Ectromelia virus encodes an anti-apoptotic protein that regulates cell death

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ABSTRACT

Apoptosis serves as a powerful defense against damaged or pathogen-infected cells. Since apoptosis is an effective defense against viral infection, many viruses including poxviruses, encode proteins to prevent or delay apoptosis. Here we show that ectromelia virus, the causative agent of mousepox encodes an anti-apoptotic protein EVM025. Here we demonstrate that expression of functional EVM025 is crucial to prevent apoptosis triggered by virus infection and staurosporine. We demonstrate that the expression of EVM025 prevents the conformational activation of the pro-apoptotic proteins Bak and Bax, allowing the maintenance of mitochondrial membrane integrity upon infection with ECTV. Additionally, EVM025 interacted with intracellular Bak. We were able to demonstrate that EVM025 ability to inhibit Bax activation is a function of its ability to inhibit the activity of an upstream BH3 only protein Bim. Collectively, our data indicates that EVM025 inhibits apoptosis by sequestering Bak and inhibiting the activity of Bak and Bax.

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Introduction

Apoptosis, also known as programmed cell death, is a tightly regulated mechanism of cell death that is critical for the disposal of unwanted cells and defense against pathogen infection (Gerschenson and Rotello, 1992; McIlwain et al., 2013; Taylor et al., 2008). Apoptosis can be triggered through two distinct pathways, the extrinsic and the intrinsic pathway. The mitochondrion acts as a critical control point for the intrinsic pathway (Green, 2005; Tait and Green, 2010). During cellular stress leading to intrinsic apoptosis, mitochondria undergo a loss of the mitochondrial outer membrane potential (MOMP) and release death promoting proteins such as cytochrome c, SMAC/Diablo, and Omi/Htr2A (Chipuk et al., 2010; Cleland et al., 2010; Hengartner, 2000). These death promoting molecules result in the activation of the apoptosome leading to cell death (Cullen and Martin, 2009; Green, 2005; McIlwain et al., 2013).

The mitochondrial pathway is controlled by both pro- and anti-apoptotic members of the Bcl-2 family that are characterized by the presence of Bcl-2 homology (BH) domains (Chipuk et al., 2010; Taylor et al., 2008). Anti-apoptotic Bcl-2 proteins including Bcl-2, Bcl-xL, Mcl-1, Bcl-w, and A1 (Chipuk et al., 2010). Members of the anti-apoptotic Bcl-2 family contain a mitochondrial targeting sequence that functions to localize it to the mitochondria. Bak

and Bax are two pro-apoptotic proteins that play a pivotal role in triggering apoptosis, these are frequently kept inactive by anti-apoptotic members such as Bcl-2, Bcl-xL, and Mcl-1 (Westphal et al., 2011; Wolter et al., 1997). In the absence of an apoptotic stimulus Bax is predominantly found in the cytoplasm or loosely associated with intracellular membranes. Upon activation Bax undergoes a conformational change that exposes the N-terminus, and Bax re-localizes to the outer mitochondria membrane (Annis et al., 2005; Antonsson et al., 1997; Wolter et al., 1997). In contrast, Bak is normally found associated with the outer mitochondrial membrane. Once activated, Bak and Bax form high molecular weight homo-oligomers that facilitate the release of molecules like cytochrome c and SMAC/Diablo (Antignani and Youle, 2006; Cleland et al., 2011; Karbowski et al., 2006). Cells deficient in Bax and Bak are protected from mitochondrial apoptosis (James et al., 2007).

Since apoptosis is an important anti-viral defense it is not surprising that many viruses have developed strategies to prevent or delay apoptosis (Benedict et al., 2002; Teodoro and Branton, 1997). For example, a number of viruses encode Bcl-2 homologs that function at the mitochondria and inhibit the release of cytochrome c (Cuconati and White, 2002; Galluzzi et al., 2008; Polster et al., 2004). Additionally, several viruses encode mitochondrial proteins that inhibit apoptosis, but lack obvious sequence homology with the cellular Bcl-2 family members; these include K7, encoded by Kaposi's sarcoma virus, and vMIA, encoded by human cytomegalovirus (Cuconati and White, 2002; Kuida et al., 1995; Polster et al., 2004). The Poxviridae are large DNA viruses that encode multiple proteins that interfere with cell signaling pathways. Importantly, poxviruses

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encode many anti-apoptotic proteins, including M11L from myxoma virus, FPV039 from fowlpox virus, DPV002 from deerpox virus, SPPV014 from sheeppox virus, and CrmA encoded by cowpox virus (Banadyga et al., 2007, 2011; Everett et al., 2000; Miura et al., 1995; Okamoto et al., 2012; Taylor and Barry, 2006). Cowpox virus-encoded cytokine response modifier A (CrmA), also known as Spi-2, inhibits both Fas- and tumor necrosis factor (TNF) induced apoptosis by inhibiting the activity of caspase 1 and caspase 8 (Dbaibo et al., 1997; Miura et al., 1995). Previously, our lab identified F1L, a unique anti-apoptotic protein encoded by vaccinia virus (VACV) (Wasilenko et al., 2003). F1L localizes to the outer mitochondrial membrane and inhibits cytochrome c release in response to a wide variety of apoptotic stimuli (Stewart et al., 2005; Wasilenko et al., 2005). Although F1L lacks obvious sequence homology to Bcl-2 family members, F1L inhibits the activation of both Bak and Bax by binding to Bak and the BH3 only protein Bim (Campbell et al., 2010; Taylor et al., 2006; Wasilenko et al., 2005). The crystal structure of F1L from the vaccinia virus-modified strain Ankara indicated that despite lack of apparent sequence homology to cellular Bcl-2 proteins, F1L adopts a Bcl-2-like fold (Kvansakul et al., 2008).

Ectromelia virus (ECTV) is the causative agent of mousepox and a member of the Orthopoxvirus family that is closely related to VACV and variola virus (Esteban and Buller, 2005). Being a member of the Orthopoxvirus family, we suspected that ECTV also encoded an inhibitor of apoptosis. Using genome analysis we identified EVM025. Based on the C-terminal homology between EVM025 and F1L, we hypothesized that EVM025 could inhibit apoptosis. Here we report that human and mouse cells infected with ECTV are resistant to staurosporine (STS) and UV-induced apoptosis. We generated a recombinant ECTV lacking a functional EVM025 that replicated efficiently in tissue culture but was unable to protect against staurosporine-induced apoptosis. Expression of EVM025 is critical for maintaining the mitochondrial membrane potential and inhibiting apoptosis during viral infection. EVM025 localizes to the mitochondria through the C-terminal transmembrane domain and prevents Bak activation. EVM025 prevents Bax activation by sequestering the BH3-only protein Bim. Collectively, our data suggest that the ECTV protein EVM025 is a inhibitor of the intrinsic apoptosis pathway, which functions together with other viral anti-apoptotic proteins to prolong the life of an infected cell.

Results

Ectromelia virus infection inhibits apoptosis

Viruses have evolved a number of strategies to inhibit the cellular apoptosis pathway for continued survival in a host. ECTV
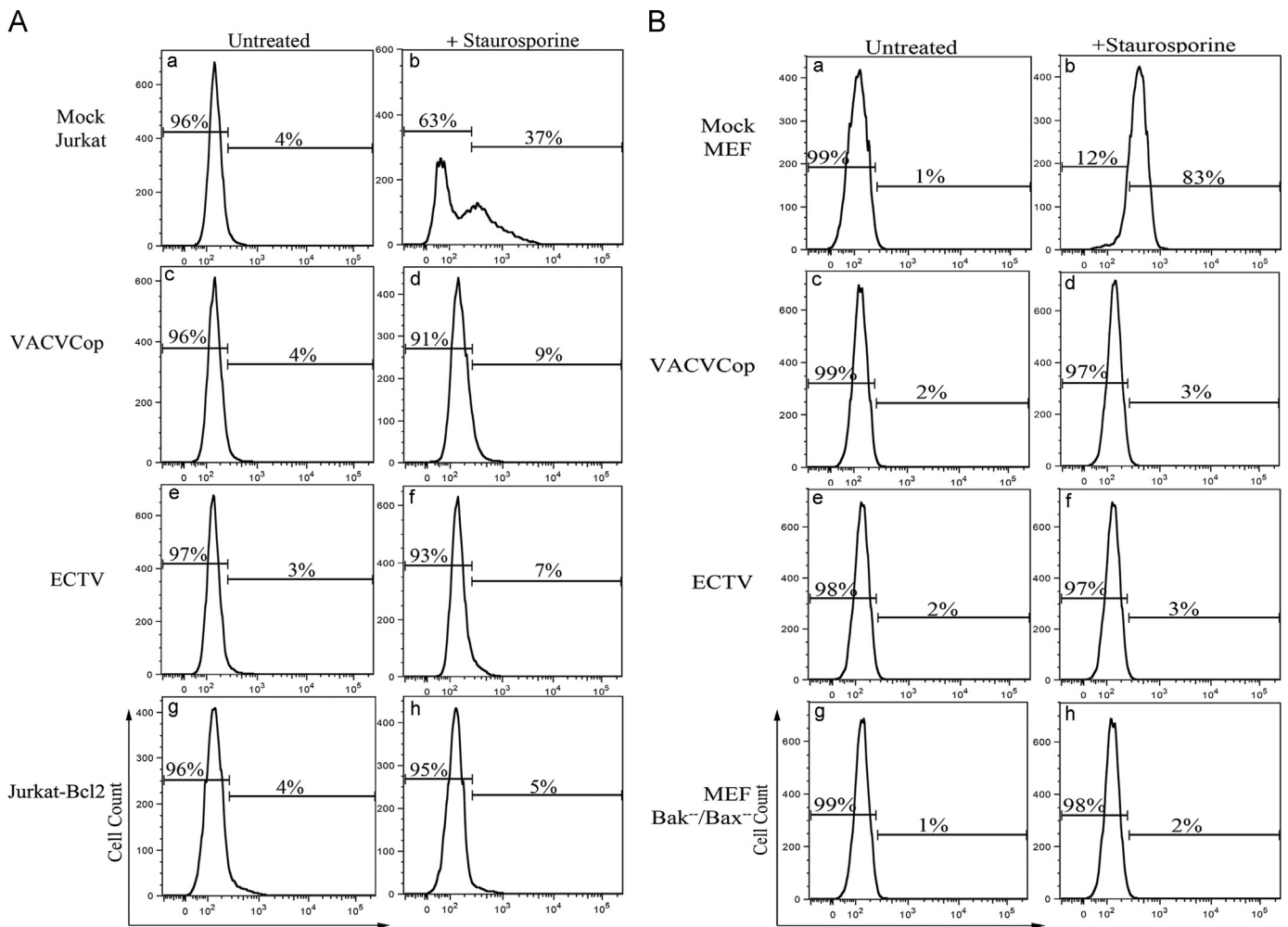

Fig. 1. ECTV infection inhibits apoptosis. (A) Jurkat cells were either mock-infected (a, b), infected with VACVcop (c, d) or infected with ECTV (e, f) at an MOI of 5. Jurkat cells overexpressing Bcl-2 were also mock-infected (g, h). After 6 h of infection cells were either treated with 2 μM STS for 120 min to induce apoptosis (b, d, e, h) or mock-treated (a, c, e, g). (B) MEF cells were either mock-infected (a, b), infected with VACVCop (c, d) or ECTV (e, f) at an MOI of 5. MEFBak$^{-/-}$Bax$^{-/-}$ cells were mock-infected (g, h). Six hours post-infection cells were treated with 3 μM STS for 120 min to induce apoptosis. Apoptosis was assessed by TUNEL fluorescence. Untreated controls showed low levels of TUNEL fluorescence, while mock-infected cells exposed to Staurosporine (STS) were TUNEL positive due to DNA fragmentation. Data are representative of at least three independent experiments.

encodes a known anti-apoptotic protein, CrmA, that inhibits caspase-8 activation following treatment with TNFα or FasL, but CrmA is unable to inhibit the intrinsic apoptotic cascade (Dbaibo et al., 1997; Miura et al., 1995). To determine whether ECTV has the ability to inhibit the intrinsic apoptosis pathway, we treated poxvirus-infected cells with STS (staturosporin), a compound that exclusively triggers induction of the intrinsic apoptosis pathway. Jurkat cells were either mock-infected (Fig. 1A, panels a and b), infected with VACVCop (Fig. 1A, panels c and d), or ECTV (Fig. 1A, panels e and f), and treated with 2 μM STS to induce apoptosis (Fig. 1A, panels b, d, f, and h). Jurkat cells constitutively expressing Bcl-2 were used as a positive control (Fig. 1A, panels g and h). Mock-infected Jurkat cells, cells infected with VACVCop or ECTV, and Jurkat-Bcl-2 were left untreated and displayed basal levels of DNA fragmentation indicative of healthy cells (Fig. 1A, panels a, c, e, and g). Jurkat cells treated with STS exhibited an increase in fluorescence that is characteristic of DNA fragmentation and apoptosis (Fig. 1A, panel b). As previously observed, infection with VACVCop inhibited STS induced DNA fragmentation (Fig. 1A panels c and d). Additionally, cells infected with ECTV were also protected against 2 μM STS induced apoptosis (Fig. 1A panel d and e). Similarly, infection of human and mouse cell with ECTV inhibited UV-induced apoptosis (data not shown). These data indicate that ECTV is capable of inhibiting apoptosis in human cell lines.

Since mice are the natural hosts of ECTV, we assessed the ability of ECTV to block cell death in murine cells (MEFs). MEFBak$^{-/-}$Bax$^{-/-}$ cells were used as a positive control since they are resistant to mitochondrial apoptosis (Lindsten et al., 2000; Wei et al., 2001). Untreated MEF and MEFBak$^{-/-}$Bax$^{-/-}$ cells, as well as MEF cells infected with VACVCop or ECTV were stained with TUNEL, and displayed minimal basal levels of DNA fragmentation (Fig. 1B, panels a, c, e, and g). MEF cells treated with STS exhibited an increase in TUNEL fluorescence characteristic of apoptosis (Fig. 1B, panel b). Infection with either VACVCop or ECTV significantly inhibited STS-induced DNA fragmentation in MEF cells (Fig. 1B). It was also observed that ECTV was able to inhibit apoptosis triggered by UV (data not shown). These data suggest that ECTV is capable of inhibiting apoptosis triggered by stimuli that are specific to the intrinsic pathway in both human and murine cell lines.

Intrinsic apoptosis is associated with the activation and cleavage of caspase-3 and poly-ADP ribose polymerase (PARP) (Duriez and Shah, 1997). Caspase-3 is an initiator caspase, while PARP is a nuclear protein that is cleaved upon caspase activation (Duriez and Shah, 1997; Gobeil et al., 2001). To confirm that ECTV can inhibit the intrinsic apoptotic cascade, we monitored caspase-3 and PARP activation following treatment with STS. Jurkat cells were mock-infected, infected with VACVCop or ECTV, at an MOI of 5. Six hours post-infection, cells were treated with 2 μM STS for 2, 4 and 6 h to trigger apoptosis. Activated caspase-3 and cleaved PARP were detected in mock-infected Jurkat cells treated with STS two hours post-treatment, and PARP was completely processed into the cleaved form following four hours of STS treatment (Fig. 2). In contrast, infection of Jurkat cells, with either VACVCop or ECTV, prevented the activation of caspase-3 and PARP following STS treatment. These data suggest that ECTV infection can block apoptosis somewhere upstream of caspase-3 activation.

ECTV encodes EVM025 a homologue of VACV F1L

VACV encodes F1L, an inhibitor of mitochondrial apoptosis (Campbell et al., 2010; Stewart et al., 2005; Taylor et al., 2006; Wasilenko et al., 2005), and orthologues of F1L are only found within members of the Orthopoxviridae (Fig. 3). EVM025, the ectromelia virus orthologue, is 95% identical to F1L, but encodes a large N-terminal extension comprised of up to 30 copies of an eight
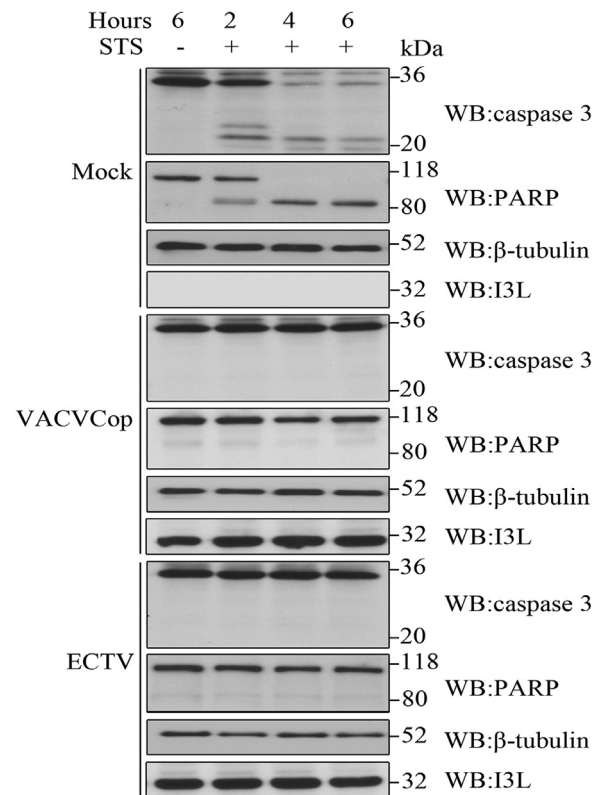

Fig. 2. ECTV protects cells from staurosporine-induced apoptosis. Jurkat cells were mock-infected, infected with VACVCop, and ECTV at an MOI of 5. Six hours post-infection cells were treated with 2 μM STS. Samples were collected at 2, 4, and 6 h post-STS treatment and cells were lysed in 1% NP40 cell lysis buffer. Samples were subjected to SDS-PAGE and immunoblotted for PARP and caspase 3 to assess apoptosis. β-tubulin was used as a loading control and I3L as a control for infection.

amino acid motif, "Asp-Asn-Gly-Ile-Val-Gln-Asp-Ile" (DNGIVQDI). This eight amino acid motif is also present twenty-five times in EVM025 from ECTV strain Naval, and so far is unique to EVM025 of ectromelia virus as it is absent in other orthopoxvirus F1L orthologues, nor is it present in any other known cellular or viral proteins. Intriguingly, variola virus (VARV) Garcia also contains a large N-terminal extension, consisting of 36 aspartic acid-rich repeats (DDI) that are absent in VARV Bangladesh, and the function of these repeat regions is currently unknown. The C-terminal amino half of EVM025 shares 95% sequence identity with VACV Cop F1L, although there are multiple substitution mutations, as well as a single DI insertion mutation (Fig. 3). Given the high degree of sequence identity between F1L and EVM025, we hypothesized that EVM025 might also act as an anti-apoptotic protein to facilitate ECTV pathogenesis.

EVM025 expression protects cells against intrinsic apoptosis

To further elucidate the anti-apoptotic mechanism of EVM025 and its importance in modulating the cellular apoptotic response during virus infection, we generated two recombinant viruses; one virus that is devoid of EVM025 (ECTVΔ025), and a double deletion virus that is missing both EVM025 and CrmA (ECTVΔ025/ΔCrmA). The genetic profile and purity of the knockout and revertant virus was confirmed by PCR in MEF cells (Fig. 4).

To determine the contribution of EVM025 during virus infection, MEF cells were mock-infected or infected with ECTV, ECTVΔ025, ECTVΔCrmA, ECTVΔ025/ΔCrmA and ECTVΔ025rev at a MOI of 5. After 6 h of infection the cells were treated with 2 μM STS for an additional 2 h to trigger apoptosis, and the mitochondrial membrane potential was monitored using TMRE staining
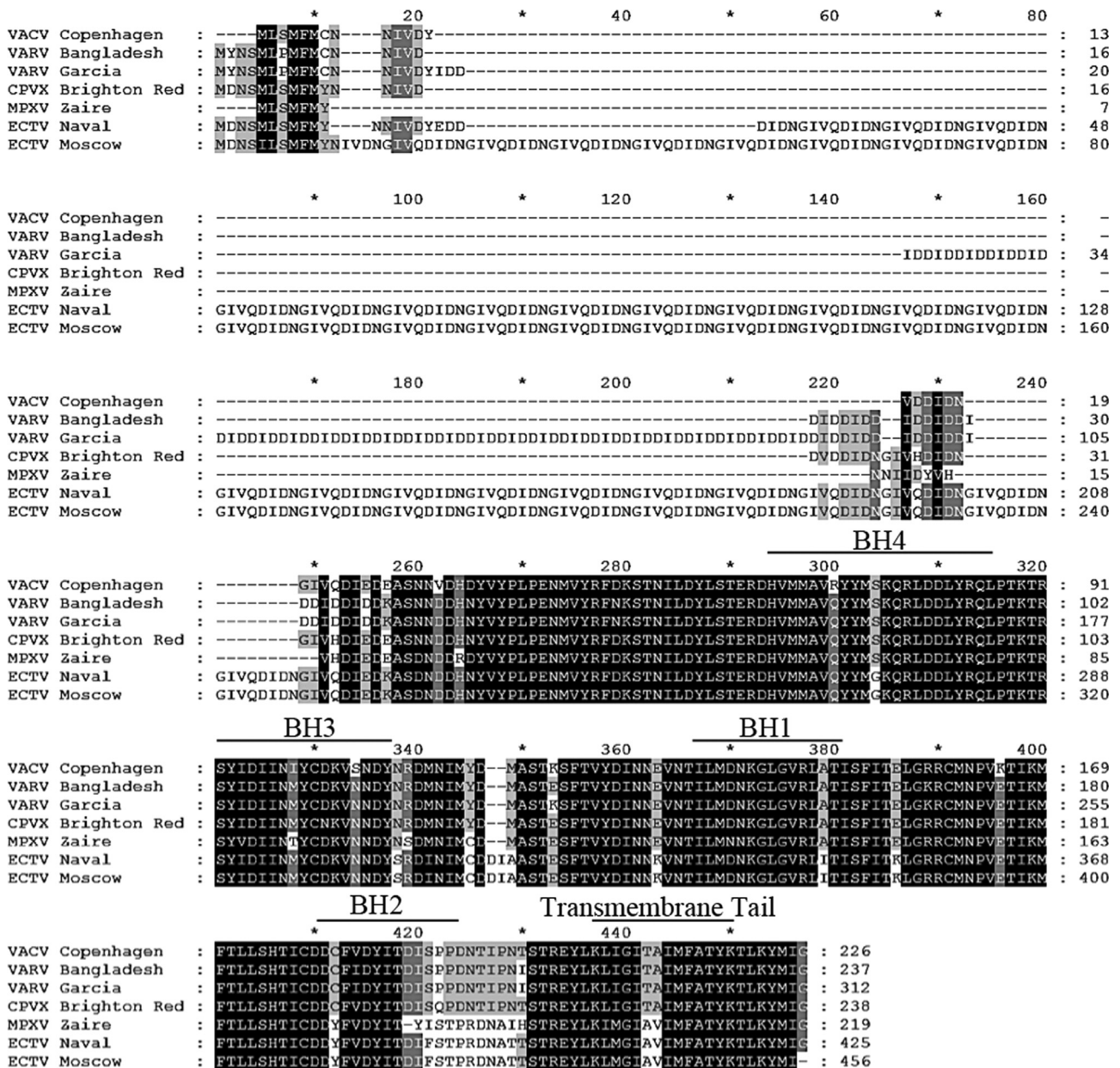
Fig. 3. Sequence alignment of EVM025 and its orthologs. Sequence alignment of the following orthologs: VACVCop F1L (AAA48014), VARV Bangladesh C5L (AAA60773), VARV Garcia E1L (CAB54625), CPXV Brighton Red CPXV048 (NP_619836.1), MPXV Zaire C7L (AAY97031), ECTV strain Naval EVM025 (VP0042282), and ECTV strain Moscow EVM025 (NP_671543). Sequences alignments were generated using the ClustalW program with a BLOSUM32 matrix. The conserved BH (BH homology) domains and transmembrane tail of the proteins are marked with the over-lines. The black shaded regions are amino acids conserved though all the proteins. The gray shaded regions are amino acids conserved among most proteins.

(Ehrenberg et al., 1988; Farkas et al., 1989). In the absence of STS treatment, mock-infected cells retained TMRE in healthy respiring mitochondria and demonstrated high levels of TMRE fluorescence (Fig. 5A, panel a). In contrast, mock-infected cells treated with STS showed a clear loss of TMRE fluorescence, indicative of the loss of inner mitochondrial membrane potential and apoptosis (Fig. 5A, panel a). MEF cells infected with ECTV were protected against the loss of inner mitochondrial membrane potential following STS treatment (Fig. 5A, panel b). Interestingly, loss of TMRE fluorescence was observed in MEF cells infected with ECTVΔ025 and subsequently treated with STS (Fig. 5A, panel c). Similar to ECTV, MEF cells infected with ECTVΔCrmA and ECTVΔ025rev were protected against loss of mitochondrial respiration upon STS treatment. However, MEF cells infected with ECTVΔ025/ΔCrmA were unable to maintain the inner mitochondrial membrane

potential. These data indicate that EVM025, and not CrmA, is required for ECTV to inhibit STS-induced apoptosis.

Following loss of mitochondrial membrane potential, cytochrome c is released which triggers the activation of the apoptosome leading to intrinsic apoptosis (Willis and Adams, 2005). We next assessed the role of EVM025 in preventing cytochrome c release during virus infection. Jurkat cells were infected with ECTV, ECTVΔ025, ECTVΔCrmA, and ECTVΔ025/ΔCrmA, treated with 2 μM STS and analyzed at 2, 4, and 6 h post-stimulation (Fig. 5B) for cytochrome c release analysis by western blot. While cytochrome c was rapidly released from mitochondria into the cytosol in mock-infected cells treated with STS, cytochrome c was retained in the mitochondria of ECTV-infected Jurkat cells, indicating that ECTV protected cells from STS-induced cytochrome c release. Similar protection was also observed in Jurkat cells
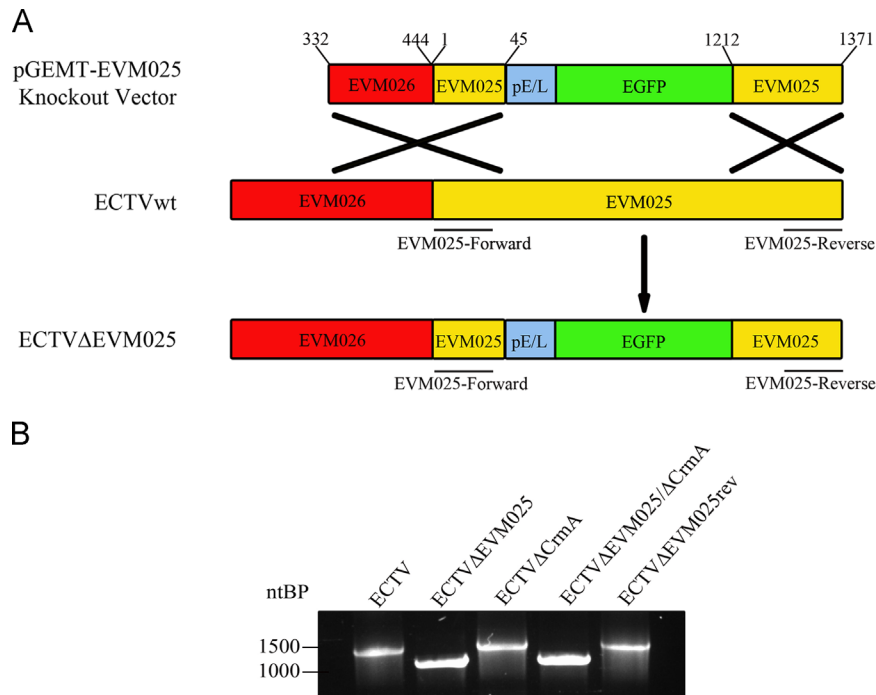
Fig. 4. Characterization of the recombinant ECTV devoid of EVM025. (A) Schematic diagram of the strategy employed for the generation of ECTVΔ025 virus. The binding site of the EVM025 sequencing primers as also indicated in the figure. (B)Viral DNA was extracted from BGMK cells infected with ECTV, ECTVΔEVM025, ECTVΔCrmA, ECTVΔEVM025/ΔCrmA, and ECTVΔEVM025rev. Successful deletion of EVM025 was confirmed by PCR using EVM025-forward primer and EVM025-reverse primer.

infected with ECTVΔCrmA. In contrast, cells infected with ECTVΔ025 or ECTVΔ025/ΔCrmA and treated with STS demonstrated abundant cytosolic cytochrome c. Together these data suggest that EVM025 is important in preserving the mitochondrial membrane integrity to block apoptosis.

Jurkat cells were analyzed for STS-induced PARP cleavage after infection with ECTV, ECTVΔ025, ECTVΔCrmA, and ECTVΔ025/ΔCrmA. Mock-infected Jurkat cells rapidly processed full length PARP into the cleaved form upon STS treatment. Four hours post-STS treatment full-length PARP was completely converted into the 89 kDa cleaved form (Fig. 5C). Infection was confirmed using an antibody to the early ECTV protein I3L, and β-tubulin was used as a loading control (Fig. 5C). As expected, ECTV and ECTVΔCrmA protected against STS-induced PARP cleavage. However, infection with ECTVΔ025 and ECTVΔ025/ΔCrmA was unable to protect against STS-induced PARP cleavage.

In addition to assessing the ability of ECTV to block STS-induced apoptosis, we also asked whether EVM025 was sufficient to protect against infection-induced cell death, as virus infection can induce apoptosis. MEF cells were either mock-infected or infected with ECTV, ECTVΔ025, ECTVΔCrmA, or ECTVΔ025/ΔCrmA. MEF cells infected with ECTV and ECTVΔCrmA showed no change in the amounts of full length PARP for 48 h post-infection. In contrast, MEF cells infected with ECTVΔ025 or ECTVΔ025/ΔCrmA induced PARP cleavage as early as 12 h post-infection, and by 36 h post-infection, no full length PARP was detected in these infected cells. These data indicate that EVM025 prevents the induction of apoptosis during ECTV infection. This experiment highlights the importance of EVM025 during in-vitro infection.

EVM025 is a tail-anchored protein that localizes to the mitochondria

The anti-apoptotic protein Bcl-2 possesses a C-terminal tail anchor that targets proteins to the mitochondrial membrane (Chipuk et al., 2010; Youle and Strasser, 2008). Sequence analysis of EVM025 confirmed the presence of a thirteen amino acid hydrophobic domain flanked by positively charged lysines at positions 436 and 450 in EVM025, suggesting that EVM025 localizes to

the mitochondria. To evaluate whether EVM025 localizes to the mitochondria, HeLa cells were transfected with either pEGFP-C3, pEGFP-EVM025, or pEGFP-EVM025($\Delta438-453$), a mutant construct that lacked the predicted transmembrane domain. Sixteen hours post-transfection cells were stained with MitoTracker to visualize the mitochondria (Fig. 6). Cells transfected with the control vector pEGFP showed a diffuse pattern of GFP expression (Fig. 6, panel e). In contrast, cells transfected with pEGFP-EVM025 displayed a reticular fluorescence pattern that co-localized with mitochondrial staining (Fig. 6, panel g). When the predicted transmembrane domain was removed (EVM025($\Delta438-453$)), a diffuse staining pattern similar to the control vector was observed (Fig. 6, panel j). These data suggest that like Bcl-2, EVM025 is a mitochondria-localized protein, and this localization is dependent on the C-terminal transmembrane domain.

EVM025 interacts with Bak to prevent Bak activation

The activation of Bak is an essential step in the intrinsic apoptotic pathway that precedes the release of cytochrome c from the mitochondria (Antignani and Youle, 2006; Westphal et al., 2011). Following an apoptotic stimulus, Bak undergoes a conformational change that results in the exposure of the N-terminus, resulting in destabilization of the outer mitochondrial membrane potential, loss of mitochondrial membrane integrity, and the release of molecules like cytochrome c and SMAC/Diablo (Chipuk et al., 2010). To determine whether EVM025 could prevent the conformational change of Bak, we assessed Bak activation following apoptotic stimulation. Jurkat cells were either mock-infected or infected with ECTV, ECTVΔCrmA, ECTVΔ025, or ECTVΔ025/ΔCrmA, and activation of Bak and exposure of the Bak N-terminus was induced by STS treatment (Fig. 7A). Exposure of the Bak N-terminus during Bak activation was detected with a conformation-specific anti-Bak AB-1 antibody and analyzed by flow cytometry. As expected, uninfected cells treated with STS exhibited a significant increase in fluorescence intensity (Fig. 7A, panel a), indicating the conformational change and activation of in Bak. Infection of Jurkat cells with ECTV

or ECTVΔCrmA, on the other hand, inhibited the exposure of the Bak N-terminus following STS treatment (Fig. 7A, panel b). Infection with ECTVΔ025 and ECTVΔ025/ΔCrmA did not inhibit STS-induced Bak activation (Fig. 7A, panels c and d), suggesting that EVM025 is necessary to inhibit the activation of Bak in infected Jurkat cells. We next determined if EVM025 could interact with the pro-apoptotic molecule Bak. BMK cells were transfected with pEGFP, pEGFP-Bcl-2, or pEGFP-EVM025. Cells were lysed in 2% CHAPS, a detergent known to preserve the conformational integrity of the Bcl-2 family members (Yao et al., 2014). Immuno-complexes were precipitated using a GFP antibody and detected using anti-Bak antibody (Fig. 7B). Interestingly, EGFP-EVM025 associated with endogenous Bak, while no Bak co-immunoprecipitation was seen with GFP alone. As expected, pEGFP-Bcl-2 also co-precipitated Bak, as has been demonstrated previously. Overall, our data suggests that EVM025 can associate with endogenous Bak.

Next, we asked whether EVM025 could inhibit the induction of apoptosis following Bak over-expression. Ectopic expression of Bak saturates the mitochondrial membrane resulting in the activation of the apoptotic cascade (Chittenden et al., 1995). HeLa cells were co-transfected with HA-Bak, along with pEGFP, pEGFP-EVM025, or pEGFP-Bcl-2. Cells transfected with empty EGFP underwent a loss of TMRE fluorescence following over expression of HA-Bak (Fig. 7C). Cells transfected with EGFP-EVM025, on the other hand, were protected from Bak-induced apoptosis (Fig. 7C). Similar results were observed in cells transfected with EGFP-Bcl-2 (Fig. 7C), a protein known to inhibit Bak-induced apoptosis. Overall, these data indicate that EVM025 directly interacts with Bak and this interaction prevents the activation of Bak-induced apoptosis.

EVM025 prevents apoptosis induced by Bax

The other key pro-apoptotic mitochondrial regulator, Bax, exists in an inactive form in the cytoplasm and undergoes a conformational change in response to an apoptotic stimulus, resulting in the exposure of its N-terminus and localization to the mitochondria (Oltvai et al., 1993). Activated Bax undergoes homo-oligomerization facilitating mitochondrial damage and ultimately results in the release of cytochrome c (Annis et al., 2005). To determine if EVM025 could inhibit Bax activation, we used flow cytometry to monitor the conformational change of activated Bax. Jurkat cells were either mock-infected, or infected with ECTV, ECTVΔCrmA, ECTVΔ025, or ECTVΔ025/ΔCrmA. Six hours post-infection, the activation of Bax was induced by STS treatment, and the conformational change in Bax was detected by staining with anti-Bax6A7, an antibody that recognizes the Bax N-terminus, a domain only exposed during apoptosis. Upon STS treatment of uninfected Jurkat cells, a significant increase in anti-Bax 6A7 fluorescence was seen as compared to untreated cells, indicating that Bax was activated and underwent a conformational change exposing its N-terminus (Fig. 8A, panel a). Conversely, cells infected with ECTV (Fig. 8A, panel b) or ECTVΔCrmA (Fig. 8A, panel c) completely inhibited the activation of Bax upon STS treatment. In contrast, cells infected with ECTVΔ025 (Fig. 8A, panel d) or ECTVΔ025/ΔCrmA (Fig. 8A, panel e) were not protected from STS-induced Bax activation, suggesting that EVM025 can inhibit Bax activation.

Next we determined if EVM025 could inhibit apoptosis induced by Bax over-expression. HeLa cells were co-transfected with HA-Bax, and pEGFP, pEGFP-Bcl-2, or pEGFP-EVM025, and apoptosis was quantified in transfected cells by measuring mitochondrial membrane potential TMRE (Fig. 8B). Overexpression of Bax resulted in the artificial activation of Bax and subsequent loss of the mitochondrial membrane potential in 45% of EGFP-transfected cells. Interestingly, transfection of EGFP-EVM025 protected the cells against Bax-induced apoptosis. Similarly, transfection of EGFP-Bcl-2 significantly inhibited apoptosis induced by Bax over-expression, as expected. We failed to detect any interaction between EVM025 and Bax (data not shown).

The lack of interaction between Bax and EVM025 suggests that EVM025 functions by interacting with a BH3-only protein upstream of Bax to prevent its activation.

EVM025 inhibits Bim-induced apoptosis

EVM025 expression inhibited Bax activation, but no direct interaction between EVM025 and Bax was detected, suggesting that EVM025 may function upstream of Bax. The BH3-only protein Bim acts as a direct upstream activator of Bax, and is activated during cellular stress such as virus infection (Bouillet et al., 1999; Connor et al., 1998). To examine the possible interaction between EVM025 and BimL, HeLa cells were co-transfected with FLAG-BimL and either EGFP, EGFP-Bcl2, or EGFP-EVM025, and immunoprecipitated with anti-GFP (Fig. 9A). Western blotting with anti-Bim demonstrated that both EGFP-EVM025 and EGFP-Bcl-2 co-precipitated with BimL, whereas no interaction was observed with EGFP. These data indicate that EVM025 can associate with BimL.

We next sought to determine if EVM025 could prevent apoptosis induced by BimL over-expression. HeLa cells were co-transfected with Flag-BimL and pEGFP, pEGFP-Bcl-2 or pEGFP-EVM025 for 12 h, and apoptosis was assessed by staining with TMRE and flow cytometry (Fig. 9B). In cells transfected with pEGFP, and Flag-BimL, approximately 40% of the cells showed a loss of TMRE staining. Conversely, only 3% of cells showed a loss of TMRE when Flag-BimL was co-expressed with EGFP-Bcl-2, a cellular antagonist of BimL protein. Similar results were observed in cells transfected with BimL and EVM025, where only 7% of the transfected population showed a loss of TMRE staining. These results indicate that EVM025 interacts with the BH3 only protein BimL and prevents BimL-induced apoptosis. Thus it is likely that EVM025 inhibits the activation of the pro-apoptotic protein Bax by interacting and inhibiting the activity of its upstream regulator Bim.

Discussion

Apoptosis is a critical tool used by the cell to block virus replication, and it is not surprising that many viruses have evolved strategies to overcome or delay cell death (Barry et al., 2004; Benedict et al., 2002; Galluzzi et al., 2008). Human and murine cytomegaloviruses inhibit the apoptotic pathway through a unique apoptosis inhibitor, vMIA (Norris and Youle, 2008). The gammaherpesviruses Kaposi's sarcoma-associated herpesvirus (KSHV), Epstein Barr virus (EBV), and murine gammaherpesvirus-68 (MHV-68) encode viral homologs of the cellular anti-apoptotic protein Bcl-2 (v-Bcl-2) to inhibit the intrinsic apoptosis pathway (Barry et al., 2004; Benedict et al., 2002; Cuconati and White, 2002; Galluzzi et al., 2008; Kepp et al., 2009; Norris and Youle, 2008; Pogo et al., 2003; Teodoro and Branton, 1997). Other viruses such as African swine fever virus (ASFV) and Adenovirus also encode vBcl-2 homologs that inhibit apoptosis. Poxviruses are renowned for their ability to manipulate the apoptotic pathway, and they encode a variety of distinct proteins that interfere with the Bcl-2 family of proteins (Cuconati and White, 2002; Taylor and Barry, 2006). Avipoxviruses encode obvious cellular Bcl-2 homologs, while several other members of the poxvirus family encode anti-apoptotic proteins that lack sequence similarity to cellular Bcl-2 proteins (Banadyga et al., 2007; Tulman et al., 2004). Previous work performed in our laboratory led to the identification of F1L in vaccinia virus (Wasilenko et al., 2003). VACV F1L consists of divergent Bcl-2 homology domains and replaces cellular anti-apoptotic proteins, Mcl-1 and Bcl-2, during infection to prevent apoptosis (Campbell et al., 2010).

Ectromelia virus (ECTV), more commonly known as mousepox, is a member of the Orthopoxviridae that was first described in 1930 (Esteban and Buller, 2005). A majority of our understanding

regarding orthopoxviruses has come through the use of VACV, whose natural host is unknown. ECTV, however, is a natural pathogen of mice. Understanding the virulence of ECTV will help improve our understanding of poxvirus infection. To date, no work has investigated the role of EVM025 in apoptotic inhibition during ectromelia virus infection. Our data confirm that ECTV encodes a
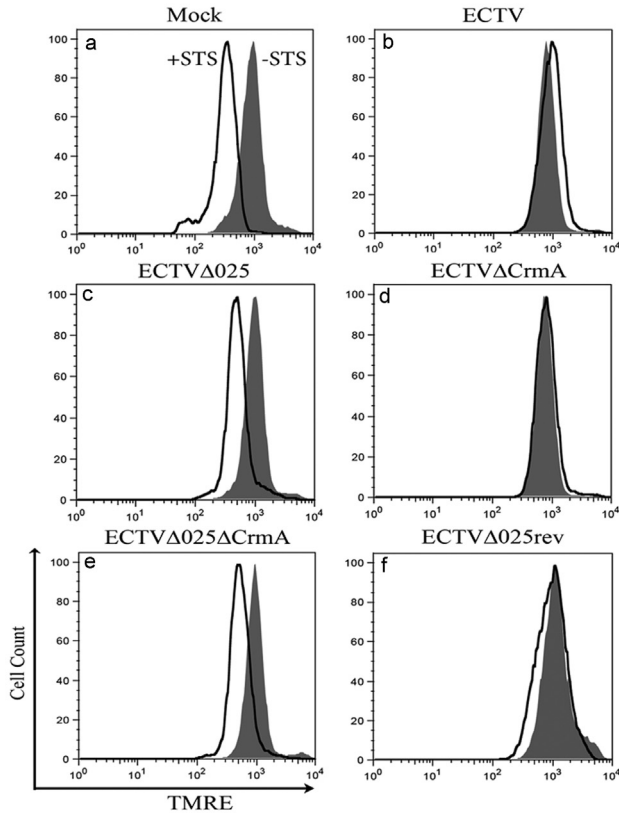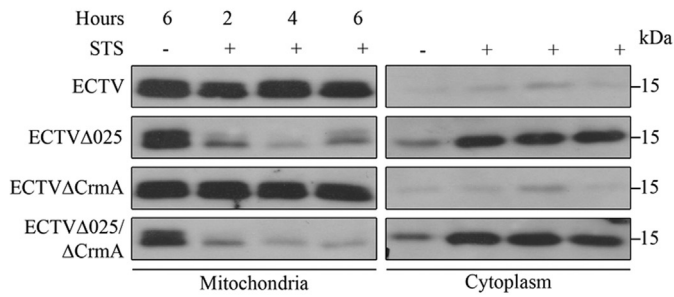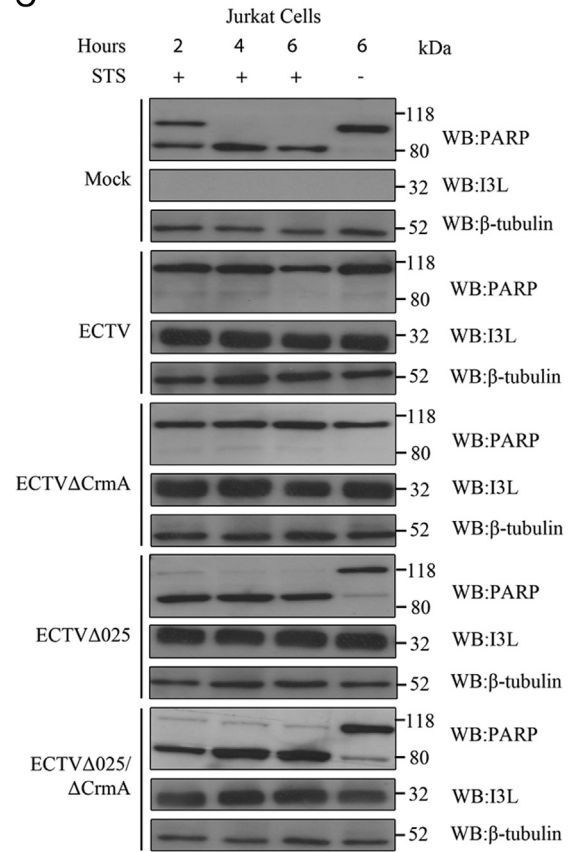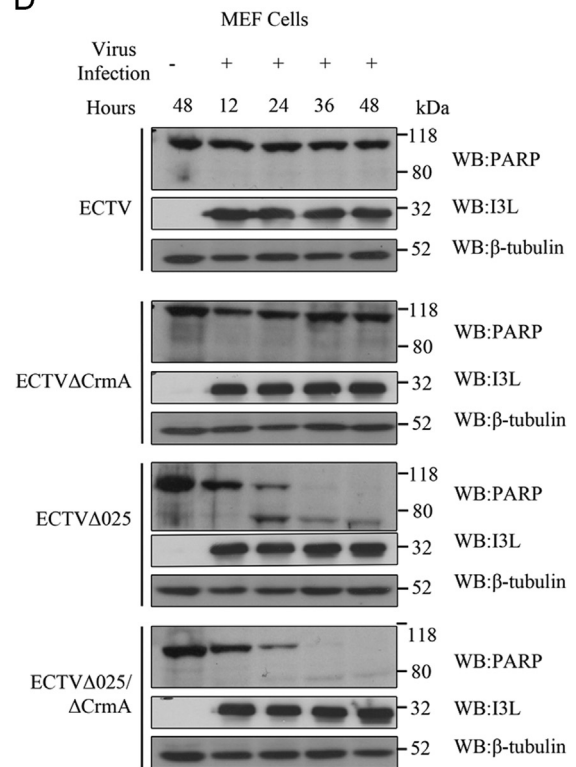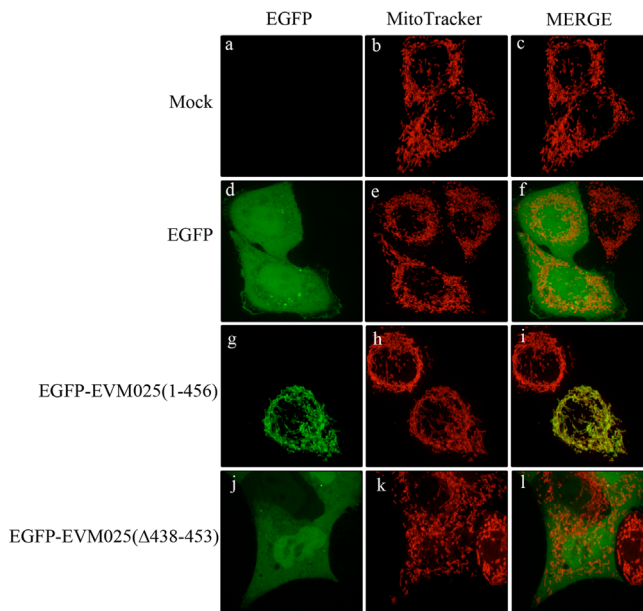
Fig. 6. EVM025 localizes to the mitochondria. MEF cells were mock-infected transiently transfected with pEGFP, pEGFP-EVM025(1-456) or pEGFP-EVM025 (Δ438453). Fourteen hours post-transfection the mitochondria were stained with 15 ng/ml of MitoTracker Red CMXRos (Invitrogen Corp.). The cells were observed under a spinning disc confocal microscope.

functional mitochondrial inhibitor of apoptosis, EVM025, which inhibits cell death induced at the mitochondria via the intrinsic pathway. Both human and murine cells infected with wild-type ECTV, but not ECTVΔ025 viruses, were inhibited from apoptosis induced by multiple stimuli. We also found that EVM025 blocks apoptosis at the mitochondria, much like VACV F1L poxviral inhibitors of apoptosis, including M11L and F1L, are localized at the mitochondria (Everett et al., 2000; Stewart et al., 2005). We were able to establish that similar to F1L, EVM025 also localizes to the mitochondria via a C-terminal hydrophobic tail (Fig. 6).

Many of the poxviral mitochondrial anti-apoptotic proteins, such as M11L, F1L, FPV039 and DPV022, are believed to function primarily by inhibiting the activation of Bak and Bax (Banadyga et al., 2007, 2011, 2009; Everett et al., 2002; Taylor et al., 2006; Wasilenko et al., 2005). Our data suggests that the expression of EVM025 was similarly sufficient to prevent the activation of Bak and Bax (Figs. 7 and 8). EVM025 was able to interact directly with endogenous Bak, but not with Bax. This is similar to results previously observed with F1L. In contrast, M11L, DPV022, and FPV039 are able to interact with both Bak and Bax to prevent their activation during virus infection. Since EVM025 inhibits the activation of Bax in the absence of detectable interaction, we hypothesized that EVM025 may function by interacting with and inhibiting

BH3-only proteins that act upstream of Bax activation. One such upstream activator of Bax is the BH3-only protein Bim. Bim exists as three isoforms, BimS, BimL and BimEL, and is able to trigger apoptosis in response to various stimuli. EVM025 expression significantly inhibited BimL-induced loss of mitochondria membrane potential, a hallmark of apoptotic cells, and EVM025 was also seen to weakly interact with ectopically expressed BimL (Fig. 9). It is interesting to note that previous work done on F1L demonstrates that F1L is able to bind to BimL strongly. This is not the case with EVM025. Addition work needs to be done to determine the ability of EVM025 to interfere with function of other BH3-only proteins. The orthopoxviral proteins F1L and EVM025 appear to function differently from the other poxviral proteins (M11L, FPV039) despite their similar anti-apoptotic phenotype suggesting different a different biochemical mechanism (Banadyga et al., 2007, 2009).

Sequence analysis of genomes of orthopoxviridae revealed that orthologues of F1L were found in multiple members of the family (Fig. 3). Our analysis showed that F1L orthologs from VARV Garcia, ECTV Moscow, and ECTV Naval have a distinct repetitive sequence at the amino terminus of the protein. The repetitive sequence is absent in other members of the orthopoxviridae family. EVM025 consists of a long N-terminal repeat region consisting of an eight amino acid motif 'DNGIVQDI' repeated 30 times. Similar to this, VARV Garcia contains multiple 'DDI' repeats at the N-terminus. These repeat regions share no sequence homology with any known cellular or viral proteins, and display no similarity to predicted secondary structure. Although the functional importance of these repeated regions remains unknown, the repeat regions are absent in VACV Copenhagen, VARV Bangladesh, MPXV Zaire, and CPVX Brighton Red, suggesting that they were lost or gained during evolution. Additional studies are required to improve our understanding of the functional and evolutionary importance of these regions. Despite the variability present at the N-terminal region of the F1L orthologues, F1L and EVM025 share 95% sequence homology at the C-termini (Fig. 3). Based on the C-terminal similarity it is likely that ECTV EVM025 will fold in a manner similar to F1L. Previous work has shown that F1L, despite lacking obvious sequence similarity with cellular Bcl-2, folds in a manner similar to cellular Bcl-2 protein (Campbell et al., 2010; Kvansakul et al., 2008). The predicted divergent BH domains of F1L are highly conserved between EVM025 and other orthologues of the orthopoxviridae family. Poxviruses have recently been shown to encode a variety of proteins that adopt a Bcl-2-like conformation (Gonzalez and Esteban, 2010; Graham et al., 2008). A52R and B14R both share limited sequence similarity with cellular Bcl-2 proteins but fold like the Bcl-2 proteins. Despite their secondary structure, A52R and B14R are involved in inhibiting the NF-κB pathway but not apoptosis (Graham et al., 2008). Several other vaccinia viral proteins, such as A46R, K7R, N1L and C1L, have all been reported to possess Bcl-2-like folds, and thus could be grouped within the same protein family (DiPerna et al., 2004; Gonzalez and Esteban, 2010; Graham et al., 2008). Of these proteins, however, only N1L has been shown to have any ability to inhibit apoptosis (Cooray et al., 2007). Other studies has shown that N1L is involved in the modulation of NFκB pathway and not apoptosis (DiPerna et al., 2004). The role of

Fig. 5. EVM025 ORF is required to inhibit apoptosis during infection. (A) EVM025 protects cells from STS-induced loss of the outer mitochondrial membrane potential. Jurkat cells were mock-infected (a), infected with ECTV (b), ECTVΔ025 (c), ECTVΔCrmA (d), ECTVΔ025/ΔCrmA (e), or ECTVΔ025rev (f) at an MOI of 5. Six hours post-infection cells were treated with 2 μM staurosporine for 90 mins or mock-treated and stained with 2 μM of TMRE. Untreated cells, (shaded histogram); staurosporine treated cells, open histogram. Data are representative of three independent experiments. Apoptosis was assessed by TMRE fluorescence, which measures loss of the inner mitochondrial membrane potential. (B) EVM025 expression inhibits STS induced cytochrome c release. Jurkat cells were infected with ECTV, ECTVΔ025, ECTVΔCrmA, and ECTVΔ025ΔCrmA at an MOI of 5 for 6 h. Six hours post-infection, the samples were treated with 2 μM STS. Cells were collected at 2, 4, and 6 h post-STS treatment. The cytosolic fraction was obtained by permeabilization using digitonin. The pellet fraction was resuspended in 0.1% Triton lysis buffer. Both the pellet and the supernatant fraction were immunoblotted with anti-cytochrome c. (C) EVM025 expression inhibits STS induced apoptosis. Jurkat cells were mock-infected, infected with ECTV, ECTVΔCrmA, ECTVΔ025, or ECTVΔ025/ΔCrmA at an MOI of 5. Six hours post-infection, samples were treated with 2 μM STS. Cells were collected at 2, 4, and 6 h post STS treatment, and cells were lysed in SDS sample buffer containing 8 M urea. Samples were subjected to SDS-PAGE and immunoblotted for PARP to determine the trigger of apoptosis. β-tubulin was used as a loading control and I3L as a control of infection. (D) EVM025 inhibits virus induced PARP cleavage. MEF cells were mock-infected or infected with ECTV, ECTVΔCrmA, ECTVΔ025, or ECTVΔ025/ΔCrmA at an MOI of 5. Cells were collected 12, 24, 36 and 48 h post-infection, and lysed in SDS-PAGE sample buffer containing 8 M urea. Samples were subjected to SDS-PAGE and immunoblotted for PARP to determine virus induced apoptosis. β-tubulin was used as a loading control and I3L as a control for infection.
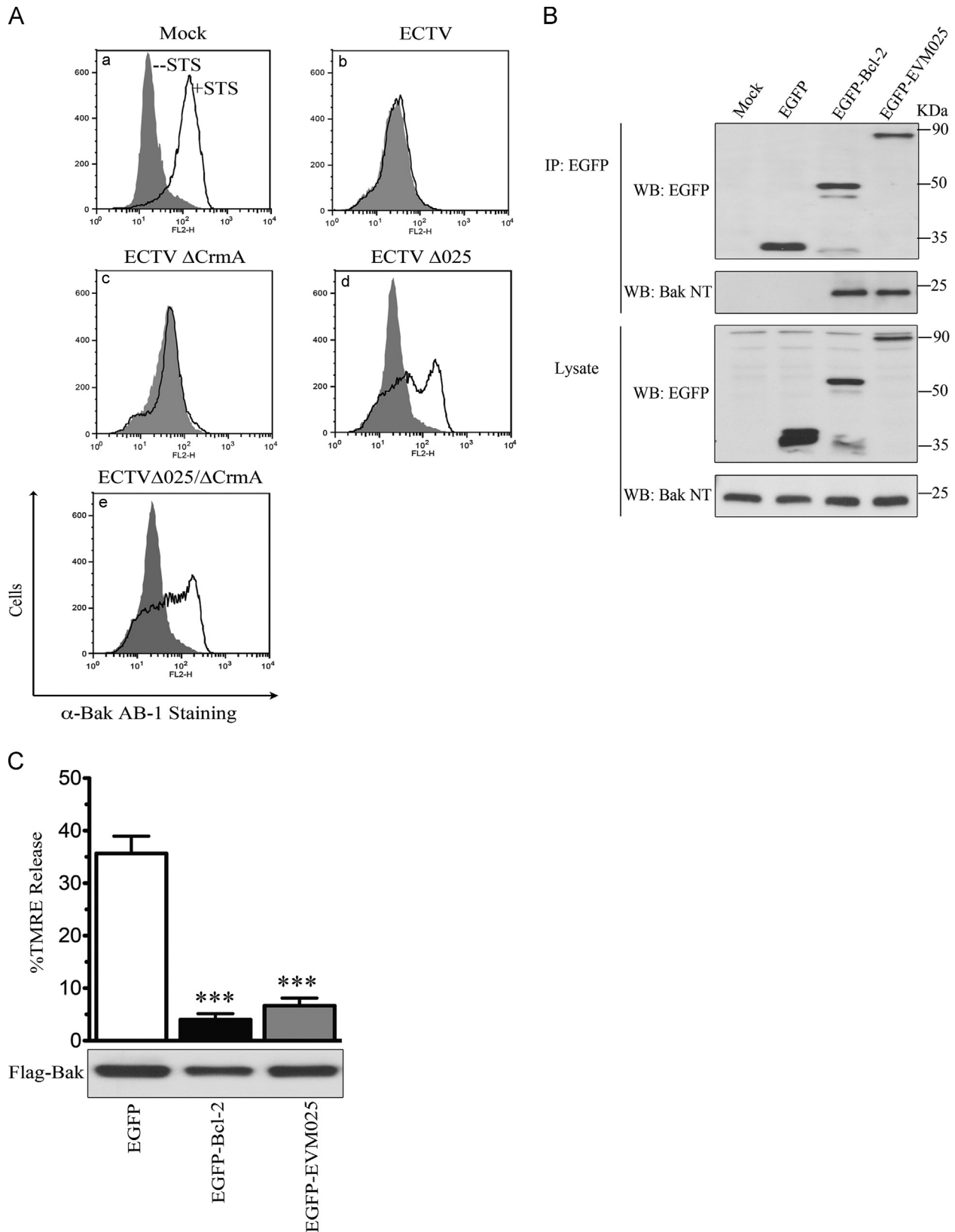
Fig. 7. EVM025 expression inhibits Bak activation. (A) EVM025 expression inhibits Bak activation. Jurkat cells were mock-infected (a), infected with ECTV (b), ECTVΔCrmA (c), ECTVΔ025 (e) or ECTVΔ025/ΔCrmA (f) at an MOI of 5 for 4 h, followed by treatment with 0.25 μM STS for 2 h to induce apoptosis. Exposure of the N-terminus of Bak was monitored by flow cytometry using a conformation-specific N-terminal Bak Ab-1 antibody. Untreated cells, (shaded histogram); STS treated cells (open histogram). Data are representative of at least three independent experiments. (B) EVM025 interacts with endogenous Bak. BMK cells were transfected with pEGFP, pEGFP-Bcl-2, or pEGFP-EVM025. Cells were lysed in CHAPs buffer and cellular lysates were immunoprecipitated (IP) with anti-GFP. The immuno-precipitates were immunoblotted with anti-Bak NT or anti-GFP antibody. Cell lysates were immunoblotted with anti-EGFP to check the expression of the EGFP constructs, and anti-Bak NT to determine the levels of endogenous Bak. (C) EVM025 expression inhibits Bak induced apoptosis. HeLa cells were co-transfected with pEGFP, pEGFP-Bcl-2, and pEGFP-EVM025 and pcDNA-Flag-Bak for 16 h stained with 50 nM TMRE to label mitochondria, and apoptosis was analyzed in EGFP-positive cells using two-color flow cytometry. Assays were performed in triplicate and were quantified as the mean percentage of EGFP-positive cells (± S.D.) demonstrating a loss of TMRE uptake. Flag-Bax expression levels from transfected cells were analyzed by Western blotting with an anti-Flag antibody. ***$P < 0.001$.
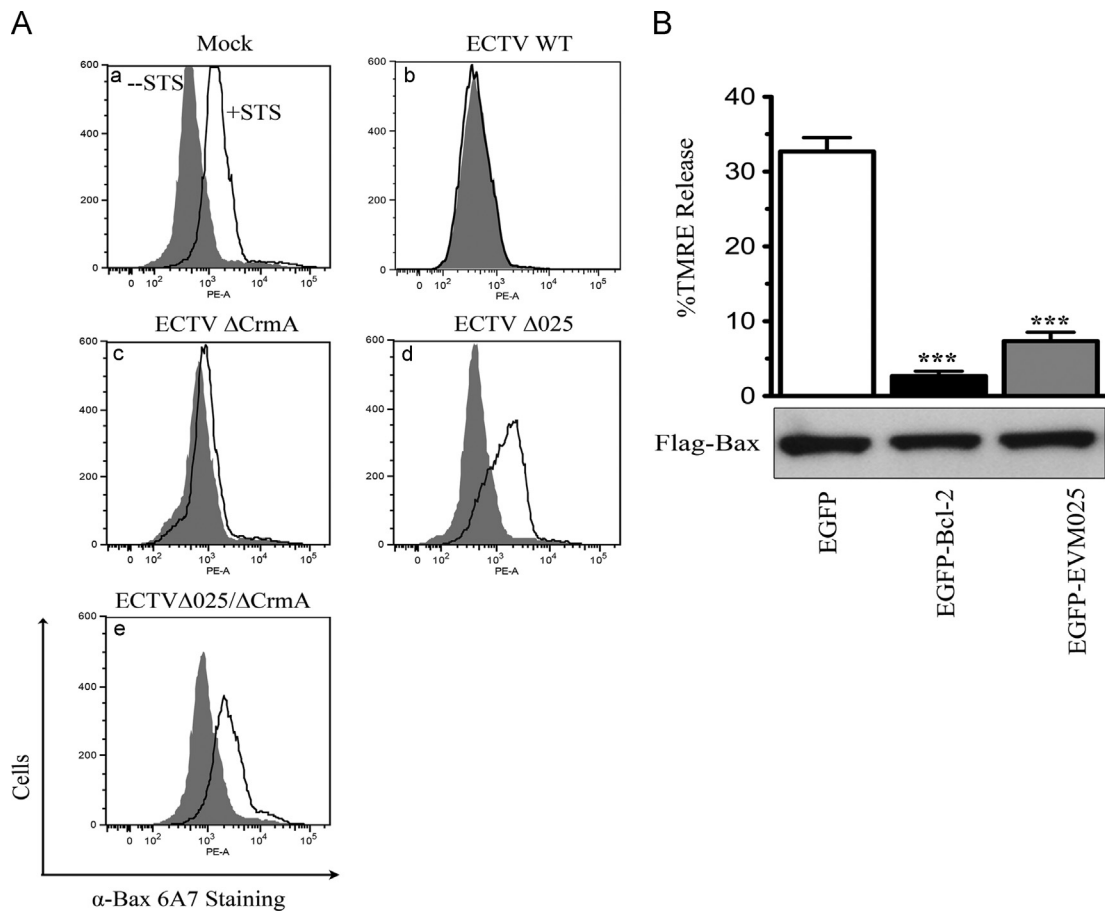
Fig. 8. EVM025 expression inhibits Bax activation. (A) EVM025 expression inhibits Bax activation. Jurkat cells were mock-infected (a), infected with ECTV (b), ECTVΔCrmA (c), ECTVΔ025 (d) or ECTVΔ025/ΔCrmA (e) at an MOI of 5 for 4 h, followed by treatment with 0.25 μM STS for 2 h to induce apoptosis. Exposure of the N-terminus of Bax was monitored by flow cytometry using a conformation-specific N-terminal Bax 6A7 antibody. Untreated cells, (shaded histogram); STS treated cells (open histogram). Data is the representative of at least three independent experiments. (B) EVM025 expression inhibits Bax induced apoptosis. HeLa cells were co-transfected with pEGFP, pEGFP-Bcl-2, and pEGFP-EVM025 and pcDNA-Flag-Bax for 16 h stained with 50 nM TMRE to label mitochondria, and apoptosis was analyzed in EGFP-positive cells using two-color flow cytometry. Assays were performed in triplicate and were quantified as the mean percentage of EGFP-positive cells (± S.D.) demonstrating a loss of TMRE uptake. Transfected Flag-Bax expression levels from transfected cells were analyzed by Western blotting with anti-Flag antibody. ***$P < 0.001$.

these or any other putative anti-apoptotic proteins remains to be seen. Our data, however, confirm the hypothesis that EVM025 is the predominant anti-apoptotic inhibitor, as EVM025 knock-out viruses are completely unable to block apoptosis induced by multiple intrinsic pro-apoptotic stimuli (Figs. 4, 7 and 8), thereby casting some doubt on the ability of other ectromelia virus proteins to also contribute to the inhibition of apoptosis. We hope that the work done in this article helps setup the foundation to address the importance of EVM025 in a mouse model.

EVM025 is a potent anti-apoptotic poxviral protein, similar to F1L. EVM025 has a distinct N-terminal end with an unknown function. EVM025 shares little similarity to cellular Bcl-2 proteins, yet effectively inhibits apoptosis. It is evident from this study that EVM025 is evolutionarily related to F1L, at some point in the evolution the N-terminus of EVM025 was lost, and subtle differences arose. But the basic function still remains to inhibit apoptosis to achieve the final goal of keeping the infected cell alive.

Materials and methods

Cells and viruses

HeLa cells were obtained from the American Type Culture Collection (ATCC), Human embryonic kidney 293T (HEK293T),

Baby mouse kidney (BMK) and CV-1 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Life Technologies), 2 mM L-glutamine (Life Technologies), 50 U/ml penicillin (Life Technologies), and 50 μg/ml streptomycin (Life Technologies). Wild type and Bak$^{-/-}$Bax$^{-/-}$ deficient mouse embryonic fibroblasts were provided by Dr. S. Korsmeyer and cultured in DMEM containing 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 100 μM MEM non-essential amino acid solution (Life Technologies). Baby green monkey kidney (BGMK) cells were obtained from ATCC and maintained in DMEM containing 10% newborn calf serum (NCS) (Life Technologies), 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Jurkat cells were maintained in Roswell Park Memorial Institute 1640 medium (RPMI) (Life Technologies) supplemented with 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 100 μM β-mercaptoethanol (Bioshop Canada Inc.). Jurkat cells stably expressing Bcl-2 were generated as previously described (Heibein et al., 2000). Vaccinia virus (VACV) strain Copenhagen (VACVCop) was provided by Dr. G. McFadden (University of Florida, Gainesville, Florida). Ectromelia virus strain Moscow (ECTV), and ECTVΔCrmA were provided by Dr. M. Buller (St. Louis University, St. Louis, Missouri). All viruses were propagated in BGMK cells and purified as previously described (Stuart et al., 1991).
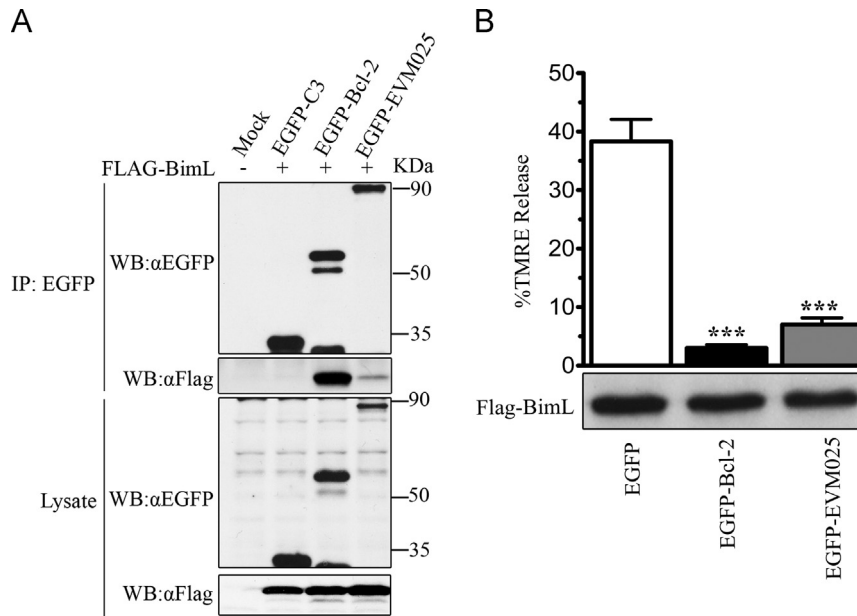
Fig. 9. EVM025 interacts with BimL and inhibits the apoptosis induced by BimL overexpression. (A) EVM025 interacts with BimL. HeLa cells were transfected with pEGFP, pEGFP-Bcl-2, and pEGFP-EVM025 and pcDNA-Flag-BimL for 14 h. The cells were lysed in 2% CHAPS buffer, lysates were immunoprecipitated (IP) with anti-GFP (goat) antibody and subjected to western blotting with anti-GFP or anti-Flag antibody to detect the interaction. Whole cell lysates were subjected to western blotting with the anti-GFP or anti-Flag antibody to detect the expression levels of the transfected proteins. (B) EVM025 expression inhibits BimL overexpression induced apoptosis. HeLa cells were co-transfected with pEGFP, pEGFP-Bcl-2, and pEGFP-EVM025 and pcDNA-Flag-BimL for 16 h. Transfected cells were stained with 50 nM TMRE to label mitochondria, and apoptosis was analyzed in EGFP-positive cells using two-color flow cytometry. Assays were performed in triplicate and were quantified as the mean percentage of EGFP-positive cells ($\pm$ S.D.) demonstrating a loss of TMRE uptake. Flag-Bax expression levels from transfected cells were analyzed by Western blotting with anti-Flag antibody. ***$P < 0.001$.

Generation of recombinant viruses

To generate an ECTV virus lacking EVM025, we cloned enhanced green fluorescent protein (EGFP) under the control of a synthetic poxviral early/late promoter (E/L) with flanking BglII sites. The poxviral early/late promoter (pE/L) and EGFP was amplified from pSC66-EGFP using primers E/LSynFor and EGFP (BglII) and the final product was cloned into pGEM-T (Promega). To generate the EVM025 knockout cassette, a region of EVM025 containing 158 base pairs upstream of EVM025 and the first 45 base pairs of the 5′ end of EVM025 was amplified using the forward primer EVM025koF1 5′-AGAATAGCTCAGCTAATCTAT-3′ and reverse primer EVM025koR1 5′-AATGCAGATCTGGATCTACGATATTATACATAAACATCGA-3′. The downstream region consisting of the last 159 base pairs of EVM025 was amplified using the forward primer EVM025koF2 5′-GATCCAGATCTGCATTTCGCATACTATATGCGATGAT-3′ and reverse primer EVM025koR2 5′-TGGATCCTTATCATATCATGTATTTGAG-3′. EVM025-koR1 and EVM025koF2 both contain an overhanging linker region that encodes a BglII restriction site. The PCR products were included in an overlapping PCR reaction to obtain a continuous fragment of the upstream and downstream fragments of EVM025 separated by the BglII restriction site. The 347 base pair fragment was cloned into a pGEMT vector (Life Technologies). An EGFP cassette under the control of a synthetic early/late poxviral promoter was inserted using the BglII restriction site between the upstream and downstream EVM025 fragments (pGEMT-EVM025/b-EGFP). This effectively encodes a disrupted non-functional version of EVM025. ECTV wild type was used to generate ECTVΔ025 by homologous recombination lacking approximately 80% of the EVM025 ORF.

In order to generate a version of ECTV deficient in CrmA and EVM025, we generated an EVM025 knockout using the vector described above in the background strain ECTVΔCrmA. BGMK cells (1×10^6) were infected with ECTV at an MOI of 0.05 and transfected with pGEMT-EVM025/d-EGFP using Lipofectamine 2000. Recombinant viruses were selected by EGFP fluorescence

and plaque purified. The disrupted EVM025 open reading frame was confirmed by PCR and sequencing using the primers EVM025-forward-ATGGACAATAGTATTTTGTCG and EVM025-reverse-ATCATATCATGTATTTGAG.

ECTVΔ025 was used as the backbone to generate the EVM025 revertant virus (ECTVΔ025rev). The wildtype EVM025 gene was amplified using the forward primer EVM025koF1 5′-AGAATAGCT-CAGCTAATCTAT-3′ and the reverse primer EVM025koR2 5′-TGGA-TCCTTATCATATCATGTATTTGAG-3′. BGMK cells (1×10^6) were infected with ECTV at an MOI of 0.05 and transfected with 10 μg amplified PCR product using Lipofectamine 2000 (Life Technologies). Recombinant viruses were selected by picking white plaques, and the presence of the wild-type EVM025 open reading frame was confirmed by PCR and sequencing.

Sequence alignments

The following sequences were used: VACVCop F1L (AAA48014), VARV Bangladesh C5L (AAA60773), VARV Garcia E1L (CAB54625), MPXV Zaire C7L (AAY97031), CPXV Brighton Red CPXV048 (NP_619836.1), ECTV strain Moscow EVM025 (NP_671543). ClustalW using the BLOSUM32 matrix was used to perform the multiple alignments (Larkin et al., 2007).

PARP cleavage

To study poly-ADP ribose polymerase (PARP) cleavage, Jurkat cells (1×10^6) were mock-infected or infected with VACVCop, ECTV, ECTVΔCrmA, ECTVΔ025, and ECTVΔ025/ΔCrmA at an MOI of 5. Six hours post-infection Jurkat cells were treated with 2 μM staurosporine (STS) (Sigma-Aldrich) and harvested six hours post-STS treatment. Similarly, MEF cells (1×10^6) were infected with ECTV, ECTVΔCrmA, ECTVΔEVM025, and ECTVΔ025/ΔCrmA at an MOI of 5 and harvested at 8, 12, 24, and 48 h post-infection. Cells were lysed in SDS-PAGE sample buffer containing 8 M urea.

Samples were subjected to SDS-PAGE and immunoblotted with anti-PARP (BD PharMingen), anti-β-tubulin (EMC Bioscience), and anti-I3L, kindly provided by Dr. D. Evans (University of Alberta, Edmonton, Alberta).

Cytochrome c release

Jurkat cells (1 × 106) were either mock-infected or infected with VACVCop or ECTV at an MOI of 5. Six hours post-infection the cells were treated with 2 μM STS and harvested at the indicated time points. Cells were permeabilized in lysis buffer containing 75 nM NaCl, 1 mM NaH2PO4, 250 mM sucrose, and 190 μg/ml of digitonin (Sigma-Aldrich). Lysates were incubated on ice for 10 min. Mitochondrial and cytoplasmic fraction were separated by centrifugation for 10 min. The mitochondrial pellet was resuspended in Triton-X-100 lysis buffer containing 25 mM Tris pH8.0 and 0.1% Triton-X-100 (Fisher Scientific). Samples were subjected to SDS-PAGE and blotted with anti-cytochrome c (BD PharMingen) and anti-Bak NT (Upstate) antibodies.

Detection of DNA fragmentation by TUNEL

DNA fragmentation was assessed by using the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling method (TUNEL) (Roche Diagnostics). Jurkat cells (2×10^6), Jurkat Bcl-2 cells (2×10^6), MEF cells (1×10^6), and MEFBak$^{-/-}$Bax$^{-/-}$ cells (1×10^6) were infected at an MOI of 5 for 6 h. Apoptosis was stimulated using 2 μM STS for 2 h for Jurkat cells, or 3 h for MEF cells. Cells were fixed with 2% (w/v) paraformaldehyde in PBS for 10 min at room temperature and permeabilized with 0.1% Triton-X-100 and 0.1% sodium citrate for 10 min on ice. Cells were stained for 1 h at 37 °C with 25 U of terminal deoxynucleotidyltransferase according to manufacturer's instructions (Roche Diagnostics). Cells were washed with PBS containing 1% (v/v) FBS and analyzed on a BD LSRFortessa flow cytometer. In each experiment 30,000 cells were analyzed. TUNEL positive cells were counted through the FL-2 channel equipped with a 561 nm filter (586/15 nm band pass), and data were analyzed with FlowJo (Tree Star, Inc.).

Mitochondrial membrane potential

Changes in mitochondrial membrane potential were assessed by staining Jurkat cells with tetramethylrhodamine ethyl ester (TMRE) (Life Technologies). MEF cells (1×10^6) were either mock-infected or infected with VACVCop, ECTV, ECTVΔ025, ECTVΔCrmA, ECTVΔ025/ΔCrmA, or ECTVΔ025rev at an MOI of 5. After 6 h of infection, the cells were treated with 2 μM STS for 2 h. MEF cells were stained with 0.3 μM TMRE for 30 min at 37 °C. Cells were washed with PBS containing 1% (v/v) FBS and analyzed using a BD LSRFortessa flow cytometer. TMRE positive cells were measured using the FL-2 channel equipped with a 586/15 nm filter and data were analyzed with FlowJo (Tree Star, Inc.).

HeLa cells (1×10^6) were transfected with EGFP, EGFP-Bcl-2, and EGFP-EVM025, using Lipofectamine 2000 for 12 h, and apoptosis was induced by co-expressing 1 μg of HA-Bak, or HA-Bax for 12 h. TMRE positive cells were measured through the FL-2 channel equipped with a 586/15 nm filter and GFP positive cells were measured through the FITC channel equipped with 530/30 nm filter. Data was acquired by counting 30,000 cells was performed on a BD LSRFortessa. The percentage of cells undergoing mitochondrial dysfunction was determined by dividing the total number of EGFP positive and TMRE negative cells by the total number of EGFP positive cells. Standard deviations were determined from three independent experiments.

Bak and Bax activation

Jurkat cells (1×10^6) were infected with ECTV, ECTVΔ025, ECTVΔCrmA, ECTVΔ025/ΔCrmA, or ECTVΔ025rev at an MOI of 5 for 6 h. Following infection, Jurkat cells were treated with 0.25 μM STS for 2 h and fixed with 0.25% paraformaldehyde. Cells were permeabilized using 500 μM digitonin and stained with 2 μg/ml Bak AB-1 antibody (EMD Millipore) or 2 μg/ml of an isotype control antibody specific for NK1.1 provided by Dr. K. Kane (University of Alberta, Edmonton, Alberta). The cells were counter stained with a Phycoerythrin-conjugated antibody (Jackson ImmunoResearch) before analysis on a BD LSRFortessa flow cytometer using the FL-2 channel equipped with a 586/15 nm filter. Data were analyzed using FlowJo (TreeStar, Inc.).

Jurkat cells were intentioned ECTVΔ025, EVCTΔCrmA, ECTVΔ025/ΔCrmA, or ECTVΔ025rev at a MOI of 5 for 8 h. Following infection, the cells were treated with 2.5 μM STS for 2 h and then fixed with 0.25% paraformaldehyde. Cells were permeablized using 500 μM digitonin and stained with 3 μg/ml Bax6A7 antibody (BD PharMingen) or 2 μg/ml of an isotype control antibody specific for NK1.1. Phycoerythrin-conjugated antibody was used to counterstain cells before analysis on BD LSRFortessa using the FL-2 channel equipped with a 586/15 nm filter and 20,000 cells were analyzed using FlowJo (TreeStar, Inc.).

Confocal microscopy

HeLa cells were transiently transfected with 2 μg of pEGFP (empty vector control), EGFP-EVM025 and EGFP-EVM025 (Δ1−255) for 14 h using Lipofectamine 2000 (Life Technologies). The Mitochondria were labeled with 15 ng/ml of MitoTracker Red CMXRos (Life Technologies). Live cells were examined using the AIVI Spinning Disk Confocal at 489 nm to detect EGFP fluorescence and 543 nm to detect MitoTracker.

Immunoprecipitation

To detect an interaction between EVM025 and endogenous Bak, BMK cells (5×106) were transfected with 5 μg of pEGFP-C3 (Clontech Laboratory Inc.), pEGFP-Bcl-2, or pEGFP-EVM025 using X-tremeGENE HP DNA reagent (Roche Diagnostics). Transfected cells were lysed 16 h post-transfection in 2% CHAPS (Sigma-Aldrich) containing 150 mM NaCl, 50 mM Tris pH8.0 and Complete Protease Inhibitors (Roche Diagnostics). Cell lysates were immunoprecipitated with anti-goat GFP antibody kindly provided by Dr. L. Berthiaume (University of Alberta, Edmonton, Alberta). Immune complexes were isolated using protein A-Sepharose (GE Healthcare). Isolated immune complexes were subjected to Western blot analysis. To study the interaction of Bax and BimL with EVM025, HeLa cells were transfected with 4 μg of pEGFP-C3, pEGFP-Bcl-2, or pEGFP-EVM025 and 2 μg pcDNA3-HA-Bax or pcDNA3-Flag-BimL using 5 μl of Lipofectamine 2000 (Life Techonologies). Transfected cells were lysed 16 h post-transfection in 2%CHAPS containing 150 mM NaCl, 50 mM Tris pH 8.0 and Complete Protease Inhibitors. Cell lysates were immunoprecipitated with anti-goat GFP antibody. Immune complexes were isolated using protein A-Sepharose. The isolated immune complexes were subjected to Western blot analysis.

Western blotting

Cell lysates were subjected to SDS polyacrylamide gel electrophoresis analysis and transferred to a polyvinylidene difluoride membrane (GE Healthcare). Following transfer, the membranes were stained with antibodies to detect the indicated proteins. Antibodies used for immunoblotting were as follows: mouse anti-cytochrome

c (clone 6H2.B4; BD PharMingen), mouse anti-PARP (BD PharMingen), mouse anti-Bak (BD PharMingen), mouse anti-EGFP (Covance), mouse anti-FLAG M2 (Sigma), mouse anti-β-tubulin (ECM Bioscience), and anti-I3L serum was provided by Dr. D. Evans (University of Alberta, Edmonton, Alberta). Membranes were blotted with donkey anti-mouse or anti-rabbit horseradish peroxidase-conjugated antibodies (Jackson ImmunoResearch). Proteins were visualized using enhanced chemiluminescence according to the manufacturer's protocol (GE Healthcare).

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