Vaccinia virus, the prototypic member of the orthopoxvirus genus, encodes the mitochondrial-localized protein F1L that functions to protect cells from apoptotic death and inhibits cytochrome c release. We previously showed that F1L interacts with the pro-apoptotic Bcl-2 family member Bak and inhibits activation of Bak following an apoptotic stimulus (Wasilenko, S. T., Banadyga, L., Bond, D., and Barry, M. (2005) J. Virol. 79, 14031–14043). In addition to Bak, the pro-apoptotic protein Bax is also capable of initiating cytochrome c release suggesting that vaccinia virus infection could also inhibit Bax activity. Here we show that F1L inhibits the activity of the pro-apoptotic protein Bax by inhibiting oligomerization and N-terminal activation of Bax. F1L expression also induced the subcellular redistribution of Bax to the mitochondria and the insertion of Bax into the outer mitochondrial membrane. The ability of F1L to inhibit Bax activation does not require Bak, because F1L expression inhibited cytochrome c release and Bax activation in Bak-deficient cells. No interaction between Bax and F1L was detected during infection, suggesting that F1L functions upstream of Bax activation. Notably, F1L was capable of interacting with the BH3-only protein BimL as shown by co-immunoprecipitation, and F1L expression inhibited apoptosis induced by BimL.

These studies suggest that, in addition to interacting with the pro-apoptotic protein Bak, F1L also functions to indirectly inhibit the activation of Bax, likely by interfering with the pro-apoptotic activity of BH3-only proteins such as BimL.

Apoptosis is a tightly controlled process that plays a critical role in development, tissue homeostasis, and the recognition and removal of virus-infected cells (1). A family of intracellular cysteine proteinases, known as caspases, largely mediates apoptosis and is activated via either the intrinsic or extrinsic pathway. The extrinsic pathway is primarily initiated via death receptor activation, whereas the intrinsic pathway is initiated from within the cell and specifically requires the involvement of the mitochondria (1–3). Mitochondria act as a pivotal regulator within cells, serving to both amplify and initiate apoptosis. Induction of apoptosis leads to the loss of the mitochondrial inner membrane potential and permeabilization of the outer mitochondrial membrane culminating in the release of a number of pro-apoptotic factors, including cytochrome c, SMAC/DIABLO, and Omi/Htr2A, which serve to promote apoptotic death (2, 3).

The mitochondrial events during apoptosis are tightly coordinated by the Bcl-2 family of proteins, all of which contain at least one Bcl-2 homology (BH) domain and function to either inhibit or promote apoptosis (4). The Bcl-2 family includes anti-apoptotic members, such as Bcl-2 and Bcl-xL, and a large group of pro-apoptotic family members. The pro-apoptotic members of the Bcl-2 family are further subdivided into the multidomain pro-apoptotic proteins, which include Bak and Bax, and the BH3-only proteins, which trigger the activation of Bak and Bax. Cells deficient in both Bak and Bax are completely resistant to cytochrome c release, demonstrating the collective importance of these two pro-apoptotic proteins (5, 6). As such, the activation of Bak and Bak must be tightly regulated to suppress death (5, 6). Bak constitutively localizes to the mitochondria where it is held in an inactive state by voltage-dependent anion channel-2 and the anti-apoptotic Bcl-2 family member Mcl-1 (7, 8). Following an apoptotic stimulus, Bak undergoes a conformational change revealing an N-terminal epitope that ultimately results in the formation of high molecular weight oligomers of Bak (9–11). In contrast, Bax is normally found in the cytoplasm or loosely associated with intracellular membranes, and apoptotic stimuli induce a conformational change in Bax that permits insertion of Bax into the outer mitochondrial membrane (12–15). Similar to Bak, this conformational change in Bax reveals an N-terminal epitope, and Bax inserts into the outer mitochondrial membrane as high molecular weight oligomers, facilitating the release of cytochrome c (16).

Current theories suggest that Bax and Bak induce apoptosis and cytochrome c release by either facilitating the formation of a pore in the outer mitochondrial membrane or by modulating pre-existing pores (17, 18).

The BH3-only proteins (Bid, Bim, Bad, Bik, Noxa, Puma, Bmf, and Hrk) are essential death sensors that respond to pro-

The Vaccinia Virus Protein F1L Interacts with Bim and Inhibits Activation of the Pro-apoptotic Protein Bax*

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apoptotic signals and ultimately activate Bak and Bax (19, 20). Following an apoptotic stimulus, BH3-only proteins are activated through transcriptional and post-translational mechanisms and exert their pro-apoptotic effects by either directly activating Bak and Bax, or by binding and inhibiting the anti-apoptotic Bcl-2 family members (19, 20). BH3-only proteins have therefore been referred to as either “direct activators” of Bak and Bax or as “death sensitizers” (21–26). Bid and Bim are believed to directly activate Bak and Bax, whereas the remaining BH3-only proteins bind and inhibit the anti-apoptotic Bcl-2 family members, thereby allowing Bak and Bax activation (21–26).

To counteract apoptosis and prolong virus survival, a large number of viruses encode anti-apoptotic proteins, many of which maintain mitochondrial integrity by specifically inhibiting the activation of Bak and Bax (27–29). Although a number of viruses encode obvious Bcl-2 homologues, a subset of viral anti-apoptotic proteins that function at the mitochondria, but lack obvious homology to Bcl-2, have recently been identified (27–29). Included among these are vMIA, encoded by human cytomegalovirus, and M11L, encoded by myxoma virus (30–35). We recently identified an additional novel anti-apoptotic protein, F1L, encoded by vaccinia virus (36–38). F1L possesses a C-terminal hydrophobic transmembrane anchor that is necessary and sufficient for mitochondrial localization (38). Despite lacking obvious homology to anti-apoptotic members of the Bcl-2 family, we have shown that F1L constitutively interacts with the pro-apoptotic Bcl-2 family member Bak and inhibits the activation of Bak following an apoptotic stimulus (36). Here we report that, in addition to inhibiting Bak, F1L is also able to inhibit Bax activation. F1L expression prevented activation, oligomerization, and insertion of Bax in the mitochondrial outer membrane. However, no interaction between F1L and Bax was detected, suggesting that F1L exerts its anti-apoptotic function upstream of Bax activation. Significantly, F1L efficiently inhibited apoptosis induced by the long form of the BH3-only protein Bim (BimL) and interacted with BimL. These results suggest that F1L can indirectly inhibit Bax activation via the interaction with BH3-only proteins, which are essential for transducing pro-apoptotic signals through Bak and Bax.

**EXPERIMENTAL PROCEDURES**

*Cells and Viruses—*HeLa, HEK293T, and Bak−/−, Bim−/−, Bak−/−/Bax−−/− baby mouse kidney (BMK) cells (kindly provided by E. White, Rutgers University, Piscataway, NJ) were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 2 mM l-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin (39). Bak−/− and Bak−/−/Bax−−/− mouse embryonic fibroblasts (MEFs) were a generous gift from S. Korsmeyer (Harvard Medical School, Boston, MA) and were cultured in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, 2 mM l-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, plus 100 μM minimal essential medium non-essential amino acids (Invitrogen). HeLa cells stably expressing Bcl-2 were generated as described (40). Recombinant vaccinia virus strain Copenhagen expressing enhanced green fluorescent protein (EGFP), VV(Cop)EGFP, was kindly provided by G. McFadden (Robarts Research Institute, London, Ontario, Canada).

*Virus Production—*Virus expressing FLAG-F1L was generated by insertion of EGFP into the F1L open reading frame as previously described (36). A recombinant vaccinia virus expressing FLAG-F1L was generated as described (38). Cells were infected at an m.o.i. of 10 pfu/cell for the indicated times. To induce apoptosis, cells were either subjected to 200 mJ/cm2 of UV-C, 10 ng/ml TNF-α (Roche Diagnostics) and 5 μg/ml cycloheximide, or 1 μM staurosporine (Sigma-Aldrich).

*Transfections—*pEGFP-F1L, pEGFP-F1L-(206–226), and pEGFP-Bcl-2 were generated as previously described (37, 38). pcDNA3-FLAG-BimL was kindly provided by R. Davis (University of Massachusetts Medical School, Boston, MA). HeLa cells were transfected with either 1 or 2 μg of plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s specifications. For co-immunoprecipitations, cells were transfected with 1 μg of pcDNA3-FLAG-BimL in combination with 2 μg of pEGFPc3 (Clontech), pEGFP-F1L, pEGFP-F1L-(206–226), or pEGFP-Bcl-2 for 16 h.

*Confocal Microscopy—*HeLa cells were fixed with 4% paraformaldehyde, permeabilized with 0.04% saponin, and blocked with 30% normal goat serum (Invitrogen). Bak activation was detected by staining with anti-Bax (6A7) (BD Biosciences) at 1:500 (12, 41), and Bak subcellular redistribution was detected by staining with rabbit polyclonal anti-Bax (BD Biosciences) at 1:200. Bim localization was detected using anti-Bim (Calbiochem) at 1:500. Primary antibodies were detected using anti-rabbit Alexa546 or anti-mouse Alexa546 (Molecular Probes) at a 1:300 dilution and analyzed using laser scanning microscopy. Data were quantified by counting 200 cells per experiment. The means ± S.D. from three replicate experiments are shown.

*Measurement of Mitochondrial Membrane Potential—*Alterations in the mitochondrial membrane potential were detected by staining cells with tetramethylrhodamine ethyl ester (TMRE, Molecular Probes) (42). Cells were stained with 0.2 μM TMRE, and fluorescence was detected with a BD Biosciences FACScan through the FL-2 channel. Loss of the mitochondrial membrane potential in EGFP-positive cells was measured as a decrease in TMRE fluorescence by two-color flow cytometry as described (38). Data were acquired on 20,000 cells per sample and analyzed with CellQuest software. Loss of the inner mitochondrial membrane potential was calculated as (number of EGFP+ TMRE− cells/total number of EGFP+ cells) × 100, and data are represented as the mean ± S.D. from three replicate experiments.

*Gel-filtration Chromatography—*Gel-filtration chromatography was performed as described previously (36). Briefly, HeLa cells (1 × 107) were either mock infected or infected with either VV(Cop)EGFP or VV(Cop)ΔF1L at an m.o.i. of 10 for 8 h, subjected to UV light, and allowed to recover for 5 h. Cells were lysed in CHAPS lysis buffer containing 2% CHAPS, 137 mM NaCl, 0.2 mM dithiothreitol, 20 mM Tris (pH 7.4), and disrupted by passage through a 22-gauge needle. Lysates were centrifuged for 15 min at 18,000 × g, and supernatants were loaded at a flow rate of 0.1 ml/min on a Superose6 HR (10/30) column (GE Healthcare) equilibrated in 1% CHAPS, 137 mM NaCl, 0.2 mM dithiothreitol, 20 mM Tris (pH 7.4). The column was calibrated
using thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), and ovalbumin (43 kDa). Fractions of 150 μl were collected, and aliquots were analyzed by Western blotting with anti-Bax(N20) (BD Biosciences).

Mitochondrial Isolation—Mitochondria were isolated as described (36). HeLa cells (1 × 10^7) or Bak−/− MEFs (1 × 10^7) were infected at an m.o.i. of 10 with either VV(Cop)EGFP or VV(Cop)ΔF1L for 8 h. Cells were washed with 0.1M Na2CO3 (pH 12) for 20 min on ice, and the supernatant containing the mitochondria was centrifuged at 10,000 × g for 15 min at 4 °C to isolate mitochondria. To remove loosely associated proteins, mitochondrial pellets were washed with 0.1M Na2CO3 (pH 12) for 20 min on ice, and spun at 100,000 × g for 35 min at 4 °C. Mitochondria were resuspended in hypotonic lysis buffer containing 2% CHAPS, and protein concentrations were determined using a standard BCA test (Pierce). To induce cytochrome c release in purified mitochondria, increasing amounts of recombinant tBid (R&D Biosystems) were added to 50 μg of mitochondria and incubated for 45 min at 30 °C. Mitochondria were harvested, lysed in 2% CHAPS lysis buffer, and activated Bax was immunoprecipitated with anti-Bax(6A7).

Detection of Apoptosis in Baby Mouse Kidney Cells—Wild-type and Bim−/− BMK cells were infected with VV(Cop) or VVΔF1L in the absence and presence of 100 μM z-VAD-fmk. Apoptosis was determined by counting the number of adherent cells. The percentage of adherent cells (% cell survival) was normalized to the number of adherent cells following infection with VV(Cop) (±S.D.). Standard deviations were calculated from three independent experiments. To detect poly-ADP-ribose polymerase (PARP) cleavage, floating and adherent cells were harvested, lysed in SDS-PAGE sample buffer containing 8 M urea, and analyzed by Western blotting with anti-PARP (1:1000, BD Pharmingen). PARP cleavage products were quantified using ImageQuant™ ML (GE Healthcare).

Immunoprecipitations and Western Blotting—To immunoprecipitate activated Bax, cells were lysed in 2% CHAPS lysis buffer (2% CHAPS, 137 mM NaCl, 20 mM Tris, pH 7.4, Complete mini proteasome inhibitor (Roche Diagnostics)) for 1 h at 4 °C, and soluble protein fractions were incubated with 1 μg of anti-Bax(6A7) (BD Pharmingen) overnight at 4 °C. Immune complexes were isolated with protein A-Sepharose (GE Healthcare) for 2 h at 4 °C, washed, and resuspended in SDS-PAGE sample buffer. FLAG immunoprecipitations were carried out in either 2% CHAPS or 1% Triton X-100 using anti-FLAG (M2) (Sigma-Aldrich). Endogenous Bim immunoprecipitations were carried out in 2% CHAPS lysis buffer using anti-Bim (Calbiochem). Immune complexes were isolated with protein A beads (GE Healthcare) and resuspended in SDS-PAGE sample buffer. EGFP immunoprecipitations were performed as previously described using goat-anti-EGFP (L. Berthiaume, University of Alberta, Edmonton, Canada) (36), and complexes were purified with protein G beads (GE Healthcare). Protein samples were separated by either 8% or 15% SDS-PAGE and transferred to either nitrocellulose or polyvinylidene fluoride membrane (GE Healthcare). Antibodies used were as follows: anti-Bax(N20) (BD Biosciences), anti-Bak NT (Upstate Biotechnology Inc.), anti-cytochrome c (clone 7H8.2C12, BD Biosciences), anti-manganese superoxide dismutase (clone 110, Stressgen Bioreagents), anti-Bim (Calbiochem), anti-FLAG M2 (Sigma-Aldrich), and anti-EGFP (Covance). Proteins were visualized with enhanced chemiluminescence as per the manufacturer’s instructions (GE Healthcare).
transient expression of F1L in the absence of virus infection also inhibited Bax activation, because HeLa cells expressing an EGFP-tagged version of F1L demonstrated inhibition of UV-induced Bax activation as measured by anti-Bax(6A7) positivity (Fig. 3A, panels a–c). In contrast, HeLa cells transiently transfected with EGFP-F1L-(206–226), a truncated version of F1L containing only the mitochondrial localization signal of F1L that fails to inhibit apoptosis (38), displayed significant UV-induced anti-Bax(6A7) reactivity (Fig. 3A, panels d–f). Furthermore, EGFP-F1L-(206–226), which localizes to the mitochondria (38), co-localized with activated Bax, indicating that the activated Bax was located at the mitochondria (Fig. 3A, panels d–f). Results were quantified as shown in Fig. 3B. These results indicate that F1L expression inhibits the conformational change in Bax initiated by either virus infection or extrinsic stimuli.

**F1L Inhibits Bax Oligomerization**—Following an apoptotic trigger, Bax oligomerizes into high molecular weight complexes to facilitate cytochrome c release (16, 46). Because F1L expression inhibited Bax N-terminal activation, we investigated the ability of F1L to also inhibit the oligomerization of Bax. HeLa cells were either mock infected or infected with wild-type VV(Cop) or VV(Cop)ΔF1L. Following treatment with UV light, cells were lysed in 2% CHAPS, a detergent that has been shown to maintain the conformation and oligomerization state of Bax (41, 47), and protein complexes were separated based on size by gel-filtration chromatography. In the absence of UV light, Bax was detected in mock infected cells as an inactive monomer that eluted in fractions below 43 kDa in size (Fig. 4). In contrast, cells treated with UV light exhibited a dramatic shift in the majority of Bax to higher molecular weight fractions in excess of 149 kDa and a loss of monomeric Bax, indicative of Bax oligomerization (Fig. 4). In lysates from HeLa cells overexpressing Bcl-2 and HeLa cells infected with VV(Cop), Bax predominantly eluted in low molecular weight fractions below 43 kDa, even following treatment with UV light, indicating that both Bcl-2 and vaccinia virus infection inhibited Bax oligomerization (Fig. 4). However, in cells infected with VV(Cop)ΔF1L, Bax eluted as a high molecular weight oligomer following UV treatment (Fig. 4), and virtually no Bax was retained in the low molecular weight fractions, unlike cells infected with VV(Cop). This indicated that F1L not only inhibited the conformational change in Bax but also inhibited Bax oligomerization, and these results did not appear to be influenced by changes in the expression of Bax during infection, because infection with either VV(Cop) or VV(Cop)ΔF1L had no effect on Bax protein expression over time (data not shown).

**F1L Inhibits Bax Recruitment to the Mitochondria and Insertion into the Outer Mitochondrial Membrane**—Given that F1L expression clearly inhibited the conformational alteration and the oligomerization of Bax, we next investigated whether or not F1L prevented Bax localization and insertion into the mitochondrial membrane following an apoptotic stimulus (12–15). Another viral mitochondrial-localized inhibitor of apoptosis, vMIA, encoded by human cytomegalovirus, actually recruits Bax to the mitochondria but inhibits the pro-apoptotic effects of Bax (30, 34). To determine the effect of F1L on Bax integration into the outer mitochondrial membrane, mitochondrion...
F1L Inhibits Bax Activation

**FIGURE 2. F1L inhibits Bax activation induced by an apoptotic stimulus.** A, HeLa cells or HeLa cells that overexpress Bcl-2 were treated with UV light to induce apoptosis, and Bax activation was monitored by staining with anti-Bax(6A7) to detect the conformationally active form of Bax. B, HeLa cells were infected with either VV(Cop)EGFP or VV(Cop)ΔF1L at an m.o.i. of 10 for 8 h. Cells were treated with UV light to induce apoptosis, and 5 h post-treatment they were fixed and stained with anti-Bax(6A7). C, infected cells were visualized by EGFP fluorescence, and the mean percentage of cells demonstrating anti-Bax 6A7 positivity (± S.D.) was quantified. D, HeLa cells were infected with either VV(Cop)EGFP or VV(Cop)ΔF1L, and Bax activation was induced with either UV light or TNF-α. Active Bax was immunoprecipitated using anti-Bax(6A7), and immunoprecipitates and whole cell lysates were analyzed by Western blotting with anti-Bax(N20).

**FIGURE 3. Transient expression of F1L inhibits UV-induced Bax N-terminal exposure.** A, HeLa cells were transfected with either pEGFP-F1L or pEGFP-F1L (206–226) for 16 h and treated with UV light, and fixed cells were stained with anti-Bax(6A7) to detect activated Bax. B, results were quantified as a mean percentage (± S.D.) of EGFP-positive cells that also were anti-Bax(6A7)-positive.

were isolated and treated with a sodium carbonate alkali wash to remove loosely associated proteins (48). Mitochondria from mock infected HeLa cells displayed loosely associated Bax that was removed following the alkali wash (Fig. 5A, lanes 1 and 2). Following treatment with UV light, however, Bax remained membrane-associated following the alkali wash, indicative of Bax integration into the outer mitochondrial membrane (Fig. 5A, lanes 3 and 4). Bak, on the other hand, is constitutively integrated into the outer mitochondrial membrane and was not removed following the alkali wash (Fig. 5A, lanes 2 and 4). HeLa cells overexpressing Bcl-2, as well as cells infected with VV(Cop), displayed no insertion of Bax into the mitochondrial membrane following treatment with UV light (Fig. 5D, lanes 3 and 4). Significantly, we also detected some Bax integrated in the mitochondrial membrane from cells infected with VV(Cop)ΔF1L alone, indicative of the ability of this virus to initiate apoptosis and induce Bax activation (Fig. 5D, lane 2). These results indicated that F1L expression inhibits the insertion of Bax into the mitochondrial outer membrane during apoptosis.

To examine the subcellular distribution of Bax, cells were stained with a polyclonal anti-Bax antibody, which, unlike anti-Bax(6A7), detects Bax in the cytosol and at the mitochondria in both apoptotic and non-apoptotic cells (41). Mock infected HeLa cells stained with the polyclonal anti-Bax antibody demonstrated a diffuse cytoplasmic staining pattern for Bax when visualized by confocal (Fig. 6A, panels a–c). The diffuse cytoplasmic staining of Bax was also detected in cells infected with VV(Cop)EGFP (Fig. 6A, panels d–f). Cells infected for 24 h with VV(Cop)ΔF1L, however, demonstrated a noticeable punctate staining pattern, indicative of Bax activation and recruitment to the mitochondrial membrane reflecting the ability of this virus to activate Bax and induce apoptosis (Fig. 6A, panels g–i). This punctate staining pattern was observed to be mitochondrial in nature, because apoptotic cells transfected with EGFP-F1L (206–226), which targets EGFP to the mitochondria, demonstrated a distinct co-localization between EGFP and anti-Bax (Fig. 6A, panels s–u). A similar recruitment to the mitochondria was detected with the polyclonal Bax antibody following UV light treatment (Fig. 6A, panels j–l). Significantly, in cells infected with VV(Cop) and treated with UV light, Bax remained cytosolic and diffuse (Fig. 6A, panels m–o), whereas cells infected with VV(Cop)ΔF1L and treated with UV light showed Bax recruitment to the mitochondria (Fig. 6A, panels p–r). The results of these experiments were quantified by counting cells (Fig. 6B). These results indicate that F1L inhibited the insertion
of Bax into the outer mitochondrial membrane as well as the subcellular redistribution of Bax to the mitochondria, suggesting that F1L functions upstream of both Bax activation and the subsequent relocalization of Bax to the mitochondria.

**F1L Inhibits Cytochrome c Release and Bax Activation in the Absence of Bak**

The pro-apoptotic proteins Bak and Bax are critical for the induction of mitochondrial-mediated apoptosis, because cells devoid of both Bak and Bax are unable to execute cytochrome c release (5, 6). Because cells deficient in either Bax or Bak are still capable of cytochrome c release from mitochondria, albeit at a slower rate, viral mitochondrial inhibitors of apoptosis likely function by inhibiting the effects of both Bak and Bax (5, 6). Because we had previously shown that F1L associates with and inhibits the activation of Bak (36), we asked if the interaction between F1L and Bak was absolutely required for the inhibition of cytochrome c release. To address this question, mitochondria were purified from Bak-deficient mouse embryonic fibroblasts (Bak<sup>-/-</sup> MEFs) and used in an in vitro cytochrome c release assay (5). Bak<sup>-/-</sup> MEFs were infected with VV(Cop)EGFP or VV(Cop)<sup>ΔF1L</sup>, and purified mitochondria were treated with increasing amounts of the active recombinant BH3-only protein Bid (tBid) to induce cytochrome c release (11, 46, 49). Supernatant and pellet fractions were then blotted for cytochrome c, and pellet fractions were also blotted for Bax and the mitochondrial protein manganese superoxide dismutase as controls. Mitochondria from mock infected, Bak-deficient MEFs treated with increasing amounts of tBid, demonstrated a loss of cytochrome c from the pellet fraction and accumulation in the supernatant fraction (Fig. 7). Mitochondria from Bak<sup>-/-</sup> MEFs infected with VV(Cop)EGFP showed clear inhibition of cytochrome c translocation from the pellet to the supernatant fraction following treatment with tBid (Fig. 7). In contrast, mitochondria isolated from VV(Cop)<sup>ΔF1L</sup>-infected Bak<sup>-/-</sup> MEFs were unable to inhibit tBid-induced cytochrome c release (Fig. 7), indicating that F1L expression during vaccinia virus infection was able to inhibit cytochrome c release in the absence of Bak. Mitochondria purified from MEFs lacking both Bak and Bax (Bak<sup>-/-</sup>/Bax<sup>-/-</sup> MEF) did not display cytochrome c translocation into the supernatant (Fig. 7), highlighting the importance of Bak or Bax in facilitating cytochrome c release. Because these cells lack Bak, tBid-induced cytochrome c release likely occurs through Bax activation. To assess the ability of F1L to inhibit tBid-induced Bax activation in Bak-deficient cells *in vitro*, mitochondria were purified from cells infected with either VV(Cop) or VV(Cop)<sup>ΔF1L</sup>. Following treatment with tBid, activated Bax was detected by immunoprecipitation with anti-Bax(NT).

Using Bak<sup>-/-</sup> MEFs and Bak-deficient baby mouse kidney cells (Bak<sup>-/-</sup> BMKs) (39), we next assessed whether prolonged...
Infection with VV(Cop) or VV(Cop)/H9004 F1L induced Bax activation. As expected, mock infected Bak−/− BMFs and Bak−/− BMK cells showed no Bax activation, as assessed by immunoprecipitation with anti-Bax(6A7) (Fig. 8A). Infection with VV(Cop) resulted in minimal or no active Bax immunoprecipitated with anti-Bax(6A7) from the Bak−/− BMFs (Fig. 8A). Infection with VV(Cop) also inhibited staurosporine-induced Bax activation.
in Bak\(^{-/-}\) BMKs as assessed by immunoprecipitation with anti-Bax(6A7) (Fig. 8B). Infection with VV(Cop)ΔF1L, however, was unable to inhibit staurosporine-induced Bax activation in Bak\(^{-/-}\) BMKs, indicating that F1L can inhibit Bax activation and, more importantly, that Bak is not required for this inhibition.

**F1L Fails to Interact with Bax during Infection**—Bcl-2-family members mediate many of their pro- or anti-apoptotic effects through interactions with other Bcl-2 family members. Because our data demonstrated that F1L was capable of inhibiting Bax activation we asked whether F1L achieved this by directly interacting with Bax. Although F1L is a mitochondria-localized protein and we fail to see recruitment and integration of Bax in the mitochondrial membrane in the presence of F1L, there remained a possibility that F1L could function through interaction with Bax. To investigate a possible interaction between Bax and F1L, HeLa cells were infected with a recombinant vaccinia virus expressing a FLAG-tagged version of F1L, VVFLAG-F1L, and treated with either UV light or TNF-α. Cells were subsequently lysed in CHAPS buffer to prevent alterations in Bax conformation and immunoprecipitated with anti-FLAG (41, 47). In all cases, FLAG-F1L co-immunoprecipitated with endogenous Bak (Fig. 9A), demonstrating that the previously described interaction between Bak and F1L was maintained following treatment with an apoptotic stimulus (36). Interestingly, no observable interaction was seen between endogenous Bak and F1L either before or after induction with an apoptotic stimulus (Fig. 9A). Recently, it has been reported that a soluble version of F1L interacts with a peptide encompassing the BH3 domain of Bax (50). However, because F1L expression inhibits the conformational change in Bax, it is unlikely that the BH3 domain of Bax is exposed. Nevertheless, F1L may be capable of interacting with active Bax. To confirm this prediction, we performed co-immunoprecipitations under conditions that artificially activate Bax. The zwitterionic detergent CHAPS maintains Bax in its natural conformation, whereas Triton X-100 causes Bax to unfold into an “active” state (41, 47). HeLa cells were infected with vaccinia virus expressing FLAG-F1L and lysed in Triton X-100 to induce a conformational change in Bax. Immunoprecipitation with anti-FLAG demonstrated a significant amount of co-precipitated Bax when cells were lysed in Triton X-100 but not in CHAPS, suggesting that F1L has the capacity to interact with Bax only following an activating conformational change (Fig. 9B). Similar results were seen using Bak-deficient BMKs, because lysis in Triton X-100 permitted an interaction between F1L and Bax, but no interaction was detected when cells were lysed in CHAPS, even upon addition of staurosporine to induce apoptosis (Fig. 9C). These results demonstrate that F1L is capable of interacting with Bax in the absence and presence of an apoptotic trigger. In contrast, F1L does not interact constitutively with Bax but, instead, inhibits Bax activity independent of any interaction with Bax prior to a conformational change in Bax.

**F1L Interacts with BimL and Inhibits BimL-induced Apoptosis**—We have shown that F1L expression inhibits the conformational change in Bax and integration into the outer mitochondrial membrane independent of an interaction with Bax. Because the BH3-only proteins are essential for the activation of Bax and Bak we hypothesized that F1L may inhibit Bax by interacting with BH3-only proteins (19, 20). The BH3-only protein Bim functions as a direct activator of Bax (22, 25, 51, 52), and previous observations indicate that a peptide encompassing the BH3 domain of Bim strongly interacts with F1L, suggesting that F1L may exert its anti-apoptotic activity through interaction with full-length Bim (50). To examine the possibility that F1L interacts with BimL, HEK293T cells were co-transfected with FLAG-BimL and either EGFP, EGFP-F1L, EGFP-Bcl-2, or EGFP-F1L-(206–226), which contains only the 20-amino acid mitochondrial targeting sequence for F1L. Co-transfected cells were subjected to immunoprecipitation with anti-EGFP. Western blotting with anti-Bim demonstrated that both EGFP-F1L and EGFP-Bcl-2 co-precipitated a significant amount of BimL, whereas no interaction was observed with EGFP or EGFP-F1L-(206–226) (Fig. 10A). Reciprocal co-im-

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**FIGURE 9. F1L fails to interact with Bax, following an apoptotic stimulus.** 
A, HeLa cells were infected with VV:FLAG-F1L for 8 h, and treated with either UV light or TNF-α for 3 or 6 h, respectively. Following apoptotic induction, cells were lysed in 2% CHAPS lysis buffer and immunoprecipitated with anti-FLAG (M2). Immunoprecipitates and lysates were analyzed by Western blotting with either anti-FLAG-HRP to detect F1L, anti-Bak(NT), or anti-Bax(N20). B, HeLa cells were infected with VVFLAG-F1L, lysed in either 1% Triton X-100 (TX-100) or 2% CHAPS buffer, F1L interaction with Bak and Bax was analyzed by co-immunoprecipitation with anti-FLAG, and Western blotting with FLAG-HRP, to detect F1L, or anti-Bak(NT) or anti-Bax(6A7). C, Bak\(^{-/-}\) BMKs cells were infected with VVFLAG-F1L in the absence or presence of 1 μM staurosporine (STS). Cells were lysed in either 1% Triton X-100 (TX-100) or 2% CHAPS, and interaction between F1L and Bax was detected by co-immunoprecipitation with anti-FLAG and Western blotting with anti-Bax(N20).
munoprecipitations were performed using anti-FLAG and Western blotting with anti-EGFP, which again showed that FLAG-BimL interacted with EGFP-F1L and EGFP-Bcl-2 but not with EGFP and EGFP-F1L-(206–226) (Fig. 10B). The lack of interaction between EGFP-F1L-(206–226) and FLAG-BimL was observed despite obvious co-localization at the mitochondria (Fig. 11B). Consistent with these observations, cells co-transfected with BimL and EGFP-F1L or EGFP-Bcl-2 were resistant to BimL-induced depolarization of the mitochondria membrane potential as measured by the membrane potential dye TMRE (Fig. 11A). In contrast, cells transfected with EGFP or EGFP-F1L-(206–226) underwent BimL-induced loss of the membrane potential indicating no ability to inhibit apoptosis (Fig. 11A).

To investigate further the interaction between F1L and BimL, we assessed the ability of F1L to interact with endoge-
Bim Is Required For Vaccinia Virus-induced Apoptosis—To assess whether Bim is involved in virus-induced apoptosis, wild-type or Bim"/- BMKs were infected with VV(Cop) or VV(Cop)ΔF1L, and apoptosis was assessed by counting surviving adherent cells. WT BMKs infected with VV(Cop) displayed limited amounts of cell death with the majority of infected cells still adherent (Fig. 12A). VV(Cop)ΔF1L-infected WT BMKs demonstrated a significant reduction in cell survival at 10 and 15 h post infection, as assessed by a loss of adherent cells. Pre-treatment with the broad-spectrum caspase inhibitor z-VAD-fmk inhibited cellular loss, indicating that cell death was the result of caspase-dependent apoptosis (Fig. 12A). In contrast, cells deficient in Bim demonstrated an increased resistance to apoptosis induced by VV(Cop)ΔF1L infection compared with the WT BMKs infected with the same virus (Fig. 12A). Accordingly, VV(Cop)ΔF1L-induced cleavage of PARP was also retarded in the Bim"/- cells compared with WT BMKs (Fig. 12B). Densitometric analysis of PARP revealed that in WT BMKs infected with VV(Cop)ΔF1L nearly 80% of PARP existed as a cleaved form, whereas only 40% of PARP was cleaved in Bim"/- BMKs infected with VV(Cop)ΔF1L. These observations suggest that the pro-apoptotic activity of Bim is required for efficient vaccinia virus-induced apoptosis, and F1L expression inhibits this activity.

DISCUSSION

The activity of Bak or Bax is critical for the release of cytochrome c from the mitochondrion and subsequent death of the cell (4–6). As such, viruses have evolved strategies to inhibit the pro-apoptotic activity of Bak and Bax, thereby ensuring successful virus propagation (27, 29, 53). We recently identified F1L as a potent anti-apoptotic protein encoded by vaccinia virus, the prototypic member of the poxvirus family (36–38, 44). F1L localizes predominantly to the outer mitochondrial membrane via a C-terminal mitochondrial targeting sequence where it functions to inhibit mitochondrial membrane permeabilization and the release of cytochrome c during vaccinia virus infection (36–38). We and others have shown previously that F1L constitutively interacts with and inhibits the activation of the pro-apoptotic protein Bak, suggesting that F1L regulates apoptosis by directly interfering with Bak (36, 45). Because both Bak and Bax are activated following an apoptotic stimulus, it would be advantageous for a virus to inhibit the activity of both of these proteins to ensure efficient inhibition of apoptosis. Therefore, we hypothesized that, in addition to inhibiting Bak activity, vaccinia virus would also encode a mechanism to inhibit the activity of Bax.

Unlike Bak, which is constitutively inserted into the outer mitochondrial membrane, Bax is normally found within the cytoplasm or loosely associated with the mitochondrial membrane (12–15). Following an apoptotic stimulus, Bax undergoes a conformational change that can be detected using N-terminal conformation-specific antibodies, such as anti-Bax(6A7) (12, 41). Infection of HeLa cells with a recombinant vaccinia virus deficient for F1L resulted in the activation of Bax as detected by anti-Bax(6A7) reactivity (Fig. 1). In contrast, no Bax activation was detected upon infection with a vaccinia virus that expressed a functional F1L, indicating that F1L expression inhibited Bax activation during virus infection (Fig. 1). The apoptosis-induced conformational change in Bax is associated with the oligomerisation and insertion of Bax into the outer mitochondrial membrane, resulting in the release of cytochrome c (16, 46). F1L expression blocked both the conformational change and oligomerization of Bax (Figs. 1–4). Moreover, F1L inhibited the subcellular redistribution and insertion of Bax into the outer mitochondrial membrane clearly indicating that F1L inhibited the activity of Bax in our assays (Figs. 5 and 6). We further showed that F1L-mediated inhibition of cytochrome c release does not require Bak, because Bak"/- MEFs were protected from tBid-induced cytochrome c release when infected with VV(Cop)EGFP, but not VV(Cop)ΔF1L (Fig. 7). Additionally, F1L expression also inhibited virus- and staurosporine-induced Bax activation in the absence of Bak, as measured by
F1L Inhibits Bax Activation

anti-Bax(6A7) immunoprecipitations in Bak−/− MEFs and Bak−/− BMKs (Fig. 8). These observations clearly indicated that, in addition to interacting with and inhibiting the activation of Bak, F1L is also capable of inhibiting Bax activity via a Bak-independent mechanism.

Although F1L strongly inhibited Bax activation, we failed to detect an interaction between F1L and Bax following an apoptotic stimulus (Fig. 9). Even in the absence of Bak expression, no interaction between F1L and Bax was detected (Fig. 9). An interaction, however, was detected in the presence of the non-ionic detergent Triton X-100, which artificially induces a conformation change in Bax, indicating that F1L possessed an innate ability to interact with conformationally active Bax (Fig. 9) (41, 47). We speculate, therefore, that if activated Bax were present during infection, F1L may interact with and inhibit Bax. However, because F1L inhibited Bax in the absence of any detectable interaction, we hypothesized that F1L may function by inhibiting BH3-only proteins that play a key role in activating Bax (19, 20). In support of this idea, we show that full-length F1L interacted with both ectopically expressed and endogenous BimL (Figs. 10A, 10B, and 11C). Notably, the mitochondria-targeted hydrophobic tail construct EGFP-F1L-(206–226) failed to co-immunoprecipitate BimL (Fig. 10, A and B), despite displaying dramatic co-localization to the mitochondria with BimL (Fig. 11B). F1L expression also inhibited BimL-induced apoptosis as measured by the loss of the inner mitochondrial membrane potential, a hallmark feature of apoptotic cells (Fig. 11A). In support of our findings, Fischer and colleagues (50) recently demonstrated that a soluble version of F1L, which lacked the mitochondrial targeting motif, interacted with a peptide encompassing the BH3 domain of Bim. Taken together, these data support a scenario by which F1L inhibits Bax activation by binding and sequestering BH3-only proteins, such as BimL. Indeed, we observed that Bim-deficient cells infected with VV(Cop)ΔF1L demonstrated reduced levels of apoptosis compared with wild-type cells, suggesting that the pro-apoptotic activity of Bim plays a role in virus-induced cell death (Fig. 12). As apoptosis was not completely abolished in Bim-deficient BMKs, it is likely that multiple BH3-only proteins are activated upon virus infection.

The BH3-only proteins are potent activators of Bak and Bax and are themselves activated by post-translational modification, proteolytic cleavage, or transcriptional regulation following an apoptotic trigger (19, 20). BH3-only proteins activate Bak and Bax either through direct stimulation, or indirectly by inhibiting anti-apoptotic Bcl-2 family members, which retain Bak and Bax in an inactive state (19, 20). Bim and tBid have the capacity to directly bind and activate Bak and Bax (52, 54), whereas the remaining BH3-only proteins reportedly bind and repress the anti-apoptotic Bcl-2 family members, thereby “sensitizing” the cell by releasing and indirectly activating Bak and Bax (23–26). Several reports have clearly shown that the pro-survival Bcl-2 family members (i.e. Bcl-2 and Bcl-xL) display dramatically different affinities for the various BH3-only proteins (23–25). In this study, we show that F1L is capable of interacting with the BH3-only protein BimL (Figs. 10 and 11); however, whether F1L is able to interact with other BH3-only proteins is currently unknown. Considering that F1L expression inhibited tBid-induced cytochrome c release in vitro (Fig. 7), and Bax activation induced by virus infection or staurosporine (Fig. 8), it is likely that F1L functions to inhibit the pro-apoptotic activity of multiple BH3-only proteins. It is interesting to speculate that F1L, similar to cellular anti-apoptotic Bcl-2 family members, may target and inhibit only a subset of BH3-only proteins. Further studies are required to determine if F1L interacts with other BH3-only proteins to further define the anti-apoptotic mechanism of F1L.

Despite displaying a lack of overall sequence homology to anti-apoptotic Bcl-2 family members, the inhibition of Bax by F1L is similar to the ability of Bcl-2 to inhibit Bax (55–57). Although F1L lacks overall sequence homology to Bcl-2 family members, it has been suggested that F1L contains a “Bcl-3-like domain” (45). This region is quite divergent from the BH3 domains of known Bcl-2 family members. It is possible that, despite a lack of obvious sequence homology, F1L may structurally resemble Bcl-2 proteins, potentially explaining the anti-apoptotic properties of F1L. If F1L does possess regions similar to BH-domains, a closer examination of these regions would provide significant insight into the function of both F1L and cellular Bcl-2 family members.

The anti-apoptotic mechanism of action of F1L is divergent from other viral inhibitors of apoptosis. For example, the anti-apoptotic protein M11L, encoded by the poxvirus myxoma virus, constitutively interacts with Bak and inhibits the N-terminal activation of Bak similar to the function of F1L (35, 58). Myxoma virus infection, however, results in the recruitment of Bax to the mitochondria where M11L interacts with Bax (58). The adenovirus protein E1B19K interacts with Bax and the conformationally active form of Bax (59, 60), yet Bax oligomerization and insertion into the mitochondria are inhibited by E1B19K expression (61). Domain-swapping studies with the BH3-domain of Bax indicate that the interaction between E1B19K and the BH3-domain of Bax is required for E1B19K to inhibit Bax (62). vMIA, encoded by human cytomegalovirus, functions by sequestering Bax at the mitochondrial membrane in an oligomerized state (30, 33, 34). Despite the recruitment of Bax to the mitochondria, vMIA inhibits cytochrome c release and apoptosis (30, 34). To date, neither vMIA nor M11L have been shown to interact with BH3-only proteins, and E1B19K has only been shown to interact with Nbk/Bik (63). Our observation that F1L interacts with BimL is the first report of a viral apoptotic inhibitor that can act as a bona fide BimL binding partner. Our findings reveal that F1L functions to protect cells from apoptosis by inhibiting the activity of both Bax and Bax via different mechanisms. Although F1L interacts with Bax to inhibit the activity of Bak, Bax activity is not inhibited directly by F1L. Instead, our data support the idea that F1L also inhibits the activity of BH3-only proteins that function as key activators of Bax and Bak. We anticipate that the ongoing study of viral anti-apoptotic proteins, such as F1L, will provide important information regarding the mechanisms of apoptotic death and the mechanisms that pathogens use to interfere with apoptosis.

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