The Vaccinia Virus-Encoded Bcl-2 Homologues Do Not Act as Direct Bax Inhibitors

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Many viruses, including members of several poxvirus genera, encode inhibitors that block apoptosis by simultaneously binding the proapoptotic Bcl-2 proteins Bak and Bax. The *Orthopoxvirus* vaccinia virus encodes the Bcl-2-like F1 protein, which sequesters Bak but not Bax. However, N1, a potent virulence factor, is reported to be antiapoptotic and to interact with Bax. Here we investigated whether vaccinia virus inhibits Bak/Bax-dependent apoptosis via the cooperative action of F1 and N1. We found that Western Reserve (WR) and Δ N1L viruses inhibited drug- and infection-induced apoptosis equally. Meanwhile, infections with Δ F1L or Δ N1L/F1L virus resulted in similar levels of Bax activation and apoptosis. Outside the context of infection, N1 did not block drug- or Bax-induced cell death or interact with Bax. In addition to F1 and N1, vaccinia virus encodes further structural homologs of Bcl-2 proteins that are conserved in orthopoxviruses, including A46, A52, B14, C1, C6, C16/B22, K7, and N2. However, we found that these do not associate with Bax or inhibit drug-induced cell death. Based on our findings that N1 is not an antiapoptotic protein, we propose that the F1 orthologs represent the only orthopoxvirus Bcl-2 homolog to directly inhibit the Bak/Bax checkpoint.

Virus detection by the infected host cell often results in the induction of cell death as a means to limit viral spread. For this reason, viruses encode a variety of proteins to prevent cell death, including inhibitors of the intrinsic pathway of apoptosis (26, 44, 48, 52). This pathway, which is activated by a range of cellular stresses, is regulated and integrated by members of the B-cell lymphoma 2 (Bcl-2) family of proteins (61). These proteins share up to four conserved alpha-helical regions, called Bcl-2 homology (BH) motifs, that are involved in determining the interaction specificity between Bcl-2 family members (61). The Bcl-2 family consists of three subfamilies: the antiapoptotic proteins, such as Bcl-2, Bcl-xL, and Mcl-1, which contain four BH motifs; the proapoptotic proteins Bak and Bax, which have BH1 to -3 motifs; and the proapoptotic BH3-only proteins (61). Various death triggers, including DNA damage, growth factor deprivation, and viral infection, lead to the induction or posttranslational activation of BH3-only proteins in the cytosol (46). These in turn inhibit the antiapoptotic Bcl-2 family members, resulting in activation of the proapoptotic members Bax and Bak in the outer mitochondrial membrane (61). In addition, the BH3-only proteins Bim and Puma may also activate Bax directly (25, 38). Activated Bak and Bax hetero- and homo-oligomerize to induce permeabilization of the outer mitochondrial membrane, leading to the release of factors, including cytochrome c, and the assembly of the apoptosome (1, 61). In turn, caspase-9 is processed in the apoptosome complex, which leads to the activation of caspase-3 and -7. This core pathway is crucial for the dismantling of the cell during apoptosis (1, 61).

Given their importance in controlling the initiation of apoptosis, it is no surprise that viruses often directly target cellular Bcl-2 proteins (26, 44). In particular, many viruses efficiently inactivate both Bak and Bax by using a variety of different strategies. For instance, rodent cytomegaloviruses inhibit Bax and Bak by using vMIA and vIBO, respectively (5, 13, 42). vMIA and vIBO bear no sequence similarity to cellular Bcl-2 proteins, and they bind their targets in a manner that is distinct from that of host Bcl-2 proteins (13, 41). A more prevalent strategy is found in a variety of viruses, including adenovirus and gammaherpesviruses (19, 30). In these

viruses, Bax and Bak are inactivated by one or more viral proteins that contain primary sequence homology to the BH1 to -4 domains (19, 30). These include adenovirus E1B-19k, Epstein-Barr virus BALF1 and BHRF1, herpesvirus saimiri ORF16, gammaherpesvirus 68 (gHV-68) M11, and African swine fever virus A179L, which all engage Bax and Bak to inhibit their activity (19, 26, 30).

Such sequence homologs of Bcl-2 proteins are not found among poxviruses, apart from avipoxviruses such as fowlpox virus (2, 54). The fowlpox virus protein FPV039 contains highly conserved BH1 and BH2 motifs, as well as a cryptic BH3 motif, which is important for its inhibitory activity (7). In addition to Bak and Bax, FPV039 also binds the BH3-only proteins BimL and Bik (9). Among other poxvirus genera, the *Parapoxvirus* protein ORF125 binds Bax but not Bak, as well as five different BH3-only proteins (59, 60). The *Leporipoxvirus* myxoma virus encodes M11L, a potent antiapoptotic protein that interacts with Bax, Bak, and Bim, as well as with a component of the mitochondrial permeability transition pore complex (23, 51, 55). Beyond these poxvirus genera, the capri-, lepori-, sui-, and yatapoxviruses encode orthologs of myxoma virus M11L, while deerpox virus (*Cervidovirus*) DPV022 is a sequence intermediate between M11L and vaccinia virus F1 (see below) that binds both Bak and Bax (8). The genomes of orthopoxviruses such as vaccinia virus do not encode any obvious Bcl-2 sequence homologs or M11L-like proteins. Instead, vaccinia virus encodes F1, which is anchored in the outer mitochondrial membrane via a C-terminal transmembrane domain (43, 50). Loss of F1L from the genomes of the vaccinia virus Western Reserve (WR), Copenhagen (COP), and attenuated modified virus Ankara (MVA) strains increases cell death in culture (24, 43,

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56). F1 blocks both drug- and infection-induced cell death through an interaction with Bak (43, 56, 57). However, F1 does not bind Bax yet can partially inhibit Bax-dependent cell death, possibly due to interactions with caspase-9 or Bim (53, 62).

Structural studies showed that despite lacking significant primary sequence similarity to each other or to Bcl-2 proteins, both F1 and M11L have a recognizable Bcl-2-like fold (21, 35, 36). Moreover, the structures of vaccinia virus A52, B14, K7, and N1 revealed that the virus encodes further structural Bcl-2 homologs (3, 17, 28, 33). A46, C1, C6, C16/B22, and N2 were also recently predicted to belong to the same family of proteins (27). A46, A52, B14, K7, and N1 inhibit specific proteins in innate immunity signaling cascades that lead to the induction of NF- κ B and IRF3 transcriptional activity (12). Only N1, however, was reported to be antiapoptotic (17). N1 localizes to the cytosol and contributes to vaccinia virus virulence in intradermal and intranasal mouse models of infection (10). Recently developed N1 antagonists limit virus growth in cells in culture, suggesting the protein as a possible antiviral drug target (15). N1 forms a homodimer whose interface involves the amino terminus of the protein but not the hydrophobic groove that would accommodate BH3 helices (3, 17). Both viral and transient expression of N1 was found to prevent drug-induced apoptosis (17). Furthermore, N1 could be coimmunoprecipitated with endogenous Bax, Bad, and Bid, but not with Bak and Bim, from infected cell extracts (17).

Taken together, these results suggest that F1 and N1 inhibit Bak and Bax, respectively, during infection (6). If so, unlike the case for other poxvirus genera but similar to the case, for instance, for rodent cytomegaloviruses, the inactivation of Bak and Bax would be executed by two separate proteins. In this study, we tested this hypothesis and found it not to be the case. We report that N1 does not inhibit apoptosis in or outside the context of infection and that it does not bind Bax. We also found that none of the other known Bcl-2-like proteins encoded by vaccinia virus are antiapoptotic. We therefore propose that F1 is the only antiapoptotic Bcl-2-like protein encoded by the vaccinia virus genome and that the principal function of N1 is to inhibit the innate immunity signaling pathways that activate the NF- κ B and IRF3 transcription factors.

MATERIALS AND METHODS

Cells, viruses, and infections. HeLa cells were grown in complete minimal essential medium (MEM) supplemented with 10% fetal bovine serum. BSC-1, HEK293T, HEK293-T-Rex, and HeLa-T-Rex cells (Invitrogen) were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum. HeLa T-Rex HA-Bax and HEK293 T-Rex N1L cells were generated by inserting hemagglutinin-Bax (HA-Bax) and N1L, respectively, into the pCDNA4/TO vector (Invitrogen), followed by transfection and selection using Zeocin (500 μ g/ml). Resistant clones were pooled and characterized. To induce expression, cells were treated with doxycycline (0.5 μ g/ml) for 20 h. To generate the Δ N1L virus, we removed nucleotides 1 to 275 of the N1L open reading frame in strain WR by using a replacement strategy with Cherry fluorescent protein driven by a synthetic early/late promoter to enable selection (18). Subsequent removal of the FIL locus in this virus generated the Δ N1L/F1L virus. This was achieved by our previous replacement strategy (43), using green fluorescent protein (GFP) driven by a synthetic early/late promoter as a selectable marker. All resulting knockout viruses were isolated by plaque purification and verified by sequencing and Western blot analysis. All viruses were propagated in BSC-1 cells. Plaque sizes were determined by measuring the diameters of at least 125 single plaques from low-density titrations at 48 h postinfection (hpi), using NIH ImageJ soft-

ware (<http://rsbweb.nih.gov/ij/>). HeLa cells were plated on fibronectin-coated dishes and infected at a multiplicity of infection (MOI) of 2 in Opti-MEM serum-free medium for 1 h with the indicated viruses. After 1 h, they were washed once and returned to complete medium for the indicated length of time. Infected cells were treated with 1 μ M staurosporine (STS) at 5 hpi for the indicated times before collection of detached and adherent cells for subsequent analysis.

Apoptosis assays. HeLa cells (2.5×10^5) were plated onto coverslips in 6-well plates 24 h prior to Effectene (Qiagen)-mediated transfection of pEGFP-C1 (0.1 μ g) (Clontech) and pCDNA4-Flag-vBcl2 (0.3 μ g) according to the manufacturer's recommendations. At 24 h posttransfection, cells were incubated with 1 μ M STS for 4 h, followed by paraformaldehyde fixation and Hoechst 33342 staining. Nuclear fragmentation was determined in a minimum of 150 GFP-positive cells per experiment. Data from three independent experiments are expressed as means $\pm$ standard errors of the means. To assess mitochondrial potential, HeLa-T-Rex and HeLa-T-Rex-HA-Bax cells (5×10^5) were transferred to 6-well plates 24 h prior to Effectene (Qiagen)-mediated transfection with 0.1 μ g CB6-EGFP and 0.3 μ g pCDNA4/TO, pCDNA4/TO-N1L, or pCDNA4/TO-Flag-M11L. Cells were cultured in the presence of 10 μ M Z-VAD-fmk (Enzo Life Sciences). At 20 h posttransfection, cells were induced with doxycycline (0.5 μ g/ml; Sigma-Aldrich) for 20 h. Floating and adherent cells were collected and stained with 40 nM tetramethylrhodamine ester (TMRE; Molecular Probes) for 30 min at 37°C, followed by the addition of 5 μ l/ml 4',6-diamidino-2-phenylindole (DAPI) (200- μ g/ml stock; Sigma-Aldrich) immediately prior to flow cytometry analysis. Samples were analyzed on an LSR Fortessa flow cytometer (BD Biosciences, San Jose, CA), with TMRE fluorescence collected through a 582/15-nm-band-pass filter following excitation with a 561-nm laser. DAPI fluorescence was detected with a 450/50-nm-band-pass filter following 404-nm laser excitation, and GFP fluorescence was detected with a 530/30-nm-band-pass filter after 488-nm laser excitation. Cell doublets and debris were excluded on the basis of their scatter characteristics, and at least 10,000 single cells were collected. Analysis was performed using FlowJo software (TreeStar, OR). UV irradiation (200 mJ/cm²) was performed on HEK293 T-Rex E.V. and N1L cells that had been induced by addition of doxycycline (0.5 μ g/ml; Sigma-Aldrich) for 20 h. At 4 h postirradiation, cell lysates were prepared and Western blot analysis using anti-poly(ADP-ribose) polymerase (anti-PARP) antibodies was performed.

Immunoprecipitations. HeLa cells were lysed in 1% CHAPS lysis buffer [20 mM Tris-HCl (pH 8.0), 140 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) (wt/vol), 10% glycerol] containing 1 $\times$ Complete protease inhibitors (Roche) for 1 h at 4°C. The soluble protein fractions were incubated with agarose resin covalently coupled to monoclonal anti-Flag (M2; Sigma-Aldrich) or anti-Bax (0.N.18; Santa Cruz) for 1 h at 4°C, followed by four washes in lysis buffer. Alternatively, after 1 h of incubation with mouse monoclonal anti-N1 or anti-GFP (3E1; Cancer Research UK), protein A/G agarose resin (Pierce) was added for 1 h before four washes with lysis buffer and processing for Western blotting.

Western blotting and antibodies. Cell lysates were separated in Nu-PAGE 4 to 12% Bis-Tris precast gels (Invitrogen) and transferred to nitrocellulose membranes (Invitrogen). The following antibodies were used for Western blotting according to standard protocols: anti-PARP, anti-active caspase-3, and anti-Bad, from Cell Signaling Technology; anti-Bax NT, from Upstate Biotechnology; anti-Bid (FL-195), from Santa Cruz Biotechnology; and anti-actin (AC74), anti-alpha-tubulin (B512), anti-Flag, and anti-HA, from Sigma-Aldrich. Antibodies against the viral proteins A27, A36, and F1 have been described previously (31, 43, 47). Secondary antibodies for Western blots were goat anti-rabbit or anti-mouse IgG coupled to horseradish peroxidase (Bio-Rad). Polyclonal anti-N1 antibodies used for immunoblotting were generated from N1 produced in *Escherichia coli*. N1L cDNA was amplified by PCR from vaccinia virus Western Reserve genomic DNA and subcloned into pMW172-GST-3C to generate *E. coli* T7 expression clones expressing glutathione S-transferase

(GST)-tagged N1. The expression clones were transformed into *E. coli* strain BL21(DE3), and exponentially growing cultures were induced for 4 h with 200 μ M IPTG (isopropyl- β -D-thiogalactopyranoside). Soluble fractions were produced by lysing bacteria in bacterial lysis buffer (BLB; 50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 25% [wt/vol] sucrose) by standard methods. GST-3C-N1 was bound to glutathione Sepharose beads by incubation of the soluble fractions in GSTbind buffer (BLB with 0.5% CHAPS, 350 mM NaCl, 1.5 mM MgCl₂) for 1 h. Following washes in GSTwash buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 0.5% CHAPS, 1 mM EDTA, 1.5 mM MgCl₂), the resin was incubated with 10 mM glutathione. Released GST-3C-N1 was incubated with PreScission protease (GE Healthcare) at 4°C for 16 h in 3C-Cleavage buffer (50 mM Tris, pH 7.5, 150 mM NaCl) before the addition of glutathione Sepharose beads. The resulting soluble fraction containing N1 was dialyzed into phosphate-buffered saline (PBS) and used for immunization of rabbits to generate anti-N1 antibodies. Mouse monoclonal anti-N1 antibodies used for immunoprecipitation and immunofluorescence studies were generated by the London Research Institute (LRI) monoclonal core facility, using purified N1 protein.

Immunofluorescence. For immunofluorescence analysis, cells were plated on fibronectin-coated coverslips and fixed in 4% paraformaldehyde. After permeabilization using 200 μ g/ml digitonin (Sigma-Aldrich) in PBS, coverslips were incubated with anti-cytochrome *c* (6H2.B4; BD PharMingen), anti-HA (HA-7; Sigma-Aldrich), or anti-active Bax (6A7; Trevigen) primary antibody for 1 h at room temperature. This was followed by incubation with anti-mouse Cy5- or Alexa 488-conjugated secondary antibody (Invitrogen). For quantitation of nuclear condensation, cells were incubated for 10 min with 2 μ M Hoechst dye before fixation for 1 min in 4% paraformaldehyde and for 10 min in ice-cold methanol. Cells were processed for immunofluorescence analysis as previously described (4). Images were collected using Plan-Neofluar 25 $\times$ and 10 $\times$ lenses (Carl Zeiss, Germany) on a Zeiss Axioplan 2 microscope controlled by MetaMorph software (Molecular Devices Corporation) and using a CoolSNAP HQ camera (Photometrics). Figures were pseudocolored as indicated for ease of interpretation and were prepared using the Adobe Photoshop and Illustrator packages. All immunofluorescence quantitative data represent over 250 cells per condition for at least three independent experiments. Data are presented as means $\pm$ standard errors of the means and were analyzed by analysis of variance (ANOVA) with a Bonferroni posttest, using Prism 4.0 (GraphPad software).

Cell viability assay. HEK293 T-Rex N1L cells (2.5×10^4 /well) were seeded into 96-well plates 24 h prior to addition of doxycycline (0.5 μ g/ml; Sigma-Aldrich) for 24 h. STS was added to the wells at the indicated concentrations, and after 6 h, the CellTiter-Blue cell viability reagent (Promega) was added (3 μ l per well). After incubation for 1.5 h at 37°C, the fluorescence was measured at an excitation wavelength of 560 nm and an emission wavelength of 590 nm, using an Envision 2102 multilabel reader (PerkinElmer). Each condition was assayed five times, and results are expressed as percentages of the value for untreated control cells. HeLa cells (1.5×10^4 /well) were seeded into 96-well plates and reverse transfected with the indicated plasmids at a total of 100 ng/well by using Effectene transfection reagent according to the manufacturer's instructions. The transfection mix was added to each well in a 50- μ l volume, to which 150 μ l cell suspension was added. Cell viability was determined 48 h later by using the CellTiter-Blue cell viability reagent (Promega).

RESULTS

N1 does not cooperate with F1 to limit drug- and infection-induced apoptosis. To investigate whether N1 can cooperate with F1 to block apoptosis at the early stages of infection, we examined the expression profiles of these proteins. We found that F1 was expressed from 2 h postinfection, while N1 was observed from 3 h postinfection, consistent with a possible cooperation between N1 and F1 in blocking apoptosis in the early stages of infection (Fig. 1A). Next, we disrupted the N1L open reading frame in the WR

genome. Using the resulting Δ N1L virus, we subsequently deleted its F1L locus to generate a Δ N1L/F1L virus. The gene deletions were verified by DNA sequencing, and the presence or absence of N1 and F1 in the corresponding virus was confirmed by immunoblotting (Fig. 1B). Previous studies have found that disruption of neither the N1L nor F1L open reading frame leads to attenuated virus growth in cell culture (10, 43, 56). Consistent with this, infections with WR, Δ F1L, and Δ N1L viruses as well as with the Δ N1L/F1L virus showed similarly sized plaques, unlike the Δ VGF/F1L virus, which generated smaller plaques (Fig. 1C) (45).

Infection with WR, but not Δ F1L virus, can provide temporary protection against STS-induced apoptosis (43, 56). The Δ N1L virus is also attenuated in its ability to protect against STS-induced cell death (17). To investigate whether N1 and F1 collaborate to protect against apoptosis, we infected HeLa cells with the WR, Δ N1L, Δ F1L, and Δ N1L/F1L viruses for 5 h and subsequently incubated the cells with STS for 2 or 4 h. STS treatment of noninfected cells resulted in increasing levels of apoptosis, as determined by cleavage of the apoptotic marker PARP (Fig. 2A). Similar levels of cleaved PARP were observed in Δ F1L and Δ N1L/F1L virus-infected cells (Fig. 2A). In contrast to previous findings (17), we found that cells infected with the WR and Δ N1L viruses showed equally robust protection against STS-induced apoptosis (Fig. 2A).

We next asked whether N1 and F1 collaborate in inhibiting virus-induced cell death. Infection of HeLa cells with the WR and Δ N1L viruses at an MOI of 2 for 20 h resulted in undetectable levels of PARP and caspase-3 cleavage (Fig. 2B). In contrast, Δ F1L and Δ N1L/F1L virus infections induced cleavage of these markers, albeit to a lesser extent than Δ VGF/F1L virus infection (Fig. 2B). Analysis of the percentage of fragmented nuclei confirmed that a loss of F1, but not N1, results in increased apoptosis (Fig. 2C). To further confirm the progression of apoptosis, we analyzed the release of cytochrome *c* from mitochondria in infected cells. Immunofluorescence analysis of infected cells revealed that in contrast to the case for the WR and Δ N1L viruses, a large number of cells infected with the Δ F1L and Δ N1L/F1L viruses had cytoplasmic cytochrome *c* (Fig. 2D and E). Quantitative analysis of these experiments revealed that Δ N1L virus infection did not increase apoptosis over WR levels, while infection with Δ N1L/F1L virus did not increase apoptosis over the levels observed with Δ F1L virus, unlike the positive control (Δ VGF/F1L virus) (Fig. 2D).

Since N1 interacts with Bax, we extended our analysis to determine the activation status of Bax in cells infected with our knockout viruses (17). Quantitative immunofluorescence with an antibody specific to the active Bax conformation showed comparable increases in the number of cells positive for active Bax during Δ F1L and Δ N1L/F1L virus infections and few positive cells in WR and Δ N1L virus-infected cells (Fig. 3A and B). Using the conformation-specific antibody, we also immunoprecipitated active Bax from cells infected with the different recombinant viruses (Fig. 3C). In agreement with our immunofluorescence analyses, we found that there was no detectable active Bax in WR or Δ N1L virus-infected cells. In contrast, similar levels of active Bax were found in Δ F1L and Δ N1L/F1L virus-infected cell lysates (Fig. 3C). Together, these results show that the loss of N1L from the vaccinia virus genome does not lead to increased cell death or Bax activation during infection.

N1 expression does not prevent Bax-induced cell death. Our results obtained using knockout viruses contradict previous find-

FIG 1 Loss of N1 does not decrease plaque size in the absence of F1. (A) Western blot analysis of whole-cell lysates of WR-infected cells probed with antibodies against F1, N1, A36 (early viral protein), A27 (late viral protein), and actin at the indicated times postinfection. (B) Western blot analysis of HeLa cell lysates infected with the indicated viruses for 20 h reveals the absence of N1 and F1 proteins after deletion of the indicated genes. (C) Representative images of plaque formation by the indicated viruses in BSC-1 cells visualized with crystal violet at 4 days postinfection. Quantitative analysis of plaque sizes of the indicated viruses shows a reduced plaque size in Δ VGF/F1L virus-infected cells. The error bars represent standard errors of the means ($n = 125$ for each virus). ***, $P < 0.001$.

ings showing that N1 inhibits drug-induced apoptosis (17). To try to resolve these differences, we extended our analysis by examining the ability of N1 to block Bax-induced cell death outside the context of infection. Overexpression of Bax leads to the activation and oligomerization of the molecule, resulting in cell death (40). To examine whether N1 can inhibit Bax-induced cell death, we generated a stable cell line that expresses HA-Bax upon induction with doxycycline. Immunofluorescence and Western blot analysis showed that few cells expressed HA-Bax or became positive for active Bax in the absence of doxycycline (Fig. 4A and B). Upon addition of doxycycline, however, a large number of cells were positive for HA and active-Bax epitopes (Fig. 4A and B). To determine the consequence of Bax induction in our cells, we performed a time course experiment to analyze Bax expression and PARP cleavage. From around 8 h postinduction, a significant amount of HA-Bax was observed, which was maintained for the 24-h duration of the time course (Fig. 4C). Concomitant with this, a clear increase in cleaved PARP was detected by Western blot

analysis, confirming the ability of HA-Bax induction to induce cell death (Fig. 4C). This system allowed us to specifically examine whether N1 can inhibit the apoptotic machinery at the level of or downstream of Bax outside the context of infection.

We performed all experiments with an untagged version of N1 to avoid disrupting the structural integrity of the N1 dimer (17). We cotransfected HeLa-T-Rex-empty vector (E.V.) and HA-Bax cells with plasmids expressing GFP as well as N1 or Flag-M11L, or with empty vector, before treating cells with doxycycline for 20 h. To assess apoptosis in these cells, we performed flow cytometric analysis to determine the proportion of GFP-positive cells that had lost their mitochondrial potential (TMRE negative) but had not yet become membrane compromised (DAPI negative) (Fig. 4D and E). After mock treatment, HeLa-T-Rex HA-Bax cells showed a modest increase in apoptosis compared to HeLa-T-Rex E.V. cells (Fig. 4F). This increase was blocked by the expression of Flag-M11L but not by N1, suggesting that the increased apoptosis was probably due to low-level HA-Bax expression in the absence

FIG 2 F1, but not N1, inhibits apoptosis. (A) HeLa cells were infected for 5 h with the indicated viruses at an MOI of 2 before treatment with 1 μ M STS for 2 and 4 h. Western blot analysis of whole-cell lysates by using antibodies against PARP, F1, N1, and tubulin is shown. (B) Western blot analysis of whole-cell lysates from floating and adherent HeLa cells infected with the indicated viruses for 20 h at an MOI of 2, using antibodies against PARP, caspase-3, A27, and tubulin. (C) Quantitative analysis of nuclear condensation in HeLa cells infected with the indicated viruses for 20 h. At least 250 cells were counted from two independent coverslips. Averages were determined for three independent experiments, and error bars represent standard errors of the means. ***, $P < 0.001$. (D) Quantitative immunofluorescence analysis of the percentage of HeLa cells exhibiting cytosolic cytochrome *c* after infection for 20 h with the indicated viruses. At least 250 cells were counted in 3 independent experiments, and error bars represent standard errors of the means. ***, $P < 0.001$. (E) Representative immunofluorescence images of HeLa cells infected with the indicated viruses and stained with cytochrome *c* antibody. Arrows illustrate cells with cytoplasmic cytochrome *c*.

of doxycycline (Fig. 4F). After induction with doxycycline, a large increase in apoptosis was observed in HeLa-T-Rex HA-Bax but not HeLa-T-Rex E.V. cells (Fig. 4F). Once again, this increase was completely negated by the expression of Flag-M11L but not N1 (Fig. 4F). To further corroborate these results, we cotransfected HeLa cells with increasing amounts of HA-Bax and a constant

amount of N1L, Flag-M11L, or empty vector. At 48 h posttransfection, assessment of cell viability revealed that a significant percentage of control cells died in response to expression of HA-Bax (Fig. 4G). Cells expressing N1 showed a similar sensitivity to HA-Bax-induced cell death. In contrast, Flag-M11L expression could partially abrogate this cell death (Fig. 4G). Together, these obser-

FIG 3 Loss of N1 does not increase Bax activation during infection. (A) Representative immunofluorescence images of HeLa cells infected for 20 h with the indicated viruses and stained with antibody (6A7) to active Bax. The appearance of activated Bax was observed predominantly in cells infected with the Δ F1L and Δ N1L/F1L viruses. (B) Quantitative analysis of Bax activation in cells infected for 20 h with the indicated viruses. The percentage of cells exhibiting staining of active Bax was determined for at least 250 cells in three independent experiments. Error bars represent standard errors of the means. ***, $P < 0.001$. (C) Western blot analysis reveals that activated Bax can be immunoprecipitated (IP) readily from HeLa cells infected with the Δ F1L and Δ N1L/F1L viruses but not the WR and Δ N1L viruses.

variations suggest either that the reported N1-Bax interaction is not sufficient to block Bax activation or that N1 does not bind Bax. Using our doxycycline-inducible HeLa-HA-Bax cell line, we tested whether virally expressed N1 could interact with endogenous or overexpressed and activated Bax. Using a monoclonal anti-N1 antibody to specifically immunoprecipitate N1 from vaccinia virus-infected cells, we observed that Bax did not interact with N1 (Fig. 5A). In the reciprocal experiment, active Bax was immunoprecipitated in larger quantities from doxycycline-treated cells than from uninduced cells, but no N1 was observed to coimmunoprecipitate in either case (Fig. 5B). The reported interaction between N1 and endogenous Bax, Bad, and Bid (17) was observed by use of a buffer containing 1% NP-40, a detergent known to induce interactions between Bcl-2 proteins (32). We found, however, that N1 still did not coimmunoprecipitate Bax, even with 1% NP-40- or 1% Triton-based buffers (data not shown). Furthermore, N1 was not associated with Bad or Bid in vaccinia virus-infected cell lysates (Fig. 5C and D).

While these results show that N1 does not bind Bax or block Bax-induced cell death, they do not formally exclude the possibility that N1 can inhibit apoptosis via an alternative mechanism. To determine whether this could be the case, we tested whether N1 can inhibit STS- and UV-induced cell death. For this purpose, we generated doxycycline-inducible HEK293-N1L and empty vector (E.V.) cells. Immunofluorescence and Western blot analyses showed that N1 could be detected only after addition of doxycycline (Fig. 6A and B). UV irradiation-induced PARP cleavage was not inhibited by N1 expression in doxycycline-treated HEK293-N1L cells (Fig. 6C). The expression of N1 also did not inhibit HEK293-N1L cell death induced by increasing concentrations of STS (Fig. 6D). Together, these results demonstrate that, as in the context of infection, N1 does not protect against drug- or UV-induced cell death.

Vaccinia virus-encoded Bcl-2 homologs do not interact with Bax or block apoptosis. Since N1 does not bind Bax or inhibit its activation, our data may imply that orthopoxviruses lack a direct

FIG 4 N1 expression does not inhibit Bax-induced cell death. (A) Representative immunofluorescence images of HeLa-T-Rex-HA-Bax cells stained with antibodies to HA (top) or active Bax (bottom) (green), before (left) or after (right) induction with doxycycline. Cells were highlighted by nuclear staining with Hoechst 33342 (red). (B) Western blot analysis of HeLa-T-Rex-empty vector (E.V.) and -HA-Bax cell lysates showing that HA-Bax is induced by doxycycline addition for 20 h. (C) Western blot analysis of doxycycline-induced HA-Bax expression and PARP cleavage in HeLa-T-Rex-HA-Bax cells over 24 h. (D) Gating strategy used in flow cytometry analysis of apoptosis. Panel I shows a region that includes all singlet events, based on their side scatter width value. This region was then applied to panel II, and whole cells were gated as shown, on the basis of their forward and side scatter signals. This cell population was then used in panel III to define GFP-positive cells. (E) Each plot displays only events that satisfy all three criteria in panel D. For each cell line and condition, TMRE fluorescence (x axis) is shown against DAPI fluorescence (y axis). Live cells are defined as TMRE^{high} and DAPI negative, apoptotic cells are TMRE^{low} and DAPI negative, and dead cells are DAPI positive. Regions were set using the HeLa-GFP control and applied to all other plots. (F) Quantitative flow cytometry analysis of apoptosis in HeLa-T-Rex-empty vector (black bars) and HeLa-T-Rex-HA-Bax (white bars) cells expressing the indicated proteins for 24 h followed by induction with doxycycline for 20 h. Apoptosis was assessed by mitochondrial dysfunction analysis using TMRE fluorescence and was quantified as the percentage of GFP-positive cells that were both TMRE and DAPI negative. Western blots show the expression of N1 and Flag-M11L under the different conditions. Error bars represent standard errors of the means for 3 independent experiments. **, $P < 0.01$. (G) Cell viability analysis of HeLa cells transfected with increasing amounts of HA-Bax plasmid and constant amounts of empty vector or N1L or Flag-M11L plasmid (in nanograms), as indicated. Western blots show the presence of N1 and Flag-M11L in the lysates derived from the remaining adherent cells. The data represent means $\pm$ standard errors of the means for three independent experiments performed in quadruplicate. **, $P < 0.01$; ***, $P < 0.001$.

FIG 5 N1 does not interact with Bax. (A and B) HeLa-T-Rex-HA-Bax cells were induced with doxycycline for 20 h and then infected with WR for 6 h. Immunoprecipitation of N1 with mouse monoclonal anti-N1 (A) and anti-active Bax agarose (0.N.18) (B) was performed with infected cell lysates. Western blots of the immunoprecipitates and the whole-cell lysates (input) were probed using anti-Bax (Bax NT) and polyclonal anti-N1 antibodies. (C and D) HEK 293 cells infected with WR for 16 h at an MOI of 2 were lysed and subjected to immunoprecipitation with mouse monoclonal GFP or N1 antibody. Western blots of the immunoprecipitates and the cell lysates (input) were probed using antibodies against Bad (C) and Bid (D).

Bax inhibitor. However, vaccinia virus encodes a number of confirmed and predicted structural Bcl-2 homologs that may have the capacity to bind Bax (27). To test whether any of these proteins could interact with Bax, we performed coimmunoprecipitation experiments using Flag-tagged versions of these Bcl-2 homologs (Fig. 7A). These revealed that M11L, but none of the additional Bcl-2 homologs, interacted with endogenous Bax during infection (Fig. 7A). Next, we tested whether any of these proteins have antiapoptotic activity. In transient expression experiments, STS-induced cell death was inhibited by expression of M11L. In contrast, none of the additional Bcl-2 homologs showed significant inhibition (Fig. 7B). Together, these results indicate that F1 is currently the only vaccinia virus Bcl-2 homolog with antiapoptotic activity.

DISCUSSION

Viruses, especially those with a protracted replication cycle, have evolved strategies to inhibit the mitochondrial checkpoint of apoptosis. The proapoptotic Bcl-2 proteins Bak and Bax are the functionally redundant gatekeepers of this checkpoint, which is why many viruses encode inhibitors that target both proteins (19, 26, 30, 44). Orthopoxviruses such as vaccinia virus encode F1, which was found to bind to Bak and Bim but not Bax (43, 53, 56). Subsequently, vaccinia virus N1 was reported to interact with endogenous Bax, Bad, and Bid (17). Since these cellular Bcl-2 proteins are complementary to one another, we set out to investigate whether F1 and N1 cooperate to inactivate both Bak and Bax to achieve efficient inhibition of infection-induced apoptosis. To test this hypothesis, we generated recombinant viruses lacking N1L, F1L, or both genes. Using these viruses, we found that the presence

of F1, but not N1, was required for vaccinia virus to inhibit STS- or infection-induced apoptosis. Moreover, we showed that no cooperativity occurred between N1 and F1 in these experimental settings. Outside the context of infection, N1 blocked neither STS- or UV-induced cell death nor apoptosis induced by overexpression of Bax, consistent with our finding that N1 and Bax do not interact.

Our results contradict previous findings (17, 28). We believe that this contradiction is explained in part by differences in experimental design. We and others examined the consequences of STS-induced cell death 5 h after infection to allow sufficient expression of N1 and F1 (43, 56). In contrast, Cooray et al. added STS at 2 h postinfection and analyzed the presence of cell death markers 1 and 2 h later (17). When we added STS at the same early time point, we found that not even WR provided any protection against STS-induced apoptosis (data not shown). This is consistent with the expression kinetics of N1 and F1, which are first detected from 3 and 2 hpi, respectively (Fig. 1A). Second, in contrast to the observations of Cooray et al., we found that N1 and Bax do not coimmunoprecipitate. We have no explanation for why we see different results, but our data are consistent with another study that also failed to observe an interaction between GFP-N1 and HA-Bax (9). While N1, like F1, is conserved among orthopoxviruses, it is noteworthy that N1L-like genes are also carried by lepori-, capri-, and cervidpoxviruses (27). Since these genera also contain either DPV022 or M11L homologues, Bax and Bak are presumably already inhibited (www.poxvirus.org). Encoding an additional Bax inhibitor in these genomes would thus be redundant, further supporting an alternative role for N1.

What is the primary function of N1 if it is not an antiapoptotic protein? Vaccinia virus N1 is a potent virulence factor in mice (10). Nevertheless, the mechanism underlying its function is as yet unclear. N1 was reported to be a multifunctional inhibitor of the NF- κ B and IRF pathways, which may explain how it is a strong virulence factor in mice (20, 28). Outside the context of infection, N1 can block induction of NF- κ B upon treatment with interleukin-1 α (IL-1 α), though some controversy exists over its ability to block tumor necrosis factor alpha (TNF- α)-induced NF- κ B activation (20, 28). Both reports further found that N1 can block TRAF2-induced NF- κ B activation, but they disagree about its effect on TRAF6-induced activation (20, 28). Moreover, the interaction of N1 with the IKK complex was not substantiated by subsequent experiments (16, 20). Toll-like receptor stimulation of NF- κ B and IRF3 signaling was blocked by N1, which can be explained partially by N1 binding to TBK1 and IKK ϵ (20). However, we have not been able to detect any interaction of N1 with overexpressed TBK1 or IKK ϵ (data not shown). These contradictory observations are not easily reconciled. Recently, infection of mice with an ectromelia virus lacking the N1L gene was shown to lead to an attenuation that was dependent on T cells but not B cells (29). Moreover, this attenuation did not require Toll-like receptor, RIG-I-like, or type I interferon signaling, which is inconsistent with the above-mentioned studies of cell lines (29). Clearly, the definitive mechanism of N1 action awaits further clarification.

In addition to F1 and N1, further structural Bcl-2-like proteins have recently been described for vaccinia virus (28, 33). The three-dimensional structures of A52, B14, and K7 reveal that they adopt a Bcl-2-like fold (28, 33). Like K7, A52 and B14 were shown to have their characteristic hydrophobic groove made up of helices α 2 to α 5 in a "closed" conformation, making the proteins unable

FIG 6 N1 does not inhibit STS-induced cell death. (A) Western blot analysis of cell lysates from HEK293-T-Rex-empty vector (E.V.) and -N1L cells showing the level of N1 induction after 20 h of doxycycline treatment. (B) Representative immunofluorescence images of HEK293-T-Rex-E.V. (left) and HEK293-T-Rex-N1L (right) cells stained with monoclonal anti-N1 antibody (green), before (top) or after (bottom) induction with doxycycline for 20 h. Cells were highlighted by nuclear staining with Hoechst 33342 (red). (C) Western blot analysis of cell lysates from HEK293-T-Rex-empty vector (E.V.) and -N1L cells treated with doxycycline or vehicle for 20 h, followed by UV irradiation at 200 mJ/cm². Cell lysates were prepared 4 h after irradiation, and apoptosis induction was assessed by Western blot analysis of PARP cleavage. (D) Cell viability analysis of HEK293-T-Rex-E.V. (left) and HEK293-T-Rex-N1L (right) cells induced or not with doxycycline for 20 h and treated for 6 h with increasing concentrations of STS. Black bars show cells not treated with STS. Graphs show data from a representative experiment, with each bar showing the mean and standard error of the mean for quintuplicate samples.

to accommodate incoming BH3 motif-containing helices (28, 33). Consistent with this, A52, B14, and K7 do not have any antiapoptotic activity (28, 49). Recent bioinformatic analysis predicted that A46, C1, C6, C16/B22, and N2 are also structural Bcl-2 homologs (27). No experimental data are currently available concerning the roles of C1, C6, C16/B22, and N2. Since we found that N1 does not bind Bax, we thought it possible that other members of the Bcl-2 family in vaccinia virus might contain a Bax-interacting surface. We tested all described Bcl-2-like proteins, with the exception of C16/B22, which is not encoded in the WR genome. None of these proteins were found to coimmunoprecipitate with Bax during infection. Moreover, none of these Bcl-2 homologs could inhibit drug-induced cell death.

It is unclear how vaccinia virus Bcl-2 proteins can bind such a wide variety of host proteins by using a similar fold (6). The amino terminus of F1 was recently described to contain a binding site for caspase-9 (62). Interestingly, A46 and C1 also contain amino-terminal extensions that do not share obvious sequence similarity with one another (27). It therefore seems possible that, at least in some family members, this extension could allow specific, novel binding surfaces to be added to the core Bcl-2 fold. This would by no means preclude the core Bcl-2 fold from acquiring binding affinities for host proteins that are not found among cellular Bcl-2

proteins. In fact, recent analysis of B14 found that its dimerization and IKK β binding surfaces overlap with the Bcl-2 core fold (11).

Here we show that vaccinia virus, the model *Orthopoxvirus*, does not appear to contain a direct inhibitor of Bax among the Bcl-2 homologs so far identified as being encoded by its genome. In contrast, poxvirus genera containing orthologs of myxoma virus M11L, fowlpox virus FPV039, or deerpox virus DPV022 inhibit both Bak and Bax. It is established that Bak and Bax are functionally redundant in their antiapoptotic activity (37, 58). This then raises the question of whether orthopoxviruses inhibit the Bak/Bax checkpoint less efficiently than viruses of other genera. We propose that this is not the case. First, even though F1 does not bind directly to Bax, it inhibits Bax activation (43, 53). While F1 acts mainly through Bak (24, 43), it also binds the BH3-only protein Bim (53). It has been shown that, at least in some contexts, Bim can directly activate Bax (38). During vaccinia virus infection, inhibition of Bim is partially responsible for the F1 antiapoptotic activity in MEFs (53). We found, however, that RNA interference (RNAi)-mediated ablation of Bim in HeLa cells did not attenuate Δ F1L virus-induced cell death (45). In contrast, we found that RNAi-mediated Bad knockdown nearly completely blocked Δ F1L virus-induced cell death, although we have not found any association between F1 and Bad (45; data not shown). In a recent study

FIG 7 Vaccinia virus Bcl-2 homologs do not interact with Bax or inhibit apoptosis. (A) HeLa cells infected with WR were transfected with the indicated pE/L-Flag constructs at 2 hpi and lysed 18 h later. Western blots of immunoprecipitates obtained using Flag antibody agarose (M2) and whole-cell lysates (input) were probed with antibodies against Bax, N1, and A36. The asterisk denotes the antibody light chain. (B) HeLa cells transfected with pEGFP and the indicated pCDNA4-Flag constructs were treated at 24 h posttransfection with STS for 4 h. The percentage of apoptosis was determined by quantification of fragmented nuclei in at least 150 GFP-positive cells in three independent experiments. Error bars represent standard errors of the means. ***, $P < 0.001$.

using a variety of primary cell lines derived from knockout mice, it was shown that Noxa is the predominant BH3-only protein mediating MVA- Δ F1L-induced cell death, while a minor role was reported for Bim (22). However, no role for Bid, Puma, or Bad was found (22). Presumably, the differences between our results and those of Eitz Ferrer et al. (22) reflect the use of RNAi compared to primary cell lines. Nevertheless, it would be interesting to observe whether Bax activation is increased after infection with viruses encoding an F1 protein that cannot bind Bim. More recently, F1 was also found to inhibit apoptosis via a caspase-9 binding site at its amino terminus, but to what extent this interaction contributes to F1 function during infection is still unexplored (62).

In the absence of direct Bax inhibition, orthopoxviruses may also boost their antiapoptotic capacity through additional viral proteins that target cell death in cooperation with F1 homologs. In

fact, TNF receptor (TNFR)/Fas death receptor signaling, interferon signaling, and caspase activation are inhibited in various ways by poxvirus proteins, and these could provide such added protection (6, 48, 52). Furthermore, activation of prosurvival signaling pathways after infection can cooperate to delay infection-induced death. For instance, we have shown that the loss of vaccinia growth factor (VGF), a vaccinia virus-encoded epidermal growth factor receptor (EGFR) agonist, exacerbates the cell death phenotype of the Δ F1L virus in a more-than-additive manner (45). This is due to the loss of EGFR-dependent MEK activation, although how this cooperates with F1 is as yet unclear.

Lastly, if Bak and Bax are not functionally redundant in all contexts, then this might explain why direct Bax inhibition may be less essential for orthopoxviruses. Indeed, some evidence supports this possibility, including observations made during infection (14, 34, 39). If so, poxviruses may have tailored the binding specificities of their viral Bcl-2 proteins for Bak or Bax to maximize their infectivity and subsequent spread. Although it remains to be established what determines the specificity of poxvirus Bcl-2 homologs, it is clear that the substantial selective pressures on viral genomes have secured proteins that are up to the task.

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