The N-terminus of the Vaccinia Virus protein F1L is an Intrinsically Unstructured Region that is not involved in Apoptosis Regulation

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ABSTRACT

Subversion of host cell apoptotic responses is a prominent feature of viral immune evasion strategies to prevent premature clearance of infected cells. Numerous poxviruses encode structural and functional homologs of the Bcl-2 family of proteins, and vaccinia virus harbors anti-apoptotic F1L that potently inhibits the mitochondrial apoptotic checkpoint. Recently F1L has been assigned a caspase-9 inhibitory function attributed to an N-terminal α-helical region of F1L spanning residues 1-15 (1) preceding the domain-swapped Bcl-2-like domains. Using a reconstituted caspase inhibition assay in yeast we found that unlike AcP35, a well-characterized caspase-9 inhibitor from the insect virus Autographa californica, F1L does not prevent caspase-9 mediated yeast cell death. Furthermore, we found that deletion of the F1L N-terminal region does not impede F1L anti-apoptotic activity in the context of a viral infection. Solution analysis of the F1L N-terminal regions using small-angle X-ray scattering indicates that the region of F1L spanning residues 1-50 located N-terminal from the Bcl-2 fold is an intrinsically unstructured region. We conclude that the N-terminus of F1L is not involved in apoptosis inhibition, and may act as a regulatory element in other signaling pathways in a manner reminiscent of other unstructured regulatory elements commonly found in mammalian pro-survival Bcl-2 members including Bcl-xL and Mcl-1.
important regulators of intrinsic or mitochondrially mediated apoptosis. The Bcl-2 family of proteins comprises pro-survival and pro-apoptotic members, which are characterized by the presence of Bcl-2 homology (BH) domains (5). Pro-survival Bcl-2 proteins such as Bcl-2, Bcl-xL and Mcl-1 maintain cell viability until their inactivation by BH3-only proteins such as Bim, Bad or Puma (6). BH3-only proteins are up-regulated after cellular insults including exposure to cytotoxic drugs or UV light, and activate the cellular apoptotic machinery (7,8). BH3-only proteins only harbor the alpha helical BH3 domain, which binds to a canonical binding groove on pro-survival Bcl-2 members (9,10), although recent evidence suggests that they may also bind transiently to an alternative site on multi-domain pro-apoptotic Bcl-2 such as Bax (11) and Bak (12). Up-regulation of BH3-only proteins leads to the activation of the essential pro-apoptotic proteins Bak and Bax (13) which drive MOMP (mitochondrial outer membrane permeabilization) (14), thus leading to the release of cytochrome C. Cytochrome C together with Apaf-1, the initiator caspase-9 and ATP form the apoptosome (15). Current models suggest that caspase-9 initially contributes to apoptosome formation in its uncleaved and inactive pro-form (16,17), and is activated via dimerization at the apoptosome platform that enables auto-activation via proteolysis. Activated caspase-9 then proteolytically activates the downstream effector caspases-3 and 7 (18), which ultimately leads to the destruction of the cell.

The importance of the Bcl-2 family of proteins in apoptosis regulation is reinforced by the observation that numerous viruses encode recognizable sequence homologs of Bcl-2 to subvert premature host cell apoptosis. These include Epstein-Barr virus BHRF1 (19), adenovirus E1B19K (20), Kaposi Sarcoma Herpesvirus KsBcl-2 (21), Fowlpoxvirus FPV039 (22,23) and herpesvirus saimiri vBcl-2 (24), and structural studies of some of these confirmed that they adopt a Bcl-2 fold (25-27). However a number of viral proteins have been identified that shared no discernible sequence identity to known inhibitors of apoptosis. These include myxoma virus M11L (28), cytomegalovirus vMIA (29) and its mouse counterpart m38.5 (30-32), deerpoxvirus DPV022 (33), sheeppoxvirus SPPV14 (34) and vaccinia virus F1L (35) and N1L (36). Structural studies of M11L (37,38) revealed that although it lacks sequence similarity to the Bcl-2 family of proteins it adopts a Bcl-2-like fold, and engages BH3 ligands utilizing the canonical BH3-domain binding groove (38). Vaccinia virus N1L was shown to also adopt a Bcl-2 like fold (39,40), which enabled it to assume dual-functionality by mediating intrinsic apoptosis via the canonical Bcl-2 binding groove as well as NF-kB signaling via an additional non-canonical site (41). Similarly, Deerpoxvirus DPV022 was shown to be a Bcl-2 like protein, albeit with a dimeric topology due to a domain swap (42).

Vaccinia virus encodes anti-apoptotic F1L, which has been shown to act on the intrinsic pathway of apoptosis (43). F1L is able to engage Bim (44,45) and Bak (46,47), and inhibits Bak activation by functionally replacing Mcl-1 during infection (48). Furthermore, F1L is able to inhibit Bax mediated apoptosis (44), presumably via an indirect mechanism since F1L appears to not engage Bax in the cellular context. Recently, the interaction of F1L with Bim has been shown to be the primary mechanism underlying F1L mediated inhibition of apoptosis in the context of a live viral infection (49). Although F1L lacks discernible sequence identity to the Bcl-2 family of proteins, the crystal structure of F1L revealed that it adopts a Bcl-2 fold in a domain-swapped dimer configuration (49,50). In its entirety, F1L from vaccinia virus (MVA) comprises 222 residues, of which only residues 57-190 form the canonical Bcl-2 like
domain. Although no experimental structural data is available for the N-terminal region of F1L, recent biochemical studies suggested a role in caspase inhibition for the N-terminal region preceding the Bcl-2 fold (51), as well as a role in modulating inflammasome regulation (52). Subsequent molecular modeling proposed the formation of two alpha helical segments (1) at the extreme N-terminus of F1L that engage in an inhibitory substrate complex with caspase-9, thus abrogating caspase-9 activity.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification - The coding sequence corresponding to MVA F1L amino acids 1-202 (Uniprot accession number O57173) was cloned into the pETDuet-1 vector. Protein expression was induced in *Escherichia coli* BL21(DE3) pLysS cells with 0.5mM IPTG for 4 hours at 37 °C. The cells were harvested and lysed using 0.2mM silica beads in the FastPrep instrument (MP Biomedicals) for 4 x 20 sec cycles. Cellular debris was removed via centrifugation at 16000 g for 20 minutes and filtered through a 0.22µm syringe filter. MVA F1L was purified with 2 x 1ml HiTrap column charged with nickel (GE Healthcare). The protein was further purified via size exclusion chromatography on a HiLoad 16/60 Superdex 75 prep grade column (GE Healthcare) into the final buffer of 25mM HEPES pH 7.5, 150mM NaCl and 5mM DTT. MVA F1L protein was concentrated to 3.9 mg/ml, flash frozen and stored at 193K.

Envelope Modeling - The scattering images were integrated, averaged, and calibrated against water using software specific to the beamline (53). The radius of gyration (R_g) and the forward scattering I(0) were determined by Guinier approximation using PRIMUS from ATSAS (54,55). Rigid body modeling was performed on the processed data using BUNCH (56) and CORAL (54). Both models were generated using a known crystal structure of MVA F1L (PDB accession code: 4d2m) lacking 50 amino acids at the N-terminus and 15 at the C-terminus. P2 symmetry was imposed to generate a dimer and the scattering curve of the 1.80mg/mL data was used. 14 residues were added to the sequence of F1L corresponding to the pDuet purification tag.

Yeast colony assays - *Saccharomyces cerevisiae* W303α cells were transformed with either pGALL(LEU2), pGALL(LEU2)-HA-Bax or pGALL(LEU2)-His6-Bak, or co-

transformed with pGALL(HIS3)-Apafl-11-530, pGALL(LEU2)-caspase-9 and pGALL (URA3)-caspase-3 or the corresponding empty vectors. Yeast bearing these plasmids were then transformed with either pGALL(TRP1), pGALL(TRP1)-Bcl-xL, pGALL(TRP1)-VACV(COP)-F1L or pGALL (TRP1)-AcP35. The pGALL(TRP1) and pGALL(LEU2) vectors place genes under the control of a galactose inducible promoter. Cells were spotted as 5-fold serial dilutions onto medium containing 2% w/v galactose (inducing), which induces protein expression, or 2% w/v glucose (repressing), which prevents protein expression, as previously described (59). Plates were incubated for 48 h (glucose) or 72 h (galactose) at 30˚C and then photographed.

**Cell lines** - HEK 293T and HeLa cells, both obtained from the American Type Culture Collection (ATCC), were maintained at 37°C and 5% CO2 in Dulbecco’s modified Eagle’s Medium (DMEM) (Invitrogen Corp.) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen Corp.), 200 µM l-glutamine (Invitrogen Corp.), 50 U of penicillin (Invitrogen Corp.) per mL, and 50 µg of streptomycin (Invitrogen Corp.) per mL.

**Plasmid Construction** – pGALL (TRP1)-VACV(COP)-F1L was generated by subcloning from synthetic cDNA encoding for full length wild-type VACV(COP)-F1L (Genscript) using available BamHI and EcoRI sites. pGALL(LEU2), pGALL(LEU2)-caspase-9, pGALL(HIS3)-Apafl-11-530, pGALL (URA3)-caspase-3, pGALL(TRP1), pGALL (TRP1)-Bcl-xL and pGALL(TRP1)-AcP35 have been previously described (60-62). pGALL(LEU2)-HA-Bax was kindly provided by Jamie Fletcher. FLAG-F1L(43-226), FLAG-F1L(50-226), and FLAG-F1L(60-226) were amplified by polymerase chain reaction (PCR) using codon-optimized pcDNA3-FLAG-F1L as a template and PWO (Pyrococcus woesei) polymerase (Roche). The forward primers used, all containing a BamHI restriction site, were 5'-GGATCCATGGAAGACTAAAGGACGATGAC GACAAGGAGACTGTTGACCCGATTCC -3' for FLAG-F1L(43-226), 5'-GGATCCATGGAAGACTAAAGGACGATGAC GACAAGGAGACTGTTGACCCGATTCC -3' for FLAG-F1L(50-226), and 5'- GGATCCATGGAAGACTAAAGGACGATGAC GACAAGGAGACTGTTGACCCGATTCC -3' for FLAG-F1L(60-226). The reverse primer used for all three constructs was 5'-GAATTCTAGCCGACATGATCTTCAAG -3' containing an EcoRI restriction site.

The three PCR products were subcloned into the shuttle vector pGemT (Promega) followed by an additional subcloning step into the final destination vector pcDNA3 (Invitrogen Corp.).

**Transient Transfection** - Transfection of HEK 293T and HeLa cells (1 × 106) seeded in 6cm cell culture dishes (Corning Inc.) was done using Lipofectamine 2000 (Invitrogen Corp.) according to the manufacturer’s instructions. Unless otherwise stated, cells were transfected with 2 µg pcDNA3-FLAG-A6L, pcDNA3-FLAG-BakΔBH3, pcDNA3-FLAG-F1L, pcDNA3-FLAG-F1L(43-226), pcDNA3-FLAG-F1L(50-226), or pcDNA3-FLAG-F1L(60-226), 1 µg pcDNA3-HA-Bak, or 0.5 µg pEGFP-C3. Transfected cells were supplemented with 20% FBS (DMEM, 20% FBS, 200 µM l-glutamine) 2 hours post-transfection and maintained at 37°C and 5% CO2.

**Whole Cell Lysates** - To determine the expression levels of the F1L N-terminal truncation mutants, HEK 293T cells were mock transfected or transiently transfected with pcDNA3-FLAG-BakΔBH3, pcDNA3-FLAG-F1L, pcDNA3-FLAG-F1L(43-226), pcDNA3-FLAG-F1L(50-226), or pcDNA3-FLAG-F1L(60-226). Following an 18 hour transfection period, cells were washed with phosphate-buffered saline (PBS) and suspended in 150 µl of SDS loading buffer.
containing 0.06M Tris pH 6.8 (Invitrogen Corp.), 2% SDS (Fischer Scientific), 32% glycerol (Anachemia), 0.05M betamercaptoethanol (Bioshop), and 0.005% bromophenol blue (BioRAD). Samples were analyzed by SDS-PAGE and immunoblotting.

**Confocal Microscopy** - To determine the subcellular localization of the F1L N-terminal truncation mutants, HeLa cells were seeded onto 18mm coverslips (Fisher Scientific) in 3.5cm diameter culture dishes (Corning Inc.). After 24 hours, $5 \times 10^5$ cells were transfected with pcDNA3-FLAG-A6L, pcDNA3-FLAG-F1L, pcDNA3-FLAG-F1L (43-226), pcDNA3-FLAG-F1L (50-226), or pcDNA3-FLAG-F1L (60-226). After a 12 hour transfection period the cells were fixed in 4% paraformaldehyde (Sigma-Aldrich), permeabilized in 1% NP-40 (Sigma-Aldrich), and blocked in 30% goat serum (Invitrogen Corp.). Cells were then stained with polyclonal rabbit anti-Flag M2 antibody (Sigma-Aldrich) at a dilution of 1:200 and monoclonal mouse anti-cytochrome c antibody (BD Pharmingen) at a dilution of 1:150. Signals were amplified with Alexa Fluor 488 conjugated donkey anti-mouse antibody (Invitrogen Corp.) and Alexa Fluor 546 conjugated donkey anti rabbit antibody (Invitrogen Corp.), both at a dilution of 1:400. Coverslips were mounted using mounting solution containing DAPI stain and visualized with a Zeiss Axiovert laser scanning microscope.

**Immunoprecipitation** - To detect the interaction between the N-terminal truncation mutants of F1L with Bak, HEK 293T cells were co-transfected with pcDNA3-HA-BAK along with pcDNA3-FLAG-A6L, pcDNA3-FLAG-F1L, pcDNA3-FLAG-F1L (43-226), pcDNA3-FLAG-F1L (50-226), or pcDNA3-FLAG-F1L (60-226). After 18 hours, transfected cells were lysed for 1.5 hours in 2% CHAPS lysis buffer containing 2% (w/v) CHAPS (Sigma-Aldrich), 150 mM NaCl, 50 mM Tris pH 8.0 (Invitrogen Corp.), and EDTA-free proteinase inhibitor (Roche). FLAG-tagged constructs in the cell lysates were immunoprecipitated with monoclonal mouse anti-FLAG M2 antibody (Sigma-Aldrich) (1:4000 dilution) for 2 hours, followed by precipitation of the immune complexes with lysis buffer-equilibrated protein G-sepharose beads (GE Healthcare) for 1 hour. Beads were washed three times in 2% CHAPS lysis buffer and resuspended in 50µl of SDS gel loading buffer. Lysate samples were acetone precipitated and suspended in 50µl of SDS gel loading buffer. The proteins were analyzed by loading 40% of each sample on SDS-PAGE gels and blotted for FLAG and Bak.

**Apoptosis Assay** - To determine the ability of the F1L N-terminal truncation mutants to protect against apoptosis, HeLa cells were co-transfected with pcDNA3-FLAG-A6L, pcDNA3-FLAG-F1L, pcDNA3-FLAG-F1L (43-226), pcDNA3-FLAG-F1L (50-226), or pcDNA3-FLAG-F1L (60-226) along with pEGFP-C3 at a ratio of 4:1 (FLAG:EGFP) which served as a marker of transfection. After an 18 hour transfection period, cells were treated with 10 ng/ml of tumor necrosis factor α (TNFα) (Roche) along with 5 µg/ml of cycloheximide (CHX) to induce apoptosis. Cells were then stained with 0.2 µM tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes) for 30 minutes. The cells were subsequently washed twice with PBS supplemented with 1% FBS and analyzed by flow cytometry. Flow cytometric analysis was performed on a Becton Dickinson FACScan with EGFP fluorescence measured through the FL-1 channel equipped with a 489nm filter (band pass, 42nm) and TMRE fluorescence measured through the FL-2 channel equipped with a 585nm filter (band pass, 42nm). Data were acquired on 20000 cells per sample with fluorescence signals at logarithmic gain. Standard deviations were generated from three independent experiments. To assess the expression of the
FLAG-tagged constructs in the presence of EGFP, whole cell lysates were harvested after an 18 hour transfection period and analyzed by SDS-PAGE and immunoblotting.

SDS-PAGE and Immunoblotting - Cellular lysates were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Lysates were suspended in SDS loading buffer, boiled for ten minutes, and run on 15% polyacrylamide gels. Proteins were then transferred to a polyvinylidene fluoride membrane (GE Healthcare) using a semi-dry transfer apparatus (TYLER Research Instruments) for 2 hours at 420 mA. Membranes were blocked in 5% skim milk powder in TBST (tris-buffered saline with 0.1% Tween) overnight at 4°C. The membranes were probed with monoclonal mouse anti-FLAG M2 antibody (1:5000) (Sigma-Aldrich) to detect FLAG-tagged constructs or polyclonal rabbit anti-Bak NT antibody (1:500) (Upstate) to detect Bak. Horseradish peroxidase-conjugated secondary donkey anti-mouse or donkey anti-rabbit antibody (Jackson Immunoresearch) was then used at a concentration of 1:25000. Proteins were visualized by chemiluminescence after treatment with ECL reagent (GE Healthcare).

RESULTS
To understand the function of the N-terminal region of F1L preceding the Bel-2 fold we investigated the solution structure of F1L with an intact N-terminus using small-angle X-ray scattering (Table 1). Recombinant MVA F1L (1-202) was measured at six concentrations, ranging from 0.12 to 3.6 mg/mL. The scattering curve profile is conserved throughout the concentration range tested with the exception of the highest concentration in which inter-particle interference is observed (Fig. 1A and Table 2). The scattering conforms to a straight line in the low q region on a Guinier plot (Fig. 1B) and the calculated radius of gyration does not vary significantly with the measured concentration range, suggesting an absence of significant concentration effects from the second highest concentration (Table 2).

The calculated molecular mass from I(0) on the absolute scattering scale across the concentration range is approximately 53 kDa, corresponding to a dimeric oligomerization state (Table 2). The dimer obtained was expected and previously observed in the known MVA F1L crystal structures (49,50). Details of the scattering analysis are summarized in Table 1. The experimentally determined Rg for F1L is 34 Å, compared to the calculated Rg from the crystal structure of F1L Bel-2 fold of 20.89 Å.

Since partial crystal structures of MVA F1L were available (49,50), we attempted to model the missing regions of the available structures using a rigid-body modeling approach. Modeling was carried out with CORAL (54) and BUNCH (56) using the monomeric F1L structure (51-186, PDB ID:4d2m) and generating the dimer via imposition of a P2 symmetry. A model for F1L obtained using BUNCH fits the experimental scattering data poorly, as indicated by a $\chi$ of 2.7 (data not shown). In contrast, a model calculated using CORAL resulted in an improved fit of the scattering curves with a value of $\chi$ of 1.96 (Fig. 2). In the model both F1L N-termini protrude away from the Bel-2 fold in an extended configuration spanning residues 1-50 in addition to the N-terminal hexahistidine tag (Fig 2). The shape of both F1L N-termini in the model suggest an absence of ordered secondary structure, thus rendering F1L residues 1-50 unfolded.

We next examined the ability of F1L to inhibit Apaf-1-activated apoptosis using a model system based on Saccharomyces cerevisiae. In this assay, expression of a constitutively active form of Apaf-1 together with both caspase-9 and caspase-3 results in yeast death (61), which can be efficiently
rescued by the overexpression of caspase inhibitors. Co-expression of full-length VACV(COP)F1L with Apaf-1, caspase-9 and caspase-3 did not protect yeast cells from cell death (Fig. 3A), when compared to the established potent pan-caspase inhibitor AcP35 (63). In contrast, in a complementary yeast-based assay where yeast growth arrest is induced by overexpression of Bak or Bax (64), VACV(COP)F1L was able to rescue yeast growth arrest during Bak overexpression, similar to mammalian Bcl-xL, suggesting that the lack of anti-caspase activity of F1L is not due to a lack of expression (Fig. 3B).

We then sought to define the contribution that the 60 N-terminal residues make to F1L mediated apoptosis inhibition. We generated FLAG-tagged F1L constructs spanning residues 43-226, 50-226 and 60-226 as well as full-length F1L. All constructs were expressed at comparable levels in HEK293T cells (Fig 4A), and fluorescence microscopy revealed that all constructs co-localized to mitochondria (Fig 4B), suggesting that the N-terminal part of F1L does not play a role in determining its subcellular localization. Next we determined the ability of N-terminally truncated VACV(COP)F1L to protect against TNFα induced apoptosis using flow cytometry. FLAG-F1L(43-226), FLAG-F1L(50-226), FLAG-F1L(60-226) and FLAG-F1L(1-226) inhibited TNFα induced cell death with comparable potency when compared to a control protein (vaccinia virus A6L), suggesting that deletion of the N-terminal 60 residues has no bearing on F1L-mediated inhibition of apoptosis (Fig 4C). Furthermore, co-immunoprecipitation experiments revealed that all F1L truncations retained their ability to bind Bak, suggesting that the truncation mutants are folded and active (Fig 4D).

DISCUSSION

Viruses utilize a range of strategies when subverting premature host cell apoptosis, including receptor homologs, IAPs, Bcl-2 homologs and direct caspase inhibitors. Although the vast majority of these effector molecules have been shown to fulfill only a single purpose to date, recently emerging evidence is pointing to the potential for multifunctionality (5), as showcased by vaccinia virus N1. In addition to being an inhibitor of the intrinsic apoptosis pathway (39,40), N1L also inhibits NF-κB, with this dual functionality being mediated via two independent binding sites on N1 (41). N1 dimerisation has proven crucial for the NF-κB inhibition, having no effect on apoptosis regulation and BH3-only protein binding.

Similarly, vaccinia virus F1L, an established Bcl-2 like anti-apoptotic protein, has been assigned additional functions: the ability to inhibit caspase-9 (51), as well as a role in inflammasome activation (52). According to previous studies, F1L inhibits the recruitment of procaspase-9 to Apaf-1 through its binding to caspase-9 by its N-terminal residues. These results were further supported by data that suggests that F1L N-terminal 15-residues motif is key for caspase-9 inhibition in vitro.

Since these activities were identified in an F1L N-terminal region of unknown structure, we investigated MVA F1L with its intact N-terminus using small angle X-ray scattering. Our structural analysis indicates that the MVA F1L N-terminal residues 1-50 prior to the Bcl-2 like domain form an extended unfolded region preceding the Bcl-2 globular fold previously described.

Since this F1L N-terminal domain should in principle be able to access the caspase-9 active site, we next investigated the ability of VACV(COP)F1L to inhibit caspase-9 in a reconstituted caspase-3/caspase-9/Apaf-1 system that efficiently mimics caspase activity in yeast. However, we were unable to observe any inhibition of caspase-9 by VACV(COP)F1L, in contrast to the established pan-caspase inhibitor AcP35, a
potent caspase inhibitor from the insect virus *Autographa californica* (63,65). Unlike VACV(COP)F1L, AcP35 fully prevented yeast death in this system, suggesting that VACV(COP)F1L is not a caspase-9, or indeed a caspase-3/Apaf-1 inhibitor. Furthermore, VACV(COP)F1L was able to rescue yeast from Bak induced growth arrest in a complementary yeast assay, suggesting that F1L is efficiently expressed in yeast in an active form. Lastly, mutant VACV(COP)F1L that lacked the N-terminal section implicated in caspase-9 inhibition showed no discernible effect on the efficiency and potency of VACV(COP)F1L mediated inhibition of apoptosis in cellular systems.

Mammalian pro-survival Bcl-2 family members have been shown to harbor intrinsically disordered regions (IDRs) in addition to a folded Bcl-2 domain, which have been assigned important regulatory functions. IDRs in the Bcl-2 proteins frequently bear multiple regulation sites, such as those for phosphorylation, de-amidation, and ubiquitination (66). Although the presence of IDRs is a recurring feature of the Bcl-2 family, their location within the protein varies. Bcl-2 and Bcl-xL both contain large IDRs of ~50 residues as insertions between the α1 and α2 helices, whereas in other family members such insertions are substantially shorter. In contrast, the loop in Mcl-1 connecting α1 and α2 is structured, with an extended IDR of low complexity spanning 160 residues N-terminally to be found prior to the Bcl-2 like fold (67). In Boo (68) and Bcl-B (69) an IDR insertion has been identified that connects the α5 and α6 helices. Viral Bcl-2 proteins appear to be largely free of IDRs (5). In BHRF1, the loop connecting α1 and α2 is unstructured in solution (26) but adopts a short helix in the crystal structure (27). All other structures of viral Bcl-2 proteins indicate a highly compact architecture with predominantly short loops connecting the alpha helical secondary structure elements. We now show that vaccinia virus F1L is a notable exception to this general observation, since it harbors a 60 residue unfolded region N-terminal to the Bcl-2 fold. The N-terminal 60 residues in F1L do not display substantial sequence variations amongst F1L ORFs from a range of vacciniaviruses or related poxviruses, suggesting that the majority if not all N-terminal regions in the various F1L ORFs are unstructured (Fig. 5).

A number of F1L homologs have been identified within the poxviridae, including ectromelia virus EVM025, monkeypox virus C7L and variola virus F1L, however only EVM025 and variola virus F1L have been studied in any detail. Both EVM025 and variola virus F1L were shown to be apoptosis inhibitors, with EVM025 inhibiting Bak directly, whereas Bax mediated apoptosis was inhibited via the sequestration of Bim (70). In contrast, variola virus F1L was only able to inhibit Bak mediated apoptosis, and did not show any ability to engage Bim (71). Both EVM025 and variola virus F1L harbor long N-terminal extensions prior to their Bcl-2 fold, however neither has been functionally characterized.

We have shown that the N-terminal region prior to the Bcl-2 fold in vaccinia virus F1L adopts an extended, unstructured configuration. Furthermore, biochemical and cellular assays suggest that this region is unable to functionally inhibit caspase-9. The lack of impact of deletion of the extended N-terminal region of F1L on its ability to inhibit apoptosis suggests that any capability of this region to inhibit caspases is vestigial, and only plays a minor role in modulating apoptosis.
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Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: SC and BM designed, performed and analyzed the experiments shown in Figure 1 and 2 and contributed to writing the manuscript. RB and SC designed, performed and analyzed the experiments shown in Figure 4. DP and CH designed, performed and analyzed the experiments shown in Figure 3. MB designed experiments in Figure 4, conceived the project and contributed to writing the manuscript. MK designed experiments in Figure 1 and 2, conceived the project and wrote the manuscript. All authors analyzed the results and approved the final version of the manuscript.

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FOOTNOTES
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FIGURE LEGENDS

FIGURE 1. F1L 1-202 SAXS analysis and oligomeric state. A) Log plot of SAXS raw data. Concentrations are in descending order, commencing at 3.60 mg/mL followed by 1.80, 0.82, 0.43, 0.21 and 0.12 mg/mL. B) Guinier plots of SAXS data. Concentrations are as in A).

FIGURE 2. F1L 1-202 CORAL envelope and fit to the scattering data (1.80mg/mL).

FIGURE 3. F1L is not able to prevent caspases mediated yeast cell death. A) Reconstitution of the caspase activation pathway (Apaf-1, caspase-9 and caspase-3) in Saccharomyces cerevisiae. Yeast were co-transformed with constructs encoding Apaf-1, caspase-9 and caspase-3 and the indicated apoptosis regulatory proteins or empty vector, each under the control of an inducible (GAL) promoter. 5-fold serial dilutions were spotted onto inducing galactose or repressing glucose plates. Colony size indicates growth rate and colony number reflects cell viability. Each dilution was also spotted onto a control plate (glucose) to verify that equivalent numbers of each transformant were spotted. B) Yeast co-transformed with constructs encoding Bax or Bak and the indicated pro-survival proteins, each under the control of an inducible (GAL) promoter, were spotted onto inducing galactose or repressing glucose plates as 5-fold serial dilutions. A,B) images are representative of 2 independent experiments. Dotted lines indicate where an irrelevant lane was spliced out of the plate photographs.

FIGURE 4. Functional characterization of F1L truncation mutants. A) VACV(COP)F1L N-terminal truncations expression levels in HeLa cells. B) Subcellular localization of VACV(COP)F1L N terminal truncations in HeLa cells. HeLa cells were transiently transfected with empty vector (a-c), FLAG-F1L (g-i), FLAG-F1L(43-226) (j-l), FLAG-F1L(50-226) (m-o), FLAG-F1L(60-226) (p-r) or FLAG-A6L (d-f) as a control. 12 hours post-transfection cells were stained with rabbit anti-FLAG M2 antibody and imaged using a Zeiss Axiovert laser scanning microscope. Mitochondria were visualized by staining for cytochrome c. C) VACV(COP)F1L N-terminal truncations potently protect HeLa cells against TNFα-induced apoptosis. Apoptosis was induced with 10 ng/mL TNFα combined with 5 mg/mL cycloheximide. Apoptosis was assessed by quantifying TMRE fluorescence via flow cytometry, and the percentage of cells that demonstrated a loss of mitochondrial membrane potential (ΔΨm) is given on the y axis. All experiments were performed in triplicate, error bars show standard deviation. D) F1L truncations efficiently immunoprecipitate Bak. HEK293T cells were co-transfected with pcDNA3-HA-BAK as well as with pcDNA3-FLAG-A6L, pcDNA3-FLAG-F1L, pcDNA3-FLAG-F1L(43-226), pcDNA3-FLAG-F1L(50-226), or pcDNA3-FLAG-F1L(60-226). FLAG tagged F1L was immunoprecipitated with monoclonal mouse anti-FLAG M2 antibody, Bak was detected using a polyclonal rabbit anti-Bak NT antibody (a) and FLAG-F1L constructs were detected using mouse anti-FLAG M2 antibody (b). Panels (c) and (d) show whole cell lysates probed with polyclonal rabbit anti-Bak NT antibody or mouse anti-FLAG M2 antibody as loading controls, respectively.
FIGURE 5. Sequence alignment of F1L proteins N-terminus from different Orthopoxviruses. Sequence alignment of N-terminal 70 residues of MVA F1L (Uniprot number: O57173), VACV-IOC (Uniprot number: A5HD14), VACV-WR (Uniprot number: P24356), CTGV (Uniprot number: A5HDH9), MPXV-C7L (Uniprot number: Q8V547), CPXB-BR (Uniprot number: Q8QN17), VARV-YUG72 (Uniprot number: Q0N5C2) and ECTV EVM025 (Q8JLH9). Conserved regions are boxed in red. X highlighted with a green box represents the following sequence: NGIVQDIDNGIVQDIDNGIVQDIDNGIVQDIDNGIVQDIDNGIVQDIDNGIVQDIDNGIVQDIDNGIVQDIDNGIVQDIDNGIVQDIDNGIVQDIDNGIVQDIDNGIVQDIDNGIVQDIDNGIVQDIDNGIVQDIDNGIVQDIDNGIVQDIDNGIVQDIDNGIVQDIDNGIVQDIDNGIVQDIDNGIVQDIDNGIVQDIDNGIVQDIDNGIVQDIDNGIVQDIDNGIV.

TABLES

TABLE 1. Data-collection and scattering-derived parameters

| Data-collection parameters | Value |
|----------------------------|-------|
| **Instrument**             | SAXS/WAXS beamline Australian Synchrotron |
| Beam geometry (µm)         | 80 x 200 |
| Wavelength (keV)           | 12    |
| $q$ range (Å$^{-1}$)       | 0.025-0.500 |
| Exposure time (sec)        | 1 (per frame, 18 frames) |
| Concentration range (mg ml$^{-1}$) | 0.12 - 3.60 |
| Temperature (K)            | 293   |
| Structural parameters †    |       |
| $I(0)$ (cm$^{-1}$) (from Guinier) | 0.073 ± 0.000 |
| $R_g$ (Å) (from Guinier)   | 34.10 ± 0.243 |
| Molecular-mass determination† |       |
| Partial specific volume (cm$^3$ g$^{-1}$) § | 0.728 |
| Contrast ( Δρ x 10$^{10}$ cm$^{-2}$) § | 3.021 |
| Molecular mass Mr [from I(0)] (Da) | 51679 |
| Calculated monomeric Mr from sequence (Da) | 25290 |

Software employed

- **Primary data reduction**: SAXS/WAXS beamline software
- **Data processing**: PRIMUS
- **Rigid body modelling**: CORAL, BUNCH
- **Three-dimensional graphics representation**: PyMOL
- **Graphics representation**: EXCEL, SASPLOT

† Reported for 1.80 mg ml$^{-1}$ § Determined with MULCh (Whitten, A. E., Cai, S. & Trewhella, J. (2008). J. Appl. Cryst. 41, 222-226.)

TABLE 2: Summary of the SAXS data and analysis of MVA F1L 1-202 oligomeric state in solution.
| Concentration (mg ml\(^{-1}\)) | \(R_g\) (Å) | Oligomeric state |
|-------------------------------|------------|-----------------|
| 3.60                          | 34.80      | 2.20            |
| 1.80                          | 34.10      | 2.04            |
| 0.82                          | 33.70      | 2.09            |
| 0.43                          | 33.10      | 2.12            |
| 0.21                          | 31.50      | 2.14            |
| 0.12                          | 32.50      | 2.17            |
Figure 1

A

F11 - Log plot

B

F11 - Guinier region

- 3.60 mg/mL
- 1.80 mg/mL
- 0.82 mg/mL
- 0.43 mg/mL
- 0.21 mg/mL
- 0.12 mg/mL
Figure 3

A

Apaf/9/3

vector

vector

F1L

AcP35

Bcl-xL

inducing

repressing

B

His6-Bak

vector

vector

F1L

AcP35

Bcl-xL

HA-Bax

vector

vector

F1L

AcP35

Bcl-xL
Figure 4

A

| Sample                | kDa |
|-----------------------|-----|
| FLAG-A6L              |     |
| FLAG-F1L              |     |
| FLAG-F1L(43-226)      |     |
| FLAG-F1L(50-226)      |     |
| FLAG-F1L(60-226)      |     |

WB: mouse α-FLAG M2

C

% loss Δμm

| Sample                |     |
|-----------------------|-----|
| FLAG-A6L              |     |
| FLAG-F1L              |     |
| FLAG-F1L(43-226)      |     |
| FLAG-F1L(50-226)      |     |
| FLAG-F1L(60-226)      |     |

D

| Sample                | kDa |
|-----------------------|-----|
| Mock                  | a   |
| FLAG-A6                |     |
| FLAG-F1L(43-226)      |     |
| FLAG-F1L(50-226)      |     |
| FLAG-F1L(60-226)      |     |
| HA-Bak                |     |

IP: Mouse α-FLAG M2
WB: Rabbit α-BakNT

Lysate: WB: Mouse α-FLAG M2
The N-terminus of the Vaccinia Virus protein F1L is an Intrinsically Unstructured Region that is not involved in Apoptosis Regulation
Sofia Caria, Bevan Marshall, Robyn-Lee Burton, Stephanie Campbell, Delara Pantaki-Eimany, Christine J. Hawkins, Michele Barry and Marc Kvansakul

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