An Anti-apoptotic Viral Protein That Recruits Bax to Mitochondria*

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The viral mitochondria-localized inhibitor of apoptosis (vMIA), encoded by the UL37 gene of human cytomegalovirus, inhibits apoptosis-associated mitochondrial membrane permeabilization by a mechanism different from that of Bcl-2. Here we show that vMIA induces several changes in Bax that resemble those found in apoptotic cells yet take place in unstimulated, non-apoptotic vMIA-expressing cells. These changes include the constitutive localization of Bax at mitochondria, where it associates tightly with the mitochondrial membrane, forming high molecular weight aggregates that contain vMIA. vMIA recruits Bax to mitochondria but delays relocation of caspase-8-activated truncated Bid-green fluorescent protein (GFP) (t-Bid-GFP) to mitochondria. The ability of vMIA and its deletion mutants to associate with Bax and to induce relocation of Bax to mitochondria correlates with their anti-apoptotic activity and with their ability to suppress mitochondrial membrane permeabilization. Taken together, our data indicate that vMIA blocks apoptosis via its interaction with Bax. vMIA neutralizes Bax by recruiting it to mitochondria and “freezing” its pro-apoptotic activity. These data unravel a novel strategy of subverting an intrinsic pathway of apoptotic signaling.

Apoptosis is mediated through two main pathways, the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway. The extrinsic pathway is initiated by ligation of a plasma membrane death receptor, which results in a stepwise recruitment of adapters and initiator caspases (in particular, caspase-8) into the death-inducing signaling complex. In some cells (type I), activation of caspase-8 directly triggers activation of the caspase cascade (1), whereas in other cells (type II), caspase-8 mediates apoptosis only via proteolytic processing of BID (2, 3), which in turn leads to mitochondrial membrane permeabilization (MMP)1 and consequently to the amplification of the apoptotic signal. The intrinsic pathway is induced by various apoptotic stimuli that converge on mitochondria to trigger MMP (4). This permeabilization causes the release of mitochondrial pro-apoptotic factors, which in turn activate the caspase pathway.

MMP is tightly controlled, negatively and positively, by the proteins of the Bcl-2 family. Anti-apoptotic proteins such as Bcl-2, Bcl-xL, and Mcl-1 inhibit MMP, whereas pro-apoptotic Bcl-2 homologues such as Bax, Bak, and BH3-only proteins such as Bid and Bad enhance MMP (3–5). Recent evidence suggests that many stress signals trigger apoptosis, at least in part, by activation of pro-apoptotic BH3-only proteins (3). BH3-only proteins seem to induce MMP by two different pathways: either by direct activation of Bax/Bak (e.g. Bid or Bim) or by inhibition of anti-apoptotic activity of Bcl-2/Bcl-XL (e.g. Bad, Bik) (3, 6).

Bax is one of the key inducers of MMP (7). This protein is predominantly cytosolic in a conformation in which its hydrophobic α-5/6 helix and its N-terminal helix-1 are hidden within a hydrophilic globular structure. Upon pro-apoptotic signaling, for instance following exposure to t-Bid, Bax undergoes a conformation change that exposes its N terminus and, possibly, its BH3 domain (8, 9). This allows its translocation and its tight association with the mitochondria, apparently through integration into the outer membrane, forming homo-oligomers and hetero-oligomers with Bak (3, 10, 11). The mechanism of the association of Bax with the mitochondrial outer membrane during apoptosis is not well understood. Some data suggest that Bax interaction with cardiolipin (a mitochondria-specific lipid) is sufficient to trigger MMP (12). On the other hand, Bax has been shown to interact (directly or indirectly) with several mitochondrial proteins, voltage-dependent anion channel (13) and the adenine nucleotide translocase (14), although these findings are controversial (12, 15–17). In any case, most of the available evidence indicates that oligomerization of Bax and its tight association with the outer mitochondrial membrane lead to MMP (12, 15, 16, 18).

The abbreviations used are: MMP, mitochondrial membrane permeabilization; CMV, human cytomegalovirus; CHX, cyclohexamide; GFP, green fluorescent protein; EGFP, enhanced GFP; MEF, murine embryonic fibroblasts; STS, staurosporine; t-Bid, truncated Bid; vMIA, viral mitochondria-localized inhibitor of apoptosis; vMIA1, Myc-tagged vMIA; TRS, 2-[2-hydroxy-1-bis(hydroxymethyl)aminoethane sulfonic acid]; CHAPS, 3-(cholamidopropyl)dimethylammonium)-1-propane sulfonate; Z, benzoylformic acid. 
Apoptotic elimination of virally infected cells is one of the basic anti-viral responses of multicellular organisms. During evolution, viruses have developed a variety of strategies to suppress the extrinsic and/or the intrinsic pathways. Thus, some viral proteins block the extrinsic pathway by suppressing the activation of initiator caspases such as viral Flice-like inhibitory proteins (FLIPs) encoded by y-herpesviruses (19), vICA homologues of β-herpesviruses that associate with and prevent death-inducing signaling complex-dependent activation of pro-caspase-8 (20), CrmA of poxvirus, a serpin that inhibits the enzymatic activity of caspase-8 (21), and the Serp-2 serpin of myxoma virus (22). Other viruses encode inhibitors of the intrinsic pathway in particular structural and functional Bcl-2 homologues. Examples of structural homologues of Bcl-2 include KScbd-2, a Bcl-2 homologue from human herpesvirus (HHV8), which resembles Bcl-2 and Bcl-xL in its three-dimensional structure and, likely, in its function (23). Another cell death suppressor BHRF1, encoded by Epstein-Barr virus, shares some homologies with Bcl-2, but its mechanism of cell death suppression remains unclear (24). The EIB19K cell death suppressor of adenovirus shares only a modest amino acid sequence homology with Bcl-2 and appears to suppress cell death via its interaction with Bak and Bax, preventing their oligomerization (25, 26). Finally, a number of viruses intercept both the intrinsic and the extrinsic pathways. This applies to Myxoma virus, which encodes both M11L (27) and Serp-2 (22) and the cytomegalovirus with viral mitochondria-localized inhibitor of apoptosis (vMIA) (28) and vICA (20).

The vMIA is encoded by exon 1 of the immediate early UL37 gene of human cytomegalovirus (CMV). Although vMIA shares no sequence homology with Bcl-2, it has functional similarities with Bcl-2: it localizes at mitochondria, inhibits MMP, and is a potential cell death suppressor (29-31). Two domains of vMIA are necessary and, together, sufficient for its cell death-suppressing activity, its N-terminal domain (amino acids 5-34), and a segment between amino acids 117 and 147 (32). The N-terminal domain targets vMIA to mitochondria, whereas the function of the second domain as well as the molecular mechanism of cell death suppression by vMIA until now remained unknown.

Here we report that vMIA inhibits apoptosis through a unique mechanism distinct from that of any previously characterized cell death suppressor-targeting MMP. We show that vMIA, through an association with Bax, recruits Bax to mitochondria, inducing its oligomerization and tight association with mitochondrial outer membrane while inhibiting its pro-apoptotic function. Our findings thus reveal a yet undescribed viral strategy for interfering with the mitochondrial apoptotic signaling pathway.

MATERIALS AND METHODS

Cell Lines, Culture Conditions, and Transfection/Injection—HeLa and BJAB cells were stably transfected with UL37 exon 1 (vMIA), a deletion mutant of vMIA (vMIA), or empty vector, pcDNA3 (neo), as described elsewhere (29). Murine embryonic fibroblasts (MEF) were a generous gift of stanley Korsmeyer (Harvard University, Boston, MA), and human colorectal carcinoma HCT116/Bax (vMIA) and HCT116/Bax (Bax) otherwise isogenic cells were a generous gift of Bernd Vogelstein (The John Hopkins Oncology Center, Baltimore, MD). The MRC-5 human embryonic pulmonary fibroblasts (MEF) were cultured in Dulbecco’s modified Eagle’s medium and RPMI 1640 (PAALaboratories), respectively, supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 10 mM HEPEs, and 100 units/ml penicillin/ streptomycin at 37°C under 5% CO2. Transient transfections of HeLa cells, MEFs, and HCT116 cells with vectors for Myc-tagged vMIA or its deletion mutants (32), a Bax-GFP fusion gene (kindly provided by Dr. Shigemi Matsuyama (33)), pEGFP only and pEGFP-Bid (Clontech), vMIA mutants (32), or an empty vector, pcDNA3.1, were performed using the LipofectAMINE 2000 reagent (Invitrogen).

Immunofluorescence and Confocal Microscopy—Cells grown on coverslips were fixed with paraformaldehyde (4% w/v) and permeabilized with 0.1% SDS in phosphate-buffered saline (34, 35). Cells were then stained for the detection of cytochrome c (monoclonal antibody 6H2.B4 from Pharmingen) and the c-Myc epitope of tagged vMIA (monoclonal antibody 9E10, Santa Cruz Biotechnology). To detect subcellular localization of Bax, cells were stained with either mouse anti-Bax (32), rabbit polyclonal anti-Bax antiseraum (N20, Santa Cruz Biotechnology), or rabbit polyclonal vMIA (29). Epifluorescence analyses were performed using a Leica microscope (DMI202), and confocal analyses were performed using an LSM 510 Zeiss microscope equipped with a 63× objective (oil immersion).

Preparation of Mitochondria—Cells resuspended in a homogenization buffer (300 mM sucrose, 10 mM TES, 300 μM EGTA) supplemented with a mixture of protease inhibitors (Roche Applied Science) were disrupted by cavitation (PAR system; 150 p.s.i., 2 × 15 min) and centrifuged at 900 × g for 15 min twice to remove nuclei and unbroken cells. The supernatant was then centrifuged at 8000 × g for 15 min, and the pellet was used as a fraction enriched in mitochondria (“mitochondrial fraction”). The supernatant was further centrifuged at 100,000 × g for 1 h to yield the cytosolic cell fraction.

Detection of Tight Association of Bax with Mitochondrial Membranes—The mitochondrial fraction was resuspended in 10 mM Na2CO3, pH 12, to a final protein concentration of 1 mg/ml and incubated on ice for 20 min. Subsequently, the sample was centrifuged at 100,000 × g for 1 h. The supernatant, which contained loosely attached proteins, was recovered, and CHAPS was added to a final concentration of 2%. The pellet, which contained tightly membrane-associated proteins, was suspended in the homogenization buffer containing 2% CHAPS, incubated on ice for 1 h, sonicated, and centrifuged at 100,000 × g for 30 min.

Cell Filtration Analysis—Cells were cultured on a Superdex 200 column (16/60 and 10/30, Amersham Biosciences), as described before (10). Briefly, the column was equilibrated in 25 mM HEPES-NaOH, 300 mM NaCl, 200 μM dithiothreitol, 2% (w/v) CHAPS, pH 7.5, and run at a flow rate of 1 ml/min. The column was calibrated using gel filtration standard proteins from Amersham Biosciences. Fractions of 2 ml were collected. The proteins present in these fractions were precipitated with trichloroacetic acid (10%) in water and subjected to immunoblot analysis.

Cell-free Assay of Bax Incorporation into Mitochondria—The association of Bax or vMIA with purified rat liver mitochondria was determined (36). Briefly, proteins labeled with [35S]Met (Amersham Biosciences) were synthesized from cDNA using the TNT-coupled transcription/translation system (Promega). Labeled proteins were incubated with mitochondrial fractions at 37°C for 1 h in homogenization buffer. Radiolabeled proteins bound to the mitochondria were recovered in the pellet after centrifugation of the incubation mixture for 10 min at 4°C at 8000 × g. [35S]Met-labeled vMIA associated with isolated mitochondria was subjected to SDS-PAGE followed by quantitation of the radioactivity in a PhosphorImager (Amersham Biosciences) using the IPLab program (Signal Analytics, Vienna, VA).

Cell-free Association of vMIA with Mitochondria—HeLa cells were lysed in buffer 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, supplemented with either 1% Triton X-100 or 0.5 M urea in the presence of 4°C (100 μM) anti-Myc antibody to remove nuclei and cell debris, and the supernatants were rotated overnight at 4°C with the 9E10 anti-Myc antibody covalently bound to Affi-Prep10 beads (29). The beads were then washed in lysis buffer, and proteins were eluted from
beads in non-reducing NuPAGE (Invitrogen) sample buffer and then separated by SDS-PAGE (Invitrogen) under reducing conditions. Cell lysates were also run as controls. The gels were then analyzed by a standard immunoblot protocol using anti-Bax antibody (N20, Santa Cruz Biotechnology) and the ECL (enhanced chemiluminescence) detection system (Amersham Biosciences).

RESULTS AND DISCUSSION

Bax Is Predominantly Localized at Mitochondria of Non-apoptotic vMIA Cells—We examined the localization of Bax in two clones (clones 3 and 8) of HeLa cells stably transfected with Myc-tagged vMIA and HeLa/pDNA3 cells (Neo) stably transfected with the empty vector were stained with an antibody against Bax (red fluorescence) and an antibody against cytochrome c (cyt.c) (green fluorescence) and analyzed by confocal microscopy. Yellow color in the overlay of these two images indicates co-localization of Bax and cytochrome c (presumably in mitochondria). Another HeLa/vMIA clone 8 was similarly analyzed by confocal microscopy and showed the same pattern of staining. B, Bax colocalizes with vMIA in transiently transfected MEFs. Cells were transiently transfected with vMIAt or an empty vector (wild type (WT)), stained with an antibody against Bax (red fluorescence) and an antibody against vMIA (green fluorescence), and analyzed by confocal microscopy. The patterns of intracellular distribution of vMIA and of Bax and their colocalization is representative of the whole transfected population. Efficiency of transfection varies between 35 and 45% in two independent experiments. C, co-localization of Bax and vMIA in MRC5 normal human fibroblasts infected with human cytomegalovirus (AD169var-ATCC) 24 h after infection. Cells were permeabilized and stained with a polyclonal anti-vMIA antibody. No immunofluorescent staining was observed with preimmune serum (data not shown). D, vMIA expression correlates with the mitochondrial localization of Bax. Transiently transfected HeLa cells were collected at 2, 4, 6, and 17 h after transfection and fixed for the staining. At the indicated times, the percentage of cells showing a positive staining for vMIA narrowed 10, 20, 55, and 60%, respectively. Of note, we observed a clear correlation between the vMIA mitochondrial expression in individual cells and the mitochondrial localization of Bax. The patterns of intracellular distribution of vMIA and of Bax, and their colocalization, are representative of the whole transfected population.
To study the kinetics of vMIA expression and Bax relocation to mitochondria, we examined the time course of both vMIA and Bax staining in transiently transfected HeLa cells. As shown in Fig. 1D, 2 h after transfection in the absence of vMIA protein, Bax shows a diffuse distribution in the cytoplasm, whereas from 4 h after transfection to 17 h, we noted that even low expression level of vMIA (at 4 h) was sufficient to trigger significant relocation of Bax to mitochondria.

Next, we determined whether exposure of vMIA-expressing cells to various apoptotic stimuli induced apoptosis. HeLa/vMIA#3, were exposed to staurosporin (STS) and analyzed by immunofluorescence confocal microscopy for the intracellular distribution of Bax and cytochrome c. Representative images of the distribution of Bax (Fig. 2A, red fluorescence) and cytochrome c (Fig. 2A, green fluorescence) in HeLa/vMIA#3 cells (vMIA) and HeLa/pcDNA3 cells (Neo) that have or have not been exposed to STS are shown in Fig. 2A. In non-treated Neo cells, Bax staining is diffuse and is not colocalized with cytochrome c. Following the exposure of Neo cells to STS, Bax distribution becomes punctate and co-localized with cytochrome c (Fig. 2A, yellow) in mitochondria, which induces cytochrome c release (Fig. 2A, inset). In HeLa/vMIA#3 cells, Bax is constitutively co-localized with cytochrome c in granular structures irrespective of whether the cells were or were not exposed to STS (Fig. 2A). Cells were then exposed to STS, cis-platin, oligomycin + carbonyl cyanide p-trifluoromethoxy-phenylhydrazine, or anti-CD-95 + CHX. To quantitate the percentage of cells undergoing apoptosis, cytochrome c staining and chromatin condensation were examined either 3 or 16 h later. In vMIA-expressing cells, both cytochrome c release and chromatin condensation were blocked (Fig. 2B). Anti-apoptotic effects of vMIA were subsequently tested in transiently transfected cells (vMIA) in comparison with vMIA#3. As shown in Fig. 2, C and D, the transient expression of vMIA was effective in inhibiting both cytochrome c release and chromatin condensation induced by CD-95 ligation.

The subcellular distribution of Bax in HeLa/Neo and HeLa/vMIA that had or had not been exposed to STS was also determined by cell fractionation. As shown in Fig. 2E, exposure of HeLa/Neo cells to STS resulted in redistribution of Bax from the cytosol to the mitochondria/heavy membrane-enriched fraction and release of cytochrome c, in accord with previous observations (10). In contrast, both Bax and cytochrome c predominantly associated with the mitochondria-enriched fraction of vMIA cells that had or had not been treated with STS. One important observation made in these experiments was that in vMIA-expressing cells, Bax is constitutively localized to mitochondria, but in response to an apoptotic stimulus, MMP is not triggered, and the cells do not undergo apoptosis.

Then, we asked whether transient expression of ectopic Bax would behave as endogenous Bax. We transiently transfected HeLa/vMIA cells (clone 3) and control HeLa/Neo cells with human Bax fused in its C terminus to the green fluorescent protein (GFP) (33). To avoid the spontaneous translocation of Bax-GFP to mitochondria, we titrated the Bax-GFP plasmid. Twenty-four hours after transfection of a low concentration of Bax-GFP plasmid, in HeLa Neo cells, Bax-GFP shows a diffuse distribution, whereas in vMIA cells, the pattern colocalized with mitochondria without cytochrome c release in both cases (Fig. 3A). Then, to test the ability of Bax-GFP to induce cytochrome c release, we added STS (1 μM) to some of the transfected cultures, further incubated cells for 3 h in the presence of 50 μM Z-VAD-fmk (to delay cell death), and examined the intracellular distribution of Bax-GFP under a fluorescent microscope (Fig. 3A). A large fraction of Bax-GFP was relocated to mitochondria, and in the majority of cells, cytochrome c was released into the cytoplasm, indicating that these cells were undergoing apoptosis (Fig. 3B). In vMIA-expressing cells, both Bax-GFP and cytochrome c were localized to mitochondria, irrespective of whether the cells had or had not been exposed to STS.

To further investigate whether vMIA can suppress apoptosis induced by overexpression of Bax-GFP, we used higher concentrations of the plasmid where Bax-GFP spontaneously relocated to mitochondria in 30% of HeLa/Neo cells. This phenomenon coincided with cytochrome c release and chromatin condensation in HeLa/Neo cells, whereas in vMIA-expressing cells, cytochrome c retained its granular localization, and chromatin remained intact (Fig. 3, C and D). These results indicate that (i) ectopically expressed Bax translocates spontaneously to mitochondria in vMIA cells and (ii) high doses of Bax-GFP that are sufficient to kill Neo cells were not toxic for vMIA cells.

**Apoptosis Inhibition by vMIA Is Associated with Mitochondrial Localization of Bax:**—Two segments of the vMIA protein (Tyr-5–Leu34 and Asp-118–Arg-147) contain domains that together are essential for its anti-apoptotic function (32). The first region (amino acids 2–23) is necessary and sufficient for the mitochondrial localization of vMIA, whereas the molecular function of the second domain (amino acids 118–147) has not been elucidated prior to this study. A “mini-vMIA” consisting of these two segments was shown to be a functional cell death suppressor (32). We have tested the ability of functionally deficient vMIA deletion mutants to induce relocation of Bax to mitochondria (Fig. 4A). These experiments were done in transient transfection assays with HeLa cells. Apoptosis was detected by cytochrome c release and chromatin condensation. First, we confirmed that vMIA and mini-vMIA protected transiently transfected HeLa cells against STS-induced apoptosis, whereas three mutants with a deletion either within the mitochondria-targeting domain (Δ2–23) or within the second anti-apoptotic domain (Δ115–130 and Δ131–147) were inefficient in preventing apoptosis (Fig. 4B). In accord with the literature (10), we found that a certain amount of endogenous Bax in HeLa cells is normally associated to mitochondria (Figs. 2E and 4C). Of note, this result is not in contradiction with immunofluorescence analysis (Fig. 1A). This only reflects the difference in the sensitivity of immunofluorescence versus subcellular fractionation in detecting low levels of Bax. Only the functional cell death suppressors, full-length vMIA and mini-vMIA, induced a significant relocation of Bax to mitochondria, whereas functionally inactive vMIA Δ2–23, vMIA Δ115–130, and vMIA Δ131–147 failed to trigger the relocation of Bax, as shown both in immunofluorescence microscopic observations (Fig. 4A) and by immunoblotting analysis of cellular fractions (Fig. 4C). These experiments demonstrate a strong correlation between the anti-apoptotic function of vMIA and its ability to relocate Bax to mitochondria.

**vMIA Causes Tight Association of Oligomeric Bax with Mitochondria:**—Prior to inducing permeabilization of mitochondria in cells undergoing apoptosis, Bax becomes strongly associated with mitochondria, possibly integrating into the outer mitochondrial membrane, and forms either homo- or heterooligomeric complexes (3, 8–11, 38–40). These events were not observed in Bel-2-expressing cells exposed to pro-apoptotic stimuli, indicating that Bel-2 blocked apoptosis either at or upstream of this step (6, 38). Since in vMIA-expressing cells Bax was predominantly localized at mitochondria, we tested whether Bax was oligomerized in these cells and whether its association with mitochondria was tight or loose. For these experiments, we prepared mitochondria-enriched membrane fractions from HeLa or BJAB/vMIA and BJAB/pcDNA3 cells. In addition, to examine
whether the status of Bax oligomerization and association with mitochondria was changed during apoptosis, we also prepared mitochondria-enriched membrane fractions from BJAB/vMIA and BJAB/pcDNA3 cells that had been exposed to STS. To separate loosely attached Bax (Fig. 5A, Att) from tightly associated Bax (Fig. 5A, Ins), possibly integrated into the mem-

**Fig. 2.** vMIA inhibits the release of cytochrome c (cyt.c) from mitochondria despite the local presence of Bax. A. HeLa/vMIA#3 cells and HeLa/pcDNA3 cells (Neo) were incubated or not with staurosporine (1 μM) for 3 h. Cells were then stained for immunodetection of Bax (red) and cytochrome c (green) and then examined by laser fluorescence confocal microscopy. Co, control. B, vMIA suppresses cytochrome c release from mitochondria and nuclear condensation in HeLa/vMIA#3 cells that were treated for 16 h with STS (250 nM), cisplatin (50 μM), or oligomycin (5 μM) plus carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (1 μM) or anti-CD-95 (4 μg/ml) and CHX (2 μg/ml). The percentage of cells exhibiting a diffuse cytochrome c staining was determined as in panel A. Results are representative of three independent experiments (400 cells were counted in each sample). C, vMIA suppresses apoptosis in stably (HeLa/vMIA#3) or transiently (vMIA/t) transfected HeLa cells with or without staurosporine (STS). Cells were treated with either 3 or 6 μg/ml anti-CD-95 and stained for immunodetection of cytochrome c (red) and Myc-tag of vMIA (green) and counterstained with Hoechst 33342. The scale bar corresponds to 10 μm. D, the percentage of cytochrome c release and chromatin condensation was determined as described in the legend for panel B. E, Bax is localized in mitochondria of HeLa/vMIA#3 cells before (Co) and after treatment with STS. Cells were subjected to subcellular fractionation, and the cytosolic as well as the mitochondrial (Mito) fractions were blotted for immunodetection of Bax and cytochrome c.
brane (10, 42), these membrane fractions were treated with 0.1 M Na₂CO₃, as described under "Materials and Methods," and then mitochondria-containing pellets were examined by immunoblot analysis. As expected (Fig. 5A), Bax was only loosely attached to the mitochondrial membranes isolated from BJAB/pcDNA3 cells prior to induction of apoptosis but became strongly associated with the membranes following exposure of cells to STS. In contrast, in vMIA-expressing cells, Bax was constitutively tightly associated with mitochondrial membranes irrespective of whether the cells had or had not been exposed to STS.

We also examined the oligomerization status of Bax in HeLa and BJAB cells stably transfected with pcDNA3 or vMIA by size exclusion chromatography of CHAPS-solubilized mitochondrial fractions (Fig. 5B and not shown). Bax extracted from healthy HeLa/pcDNA3 cells migrated as a monomer with the apparent molecular size of slightly above 20 kDa. Upon treatment with STS, most of the extracted Bax was found in large complexes with an apparent molecular mass mostly above 90 kDa. In contrast, a major fraction of Bax extracted from healthy vMIA-expressing cells eluted as part of large complexes, and this pattern of Bax migration did not change after exposure to STS. These experiments demonstrated that vMIA-induced relocation of Bax at mitochondria was accompanied by either homo-, or possibly, hetero-oligomerization of Bax and its tight association with mitochondria.

vMIA Interacts with Bax and Facilitates Its Tight Association with Mitochondria—To test whether Bax physically associates with vMIA, we immunoprecipitated vMIA with anti-Myc antibody from CHAPS or Triton X-100 lysates of BJAB or HeLa cells stably transfected either with Myc-tagged vMIA or with the empty vector and then examined these immunoprecipitates for the presence of Bax by immunoblot analysis (Fig. 6A). These experiments detected Bax in either CHAPS or Triton X-100 immunoprecipitates from HeLa or BJAB/vMIA cell lysates but not from control HeLa or BJAB/pcDNA3 cell lysates (Fig. 6A). This indicated that, indeed, Bax formed a complex with vMIA detected with either of these two detergents. Furthermore, when we immunoprecipitated vMIA deletion mutants from Triton X-100 lysates of BJAB cells, only wild type vMIA and functionally active mini-vMIA (Δ35–112/Δ148–163 deletion mutant) formed a complex with Bax. The ability of mini-vMIA
consisting essentially of only the two anti-apoptotic domains to associate with Bax. In transfected cells, HeLa cells were transiently transfected with various vMIA mutants. Twenty-four hours after transfection, cells were fixed, permeabilized, and stained for detection of active Bax (red) and vMIA (green) followed by laser fluorescence confocal microscopy. The scale bar indicates 10 μm. WT, wild type. B, inhibition of apoptosis by different vMIA mutants. Cells transfected as in panel A were cultured in the absence or presence of staurosporine (1 μM, 3 h), and the percentage of cells exhibiting cytochrome c (Cyt.c) release, the translocation of Bax to mitochondria, or chromatin condensation was determined in transfected cells (30–40% of total cells). 400 cells were counted in each sample. Results are presented as means of three experiments. C, mitochondrial (Mito) localization of Bax in vMIA mutants correlates with the anti-apoptotic function of vMIA. Cells were transfected with vector only (Neo), wild type vMIA, or various vMIA mutants. Twenty-four hours later, the transfected cells were fractionated into the cytosol and mitochondria. These fractions were blotted for immunodetection of Bax. Equal loading was confirmed by Coomassie staining (not shown).

To further characterize the putative physical interaction between Bax and vMIA, we used an in vitro translation system of vMIA and two different forms of Bax. Bax Δ2–20 (also called Bax Δ2–37) is a newly described splice variant of Bax lacking the N-terminal 20 amino acids. This Bax variant is constitutively associated with mitochondria and highly apoptogenic (43). Bax Δ2–37, which lacks the mitochondrial-targeting signal contained in the α-1 helix (amino acids 19–37) (44), is constitutively cytosolic, fails to induce apoptosis, and cannot be activated to relocate to mitochondria. These two forms of Bax thus represent the two opposites of the spectrum of the conformation adopted by wild type Bax, activated (Bax Δ2–20), and inactive Bax (Δ2–37). Both forms of Bax interact with in vitro translated vMIA (Fig. 6C). Next, we analyzed the capacity of vMIA to stimulate the tight association of Bax with mitochondria. In vitro translated vMIA readily incorporated into the mitochondrial fraction, and this was also observed for Bax Δ2–20 (Fig. 6D, middle row). In contrast, in vitro translated Bax Δ2–37 did not incorporate into the mitochondrial fraction. However, when co-incubated with vMIA (Fig. 6D, bottom row), Bax Δ2–37 was incorporated into the mitochondrial fractions as efficiently as Bax Δ2–20 (Fig. 6D). As a result, it appears that vMIA can stimulate tight association of Bax with mitochondria.

Bax Modified by vMIA Becomes Refractory to the Action of t-Bid—vMIA does not prevent caspase-8-mediated cleavage of Bid during CD-95-mediated apoptosis of HeLa cells and acts downstream of this event but upstream of permeabilization of mitochondria (29). Formation of the t-BID/Bax complex, a crit-
vMIA and Bax

vMIA and Bax is shown. A, HeLa and BJAB cells stably expressing or not vMIA were lysed in lysis buffer containing either Triton X-100 (TX 100) or CHAPS as described under “Materials and Methods” and subjected to immunoprecipitation with anti-Myc antibody followed by SDS-PAGE and immunoblot detection of Bax. Samples of cell lysates prior to immunoprecipitation are labeled as L, and samples of immunoprecipitated vMIA are labeled as IP. Note that Bax co-immunoprecipitated with vMIA equally well from CHAPS- and Triton X-100 lysates. B, BJAB cells stably transfected with pcDNA3, Myc-tagged full-length vMIA, or various deletion constructs of vMIA lysed with Triton X-100-containing lysis buffer and immunoprecipitated with anti-Myc antibody followed by SDS-PAGE and immunoblot detection of Bax. Note that Bax co-immunoprecipitated only with full-length vMIA and mini-vMIA. As for full-length vMIA, mini-vMIA coimmunoprecipitation with Bax was confirmed in CHAPS-containing lysis buffer. WT, wild type. C, direct interaction between vMIA and Bax ΔΔ–20 or Bax ΔΔ–37. Four fmol of in vitro translated vMIA was incubated with 4 fmol of His-tagged Bax ΔΔ–20 or Bax ΔΔ–37 protein immobilized on nickel-nitrilotriacetic acid agarose beads or the agarose beads alone (control, Co). The input of in vitro translated vMIA (25% of the amount added to the beads) and the amount of 35S-labeled proteins bound to the beads was determined by Phosphorimager after SDS-PAGE. Similar data were obtained in three independent experiments. D, mitochondrial relocation of in vitro translated Bax protein in the absence or presence of vMIA. The indicated Bax mutants were generated in an in vitro transcription/translation system, and 25% of samples were run as input controls. The remaining samples were subjected to a mitochondrial association assay, either alone (single) or after co-incubation with vMIA (co-inc.), as indicated in the panel. The results shown in panel D are representative for three independent assays. Note that mitochondrial association of the Bax ΔΔ–20 mutant is not influenced by vMIA, whereas that of Bax ΔΔ–37 strictly depends on vMIA. Bax ΔΔ–37, which lacks the mitochondrial-targeting signal contained in the α-1 helix (amino acids 19–37) (44), fails to associate with mitochondria and thus serves as internal control for the specificity of association with mitochondria.

Concluding Remarks—A major finding of our study is that the viral inhibitor of apoptosis vMIA neutralizes Bax by recruiting it to mitochondria and “freezing” its pro-apoptotic activity. We showed that the triggering of Bax relocation to mitochondria by vMIA is a general phenomenon, observed in different human and mouse cell lines and in human fibroblasts infected with human CMV.
Our data support a correlation between the anti-apoptotic function of vMIA and its ability to relocate/interact with Bax at mitochondria. Indeed, among different deletion mutants of vMIA, only mini-vMIA (a truncated protein consisting of the two segments of vMIA that are required for mitochondrial localization and anti-apoptotic function) maintained the ability to relocate/interact with Bax at mitochondria. The two vMIA mutants with deletions in the C terminus domain of vMIA (Δ115–130 and Δ131–147) showed mitochondrial localization but were ineffective in preventing apoptosis and failed to relocate/associate with Bax. These results suggest that the C terminus domain of vMIA-(115–147) was required for cell death suppressing by favoring the association of vMIA with Bax.

Previously published experiments with recombinant Bax protein demonstrated that this protein is capable of forming higher order structures with membrane-permeabilizing properties and thus destabilizes lipid bilayers (49), induces the formation of non-specific ion channels in synthetic lipid bilayers, and forms cytochrome c-permeable conduits in liposomes (50). These effects produced by recombinant Bax protein coincided with its oligomerization, and it has been tacitly concluded from these studies that relocation of Bax to mitochondria accompanied by its oligomerization within the mitochondrial membrane is sufficient to mediate the MMP reaction (3, 12, 16, 45, 51). The data reported in this study provide a seemingly contradicting example. vMIA stimulates relocation of Bax to mitochondria, its oligomerization, and tight association with mitochondria, possibly due to its integration into the mitochondrial outer membrane. Although this status of Bax normally correlates with apoptotic MMP, MMP is blocked in vMIA-expressing cells. This is reminiscent of the description of the translocation and formation of dimer/complexes of Bax at mitochondria without MMP, occurring in a taxol-resistant cell line (41).

How vMIA recruits Bax to mitochondria is an ongoing conundrum. Newly translated vMIA could act as a co-chaperone for Bax during its relocation to mitochondria. Alternatively, vMIA, which is already present on mitochondria, could recruit cytosolic Bax. How can we explain, in speculative terms, that Bax can oligomerize without spontaneously triggering MMP? It is possible that vMIA keeps Bax in an inactive state. In favor of this hypothesis, we found that the recruitment of t-Bid into mitochondria is delayed in vMIA-expressing cells. However, it is also quite possible that oligomerization of Bax itself is not sufficient to create the pores responsible for MMP and that interactions with additional mitochondrial proteins are required (16). At present, it is not clear whether Bax oligomers formed in the presence of vMIA have the same characteristics.

Fig. 7. vMIA delays translocation of t-Bid to mitochondria. A, cell extracts were prepared from Bid-GFP transfected cells, treated or not with anti-CD-95 + CHX, and subjected to anti-GFP immunoblotting. CD-95 ligation causes similar levels of Bid activation in different cells. B, vMIA-mediated inhibition of t-Bid relocation to mitochondria. Cells treated as in panel A were examined by fluorescence microscopy to detect cytochrome c (cyt.c) (red) and the accumulation of the Bid/t-Bid-GFP fusion protein (green) in mitochondria. C, quantitation of t-Bid translocation. The percentage of cells exhibiting a clearly punctate pattern of GFP fluorescence among the transfected population was determined as in panel B. The percentage of apoptosis was determined by calculating the number of cells exhibiting a diffuse pattern of cytochrome c. Results are means of three different experiments.

Fig. 8. vMIA suppresses apoptosis as efficiently as Bax−/− cells. HCT116/Bax−/− cells and HCT116/Bax+/− cells were transiently transfected with vMIA, and 24 h later, the cells were treated with anti-CD-95 + CHX for 6 h and fixed for cyt c (Cyt.c) and nuclear staining. Error bars represent standard deviation of three independent experiments (400 cells were counted in each sample). WT, wild type.
as those formed after Bax activation during apoptosis. Additional studies are clearly needed to answer this question. Moreover, since vMIA has no sequence homology with other anti-apoptotic members of the Bcl-2 family, we will address in the future whether vMIA specifically targets Bax or may interact with other pro-apoptotic family members.

vMIA is an obligatory virulence factor for human cytomegalovirus and cells infected by cytomegalovirus, in particular, connective tissues that are abundant in Bax. Based on this, one can speculate that inhibitors of the Bax-vMIA interaction would constitute a new kind of treatment of cytomegalovirus infection, especially in immunocompromised individuals.

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