The permeability transition pore triggers Bax translocation to mitochondria during neuronal apoptosis

TA Precht[1,2], RA Phelps[1,2], DA Linseman[1], BD Butts[1], SS Le[1], TA Laessig[1], RJ Bouchard[1] and KA Heidenreich*,[1]

[1] Department of Pharmacology, University of Colorado Health Sciences Center and the Denver Veterans Affairs Medical Center, Denver, CO, USA
[2] These authors contributed equally to this paper
* Corresponding author: KA Heidenreich, Department of Pharmacology, University of Colorado Health Sciences Center at Fitzsimons, PO Box 6511, Mail stop 8303, Aurora, CO 80045-0511, USA;
E-mail: Kim.Heidenreich@UCHSC.edu

Abstract

Cerebellar granule neurons (CGNs) require depolarization for their survival in culture. When deprived of this stimulus, CGNs die via an intrinsic apoptotic cascade involving Bim induction, Bax translocation, cytochrome *c* release, and caspase-9 and -3 activation. Opening of the mitochondrial permeability transition pore (mPTP) is an early event during intrinsic apoptosis; however, the precise role of mPTP opening in neuronal apoptosis is presently unclear. Here, we show that mPTP opening acts as an initiating event to stimulate Bax translocation to mitochondria. A C-terminal (α9 helix) GFP-Bax point mutant (T182A) that constitutively localizes to mitochondria circumvents the requirement for mPTP opening and is entirely sufficient to induce CGN apoptosis. Collectively, these data indicate that the major role of mPTP opening in CGN apoptosis is to trigger Bax translocation to mitochondria, ultimately leading to cytochrome *c* release and caspase activation.
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Introduction

The normal development of the nervous system involves generation, as well as, degeneration of neurons to maintain tissue homeostasis.[1] The balance of these processes is controlled in part by apoptosis, a type of programmed cell death. Dysregulated apoptosis is thought to contribute to a variety of neurodegenerative diseases.[2] An established *in vitro* model for investigating the pathways of neuronal apoptosis is cultured rat cerebellar granule neurons (CGNs).[3] When cultured CGNs are deprived of serum and depolarizing extracellular potassium, cell death proceeds by an intrinsic, mitochondrial cascade that involves cytochrome *c* release and caspase activation.[4,5]

The intrinsic apoptotic pathway is regulated by the Bcl-2 family of proteins.[6] Bcl-2 family members possess one or more of four Bcl-2 homology (BH) domains. The precise complement of these structural domains determines the pro- or antiapoptotic activity of Bcl-2-related proteins.[7] For example, family members that possess exclusively BH3 domains are proapoptotic, and have multiple mechanisms of action. Some BH3-only proteins, such as Bad and Bik, promote the intrinsic death pathway by sequestering Bcl-2 and inhibiting its antiapoptotic effects[8] by displacing Bid and Bim.[9] Bid and Bim can act directly to activate Bax, a multidomain proapoptotic Bcl-2 family member.[10] In response to undefined apoptotic signals, Bax translocates to the mitochondria where it oligomerizes into a heteromeric protein channel. This putative Bax channel inserts into the outer mitochondrial membrane and releases apoptotic factors, such as cytochrome *c*, eventually leading to caspase activation and cell death.[11]

The intrinsic apoptotic pathway is also regulated by the mitochondrial permeability transition pore (mPTP).[12] The mPTP is thought to be a large protein complex composed of the voltage-dependent anion channel (VDAC), the adenine nucleotide translocator (ANT), and cyclophilin D.[13] A recent study in hepatocytes has shown that the ANT may not be required to form a functional mPTP, but might play a role in regulation of the pore.[14] The components of the mPTP are associated with the mitochondrial inner membrane and form a pore that is permeable to low molecular weight ($\leqslant 1500$ Da) solutes and ions, ultimately leading to mitochondrial membrane depolarization and swelling of the mitochondrial matrix.[15] Whether BH3-only proteins are directly involved in the formation or function of the mPTP during neuronal apoptosis has not been elucidated. Likewise, the potential role of the mPTP in the regulation of Bax translocation during neuronal apoptosis is presently unclear.

De Giorgi *et al.*[16] have previously shown that formation of the mPTP indirectly signals Bax to move to mitochondria in the non-neuronal COS cell line. Under these conditions, the release of cytochrome *c* is dependent upon Bax movement, multimer formation, clustering, and insertion into the outer mitochondrial membrane. In contrast, in glioma cells, mPTP opening, indicated by a loss of mitochondrial membrane potential and matrix swelling, does not occur until very late

phases of cell death after cytochrome *c* release.[17] Similarly, Krohn et al.[18] previously showed that staurosporine-induced cytochrome *c* release and caspase activation occurs in hippocampal neurons prior to any reduction in mitochondrial membrane potential. These studies indicate that the mPTP may or may not influence Bax-mediated cytochrome *c* release in neurons.

In the current study, we investigated the role of the mPTP in localizing Bax to mitochondria in CGNs undergoing apoptosis. We utilized two specific tools to investigate the involvement of the mPTP in Bax translocation. Firstly, we used the mPTP inhibitor, cyclosporin A (CyA), which binds cyclophilin D and prevents pore formation. Secondly, we used a C-terminal point mutant of Bax α (T182A) that constitutively localizes to mitochondria and induces apoptosis. We show that mPTP opening is an initiating event to stimulate Bax translocation to mitochondria. Moreover, constitutive localization of Bax to mitochondria circumvents the requirement for mPTP opening and is sufficient to induce apoptosis.

Results

CyA blocks opening of the mPTP in CGNs subjected to trophic factor withdrawal

Primary CGNs deprived of serum and depolarizing extracellular potassium (trophic factor withdrawal) die via activation of a mitochondrial apoptotic cascade.[5] Profound mitochondrial swelling and loss of mitochondrial membrane potential are early events in CGN apoptosis that are consistent with opening of the mPTP, but the role of these mitochondrial changes in the execution of the apoptotic cascade is unclear. CyA inhibits opening of the mPTP by binding to cyclophilin D, an integral protein in the mPTP.[19] Initially, we evaluated the utility of CyA as a pharmacological tool to prevent the mitochondrial swelling and depolarization induced by trophic factor withdrawal in CGNs. CGNs incubated in control medium (containing serum and depolarizing potassium; 25K + Ser) displayed small, compact mitochondria visualized by staining with the cationic dye JC-1 (Figure 1a, left panel). In addition, CGNs maintained in control medium showed an intact mitochondrial membrane potential demonstrated by substantial tetramethylrhodamine ethyl ester (TMRE) staining (Figure 1b, left panel). When CGNs were switched for 4 h to apoptotic medium (lacking serum and depolarizing potassium; 5K−Ser), they displayed marked mitochondrial swelling (Figure 1a, middle panel) and a significant loss of mitochondrial membrane potential indicated by a substantial reduction in TMRE staining (Figure 1b, middle panel). The addition of CyA (10 μM), at the time of trophic factor withdrawal, completely blocked both mitochondrial swelling (Figure 1a, right panel) and mitochondrial depolarization (Figure 1b, right panel), consistent with its known actions as an mPTP inhibitor. To further support the CyA effect on mPTP opening, we utilized a technique involving the cell-permeable dye calcein-AM, which upon hydrolysis by cell esterases produces a green fluorescent dye. The calcein/Co^{2+} assay for PTP opening was adapted from Petronilli et al.[20] to visualize selective accumulation of calcein in mitochondria by quenching cytosolic and nuclear fluorescence with Co^{2+} addition. The
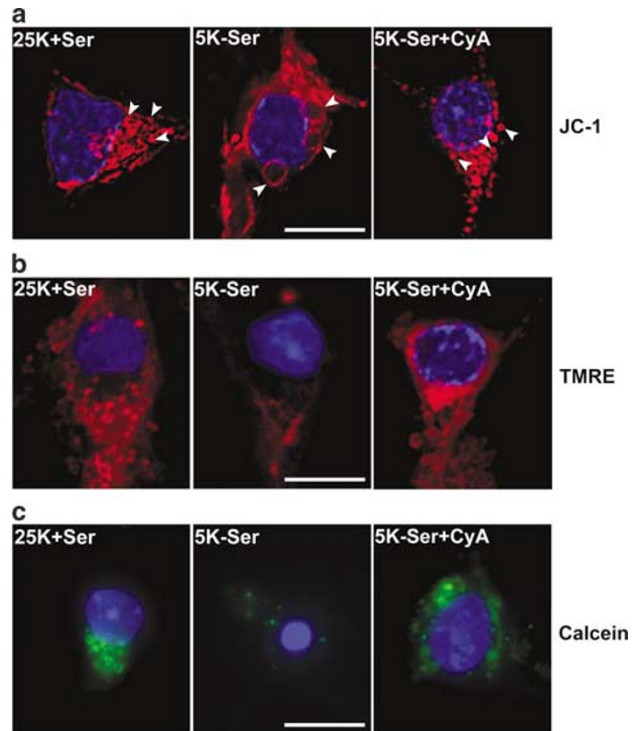

Figure 1 Trophic factor withdrawal induces CyA-sensitive mitochondrial swelling and mitochondrial membrane depolarization. Mitochondrial swelling and depolarization are indicative of the opening of the PTP. Both the marked mitochondrial swelling and depolarization are blocked by the PTP inhibitor CyA. CGNs were incubated for 4 h in either control medium containing serum and depolarizing (25 mM) extracellular potassium (25K + Ser) or apoptotic medium lacking serum and containing 5 mM extracellular potassium (5K−Ser) in the absence or presence of CyA (10 μM). (**a**) At 30 min prior to fixation, JC1 (final concentration, 2 μg/ml) and Hoechst dye were added to the cultures to stain mitochondria and nuclei, respectively. JC1 fluorescence was captured using a Cy3 filter under a × 100 oil objective. Mitochondria are indicated by the arrows. Note that CyA completely blocks the marked swelling of mitochondria induced by trophic factor deprivation. The images shown are representative of results obtained from four separate experiments. Scale bar, 10 μm. (**b**) CGNs were incubated exactly as described in (**a**). At 30 min before the end of the incubation period, tetramethylrhodamine ethylester (TMRE, 500 nM) and Hoechst were added directly to the cells. After incubation, the coverslips were inverted onto slides into a small volume of PBS containing glucose and *p*-phenylenediamine (antiquenching agent). Then, living cells were imaged under a × 100 oil objective. Nuclear staining is shown in blue; TMRE is shown in red. CGNs maintained in either control medium or apoptotic medium containing CyA displayed many distinct mitochondria with intense TMRE staining, indicative of an intact mitochondrial membrane potential (left and right panels). In contrast, CGNs incubated in apoptotic medium alone (middle panel) showed only diffuse TMRE staining, indicative of mitochondrial depolarization. The images shown are representative of three separate experiments. Scale bar, 10 μm. (**c**) CGNs were incubated exactly as described in (**a**). At 20 min prior to the end of the incubation period, cells were loaded with 1 μM calcein-AM in the presence of 1 mM Co^{2+}, ∼0.1% Pluronic F-127, and Hoechst dye. After incubation, cells were washed and mounted on slides in a small volume of PBS containing glucose and *p*-phenylenediamine. Living cells were imaged under a × 100 oil objective. Nuclear staining is shown in blue; calcein fluorescence is in green. The majority of CGNs maintained in control medium or apoptotic medium containing CyA displayed green fluorescence in distinct subcellular fractions, which was confirmed to be mitochondrial (see Materials and Methods) (left and right panels). However, CGNs maintained in apoptotic medium alone (middle panel) showed a dramatic loss of mitochondrial calcein fluorescence indicating an open mPTP. Images are representative of four separate experiments. Scale bar, 10 μm

left panel of Figure 1c illustrates that calcein fluorescence can be localized to mitochondria under control conditions (25K + Ser), which was confirmed by colocalization with TMRE (data not shown). Upon trophic factor withdrawal (5K−Ser), calcein fluorescence is substantially decreased in cells undergoing apoptosis (Figure 1c, middle panel) and this decrease can then be reversed by addition of CyA (Figure 1c right panel). Multiple regions of interest were visualized to confirm the loss and rescue of calcein fluorescence of 5K−Ser and 5K−Ser + CyA, respectively (data not shown). These data indicate that trophic factor withdrawal induces PTP opening in mitochondria and that this opening can be blocked by CyA. In addition to binding cyclophilin D, CyA inhibits the type 2B Ser/Thr phosphatase, calcineurin, which in some cells dephosphorylates and activates the proapoptotic protein Bad, an action that is mimicked by FK506.[21] FK506 (10 μM) had no effect on either mitochondrial swelling or depolarization induced in CGNs incubated in apoptotic medium (data not shown), suggesting that the ability of CyA to block mPTP opening triggered by trophic factor withdrawal in CGNs is independent of its ability to inhibit calcineurin phosphatase activity.

CyA inhibits cytochrome *c* release in CGNs subjected to trophic factor withdrawal: the effects of CyA are independent of Bim regulation

Cytochrome *c* release is a key event in the activation of the intrinsic death pathway.[13,22] Once released from mitochondria, cytochrome *c* interacts with apaf-1 and pro-caspase-9 to form an apoptosome that ultimately activates the intrinsic initiator caspase-9.[23] Caspase-9 subsequently cleaves the executioner caspase-3 resulting in the proteolysis of critical cellular proteins and apoptotic cell death.[24] To determine if opening of the mPTP is required for cytochrome *c* release in trophic factor-deprived CGNs, we analyzed the distribution of cytochrome *c* in subcellular fractions. In CGNs maintained in control medium, cytochrome *c* was localized predominantly in the mitochondrial fraction (Figure 2a, left two lanes). Following 6 h of trophic factor deprivation, a substantial portion of the cytochrome *c* was released from mitochondria and redistributed into the cytosolic fraction (Figure 2a, middle two lanes). Addition of CyA to CGNs incubated in apoptotic medium completely blocked the release of cyt *c* from the mitochondria into the cytosol (Figure 2a, right two lanes). The enrichment of the cytosolic and mitochondrial fractions was verified by immunoblotting for the integral mitochondrial membrane protein, cytochrome *c* oxidase subunit IV (COX IV) (Figure 2a, lower blot). These results indicate that inhibition of the mPTP with CyA prevents the release of cyt *c* from mitochondria in trophic factor-deprived CGNs.

Recently, induction of the BH3-only protein, Bim, has been shown to be critical for intrinsic neuronal apoptosis.[5,25,26] Immunoblot analysis of CGN extracts for Bim demonstrated that the expression of the short isoform of Bim (Bim$_s$, $\sim$15 kDa) was very low in control conditions and increased markedly upon induction of apoptosis (Figure 2b, first and second lanes). We previously reported that insulin-like growth factor-I (IGF-I), a prosurvival factor for CGNs *in vitro* and *in*
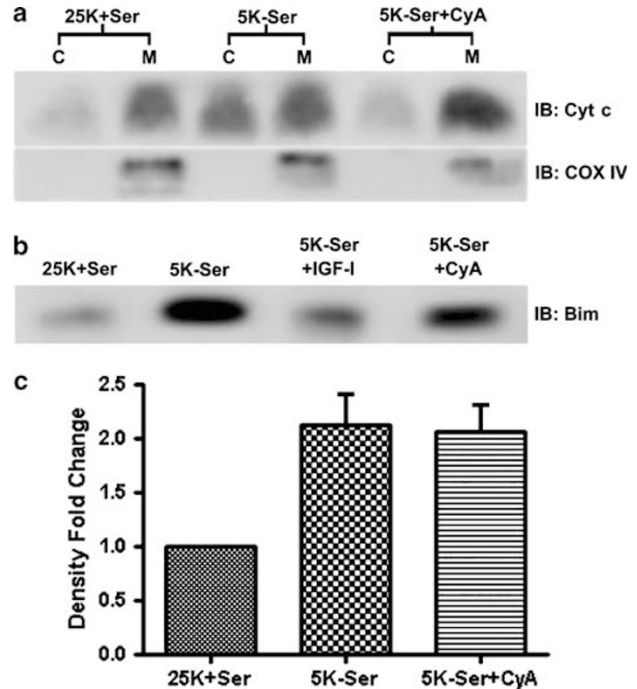

Figure 2 CyA inhibits cyt *c* release in trophic factor-deprived CGNs independent of effects on Bim. (**a**) CGNs were incubated as described in Figure 1, but for 6 h. After incubation, the cells were lysed and fractionated to separate the mitochondrial fraction (M) from the cytosolic fraction (C). The subcellular fractions were then subjected to SDS–PAGE on 15% polyacrylamide gels, and the proteins were transferred to PVDF membranes. Membranes were immunoblotted (IB) with an antibody to cytochrome *c* (cyt *c*). The blots were then stripped and reprobed with an antibody to COX IV as a specific marker for the mitochondrial fractions. The blots shown are typical of results obtained in three separate experiments. (**b**) CGNs were incubated for 6 h in either control (25K + Ser) or apoptotic (5K−Ser) medium in the absence or presence of IGF-I (200 ng/ml) or CyA (10 μM). After incubation, cells were lysed and CGN lysates were immunoblotted with an antibody that recognizes Bim. (**c**) Immunoblots of Bim were analyzed for densitometry and normalized to 25K + Ser. The bar graph represents the fold change of density for 5K−Ser and 5K−Ser + CyA (10 μM). There was no significant difference between 5K−Ser and 5K−Ser + CyA as determined by ANOVA

vivo,[27,28] inhibits CGN apoptosis without blocking either mitochondrial swelling or depolarization.[5] A primary mechanism underlying the protective effects of IGF-I is its ability to block the increased expression of Bim$_s$ induced by trophic factor withdrawal in CGNs (Figure 2b, third lane). To exclude the possibility that CyA blocks cytochrome *c* release by similarly influencing the expression of Bim, we examined CyA effects on Bim induction in CGNs incubated in apoptotic medium. Although both IGF-I[5] and CyA (Figure 2a) inhibit cytochrome *c* release, CyA did not substantially block the induction of Bim$_s$ in apoptotic medium (Figure 2b, fourth lane). Densitometric measurements of Bim immunoblots (Figure 2c) show an approximate two-fold increase in Bim expression in 5K−Ser medium when normalized to 25K + Ser. There was no significant change in Bim expression when CyA was included in the medium. Collectively, these data suggest that there are mPTP-dependent (CyA-sensitive) and Bim-dependent (IGF-I-sensitive) mechanisms that act in concert to trigger cytochrome *c* release in trophic factor-deprived CGNs.

Translocation of GFP-Bax to mitochondria during CGN apoptosis is inhibited by CyA

Since CyA had no significant effect on the induction of Bim, we investigated another potential mechanism by which the mPTP could regulate cytochrome *c* release in CGNs. We hypothesized that opening of the mPTP may trigger Bax translocation to mitochondria in trophic factor-deprived CGNs. To examine this question, we utilized a gene gun transfection method[29] to transiently transfect primary CGNs with a construct encoding for a green fluorescent protein (GFP)-Bax fusion protein.[30] We found that addition of CyA to GFP-Bax-transfected CGNs at the time of trophic factor withdrawal significantly blunted translocation of GFP-Bax to mitochondria (Figure 3a). Quantitative analysis showed that addition of CyA to apoptotic medium sustained a subcellular distribution of GFP-Bax that was indistinguishable from that observed in CGNs maintained in control medium (Figure 3b). In control conditions, $73 \pm 2\%$ of GFP-Bax-expressing cells demonstrated a diffuse localiza-
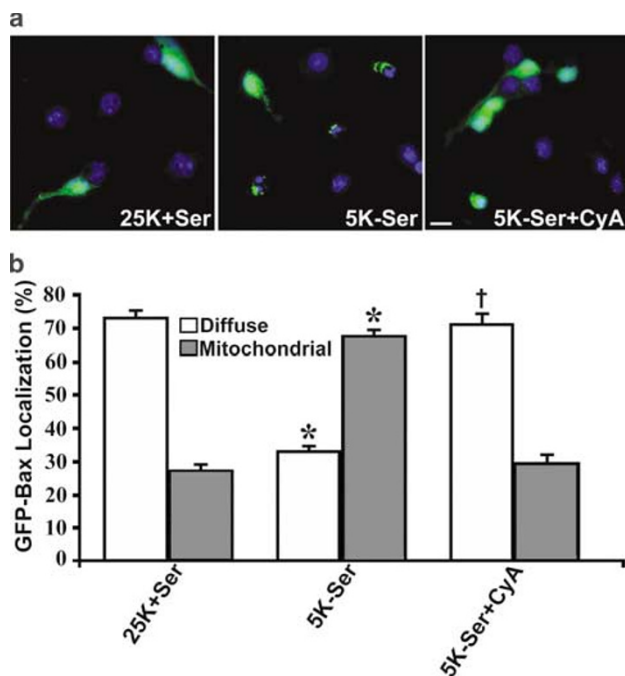

Figure 3 Inhibition of PTP opening with CyA blocks translocation of GFP-Bax to mitochondria. (**a**) CGNs were transfected with GFP-Bax using a Helios gene gun as described in Materials and Methods and were then incubated for 48 h to allow for sufficient GFP-Bax expression. CGNs were then incubated for an additional 4 h in either control (25K + Ser) or apoptotic (5K−Ser) medium in the absence or presence of CyA (10 μM). CGNs were fixed in paraformaldehyde and Hoechst dye was added to stain the nuclei. Images were captured using a × 63 oil immersion lens. GFP-Bax was captured in the FITC channel (shown in green) and Hoechst was captured in the DAPI channel (shown in blue). The images shown are representative of results obtained in three separate experiments. Scale bar, 10 μm. (**b**) Quantification of the percentage of cells demonstrating either diffuse or mitochondrial (punctate) GFP-Bax localization was performed for the conditions described in (**a**). Approximately 75–100 cells/coverslip expressed the GFP-Bax. The percentage of diffuse *versus* mitochondrial GFP-Bax-positive cells per incubation condition is shown as the mean±S.E.M. of three independent experiments, each performed in duplicate. *Significantly different from the 25K + Ser GFP-Bax distribution ($P < 0.01$). †Significantly different from the 5K−Ser GFP-Bax distribution ($P < 0.01$)

tion of the fusion protein. The fraction of CGNs displaying diffuse GFP-Bax decreased significantly ($P < 0.01$) to $33 \pm 2\%$ in apoptotic conditions, with a corresponding increase in mitochondrial localization. Inclusion of CyA, at the time of trophic factor deprivation, sustained the diffuse GFP-Bax fraction at $67 \pm 3\%$ of the GFP-Bax-expressing CGNs. Similar to its lack of effect on mPTP opening, FK506 had no effect on the translocation of GFP-Bax to mitochondria observed in apoptotic medium (data not shown). These results suggest that opening of the mPTP acts as an initiating stimulus to trigger Bax translocation to mitochondria in trophic factor-deprived CGNs.

Bax T182A constitutively localizes to mitochondria in human embryonic kidney 293 cells

Translocation of Bax from the cytosol to the mitochondria is critical for initiation of the intrinsic apoptotic cascade.[30] Once localized to mitochondria, Bax oligomerizes to form a pore in the outer mitochondrial membrane that is permeable to cytochrome *c*.[31,32] To further define the role of the mPTP in Bax localization, we created a novel mutant of Bax α that constitutively localizes to mitochondria even in the absence of an apoptotic stimulus. This mutant contains a threonine 182 to alanine substitution that is thought to expose the C-terminal helix of Bax for membrane insertion, similar to that described for an S184A mutant.[33] We transfected GFP-Bax T182A into human embryonic kidney (HEK) 293 cells and compared its localization to wild-type (WT) Bax. GFP-Bax WT showed a diffuse distribution in transfected HEK293 cells (Figure 4a, left panel), whereas GFP-Bax T182A displayed a punctate staining pattern associated with mitochondria (Figure 4a, right panel). To confirm the differential localization of WT and mutant Bax, we isolated cytosolic and mitochondrial fractions from HEK293 cells transfected with either GFP-Bax WT or GFP-Bax T182A. Immunoblotting with an antibody to GFP revealed that cells transfected with GFP-Bax WT contained approximately equal amounts of GFP-Bax in cytosolic and mitochondrial fractions (Figure 4b, first and second lanes). In contrast, cells transfected with GFP-Bax T182A expressed mutant GFP-Bax that was almost entirely localized in the mitochondrial fraction (Figure 4b, third and fourth lanes). The integrity of the fractionation procedure was confirmed by reprobing the Western blot for a mitochondrial marker, COX IV (Figure 4b, lower blot).

Bax T182A causes cytochrome *c* release, caspase activation, and apoptosis in HEK293 cells

Although we showed that GFP-Bax T182A constitutively localized to mitochondria, it was important to determine if this movement induced apoptosis via the intrinsic cascade. To accomplish this, HEK293 cells were transfected with GFP-Bax T182A and immunostained with primary antibodies recognizing cytochrome *c*, and active (cleaved) fragments of caspase-9 and -3. Healthy HEK293 cells not expressing the GFP-Bax T182A mutant (cells in field not indicated by arrows) displayed intact nuclei and cytochrome *c* staining that was mainly associated with mitochondria (Figure 5a). In contrast,
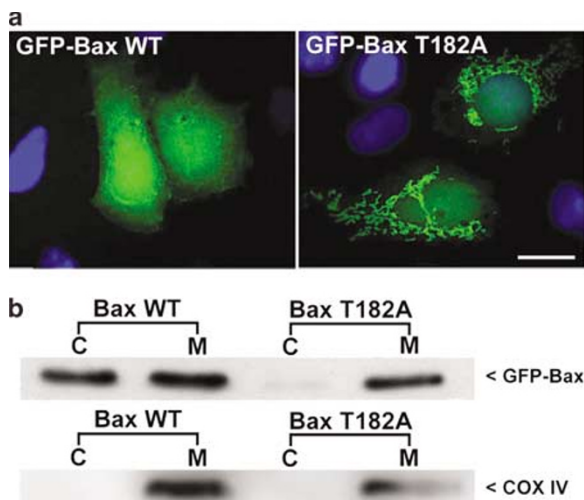
Figure 4 GFP-Bax T182A transiently expressed in HEK293 cells is constitutively localized to mitochondria. (**a**) HEK293 cells were transfected with either GFP-Bax WT or GFP-Bax T182A using the lipofectamine method. Cells were incubated for 24 h after transfection, fixed, incubated with Hoechst dye, and visualized for GFP-Bax localization. GFP-Bax WT-expressing cells showed a diffuse staining of the fusion protein throughout most of the cell. In contrast, GFP-Bax T182A-expressing HEK cells showed extremely punctate staining displayed by concentrated fluorescence within mitochondria. Cells were imaged in 2 ml of PBS with a ×63 water lens. (**b**) Confirmation of GFP-Bax T182A localization to mitochondria was completed by fractionation of HEK293 cells expressing either GFP-Bax WT or GFP-Bax T182A. Cells were lysed and fractionated to separate the mitochondrial fraction (M) from the cytosolic fraction (C) as described in Materials and Methods. Fractions of each were run on 15% SDS–polyacrylamide gels, transferred to PVDF membranes, and immunoblotted for GFP using a monoclonal antibody to detect GFP-Bax. The GFP-Bax blot confirmed that cells transfected with GFP-Bax WT display an approximately equal distribution of Bax in cytosol and mitochondria, whereas cells transfected with GFP-Bax T182A had almost all of the GFP-Bax located in mitochondria. The blot was stripped and reprobed for COX IV to confirm that the fractions were indeed cytosolic and mitochondrial as indicated

cells expressing the GFP-Bax T182A mutant (indicated by white arrows) were clearly apoptotic as seen by 4′,6-diamidino-2-phenylindole (DAPI) staining illustrating condensed and fragmented nuclei in GFP-positive cells (Figure 5a–c). GFP-Bax T182A-expressing cells showed either a diffuse cytochrome c staining pattern, indicative of mitochondria that had lost their membrane integrity, or little to no cytochrome c staining due to late phases of apoptosis where cytochrome c has completely escaped the cell (Figure 5a). In addition to cytochrome c release, active caspase-9 and -3 were detected in GFP-Bax T182A-positive cells (Figure 5b and c). The ability of Bax T182A to induce apoptosis in HEK cells was confirmed by assessing nuclear condensation in cells expressing either Bax WT or Bax T182A. HEK cells expressing GFP-Bax WT displayed a low level of apoptosis (21±5%) that was significantly greater than cells expressing GFP alone. Cells expressing GFP-Bax T182A showed a significant increase in the amount of apoptosis (46±7%), relative to WT Bax-expressing cells (Table 1). These data indicate that the constitutive mitochondrial localization of the Bax point mutant, T182A, induces HEK cell apoptosis through cytochrome c release and caspase activation.

The T182A point mutant of GFP-Bax constitutively localizes to mitochondria and induces CGN apoptosis in the absence of mPTP opening

We have demonstrated the functional result of expressing the GFP-Bax T182A mutant in HEK cells (i.e., induction of mitochondrial apoptosis). To determine if triggering Bax translocation to mitochondria is indeed the major function of mPTP opening during CGN apoptosis, we examined the effects of the Bax T182A point mutant on CGN apoptosis and mitochondrial membrane potential. In agreement with the data in HEK cells, GFP-Bax T182A demonstrated a punctate distribution when expressed in CGNs maintained in control (25K + Ser) medium (Figure 6a). Consistent with its localization to mitochondria, expression of GFP-Bax T182A in CGNs induced the release of cytochrome c (Figure 6b, upper panels) and activation of the intrinsic initiator caspase-9 (Figure 6b, middle panels) and the executioner caspase-3 (Figure 6b, lower panels). In accordance with activation of the intrinsic death pathway culminating with activation of caspase-3, GFP-Bax T182A induced CGN apoptosis at a significantly higher rate than was observed with either GFP, a measure of the baseline apoptosis induced following gene gun transfection, or WT GFP-Bax maintained in control medium (Table 1). These results demonstrate that constitutive localization of Bax to mitochondria is sufficient to induce CGN apoptosis even in the presence of trophic support.

Finally, to determine if the apoptosis induced by constitutive localization of GFP-Bax T182A to mitochondria required opening of the mPTP, we measured the mitochondrial membrane potential in CGNs expressing this point mutant. To assess the loss of membrane potential, TMRE, a dye sensitive to intact mitochondrial membrane potential, was utilized. The left panel of Figure 7a illustrates that under healthy conditions (25K + Ser) CGNs contain abundant TMRE staining, but the right panel shows that apoptotic conditions (5K−Ser) cause CGNs to have almost no TMRE staining indicative of mPTP opening. After 48 h of incubation in control medium post-transfection, transfected CGNs showed clear mitochondrial localization of GFP-Bax T182A as well as condensed and fragmented chromatin, consistent with induction of apoptosis (Figure 7b, left panel). However, even while undergoing apoptosis, transfected CGNs demonstrated an intact mitochondrial membrane potential as shown by a significant accumulation of TMRE (Figure 7b, right panel). These data show that constitutive localization of Bax to mitochondria does not by itself trigger mPTP opening, but still induces apoptosis (Figure 7c, middle bar). To further strengthen this argument, we have counted CGNs expressing GFP-Bax T182A that have mitochondrial GFP fluorescence as well as mitochondrial TMRE fluorescence. Of the cells that were GFP-positive, approximately 78±3.8% showed mitochondrial localization. Of these 78±3.8%, approximately 95±1.2% had mitochondrial TMRE fluorescence. Moreover, inclusion of CyA throughout the 48 h incubation period had no effect on GFP-Bax T182A localization to mitochondria (data not shown) or its ability to induce apoptosis (Figure 7c, right bar). In support of this, we have previously shown that an isoform of Bax, Bax σ, which lacks 12 aa near the C-terminus of the protein, does not move to mitochondria even in
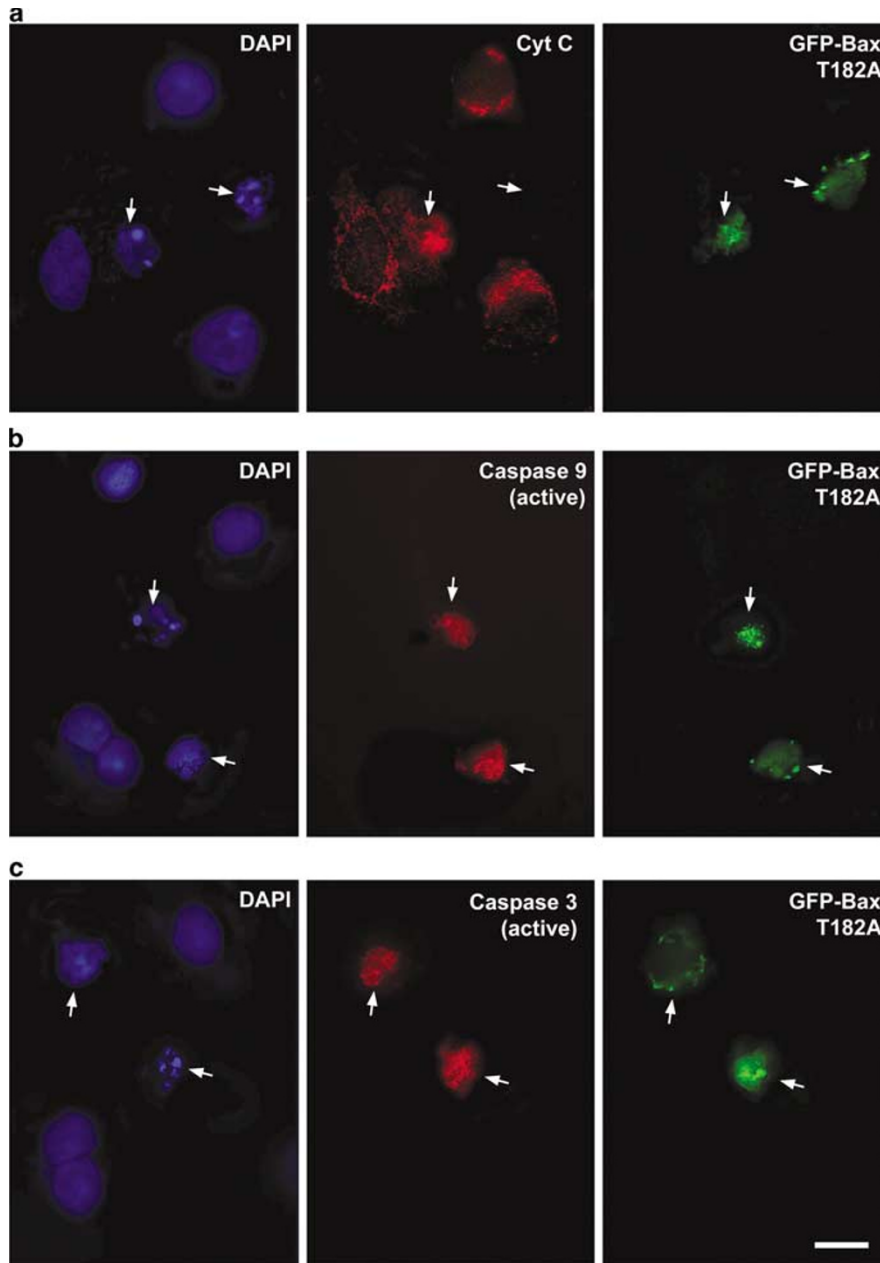
Figure 5 Expression of GFP-Bax T182A in HEK293 cells induces apoptosis by cytochrome *c* release and activation of caspase-9 and -3. HEK293 cells were transfected with GFP-Bax T182A as described in Figure 3 legend. The arrows indicate cells expressing GFP-Bax T182A. Cells were allowed to express GFP-Bax T182A for 24 h, and they were then fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and immunostained as described in Materials and Methods. (**a**) Cytochrome *c* release was observed using a rabbit polyclonal primary antibody and a rhodamine-conjugated secondary (red staining). In cells expressing GFP-Bax T182A, cytochrome *c* was either diffuse (cytosolic) or had permanently left the cell typical of apoptosis induced by the intrinsic death pathway. Cells not expressing GFP-Bax T182A showed punctate cytochrome *c* staining within mitochondria. (**b**) Caspase-9 activation is shown in cells expressing GFP-Bax T182A using a polyclonal antibody that specifically detects cleaved caspase-9. (**c**) Caspase-3 activation is shown in cells expressing GFP-Bax T182A using a polyclonal antibody that specifically detects cleaved caspase-3. Untransfected cells not expressing GFP-Bax T182A contain no active caspases (absence of staining). Cells were imaged in 2 ml of PBS with a ×63 water lens. All panels in Figure 4a–c are composites of multiple fields captured from two separate experiments

response to trophic factor withdrawal and mPTP opening (data not shown).[42] Collectively, these data indicate that the principal role of mPTP opening in CGN apoptosis is to trigger Bax translocation to mitochondria. Further, by bypassing opening of the mPTP with a Bax mutant that constitutively localizes to mitochondria, CGN apoptosis can be induced in the absence of a permeability transition and is therefore unaffected by CyA.

Discussion

In this study, we examined the hypothesis that the mPTP plays a role in signaling Bax translocation from the cytosol to mitochondria during neuronal intrinsic apoptosis. We showed that CGNs, deprived of trophic support, commit to intrinsic apoptosis that can be blocked by the addition of CyA. We also showed that Bax moves to the mitochondria following trophic

Table 1 GFP-Bax T182A induces apoptosis in HEK293 cells and CGNs

	% Apoptosis	
Constructs	**HEKs ($n=4$)**	**CGNs ($n=6$)**
GFP	1.9 ± 0.2	9.4 ± 1.7
GFP-Bax WT	21.4 ± 5.2*	21.5 ± 2.5*
GFP-Bax T182A	46.7 ± 7.5*,†	81.4 ± 2.5*,†

HEK293 cells were transfected with GFP-Bax T182A as previously described. Cells were then incubated in Hoechst dye for 30 min, scraped, centrifuged for 2 min at 14k r.p.m. to pellet all cells, washed with PBS, recentrifuged, and resuspended in 200–300 μl of PBS. A 10 μl portion of the cell suspension was then used to count four fields for each condition (GFP; GFP-Bax WT; GFP-Bax T182A) on an Olympus inverted stereomicroscope with a $\times$40 objective. GFP-expressing cells were counted as apoptotic if they had condensed nuclei and counts are reported as % apoptosis of total cells expressing GFP. The percentage of GFP-, GFP-Bax-, and GFP-Bax T182A-expressing cells that were apoptotic is shown as the mean$\pm$S.E.M. of three independent experiments. Quantification of the percent apoptosis of CGNs transfected with GFP, GFP-Bax WT, or GFP-Bax T182A was assessed after 48 h. Approximately 75–100 cells/coverslip expressed GFP, GFP-Bax WT, or GFP-Bax T182A. Each expressing cell was classified as either healthy or apoptotic using nuclear condensation and fragmentation as indicators of apoptosis. Experiments were carried out as above, except that each was performed in duplicate. *Significantly different from the GFP (baseline transfection) control ($P<0.01$). †Significantly different from the GFP-Bax WT ($P<0.01$)

factor withdrawal and this translocation is sensitive to CyA as well. To further support the hypothesis that the mPTP has a role in directing Bax movement, we showed that a Bax T182A mutant localized to mitochondria and induced apoptosis, regardless of the presence of CyA. The data presented here suggest a model for the intrinsic apoptotic pathway in CGNs that involves the mPTP signaling Bax to translocate to mitochondria and release cytochrome c.

It has been previously demonstrated that overexpression of Bax can lead to apoptosis in the absence of an internal or external apoptotic stimulus.[34] It is possible that in our model, overexpression of the Bax T182A mutant induces apoptosis by a mechanism separate from that of trophic factor withdrawal. Although these two activators of apoptosis may initiate death differently, we have shown that they converge on a common, intrinsic pathway that involves cytochrome c release and caspase-9 and -3 activation, and is sensitive to CyA.

Previous studies examining the relationship between the mPTP and Bax in apoptosis have produced contradictory results. Overall, this debate stems from three perspectives of how the intrinsic mitochondrial apoptotic cascade is initiated. First, Bax alone may be sufficient to induce cytochrome c release without opening of the mPTP.[35] Second, opening of the mPTP alone may be enough to induce cytochrome c release without Bax translocation, oligomerization, or insertion.[36] And third, it has been proposed that Bax may form a composite pore with the mPTP that is necessary to release cytochrome c.[37] In vitro studies have shown that recombinant Bax can induce cytochrome c release from isolated mitochondria, independent of the cell type specific origin, with or without opening of the mPTP.[35] However, the data presented here confirm the requirement for Bax in neuronal apoptosis and indicate that the mPTP must signal to Bax to induce its translocation to mitochondria.

A Bax-only pore model cannot explain results showing that Bax interacts with a putative component of the mPTP, the
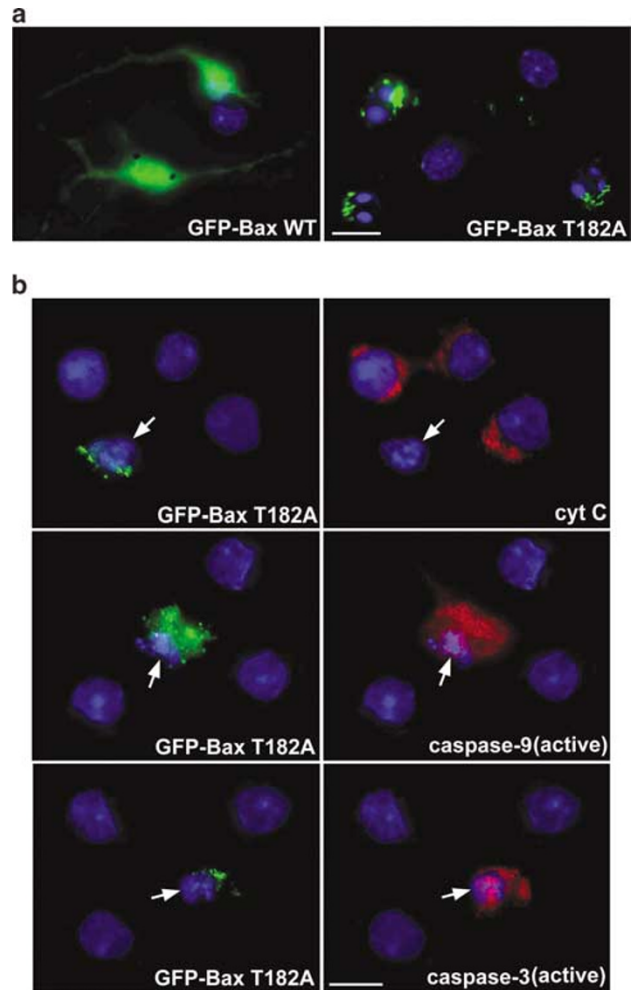

Figure 6 GFP-Bax T182A constitutively localizes to mitochondria in CGNs and induces cytochrome c release and apoptosis. (a) CGNs were transfected with either GFP-Bax WT or GFP-Bax T182A using a Helios gene gun as described in Materials and Methods and were incubated in control (25K + Ser) medium for 48 h to allow for sufficient GFP-Bax expression. Cells were fixed in paraformaldehyde and Hoechst dye was added to stain the nuclei. Images were captured using a 63 $\times$ oil immersion lens. GFP-Bax was captured in the FITC channel (shown in green) and Hoechst was captured in the DAPI channel (shown in blue). Note that the mutant Bax constitutively localized to the mitochondria even in control medium, while the WT GFP-Bax demonstrated primarily diffuse staining. The images shown are representative of results obtained in four separate experiments, each performed in duplicate. Scale bar, 10 μm. (b) CGNs were transfected with GFP-Bax T182A using a Helios gene gun as described in Materials and Methods and allowed to incubate in control (25K + Ser) medium for 48 h to allow for sufficient GFP-Bax T182A expression. After incubation, the CGNs were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 5% BSA. Cytochrome c (cyt c), active caspase-9, and active caspase-3 were immunostained by incubating the cells with polyclonal antibodies to cyt c and the active fragments of caspase-9 and -3, followed by Cy3-conjugated secondary antibodies. Fluorescent images were captured using a $\times$100 oil objective. Note that both caspase-3 and -9 were active only in the cells expressing GFP-Bax T182A and that the cyt c was released by the mitochondria only in cells expressing the mutant Bax. The images shown are representative of results obtained in three separate experiments, each performed in duplicate. Scale bar, 10 μm

VDAC.[38] Furthermore, Bax has been shown to induce opening of a VDAC channel in synthetic liposomes and this opening can be blocked by CyA.[39,40] Given the Bax–VDAC
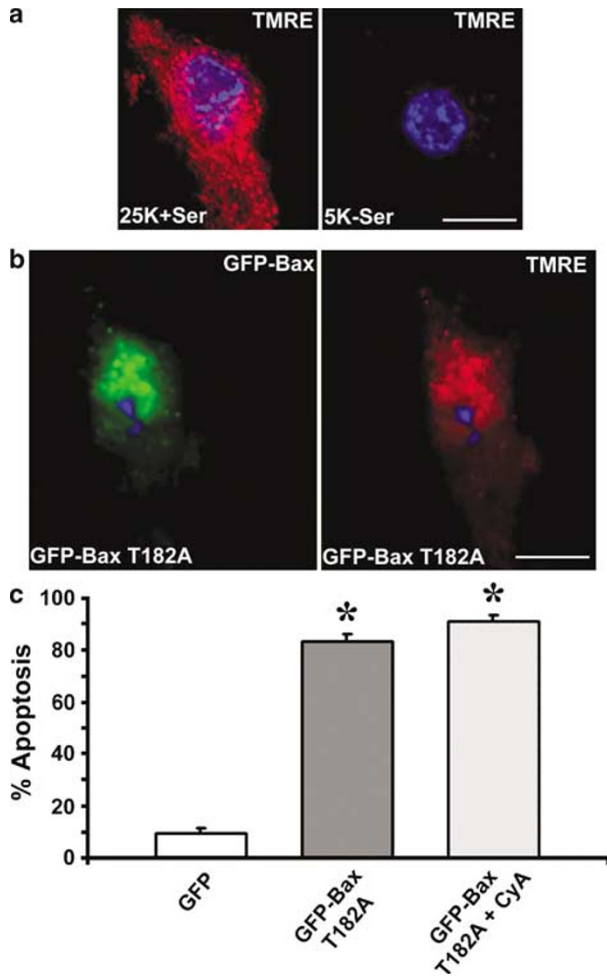
Figure 7 Constitutive localization of GFP-Bax T182A to mitochondria induces CGN apoptosis independently of mPTP opening. (**a**) Rat CGNs were treated and imaged exactly as described in Figure 1b with regard to cells stained with TMRE. After incubation with Hoechst dye and TMRE (500 nM), the coverslips were inverted onto slides into a small volume of PBS containing glucose and *p*-phenylenediamine (antiquenching agent). Living cells were then were imaged under a $\times$ 100 oil lens. Under healthy conditions (25K + Ser), cells have distinct, localized staining of TMRE displaying mitochondria with intact membrane potentials. Under apoptotic conditions (5K−Ser), cells show little to no TMRE staining, indicating that the mPTP has formed and mitochondrial membranes have lost their potential. (**b**) Rat CGNs were transfected with GFP-Bax T182A using the Helios gene gun and cells were maintained in control medium (25K + Ser). The left panel shows a cell expressing GFP-Bax T182A, identified by GFP fluorescence, imaged using the FITC channel (green fluorescence), where Bax T182A is constitutively localized to mitochondria, and as a result the cell is undergoing apoptosis. In the right panel, the same cell is stained by TMRE (red fluorescence) in a punctate distribution that colocalized with the GFP-Bax fluorescence, indicating that while the GFP-Bax is constitutively localized to mitochondria and apoptosis is induced by this localization, mitochondrial membranes still maintain their potential. (**c**) Apoptosis induced by the constitutively mitochondrial GFP-Bax T182A cannot be blocked by the addition of cyclosporin A Rat CGNs were transfected with either GFP or GFP-Bax T182A as previously described. They were allowed to incubate in control medium (25K + Ser) for 48 h in the presence or absence of CyA (10 μM) and Hoechst dye was added to stain the nuclei. Imaging for counting was performed using a 63 $\times$ oil immersion lens. Cells were considered apoptotic if their nuclei were either condensed and/or fragmented. Approximately 200 GFP-Bax-expressing cells were counted from three replicate coverslips per experiment, with each experiment performed at least three times. Cells expressing GFP-Bax T182A in the presence or absence of CyA that were scored as apoptotic are shown as the mean $\pm$ S.E.M. of three independent experiments. *Significantly different from the GFP (baseline transfection) control ($P < 0.01$)

interaction data, a VDAC/cyclophilin D-composed mPTP supports the model that a composite channel is likely to exist; however, *in vivo* colocalization of any components of the mPTP with Bax has yet to be shown.

It is presently unclear how the PTP opening triggers Bax translocation to the mitochondria. It is known that PTP opening causes shape changes in mitochondria.[5] It is also known that certain members of the Bcl-2 family, such as Bim, are associated with the dynein motor complex.[8] It is possible that an association between Bax and dynein may exist and be regulated by mitochondrial shape change. It has also been demonstrated that Bax is sequestered in the cytosol by the scaffolding protein 14-3-3 and that this interaction negatively regulates Bax movement.[41] This Bax/14-3-3 interaction might be a site for regulation by the mPTP.

In summary, we have found that CyA blocks opening of the mPTP and the release of cytochrome *c* in CGNs deprived of trophic support, without affecting Bim expression. Moreover, inhibition of the mPTP with CyA efficiently blocked Bax translocation to mitochondria. A T182A mutant of GFP-Bax that constitutively localized to mitochondria induced cytochrome *c* release, caspase-9 and -3 activation, and CGN apoptosis without inducing opening of the PTP. Further, the constitutive localization of Bax T182A to mitochondria and its ability to induce CGN apoptosis were unaffected by the addition of CyA. We conclude that the major role of the PTP during neuronal apoptosis induced by trophic factor withdrawal is to direct Bax localization to mitochondria.

Materials and Methods

Materials

cDNA encoding for the α-isoform of human Bax protein fused to GFP (GFP-Bax) was kindly provided by Dr. Richard Youle (NIH, Bethesda, MD, USA). FK506 and CyA were purchased from Calbiochem (La Jolla, CA, USA). Hoechst dye no. 33258, $CoCl_2 \cdot 6H_2O$, DAPI, and recombinant human IGF-I were obtained from Sigma (St. Louis, MO, USA). Calcein-AM, Pluronic F-127, JC-1, and TMRE were obtained from Molecular Probes (Eugene, OR, USA). The Helios gene gun, gold (0.6 μm diameter) microcarrier beads, and calcium phosphate reagents for plasmid DNA precipitation were obtained from Bio-Rad (Hercules, CA, USA). EGFP plasmid DNA was obtained from BD Biosciences/Clontech (Palo Alto, CA, USA). The GFP monoclonal antibody and the COX IV monoclonal antibody were purchased from Clontech (Palo Alto, CA, USA). The cytochrome *c* rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The caspase-3 rabbit polyclonal antibody was purchased from Promega (Madison, WI, USA). The caspase-9 rabbit polyclonal antibody was obtained from Cell Signaling Technology (Beverly, MA, USA). Cy3-conjugated secondary antibodies for immunocytochemistry were purchased from Jackson Immunoresearch Laboratories (West Grove, PA, USA). Horseradish peroxidase-linked secondary antibodies and reagents for enhanced chemiluminescence detection were obtained from Amersham Pharmacia Biotechnologies (Piscataway, NJ, USA).

Site-directed mutagenesis of Bax

Mutants of human GFP-Bax α were created using a QuickChange site-directed mutagenesis kit (Stratagene) according the manufacturer's

recommended procedure. Human GFP-Bax α point mutations were created in the following way: the T182A substitution mutation was obtained with the mutagenic forward primer 5′-GGGAGTGCTCGCCGCC TCACTCACCATCTGG-3′ and its complementary reverse primer. Primers were supplied by Invitrogen (Carlsbad, CA, USA) and IDT (Coralville, IA, USA).

Rat cerebellar granule neuron cultures

Rat CGNs were isolated from 7-day-old Sprague–Dawley rat pups (15–19 g) as described previously.[3] Briefly, neurons were plated at a density of 4.0×10^6 cells/35-mm well in basal modified Eagle's medium containing 10% fetal bovine serum, 25 mM KCl, 2 mM L-glutamine, and penicillin (100 U/ml)–streptomycin (100 μg/ml) (Invitrogen, Grand Island, NY, USA). Cytosine arabinoside (10 μM) was added to the culture medium 24 h after plating to limit the growth of non-neuronal cells. Using this protocol, the cultures were $\sim 95\%$ pure for granule neurons. In general, experiments were performed after 7 days in culture.

Helios gene gun transfection

Gold (0.6 μm diameter) microcarrier beads were coated with the desired plasmid DNA encoding for either GFP, GFP-Bax WT, or GFP-Bax T182A mutant using a calcium phosphate precipitation method described by the Helios gene gun manufacturer (Bio-Rad). The gold beads were then coated on the inside of plastic tubing and the tubing was cut into individual cartridges. Helium at a pressure of 100 psi was shot through the tubing to deliver the plasmid-coated beads into the CGN cultures.

HEK293 cell culture and transfection

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum, 10 μg/μl of streptomycin, and 10 IU/ml of penicillin. The cultures were incubated at 37°C and in an atmosphere of 5% CO_2. HEK293 cells were transfected with 0.5–2 μg of DNA using the lipofectamine method according to the manufacturer's procedure (Invitrogen).

Cell fractionation

After incubation of CGNs in either control or apoptotic medium ($\pm 10 \mu$M CyA), the medium was removed and CGNs were washed once with 2 ml of ice-cold PBS (pH 7.4). Cells were then placed on ice and scraped into cytosolic extraction buffer (200 μl/35-mm well) containing 1 $\times$ protease inhibitor cocktail, 1 mM DTT, and 4 ml PBS, each components of the BioVision cytochrome c releasing apoptosis assay kit (Alexis Biochemicals). The lysates were then removed from the wells and each sample was subjected to 35 strokes in a dounce homogenizer on ice.

HEK293 cells expressing GFP-Bax WT or GFP-Bax T182A were lysed and treated as previously described for CGNs. Cells were washed with 1 $\times$ PBS, incubated with 200 μl of cytosol extraction buffer for 20 min, scraped and harvested, and homogenized with a dounce homogenizer.

The lysates from CGNs or HEK cells were centrifuged at 700 $\times$ g for 10 min, the supernatant was transferred to a new tube, centrifuged again at 10 000 $\times$ g for 30 min, and this supernatant was transferred to a new tube labeled cytosolic fraction. The pellet was resuspended in mitochondria extraction buffer provided by Alexis containing DTT (1 M) and protease inhibitors, and then labeled mitochondrial fraction. Fractions were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) using 15% gels and then subjected to Western blotting, probing initially for

cytochrome c or GFP-Bax and then reprobing for COX IV (mitochondrial marker).

Immunoblot analysis

Nonspecific binding sites were blocked in PBS (pH 7.4) containing 0.1% Tween 20 (PBS-T) and 1% BSA for 1 h at room temperature. Primary rabbit polyclonal antibodies were diluted in blocking solution (final dilution of 1 : 250–1 : 1000) and incubated with the membranes for 1 h. Excess primary antibody was removed by washing the membranes three times in PBS-T. The blots were then incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody diluted in PBS-T (final dilution of 1 : 5000) for 1 h and subsequently washed three times with PBS-T. Immunoreactive proteins were detected by enhanced chemiluminescence. In some experiments, membranes were reprobed after stripping in 0.1 M Tris-HCl (pH 8.0), 2% SDS, and 100 mM β-mercaptoethanol for 30 min at 52°C. The blots were rinsed twice in PBS-T and processed as above with a different primary antibody. Autoluminograms shown are representative of at least three different experiments. Densitometry measurements were performed on Bim_s immunoblots normalizing to 25K + Ser and represented as a fold change of density. Data analysis was performed using ANOVA two-factor statistics.

Measurement of mitochondrial swelling

CGNs were plated onto polyethyleneimine-coated glass coverslips at a density of approximately 2×10^5 cells/coverslip. CGNs were incubated in either control medium or apoptotic medium in the absence or presence of CyA. JC-1 (final concentration of 2 μg/ml) and Hoechst dye were added directly to the culture medium 30 min prior to fixation to stain mitochondria and nuclei, respectively. JC-1 fluorescence was captured in paraformaldehyde-fixed cells using a Cy3 filter under a $\times 100$ oil objective.

Assessment of mitochondrial membrane potential

CGNs plated on glass coverslips were incubated in either control or apoptotic medium ($\pm$CyA). TMRE (final concentration, 500 nM) and Hoechst dye were added directly to the cells 30 min prior to the end of the incubation period. Following incubation, coverslips were inverted onto slides into a small volume of phenol red-free medium containing TMRE (500 nM). Living cells were then imaged using a Cy3 filter to detect TMRE fluorescence under a $\times 100$ oil objective. All images were acquired at equal exposure times for TMRE fluorescence to assess the relative mitochondrial membrane potentials. Counts of CGNs expressing GFP-Bax T182A with and without intact TMRE fluorescence were performed as described above and at least 100 cells were counted in three experiments.

Calcein/Co^{2+} assay for PTP opening

CGNs plated on glass coverslips were incubated in either control or apoptotic medium ($\pm$CyA) for 4 h. At 20 min prior to the end of the incubation, cells were loaded with calcein-AM to a final concentration of 1 μM in the presence of 1 mM $CoCl_2$, $\sim 0.1\%$ Pluronic F-127, and Hoechst dye. After the 20 min incubation, calcein-AM cells were washed four times with ice-cold 1 $\times$ PBS. Coverslips were inverted onto slides in a small volume of phenol red-free medium containing glucose and loosely mounted. Living cells were imaged immediately using a FITC filter to detect calcein fluorescence under a $\times 100$ oil objective. Calcein fluorescence was confirmed to be mitochondrial by colocalizing with TMRE (final concentration, 500 nM).

Preparation of CGN cell extracts

CGNs were incubated in either control or apoptotic medium ($\pm$CyA). The culture medium was aspirated, CGNs were washed once with 2 ml of ice-cold PBS (pH 7.4), and cells were then placed on ice and scraped into lysis buffer (200 μl/35-mm well) containing 20 mM HEPES (pH 7.4), 1% Triton X-100, 50 mM NaCl, 1 mM EGTA, 5 mM β-glycerophosphate, 30 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 1 mM phenyl-methylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Cell debris was removed by centrifugation at 6000 $\times$ g for 3 min and the protein concentration of the supernatant was determined using a commercially available protein assay kit (Pierce Chemical Co., Rockford, IL, USA). Aliquots ($\sim$150 μg) of supernatant protein were diluted to a final concentration of 1$\times$ SDS–PAGE sample buffer, boiled for 5 min, and electrophoresed through 15% polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA, USA) and processed for immunoblot analysis.

Quantification of GFP-Bax localization

At 48 h after Helios gene gun transfection, approximately 2% of the CGNs expressed the transgene. Cells were then subjected to trophic factor withdrawal for 4 h, followed by Hoechst staining and fixation in paraformaldehyde. Two distinct patterns of GFP-Bax expression were observed – diffuse and punctate (mitochondrial). The number of GFP-Bax-expressing cells demonstrating either a diffuse or mitochondrial localization was assessed by counting 50–100 transfected cells per coverslip. Three replicate coverslips were scored per treatment group in a given experiment and each experiment was performed at least three times. The images shown are representative of results obtained from at least three independent experiments.

Quantification of CGN apoptosis

Apoptosis was induced either by removing serum and decreasing the extracellular potassium concentration from 25 to 5 mM or by transfecting CGNs with the GFP-Bax T182A point mutant followed by incubation for 48 h in control medium. After the induction of apoptosis, CGNs were fixed with 4% paraformaldehyde and nuclei were stained with Hoechst dye. Cells were considered apoptotic if their nuclei were either condensed or fragmented. In general, approximately 500 cells from at least two fields of a 35-mm well were counted. For transfection experiments, approximately 200 GFP-Bax (WT or mutant)-expressing cells were counted from three replicate coverslips per experiment, with each experiment performed at least three times. Data are presented as the percentage of cells in a given treatment group that were scored as apoptotic.

Quantification of HEK293 apoptosis

HEK293 cells were transfected with GFP-Bax WT or GFP-Bax T182A. Hoechst dye was added to each 35 mm well of HEK cells (1 μl/1 ml of media) and allowed to incubate for 20 min. Cells were scraped off the dish, centrifuged, and resuspended in 1$\times$ PBS. Cells were washed again with 1$\times$ PBS, resuspended again in PBS, and applied to microscope slides. Counting was carried out on an Olympus phase contrast inverted microscope using epi-illuminescence at $\times$40 magnification. Cells were scored as apoptotic if their nuclei were condensed or fragmented, and expression of Bax was confirmed by GFP fluorescence. HEK293 apoptosis was quantified using two replicate dishes per experiment, with each experiment performed at least three times. Data represent the percentage of cells in a given experiment that were scored as apoptotic.

Immunocytochemistry

HEK293 cells used for visualizing cytochrome c release and caspase activation were fixed in 4% paraformaldehyde. Cells were washed once with PBS (pH 7.4) then permeabilized and blocked with PBS containing 5% BSA and 0.2% Triton X-100 for 1 h. Cells were washed once with PBS (pH 7.4) and allowed to incubate at room temperature with either the rabbit anti-cytochrome c, rabbit anti-caspase-3, or rabbit anti-caspase-9 primary antibodies (1:500) for 1 h, each diluted in PBS containing 0.2% Triton X-100 and 2% BSA. Primary antibodies were aspirated and the cells were washed five times with PBS. Cells were then incubated at room temperature for 1 h with the appropriate rhodamine-conjugated or fluorescein isothiocyanate-conjugated secondary antibody (1:500) and DAPI. Cells were imaged in 2 ml of PBS and captured with a Zeiss Axioplan 2 digital microscope equipped with a Cooke Sensicam deep-cooled CCD camera. Images were analyzed and subjected to digital deconvolution using the Slidebook software program (Intelligent Imaging Innovations Inc., Denver, CO, USA). Rat CGNs were treated exactly as described above, with the following exceptions. CGNs were cultured on polyethyleneimine-coated glass coverslips at a density of approximately 2 $\times$ 10^5 cells/coverslip. Cells were then incubated for approximately 16 h at 4°C with primary rabbit polyclonal antibodies to either cytochrome c, active caspase-9, or active caspase-3, each diluted in PBS containing 0.2% Triton X-100 and 2% BSA. After addition of secondary antibody, CGNs were then washed five more times with PBS, and coverslips were adhered to glass slides in mounting medium (0.1% p-phenylenediamine in 75% glycerol in PBS). Fluorescent images were captured using a $\times$63 oil immersion objective.

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