Fowlpox Virus Encodes a Bcl-2 Homologue That Protects Cells from Apoptotic Death through Interaction with the Proapoptotic Protein Bak

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Poxviruses are renowned for encoding numerous immunomodulatory proteins capable of undermining potent immune defenses. One effective barrier against infection is apoptosis, a process controlled at the mitochondria by pro- and antiapoptotic members of the highly conserved Bcl-2 family of proteins. Although poxviruses are known to encode an array of effective inhibitors of apoptosis, members of the Avipoxvirus genus, which includes fowlpox virus, encode proteins with Bcl-2 homology. Here, we show that FPV039, a fowlpox virus protein with limited Bcl-2 homology, inhibited apoptosis in response to a variety of cytotoxic stimuli, including virus infection itself. Similar to other antiapoptotic Bcl-2 proteins, FPV039 localized predominantly to the mitochondria in both human and chicken cells and protected human cells from tumor necrosis factor alpha-induced loss of the mitochondrial membrane potential. In addition, coimmunoprecipitation revealed that FPV039 interacted constitutively with the proapoptotic Bcl-2 protein, Bak, in both human and chicken cells. Concordantly, FPV039 also inhibited apoptosis induced by the transient overexpression of Bak. To confirm these results in the context of virus infection, we generated a recombinant vaccinia virus lacking F1L, the endogenous apoptotic inhibitor in vaccinia virus, and expressing FPV039. In the context of vaccinia virus infection, FPV039 retained the ability to localize to the mitochondria and interacted with Bak. Moreover, FPV039 prevented the activation of Bak and protected infected cells from apoptosis induced by staurosporine and virus infection. Together, our data indicate that FPV039 is a functional Bcl-2 homologue that inhibits apoptosis by neutralizing the proapoptotic Bcl-2 family member Bak.

Apoptosis is a complex and highly structured mechanism of cell death that is evolutionarily conserved throughout all multicellular organisms and functions to eliminate redundant, damaged, infected, or otherwise unwanted cells (19, 42). The network of biochemical pathways that regulate apoptosis converges on the activation of caspases, a group of cysteine proteases that cleave various cellular proteins and lead to the systematic dismantling of the cell (49, 71). Although the activation of caspases and, concordantly, the commitment to cell death are multifaceted and largely circuitous, the mitochondrial cascade (33, 34, 77). Within the mitochondrion is sequestered a pool of apoptogenic proteins, including cytochrome c, that when released into the cytosol activate caspases and induce apoptosis (33, 77). Thus, the ability to regulate the integrity of the mitochondrion is paramount in controlling apoptosis.

The highly conserved family of Bcl-2 proteins is ultimately responsible for regulating apoptosis at the mitochondrion (1, 17). Within this family exist both pro- and antiapoptotic members whose cooperative or antagonistic interactions collectively determine the fate of the cell (1, 17). These interactions depend on the presence of at least one of four highly conserved Bcl-2 homology (BH) domains as well as domains necessary for localization to the outer mitochondrial membrane. Antiapoptotic members of the Bcl-2 family possess four BH domains whereas Bak and Bax, proapoptotic members of the family, possess only BH domains 1, 2, and 3 (BH1, BH2, and BH3, respectively) (1, 17). BH3-only proteins, a second subset of proapoptotic Bcl-2 proteins, bear little homology to other Bcl-2 family members and contain only BH3 domains (65, 84). Structural analyses of these proteins have revealed that the BH1, BH2, and BH3 domains comprise α-helices that form a hydrophobic cleft capable of binding the BH3 α-helix of other Bcl-2 family members, thus forming the basis of the homo- and heterotypic interactions that characterize this family (57).

As upstream regulators of apoptosis, the BH3-only proteins are activated in response to a variety of cellular stresses, and they trigger apoptosis by repressing antiapoptotic Bcl-2 proteins and activating the proapoptotic Bcl-2 proteins, Bak and Bax (65, 84). Following activation, Bak and Bax undergo a series of conformational changes that result in their oligomerization (4, 35, 36, 81). Through a still largely undefined process, these oligomers induce the loss of the mitochondrial membrane potential and facilitate the release of apoptogenic proteins, such as cytochrome c (3, 20, 29). Bak and Bax are thus crucial to the induction of apoptosis; without Bax and Bak, mitochondrial membrane permeabilization and release of cytochrome c cannot occur (51, 82, 89). Conversely, antiapoptotic Bcl-2 family members, exemplified by Bcl-2 and Bcl-xL, interact at the mitochondria with Bak, Bax, and BH3-only proteins, to hold them in inactive conformers, thereby preventing apoptosis (1, 17). Together, the balance of interactions...
among the Bcl-2 family members determines whether or not a cell is to live or die (1, 17).

Apoptosis is a critical defense against virus infection, and many viruses, therefore, have evolved mechanisms to interfere with apoptosis (9, 60, 72). For example, a large number of viruses encode Bcl-2 homologues that function at the mitochondria to inhibit the release of cytochrome c (18, 41, 58). Additionally, some viruses encode mitochondrion-localized proteins that inhibit apoptosis but exhibit no obvious sequence homology to Bcl-2 proteins (25, 31, 80). Poxviruses, by virtue of their relatively large genomes, encode numerous immunomodulatory proteins, including several that are capable of inhibiting cytochrome c release (69). Our laboratory recently identified F1L as a unique vaccinia virus (VV) protein that localizes to the mitochondria and constitutively interacts with Bax to inhibit apoptosis, despite lacking obvious sequence homology to cellular Bcl-2 proteins (64, 70, 78–80). Another novel poxviral inhibitor of apoptosis, M11L, encoded by myxoma virus, also interacts constitutively with Bak to inhibit apoptosis (25, 75). M11L, like F1L, lacks obvious sequence homology to cellular Bcl-2 proteins; however, the three-dimensional structure of M11L bears striking similarity to that of cellular Bcl-2 proteins (22, 50). F1L orthologues are found in nearly all poxvirus genera, whereas Orthopoxvirus, among other genera, whereas Orthopoxvirus, among other genera, lacks obvious homology within the BH3 and BH4 domains, which is conserved among cellular antiapoptotic Bcl-2 family members, however, FPV039 lacks critical Bcl-2 homology domains and the relatively small size of FPV039 well as a putative C-terminal transmembrane domain. Unlike cellular antiapoptotic counterparts (2), the lack of critical Bcl-2 homology domains and the relatively small size of FPV039 therefore distinguish it from cellular antiapoptotic Bcl-2 family members.

Given the unique homology between FPV039 and cellular Bcl-2 proteins, we sought to determine what role FPV039 played in the modulation of apoptosis. We report that FPV039 localized to the mitochondria, where it maintained the integrity of the mitochondria by constitutive interaction with the proapoptotic protein Bak. FPV039 expressed in the context of viral infection retained interaction with Bak and rendered cells resistant to apoptosis. Furthermore, infection with a recombinant VV expressing FPV039 prevented Bak conformational change, a prerequisite for cytochrome c release and apoptotic death of the cell.
Measurement of mitochondrial membrane potential. Changes in the mitochondrial membrane potential were quantified by staining with tetramethylrhodamine ethyl ester (TMRE) (Invitrogen Life Technologies) (24, 53). HeLa cells (1 × 10⁶) were transfected with pEGFP-C3, pEGFP-HubCl-2, pEGFP-FIL, pEGFP-FPV039(1–176), pEGFP-FPV039(1–94), pEGFP-FPV039(1–141), or pEGFP-FPV039(142–176) using Lipofectamine 2000, and apoptosis was induced by treating cells with 10 ng/ml of tumor necrosis factor alpha (TNF-α) (Roché Diagnostics) and 5 μg/ml of cycloheximide (ICN Biomedical Inc.) for 6 h or by transfecting cells with 2 μg of pcDNA3-HA-Bak (where HA is hemagglutinin) for 18 h. Following treatment, cells were stained with 0.2 μM TMRE (24, 53). Similar experiments were performed in the context of viral infection. Jurkat cells or Jurkat Bcl-2-overexpressing cells (1 × 10⁶) were infected for 8 h at an MOI of 10. Cells were analyzed by two-color flow cytometry (FACSscan; Becton Dickinson) with TMRE fluorescence measured through the FL-2 channel equipped with a 585-nm filter (42-nm band-pass filter), and EGFP fluorescence was measured through the FL-1 channel equipped with a 489 nm filter (42-nm band-pass filter). Data were acquired on 20,000 cells per sample with fluorescence signals at logarithmic gain, and analysis was performed using CellQuest software. The percentage of killing was calculated as the number of EGFP⁺ TMRE⁺ cells divided by the total number of EGFP⁺ cells, and standard deviations were generated from three independent experiments.

Conformational analysis of Bak by flow cytometry. Jurkat cells (1 × 10⁶), Jurkat Bcl-2 cells, or Jurkat Bak⁻/⁻/Bax⁻/⁻/Bak-/-/Bax-/- cells were infected with VV-EGFP, VVAFIL, VVFIL-Flag-FPV039(1–176), or VVFIL-Flag-FPV039(1–94) for 4 h at an MOI of 10. Following infection, cells were exposed to 250 μM staurosporine (Sigma-Aldrich) for 2 h and then fixed in 0.25% paraformaldehyde. Cells were incubated with 2 μg/ml anti-Bak antibody (Ab-1; Oncogene Research Products) (35, 36) or 2 μg/ml of an isotype control antibody specific for NK1.1 (PK136) (48) and counterstained with phycoerythrin-conjugated anti-mouse antibody (Jackson ImmunoResearch Laboratories Inc.). Antibody staining was analyzed by flow cytometry (FACSscan; Becton Dickinson) with fluorescence measured through the FL-1 channel equipped with a 585-nm filter (42-nm band-pass filter).

Immunoprecipitations and immunoblotting. HEK 293T cells (1 × 10⁶) were transfected with 2 μg of pEGFP-C3, pEGFP-FIL, pEGFP-FPV039(1–176), pEGFP-FPV039(1–94), or pEGFP-FPV039(1–141) and cotransfected with 2 μg of pcDNA3-HA-Bak using Lipofectamine 2000. Cells were lysed in 2% CHAPS (3-[3-cholamidopropyl]-dimethylammonio)-1-propanesulfonate) (Sigma) supplemented with EDTA-free protease inhibitor (Roche Diagnostics), followed by precipitation using goat anti-GFP antibody (Luc Berthiaume, University of Alberta, Edmonton, Alberta, Canada). Similar experiments were performed in the context of viral infection. HeLa or LMH cells (7 × 10⁶) were infected with VV-EGFP, VVAFIL, VVFIL-Flag-FPV039(1–176), or VVFIL-Flag-FPV039(1–94) at an MOI of 5 for 6 h or, in the case of VVFIL-Flag-FPV039(1–94), 16 h. Cells were lysed in 2% CHAPS (Sigma) supplemented with EDTA-free protease inhibitor (Roche Diagnostics), followed by precipitation using rabbit anti-BakNT (Upstate) or mouse anti-Flag M2 (Sigma) antibody.

Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose or polyvinylidene difluoride membranes. The following antibodies were used for detection: mouse anti-GFP (1: 5,000; Cedarlane Laboratories Ltd.), mouse anti-HA (clone 12CA5) (1:4,000; Roche Diagnostics), rabbit anti-BakNT (1:2,000; Upstate), mouse anti-Bak (1:500, Pharmingen), mouse anti-Flag M2 (1:5,000; Sigma), and mouse anti-Flag-horseradish peroxidase (HRP) (1:2,000; Sigma). Proteins were visualized using enhanced chemiluminescence according to the manufacturer’s directions (GE Healthcare).

RESULTS

Fowlpox virus encodes a Bcl-2 homologue. Genomic sequencing of fowlpox virus revealed a putative Bcl-2 homologue encoded by the open reading frame FPV039 (2). Similar to most antiapoptotic Bcl-2 proteins, FPV039 is predicted to contain a series of α-helices that closely align with those determined from the structure of Bcl-2 (Fig. 1A). In addition, FPV039 possesses conserved BH1 and BH2 domains; however, FPV039 lacks obvious BH3 and BH4 domains and is unusually short, owing to deletion of an unstructured loop region present in Bcl-2 (Fig. 1A and B). Alignment of the BH1 domain of FPV039 with several cellular Bcl-2 proteins indicates that FPV039 possesses a highly conserved BH1 domain characterized by the presence of the NWGR sequence (amino acids 82 to 85) (Fig. 1B). Likewise, the BH2 domain of FPV039 bears sequence homology to the same domain in cellular Bcl-2 proteins (Fig. 1B). Importantly, G84 and R85 of the BH1 domain and W128 of the BH2 domain in FPV039 correspond to the same residues in cellular Bcl-2 proteins that are critical for antiapoptotic function (37, 87). Although FPV039 lacks key residues usually conserved within the BH3 domain, α-helix 2 of FPV039 corresponds with an α-helix in Bcl-2 that defines the BH3 domain (Fig. 1A). Similarly, α-helix 1 in Bcl-2, which contains the BH4 domain, also aligns with an α-helical region in FPV039, suggesting that despite lacking obvious sequence homology, FPV039 may be structurally and functionally homologous to Bcl-2. These discrepancies in sequence conservation between FPV039 and Bcl-2 may highlight substantial, and possibly critical, functional differences.

FPV039 is a tail-anchored protein that localizes to the mitochondria. Proapoptotic Bcl-2 proteins possess C-terminal “tail anchors” that typically target them to the endoplasmic reticulum membrane or the outer mitochondrial membrane (11, 61). Sequence analysis of FPV039 demonstrated the presence of a 26-amino-acid hydrophobic domain flanked by positively charged lysines at positions 141 and 169 of FPV039, suggesting that FPV039 may localize to intracellular membranes (Fig. 1A). Therefore, to determine the subcellular localization pattern of FPV039, HeLa cells were transiently transfected with pEGFP, pEGFP-FIL, or pEGFP-FPV039(1–176) (Fig. 2A) and analyzed by confocal microscopy (Fig. 2B). Cells transfected with pEGFP alone showed a diffuse fluorescence pattern indicative of uniform distribution throughout the cell that did not display colocalization with MitoTracker, a mitochondrion-specific dye that labels the mitochondria (Fig. 2B, frames a to c). As a control, cells transfected with pEGFP-FIL, which expresses an EGFP-tagged version of the VV-encoded F1 protein (64), showed a punctate fluorescence pattern that overlapped with MitoTracker (Fig. 2B, frames d to f). Similarly, cells expressing wild-type EGFP-FPV039(1–176) displayed a punctate fluorescence pattern that colocalized with MitoTracker, indicating that FPV039 also localized to the mitochondria (Fig. 2B, frames g to i). Deletion of the region in FPV039 predicted to encode an α-helical BH3 domain in other antiapoptotic Bcl-2 proteins did not affect localization of EGFP-FPV039(Δ41–54) to the mitochondria (Fig. 2B, frames j to l) since this construct retains the C-terminal transmembrane domain.

To determine whether the C-terminal domain of FPV039 is necessary for mitochondrial localization of FPV039, three mutated constructs were generated (Fig. 2A). Cells transfected with pEGFP-FPV039(1–94), which is truncated immediately after the BH1 domain and therefore lacks the downstream BH2 domain and putative transmembrane domain, showed a diffuse cytoplasmic distribution, which did not colocalize with MitoTracker (Fig. 2B, frames m to o). These results suggested that the C-terminal 82 amino acids were important for the mitochondrial localization of FPV039. A second mutant construct, pEGFP-FPV039(1–141), which lacks only the 35 C-terminal amino acids was used to refine the domain required for mitochondrial localization. Cells transfected with pEGFP-
FPV039(1–141) displayed diffuse localization throughout the cell and failed to colocalize with MitoTracker (Fig. 2B, frames p to r). In contrast, cells transfected with pEGFP-FPV039(142–176), a construct comprised of only the C-terminal 35 amino acids appended to the C terminus of EGFP, displayed a punctate green fluorescence pattern similar to pEGFP-F1L and pEGFP-FPV039(1–176) (Fig. 2B, frames s to u). Colocalization with MitoTracker indicated that the C-terminal 35 amino acids of FPV039 are necessary and sufficient for promoting localization to the mitochondria.

Because fowlpox virus is a natural pathogen of various poultry species (27, 73), including chickens, we wanted to confirm the localization of FPV039 in a physiologically relevant context. Chicken LMH cells were transfected with pEGFP-FPV039(1–176), pEGFP-FPV039(1–94), pEGFP-FPV039(1–141), or pEGFP-FPV039(142–176) (Fig. 2A) and analyzed by confocal microscopy (Fig. 2B). The colocalization observed in LMH cells precisely reflected the colocalization of the same constructs observed in HeLa cells (Fig. 2B and C). EGFP-FPV039(1–176) and EGFP-FPV039(142–176) localized predominantly to the mitochondria (Fig. 2C, frames a to c and j to l). Conversely, EGFP-FPV039(1–94) and EGFP-FPV039(1–141) exhibited a cytoplasmic distribution that did not colocalize with the mitochondria (Fig. 2C, frames d to i). Together, these data indicate that FPV039 is capable of localizing to the mitochondria in human and chicken cells.

FPV039 inhibits TNF-α-induced loss of mitochondrial membrane potential. The apparent sequence homology between FPV039 and cellular Bcl-2 proteins coupled with the observed localization of FPV039 to the mitochondria suggested that FPV039 may play a role in modulating apoptosis. To determine whether FPV039 was sufficient to inhibit apoptosis, HeLa cells were transfected with pEGFP, pEGFP-HuBcl-2, pEGFP-F1L, pEGFP-FPV039(1–176), or pEGFP-FPV039(142–176). Transiently transfected cells were subsequently treated with TNF-α to induce apoptosis, and uptake of TMRE, a dye that fluorescently labels healthy, respiring mitochondria, was used as an indicator of apoptosis (Fig. 3A) (24, 53). To ensure the phenomena we were observing were specific to cells expressing the transfected proteins, EGFP-expressing cells were analyzed for TMRE fluorescence. After TNF-α treatment, 53% of cells expressing EGFP alone exhibited loss of the mitochondrial membrane potential, whereas less than 1% of cells transfected with EGFP-HuBcl-2 and EGFP-FPV039(142–176) exhibited loss of the mitochondrial membrane potential.
and only 6% of cells expressing EGFP-F1L showed a drop in TMRE fluorescence (Fig. 3A). These data agree with previous studies and indicate that both Bcl-2 and the VV-encoded protein, F1L, protect cells from TNF-α-induced apoptosis (64). Similarly, expression of EGFP-FPV039(1–176) also inhibited apoptosis, with only 2% of transfected cells exhibiting a loss in mitochondrial membrane potential after TNF-α treatment (Fig. 3A). Conversely, cells expressing EGFP-FPV039(142–176) provided no protection, with 51% of transfected cells exhibiting a loss in TMRE fluorescence (Fig. 3A).

To ensure that differences in protein expression levels were not influencing the ability of the various EGFP-tagged con-
FPV039 inhibits Bak

FPV039 interacts with the proapoptotic protein Bak and inhibits Bak-induced apoptosis. Loss of the mitochondrial membrane potential and subsequent induction of apoptosis is governed by the Bcl-2 family of proteins and requires the activity of at least one of two proapoptotic proteins, Bak or Bax (51, 82, 89). Two other poxviral antiapoptotic proteins, M11L and F1L, which, unlike FPV039, display little sequence homology to members of the Bcl-2 family, constitutively interact with and inhibit Bak activity (75, 78). Accordingly, we wanted to ascertain if FPV039 also interacted with Bak. HEK 293T cells were cotransfected with pEGFP, pEGFP-F1L, pEGFP-FPV039(1–176), or pEGFP-FPV039(1–94), or pEGFP-FPV039(Δ41–54) and HA-tagged Bak and lysed in 2% CHAPS, a detergent which preserves the conformational integrity of Bcl-2 family members (43, 44). Immunocomplexes were precipitated with an anti-GFP antibody, and subsequent Western blotting was performed with an anti-HA or anti-Bak antibody (Fig. 4). In agreement with previous studies, an interaction between EGFP-F1L and HA-Bak was detected (Fig. 4A) (78). Using this approach, an interaction between EGFP-FPV039 (1–176) and HA-Bak was also observed (Fig. 4A). Significantly, no communoprecipitation was observed in cells transfected with EGFP or HA-Bak alone, confirming the specificity of the interaction between EGFP-FPV039(1–176) and HA-Bak (Fig. 4A). To ensure that we were precipitating equal amounts of the EGFP-tagged proteins, we subjected the lysates and immunocomplexes to Western blotting with an anti-GFP antibody, and the results indicated that each EGFP-tagged protein was expressed and precipitated at approximately equal levels (Fig. 4A and B). Although EGFP alone was highly expressed and readily precipitated, this protein exhibited no interaction with HA-Bak.

Two mutant FPV039 constructs were employed to refine the domains within FPV039 required for its interaction with Bak (Fig. 4B). EGFP-FPV039(1–94) failed to interact with HA-Bak, suggesting that the BH2 domain and the transmembrane domain play critical functional roles (Fig. 4B). Additionally, although FPV039 lacks a domain with obvious homology to the BH3 domain of human Bcl-2 (Fig. 1A and B), secondary structural analysis predicted an α-helix (n2) in FPV039 (amino acids 41 to 54) that aligns with the BH3 α-helix of human Bcl-2 (Fig. 1A), suggesting that this region may be important for interaction with Bak. To determine if amino acids 41 to 54 of FPV039 were critical for interaction with Bak, we generated an EGFP-tagged version of FPV039 lacking amino acids 41 to 54, EGFP-FPV039(Δ41–54). No interaction by communoprecipitation was detected between Bak and EGFP-FPV039(Δ41–54), indicating that, despite lacking homology to the BH3 domain of several antiapoptotic Bcl-2 proteins, this region was required for Bak interaction (Fig. 4B). Together, these data demonstrate that, like cellular Bcl-2 proteins and viral proteins, such as M11L and F1L, FPV039 also interacts with Bak (1, 17, 75, 78).

Since FPV039 clearly interacted with Bak, we next wanted to determine whether FPV039 could inhibit apoptosis induced by overexpression of Bak. Ectopic overexpression of Bak saturates the mitochondria with Bak, artificially inducing its activation and leading to apoptosis (15). HeLa cells were cotransfected with pEGFP, pEGFP-HuBcl-2, pEGFP-F1L, pEGFP-FPV039(1–176), or pEGFP-FPV039(142–176) and HA-Bak. Apoptosis was quantified by staining cells with TMRE to determine the percent loss of the mitochondrial membrane potential, and EGFP-expressing cells were analyzed by flow cytometry. EGFP expression alone was unable to prevent the loss of the mitochondrial membrane potential induced by HA-Bak, with almost 30% of cells exhibiting decreased TMRE fluorescence (Fig. 5A). A loss of mitochondrial membrane potential in cells expressing EGFP-HuBcl-2 was undetectable, and less than 5% of cells expressing EGFP-F1L were significantly decreased (Fig. 5B) compared to pEGFP or pEGFP-FPV039(1–176), pEGFP-FPV039(1–94), or pEGFP-FPV039(Δ41–54) and HA-Bak. Apoptosis was quantified by staining cells with TMRE to determine the percent loss of the mitochondrial membrane potential, and EGFP-expressing cells were analyzed by flow cytometry. EGFP expression alone was unable to prevent the loss of the mitochondrial membrane potential induced by HA-Bak, with almost 30% of cells exhibiting decreased TMRE fluorescence (Fig. 5A). A loss of mitochondrial membrane potential in cells expressing EGFP-HuBcl-2 was undetectable, and less than 5% of cells expressing EGFP-F1L.

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FIG. 3. Expression of FPV039 protects cells from TNF-α-induced apoptosis. (A) HeLa cells were transfected with either pEGFP, pEGFP-HuBcl-2, pEGFP-F1L, pEGFP-FPV039(1–176), or pEGFP-FPV039(141–176) and treated with 10 ng/ml TNF-α and 5 μg/ml cycloheximide for 6 h, and apoptosis was assessed by measuring TMRE fluorescence. Standard deviations were calculated from three independent experiments. (B) Expression levels of the various transfected proteins. HeLa cells were transfected, and protein levels were determined by Western blotting (WB) with anti-GFP.

Western blotting. HeLa cells transfected with either pEGFP, pEGFP-HuBcl-2, pEGFP-F1L, or pEGFP-FPV039(1–176), or pEGFP-FPV039(141–176) and treated with 10 ng/ml TNF-α and 5 μg/ml cycloheximide for 6 h, and apoptosis was assessed by measuring TMRE fluorescence. Standard deviations were calculated from three independent experiments. (B) Expression levels of the various transfected proteins. HeLa cells were transfected, and protein levels were determined by Western blotting (WB) with anti-GFP.
exhibited a drop in TMRE fluorescence, indicating that both Bcl-2 and F1L protected against Bak-induced apoptosis (Fig. 5A). EGFP-FPV039(1–176) also inhibited Bak-induced apoptosis, with approximately only 1% of transfected cells showing a decrease in TMRE fluorescence (Fig. 5A). The activity of wild-type FPV039 contrasted sharply with that of EGFP-FPV039(142–176), where greater than 30% of cells transfected with this construct lost their mitochondrial membrane potential as a result of HA-Bak overexpression (Fig. 5A).

Protein expression levels were analyzed via Western blotting to ensure that differences in the protective abilities of the EGFP-tagged proteins were not a result of differences in expression of either the EGFP-tagged proteins or HA-Bak. EGFP and EGFP-FPV039(142–176) were robustly expressed, but neither prevented apoptosis induced by HA-Bak (Fig. 5B). EGFP-HuBcl-2, EGFP-F1L, and EGFP-FPV039(1–176) were expressed at slightly varied levels, which correlated with the ability to inhibit apoptosis (Fig. 5A and B). HA-Bak was equally expressed in all samples. Collectively, these data imply that FPV039 is capable of interacting with the proapoptotic protein Bak and preventing the induction of apoptosis.

**FPV039 interacts with endogenous Bak during VV infection.**

To further elucidate the antiapoptotic mechanism of FPV039 during virus infection, we constructed two recombinant viruses...
using the previously generated VV strain Copenhagen lacking its natural apoptotic inhibitor, F1L, VVΔF1L (78). Using homologous recombination, we generated VV/H9004 F1L-Flag-FPV039(1–176), which expresses wild-type FPV039, and VV/H9004 F1L-Flag-FPV039(1–94), which expresses a truncated FPV039 lacking the 82 amino acids immediately C-terminal of the BH1 domain (Fig. 2A).

To determine if FPV039 was able to interact with Bak in the context of infection, we infected HeLa cells with VV/H9004 F1L, VV-Flag-F1L, VV/H9004 F1L-Flag-FPV039(1–176), or VV/H9004 F1L-Flag-FPV039(1–94) and immunoprecipitated cell lysates with an anti-Flag antibody. Subsequent Western blotting of the precipitates using an anti-Bak antibody revealed that Flag-tagged FPV039(1–176) interacted with endogenous Bak during infection (Fig. 6A). Conversely, Flag-tagged FPV039(1–94), which lacks the BH2 region and the C-terminal transmembrane domain, was unable to interact with Bak during virus infection, despite being precipitated and expressed at equal or greater levels (Fig. 6A). As a control, Flag-F1L also interacted with Bak, as previously shown (78), but no interaction was observed in the lysates from cells infected with VVΔF1L, confirming the specificity of the interaction between the Flag-tagged proteins and endogenous Bak (Fig. 2A).

Western blot analysis of these samples with anti-Flag antibodies revealed that each Flag-tagged protein was precipitated equally and that equal levels were expressed in the lysates (Fig. 6A). Notably, Western blotting of lysates infected with VVΔF1L-Flag-FPV039(1–94) showed a pattern indicative of ubiquitination (Fig. 6A and B), which we routinely observed for this mutant as well as FPV039(1–176) (data not shown). To confirm the interaction, we performed the reciprocal immunoprecipitation using anti-Bak and again showed that both F1L and FPV039 interacted with Bak during infection (Fig. 6B). Western blotting of infected cell lysates indicated that endogenous Bak and each Flag-tagged protein were expressed at equal levels (Fig. 6B). Importantly, the interactions observed between virally expressed FPV039 and endogenous Bak reflect those observed between ectopically expressed EGFP-tagged proteins and HA-Bak (Fig. 4).

We next sought to confirm the ability of FPV039 to interact with chicken Bak, a presumably important and natural partner. LMH cells were infected with VVΔF1L, VV-Flag-F1L, or VVΔF1L-Flag-FPV039(1–176), and the lysates were immunoprecipitated with either an anti-Flag (Fig. 7B) or anti-Bak (Fig. 7C) antibody. Because of the paucity of reagents available to study Bcl-2 proteins in chickens, we relied on rabbit anti-human BakNT, an antibody generated against the human Bak homologue (26). Overall, chicken Bak and human Bak share approximately 60% amino acid identity; however, the immunogen used to generate rabbit anti-human BakNT was a 16-amino-acid peptide of human Bak that shares approximately 80% identity with chicken Bak (Fig. 7A). Indeed, anti-BakNT detected a single band from LMH whole-cell lysates running at the predicted size of chicken Bak (data not shown). Immunoprecipitation of cell lysates with an anti-Flag antibody, followed by Western blotting with anti-BakNT, revealed an interaction between chicken Bak and both Flag-F1L and Flag-FPV039(1–176) (Fig. 7B). No interaction was observed in the lysates from cells infected with VVΔF1L (Fig. 7B). Western blotting the immunoprecipitates with anti-Flag revealed that
Flag-F1L and Flag-FPV039(1–176) were precipitated in equal amounts, and Western blotting of infected cell lysates showed that the Flag-tagged proteins and endogenous chicken Bak were equally expressed (Fig. 7B). To confirm the interaction, a reciprocal immunoprecipitation was performed. Infected cell lysates were immunoprecipitated with anti-BakNT and subsequently Western blotted with an anti-Flag antibody (Fig. 7C). As expected, both Flag-F1L and Flag-FPV039(1–176) interacted with chicken Bak, and no interaction was observed in lysates from cells infected with VVΔF1L. Cellular lysates were immunoprecipitated (IP) with anti-Flag and Western blotted (WB) with anti-Flag or anti-BakNT. Both F1L and FPV039 interact with endogenous levels of chicken Bak. Lysates were Western blotted with anti-BakNT or anti-Flag to determine expression of endogenous Bak and Flag-tagged proteins, respectively. (C) LMH lysates were subjected to reciprocal immunoprecipitation (IP) with anti-BakNT followed by Western blotting (WB) with anti-BakNT or anti-Flag to detect an interaction. Lysates were Western blotted with anti-BakNT or anti-Flag to determine protein expression. The immunoglobulin light chain (Ig LC) is indicated by an arrow.

Flag-F1L and Flag-FPV039(1–176) were precipitated in equal amounts, and Western blotting of infected cell lysates showed that the Flag-tagged proteins and endogenous chicken Bak were equally expressed (Fig. 7B). To confirm the interaction, a reciprocal immunoprecipitation was performed. Infected cell lysates were immunoprecipitated with anti-BakNT and subsequently Western blotted with an anti-Flag antibody (Fig. 7C). As expected, both Flag-F1L and Flag-FPV039(1–176) interacted with chicken Bak, and no interaction was observed in lysates from cells infected with VVΔF1L (Fig. 7C). Anti-BakNT precipitated approximately equal amounts of Bak in mock-, VV-Flag-F1L-, and VVΔF1L-Flag-FPV039(1–176)-infected cells. More Bak was immunoprecipitated in VVΔF1L-infected cells, but, as expected, no protein coprecipitated (Fig. 7C). Both Flag-tagged proteins and endogenous Bak were expressed at equal levels (Fig. 7C). The results in chicken cells precisely mirror those obtained in human cells and lend further support to the idea that FPV039 is a functional Bcl-2 homologue.

**FPV039 inhibits apoptosis induced by virus infection.** We have previously shown that the VV protein F1L is a potent inhibitor of apoptosis and that, in the absence of F1L, VV infection induces apoptosis (78). To ascertain whether FPV039 could functionally replace F1L as an inhibitor of VV-induced apoptosis, Jurkat cells were infected with either VV-EGFP, VVΔF1L, VVΔF1L-Flag-FPV039(1–176), or VVΔF1L-Flag-FPV039(1–94), and the ability of these viruses to inhibit apoptosis was quantified using two-color flow cytometric analysis. Apoptosis was measured by quantifying the loss of the inner mitochondrial membrane potential using TMRE staining in EGFP-positive cells since each virus expressed EGFP. Using this experimental approach, the majority of cells infected with VV-EGFP, which encodes F1L, and VVΔF1L-Flag-FPV039 (1–176) maintained their mitochondrial membrane potential following infection for 8 h (Fig. 8a and c). Conversely, 23% of cells infected with VVΔF1L, which induces apoptosis, exhibited a loss of the inner mitochondrial membrane potential (Fig. 8b) (78). Similar to F1L, expression of FPV039 inhibited apoptosis induced by VV lacking the antiapoptotic protein F1L. Infection with VVΔF1L-Flag-FPV039(1–94), however, failed to completely protect cells from virus-induced apoptosis, perhaps offering partial protection (Fig. 8d). Jurkat cells over-expressing Bcl-2 were completely resistant to apoptosis induced by either VVΔF1L or VVΔF1L-Flag-FPV039(1–94) (Fig. 8f and h). Additionally, both VV-EGFP and VVΔF1L-Flag-FPV039(1–176), but not VVΔF1L or VVΔF1L-Flag-FPV039(1–94), protected cells from apoptosis induced by staurosporine, a potent apoptotic stimulus (data not shown). Together, these data demonstrate that FPV039, despite lack-
FPV039 inhibits virus-induced apoptosis. Expression of FPV039 inhibits apoptosis induced by VVΔF1L. Jurkat (a to d) or Jurkat cells overexpressing Bcl-2 (e to h) were infected with VV-EGFP, VVΔF1L, VVΔF1L-Flag-FPV039(1–176), or VVΔF1L-Flag-FPV039(1–94) at an MOI of 10. After 8 h, apoptosis was quantified via two-color flow cytometry by measuring loss of the inner mitochondrial membrane potential as detected by TMRE fluorescence. Data were acquired on 20,000 cells per sample, and the data shown are representative of three or more independent experiments.

**DISCUSSION**

Apoptosis comprises an important and powerful part of the innate and acquired immune system (19, 42). As obligate intracellular parasites, viruses depend on healthy host cells to complete their replication cycle and facilitate a productive infection; accordingly, many viruses encode proteins that interfere with apoptosis (9, 60). Poxviruses are renowned for encoding numerous immunomodulatory proteins capable of undermining or subverting the immune barriers to infection, including apoptosis (69). Notably, however, fowlpox virus and canarypox virus, both of the *Avipoxivirus* genus, encode proteins with obvious Bcl-2 sequence homology (2, 74). We now provide evidence indicating that FPV039 functions at the mitochondria to prevent the induction of apoptosis.

The highly conserved family of Bcl-2 proteins regulates the commitment to apoptosis at the mitochondria (1, 17). Not surprisingly, several viruses encode Bcl-2 homologues that specifically localize to the mitochondria and inhibit apoptosis (18, 41, 58). FPV039, encoded by fowlpox virus, was initially identified as a Bcl-2 homologue based on sequence analysis that revealed the presence of obvious BH1 and BH2 domains and a series of α-helices, similar to members of the cellular Bcl-2
family (Fig. 1A) (2). However, key amino acids typically found within the BH3 and BH4 domains of cellular Bcl-2 proteins are not conserved within FPV039 (Fig. 1) (2). As such, FPV039 differs markedly from cellular antiapoptotic Bcl-2 proteins, such as Bcl-2 and Bcl-xL, which typically possess all four BH domains (1, 17). In general, virus-encoded Bcl-2 (vBcl-2) homologues exhibit considerably less conservation within the BH3 and BH4 domains (18, 41, 58). For example, the Kaposi’s sarcoma herpesvirus (KSHV) Bcl-2 homologue possesses conserved BH1 and BH2 domains but possesses poorly conserved BH3 and BH4 domains (14). Similarly, A179L, encoded by African swine fever virus, also possesses highly conserved BH1 and BH2 domains but lacks BH3 and BH4 domains (54, 86). In fact, of all vBcl-2 proteins identified, FPV039, KSHV Bcl-2, and A179L possess the most highly conserved BH1 domains and are the only vBcl-2 proteins to retain the entire NWGR sequence that is unfailingly conserved and functionally critical for cellular Bcl-2 proteins (Fig. 1B) (40).

Similar to Bcl-2, FPV039 is comprised of a series of α-helices that closely align with those determined from the structure of Bcl-2 (Fig. 1A) (56). The cellular multidomain Bcl-2 proteins adopt a similar fold comprised of at least eight α-helices, and the conserved BH domains, in turn, correspond to or overlap with these α-helices (56, 57). BH1, BH2, and BH3 contribute to the formation of a hydrophobic pocket that binds the α-helical BH3 domain of other Bcl-2 proteins, thus facilitating the homo- and heterotypic interactions that occur among all Bcl-2 family members (56, 57). Interestingly, two of the predicted α-helices possessed by FPV039 correspond with the BH3- and BH4-containing α-helices of Bcl-2 (Fig. 1A), suggesting that these α-helices in FPV039 are structurally homologous. Indeed, the three-dimensional structure of the KSHV Bcl-2 homologue exhibits a hydrophobic pocket similar to that possessed by cellular Bcl-2 and Bcl-xL, even though KSHV Bcl-2 lacks obvious BH3 and BH4 domains (46). Additionally, deletion of amino acids 41 to 54 in FPV039, which align with the BH3 domain of human Bcl-2, resulted in the inability of FPV039 to interact with Bak, providing evidence that this region within FPV039 is critical for function (Fig. 4B).

The absence of conserved BH3 and BH4 domain sequences in FPV039 may reflect a divergence on the part of FPV039 from negative regulation. Peptides corresponding to several BH3 domains are intrinsically proapoptotic and, like BH3-only proteins, capable of inducing apoptosis (65, 84). As such, strict conservation of the BH3 domain may be a disadvantage to the virus. Moreover, the BH3 domain plays an important role in the binding specificity of Bcl-2 proteins, and modification of this domain may enable FPV039 to interact more promiscuously with Bcl-2 family members or, conversely, increase the specificity of interaction. The lack of BH4 sequence conservation in FPV039 may reflect the loss of an additional phosphorylation site typically present in the BH4 domain of Bcl-2, which negatively regulates Bcl-2 (38). Furthermore, similar to other vBcl-2 homologues, FPV039 lacks the unstructured “loop” domain, normally present between the BH3 and BH4 domains of Bcl-2 and Bcl-xL, making FPV039 a comparably smaller pro-
tein (Fig. 1A). This loop acts as a negative regulatory domain, sensitive to inhibitory phosphorylation (55, 85) and caspase-3-mediated cleavage, which converts Bcl-2 and Bcl-xL from antiapoptotic to proapoptotic proteins (7, 13, 16, 32). Several vBcl-2 homologues bypass this level of control and are resistant to caspase cleavage (8, 18, 41, 58). Indeed, the various levels of negative regulation imposed on cellular Bcl-2 family members has likely placed a strong evolutionary pressure on vBcl-2 proteins, resulting in the preservation of only the minimal functional elements and the loss of regulatory elements. The absence of complete sequence homology in functional vBcl-2 homologues suggests that a strict conservation of amino acids is not necessary for these proteins to inhibit apoptosis.

The subcellular location of FPV039, along with the predicted secondary structure and sequence homology to cellular Bcl-2, clearly suggested that, like cellular Bcl-2 proteins, FPV039 functions through interaction with members of the Bcl-2 family, which tightly regulates the mitochondrial events leading to apoptosis (1, 17). Specifically, Bak and Bax, the two proapoptotic proteins absolutely required for the induction of apoptosis, represent a crucial lynchpin in the regulation of cell death (51, 82, 89), and vBcl-2 proteins inhibit apoptosis by interfering with the proapoptotic activity of Bak and Bax (18, 41, 58). Accordingly, we have shown that FPV039 constitutively interacts with both human and chicken Bak (Fig. 4, 6, and 7). Additionally, FPV039 inhibits apoptosis induced by Bak overexpression, as well as TNF-α treatment, in mammalian cells (Fig. 3 and 5). Moreover, FPV039 expression prevented the activation of Bak, a key step in the progression to Bak oligomerization and permeabilization of the outer mitochondrial membrane (35, 36, 81), thereby retaining Bak in an inactive and unresponsive state, unable to facilitate the induction of apoptosis (Fig. 9). Such an inhibitory mechanism has been observed previously for the adenovirus Bcl-2 homologue, E1B 19K, which also constitutively interacts with Bak and inhibits Bak activation in response to a variety of cytotoxic stimuli (26, 68). Although interaction with proapoptotic Bcl-2 family members such as Bak represents the most direct mechanism for a viral protein to prevent apoptosis, other vBcl-2 proteins, including KSHV Bcl-2, have not yet demonstrated an ability to interact with Bak but are capable of inhibiting apoptosis (14). Given that Bak is a key player in the release of mitochondrial-associated proapoptotic proteins, it is likely that other vBcl-2 proteins will also function through interaction with Bak.

Poxviruses are renowned for manipulating multiple aspects of the host immune response and encode several other antiapoptotic proteins that, similar to FPV039, mediate their effect by interfering with Bcl-2 family members (69). Two well-characterized poxvirus proteins, M11L from myxoma virus and F1L from VV, bear no sequence homology to each other and lack obvious sequence homology to cellular Bcl-2 proteins, yet they function analogously to Bcl-2 proteins in their ability to inhibit apoptosis (25, 28, 59, 67, 70, 75, 78, 80). Both F1L and M11L constitutively interact with and inhibit the activity of Bak (59, 75, 78). Likewise, a recently characterized protein, ORFV125, encoded by the orf virus, inhibits apoptosis and prevents the activation of Bak (83). FPV039, like F1L and M11L, inhibits apoptosis by interacting with Bak and inhibiting Bak activation (59, 75, 78). Indeed, we have shown that FPV039 can functionally replace F1L in VV to inhibit virus-induced apoptosis (Fig. 8).

Despite the lack of sequence homology to cellular Bcl-2 proteins, the recently solved three-dimensional structure of M11L demonstrates a strikingly similar structure to Bcl-xL, explaining the basis for the molecular interaction with Bak (22, 50). In addition to interacting with Bak, M11L also interacts with the proapoptotic protein Bax, suggesting that FPV039 may perform a similar function to efficiently inhibit apoptosis (67). Conversely, in a manner similar to F1L, FPV039 may not directly interact with Bax but, instead, may inhibit Bax activity by binding directly to members of the BH3-only family of proteins, which serve as activators of Bak and Bax (65, 84).

Interacting with, and inhibiting the activity of, BH3-only proteins might be an equally protective mechanism by which FPV039 inhibits apoptosis. E1B 19K, encoded by adenovirus, interacts with Bik (39), and we have recently shown that F1L interacts with Bim (70), thus supporting the possibility that inhibitors of apoptosis, whether homologous to Bcl-2 proteins or not, can prevent cell death by interacting with BH3-only proteins. Further studies are therefore required to define the antiapoptotic mechanism of FPV039 and the range of interactions FPV039 exhibits. The fact that FPV039, F1L, and M11L all inhibit apoptosis by modulating Bak yet lack sequence homology clearly implicates Bak as a critical protein in the apoptotic cascade.

It is possible that the differences among FPV039, F1L, and M11L can be accounted for by convergent evolution, whereby the ancestral genes for these proteins were all acquired independently and subsequently evolved to inhibit apoptosis via similar mechanisms. Interestingly, however, FPV039, F1L, and M11L are all found at a similar location within the genome and are flanked by a gene encoding the dUTPase (2, 12, 30), suggesting that these three genes, despite bearing no sequence similarity, may be the products of divergent evolution from a common progenitor. A similar phenomenon has been observed in human and murine cytomegalovirus. Both human and murine cytomegaloviruses encode viral mitochondrial inhibitors of apoptosis that lack sequence similarity but are encoded by open reading frames in homologous positions in the viral genome (52). The lack of sequence homology between FPV039, F1L, and M11L, however, does not obviously preclude the presence of structural homology, given that M11L adopts a Bcl-2-like structure (22, 50). Additionally, recent structural data indicate that N1L, a VV protein linked to NF-κB regulation (21), structurally resembles Bcl-2 family members and interacts with BH3 peptides, yet N1L possesses no obvious BH domains or other sequence similarity with cellular Bcl-2 proteins (5). Determination of the three-dimensional structure of F1L and FPV039 will help elucidate the evolutionary relatedness of FPV039, F1L, and M11L and determine if F1L and FPV039 physically resemble cellular Bcl-2 proteins, as we predict for FPV039 (Fig. 1).

Notably, fowlpox virus is currently being pursued as a potential vaccine vector and gene therapy tool (10, 62). Although fowlpox virus is incapable of replicating in mammalian cells and causing a productive infection, mammalian cells do support the transcription and translation of native or recombinantly inserted early genes (63). As such, understanding how FPV039 interacts with Bcl-2 proteins and inhibits apoptosis in a mammalian context may have significant implications in the development of vaccines and gene therapy vectors using fowl-
pox virus. In addition, understanding how FPV039, FIL, and M11L, three seemingly unrelated poxviral proteins, interact with Bcl-2 family members and inhibit apoptosis will provide significant advancements in our understanding of the pathways that govern cellular fate and will ultimately shed light on the mechanisms of Bcl-2 family regulation.

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