

The Vaccinia Virus F1L Protein Interacts with the Proapoptotic Protein Bak and Inhibits Bak Activation

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Many viruses have evolved strategies to counteract cellular immune responses, including apoptosis. Vaccinia virus, a member of the poxvirus family, encodes an antiapoptotic protein, F1L. F1L localizes to mitochondria and inhibits apoptosis by preventing the release of cytochrome *c* by an undetermined mechanism (S. T. Wasilenko, T. L. Stewart, A. F. Meyers, and M. Barry, *Proc. Natl. Acad. Sci. USA* 100:14345–14350, 2003; T. L. Stewart, S. T. Wasilenko, and M. Barry, *J. Virol.* 79:1084–1098, 2005). Here, we show that in the absence of an apoptotic stimulus, F1L associates with Bak, a proapoptotic member of the Bcl-2 family that plays a pivotal role in the release of cytochrome *c*. Cells infected with vaccinia virus were resistant to Bak oligomerization and the initial N-terminal exposure of Bak following the induction of apoptosis with staurosporine. A mutant vaccinia virus missing F1L was no longer able to inhibit apoptosis or Bak activation. In addition, the expression of F1L was essential to inhibit tBid-induced cytochrome *c* release in both wild-type murine embryonic fibroblasts (MEFs) and Bax-deficient MEFs, indicating that F1L could inhibit apoptosis in the presence and absence of Bax. tBid-induced Bak oligomerization and N-terminal exposure of Bak in Bax-deficient MEFs were inhibited during virus infection, as assessed by cross-linking and limited trypsin proteolysis. Infection with the F1L deletion virus no longer provided protection from tBid-induced Bak activation and apoptosis. Additionally, infection of Jurkat cells with the F1L deletion virus resulted in cellular apoptosis, as measured by loss of the inner mitochondrial membrane potential, caspase 3 activation, and cytochrome *c* release, indicating that the presence of F1L was pivotal for inhibiting vaccinia virus-induced apoptosis. Our data indicate that F1L expression during infection inhibits apoptosis and interferes with the activation of Bak.

Apoptosis is an evolutionarily conserved pathway that is important for development, cellular homeostasis, and protection from microbial pathogens (38). Although the central tenets of the apoptotic process involve the activation of a family of cysteine proteases, referred to as caspases, mitochondria act as a critical control point during apoptosis (22, 55). Following an apoptotic insult, mitochondria undergo loss of the inner mitochondrial membrane potential and release of an array of death-promoting proteins, including apoptosis-inducing factor, endonuclease G, SMAC/Diablo, HtrA2/Omi, and cytochrome *c* (22, 55).

The mitochondrial component of the apoptotic cascade is tightly regulated by members of the Bcl-2 family (10, 25, 44). The Bcl-2 family consists of both anti- and proapoptotic members that function to either maintain mitochondrial integrity or stimulate the release of cytochrome *c* (10, 25, 44). Bcl-2 family members are typically characterized as containing one or more Bcl-2 homology (BH) domains (10, 25). Bak and Bax, two proapoptotic members of the Bcl-2 family, are activated in response to apoptotic stimuli and play pivotal roles in generating apoptotic death (34, 59). In the absence of an apoptotic trigger, Bax is predominantly cytoplasmic or loosely associated with intracellular membranes (60). Following an apoptotic trigger, Bax undergoes a series of conformational changes, which

include exposure of the N terminus and liberation of the C-terminal transmembrane domain, resulting in mitochondrial membrane insertion, followed by subsequent homo-oligomerization and release of cytochrome *c* (2, 7). In contrast to Bax, the majority of Bak normally resides at the mitochondria. Similar to Bax activation, apoptosis triggers a multistep activation of Bak, resulting in a conformational change in Bak and subsequent homo-oligomerization (24, 58). Antiapoptotic members of the Bcl-2 family, such as Bcl-xL and Bcl-2, prevent Bax and Bak oligomerization, thereby interfering with cytochrome *c* release (25, 44). Cells deficient in Bax and Bak are protected from apoptosis initiated by a wide range of stimuli, clearly establishing the importance of Bak and Bax in apoptotic cells (34, 59).

To inhibit apoptosis, many viruses encode proteins that function directly at the mitochondrial checkpoint (5, 6). For example, a number of viruses encode obvious Bcl-2 homologues that function at the mitochondria to inhibit the release of cytochrome *c* and apoptosis (13, 26, 40). More recently, however, novel viral proteins that lack homology to Bcl-2 have been described that also function to inhibit the release of cytochrome *c* (5, 6). These include K7, encoded by Kaposi's sarcoma-associated herpesvirus; vMIA (viral mitochondrion-localized inhibitor of apoptosis), encoded by human cytomegalovirus (HCMV); and M11L, encoded by myxoma virus (16, 21, 54). K7 is related to a spliced version of human survivin but also contains a portion of a baculovirus inhibitor of apoptosis repeat domain and a putative BH2 region (54). K7 localizes to mitochondria and interacts with activated caspase 3, Bcl-2, and calcium-modulating cyclopilin ligand, resulting in the regula-

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tion of cellular calcium levels and effectively protecting cells from apoptosis (19, 54). vMIA encoded by HCMV inhibits release of cytochrome *c* by a unique mechanism (21). The expression of vMIA causes the constitutive mitochondrial localization of Bax and the formation of Bax oligomers; however, despite demonstrating Bax oligomerization, vMIA effectively inhibits cytochrome *c* release (3, 41). M11L, encoded by the poxvirus myxoma virus, localizes to mitochondria and inhibits the release of cytochrome *c* (16, 17). M11L constitutively interacts with Bak and the peripheral benzodiazepine receptor, a component of the permeability transition pore, which may be involved in cytochrome *c* release (17, 29, 52). M11L homologues exist in a subset of poxviruses, including members of the genera *Leporipoxvirus*, *Suipoxvirus*, *Capripoxvirus*, and *Yatapoxvirus*. At present, the only poxviruses known to contain obvious Bcl-2 homologues are fowlpox virus and canarypox virus, two members of the genus *Avipoxvirus* (1, 51). The FPV039 open reading frame in fowlpox virus was initially identified based upon the presence of obvious BH1 and BH2 domains and is predicted to localize to the mitochondria and inhibit cytochrome *c* release (1).

We recently identified F1L as an antiapoptotic protein expressed during vaccinia virus (VV) infection (47, 57). F1L lacks obvious sequence homology to M11L, as well as cellular members of the Bcl-2 family. F1L localizes to the mitochondria and inhibits apoptosis by blocking the release of cytochrome *c* and preventing the loss of the inner mitochondrial membrane potential by an unknown mechanism (47, 57). We now report that F1L interacts with the proapoptotic protein Bak. F1L expression inhibits both Bak oligomerization and the initial N-terminal exposure of Bak, two necessary features for Bak proapoptotic function. Notably, a recombinant vaccinia virus with F1L deleted induces apoptosis upon infection, indicating that the presence of F1L is necessary to inhibit apoptosis initiated by virus infection.

MATERIALS AND METHODS

Cells and viruses. Jurkat and HeLa cells were cultured as previously described (47, 57). Bak- and Bax-deficient Jurkat cells were a gift from H. Rabinowich (University of Pittsburgh School of Medicine, Pittsburgh, PA) (53). CV-1 and HEK 293T cells were grown in Dulbecco's modified Eagle's medium (Gibco Invitrogen Inc.) supplemented with 10% fetal bovine serum (Gibco Invitrogen Inc.), 2 mM L-glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin. Murine embryonic fibroblasts (MEFs) were provided by S. Korsmeyer. (Harvard Medical School, Boston, MA) and cultured as described previously (59). Vaccinia virus strain Copenhagen [VV(Cop)] and VV(Cop)-enhanced green fluorescent protein (EGFP) were provided by G. McFadden (Robarts Research Institute, London, Ontario, Canada). Vaccinia virus strain Western Reserve (WR) expressing a Flag-tagged F1L [VV(WR)Flag-F1L] was generated as described previously (47). Viruses were routinely grown in baby green monkey kidney (BGMK) cells. Single-step growth curves were generated by infecting CV-1 cells at a multiplicity of infection (MOI) of 2 for 1 h. Infected cells were harvested at 0, 4, 8, 12, and 24 h postinfection, and viral titers were determined by plaque formation on CV-1 cells.

Generation of F1L deletion virus. F1L gene fragments corresponding to the first 180 nucleotides and the last 166 nucleotides were amplified by PCR using the following primer pairs: F1L(F), 5'-CTCGAGATGTTGTCGATGTTTATG, and F1L 2.2, 5'-AATGCAGATCTGGATCTGATAGATAATCGAGTATGT-3'; F1L(R), 5'-GGATCCTTATCCTATCATGTATTT-3', and F1L2.3, 5'-GATCCAGATCTGCATTCTATCGCATACTATCGCATACTATATGCGA-3'. The resulting amplified gene fragments contained a complementary linker with a BglIII restriction site. The F1L gene fragments served as templates for overlapping PCR with primers F1L(F) and F1L(R) to generate an F1L fragment, F1Lf/b, containing a BglIII restriction site, which was subsequently cloned into pGEMT

(Promega) to generate pGEMT-F1Lf/b. EGFP under the control of an early/late poxvirus promoter was amplified by PCR from pSC66-EGFP using primers E/L-Syn(*BglIII*), 5'-AGATCTAAAAATTGAAATTTTATTTT-3', and EGF-PR(*BglIII*), 5'-AGATCTTTACTGTACAGCTCGTCCATGCC-3'. The resulting EGFP gene fragment was subcloned into pGEMT-F1Lf/b via the internal BglIII restriction site to generate pGEMT-F1Lf/b-EGFP. VV(Cop) was used to generate VV(Cop)ΔF1L by homologous recombination, as previously described (14). BGMK cells (10^6) were infected with VV(Cop) at an MOI of 0.05 and transfected with pGEMT-F1Lf/b-EGFP using Lipofectin (Invitrogen Life Technologies). Recombinant viruses were selected by EGFP fluorescence and plaque purified, and the presence of the disrupted F1L open reading frame was confirmed by PCR.

Gel filtration analysis. Jurkat cells (10^7) were lysed in CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} lysis buffer containing 2% (wt/vol) CHAPS (Sigma Chemical Co.), 300 mM NaCl, 20 mM Tris, pH 7.4, 2 mM EDTA, and 0.2 mM dithiothreitol. Cell lysates were centrifuged at $16,000 \times g$ for 15 min, and the soluble fractions were retained. Gel filtration chromatography was performed at 4°C using a Superose6 HR (10/30) column (GE Healthcare) equilibrated with 1% CHAPS lysis buffer. The Superose6 column was calibrated with thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), and ovalbumin (43 kDa) (GE Healthcare). Cell lysates (1.3 mg) were applied to the column and eluted at a flow rate of 0.150 ml/minute. Fractions (150 µl) were collected and analyzed by Western blotting for the presence of Bak.

Immunoaffinity purification. Immunoaffinity experiments were performed on a HiTrap *N*-hydroxy-succinimide-activated high-performance affinity column (GE Healthcare) coupled with 9.6 mg of protein G-purified rabbit anti-F1L antibody according to the manufacturer's protocol (GE Healthcare) (47). HeLa cells (10^7) were either mock infected or infected with VV(WR)Flag-F1L and lysed in CHAPS lysis buffer containing 2% (wt/vol) CHAPS, 137 mM NaCl, and 20 mM Tris, pH 7.4. Cell lysates were applied to the anti-F1L affinity column and washed with 2% CHAPS lysis buffer. Bound proteins were eluted over a total volume of 5 ml using a linear gradient with elution buffer containing 100 mM glycine, 0.5 M NaCl, pH 2.7. One-milliliter elution fractions were collected and analyzed by Western blotting for the presence of F1L-Flag, Bak, Bax, Bcl-2, Bcl-xL, and Mcl-1.

Immunoprecipitation. HEK 293T cells (4×10^6) were transfected with 4 µg of pcDNA3-HA-Bak and pEGFP-F1L using Lipofectamine 2000 (GIBCO BRL Technologies Inc.). The cells were lysed in 2% (wt/vol) CHAPS, 150 mM NaCl, 50 mM Tris, pH 8.0, containing protease inhibitors (Roche). Cell lysates were immunoprecipitated with either goat anti-EGFP antibody (Luc Berthiaume, University of Alberta, Canada) or mouse anti-HA (12CA5). To detect interaction during virus infection, HeLa cells (8×10^6) were transfected with 14 µg of either pSC66 or pSC66-F1L-Flag and infected with VV(Cop) at an MOI of 10. Whole-cell lysates were prepared in CHAPS lysis buffer containing 2% (wt/vol) CHAPS, 137 mM NaCl, 20 mM Tris, pH 7.4, and 20 mM EDTA containing 10% glycerol, and complexes were precipitated using magnetic beads (Dynabeads M-280 Tosylactivated; Dynal Biotech) coated with either anti-Bak NT (Upstate) or anti-Flag M2 (Sigma Aldrich) as specified by the manufacturer. To detect F1L interaction with endogenous Bak in the absence of infection, 8×10^6 HeLa cells were transfected with 14 µg of either pEGFP-C3 (Clontech) or pEGFP-F1L and immunoprecipitated with goat anti-EGFP antibody.

Mitochondrion purification and cytochrome *c* release. Cellular fractionation into cytosolic and membranous/mitochondrial fractions was performed using digitonin as described previously (56). Cytochrome *c* release was monitored by Western blotting of both the supernatant and membranous fractions. Mitochondria were purified from MEFs as described previously (61). Briefly, MEFs were resuspended in hypotonic lysis buffer containing 250 mM sucrose, 20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, and 1 mM EGTA and incubated on ice for 30 min with intermittent mixing. Cells were disrupted by passage through a 23-gauge needle, and the lysates were centrifuged at $750 \times g$ for 10 min at 4°C. Mitochondria were isolated at $10,000 \times g$ for 20 min at 4°C and resuspended in 250 mM sucrose, 20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, and 150 mM NaCl. Protein concentrations were determined by bicinchoninic acid assay (Pierce Chemical Co.). To monitor cytochrome *c* release, 30 µg of purified mitochondria was incubated with 5, 10, or 15 ng caspase-8-cleaved recombinant human Bid (tBid) (R&D Systems Inc.) for 35 min at 30°C. Samples were centrifuged at $10,000 \times g$ for 15 min at 4°C to separate the mitochondrial pellet from the supernatant prior to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis analysis. Isolated mitochondria (30 µg) from MEFs treated with tBid were subjected to cross-linking using 900 µM of bismaleimido-hexane (BMH) for 30 min (Pierce Chemical Co.). Cross-linking was terminated by the addition of SDS sample buffer containing 1 mM

dithiothreitol. For limited trypsin proteolysis, 30 μ g of mitochondria treated with tBid was resuspended in buffer containing 250 mM sucrose, 20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, and 150 mM NaCl supplemented with 100 μ g/ml trypsin (Sigma Chemical Co.). Following a 20-min incubation on ice, mitochondria were collected by centrifugation at 10,000 \times g for 15 min at 4°C and resuspended in SDS sample buffer.

Confocal microscopy. The localization of Bak and F1L during virus infection was assessed by confocal microscopy. HeLa cells (5×10^5) seeded on 1-mm glass coverslips were infected with VV(WR)Flag-F1L at an MOI of 5. Following an 8-h infection, the cells were fixed at room temperature for 30 min with 3% paraformaldehyde in phosphate-buffered saline and permeabilized with 0.25% (wt/vol) Saponin (Sigma Chemical Co.). The cells were stained with 4 μ g/ml rabbit anti-Bak antibody (G23; Santa Cruz Biotechnology) and 10 μ g/ml Alexa-Fluor 546 goat anti-rabbit antibody (Invitrogen Life Technologies). To detect Flag-F1L, the cells were treated with 10 μ g/ml anti-Flag M2 monoclonal antibody directly conjugated to fluorescein isothiocyanate (Sigma-Aldrich Inc.). Images were obtained using a LSM510 scanning microscope equipped with a 43 \times oil emersion Plan-Apochromat objective.

Bak conformational analysis by flow cytometry. The conformational status of Bak was assessed as previously described (23, 24). Jurkat cells (10^6) were treated with 0.5 μ M staurosporine for 2 h, fixed with 0.25% paraformaldehyde (Sigma Chemical Co.), and stained with 2 μ g/ml anti-Bak antibody (clone TC100; Oncogene Research Products) or an antibody specific for NK1.1 (PK136) as an isotype control (28). Cells were counterstained with phycoerythrin-conjugated anti-mouse antibody (Jackson ImmunoResearch Laboratories Inc.). Antibody staining was detected through the FL-2 channel equipped with a 585 filter (42-nm band-pass). Data were acquired with fluorescence signals at logarithmic gain and were analyzed with CellQuest software.

Measurement of mitochondrial membrane potential. Changes in mitochondrial membrane potential were quantified in infected cells by staining the cells with tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes) as previously described (36, 57). Cells were loaded with TMRE by incubating them in RPMI 1640 medium containing 0.2 μ M TMRE for 30 min at 37°C. Cells were also treated with a membrane uncoupler, carbonyl cyanide *m*-chlorophenyl hydrazone (Sigma Chemical Co.), as described previously (57). TMRE fluorescence was acquired through the FL-2 channel equipped with a 585 filter (42-nm band-pass). Data were acquired with fluorescence signals at logarithmic gain. The data were analyzed with CellQuest software.

Immunoblotting. Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (GE Healthcare), and the following antibodies were used for detection: anti-cytochrome *c* (clone 7H8.2Cl2; Pharmingen), polyclonal rabbit anti-caspase 3 (56), rabbit anti-EGFP (Luc Berthiaume, Edmonton, Alberta, Canada), anti-Flag M2-horseradish peroxidase-conjugated antibody (Sigma Aldrich), anti-manganese superoxide dismutase (Mn SOD) (SOD-111; Stressgen Bioreagents), anti-Bak NT (Upstate), anti-Bax N20-horseradish peroxidase (Santa Cruz Biotechnology), anti-Bcl-2 (clone 124; Upstate), anti-Bcl-xL (Santa Cruz Biotechnology), and anti-Mcl-1 (clone RC22; Lab Vision Corp.). Proteins were visualized by chemiluminescence according to the manufacturer's directions (GE Healthcare).

RESULTS

F1L expression protects cells from apoptosis. To further understand the antiapoptotic mechanism of F1L and its importance in modulating the apoptotic response during virus infection, we generated an F1L deletion virus. Using homologous recombination, the F1L open reading frame was disrupted by insertion of EGFP to create VV(Cop) Δ F1L (14). The recombinant VV(Cop) Δ F1L virus was plaque purified on CV-1 cells, and PCR analysis indicated that the resulting VV(Cop) Δ F1L virus was pure (Fig. 1A). No obvious difference in plaque morphology or growth in a single-step growth curve was detected between the wild-type VV(Cop) and VV(Cop) Δ F1L viruses following infection (Fig. 1B and C), suggesting that F1L is dispensable for efficient virus replication during infection of CV-1 cells.

To investigate the antiapoptotic contribution of F1L during virus infection, Jurkat cells were infected with either VV(Cop)

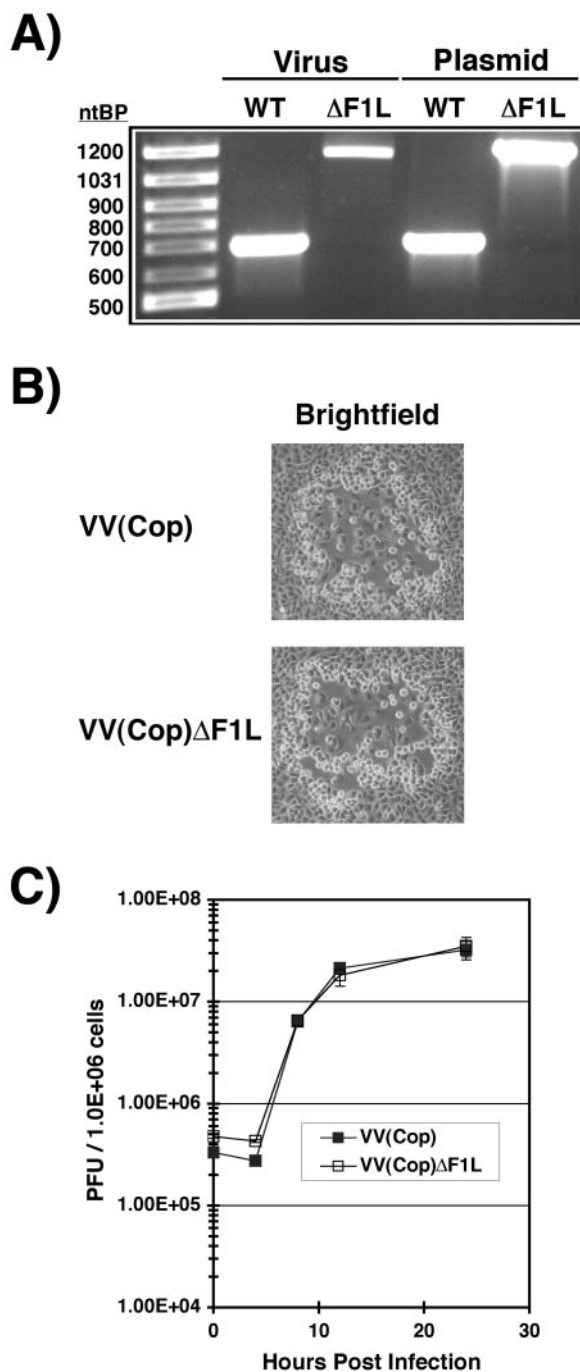


FIG. 1. Characterization of a recombinant VV devoid of F1L. (A) Agarose gel analysis of PCR products amplified from VV(Cop)- and VV(Cop) Δ F1L-infected cells compared to plasmid containing wild-type (WT) F1L and the plasmid used to generate VV(Cop) Δ F1L by homologous recombination. (B) Microscopic analysis of plaques generated from CV-1 cells infected with VV(Cop) and VV(Cop) Δ F1L. (C) Single-step growth analysis of VV(Cop) and VV(Cop) Δ F1L in CV-1 cells.

or VV(Cop) Δ F1L, and 5 hours postinfection, apoptosis was triggered by treating the cells with staurosporine. Apoptosis was measured by quantifying loss of the inner mitochondrial membrane potential by TMRE fluorescence, a hydrophobic

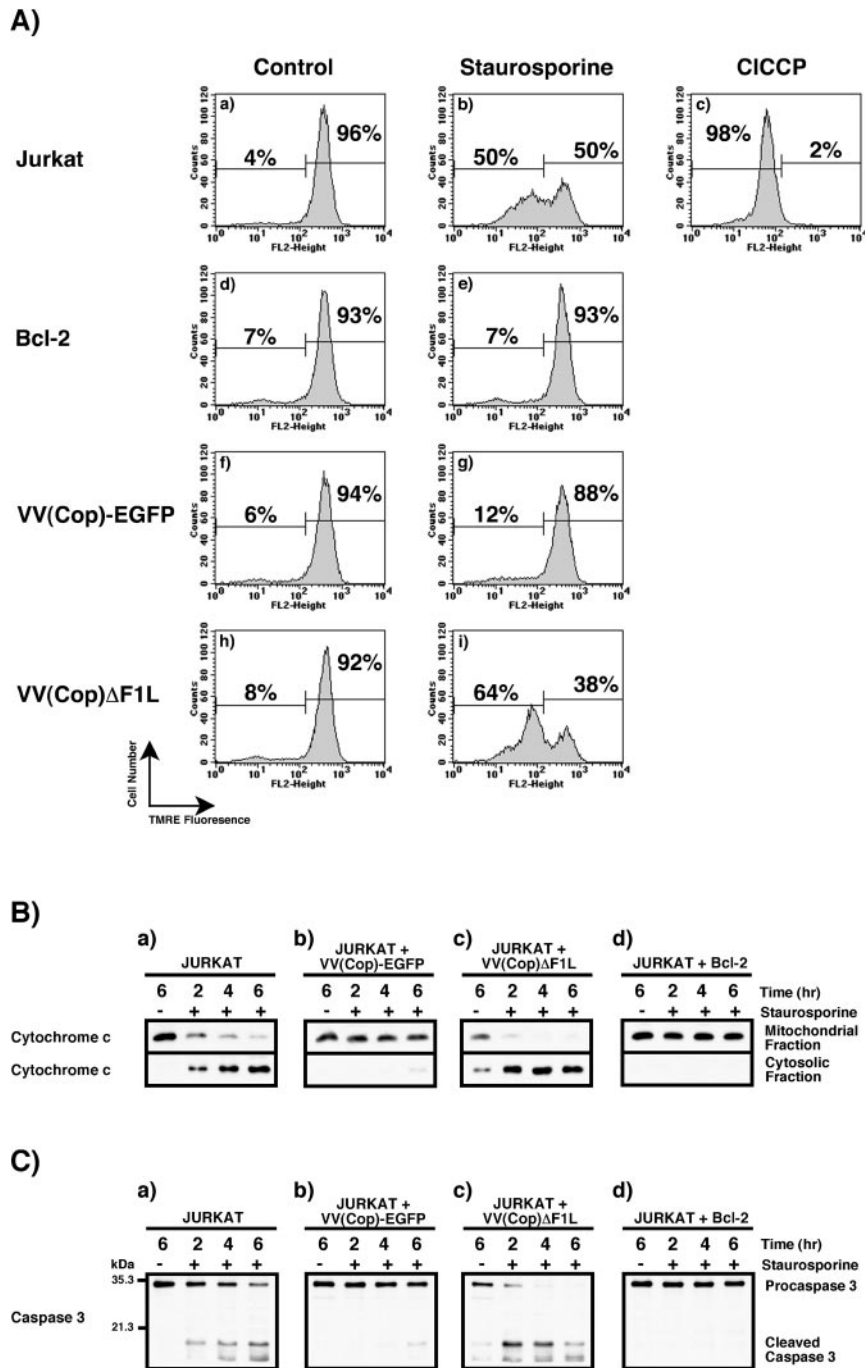


FIG. 2. VV(Cop) Δ F1L is unable to protect cells from staurosporine-induced apoptosis. (A) Jurkat cells and Jurkat cells overexpressing Bcl-2 were mock infected or infected with VV(Cop) or VV(Cop) Δ F1L at an MOI of 10 and treated with 500 nM staurosporine for 90 min to induce apoptosis. Apoptosis was assessed by TMRE fluorescence, which measures loss of the inner mitochondrial membrane potential. (B) Jurkat cells and Jurkat cells overexpressing Bcl-2 were either mock infected, infected with VV(Cop), or infected with VV(Cop) Δ F1L and treated with 500 nM staurosporine. Cytochrome *c* release was monitored by Western blot analysis. (C) Jurkat cells and Jurkat cells overexpressing Bcl-2 were either mock infected, infected with VV(Cop), or infected with VV(Cop) Δ F1L at an MOI of 10 and treated with 500 nM staurosporine. Caspase 3 activation was monitored by Western blot analysis.

cationic dye that is readily taken up by healthy respiring mitochondria (36). In the absence of staurosporine, all cell populations demonstrated high levels of TMRE fluorescence, indicative of healthy nonapoptotic cells (Fig. 2A, a, d, f, and h). Jurkat cells treated with the uncoupler carbonyl cyanide *m*-

chlorophenyl hydrazone showed a clear loss of the inner mitochondrial membrane potential (Fig. 2A, c), and treatment with staurosporine resulted in 50% of mock-infected cells losing their inner mitochondrial membrane potential (Fig. 2A, b). Jurkat cells overexpressing Bcl-2 and cells infected with

VV(Cop) showed clear inhibition of the loss of the inner mitochondrial membrane potential (Fig. 2A, e and g), while Jurkat cells infected with VV(Cop) Δ F1L no longer maintained the inner mitochondrial membrane potential, indicating that in the absence of F1L, vaccinia virus was unable to inhibit staurosporine-induced apoptosis (Fig. 2A, i).

Since infection with VV(Cop) Δ F1L was unable to inhibit loss of the inner mitochondrial membrane potential, we assessed the ability of VV(Cop) Δ F1L to inhibit both cytochrome *c* release and caspase 3 activation after treatment with staurosporine. To determine if VV(Cop) Δ F1L was also unable to inhibit the release of cytochrome *c*, Jurkat cells were infected, treated with staurosporine, and fractionated into mitochondrial and cytosolic fractions, and cytochrome *c* release was monitored by Western blotting. Mock-infected cells treated with staurosporine displayed a loss of cytochrome *c* from the mitochondrial fraction to the cytosolic fraction (Fig. 2B, a) which was completely blocked by overexpression of Bcl-2 and significantly impaired when cells were infected with VV(Cop) (Fig. 2B, b and d). Cells infected with VV(Cop) Δ F1L, however, were no longer able to inhibit cytochrome *c* release (Fig. 2B, c). Similarly, cells infected with VV(Cop) Δ F1L and treated with staurosporine were no longer able to inhibit the activation of caspase 3 (Fig. 2C, c). These results indicated that cells infected with VV(Cop) Δ F1L were incapable of inhibiting the mitochondrion-dependent apoptotic cascade, including the loss of cytochrome *c* and the loss of the inner membrane potential. Even in the absence of staurosporine, infection with VV(Cop) Δ F1L promoted the release of cytochrome *c* and caspase 3 activation (Fig. 2B, c, and C, c), indicating that F1L expression was essential during VV(Cop) infection to inhibit apoptosis induced during virus infection.

To determine if VV(Cop) Δ F1L infection initiated apoptosis, we infected Jurkat cells with either VV(Cop) or VV(Cop) Δ F1L for 15 h and assessed apoptosis by quantifying loss of the inner mitochondrial membrane potential by TMRE fluorescence (36). Mock-infected and VV(Cop)-infected Jurkat cells demonstrated 96% and 90% of the cells positive for TMRE fluorescence, respectively (Fig. 3A, a and b). In contrast, only 68% of the Jurkat cells infected with VV(Cop) Δ F1L demonstrated TMRE fluorescence, with 32% of the cells showing a dramatic decrease in TMRE fluorescence indicative of cells undergoing apoptosis (Fig. 3A, c). Infection of Bcl-2-overexpressing Jurkat cells completely inhibited VV(Cop) Δ F1L-induced apoptosis (Fig. 3A, f). To further assess the ability of VV(Cop) Δ F1L to initiate apoptosis in Jurkat cells, we monitored both cytochrome *c* release and caspase 3 activation. Jurkat cells infected with VV(Cop) showed no cytochrome *c* release or caspase 3 activation (Fig. 3B), whereas cells infected with VV(Cop) Δ F1L showed obvious release of mitochondrial cytochrome *c* and caspase 3 cleavage at 10 and 15 h postinfection (Fig. 3B). VV(Cop) Δ F1L-induced cytochrome *c* release occurred in the presence of the broad-spectrum caspase inhibitor zVAD.fmk, indicating that prior caspase activation was not a requirement for VV(Cop) Δ F1L-induced cytochrome *c* release (Fig. 3B). Additionally, the overexpression of Bcl-2 completely inhibited VV(Cop) Δ F1L-induced cytochrome *c* release and caspase 3 activation, indicating that Bcl-2 compensated for the lack of F1L to inhibit VV(Cop) Δ F1L-induced apoptosis (Fig. 3C).

Vaccinia virus F1L protein specifically interacts with Bak.

Our studies indicated that VV(Cop) inhibited apoptosis by regulating the mitochondrial checkpoint through the activity of the F1L open reading frame (47, 56, 57). F1L expression inhibits apoptosis by interfering with the release of cytochrome *c* and the loss of the inner mitochondrial membrane potential by an unknown mechanism (47, 57). Therefore, to determine the mechanism of action of F1L, we asked if F1L functioned by interacting with members of the Bcl-2 family, which tightly regulate the mitochondrial checkpoint in apoptotic cells (25, 44). Cell lysates from HeLa cells either mock infected or infected with a recombinant vaccinia virus expressing a Flag-tagged version of F1L, VV(WR)Flag-F1L, were applied to the anti-F1L immunoaffinity column. Flag-tagged F1L-interacting proteins were subsequently eluted, and potential F1L binding partners were monitored by Western blotting. Using this approach, F1L successfully eluted, as indicated by immunoblotting with an anti-Flag antibody (Fig. 4). Both mock-infected and vaccinia virus-infected lysates displayed similar expression levels of Bax, Bak, Bcl-2, Bcl-xL, and Mcl-1, whereas only the virus-infected cell lysate expressed the Flag-tagged version of F1L (Fig. 4). To determine if F1L interacted with members of the Bcl-2 family, the eluted fractions were probed with antibodies directed against various Bcl-2 family members. Under these conditions, the proapoptotic protein Bak consistently coeluted with the Flag-tagged version of F1L, while no interactions with Bax, Bcl-2, Bcl-xL, or Mcl-1 were detected (Fig. 4). These results were confirmed using an anti-Flag immunoaffinity column (data not shown).

To further confirm the interaction between F1L and Bak, we performed a series of coimmunoprecipitation assays. HEK 293T cells were cotransfected with plasmids expressing EGFP-tagged F1L and hemagglutinin (HA)-tagged Bak and lysed in 2% CHAPS, and immunocomplexes were precipitated with either anti-EGFP or anti-HA antibodies. Immunoprecipitation with anti-EGFP resulted in coimmunoprecipitation of EGFP-F1L and HA-Bak (Fig. 5A). Reciprocal immunoprecipitations performed with an anti-HA antibody also indicated that HA-Bak coprecipitated EGFP-F1L (Fig. 5A). To determine the ability of F1L to interact with endogenous Bak in the absence of infection, HeLa cells were transfected with plasmids expressing EGFP or EGFP-F1L and immunoprecipitated with an anti-EGFP antibody. Both EGFP and EGFP-F1L immunoprecipitated with the anti-EGFP antibody (Fig. 5B), and immunoblotting with an anti-Bak antibody indicated that endogenous Bak coprecipitated with F1L (Fig. 5B). To confirm that F1L was able to interact with endogenous levels of Bak during infection, HeLa cells were infected with VV(Cop) and simultaneously transfected with either pSC66 or pSC66-Flag-F1L, which places Flag-tagged F1L under the control of a poxvirus promoter. Western blot analysis indicated that Flag-F1L was expressed only in cells transfected with pSC66-Flag-F1L and infected (Fig. 5C), and antibodies directed against the Flag epitope effectively immunoprecipitated Flag-F1L and coimmunoprecipitated endogenous Bak (Fig. 5C). The interaction between Flag-F1L and Bak during virus infection was confirmed by performing reciprocal coimmunoprecipitations (Fig. 5C). To verify that F1L and Bak localized to the mitochondria during virus infection, HeLa cells were infected with VV(WR)Flag-F1L and the localization of Flag-F1L and en-

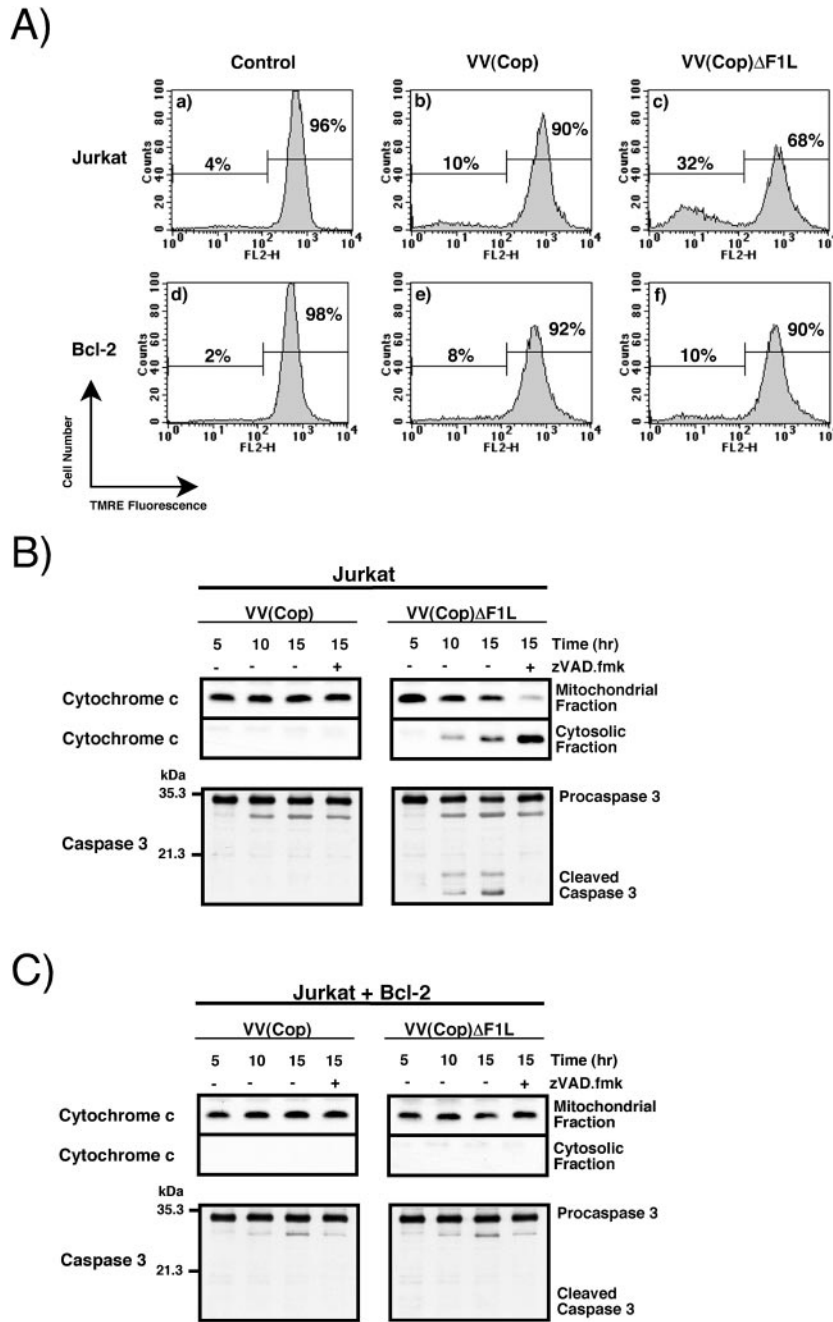


FIG. 3. VV(Cop)ΔF1L induces apoptosis in Jurkat cells. (A) Jurkat cells were infected with VV(Cop) or VV(Cop)ΔF1L at an MOI of 10 for 15 h, and apoptosis was assessed by TMRE fluorescence. (B) Jurkat cells were infected with VV(Cop) or VV(Cop)ΔF1L at an MOI of 10 for 5, 10, and 15 h. Cytochrome *c* release and caspase 3 activation were monitored by Western blot analysis. (C) Bcl-2 overexpression is sufficient to inhibit VV(Cop)ΔF1L-induced apoptosis. Jurkat cells were infected with VV(Cop) or VV(Cop)ΔF1L at an MOI of 10 for 5, 10, and 15 h in the presence and absence of zVAD.fmk. Cytochrome *c* release and caspase 3 activation were monitored by Western blot analysis.

dogenous Bak was monitored by confocal analysis. Using an antibody directed against the Flag epitope, cells infected with VV(WR)Flag-F1L demonstrated a punctate staining pattern (Fig. 6a), and a similar staining profile was detected for endogenous Bak (Fig. 6b). When the fluorescent signals generated from Flag-F1L and Bak were superimposed, colocalization was evident, demonstrating that F1L and Bak localized to the mitochondria during virus infection (Fig. 6c).

F1L inhibits staurosporine-induced Bak activation. The interaction of F1L with Bak suggested that F1L may function by interfering with the proapoptotic activity of Bak. Following an apoptotic stimulus, Bak undergoes a multistep activation process in which the N terminus becomes exposed, priming Bak for subsequent homo-oligomerization that results in the release of cytochrome *c* (24, 58). Therefore, we asked if infection with VV(Cop) affected Bak homo-oligomerization. Jurkat cells

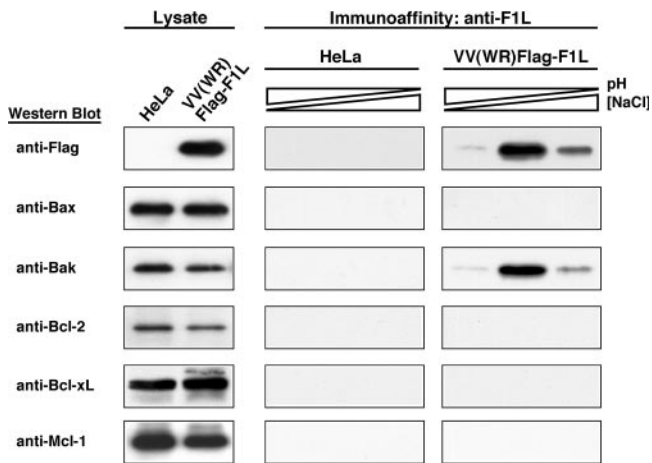


FIG. 4. F1L interacts with endogenous Bak but not other Bcl-2 family proteins. HeLa cell lysates from mock-infected cells or cells infected with VV(WR)Flag-F1L at an MOI of 10 were applied to an anti-F1L immunoaffinity column. Bound proteins were eluted by linear addition of elution buffer containing 100 mM glycine and 0.5 M NaCl, pH 2.7. The eluted fractions were monitored by Western blotting for Flag-F1L, Bax, Bak, Bcl-2, Bcl-xL, and Mcl-1.

were either mock infected or infected with VV(Cop)-EGFP or VV(Cop) Δ F1L. Following treatment with staurosporine, whole-cell lysates were solubilized in 2% CHAPS buffer and Bak oligomerization was analyzed by gel filtration chromatography, followed by immunoblotting with anti-Bak antibody. In the absence of staurosporine, Bak was detected in mock-infected cells as an inactive form ranging in size from approximately 35 kDa to 130 kDa (Fig. 7A). Treatment with staurosporine, however, resulted in loss of the lower-molecular-mass inactive form of Bak and the appearance of a higher-molecular-mass oligomeric form ranging from 200 kDa to 360 kDa (Fig. 7A). When cells were infected with VV(Cop)-EGFP and treated with staurosporine, Bak oligomerization was clearly inhibited, indicating that vaccinia virus infection interfered with Bak oligomerization (Fig. 7A). In contrast to VV(Cop)-EGFP infected cells, the formation of higher-molecular-mass Bak oligomers was not inhibited when cells were infected with VV(Cop) Δ F1L and treated with staurosporine, indicating a role for F1L in the inhibition of Bak oligomerization (Fig. 7A).

Bak oligomerization requires an initial conformational change, which is characterized by exposure of the N terminus (24, 43). To determine if vaccinia virus interfered with the initial conformational change in Bak, Jurkat cells were infected with VV(Cop)-EGFP or VV(Cop) Δ F1L and the activation of Bak was induced by the addition of staurosporine. The conformation of Bak was monitored using a conformation-specific anti-Bak antibody that recognizes the exposed N terminus, and activated Bak was detected by flow cytometry (24). As shown in Fig. 7B, all untreated cells displayed low levels of background fluorescence associated with nonspecific antibody staining. Jurkat cells treated with staurosporine and stained with anti-Bak resulted in a clear increase in fluorescence intensity, indicating an increase in epitope availability for the conformation-specific antibody (Fig. 7B, a). No increase in fluorescence intensity was detected in cells treated with staurosporine and stained with an isotype control antibody (Fig. 7B, d). As previously docu-

mented, Bcl-2 expression clearly inhibited the N-terminal exposure of Bak induced by staurosporine treatment (Fig. 7B, e) (24). To ensure we were measuring the N-terminal exposure of Bak, we performed similar experiments in Jurkat cells deficient in Bak and Bax (53). Staurosporine-treated Jurkat cells deficient in Bak and Bax also demonstrated a lack of antibody staining, clearly indicating that the assay was measuring Bak activation (Fig. 7B, h to j). In contrast to mock-infected Jurkat cells, infection with VV(Cop)-EGFP exhibited clear protection of the N-terminal exposure of Bak following staurosporine treatment (Fig. 7B, b), while infection with VV(Cop) Δ F1L and the subsequent addition of staurosporine resulted in the appearance of N-terminal Bak epitope, indicating that the presence of F1L was essential for vaccinia virus to inhibit staurosporine-induced N-terminal Bak (Fig. 7B, c). The overexpression of Bcl-2 inhibited activation of Bak in cells infected with VV(Cop) Δ F1L, indicating that Bcl-2 could functionally replace F1L (Fig. 7B, g).

F1L regulates tBid-induced Bak activation. Multiple apoptotic signals originating upstream of the mitochondria require Bak and Bax activation (25, 44, 59). A subclass of Bcl-2 family members, which contain only BH3 domains, such as Bid, initiate the activation of Bak and Bax (2, 58). Bid activation occurs through caspase 8 cleavage, resulting in the C-terminal portion of Bid (tBid) translocating to the mitochondria, initiating the homo-oligomerization of Bak and Bax and resulting in the release of cytochrome *c* (15, 33, 58). To determine the ability of F1L to modulate Bak activation induced by the BH3-only protein Bid, we utilized wild-type MEFs, MEFs deficient in Bax, and MEFs doubly deficient in Bak and Bax (59). Mitochondria purified from mock-infected wild-type MEFs and treated with increasing amounts of tBid resulted in the release of cytochrome *c* into the supernatant fraction (Fig. 8A, a), which was completely inhibited in mitochondria purified from MEFs deficient in both Bak and Bax, as previously documented (Fig. 8A, d) (34, 59). Purified mitochondria from VV(Cop)-infected wild-type MEFs and treated with tBid were protected from cytochrome *c* release (Fig. 8A, b), while mitochondria purified from wild-type MEFs infected with VV(Cop) Δ F1L were unable to inhibit cytochrome *c* release (Fig. 8A, c). In fact, when compared to mock-infected conditions, infection with VV(Cop) Δ F1L resulted in mitochondria that were more sensitive to tBid, as indicated by the requirement for smaller amounts of tBid to achieve cytochrome *c* release (Fig. 8A, c). These results indicated that VV(Cop) infection inhibited tBid-induced cytochrome *c* release mediated by Bak and Bax and that F1L was necessary for this inhibitory effect. Additionally, we infected Bax-deficient MEFs, which allowed us to exclude the proapoptotic function of Bax and focus specifically on Bak. VV(Cop) infection of MEFs deficient in Bax also provided protection from tBid-induced cytochrome *c* release (Fig. 8A, e), and the expression of F1L was necessary to inhibit Bak-induced release of cytochrome *c* (Fig. 8A, f).

To determine if F1L inhibited tBid-induced Bak homo-oligomerization, we monitored the appearance of higher-order Bak complexes. Mitochondria were isolated from MEFs deficient in Bax and were treated with tBid, and Bak oligomerization was monitored by chemical cross-linking with BMH and assessed by Western blotting. In the absence of BMH, Bak

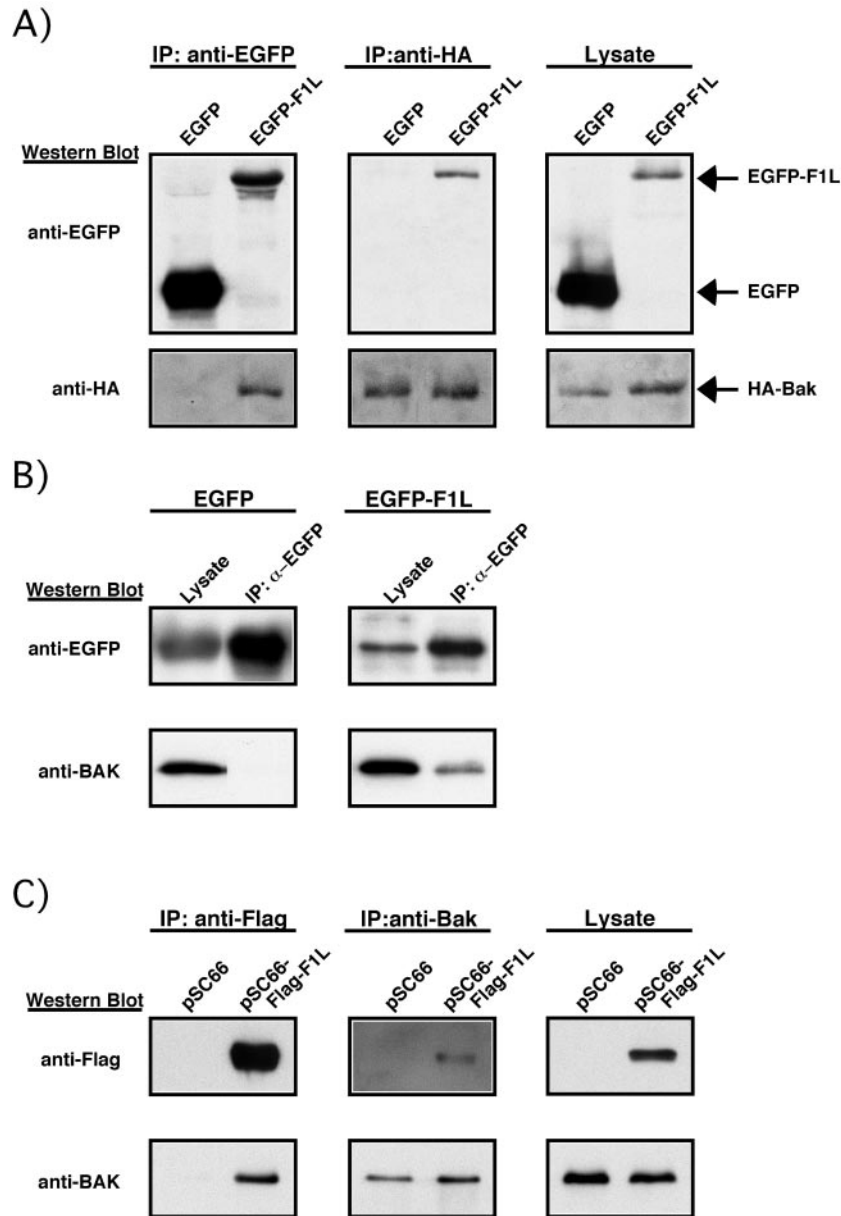


FIG. 5. F1L interacts with Bak in the presence and absence of VV(Cop) infection. (A) Ectopic expression of F1L and Bak demonstrates interaction between F1L and Bak. HEK 293T cells were cotransfected with either pEGFP or pEGFP-F1L in the presence of pcDNA-HA-Bak. EGFP-F1L interacts with HA-Bak. IP, immunoprecipitate. (B) F1L interacts with endogenous Bak. HeLa cells were transfected with pEGFP or pEGFP-F1L. EGFP-F1L, but not EGFP, interacts with endogenous Bak. (C) F1L associates with endogenous Bak during virus infection. HeLa cells were infected with VV(Cop) at an MOI of 10 and transfected with pSC66 or pSC66-Flag-F1L to express Flag-F1L during infection. F1L associates with endogenous Bak.

migrates at approximately 26 kDa (Fig. 8B, a); however, upon the addition of BMH, Bak displays a faster mobility as a result of intramolecular cross-linking (Fig. 8B) (43, 58). The addition of tBid to mitochondria isolated from Bax-deficient MEFs resulted in loss of the intramolecular cross-linked Bak species and the formation of Bak oligomers indicative of Bak activation (Fig. 8B, a) (43, 59). Mitochondria purified from Bax-deficient MEFs infected with VV(Cop) and treated with tBid showed retention of the intramolecular cross-linked species of Bak and the absence of Bak homo-oligomers (Fig. 8B, b). In contrast, Bak oligomerization and loss of the intramolecularly

cross-linked Bak species were detected in mitochondria isolated from VV(Cop) Δ F1L-infected Bax-deficient MEFs and treated with tBid, indicating that F1L was necessary to inhibit tBid-induced Bak oligomerization (Fig. 8B, c). Moreover, infection with VV(Cop) Δ F1L appeared to augment the efficacy of tBid to promote homo-oligomerization of Bak (Fig. 8B, c).

To determine if F1L was capable of inhibiting the initial tBid-induced activation of Bak, Bak conformation was assessed by limited proteolysis. In an inactive state, the N terminus of Bak is inaccessible to trypsin proteolysis; however, following an apoptotic trigger, the N terminus of Bak becomes

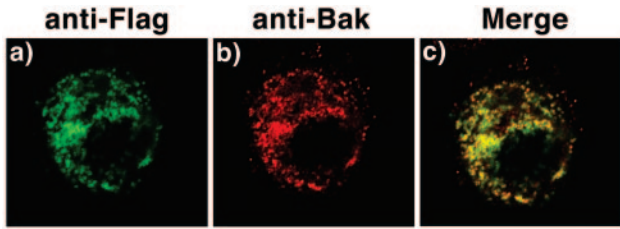


FIG. 6. F1L and endogenous Bak localize at the mitochondria during vaccinia virus infection. HeLa cells were infected with VV(WR)Flag-F1L at an MOI of 5 for 8 h. The localization of Flag-F1L was visualized with a fluorescein isothiocyanate-conjugated mouse anti-Flag antibody (a and c). Endogenous Bak was detected using an anti-Bak (G23) antibody, followed by detection with the Alexa-Fluor 546-conjugated goat anti-rabbit antibody (b and c).

exposed and susceptible to trypsin-mediated proteolysis (43, 58). As such, Bak conformational changes can be monitored as a loss of antibody reactivity during Western blot analysis using an antibody specific for the N terminus of Bak (43, 58). In the absence of trypsin, all mitochondria displayed equal levels of Bak as measured by Western blot analysis (Fig. 8C). Following treatment with trypsin, mitochondria isolated from mock-infected Bax-deficient MEFs and incubated with tBid resulted in an increase in susceptibility of Bak to proteolysis, as detected by the appearance of a lower-molecular-weight form of Bak and the eventual loss of antibody reactivity with increasing amounts of tBid (Fig. 8C). Western blot analysis of the mitochondrial matrix protein Mn SOD served as a loading control (Fig. 7C). Mitochondria isolated from VV(Cop)-infected

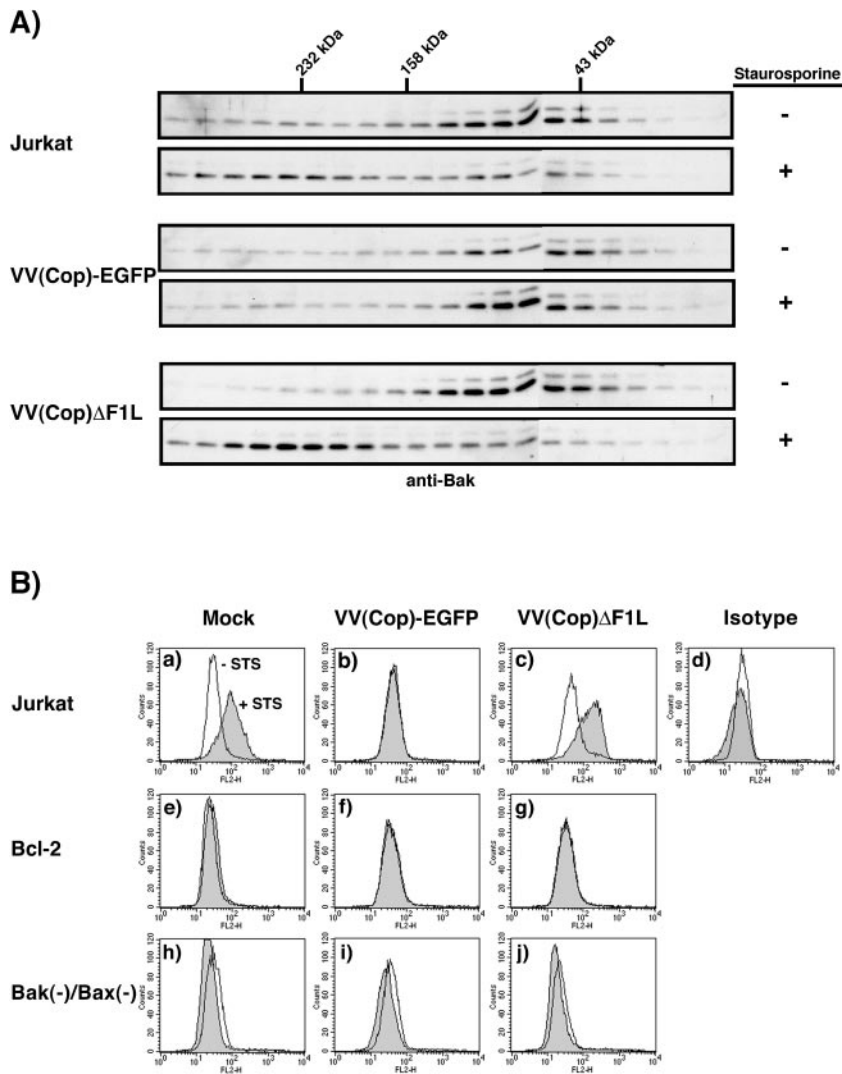


FIG. 7. F1L expression inhibits staurosporine-induced Bak activation. (A) Bak oligomerization is inhibited by VV(Cop)-EGFP infection but not infection with VV(Cop) Δ F1L. Jurkat cells were infected with VV(Cop)-EGFP or VV(Cop) Δ F1L at an MOI of 10, and 5 h postinfection, they were treated with 1 μ M staurosporine for 2 h to induce apoptosis. Bak oligomerization was assessed by gel filtration analysis and detected by Western blotting. (B) F1L expression is necessary for VV(Cop)-EGFP to inhibit the N-terminal exposure of Bak. Jurkat cells overexpressing Bcl-2, or Jurkat cells devoid of Bak and Bax were infected with VV(Cop)-EGFP or VV(Cop) Δ F1L at an MOI of 10 and treated with 250 nM staurosporine for 2 h to induce apoptosis. Exposure of the N terminus of Bak was monitored by flow cytometry using a conformation-specific N-terminal anti-Bak antibody or an isotype control antibody. Untreated cells, open histogram; staurosporine-treated cells, shaded histogram.

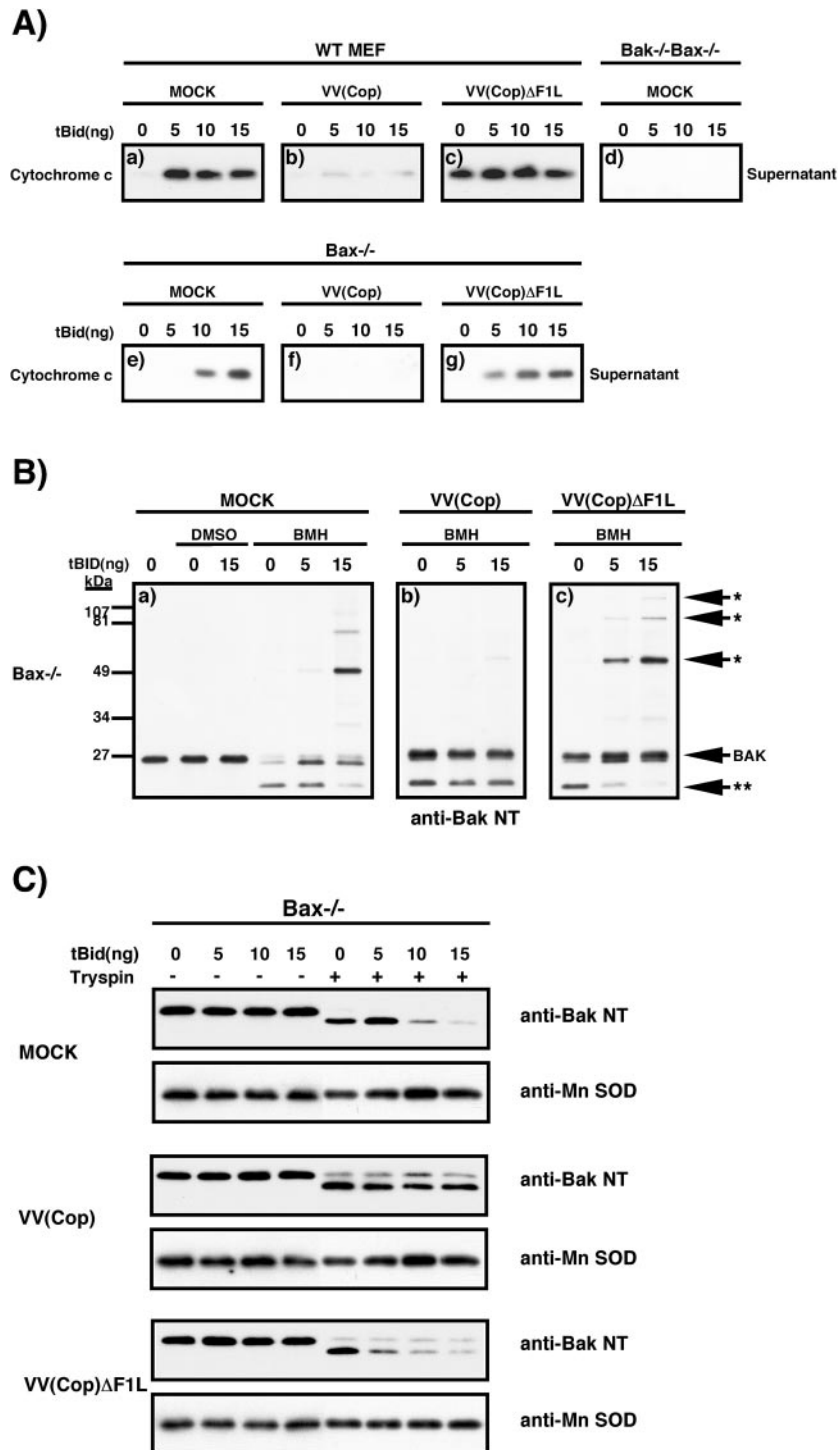


FIG. 8. F1L inhibits tBid-induced Bak activation. (A) F1L expression inhibits release of cytochrome *c* initiated by tBid. Mitochondria were isolated from wild-type (WT) MEFs, Bax-deficient (Bax^{-/-}) MEFs, and Bak- and Bax-deficient (Bak^{-/-}Bax^{-/-}) MEFs that had previously been infected with VV(Cop) or VV(Cop) Δ F1L at an MOI of 20. The mitochondria were treated with increasing amounts of tBid and assessed for cytochrome *c* release by Western blotting. (B) F1L expression is necessary to inhibit Bak oligomerization initiated by tBid. Mitochondria from Bax-deficient MEFs were treated with tBid and cross-linked with BMH. VV(Cop)EGFP infection, but not VV(Cop) Δ F1L infection, inhibits Bak oligomerization. *, Bak homo-oligomers; **, monomeric intramolecularly cross-linked Bak species. (C) F1L expression is necessary to inhibit the N-terminal exposure of Bak induced by increasing amounts of tBid. After exposure to tBid, isolated mitochondria were treated with trypsin and Bak conformation was monitored by Western blotting. As a control, the presence of Mn SOD was also monitored by Western blotting.

MEFs deficient in Bax and treated with tBid showed clear retention of trypsin-resistant Bak (Fig. 8C). However, Bak sensitivity to proteolysis was observed in mitochondria isolated from Bax-deficient MEFs infected with VV(Cop) Δ F1L (Fig. 8C), indicating that VV(Cop) infection inhibited the conformational change of Bak induced by tBid and that F1L was essential for this inhibition.

DISCUSSION

To ensure successful propagation, viruses must overcome the host innate and acquired immune responses (50). Members of the family *Poxviridae*, including vaccinia virus, the prototypic member of the family, encode numerous immunomodulatory proteins to counteract host antiviral strategies, including apoptosis (45, 46). Apoptosis is a characteristic form of cellular suicide that can be initiated by a wide variety of stimuli, resulting in the ultimate destruction of the cell (27). Members of the poxvirus family have evolved a wide range of strategies to interfere with apoptosis in order to ensure efficient virus propagation and dissemination (4, 18). We recently identified F1L as an additional antiapoptotic protein encoded by vaccinia virus (47, 57). F1L is a tail-anchored protein that localizes to the outer mitochondrial membrane and inhibits the release of cytochrome *c* and loss of the inner mitochondrial membrane potential by an unknown mechanism (47, 57). We now provide evidence that F1L expression inhibits the activation of Bak, a proapoptotic member of the Bcl-2 family.

To further understand the antiapoptotic mechanism of the F1L protein, we set out to identify potential cellular partners for F1L. We focused on the members of the Bcl-2 family, which tightly regulate the apoptotic cascade at the mitochondria. Using an anti-F1L affinity column, we found that F1L interacted with the proapoptotic Bcl-2 family member Bak but not with the proapoptotic protein Bax or with the antiapoptotic proteins Bcl-2, Bcl-xL, and Mcl-1. The interaction between F1L and Bak was confirmed by coimmunoprecipitation, which clearly demonstrated that F1L interacted with endogenous Bak in the absence and presence of infection. Bak is a proapoptotic member of the Bcl-2 family that constitutively localizes to the outer mitochondrial membrane, playing a pivotal role in cytochrome *c* release from mitochondria (34, 58, 59).

During virus infection, F1L interacts constitutively with Bak, and the expression of F1L is essential to inhibit the release of cytochrome *c*, suggesting that F1L may function by interfering with the proapoptotic function of Bak. The activation of the multidomain proapoptotic proteins Bak and Bax constitutes a critical step in the release of cytochrome *c* that is antagonized by antiapoptotic Bcl-2 family members, as well as virus-encoded proteins (25, 44, 59). In response to an apoptotic stimulus, Bak undergoes a conformational change, exposing a new epitope, followed by Bak homo-oligomerization (24, 58). Using gel filtration analysis, we found that vaccinia virus infection potently prevented Bak homo-oligomerization induced by staurosporine and that F1L was essential for this inhibition. Flow cytometric data using a conformation-specific Bak antibody also indicated that F1L blocked staurosporine-induced N-terminal exposure of Bak.

Bak and Bax activation is regulated by a subset of Bcl-2 family members referred to as BH3-only proteins, which are

activated by posttranslational modification, transcriptional up-regulation, or caspase activation (48). Caspase 8 cleavage of the BH3-only protein Bid generates a C-terminal fragment referred to as tBid that can directly activate Bak and Bax (31, 43, 58). We used recombinant tBid to test the ability of vaccinia virus and F1L to regulate the direct activation of Bak and Bax by tBid. Our data indicated that vaccinia virus infection inhibited tBid-induced cytochrome *c* release from mitochondria purified from both wild-type MEFs and Bax-deficient MEFs. The expression of F1L was absolutely essential to inhibit tBid-induced cytochrome *c* release from both cell lines, suggesting that F1L could inhibit direct activation of Bak and Bax by tBid. Using cross-linking and limited tryptic digests, we found that F1L expression during infection prevented tBid-induced Bak oligomerization and the conformational change in Bak.

Several potential mechanisms can be envisioned for F1L inhibition of Bak. The simplest explanation would be that F1L directly interacts with Bak, preventing its activation. A similar role for VDAC2 and Mcl-1 has been described in which both cellular proteins sequester Bak in an inactive conformation (9, 12). Alternatively, F1L could augment the activity of VDAC2 and Mcl-1 to keep Bak in an inactive state (9, 12). Following an apoptosis stimulus, Mcl-1 is released from Bak, allowing the activation of Bak and the subsequent destruction of Mcl-1 by the 26S proteasome (12, 32, 37). We were unable to detect an interaction between F1L and Mcl-1 (Fig. 4), suggesting that in the presence of F1L, Mcl-1 no longer interacts with Bak, resulting in the degradation of Mcl-1 following vaccinia virus infection, a possibility we are pursuing. Additionally, our data clearly demonstrated that F1L inhibits apoptosis in cells that express both Bak and Bax, suggesting that F1L may inhibit Bax activation. Bak and Bax activation is initiated either directly or indirectly by BH3-only proteins, such as Bid, and it is well established that antiapoptotic Bcl-2 proteins sequester BH3-only proteins to disrupt Bak and Bax activation (25, 44). Therefore, F1L may function by sequestering and inhibiting the activity of BH3-only proteins in a fashion similar to that proposed for Bcl-2 (8, 10, 20, 30, 48). Through this mechanism, F1L expression would ultimately inhibit activation of both Bak and Bax.

The regulation of Bak and Bax is a common theme employed by viruses. Many viruses encode obvious Bcl-2 homologues that function by inhibiting Bak and Bax activation (11, 26, 40). E1B19K, encoded by adenovirus, interacts with both Bak and Bax following N-terminal exposure of both proteins (11, 49). VMIA, encoded by HCMV, paradoxically recruits Bax to mitochondria and freezes the homo-oligomeric configuration of Bax (3, 41). Recently, M11L, encoded by myxoma virus, another member of the poxvirus family, was found to constitutively interact with Bak and prevent apoptosis by an undefined mechanism (52). Notably, F1L open reading frames are present only in members of the genus *Orthopoxvirus*, while members of the genera *Leporipoxvirus*, *Capripoxvirus*, *Suipoxvirus*, and *Yatapoxvirus* encode M11L, which functions to inhibit apoptosis at the mitochondria. In contrast, the avipoxviruses are to date the only members of the poxvirus family to encode obvious Bcl-2 family members (1, 51). Although M11L and F1L display no obvious sequence homology, both localize to the mitochondria and inhibit the release of cytochrome *c*. Data generated in our laboratory clearly show that the Bcl-2

homologue in fowlpox virus also functions at the mitochondria to inhibit apoptosis (L. Banadyga and M. Barry, unpublished data). As such, members of the poxvirus family have evolved distinct proteins that are functionally conserved to inhibit apoptosis. The modulation of Bak and Bax during poxvirus infection reflects the important role of the mitochondria in the elimination of poxvirus-infected cells. In support of this, we found that vaccinia virus lacking F1L induced an intrinsic apoptotic cascade that could be inhibited by overexpression of Bcl-2. A similar observation was recently made using modified vaccinia virus Ankara missing F1L (20). Vaccinia virus-induced apoptosis correlated with Bak activation and oligomerization and the release of cytochrome *c*. The release of cytochrome *c* induced by virus infection was caspase independent, suggesting the involvement of caspase-independent BH3-only proteins as initiators of the apoptotic cascade. Although we did not observe a growth defect upon infection of CV-1 cells infected with vaccinia virus missing F1L, regulation of the mitochondrial checkpoint to ensure successful viral replication has been observed with other viruses. For example, adenovirus relies on the expression of E1B 19K to inhibit apoptosis by regulating Bak and Bax (11). Similar results have been reported for HCMV. In the absence of vMIA expression, HCMV induces apoptosis that results in inefficient viral replication (42). Additionally, myxoma virus with M11L deleted induces apoptosis in primary monocytes and rabbit lymphocytes (RL-5 cells) (16, 35). A growth defect for the M11L-deficient virus was noted in RL-5 cells and spleen cells but not in rabbit SIRC cells (35, 39). The lack of a restricted-growth phenotype in CV-1 cells following infection with VV(Cop) Δ F1L suggested that the F1L-deficient virus was unable to induce apoptosis in these cells. In support of this, infection of CV-1 cells and BGMK cells with VV(Cop) Δ F1L resulted in limited amounts of apoptosis at 24 h postinfection, as measured by terminal deoxynucleotidyl-transferase-mediated dUTP-biotin nick end labeling assay (data not shown). We speculate that apoptosis induced by the F1L deletion virus may evoke a growth defect in cell lines other than CV-1 and BGMK cells or in primary cells, as documented for the antiapoptotic protein M11L (35, 39).

Modulating the mitochondria to control apoptosis is a general strategy employed by viruses. The observation that F1L interacts with Bak and regulates Bak function highlights the importance of Bak in regulating apoptosis and the necessity to maintain a suitable cellular environment during virus infection. Understanding the complexities of the interaction between Bak and F1L will provide important clues regarding the mechanism of cytochrome *c* release and mitochondrial permeabilization.

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