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Endogenous Bax Translocation in SH-SY5Y Human Neuroblastoma Cells and Cerebellar Granule Neurons Undergoing Apoptosis

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Abstract: Changes at the mitochondria are an early, required step in apoptosis in various cell types. We used western blot analysis to demonstrate that the proapoptotic protein Bax translocated from the cytosolic to the mitochondrial fraction in SH-SY5Y human neuroblastoma cells undergoing staurosporine- or EGTA-mediated apoptosis. Levels of mitochondrial Bax increased 15 min after staurosporine treatment. In EGTA-treated cells, increased levels of mitochondrial Bax were seen at 4 h, consistent with a slower onset of apoptosis in EGTA versus staurosporine treatments. We also demonstrate the concomitant translocation of cytochrome c from the mitochondrial to the cytosolic fractions. We correlated these translocations with changes in caspase-3-like activity. An increase in caspase-3-like activity was evident 2 h after staurosporine treatment. Inhibition of the mitochondrial permeability transition had no effect on Bax translocation or caspase-3-like activity in staurosporine-treated SH-SY5Y cells. In primary cultures of cerebellar granule neurons undergoing low K^+-mediated apoptosis, Bax translocation to the mitochondrial fraction was evident at 3 h. Cytochrome c release into the cytosol was not significant until 8 h after treatment. These data support a model of apoptosis in which Bax acts directly at the mitochondria to allow the release of cytochrome *c*.
Key Words: Apoptosis—Cerebellar granule neuron—SH-SY5Y cells—Bax—Cytochrome *c*.
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Apoptosis is physiologically essential for the elimination of excess neurons during nervous system maturation. The triggering of unregulated apoptosis has been implicated in the development of several clinically important adult neuropathologies, including cerebral ischemia (Linnick et al., 1993), Huntington's disease (Zeitlin et al., 1995; Goldberg et al., 1996), Alzheimer's disease (Nixon et al., 1994; Satou et al., 1995; Vito et al., 1996; Yamatsuji et al., 1996), amyotrophic lateral sclerosis (Troost et al., 1995), and Parkinson's disease (Ziv et al., 1996).

A growing body of evidence implicates changes at the mitochondria, particularly the release of cytochrome *c*, as an early required step in the onset of apoptosis (Kluck et al., 1997; Yang et al., 1997; Green and Kroemer, 1998). Cytochrome *c* released from mitochondria is a necessary cofactor for activation of caspase-3-like proteases (Liu et al., 1996). The cytosolic partner of cytochrome *c* in the apoptotic cascade has recently been identified as apoptosis protease-activating factor-1 (Apaf-1) (Li et al., 1997; Zou et al., 1997). Apaf-1 and cytochrome *c*, along with dATP, activate caspase-9. Activated caspase-9 mediates cleavage of pro-caspase-3 to active caspase-3 (Li et al., 1997; Zou et al., 1997), one of the principal proteases in mammalian apoptosis (Thornberry et al., 1992; Tewari et al., 1995). The mechanism by which cytochrome *c* is released from the mitochondria is unclear, but evidence is emerging supporting two main hypotheses: (a) release via the mitochondrial permeability transition (MPT) pore or (b) release due to insertion and channel formation at the mitochondrial membrane by Bax, a proapoptotic Bcl-2 family member.

Cytochrome *c* release in some cell types corresponds with the opening of the MPT pore (Hortelano et al., 1997; Susin et al., 1997). Agents that prevent MPT attenuate apoptosis in various cell types (Marchetti et al., 1996; Kroemer, 1997; Pastorino et al., 1998*b*). Transfected Bax has been shown in Jurkat T cells to act on the MPT pore to induce cytochrome *c* release (Pastorino et al., 1998*a*). However, in the same cell line, transfected

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Abbreviations used: AA, aristolochic acid; Ac-DEVD-MCA, acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin; Apaf-1, apoptosis protease-activating factor-1; BA, bongkrekic acid; BDP, breakdown product; CalpInh II, calpain inhibitor II; CGN, cerebellar granule neuron; CsA, cyclosporin A; MPT, mitochondrial permeability transition; Z-D-DCB, carbobenzoxy-Asp-$CH_2OC(O)$-2,6-dichlorobenzene.

Bax was reported to mediate directly the release of cytochrome *c* from the mitochondria without inducing MPT (Jurgensmeier et al., 1998). Several groups have also demonstrated that the release of cytochrome *c* occurs before the change in mitochondrial membrane permeability (Yang et al., 1997; Bossy-Wetzel et al., 1998).

Translocation of Bax from the cytosol to the mitochondria has been previously reported (Hsu et al., 1997) and linked to cytochrome *c* release in various cell types (Wolter et al., 1997; Eskes et al., 1998) but has never been demonstrated in neuronal cells. Bax has been reported to play a pivotal role in neuronal apoptosis. Bax expression is elevated in apoptotic neurons (Bossenmeyer et al., 1998; Tamatani et al., 1998; Wullner et al., 1998). In neurons from Bax knockout mice, Bax deletion reduces developmental apoptosis (White et al., 1998) and is essential for neurotrophic factor deprivation-induced apoptosis (Deckwerth et al., 1996).

In this report, we examine whether the induction of apoptosis in neuroblastoma cells and neurons in primary culture leads to alterations in endogenous Bax localization. We also investigated the requirement for MPT in cytochrome *c* release and induction of caspase-3-like activity in SH-SY5Y human neuroblastoma cells. SH-SY5Y cells have been used extensively in the study of neuronal death (Boix et al., 1997; Posmantur et al., 1997; Ronca et al., 1997; Fall and Bennett, 1998; Kitamura et al., 1998), particularly in aspects associated with Alzheimer's disease (Peraus et al., 1997; Sheehan et al., 1997; Uberti et al., 1997; Parvathy et al., 1998; Shen et al., 1998). Because the signal transduction pathway leading to apoptosis varies among cell types, it is important to investigate the relevance of processes such as Bax translocation and cytochrome *c* release to neuronal cells undergoing apoptosis.

MATERIALS AND METHODS

Cell culture and treatment

SH-SY5Y human neuroblastoma cells were grown on 12-well plates to confluency (~2×10^6 per well) at 37°C with 5% CO_2 in a humidified atmosphere with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cerebellar granule neurons were isolated as previously described (Nath et al., 1996*b*).

At the beginning of each experiment, cultures were washed three times with serum-free DMEM. As indicated, cells were pretreated for 1 h with *N*-acetyl-Leu-Leu-Met-aldehyde [calpain inhibitor II (CalpInh II)] or the pan-caspase inhibitor carbobenzoxy-Asp-$CH_2OC(O)$-2,6-dichlorobenzene (Z-D-DCB; Bachem).

Protein extraction and fractionation

Cells were collected at the end of the treatment period by scraping and centrifugation at 3,600 *g*. Cell pellets were washed twice with Tris-buffered saline/EDTA. Pellets were resuspended in 5 volumes of homogenization buffer: 20 m*M* HEPES (pH 7.5), 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 1 m*M* EGTA, 1 m*M* dithiothreitol, protease inhibitor cocktail (Calbiochem), and 250 m*M* sucrose. Cells were homogenized in a Dounce Teflon–glass homogenizer (15 strokes). The homogenate was centrifuged at 500 *g* for 5 min to pellet out nuclei and intact cells. Supernatants were centrifuged at 10,000 *g* for 30 min. The 10,000-*g* mitochondria-rich heavy membrane pellet was resuspended in 100 μl of homogenization buffer and represents the mitochondrial fraction. The 10,000-*g* supernatant is used as a crude cytosol. Some experiments were performed using a 100,000-*g* cytosol, and results were not different from those with the 10,000-*g* supernatant. Samples were frozen at −70°C until use. To validate each fractionation, we performed a western blot for the mitochondrial protein Bcl-2 to ensure that it was restricted to the mitochondrial fraction.

Western blotting

Protein concentration was determined with a modified Lowry procedure (Bio-Rad D-C protein assay kit). Equal amounts of protein were loaded on each lane, electrophoresed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (4–20% acrylamide gradient gel; Novex) with a Tris/glycine running buffer, and transferred to a polyvinylidene difluoride membrane (0.2 μm) by semidry electrotransfer for 2 h at 20 V. The blots were probed with anti-Bax (human or rat polyclonal; Pharmingen) or anti-cytochrome *c* (human monoclonal; Pharmingen) and a biotinylated secondary antibody and avidin-conjugated with alkaline phosphatase (Amersham Pharmacia). The blots were developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma).

Caspase-3-like activity assay

SH-SY5Y cell lysates were prepared by Triton X-100 extraction as previously described (Nath et al., 1996*b*). Caspase-3-like activity was determined by monitoring proteolysis of the fluorogenic substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-MCA; Peptide International). Whole-cell lysate was added to a buffer containing 100 μ*M* peptide substrate, 100 m*M* HEPES, 10% glycerol, 1 m*M* EDTA, and 10 m*M* dithiothreitol. Fluorescence (excitation at 380 nm and emission at 460 nm) was measured every 30 min up to 2 h using a Millipore Cytofluor 2300 fluorescence plate reader.

RESULTS

Bax translocates from cytosolic to mitochondrial fraction in SH-SY5Y cells undergoing staurosporine-mediated apoptosis

To investigate the localization of endogenous Bax in neuronal cells undergoing apoptosis, we treated SH-SY5Y human neuroblastoma cells with staurosporine, a nonspecific protein kinase inhibitor and well-established proapoptotic agent (Koh et al., 1995; Nath et al., 1996*b*; Wiesner and Dawson, 1996). Our laboratory has demonstrated that caspase activity is significantly elevated within 2 h after staurosporine treatment in SH-SY5Y cells (Posmantur et al., 1997). In control cells, Bax is primarily localized to the cytosolic fraction (Fig. 1A). Staurosporine treatment produced a time-dependent loss of Bax immunoreactivity in the cytosolic fraction and concomitant increase in the mitochondrial fraction (Fig. 1A). The decrease in cytosolic Bax is apparent within 1 h after initiation of staurosporine treatment. The increase in mitochondrial Bax was significant by 30 min after the start of treatment (Fig. 1B; 40.9 ± 6.3% maximal den-

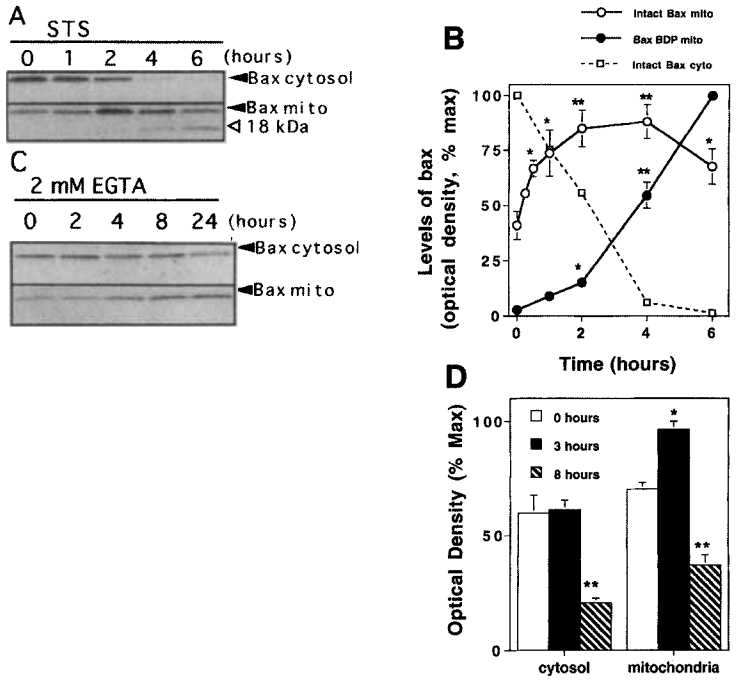

FIG. 1. Bax localization in cytosolic and mitochondrial fractions in staurosporine (STS)- and EGTA-treated SH-SY5Y cells. **A:** SH-SY5Y cells were untreated or treated for various times with 0.5 μM STS. Western blotting with Bax antibody was performed on cytosolic (**top;** 40 μg of protein per lane) and mitochondrial (**bottom;** 20 μg of protein per lane) fractions. Solid arrowhead indicates intact Bax protein (23 kDa). Open arrowhead indicates Bax fragment. **B:** Densitometric analysis of changes in levels of Bax and Bax BDP in STS-treated SH-SY5Y cells. Intact Bax (○) and Bax BDP (●) from the mitochondrial fraction are presented as mean ± SEM (bars) percentages of the maximum of optical density (n = 3). $^*p < 0.05$, $^{**}p < 0.01$ versus control by Student's t test. Owing to variation between experiments, results showing loss of Bax from the cytosol are representative of two experiments. **C:** Cells were treated with 2 mM EGTA at the time indicated and assayed by western blotting for Bax in cytosolic (**top;** 40 μg of protein/lane) and mitochondrial (**bottom;** 20 μg of protein per lane). **D:** Densitometric analysis of Bax translocation in CGNs after 3 (solid columns) and 8 h (striped columns) of low K^+. $^*p < 0.05$, $^{**}p < 0.01$ versus 0 h by Student's t test. Data are mean ± SEM (bars) values (n = 3).

sitometry units for control vs. 66.6 ± 3.7% for 30 min). Although the level of mitochondrial Bax peaked at 2–4 h, an 18-kDa Bax breakdown product (BDP) appeared beginning after 2 h of staurosporine treatment, and its level continued to increase throughout the treatment period (Fig. 1A and B). The loss of intact mitochondrial Bax at later time points may be attributable to this fragmentation.

We investigated whether a different apoptotic stimulus, the Ca^{2+} chelator EGTA, would produce Bax translocation. EGTA induces apoptosis in SH-SY5Y cells via activation of caspase-3-like proteases (McGinnis et al., 1999) and causes apoptosis in other cell types (Chiesa et al., 1998; Mizuno et al., 1998). Treatment with 2 mM EGTA led to increased mitochondrial and decreased cytosolic Bax immunoreactivity (Fig. 1C). Bax translocation occurred more slowly in EGTA-treated as compared with staurosporine-treated cells. Changes in Bax localization were not apparent until 4 h after the start of EGTA challenge and increased through 24 h. In EGTA-mediated apoptosis, no Bax fragmentation was apparent, consistent with the recent report that Bax fragmentation is due to the Ca^{2+}-dependent protease calpain (Wood et al., 1998).

To examine whether Bax translocation occurs in other neuronal cultures, we used primary cultures of rat cerebellar granule neurons (CGNs). CGNs are a homogeneous population of neurons (95%) with only a small amount of contaminating glial cells. These cells survive in vitro only when grown in 25 mM K^+ and undergo spontaneous apoptosis in 5 mM (low) K^+ (D'Mello et al., 1993). CGNs were untreated or exposed to low K^+ medium for 3 or 8 h. Cytosolic Bax immunoreactivity is significantly reduced by 8 h. Mitochondrial Bax immunoreactivity was elevated at 3 h (70.3 ± 2.8% maximal densitometry units for control vs. 96.5 ± 3.2% for 3 h; Fig. 1D) but decreased by 8 h (37 ± 4.6% maximal). Although no Bax BDP is apparent, the loss of intact Bax at 8 h may be due to proteolytic degradation. A higher level of Bax was found in the mitochondrial fraction of resting CGNs compared with resting SH-SY5Y cells. This may be attributable to a higher level of background death in the CGN primary culture.

Time course of cytochrome *c* translocation from the mitochondrial to the cytosolic fraction is consistent with Bax translocation

We subjected cytosolic and mitochondrial fractions from staurosporine- and EGTA-treated SH-SY5Y cells to western blot analysis for cytochrome *c*. For consistency, the samples used to demonstrate cytochrome *c* translocation are from the same experiment as those used to demonstrate Bax translocation (Fig. 1). Some cytochrome *c* was present in the control cytosol. Because there is little caspase-3-like activity in resting SH-SY5Y cells (Posmantur et al., 1997), the cytosolic cytochrome *c* may be either apo-cytochrome *c*, which lacks the heme group of holo-cytochrome *c* and does not activate caspase-3 (Kluck et al., 1997; Zhivotovsky et al., 1998), or cytochrome *c* that was released during sample preparation. In staurosporine-treated cells, an increase in cytosolic and concurrent loss in mitochondrial cytochrome *c* content were evident after 1 h and reached a maximum at 4–6 h (Fig. 2A). This time is consistent with the increase of Bax seen on staurosporine treatment (Fig. 1).

In EGTA-treated SH-SY5Y cells, an increase in cytosolic cytochrome *c* did not occur until 4 h after initiating

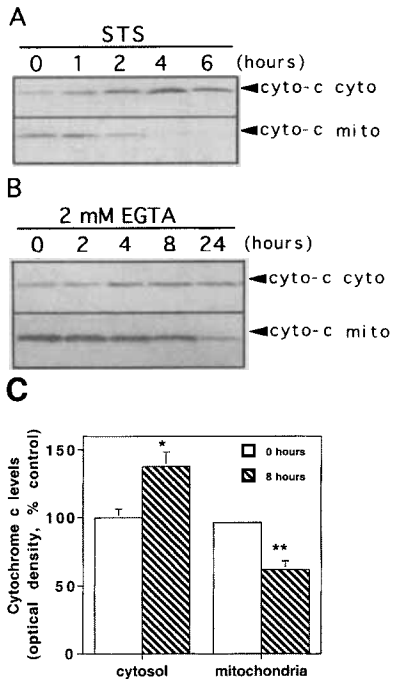

FIG. 2. Cytochrome *c* localization in mitochondrial and cytosolic fractions in staurosporine (STS)-treated SH-SY5Y cells. **A:** SH-SY5Y cells were untreated or treated for various times with 0.5 μM STS. Western blotting with cytochrome *c* antibody was performed on cytosolic (cyto; **top;** 40 μg of protein per lane) and mitochondrial (mito; **bottom;** 20 μg of protein per lane) fractions. **B:** Cells were treated with 2 m*M* EGTA. Cytosolic (cyto; **top**) and mitochondrial (mito; **bottom**) fractions were assayed by western blotting for cytochrome *c*. **C:** Densitometric analysis of changes in cytochrome *c* localization in CGNs challenged for 8 h with low K^+. Data are mean ± SEM (bars) values (n = 3). $^*p < 0.05$, $^{**}p < 0.01$ versus 0 h by Student's *t* test.

the challenge (Fig. 2B). This was consistent with the slower onset for EGTA-mediated Bax translocation. Loss of cytochrome *c* from the mitochondrial fraction was not obvious until 24 h of treatment. This may be attributed to the overall higher level of cytochrome *c* in these samples.

We also quantified changes in levels of cytochrome *c* localization in low K^+-challenged CGNs (Fig. 2C). No significant change occurred by 3 h (data not shown). By 8 h, levels of mitochondrial cytochrome *c* had significantly decreased (96.1 ± 2.1% of control vs. 62.0 ± 6.2% of control after 8 h), and cytosolic cytochrome *c* had significantly increased (99.8 ± 6.6% of control vs. 137.4 ± 11% of control after 8 h). This observation is consistent with a recent report demonstrating elevated cytosolic cytochrome *c* in CGNs after a 5-h exposure to low K^+ medium (Gleichman et al., 1998). Again, the high levels of cytochrome *c* in the cytosolic fraction of control CGNs may be attributed to a higher background death in these preparations compared with SH-SY5Y neuroblastoma cells.

Changes in Bax and cytochrome *c* localization precede caspase activation in staurosporine-treated SH-SY5Y cells

We correlated the time course for the changes in Bax and cytochrome *c* localization with the activation of caspase-3-like proteases in staurosporine-treated SH-SY5Y cells. We measured caspase-3-like activity in staurosporine-treated SH-SY5Y cells by monitoring hydrolysis of Ac-DEVD-MCA. A significant increase in caspase-3-like activity was detected 2 h after staurosporine treatment (Fig. 3A). Caspase-3-like activity became maximal at 4 h. This demonstrated that an increase in caspase-3-like activity follows increases in cytosolic cytochrome *c*, which were apparent at 1 h.

Because both calpains and caspases are activated in SH-SY5Y cells undergoing staurosporine-mediated apoptosis (Nath et al., 1996*a*,*b*; Posmantur et al., 1997), we investigated whether caspase or calpain inhibition would interfere with Bax translocation. Neither the pan-caspase inhibitor Z-D-DCB nor CalpInh II prevented the translocation of Bax (Fig. 3B). Consistent with a recent report (Wood et al., 1998), we found that Bax fragmentation is due at least in part to calpain activity. CalpInh II, but not Z-D-DCB, was able to attenuate Bax fragmentation after 6 h of staurosporine treatment. Caspase inhibition had no effect on Bax BDP formation.

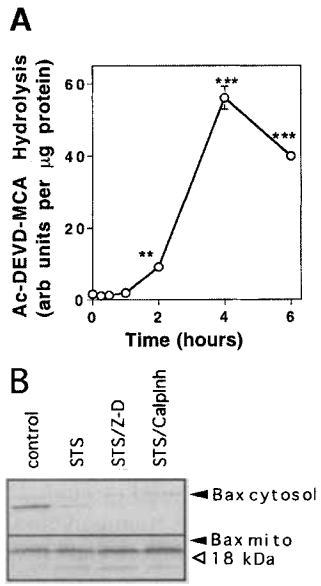

FIG. 3. Caspase activation and inhibition in staurosporine (STS)-treated SH-SY5Y cells. **A:** Whole-cell lysate from 0.5 μM STS-treated SH-SY5Y cells was collected by Triton X-100 extraction at the indicated time. Caspase-3-like activity was assayed by monitoring hydrolysis of the fluorogenic substrate Ac-DEVD-MCA as described in Materials and Methods. Data are mean ± SEM (bars) values, expressed as fluorescence units per microgram of protein (n = 4). $^{**}p < 0.01$, $^{***}p < 0.001$ versus 0 h by ANOVA with post hoc Tukey–Kramer test. **B:** SH-SY5Y cells were pretreated with 50 μM Z-D-DCB (Z-D) or 20 μM CalpInh II and then treated with 0.5 μM STS. Cytosolic (40 μg) and mitochondrial (mito; 20 μg) fractions were subjected to western blot analysis with Bax antibody.

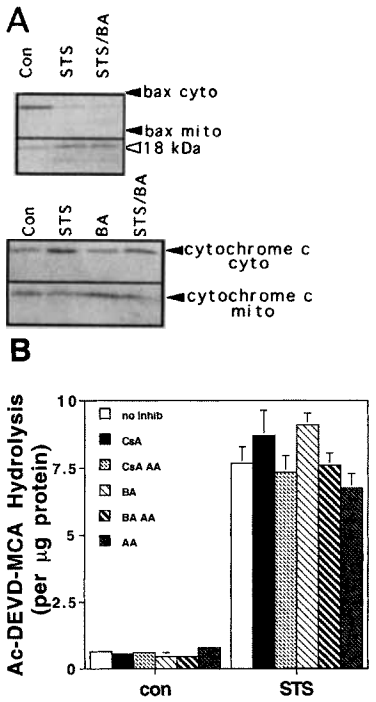

FIG. 4. Bax localization and caspase activity in staurosporine (STS)-treated SH-SY5Y cells pretreated with MPT inhibitors. **A:** SH-SY5Y cells were untreated or pretreated with 50 μM BA and challenged with 0.5 μM STS for 4 h. Cytosolic (cyto; **top;** 40 μg) and mitochondrial (mito; **bottom;** 20 μg) fractions were probed for Bax and cytochrome c immunoreactivity. **B:** Ac-DEVD-MCA hydrolysis in SH-SY5Y whole-cell lysate in cells pretreated with CsA or BA in the presence or absence of 50 μM AA. Cells were treated for 2 h with 0.5 μM STS. Con, control. Data are mean ± SEM (bars) values.

MPT inhibition does not attenuate Bax translocation or caspase activation

As Bax may exert its proapoptotic effect via induction of MPT (Pastorino et al., 1998*b*), we tested the effects of the MPT inhibitors bongkrekic acid (BA; 20–100 μM) and cyclosporin A (CsA; 100 nM–1 μM) (Marchetti et al., 1996; Pastorino et al., 1998*a*) in our system. Neither inhibitor at any concentration tested provided protection against staurosporine-mediated apoptosis (data not shown). CsA alone at 500 nM was cytotoxic in SH-SY5Y cells. Neither BA (50 μM) nor CsA (100 nM) prevented staurosporine-mediated Bax or cytochrome *c* translocation in SH-SY5Y cells (data for BA shown in Fig. 4A).

We examined whether these inhibitors affected staurosporine-mediated increases in caspase-3-like activity. SH-SY5Y cells were pretreated with CsA or BA in the presence or absence of the phospholipase A_2 inhibitor aristolochic acid (AA), which enhances the protective effect of MPT inhibitors (Pastorino et al., 1998*b*). Hydrolysis of the fluorogenic caspase-3-like peptide substrate Ac-DEVD-MCA was measured from whole-cell lysate after 2 h of staurosporine treatment. We chose to use 2 h of treatment so that early effects of MPT inhibitors on caspase activity could be detected. Staurosporine treatment on its own and with MPT inhibitors led to significantly elevated Ac-DEVD-MCA hydrolysis (Fig. 4B). There were no differences in caspase-3-like activity among treatment conditions. These data suggest that MPT does not play a role in the onset of staurosporine-mediated apoptosis in SH-SY5Y cells.

DISCUSSION

Using both human neuroblastoma cells and primary cultures of rat cerebellar granule neurons, we made the original observation that endogenous Bax translocates from the cytosol to the mitochondria in neuronal cells undergoing apoptosis. Bax translocation has been previously found in various cell types (Hsu et al., 1997; Wolter et al., 1997; Goping et al., 1998) but never before reported in neuronal cells. Furthermore, we demonstrated that Bax translocation is accompanied by release of cytochrome *c* from the mitochondria in both CGNs and SH-SY5Y neuroblastoma cells. Cytochrome *c* released from the mitochondria is a required cofactor for caspase-3 activity, and we showed that activation of caspase-3 lags behind cytochrome *c* release. Finally, using two unrelated inhibitors of MPT, we concluded that MPT is not required for cytochrome *c* release and caspase-3-like protease activity in SH-SY5Y cells.

Our finding that Bax is predominantly cytosolic in resting cells and translocates to membrane fractions during apoptosis is consistent with recent work in nonneuronal cells (Hsu et al., 1997; Wolter et al., 1997; Goping et al., 1998). The fact that apoptotic signals induced Bax translocation in both neuroblastoma SH-SY5Y cells and primary cultures of CGNs suggests that Bax translocation is a universal component of apoptosis in neuronal cells. Bax expression is known to be critical for neuronal apoptosis. For instance, cerebellar granule neurons from Bax −/− mice do not undergo apoptosis in response to 5 mM K^+ (Miller et al., 1997). The mechanism by which Bax induces apoptosis is unclear, but evidence suggests that Bax translocation could play a significant role. Bax translocation may be necessary for the formation of transportation pathways leading to the release of cytochrome *c* and/or other apoptosis-inducing factors from the mitochondria (Kroemer, 1997; Reed, 1997). Consistent with this, Bax has channel-forming capability in vitro (Schlesinger et al., 1997), and Bax insertion into the mitochondrial membrane is prevented by deletion of the carboxy tail (Wolter et al., 1997; Goping et al., 1998), which protects against staurosporine-mediated toxicity (Wolter et al., 1997). Our results demonstrating that Bax translocation precedes cytochrome *c* release in both SH-SY5Y cells and cerebellar granule cells suggest that this mechanism is functional in neurons. Because the pancaspase inhibitor Z-D-DCB was unable to prevent Bax translocation, Bax insertion into the mitochondria is upstream from caspase activation in apoptotic SH-SY5Y cells. This is consistent with the hypothesis that Bax insertion leads to the release of the caspase-3 activation

cofactor cytochrome *c* as well as other putative apoptosis-inducing factor(s). However, this need not be identical in all cells because the caspase inhibitor carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone attenuated Bax insertion in hematopoietic cells (Goping et al., 1998). Rossé et al. (1998) demonstrated that Bcl-2 overexpression did not prevent Bax translocation but did attenuate cell death, perhaps owing to a change in Bax:Bcl-2 stoichiometry within the mitochondrial membrane. Bax may heterodimerize with Bcl-2 within the mitochondrial membrane, eliminating Bcl-2's protective effects and allowing the release of cytochrome *c* through channel formation, by breaking down the outer mitochondrial membrane, or by causing swelling of the mitochondrial matrix and thereby disrupting the outer mitochondrial membrane.

We demonstrated that Bax is cleaved in a Ca^{2+}-dependent manner in SH-SY5Y cells undergoing apoptosis, probably owing to calpain activation. Calpain-mediated Bax cleavage in apoptotic leukemia cells was recently reported (Wood et al., 1998). A physiological function for Bax fragmentation has not been determined. In light of the recent findings that cleavage of Bcl-2 (Cheng et al., 1997) and Bid (Li et al., 1998; Luo et al., 1998) renders them proapoptotic, it is tempting to speculate that the Bax fragment may serve to augment proapoptotic effects, perhaps by facilitating its insertion or homodimerization within the mitochondrial membrane. However, calpain activation is not necessary for apoptosis: EGTA treatment leads to apoptosis in the absence of calpain activation (McGinnis et al., 1999). Calpain inhibition does not prevent staurosporine-mediated apoptosis in SH-SY5Y cells (Nath et al., 1996*a*). Also, Bax cleavage is not evident until late in the apoptotic process, well after cytochrome *c* release and caspase-3 activation have occurred. It is possible, however, that undetectable amounts of Bax cleavage may initiate the apoptotic process by augmenting Bax insertion into the mitochondrial membrane.

Although essential for the release of cytochrome *c* and apoptosis in some models (Marchetti et al., 1996; Kroemer, 1997; Pastorino et al., 1998*a*), MPT is not a universal requirement for apoptosis. Cytochrome *c* release occurs before the change in mitochondrial permeability in some systems (Yang et al., 1997; Bossy-Wetzel et al., 1998). Also, Bax can directly induce release of cytochrome *c* from isolated mitochondria without inducing MPT (Eskes et al., 1998; Jurgensmeier et al., 1998). We demonstrated that cytochrome *c* release and caspase-3-like protease activity do not seem to require MPT in neuronal cells.

In summary, we have demonstrated endogenous Bax translocation from the cytosol to the mitochondria in neurons undergoing apoptosis. The translocation of Bax from the cytosol to the mitochondria has been reported in various cell types (Hsu et al., 1997; Wolter et al., 1997; Gross et al., 1998) but never before in neurons. We showed concomitant cytochrome *c* release from the mitochondria, which occurs before caspase-3-like activity and is not dependent on MPT. We propose a model in which Bax inserts into the mitochondrial membrane in response to apoptotic stimuli. Bax forms channels within the mitochondrial membrane through either homodimerization or heterodimerization with Bcl-2. This transport pathway would mediate the release of cytochrome *c* (and perhaps other caspase-activation cofactors) and result in caspase-3 activation leading to neuronal apoptosis.

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