residues 64–84 of F1L interact with the BH3 domain of Bak. (**a**) Far Western blot analysis with anti-GST antibody of overlapping arrays of 18-mer peptides spanning the complete sequence of Bak (**a**, **b**) and F1L (**c**) probed with the indicated GST fusion proteins. (**a**) The Bak peptide sequences binding GST-F1LΔTM are depicted with respect to the Bak sequence. The BH3 domain in Bak is shown in bold. (**b**) In a longer exposure of (**a**), four peptides binding GST-F1LΔTM can be observed. This region covers the conserved BH1 domain (bold). Similarly, below (**c**), the F1L peptides binding GST-Bak are indicated. The predicted α-helix is shown in bold. (**d**) A sequence alignment of the BH3 domains of Bcl-XL, Mcl-1, Bcl-2, Bax and Bak with the predicted α-helices containing BH3-like domains of F1L (**a**) and M11L.[15] Residues matching the variant search profile are highlighted with black boxes. The BH3 consensus as described in Prosite (http://www.expasy.org/prosite/) and our variant BH3-like search are depicted above each residue position. Human sequences are referred to as h, vv is vaccinia virus and mv is myxoma virus

F1L prevents Bak from binding active Bax

In order to study the role of F1L independently from other viral proteins and signalling pathways stimulated by infection, we generated cells stably expressing F1L. We created a tetracycline-inducible GFP-F1L cell line, which allows inducible expression in HeLa cells. We confirmed that when GFP-F1L is induced by the addition of doxycyclin to cells, we can coimmunoprecipitate Bak with F1L. As in our previous experiments, we could not detect an interaction of F1L with Bax (Figure 6a; data not shown). We noticed in contrast to controls that induction of GFP-F1L only leads to a modest increase of GFP expression. We reasoned that F1L may be degraded outside of the viral context, resulting in lower protein levels in our stable line. To test this, we treated cells for 6 h with the proteasome inhibitor MG-132 at 1 μM, which led to a strong increase of GFP-F1L (Figure 6b). Therefore, the combination of CMV-driven induction and short-term inhibition of protein degradation produced a significant level of GFP-F1L expression with which to test its antiapoptotic properties. We confirmed that stabilization of GFP-F1L against proteolysis in these cells significantly protects cells from STS-induced cell death (Figure 6c).

Activation and (hetero)oligomerization of the proapoptotic Bcl-2 proteins Bak and Bax is required for the release of death-inducing factors from the mitochondria. We immunoprecipitated Bak from cells treated with doxycyclin and STS. In uninduced cells STS treatment led to Bax co-precipitating with Bak, which was not observed in GFP-F1L-expressing cells (Figure 6d). In the converse experiment, treatment of uninduced cells with STS led to the progressive accumulation of active Bax from as early as 2 h after treatment, as determined by the use of an active Bax-specific antibody (*N*−20), with the first active Bax detected after 2 h of treatment. Also, active Bax coimmunoprecipitates with Bak in these cells (Figure 6d). In contrast, in cells where expression of GFP-F1L is induced, Bax activation is delayed. Even after 4 h of STS treatment less active Bax is present in GFP-F1L-expressing cells. Strikingly, in these cells, no Bak protein co-precipitates with active Bax. Together, these results suggest that F1L prevents Bak undergoing heterodimerization with Bax.

F1L inhibits Bak activation during infection and after STS treatment

Together, our findings are consistent with the notion that F1L protects cells by constitutively engaging Bak. To further test whether this prevents Bak from becoming activated after an apoptotic trigger, we used size-exclusion chromatography to characterize the oligomerization state of Bak. HeLa cells were solubilized using Chaps buffer and the resulting lysates were analysed by gel filtration. In untreated HeLa cells Bak eluted over a broad range (Figure 7). This suggests that monomeric and oligomeric forms of Bak are present in normal cells, consistent with previous reports.[21,22] As expected, STS treatment led to the accumulation of Bak in high-molecular-weight fractions. Infection of HeLa with WR for 12 h results in elution of Bak over a range reminiscent of that in uninfected cells (Figure 7). HeLa cells infected with ΔF1L showed a larger proportion of high-molecular-weight and fewer low-molecular-weight Bak complexes, suggesting that F1L delays the oligomerization of Bak during vaccinia infection. Using our inducible GFP-F1L HeLa cells, we tested whether GFP-F1L is sufficient to prevent STS-induced Bak oligomerization. In STS-treated uninduced cells, Bak elutes predominantly in high-molecular-weight complexes, whereas in cells treated with doxycycline in addition to STS, Bak elutes over a broad range. This demonstrates that, even in the absence of additional viral proteins, F1L is sufficient to prevent Bak oligomerization in STS-treated cells.

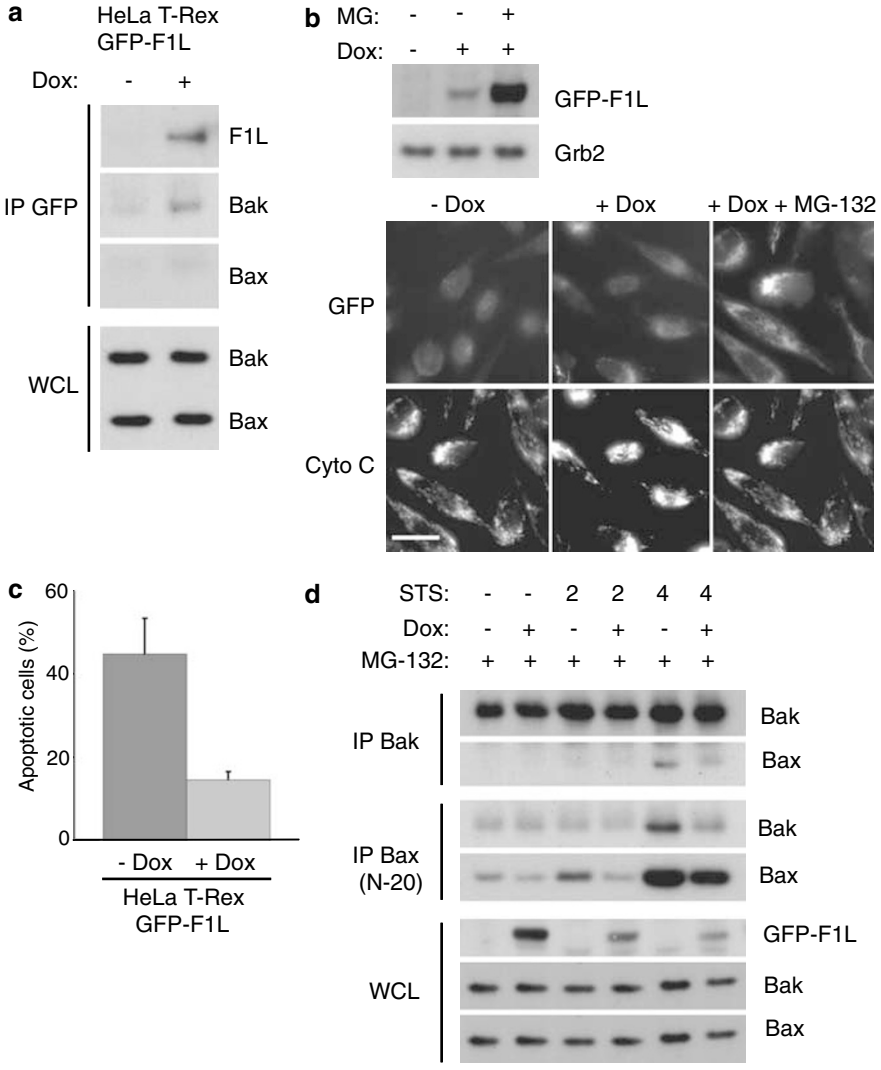

Figure 6 F1L prevents STS-induced cell death and the association of Bak with Bax. (**a**) IP of GFP-F1L from HeLa T-Rex GFP-F1L cells after induced expression using anti-GFP antibodies. Western blots of the IP and the whole cell lysate (WCL) were probed using anti-F1L, anti-Bak and anti-Bax antibodies. (**b**) Western blot analysis of HeLa T-Rex GFP-F1L treated with doxycycline overnight before addition of MG-132 reveals a strong increase in the steady-state levels of GFP-F1L. Immunofluorescence stainings using an anti-cytochrome *c* antibody show a marked induction of GFP-F1L at the mitochondria. Images were taken at identical settings. Scale bar = 10 μm. (**c**) Quantification of fragmented nuclei by staining cells on coverslips with DAPI. Single-clone HeLa T-Rex GFP-F1L cells were treated with doxycycline before addition of MG-132. Subsequently, the samples were treated with STS. These data were obtained from two independent clones. (**d**) Immunoprecipitates with anti-active Bax (N-20) or anti-Bak (NT) antibodies of HeLa treated with MG-132, doxycycline and STS as indicated were analysed by Western blot using anti-Bak and anti-Bax antibodies. WCL were analysed by immunoblotting for Bak, Bax and F1L

To further investigate the activation state of Bak during infection with WR and ΔF1L, we using antibodies directed against epitopes only exposed during Bak activation.[23] FACS analysis using the Bak Ab-1 monoclonal antibody reveals that STS treatment of mock-infected cells for 4 h resulted in pronounced Bak activation (Figure 8a). In contrast, Bak activation was largely inhibited in WR-infected cells even after 4 h of STS treatment. However, in ΔF1L-infected cells, Bak activation was even more pronounced than mock-infected cells, being readily detectable even at 2 h STS treatment (Figure 8a). These results clearly demonstrate that F1L not only binds Bak, but also prevents its activation during STS treatment.

To examine if F1L inhibits activation of Bak in the absence of an external trigger such as STS, we stained infected cells with antibodies against active Bak and cytochrome *c* (Figure 8b). In contrast to WR, infection with ΔF1L virus resulted in ~3-fold more cells staining positive for Bak activation (Figure 8c). Examination of the number of cells with fragmented nuclei and cytoplasmic cytochrome *c* also confirmed that WR-infected cells show a significant delay in apoptosis compared to those infected with ΔF1L (Figure 8c). Together, these results demonstrate that F1L prevents the conformational changes and oligomerization of Bak during vaccinia infection. Recently, it was reported that death induced by an attenuated vaccinia strain lacking F1L (MVA-ΔF1L) causes activation of Bax in addition to release of cytochrome *c* from the mitochondria.[24] We extended our antibody analysis using an equivalent antibody, which recognizes active Bax. We found that after 12 h of ΔF1L

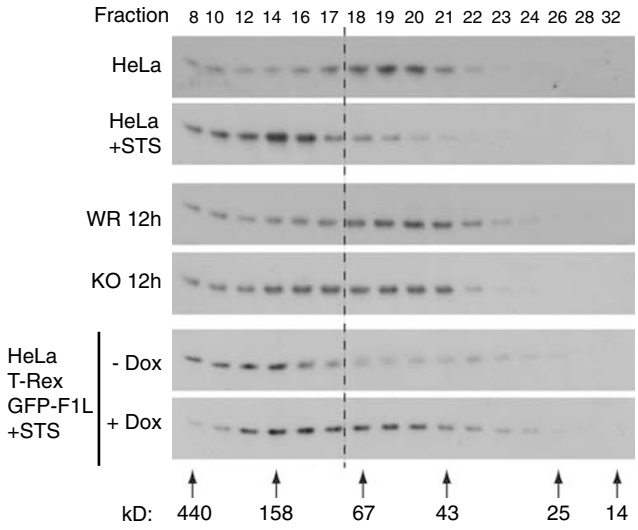

Figure 7 F1L prevents formation of high-molecular-weight Bak complexes. HeLa cells were treated with 1 μM STS for 3 h or infected with WR or ΔF1L virus for 12 h. HeLa T-Rex GFP-F1L cells were treated with or without 1 μM doxycycline overnight before addition of MG-132 for 6 h and 1 μM STS for 3 h. Cell lysates were separated by size exclusion chromatography and selected fractions were submitted to Western analysis using anti-Bak antibodies. The dashed line indicates an approximate separation between the complexes before and after apoptosis induction

infection Bax is activated, whereas few WR-infected cells are positive for active Bax (Figure 8c).

To address whether Bak is a significant interaction partner of F1L in blocking apoptosis, we depleted HeLa cells of Bak using siRNA before testing whether WR- and ΔF1L-infected cells are protected against STS-induced death (as shown in Figure 2c). Interestingly, knockdown of Bak results in a general reduction in sensitivity to STS-induced cell death (Figure 8d). Infection of these cells with ΔF1L virus does not prevent STS-induced cell death. In contrast, WR infection still confers a small protection against STS-induced death, suggesting that F1L-mediated cell death protection is largely, though not exclusively, Bak-dependent. In conclusion, we find that F1L constitutively binds Bak in the OMM, using a BH3-like domain. This interaction significantly delays Bak activation and oligomerization and protects cells against cell death during vaccinia infection.

Discussion

Apoptosis is a powerful cellular defence mechanism against viral infection. In response, viruses have developed strategies to inhibit many levels of the apoptotic signalling pathways, including at the mitochondria.[7] Our observations reveal that F1L from vaccinia virus specifically binds Bak to inhibit its activation and prolong cell viability during infection.

Bak and Bax are critical regulators of mammalian cell apoptosis, and are held in check by the antiapoptotic Bcl-2 family proteins. After an apoptotic stimulus, BH3-only proteins bind to specific antiapoptotic Bcl-2 family proteins, allowing the activation of Bak and Bax.[3,25] Bak in healthy cells is an integral membrane protein bound to mitochondria, where it associates with Mcl-1 and Bcl-XL.[25–28] Once released from sequestration by Mcl-1 and Bcl-XL, Bak becomes activated and can associate with itself as well as Bax.[11,22]

Bak and Bax are regulated in multiple ways, including the activity of BH3-only proteins. It has been suggested that two classes of BH3-only proteins exist: those that block the repression of Bak/Bax by the antiapoptotic Bcl-2 family proteins and those that bind and activate Bak/Bax directly.[4,5] Specifically, some controversy exists as to whether Bid and Bim bind Bax directly. For instance, it has been reported that Bim protein and BH3 peptides directly engage and activate Bax,[4,5,29] while others reported that they could not detect interactions between these proteins.[16]

The structures of several members of the Bcl-2 family (Bcl-2, Bcl-XL, Bcl-w, Bax, Bid) have revealed very similar structural folds.[14] Central α-helices 2–5 containing the conserved BH1–3 domains form an elongated hydrophobic groove on the surface of the protein. Cocrystallization studies of Bcl-XL with the BH3 peptides of Bak, Bad and Bim show that the peptides bind as an amphipathic α-helix and make extensive contacts with this groove.[16–18] In particular, the incoming peptides containing an α2 helix/BH3 domain have significant hydrophobic and hydrophilic interactions with residues in the α2 helix/BH3 domain and α4–5 helices/BH1 domain of Bcl-XL. These experiments very well explain how BH3-only proteins bind pro- and antiapoptotic Bcl-2 proteins. They do not however explain how two BH1–3 proteins, such as Bcl-XL and Bak, would bind one another. Unfortunately, to date there are no structures of Bak, which is constitutively inserted in the mitochondrial membrane, nor of full-length pairs of Bcl-2 family members. In our peptide scan, we found that Bak binds to a region of F1L that contains a BH3-like domain. Conversely, F1L protein binds preferentially to peptides corresponding to Bak BH3 domain, and with lower affinity to BH1 domain-containing peptides (Figure 5). It is therefore tempting to speculate that F1L might prevent Bak activation by extending its BH3-like domain-containing helix into the Bak structure to contact the Bak BH1 and BH3 domains.

Our data show that F1L binds to and prevents the activation of Bak. Supporting the notion that Bak is a critical F1L effector are studies using mouse embryo fibroblasts derived from Bak- and Bax-deficient mice.[24] These showed that the apoptotic pathway inhibited by F1L in the attenuated vaccinia strain MVA primarily functions through Bak, while a much smaller contribution via Bax was observed.[24] Interestingly, we find that in WR-, but not in ΔF1L-infected cells, the activation of both Bak and Bax is prevented (Figure 8d). However, when we treat cells with a strong apoptotic trigger such as STS, Bax does get activated in the presence of F1L, albeit with delayed kinetics. This active Bax does not however interact with Bak, presumably because Bak is already sequestered by F1L (Figure 6d). Still, under these conditions, expression of F1L prevents Bak from interacting with Bax, suggesting that F1L prevents Bak activation more efficiently than Bax activation (Figure 6d). We have been unable to detect an interaction between F1L and Bax in our assays (Figure 6a, data not shown). One explanation could be that we missed an F1L–Bax interaction in our studies because we deleted the TM domains of the Bcl-2 proteins (Figure 4a). However,

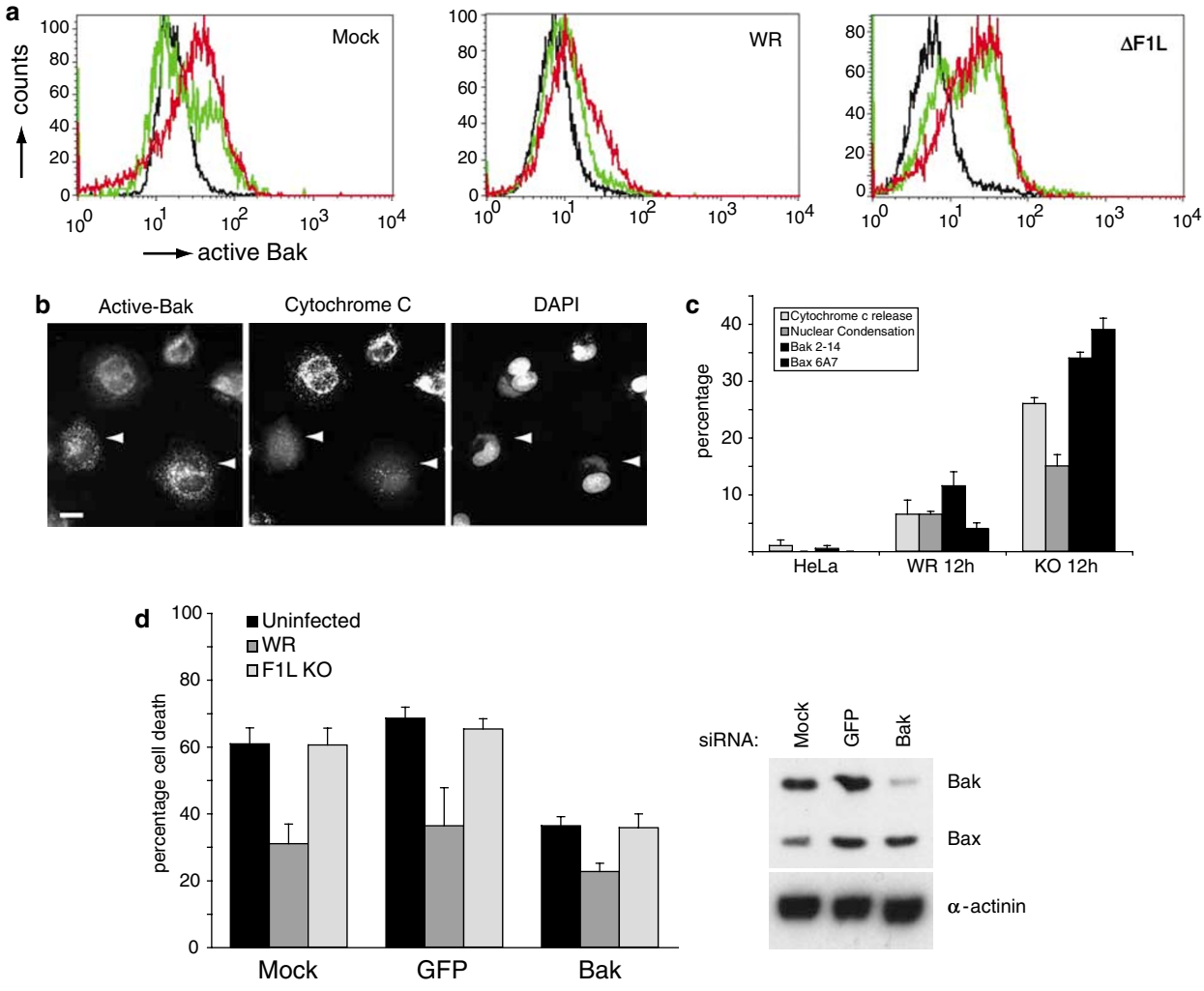

Figure 8 F1L prevents Bak activation during vaccinia infection. (**a**) Detection of active Bak by FACS analysis of Ab-1 monoclonal antibody-stained mock, WR- or ΔF1L virus-infected HeLa cells. After 4 h of infection, cells were treated with 1 μM STS for 0 (black line), 2 (green line) or 4 (red line) h. (**b**) A representative example of immunofluorescence analysis of ΔF1L-infected cells 12 h.p.i. using the conformation-specific Bak polyclonal antibody. White arrowheads indicate the cells positive for active Bak, while having cytoplasmic cytochrome C staining. DAPI staining was used to reveal perinuclear viral factories and fragmentation of cell nuclei. Scale bar = 20 μm (**c**). Quantification of immunofluorescence analysis with the active Bak polyclonal 2–14 antibody, the Bax (6A7) or cytochrome *c* monoclonal antibody and DAPI at 12 h.p.i. with mock, WR or ΔF1L virus. The data represent mean (± S.E.M.) from three independent experiments in which over 200 cells per condition were scored. (**d**) Quantitation of nuclear fragmentation by DAPI staining after treating HeLa cells infected with WR or ΔF1L virus with 1 μM STS for 4 h. HeLa were transfected with siRNA oligo's against GFP or a Bak pool for 72 h before infection. Western blot analyses of cell lysates were blotted for the presence of Bak, Bax and α-actinin

the use of TM deletions, for instance, of Bax and Bcl-XL, has been reported to lead to an increase, not a decrease, of binding promiscuity towards other Bcl-2 family members, at least in the absence of nonionic detergents.[16,30] We therefore favour the interpretation that F1L binds Bak specifically and not Bax.

So how does F1L prevent Bax activation if not via direct interaction? It has been reported that Bak and Bax are genetically redundant;[31,32] yet, in our cells Bak knockdown is sufficient to desensitize cells to STS-induced cell death (Figure 8d). This could reflect the difference in Bak/Bax dependence between cell lines, as has been reported previously.[33] We found that Bak knockdown in uninfected and ΔF1L-infected cells protects equally well from apoptosis, as infection with WR virus protects the control knockdown cells (Figure 8d). We therefore favour that acute depletion of Bak lowers the total Bak/Bax burden on the cells. In this way, Bak knockdown is analogous to locking up cellular Bak through its F1L or Bcl-XL sequestration. In both cases, lowering the amount of available Bak increases the threshold for cell death and is predicted to delay Bax activation, as is the case in our F1L-expressing and WR-infected cells (Figures 6d and 8c). Alternatively, it could indicate F1L can protect cells by engaging proteins in addition to Bak, preventing them from activating Bax. In support of this, even after Bak knockdown, infection with WR, but not ΔF1L, provides protection against STS-induced apoptosis, albeit less efficiently than in control knockdown cells (Figure 8d). Although this could be due to incomplete Bak knockdown, it has recently been shown that expression of F1L abrogated cell death induced by over-expression of the BH3-only proteins Noxa, Puma and Bim, placing F1L downstream of BH3-only protein activation.[24]

Furthermore, in binding studies using recombinant F1L lacking its TM domain (F1LΔTM) and selected BH3 domain peptides, F1LΔTM was described to have a significant affinity for Bim BH3 peptides in addition to binding Bak BH3 peptides.[24] This raises the possibility that, like cellular prosurvival proteins, F1L prevents apoptosis through a dual capacity: on the one hand binding and inhibiting Bak directly, while also targeting one or several BH3-only proteins that otherwise contribute to Bax activation. It will be interesting to see whether the F1L protein is indeed capable of binding any of the BH3-only proteins, especially during viral infection.

Other viral proteins exist that lack homology to Bcl-2 proteins, yet target Bak or Bax to inhibit the mitochondrial death checkpoint. For instance, myxoma virus M11L binds Bak, while cytomegalovirus vMIA targets Bax.[15,34,35] It is possible that viruses may contain proteins to target both Bak and Bax, depending on their host range or tissue specificity. Consistent with this hypothesis, Andoniou *et al*.[36] have recently suggested that mouse cytomegalovirus encodes proteins in addition to vMIA that specifically interfere with Bak function. Interestingly, the region of F1L that we have shown to interact with Bak exhibits only limited homology to cellular or viral BH3 domains, yet it specifically binds Bak (Figures 4 and 6). Future work is needed to examine whether binding both Bak and Bax is a common theme in apoptosis inhibition by viruses. It is perhaps unsurprising that the BH3-like domains of these viral proteins look different from their cellular counterparts, as it enables them to bind the proapoptotic Bak and Bax proteins, while avoiding binding to prosurvival Bcl-2 proteins. We find using a refined BH3 consensus algorithm including features of the F1L and putative M11L BH3-like domains that the vaccinia genome encodes two additional open reading frames that conform to our search consensus motif (Figure 5). It will be interesting to determine if these proteins can indeed modulate Bcl-2 family members and in particular have Bax-binding activity. Characterization of the molecular mechanisms of antiapoptotic proteins such as F1L, and identification of additional factors will ultimately be required to fully understand how viruses like vaccinia are so effective at preventing apoptosis during infection.

Materials and Methods

Antibodies and infections

Anti-F1L antibodies were produced in rabbits by immunization with peptides corresponding to amino acids 12–30 (for IP and IF analysis) and 181–203 (for Western analysis) of the protein coupled to KLH. Anti-F1L antibodies were affinity purified on their respective peptides. Polyclonal antibodies used were: anti-Bak (Bak NT; Upstate Biotechnology), anti-Bax (Bax NT; Upstate Biotechnology), anti-active Bax (Bax N-20; Santa Cruz) anti-Bak (2–14; Calbiochem); anti-active caspase 3 (Promega), anti-cytochrome *c* (6H2.B4, BD PharMingen), anti-GST (Sigma), anti-F17R (a kind gift of Jacomine Krijnse-Locker) and anti-A36R.[37] Mouse monoclonals used were: anti-Bak (Ab-1; Oncogene), anti-GFP (3E1, CR-UK, London), anti-polyhistidine (Sigma), anti-Hsp60 (BD Transduction Labs), anti-Bax (6A7, Trevigen) and rat monoclonal anti-B5R (19C2[38]). Infections with vaccinia virus and processing for immunoblot or immunofluorescence analysis were as described previously.[37]

Cell death assays

For sub-G1 DNA content analysis, cells were stained with propidium and analysed by FACS. The percentage of cells with fragmented DNA was calculated. The data represent at least three independent experiments. Cells grown on coverslips were infected with WR or ΔF1L and/or treated with 1 μM STS or 100 mJ/cm^2 UV and processed for staining with indicated antibodies or DAPI to allow quantification of the number of cells showing fragmented DNA. All immunofluorescence data represent over 200 cells per condition in at least three independent experiments. Alternatively, cells were trypsinized, fixed and stained for active Bak (Ab-1) before analysis by FACS. A minimum of 10^5 events was collected per sample.

siRNA downregulation of Bak

SiGENOME SMARTpool for Bak (Bak-siRNApool; sequence 1, 5-CAGAGAAUGCCUAUGAGUAUU-3; sequence 2, 5-UAUGAGUACUU CACCAAGAUU-3; sequence 3, 5-CAACCGACGCUAUGACUCAUU-3 and sequence 4, 5-CGACAUCAACCGACGCUAUUU-3) was obtained from Dharmacon. The control GFP siRNA 5-GCAAGCUGACCCUGAA GUUCAU-3/5-GAACUUCAGGGUCAGCUUGCCG-3, directed against the DNA sequence of EGFP, was obtained from Qiagen. Cells plated 1 day earlier on six-well or 10-cm culture dishes to achieve 50–60% confluence were transfected with Bak siRNApool or a control GFP siRNA (100 nM) using Lipofectamine (Invitrogen, Paisley, UK). Transfection efficiency was at least 90%, as determined by cotransfection with a rhodamine-labelled control siRNA oligo. At 72 h after transfection, cells were lysed in 2% Chaps lysis buffer and knockdown was assessed by immunoblot analysis.

E. coli protein expression, purification and affinity resin production

F1L and M11L were amplified by PCR from vaccinia WR and myxoma genomic DNA, respectively. Bcl-2, Bcl-XL, Bax and Bak were obtained from D Hancock (CR-UK, London). cDNAs were PCR amplified without their C-terminal TM domain and subcloned into pMW172-His or GST to generate His- or GST-tagged F1L *E. coli* T7 expression clones. All expression clones were transformed into *E. coli* strain BL21(DE3) and exponentially growing cultures were induced for 4 h with 200 μM IPTG. Soluble fractions were produced using standard methods. Equal amounts of soluble GST-tagged proteins were bound for 1 h to glutathione sepharose beads in 0.1% Chaps, 400 mM NaCl wash buffer (WB: 20 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1 mM EDTA). Subsequently, equal amounts of lysates containing His-F1L or His-F1L (M67P) were added to beads with bound GST-tagged proteins, incubated for 1 h and washed four times with WB containing 0.5% Chaps and 500 mM NaCl.

Pulldowns, co-IP assays and Western blotting

HeLa cells were lysed in 1% Chaps lysis buffer (20 mM Tris-HCl (pH 8.0), 140 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1% Chaps) containing 1 $\times$ complete protease inhibitors (Roche, Lewes, UK). Equal amounts of GST and GST-F1L protein were bound to gluthatione beads as described above. Precleared lysates were incubated with the beads for 1–2 h at 4°C, washed four times in lysis buffer and processed for Western blotting. For peptide pulldowns, the peptide corresponding to residues 57–78 and 57–78(M67P) were linked to agarose beads via a carboxy-terminal cysteine residue using the Sulfolink kit (Pierce, Rockford, IL, USA). For co-IP experiments, cells were lysed in 2% Chaps lysis buffer as described.[15]

The complexes were resolved by SDS-PAGE and analysed by Western blotting. HeLa T-Rex GFP-F1L cells were generated by inserting GFP-F1L into the pCDNA4/TO vector (Invitrogen), followed by transfection and selection of HeLa T-Rex cells (Invitrogen) using Zeocin (500 μg/ml). Single clones of HeLa T-Rex GFP-F1L cells were characterized and treated with doxycycline overnight before addition of MG-132 (EMD Bioscience, La Jolla, CA, USA) for 6 h. Subsequently, some samples were treated for 5 h with 1 μM STS as indicated.

Far Western analysis of peptide arrays and gel filtration analysis

Peptide arrays spanning residues 1–205 of F1L and 1–187 of Bak with 18-mer peptides and a moving window of one amino acid were produced using a Multipep synthesizer with spot upgrade (Intavis, Cologne, Germany). To probe the array, soluble GST fusion proteins were purified on gluthatione beads, eluted, dialysed and quantified according to the manufacturer's instructions (Pharmacia). Membranes were blocked for 1 h in 5% skim milk in PBT (PBS/0.1% Tween-20), incubated with 2 μg/ml purified GST fusion proteins in PBT for 2 h, washed three times, and probed with rabbit polyclonal anti-GST antibody. Primary antibodies were detected by horseradish peroxidase-conjugated anti-rabbit antibody, followed by enhanced chemiluminescence (Amersham Bioscience, Uppsala, Sweden). Gel filtration was performed at 4°C using Superdex 200 resin equilibrated in 1% Chaps lysis buffer. The column was calibrated using thyroglobulin (667 kDa), ferritin (440 kDa), aldolase (158 kDa), BSA (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (14 kDa). HeLa cells (1.5×10^7) were solubilized using 2% Chaps lysis buffer containing protease inhibitors. The sample was then centrifuged at 21 000 *g* to remove insoluble material. Samples of 50 μl were loaded onto the column and the eluate was monitored at 280 nm. Fractions of 30 μl were collected and aliquots analysed by immunoblot.

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Note added in proof

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