Vaccinia virus encodes a previously uncharacterized mitochondrial-associated inhibitor of apoptosis

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To circumvent apoptotic death, many viruses encode Bcl-2 homologous proteins that function at the mitochondria. Vaccinia virus, the prototypic member of the *Poxviridae* family, does not encode a Bcl-2 homolog but inhibits the mitochondrial arm of the apoptotic cascade by an unknown mechanism. We now report that F1L, a previously unidentified protein in vaccinia virus, is responsible for the inhibition of apoptosis. Cells infected with vaccinia virus are resistant to staurosporine-mediated cleavage of poly(ADP-ribose) polymerase, caspases 3 and 9, and release of cytochrome *c*. In contrast, a vaccinia virus deletion mutant, VV811, was unable to inhibit apoptosis; however, the antiapoptotic function was restored by expression of the F1L ORF, which is absent in VV811. Although F1L displays no homology to members of the Bcl-2 family, it localizes to the mitochondria through a C-terminal hydrophobic domain. We show that expression of F1L interferes with apoptosis by inhibiting the loss of the inner mitochondrial membrane potential and the release of cytochrome *c*.

Apoptosis is a major antiviral response used by multicellular organisms for the coordinated removal of infected cells (1, 2). Apoptosis involves the activation of a family of cysteine proteases, referred to as caspases, that mediate the ordered dismantling of infected cells (2). Recent advances have also identified mitochondria as important regulating organelles during apoptosis (3, 4). Apoptosis results in mitochondrial alterations, such as loss of the mitochondrial membrane potential ($\Delta\Psi_m$), production of reactive oxygen species, and release of proapoptotic proteins including SMAC, HtrA2, apoptosis inducing factor, endonuclease G, and cytochrome *c* (4). Mitochondrial-released cytochrome *c* initiates caspase activation through an interaction with the adapter molecule Apaf-1 subsequently promoting recruitment and activation of caspase 9 (5). Both pro- and antiapoptotic members of the Bcl-2 family tightly regulate the mitochondrial checkpoint (6).

Multiple viruses, including vaccinia virus, a member of the poxvirus family, have evolved mechanisms that target crucial components within the apoptotic cascade (7, 8). The poxvirus-encoded cytokine response modifier A (CrmA), referred to as SPI-2 in vaccinia virus, effectively inhibits caspases 1 and 8 and is one of the best studied antiapoptotic proteins (9, 10). Viruses also encode obvious Bcl-2 homologous proteins that function at the mitochondria to inhibit apoptosis (11). The importance of mitochondria during apoptosis, as well as the lack of a Bcl-2 homolog in the genome of vaccinia virus, led us to speculate that vaccinia virus would encode a unique protein to inhibit the mitochondrial component of apoptosis. Despite the lack of an obvious Bcl-2 homolog in the genome of vaccinia virus, we previously demonstrated that Jurkat cells infected with vaccinia virus strain Copenhagen (Cop), which is devoid of the functional caspase inhibitor, CrmA, were protected from apoptosis induced by staurosporine and anti-Fas (12). We now report the identification and characterization of a unique vaccinia virus-encoded protein, F1L, that localizes exclusively to mitochondria where it functions to regulate the apoptotic cascade by inhibiting loss of the inner mitochondrial membrane potential and inhibiting release of cytochrome *c*.

Materials and Methods

Cells and Viruses. Jurkat were cultured as described (12). Jurkat cells stably expressing Bcl-2 were generated as described (13). HeLa cells were grown in DMEM (GIBCO) supplemented with 10% FCS (GIBCO), 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. Recombinant vaccinia virus strain Copenhagen expressing β-galactosidase, VV65, was provided by G. McFadden (Robarts Research Institute, London, ON, Canada) (14). The deletion viruses VV811 and VV759 were a generous gift from Enzo Paoletti (Virogenetics, Troy, NY) (15).

Plasmid Construction. The F1L ORF was amplified from virus DNA by PCR by using 5′-CTCGAGATGTTGTCGATGTTTATG, containing a *Xho*I restriction site, and 5′-GGATCCTTATCCTATCATGTATTT, containing a *Bam*HI restriction site and subcloned into pEGFP-C3 (Clontech). A truncated version of F1L (1–206) was generated by PCR by using 5′-CTCGAGATGTTGTCGATGTTTATG, containing a *Xho*I restriction site, and 5′-GGATCCTTACTTTAGATATTCACGCGTGCT, containing a *Bam*HI restriction site, and subcloned into pEGFP-C3. An N-terminal-deleted version of F1L (199–226) was generated by PCR by using 5′-CTCGAGACTAGCACGCGTGAATAT, containing a *Xho*I restriction site, and 5′-GGATCCTTATCCTATCATGTATTT, containing a *Bam*HI restriction site and cloned into pEGFP-C3. For the infection/transfection assays, the plasmid pSC66 was used to express enhanced GFP (EGFP) and F1L under the control of the early/late synthetic poxviral promoter. The EGFP-coding sequence was liberated from pEGFP-N3 (Clontech) with *Sal*I and *Not*I and subsequently subcloned into pSC66. The F1L ORF was subcloned into the *Not*I site of pSC66. For construction of pSC66-F1L-FLAG-N, which contains a FLAG-tag at the N terminus, the F1L ORF was amplified by PCR with F1L-FLAG-N-*Sal*I, 5′-GTCGACATGGACTACAAAGACGATGACGACAAGTTGTCGATGTTTATGTGT, containing a *Sal*I restriction site and 5′-CTCGAGTTATCCTATCATGTATTT-3′, containing a *Xho*I restriction site. For stable expression, a copy of the F1L ORF was subcloned into the *Sal*I site of the eukaryotic expression vector BMGneo and designated BMGNeo-F1L-HA-N (16).

Generation of Stable Transfected Cell Lines. Jurkat cells (5×10^6) were stably transfected with 20 μg of BMGneo or BMGneo-F1L-HA-N by electroporation at 250 V with three 8-ms pulses (BTX, San Diego). Stable transfectants were selected in 1 mg/ml G418 (GIBCO) and subsequently cloned by serial dilution, screened for expression, and maintained in RPMI medium 1640 supplemented with 800 μg/ml G418 and 10% FCS.

Apoptosis Induction. Jurkat cells were induced to undergo apoptosis by the addition of 125 ng/ml or 250 ng/ml anti-Fas (clone CH11) (Upstate Biotechnology, Lake Placid, NY) and 1–2.5 μM staurosporine (Sigma). HeLa cells were treated with 125 ng/ml anti-Fas (clone CH11) (Upstate Biotechnology) and 5 μg/ml cycloheximide (Sigma). To inhibit caspase activity, 100 μM zVAD.fmk (Kamiya

Abbreviations: CrmA, cytokine response modifier; EGFP, enhanced GFP; TMRE, tetramethylrhodamine ethyl ester; PARP, poly(ADP-ribose) polymerase.

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Biomedical, Thousand Oaks, CA) was added 30 min before the addition of apoptotic stimuli.

Detection of DNA Fragmentation. DNA fragmentation was assessed by using the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling method (Roche Diagnostics) as described (12). Analysis was performed on a Becton Dickinson FACScan, and data were acquired on a minimum of 10,000 cells per sample.

Cellular Fractionation. Cellular fractionation into cytosolic and membranous mitochondria-containing fractions was performed as described (12). Cytochrome *c* release was monitored by immunoblotting both the supernatant and membranous fractions.

Infection and Transfection Assay. Infection and transfection of Jurkat cells in the presence of VV811 was performed as described (17). In brief, 1×10^6 cells were transfected with 1 μg of pSC66EGFP and 4 μg of pSC66-F1L or 4 μg of pSC66 DNA by using DMRIE-C (Invitrogen) according to the manufacturer's protocol. Concurrent with the transfection process, Jurkat cells were infected with VV811 at a multiplicity of infection of 1 plaque-forming unit per cell.

Measurement of Mitochondrial Membrane Potential. The changes in mitochondrial membrane potential were quantified in cells by staining cells with tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes) (18, 19). Cells were loaded with TMRE by incubating cells in media containing 0.2 μM TMRE for 30 min at 37°C. Some cells were treated with 50 μM membrane uncoupler, carbonyl cyanide *m*-chlorophenyl hydrazone (Sigma). TMRE fluorescence was acquired through the FL-2 channel equipped with a 585 filter (42-nm bandpass), and detection of EGFP-positive cells was performed on a FACScan equipped with an argon laser with 15 mV of excitation at 488 nm.

Confocal Microscopy. HeLa cells were transfected with 2 μg of pEGFP-C3, pEGFP-F1L, pEGFP-F1L (1–206), or pEGFP-F1L (199–206) by using Lipofectamine 2000 (Invitrogen). Mitochodria were labeled with 15 ng/ml Mitotracker Red CMXRos (Molecular Probes) for 30 min at 37°C. When necessary, cells were treated with 125 ng/ml anti-Fas (clone CH11) and 5 μg/ml cycloheximide (Sigma) for 3 h to induce apoptosis.

To study the localization of F1L in the presence of vaccinia virus infection, 1×10^6 HeLa cells were transfected with 5 μg of pSC66 or pSC66-F1L-FLAG-N by using Lipofectamine 2000 and simultaneously infected with VV65 at an multiplicity of infection of 5 plaque-forming units per cell. Cells were stained with 10 μg/ml mouse anti-cytochrome *c* (clone 6H2.B4) (Pharmingen) followed by the addition of 10 μg/ml Alex Fluor 546 goat anti-mouse Ab (Molecular Probes) (20). To detect the FLAG-tagged F1L, cells were treated with 10 μg/ml anti-FLAG M2 mAb directly conjugated to FITC (Sigma). Cellular nuclei and DNA-containing virus factories were stained with 1 μg/ml Hoechst 33342 (Molecular Probes).

Immunoblotting. Whole cell lysates were analyzed by electrophoresis on SDS polyacrylamide gels, and proteins were transferred to nitrocellulose membranes (Osmonics, Minnetonka, MN). Cytochrome *c* and poly(ADP-ribose) polymerase (PARP) were detected by using an anti-cytochrome *c* Ab (clone 7H8.2C12) (Pharmingen) and an anti-PARP Ab (clone C2-10) (Pharmingen) at a dilution of 1:1,000 and 1:2,000, respectively. Caspase 3 and caspase 9 were detected with polyclonal rabbit anti-caspase 3 and anti-caspase 9 serum at a dilution of 1:5,000 and 1:2,000, respectively (12). The membranes were probed with goat anti-mouse horseradish peroxidase-conjugated Ab (Bio-Rad) at 1:3,000 or goat anti-rabbit horseradish peroxidase-conjugated Ab (Bio-Rad) at 1:10,000. Proteins were visualized with a chemiluminescent detection system (Amersham Pharmacia).

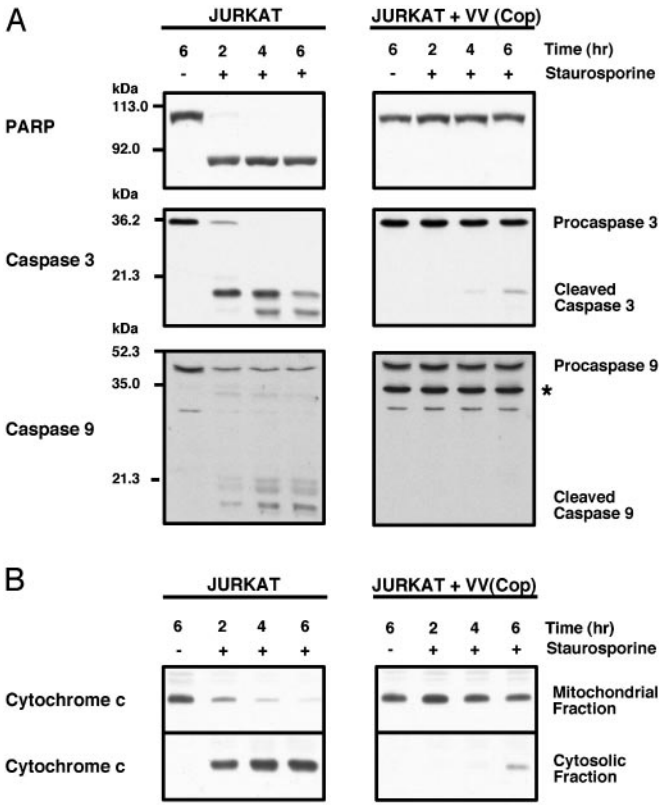

Fig. 1. Vaccinia virus (Cop) protects cells from staurosporine-induced apoptosis. (*A*) The cleavage of PARP caspases 3 and 9 were monitored by Western blot analysis. *, Crossreacting protein in vaccinia virus-infected lysates. (*B*) To detect cytochrome *c* release, cells were permeabilized with digitonin and fractionated into mitochondrial-containing membranous fractions and cytosolic fractions.

Results

Vaccinia Virus Infection Inhibits the Mitochondrial Component of the Apoptotic Cascade. The genome of vaccinia virus contains no ORFs with homology to known mitochondrial-localized apoptotic regulators (21). To determine whether vaccinia virus encoded a protein(s) that directly inhibited the mitochondrial apoptotic cascade, we initiated our studies by monitoring the ability of vaccinia virus (Cop), which is devoid of the caspase inhibitor CrmA, to inhibit postmitochondrial events. Jurkat cells were infected with vaccinia virus (Cop), and apoptosis was induced by staurosporine, a caspase-independent direct activator of the mitochondrial cascade (22, 23). As shown in Fig. 1*A*, Jurkat cells treated with staurosporine rapidly processed full-length PARP protein to its 89-kDa cleaved form, which was inhibited in the presence of vaccinia virus infection (Fig. 1*A*). To determine the effect of vaccinia virus infection on the activation of caspases 3 and 9 and the release of cytochrome *c*, infected Jurkat cells were treated with staurosporine and fractionated into membrane and cytosolic fractions. Mock-infected cells treated with staurosporine displayed activation of both caspases 3 and 9 and loss of cytochrome *c* from the membrane fraction to the cytosolic fraction (Fig. 1). In contrast, vaccinia virus infection significantly reduced the activation of both caspase 9 and caspase 3 and also prevented the translocation of cytochrome *c*, demonstrating that vaccinia virus infection blocked all postmitochondrial events tested (Fig. 1).

Vaccinia Virus Deletion Mutants Have Distinguishable Effects on Apoptosis. To identify the vaccinia virus-encoded gene(s) responsible for inhibiting apoptosis, we used two vaccinia virus (Cop)

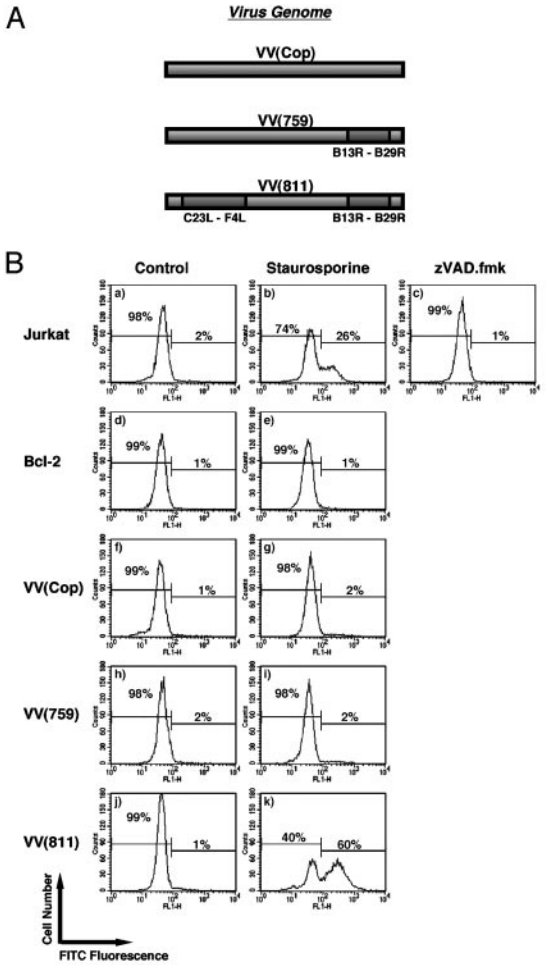

Fig. 2. Vaccinia virus deletion mutant VV811 is unable to protect cells from staurosporine-induced apoptosis. (*A*) Schematic representation of the vaccinia virus (Cop) deletion mutants. VV759 is missing ORFs B13R–B29R. VV811 is missing ORFs between B13R–B29R and C23L–F4L. (*B*) DNA fragmentation is blocked by infection with vaccinia virus (Cop) and VV759 but not VV811. (*C*) VV759 infection but not VV811 infection protects cells from apoptotic events downstream of the mitochondria. The cleavage of PARP, caspases 3 and 9, and cytochrome *c* were monitored by Western blot analysis. *, Crossreacting protein in vaccinia virus-infected lysates.

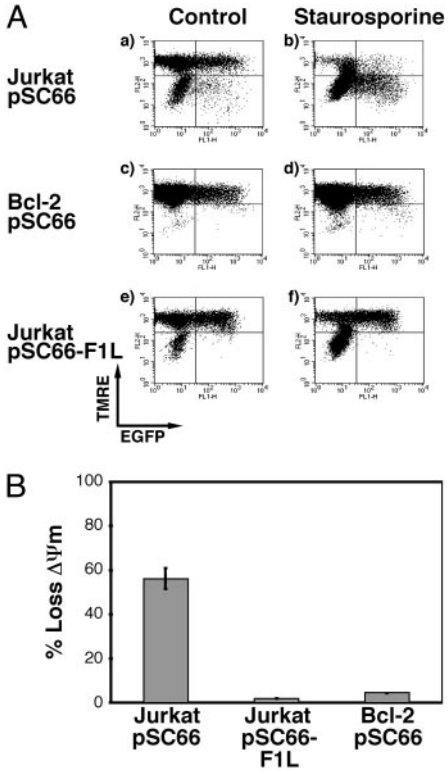

Fig. 3. The vaccinia virus F1L ORF restores the ability of VV811 to inhibit apoptosis. Jurkat cells were infected with VV811 and transfected with 1 μg of the reporter plasmid pSC66-EGFP in combination with 4 μg of pSC66 or pSC66-F1L. (*A*) At 5 h after infection/transfection, cells were treated with 1 μM staurosporine for 2 h, and the inner mitochondrial membrane potential was assessed by TMRE fluorescence. (*B*) Graphical representation of the experiment is described in *A*. SDs were calculated from three independent experiments.

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deletion viruses, VV759 and VV811 (15). VV759 is missing 18 ORFs at the right terminus of the vaccinia virus genome whereas VV811 lacks a total of 55 ORFs from both ends of the genome (Fig. 2*A*). To determine whether the deletion viruses VV759 and VV811 retained the ability to inhibit apoptosis, Jurkat cells were infected and treated with staurosporine, and apoptosis was monitored by assessing the levels of DNA fragmentation. As shown in Fig. 2*B*, treatment with staurosporine resulted in 26% of Jurkat cells positive for DNA fragmentation (Fig. 2*Bb*), which was completely inhibited by pretreatment with the broad-spectrum caspase inhibitor, zVAD.fmk (Fig. 2*Bc*). Jurkat cells overexpressing Bcl-2 were resistant to staurosporine-induced DNA fragmentation as expected (Fig. 2*Be*) (22, 23). Infection of Jurkat cells with vaccinia virus (Cop) inhibited DNA fragmentation (Fig. 2*Bg*) as did infection of Jurkat cells with VV759 (Fig. 2*Bi*). Jurkat cells infected with VV811, however, were no longer resistant to staurosporine-induced DNA fragmentation (Fig. 2*Bk*). In fact, infection with VV811 reproducibly augmented the level of DNA fragmentation detected after staurosporine treatment (Fig. 2*B*, compare *b* and *k*).

Because our data demonstrated that vaccinia virus (Cop) infection inhibited postmitochondrial events (Fig. 1), we asked whether VV811 was also capable of inhibiting these same postmitochondrial events. Jurkat cells were infected with either VV759 or VV811 and treated with staurosporine. Similar to infection with vaccinia virus (Cop) (Fig. 1), infection of cells with VV759 resulted in resistance to staurosporine-induced PARP, caspase 9, and caspase 3 cleavage, and cytochrome *c* release into the cytosolic fraction (Fig. 2*C*). In contrast, VV811 infection was unable to prevent cleavage of PARP, cleavage of caspases 3 and 9, and the release of cytochrome *c* (Fig. 2*C*). These observations clearly indicated that infection with VV811

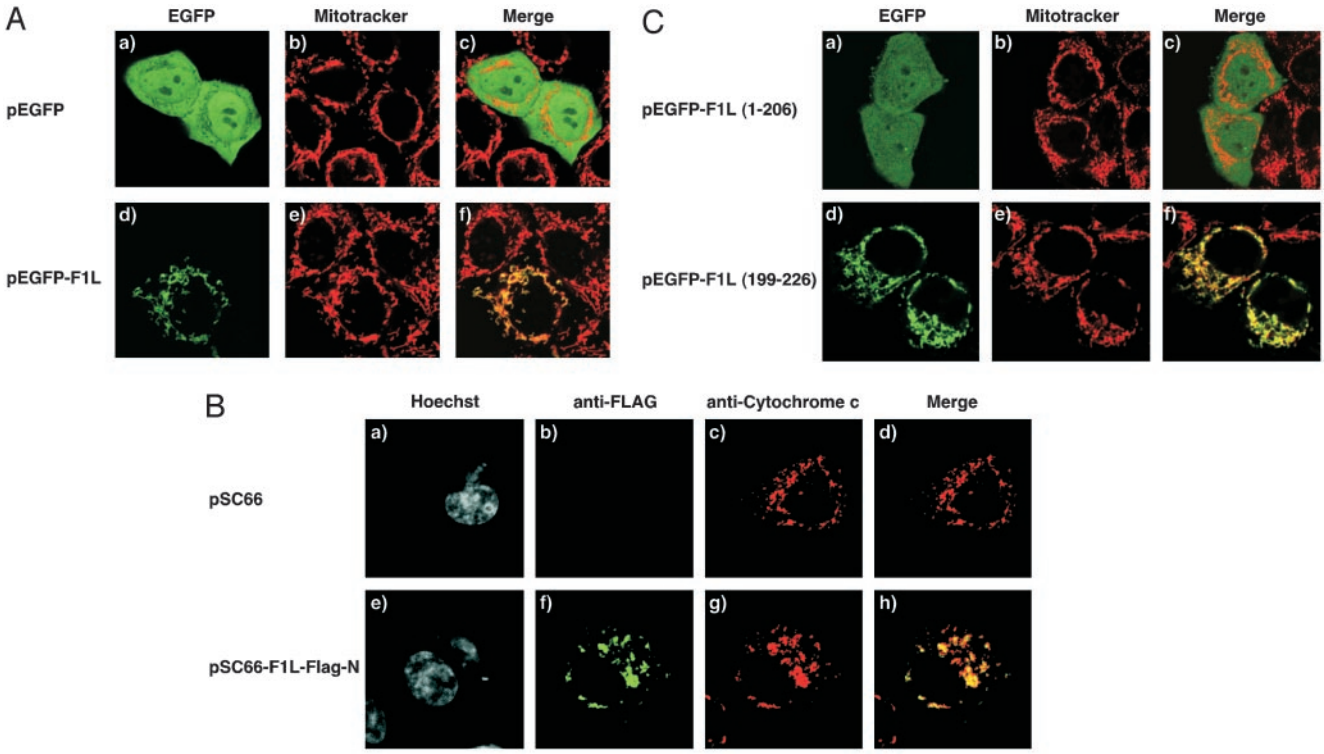

Fig. 4. The product of the vaccinia virus F1L ORF localizes to mitochondria. (*A*) Live HeLa cells were transiently transfected with either pEGFP (*a*) or an EGFP-tagged version of F1L, pEGFP-F1L (*d*) and visualized by confocal microscopy. Mitochondria were labeled with Mitotracker Red (*b* and *e*). Merged images (*c* and *f*) indicate that EGFP-F1L localizes to the mitochondria. (*B*) HeLa cells were infected with vaccinia virus strain Copenhagen and transfected with pSC66 or pSC66-F1L-FLAG-N. Mitochondria were labeled by using an anti-cytochrome *c* Ab (*c* and *g*). The nucleus and DNA-containing viral factories were labeled with Hoechst (*a* and *e*). The localization of FLAG-tagged F1L was visualized with a FITC-conjugated mouse-anti-FLAG Ab (*b* and *f*). Merged images from *f* and *g* indicate that F1L-FLAG-N localized to the mitochondria during vaccinia virus infection (*h*). (*C*) The C-terminal hydrophobic domain of F1L is necessary and sufficient for mitochondria localization. HeLa cells were transfected with a truncated version of F1L, pEGFP-F1L (1–206), or with pEGFP-F1L (199–226) containing the C-terminal region of F1L. Mitochondria were labeled with Mitotracker Red (*b* and *e*). Merging of *a* and *b* indicated that EGFP-F1L (1–205) did not localize with Mitotracker Red (*c*) whereas pEGFP-F1L (199–226) (*d*) localized to the mitochondria as indicated by the merged image (*f*).

was unable to inhibit the mitochondrial-mediated apoptotic pathway, including cytochrome *c* release, the pivotal step in the cascade (Fig. 2*C*). We observed that in the absence of staurosporine, VV811 infection resulted in some cleavage of PARP, caspase 3, and caspase 9 as well as release of cytochrome *c* (Fig. 2*C*), indicating that VV811 infection activated the apoptotic pathway in Jurkat cells.

F1L Protects VV811-Infected Cells from Staurosporine-Induced Apoptosis. We used VV811 to identify vaccinia virus ORFs necessary for inhibiting apoptosis by reintroducing ORFs into VV811-infected cells. Using this strategy, we found one vaccinia virus ORF, F1L, which could restore the inhibition of apoptosis. Jurkat cells were infected with VV811 and cotransfected with pSC66-EGFP in combination with either pSC66-F1L or pSC66. Infection with VV811, which is unable to inhibit apoptosis, drives the expression of both F1L and EGFP, which are placed under the control of a poxvirus promoter in pSC66, and EGFP expression serves as a reporter for infected and transfected cells. Apoptosis was monitored in the EGFP-positive population by assessing loss of the inner mitochondrial membrane potential by TMRE uptake (18, 19). All infected and cotransfected cells showed a population of cells that were positive for both EGFP expression and TMRE (Fig. 3*A a*, *c*, and *e*). After treatment with staurosporine, cells infected with VV811 and cotransfected with pSC66 and pSC66-EGFP demonstrated a population of cells with decreased ability to take up TMRE indicative of cells dying by apoptosis (Fig. 3*Ab*). Efficient TMRE uptake occurred in VV811-infected, pSC66-EGFP, and pSC66-cotransfected cells overexpressing Bcl-2 after treatment with staurosporine (Fig. 3*Ad*), demonstrating that Bcl-2 expression could replace the antiapoptotic protein(s) missing in VV811. Most importantly, cells infected with VV811 and cotransfected with pSC66-EGFP and pSC66-F1L, were competent for TMRE uptake in the presence of staurosporine (Fig. 3*Af*), and the results of three independent experiments are graphically represented in Fig. 3*B*. These results demonstrated that the expression of F1L could inhibit apoptosis during virus infection.

The Vaccinia Virus F1L Protein Localizes to Mitochondria. To determine the localization of F1L, an N-terminal EGFP-F1L fusion was generated and localization was visualized by confocal microscopy. Transfection of HeLa cells with EGFP alone resulted in a diffuse fluorescence signal throughout the cell (Fig. 4*Aa*), which did not colocalize with the mitochondrion specific dye Mitotracker Red (Fig. 4*Ac*). In contrast, cells transfected with EGFP-F1L displayed a punctate staining pattern that colocalized with Mitotracker Red, indicating that EGFP-F1L localized to mitochondria (Fig. 4*A d–f*).

To investigate the localization of F1L during virus infection, we generated an N-terminal Flag-tagged version of F1L under the control of a poxvirus promoter, pSC66-F1L-Flag-N. HeLa cells were infected with vaccinia virus and transfected with pSC66-F1L-Flag-N or pSC66 alone. Using an Ab specific for the Flag epitope, cells infected and transfected with the Flag-F1L construct demonstrated a staining pattern typical of mitochondrial localization, which colocalized with cytochrome *c* (Fig. 4*B g* and *h*), providing clear evidence that F1L also localizes to mitochondria during infection.

Sequence analysis of F1L demonstrated the presence of a C-terminal hydrophobic domain within F1L that was flanked by

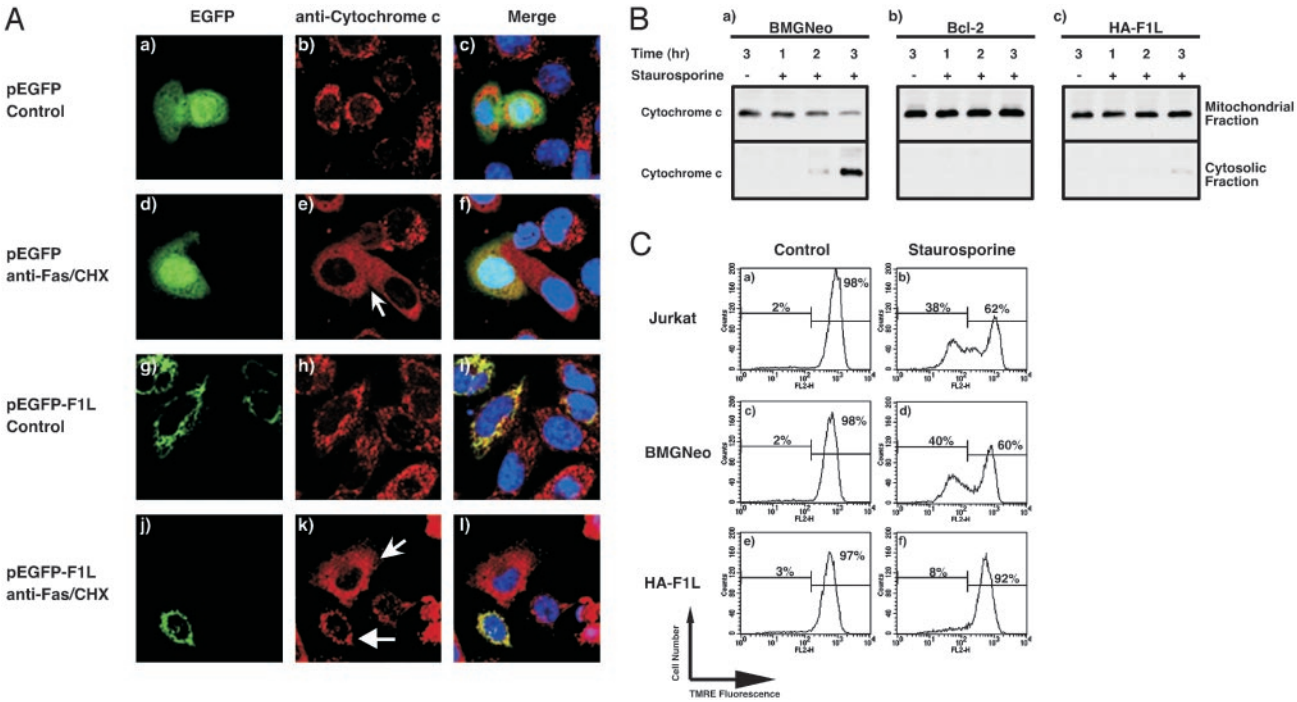

Fig. 5. F1L inhibits cytochrome *c* release from mitochondria. (*A*) HeLa cells were transfected with pEGFP (*a–f*) or with pEGFP-F1L (*g–l*). Transfected cells were treated with anti-Fas, and cytochrome *c* was detected by using anti-cytochrome *c*. DNA was stained with Hoechst to identify nuclei (*c*, *f*, *i*, and *l*). Untreated transfected cells showed mitochondrial localization of cytochrome *c* (*b*, *c*, *h*, and *i*). After treatment with anti-Fas, EGFP-transfected cells showed release of cytochrome *c* (*e* and *f*) denoted by arrow. Cytochrome c was retained in cells transfected with EGFP-F1L (*k* and *i*) denoted by arrows. (*B*) F1L expression inhibits cytochrome *c* release. Jurkat cells transfected with the BMGneo empty vector (*a* and *d*), overexpressing Bcl-2 (*b* and *e*) or expressing F1L (*c* and *f*) were treated with staurosporine. (*C*) F1L inhibits disruption of the mitochondrial membrane potential. Jurkat cells were treated with staurosporine, and the loss of the membrane potential was determined by using TMRE fluorescence.

positively charged residues and a short C-terminal hydrophilic tail. To determine whether this domain was necessary for mitochondrial localization, we generated an EGFP version of F1L, pEGFP-F1L (1–206), lacking the final 20 aa encompassing the hydrophobic domain and the short hydrophilic tail. HeLa cells transfected with pEGFP-F1L (1–206) demonstrated EGFP fluorescence throughout the cytoplasm and nucleus (Fig. 4*Ca*), which did not colocalize with Mitotracker Red (Fig. 4*C b* and *c*). This result suggested that the C-terminal domain of F1L played a critical role in mitochondrial localization. To determine whether the C-terminal domain was sufficient for mitochondrial localization, we appended the C-terminal fragment of F1L onto EGFP to create pEGFP-F1L (199–226). HeLa cells transfected with pEGFP-F1L (199–226) showed localization that mirrored mitochondria staining by Mitotracker Red (Fig. 4*C d–f*) clearly demonstrating that a mitochondrial targeting sequence was contained within amino acids 199–226 of F1L (Fig. 4*Cf*).

F1L Inhibits Apoptosis by Blocking Release of Cytochrome *c*. Evidence clearly indicates that mitochondria play a pivotal role in apotosis (3, 4). The presence of F1L at the mitochondria suggested that F1L may regulate apoptosis by regulating the inner mitochondrial membrane potential and inhibiting the release of proapoptotic proteins such as cytochrome *c*. To determine whether F1L expression could inhibit cytochrome *c* release, HeLa cells were transfected with pEGFP or pEGFP-F1L, treated with anti-Fas, and cytochrome *c* release was detected by confocal microscopy. In the absence of anti-Fas, confocal microscopy revealed a mitochondrial distribution of cytochrome *c* (Fig. 5*A b* and *h*). After the addition of anti-Fas, cells transfected with pEGFP showed a diffuse cytosolic pattern of cytochrome *c* staining indicating that cytochrome *c* was released from the mitochondria (Fig. 5*A e* and *f*). In contrast, we routinely found that cytochrome *c* was retained within the mitochondria in cells transfected with pEGFP-F1L after anti-Fas treatment (Fig. 5*A k* and *i*). In fact, surrounding cells not expressing EGFP-F1L showed cytochrome *c* release and membrane blebbing (Fig. 5*A k* and *i*). To determine whether F1L interfered with release of mitochondrial proteins, we generated Jurkat cells stably expressing F1L. Ten F1L and 10 empty vector transfected clones were assayed for resistant to DNA fragmentation after staurosporine treatment and eight of the 10 F1L clones inhibited apoptosis (data not shown). These stable F1L-expressing cell lines were used to confirm that F1L functioned to inhibit release of cytochrome *c*. Fig. 5*B* demonstrates that translocation of cytochrome *c* from the mitochondria occurred in Jurkat cells transfected with the empty vector at 2 and 3 h after treatment with staurosporine (Fig. 5*Ba*) whereas cytochrome *c* release was blocked in cells overexpressing Bcl-2 and in cells expressing F1L (Fig. 5*B b* and *c*).

To determine whether F1L expression inhibited the loss of the mitochondrial membrane potential, we treated the stable clones with staurosporine and monitored loss of the inner mitochondria membrane potential by TMRE uptake (Fig. 5*C*). As shown in Fig. 5*C*, treatment of Jurkat cells or BMGneo stable clones with staurosporine resulted in 38% and 40% of the cells showing decreased uptake of TMRE, respectively (Fig. 5*C b* and *e*). Jurkat cells expressing F1L maintained high levels of TMRE fluorescence after the addition of staurosporine (Fig. 5*Cg*), demonstrating that F1L expression functions independently of other vaccinia virus proteins to inhibit the loss of the inner mitochondrial membrane potential.

Discussion

Successful virus infection requires that viruses counteract numerous antiviral responses inflicted by the host immune system. The

Poxviridae family, of which vaccinia is a member, encode proteins aimed at interfering with the host immune response and apoptosis (8, 24). The best-studied antiapoptotic protein encoded by members of the poxvirus family is the caspase 8 inhibitor CrmA (9, 10). The importance of mitochondria in the apoptotic cascade led us to hypothesize that vaccinia virus would encode a protein to block the mitochondrial component of apoptosis. Although the genome of vaccina virus (Cop) was completely sequenced in 1990, sequence analysis failed to reveal the presence of any obvious antiapoptotic proteins that regulate the mitochondrial component, suggesting that vaccinia virus might encode a previously uncharacterized inhibitor (21). We previously demonstrated that cells infected with vaccinia virus (Cop), which is naturally devoid of the caspase inhibitor CrmA, still displayed resistance to apoptosis (12). We now report that the vaccinia virus protein F1L is responsible for inhibiting the mitochondrial arm of the apoptotic response.

Evidence now clearly indicates that mitochondria are central regulators of the apoptotic response by serving to directly initiate apoptosis in some situations and serving an amplification role in other situations (4, 25). Apoptosis results in both structural and physiological alterations to the mitochondria. We found that the expression of F1L inhibited loss of the inner membrane potential and postmitochondrial events by blocking the release of cytochrome *c*, the pivotal commitment step in the cascade.

Confocal studies revealed that F1L localized to the mitochondria during infection as well as in the absence of infection and localization to the mitochondria requires the presence of a C-terminal domain. This domain encompasses a transmembrane region flanked by positive charges and a short hydrophilic tail. By appending the C-terminal domain of F1L onto EGFP, we found that this domain was sufficient for mitochondrial localization. As such, F1L is a new member of a growing class of tail-anchored proteins that are specifically targeted to cellular membranes (26). Included in this class are proteins that target to the outer mitochondrial membrane, including Bcl-2. However, unlike Bcl-2, which localizes to the ER and nuclear membrane in addition to the mitochondria, we find that F1L localizes exclusively to the mitochondria where it functions to inhibit apoptosis (27).

To inhibit the mitochondrial component of the apoptotic cascade, a large number of viruses encode obvious Bcl-2 homologs (11). Members of the Bcl-2 family contain up to four different Bcl-2 homology domains that are important for function (6). Database searches have revealed no cellular proteins with homology to F1L. Homology between F1L and Bcl-2 was only detected within the conserved C-terminal mitochondrial localization domain. Recently, two other proteins that function to inhibit apoptosis at the mitochondria have been identified (28–30). vMIA, from human cytomegalovirus, and M11L, from myxoma virus, both localize to mitochondria and block the release of cytochrome *c* (28–30). F1L orthologs are present only in members of the *Orthopoxvirus* genus, which includes vaccinia virus, variola virus, monkeypox virus, and ectromelia virus. Members of the *Leporipoxvirus*, *Capripoxvirus*, *Yatapoxvirus*, and *Suipoxvirus* genera contain M11L orthologs. At present the only poxvirus known to contain a Bcl-2 homolog is fowlpox virus, which was identified based on the presence of conserved Bcl-2 homology domains (11, 31). All of the F1L orthologs sequenced to date display >95% amino acid identity within the final 226 aa. The region of greatest diversity occurs at the N terminus where strains of variola virus, camelpox virus, and ectromelia virus contain unique repeats. The significance of these repeats is currently unknown but cells infected with ectromelia virus are protected from apoptosis at the level of the mitochondria, suggesting that the F1L ortholog is functional (J.T. and M.B., unpublished data).

Members of the poxvirus family encode multiple antiapoptotic proteins with distinct mechanisms of action (8). This is undoubtedly reflective of the complexity of the apoptotic pathways and the wide range of cell types infected within the host. The identification of F1L in the genome of vaccinia virus clearly indicates the importance of regulating the mitochondrial apoptotic response and continues to demonstrate that vaccinia virus is striving to outplay the host immune response.

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