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BH3 Death Domain Peptide Induces Cell Type-selective Mitochondrial Outer Membrane Permeability*

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The BH3 domain is essential for the release of cytochrome *c* from mitochondria by pro-apoptotic Bcl-2 family proteins during apoptosis. This study tested the hypothesis that a Bax peptide that includes the BH3 domain can permeabilize the mitochondrial outer membrane and release cytochrome *c* in the absence of a permeability transition at the mitochondrial inner membrane. BH3 peptide (0.1–60 μM) released cytochrome *c* from mitochondria in the presence of physiological concentrations of ions in a cell type-selective manner, whereas a BH3 peptide with a single amino acid substitution was ineffective. The release of cytochrome *c* by BH3 peptide correlated with the presence of endogenous Bax at the mitochondria and its integral membrane insertion. Cytochrome *c* release was accompanied by adenylate kinase release, was not associated with mitochondrial swelling or substantial loss of electrical potential across the inner membrane, and was unaffected by inhibitors of the permeability transition pore. Cytochrome *c* release was, however, inhibited by Bcl-2. Although energy-coupled respiration was inhibited after the release of cytochrome *c*, mitochondria maintained membrane potential in the presence of ATP due to the reversal of the ATP synthase. Overall, results support the hypothesis that BH3 peptide releases cytochrome *c* by a Bax-dependent process that is independent of the mitochondrial permeability transition pore but regulated by Bcl-2.

In recent years, a ubiquitous, intrinsic apoptotic pathway has been delineated where the redistribution of death-promoting factors such as cytochrome *c* and Smac/DIABLO from the mitochondria to the cytosol constitutes the apical event in the cascade of caspase activation (1). Pro-apoptotic Bcl-2 protein family members such as Bax and Bid that are believed to be involved in triggering this mitochondrial pathway have been well studied, and regulation of the level and localization of these proteins occurs after many forms of tissue injury.

Although many molecules can target mitochondria and release cytochrome *c*, the physiological importance of these effectors and the mechanism by which they act remains controversial. For example, some reports suggest that Bax can initiate the release of cytochrome *c* through an interaction with the mitochondrial permeability transition pore (PTP)[1] and associated swelling and outer membrane rupture (2–6), whereas others provide evidence that Bax-induced cytochrome *c* release occurs in the absence of permeability transition (7–9). The ability to release cytochrome *c* without a disruption of inner membrane integrity would be consistent with observations that mitochondria in apoptotic cells are able to maintain membrane potential (10–12) and protein import (12) after the loss of cytochrome *c*.

The Bcl-2 family of proteins is defined by the inclusion of one or more Bcl-2 homology domains (BH domains). Many Bcl-2 family members have now been characterized that possess only a BH3 domain, such as Bid, Bad, Bik, Blk, Bim, DP5/Hrk, and Noxa (13, 14). The BH3 domain is critical for heterodimerization with other Bcl-2 family proteins (15, 16) and appears to be essential for death-promoting activity (13). A conformational change of Bax that exposes the BH3 domain during the induction of apoptosis has been documented (17, 18). It has also been reported that caspase cleavage of Bid, Bcl-2, and Bcl-X_L exposes the BH3 domain and converts these molecules to mediators of cytochrome *c* release and apoptosis (19–23). A more complete understanding of the function of Bcl-2 family proteins is particularly important for studies on developmental neuron death and neurodegenerative cell loss, where the involvement of Bcl-2 family proteins such as Bax, Bad, Bcl-X_L, Bcl-2, and DP5/Hrk has been implicated (13, 24).

Despite the extensive evidence highlighting the importance of the BH3 domain in the mechanism of cytochrome *c* release by Bcl-2 family proteins, there have been conflicting reports on whether short peptides derived from the BH3 region of proteins are themselves capable of releasing cytochrome *c* (3, 7, 25, 26). Discrepancies in the literature may result from differences in assay conditions, cell type, or length of peptide. Because of the pervasiveness of the BH3 domain in otherwise non-homologous proteins that possess cytochrome *c*-releasing and apoptosis-promoting activity, a BH3 peptide should serve as a useful tool for identifying and characterizing mechanisms that are common to Bcl-2 family protein function.

The present study utilized neural cells and isolated mitochondria derived from neural cells or tissue to test the hypotheses that 1) a peptide comprised of amino acids 53–86 of Bax that encompasses the BH3 domain permeabilizes the mitochondrial outer membrane and releases cytochrome *c* by a mechanism that is independent of the mitochondrial permeability transition pore but is inhibited by Bcl-2 and 2) mitochondria maintain inner membrane integrity and transmem-

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[1] The abbreviations used are: PTP, permeability transition pore; BH, Bcl-2 homology; VDAC, voltage-dependent anion channel.

brane electrical potential after the release of cytochrome *c* by the BH3 peptide.

EXPERIMENTAL PROCEDURES

Materials—Rat forebrain mitochondria were isolated according to the procedure of Rosenthal *et al*. (27), yielding a combination of both non-synaptosomal and synaptosomal mitochondria. In some experiments non-synaptosomal rat forebrain mitochondria were purified on a Percoll gradient as commonly described (28). Rat liver mitochondria were isolated by standard differential centrifugation as previously described (29). GT1–7 and PC12S mitochondria were isolated according to the method of Moreadith and Fiskum (30) with slight modifications. The BH3 peptide spanned amino acids 53–86 (^{53}DASTKKLSECLKRIGDELDSNMELQRMIAAVDTD^{86}) of Bax and was synthesized by the Wadsworth Center Biochemistry and Peptide Synthesis Core using an Applied Biosystem 431A automated peptide synthesizer as previously described (31). The control BH3 peptide was synthesized by the University of Maryland School of Medicine Biopolymer Laboratory using an automated Symphony peptide synthesizer (Protein Technologies Inc.) and spanned the identical region of Bax with a single substitution of arginine for glycine at position 67 (BH3 G67R). Peptides were prepared as dilute (1.5–15 mM) stocks in distilled water. Bongkrekic acid was obtained from Calbiochem, and cyclosporin A was obtained from Alexis Biochemicals. Monoclonal anti-Bcl-2 mouse IgG and polyclonal anti-Bax and anti-Bak rabbit IgGs were purchased from Upstate Biotechnology. Polyclonal anti-Bax rabbit IgG was also purchased from Santa Cruz. Monoclonal anti-cytochrome *c* mouse IgG and monoclonal anti-Bcl-X mouse IgG were from Pharmingen. Monoclonal anti-porin mouse IgG was from Calbiochem. Other chemicals were from Sigma, and all reagents were of the highest grade available.

Cell Culture—GT1–7 cells stably transfected with a Bcl-2-overexpressing vector or a control puromycin resistance vector (GT1–7 *Bcl-2* and GT1–7 *puro*) were generated and maintained as previously described (32). PC12S cells, a morphological variant of rat pheochromocytoma PC12 cells that retain the ability to grow in tissue culture without poly-L-lysine treatment, were maintained as described (33). Primary cerebellar granule neurons were prepared from postnatal day 8 Harlan Sprague-Dawley rats according to the procedure of Schousboe *et al.* (34). Neurons were either resuspended in Ca^{2+}-free Hank's balanced salt solution buffered to pH 7.4 with 25 mM HEPES and used on the day of isolation or maintained for 1 day in culture in basal Eagle's medium (Life Technologies, Inc.) with 10% fetal bovine serum and 1% penicillin and streptomycin before harvesting. Primary cortical astrocytes were obtained from postnatal day 1 Harlan Sprague-Dawley rats as described (35) and cultured in DMEM/F-12 (Biofluids) with 10% fetal bovine serum and 1% penicillin and streptomycin for 10 days before isolation. All cells were harvested by brief exposure to 0.05% tryspin at 37 °C followed by resuspension in culture media that contains trypsin inhibitor. Cells were maintained in Ca^{2+}-free Hanks' balanced salt solution buffered with 25 mM HEPES at pH 7.4 for up to 4 h after isolation.

Measurement of Mitochondrial Respiration—Respiration by isolated mitochondria was measured polarimetrically with a Clark-type oxygen electrode (Hansatech) (32). Mitochondria were suspended at a concentration of 0.5 mg/ml in medium maintained at 30 °C consisting of 125 mM KCl, 2 mM P_i, 1 mM $MgCl_2$, 5 mM malate, 5 mM glutamate, and 20 mM Hepes-KOH, pH 7.0. Rates of O_2 consumption are expressed as nmol of O_2/mg of mitochondrial protein/min assuming a medium O_2 content of 195 nmol O_2/ml at 30 °C. To verify acceptable functional integrity of mitochondria isolated from rat brain and liver and from cultured cells, the acceptor control ratio was determined. The acceptor control ratio was defined as the rate of ADP (0.8 mM)-stimulated O_2 consumption (State 3 respiration) divided by the State 4 rate of respiration observed in the presence of oligomycin (2.5 μg/ml), an inhibitor of the mitochondrial ATP synthase. The acceptor control ratio values ranged from 5 to 10, indicating strong energy coupling between respiration and ATP synthesis. Slightly different conditions were used to determine the effects of the BH3 peptide on State 3 respiration. Mitochondria were suspended at a concentration of 0.25 mg/ml in KCl medium (125 mM KCl, 2 mM P_i, and 20 mM HEPES, pH 7.0) that contained 4 mM $MgCl_2$, 3 mM ATP, 250 μM EGTA, 5 mM succinate, and 2 μM rotenone. State 3 respiration was initiated by the addition of 0.8 mM ADP, then BH3 peptide, vehicle control, or the artificial pore-forming peptide alamethicin was added after 1 min.

Examination of Changes in Mitochondrial Volume by Light Scattering and EM—Absorbance change of mitochondrial suspensions (0.4 mg/ml) at 660 nm was used as an indicator of mitochondrial swelling as described previously (36). Assay conditions were identical to those employed during measurements of mitochondrial oxygen consumption.

A JEOL 1200 EX electron microscope was used to examine mitochondrial volume and morphology within permeabilized cortical astrocytes and cerebellar granule neurons after control, BH3 peptide, or alamethicin treatment. Cells were incubated in KCl media containing 4 mM $MgCl_2$, 3 mM ATP, and 5 mM each of malate and glutamate. The addition of digitonin (0.01%) permeabilized the plasma membrane of cells without affecting mitochondrial membranes (37). BH3 peptide, vehicle control, or alamethicin was added 2 min after the introduction of digitonin, and cells were incubated for a total of 10 min in suspension. Samples of permeabilized cells were centrifuged at 3000 × g for 3 min, fixed overnight in a solution containing 4% formaldehyde plus 1% glutaraldehyde, and postfixed in 1% osmium tetroxide. Dehydration, cutting of sections, and visualization was performed as described (38).

Measurement of Mitochondrial Membrane Potential—Mitochondrial membrane potential was monitored with an LS-3 fluorescence spectrometer (PerkinElmer Life Sciences) by measuring fluorescent changes due to the extent of mitochondrial sequestration of the fluorescent cationic dye safranine-O (5 μM, excitation at 485 nm, emission at 586 nm) (37, 39). Isolated GT1–7 mitochondria (0.125 or 0.25 mg/ml) were incubated in KCl media including 5 mM succinate, 2 μM rotenone, 3 mM ATP, 4 mM $MgCl_2$, and 250 μM EGTA in the presence of 5 μM safranine-O. Horse heart cytochrome *c* (Sigma) was included in the assay media before the addition of mitochondria in Fig. 6, and other additions were made as indicated in the figure legends.

Determination of Cytochrome c and Adenylate Kinase Release—Cytochrome *c* and adenylate kinase release from mitochondria was determined under the conditions used for the measurement of oxygen consumption. Alamethicin treatment was used as a positive control representing maximum release (38). At 3–8 min after the addition of BH3 peptide or vehicle control (see figure legends), mitochondria or permeabilized cells were pelleted by centrifugation at 13,400 × g for 5 min, and the supernatant and pellet were assayed for the presence of cytochrome *c* by immunoblot as described (36). For quantitative comparisons, cytochrome *c* release was also determined using an enzyme-linked immunosorbent assay kit (R&D Systems) according to the instructions of the manufacturer. Adenylate kinase activity in the supernatant and pellet fractions derived from centrifugation of GT1–7 mitochondrial suspensions were assessed according to the procedure recently described for brain mitochondrial suspensions (36).

Statistical Analysis—A two-way analysis of variance with a Tukey's post-hoc test was utilized to determine statistical differences in cytochrome *c* release. A Student's t test was used to assess statistical differences in adenylate kinase release between control and BH3 peptide treatment (n = 3 determinations). $p < 0.05$ was considered significant.

Alkali Extraction and Detection of Bax, Bcl-X_L, and VDAC—The localization of mitochondrial Bax protein to sodium carbonate-extracted soluble fraction *versus* membrane fraction was determined essentially as described (40). Proteins were separated by SDS-polyacrylamide gel electrophoresis, and Bax was immunostained with primary rabbit polyclonal Δ21 anti-Bax antibody (Santa Cruz, 1:500 dilution) plus secondary anti-rabbit IgG conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, 1:4000 dilution). A primary mouse monoclonal anti-Bcl-X antibody (Pharmingen, 1:500 dilution) plus secondary anti-mouse IgG conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, 1:4000 dilution) was used to detect Bcl-X_L. VDAC was detected with a primary mouse monoclonal anti-porin antibody (Calbiochem, 1:500 dilution) plus secondary anti-mouse IgG conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, 1:4000 dilution). Peroxidase activity was detected using the enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech) and x-ray film.

RESULTS

BH3 Peptide Releases Cytochrome c from Neural Cell Mitochondria in a Dose-dependent Fashion That Is Inhibited by Bcl-2—BH3 peptide containing amino acids 53–86 of Bax was added to isolated neural GT1–7 *puro* mitochondria in a cytosolic-like media that included physiologically relevant concentrations of KCl, adenine nucleotides, and Mg^{2+}. Under these conditions, a dose-dependent release of cytochrome *c* by BH3 peptide was observed (Fig. 1, *A* and *B*). The BH3 peptide also induced a dose-dependent efflux of cytochrome *c* from isolated PC12S mitochondria (Fig. 1*C*). A BH3 peptide with a single amino acid substitution of arginine for glycine 67 (BH3 G67R)

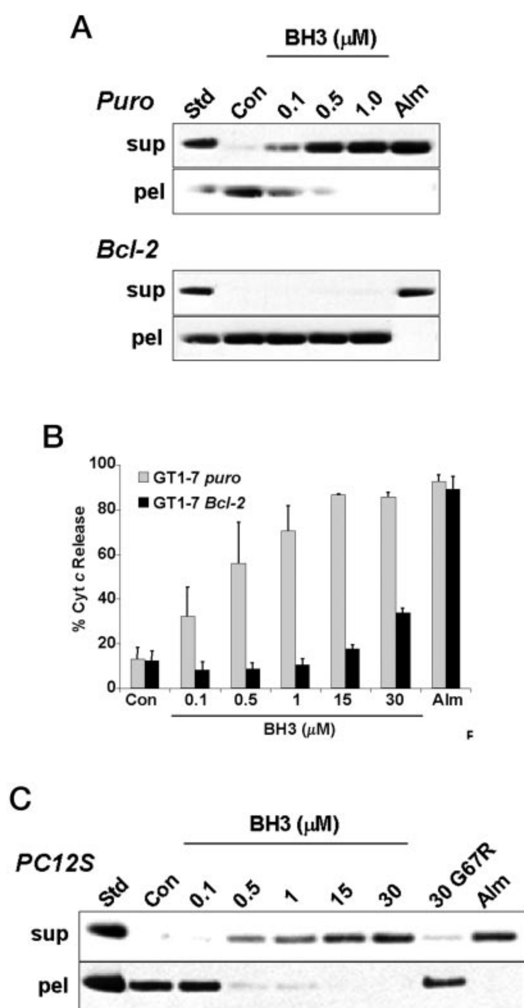

FIG. 1. **BH3 peptide induces dose-dependent release of cytochrome *c* from isolated neural cell mitochondria that is inhibited by Bcl-2 expression.** Isolated GT1–7 *puro* or *Bcl-2* mitochondria (0.25 mg/ml) were incubated at 30 °C in KCl media with 5 mM succinate, 2 μM rotenone, 4 mM $MgCl_2$, 3 mM ATP, and 250 μM EGTA for 2 min, at which time BH3 peptide, vehicle control, or alamethicin (*Alm*, 40 μg/ml) was added. For the alamethicin treatment, a second 40 μg/ml addition was performed after 3 min to ensure maximal effect. Mitochondrial suspension was centrifuged after a total of 8 min of incubation, and supernatant (*sup*) and pellet (*pel*) fractions were analyzed for cytochrome *c* (*Cyt c*) content by immunoblot (*A*) and enzyme-linked immunosorbent assay (*B*) as described under "Experimental Procedures." The amount of cytochrome *c* released from GT1–7 *Bcl-2* mitochondria was significantly less than the amount released from GT1–7 *puro* mitochondria at all BH3 peptide concentrations tested (analysis of variance, $p < 0.001$, $n = 3$). In C, PC12S mitochondria (0.25 mg/ml) were incubated under conditions identical to those described for *A* and *B*, and cytochrome *c* release was assessed by immunoblot. *30 G67R* represents a BH3 peptide with an arginine for glycine substitution at amino acid 67 that was added at a concentration of 30 μM. *Std*, standard; *Con*, control.

was ineffective at inducing cytochrome *c* release even at high concentrations (Fig. 1*C*). The artificial pore-forming peptide alamethicin (80 μg/ml) released ~90% of the mitochondrial cytochrome *c* and was employed throughout the study as a positive control for cytochrome *c* release.

The anti-apoptotic protein Bcl-2 would be expected to inhibit cytochrome *c* efflux induced by the BH3 domain peptide if the natural apoptotic mechanism of cytochrome *c* release is functioning. Bcl-2 was detected by immunoblot in mitochondria isolated from Bcl-2-overexpressing GT1–7 cells (GT1–7 *Bcl-2*), but in mitochondria isolated from cells that were control-transfected with a puromycin resistance vector (GT1–7 *puro*) Bcl-2 was barely detectable (see Fig. 4*A*). As anticipated, Bcl-2 significantly inhibited the release of cytochrome *c* by BH3 peptide at tested concentrations ranging from 0.5 to 30 μM ($p < 0.001$, Fig. 1, *A* and *B*). The effective concentration of BH3 required to achieve half-maximal cytochrome *c* efflux in GT1–7 *puro* mitochondria was between 0.5 and 1 μM, whereas less than 50% of the total cytochrome *c* was released from GT1–7 *Bcl-2* mitochondria at concentrations up to 30 μM.

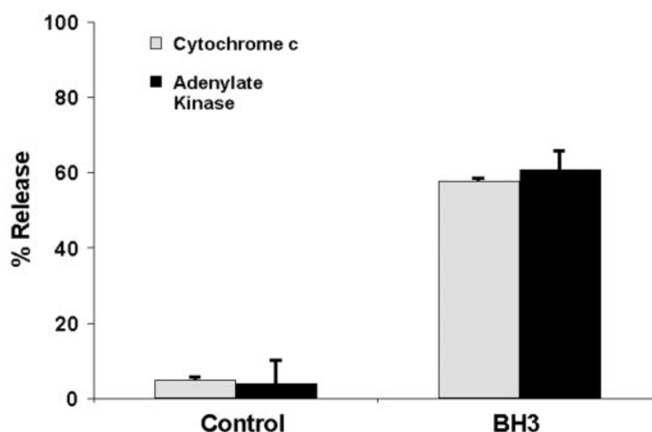

FIG. 2. **Adenylate kinase is co-released with cytochrome *c* from GT1–7 *puro* mitochondria by BH3 peptide.** GT1–7 *puro* mitochondria (0.4 mg/ml) were incubated under conditions described in Fig. 1 for 3 min, at which time BH3 peptide was added (10 μM). Mitochondrial suspension was centrifuged after an additional 3 min, cytochrome *c* content was analyzed in supernatant and pellet fractions by enzyme-linked immunosorbent assay, and adenylate kinase was assessed enzymatically as described under "Experimental Procedures." Cytochrome *c* and adenylate kinase release are expressed as a percentage of reactivity present in the supernatant compared with the total amount. The percent release of both cytochrome *c* and adenylate kinase from BH3-treated mitochondria was significant compared with vehicle control treatment (*t* test, $p < 0.001$, $n = 3$).

Cytochrome c Release by BH3 Peptide Is Not Selective—The co-release of adenylate kinase, another protein present in the mitochondrial intermembrane space, has frequently been assessed in studies on cytochrome *c* redistribution to examine the selectively of cytochrome *c* release. To determine whether the BH3-mediated release of cytochrome *c* was selective, adenylate kinase activity was measured spectrophotometrically in supernatant and pellet fractions derived from BH3 peptide-treated GT1–7 mitochondria by following a decrease in NADH absorbance. BH3 peptide released a significant percentage of adenylate kinase from GT1–7 *puro* mitochondria compared with the vehicle control (Fig. 2, $p < 0.001$). Additionally, there was a close correspondence between the percentage of adenylate kinase and the percentage of cytochrome *c* that was released, suggesting that the two proteins are released by a common mechanism. Like cytochrome *c* release, adenylate kinase release was inhibited by overexpression of Bcl-2 in GT1–7 mitochondria (data not shown).

The Release of Cytochrome c by BH3 Peptide Is Cell-specific and May Depend on the Presence of Endogenous Bax Protein—Although BH3 peptide released cytochrome *c* from isolated GT1–7 *puro* mitochondria and PC12S mitochondria with an EC_{50} between 0.5–1.0 μM, no cytochrome *c* release was observed when 60 μM BH3 peptide was added to isolated adult forebrain or liver mitochondria (Fig. 3). BH3 peptide released cytochrome *c* from mitochondria when added to GT1–7 puro cells after the plasma membranes were permeabilized by digitonin (0.005% w/v), demonstrating that the efficacy of BH3 peptide is not an artifact resulting from the procedure of cell mitochondrial isolation. BH3 peptide also released cytochrome *c* when added to digitonin-permeabilized primary cerebellar granule neurons or cortical astrocytes, showing that the cyto-

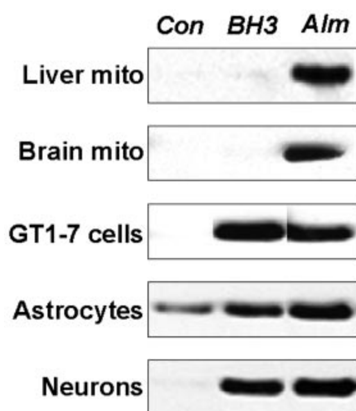

FIG. 3. **BH3 peptide induces the release of cytochrome *c* from the mitochondria of permeabilized neural cells but not isolated adult rat liver or forebrain mitochondria.** Cells or mitochondria were incubated at 37 °C in KCl media with 5 mM malate, 5 mM glutamate, 4 mM $MgCl_2$, 3 mM ATP, and 250 μM EGTA. Cells were permeabilized with 0.005–0.05% digitonin. The amount of digitonin added for each cell type was determined by a titration to produce the maximal rate of oxygen consumption. BH3 peptide (60 μM), vehicle control, or alamethicin (*Alm*, 80 μg/ml) was added at 2 min, and suspensions were separated into supernatant and pellet fractions after a 10-min total incubation. Cytochrome *c* content in the supernatant fraction was assessed by immunoblot as described under "Experimental Procedures." *Con*, control.

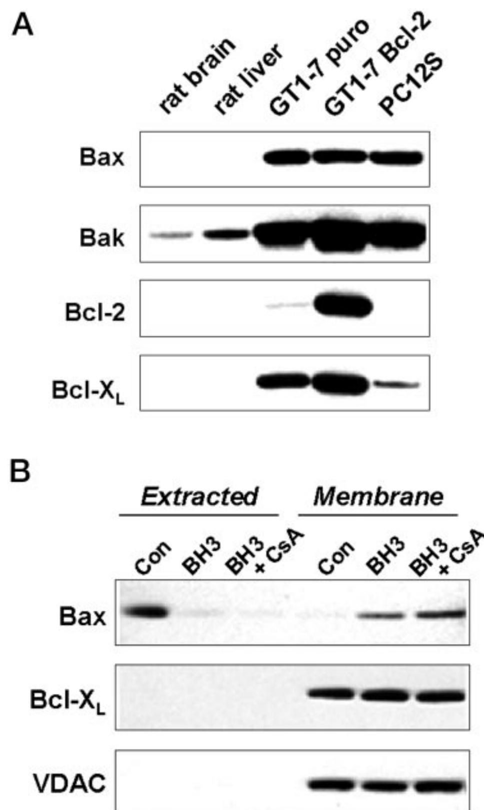

FIG. 4. **Bax protein is associated with GT1–7 mitochondria (*A*) and becomes integrally inserted into mitochondrial membrane after BH3 peptide addition (*B*).** In *A*, isolated mitochondrial (80 μg of protein) suspensions were subjected to gel electrophoresis, and the presence of Bax, Bak, Bcl-2, and Bcl-X_L were assayed by immunoblot. In *B*, after incubation with BH3 peptide under the conditions described in Fig. 1, GT1–7 *puro* mitochondria were pelleted by centrifugation and exposed to alkali extraction, as described under "Experimental Procedures." Bax, Bcl-X_L, and VDAC were detected in the soluble and membrane fractions by immunoblot. *Con*, control; *CsA*, cyclosporin A.

chrome *c*-releasing activity is not restricted to GT1–7 and PC12S cells. Cytochrome *c* was released from permeabilized cerebellar neurons exposed to BH3 peptide whether they were cultured (Fig. 3) or freshly dissociated (data not shown), confirming that culturing of cells is not a requirement for sensitivity to the BH3 domain. The differential effect of BH3 domain peptide on mitochondria from different sources indicates that the phenomenon is cell-type specific and suggests that a mitochondrial target protein may be required for BH3-mediated cytochrome *c* redistribution.

Bax, Bak, Bcl-2, and Bcl-X_L levels in mitochondria isolated from GT1–7 cells, PC12S cells, adult rat forebrain, or adult rat liver were determined by immunoblot. Endogenous Bax is found in mitochondria of GT1–7 and PC12S cells but not in rat forebrain or liver mitochondria (Fig. 4*A*). The presence of Bax protein in isolated mitochondria therefore correlates with the ability of BH3 peptide to release cytochrome *c* (Fig. 1 and 3). In contrast, Bak was present in rat forebrain and liver mitochondria where BH3 peptide was without effect. Bax integrates into the outer membrane of mitochondria during apoptosis, rendering it insensitive to alkali extraction (40). To determine whether endogenous Bax becomes integrally inserted into the mitochondrial membrane during BH3 peptide-induced cytochrome *c* release, GT1–7 *puro* mitochondria were subjected to 0.1 Na_2CO_3 treatment (pH 11.5) 6 min after BH3 peptide addition, and the extracted and membrane fractions were separated by high-speed centrifugation. All of the endogenous Bax protein in the vehicle control-treated mitochondria was extracted by sodium carbonate, indicative of a loose attachment to mitochondrial membranes (Fig. 4*B*). By contrast, the majority of Bax redistributed to the membrane fraction after BH3 peptide treatment, suggesting integral insertion. Cyclosporin A, an inhibitor of the mitochondrial inner membrane permeability transition, was unable to prevent this redistribution of Bax. The outer integral membrane proteins Bcl-X_L and VDAC were located exclusively in the membrane fraction, confirming complete separation of soluble and membrane fractions.

BH3 Peptide-mediated Cytochrome c Efflux Is Independent of Mitochondrial Permeability Transition—A combination of oxidative stress and Ca^{2+} exposure initiates cyclosporin A-sensitive swelling and cytochrome *c* release from GT1–7 mitochondria (41). In contrast to the Ca^{2+}-activated PTP, measurements of light scattering using isolated GT1–7 *puro* mitochondria and electron micrographs of mitochondria within permeabilized cortical astrocytes and cerebellar granule neurons both indicate a lack of swelling after BH3 peptide addition (Fig. 5, *A* and *B*). A large degree of mitochondrial swelling was obtained upon alamethicin addition. The PTP inhibitors cyclosporin A and bongkrekic acid were both incapable of diminishing BH3-induced cytochrome *c* efflux (Fig. 5*C*).

Membrane potential ($\Delta\Psi$) after the addition of BH3 domain peptide to GT1–7 *puro* mitochondria was monitored by safranine-O fluorescence changes. Little dequenching of dye fluorescence was observed after BH3 peptide addition compared with the complete dequenching observed upon treatment with the protonophore uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (*FCCP*), indicating that the mitochondria retained substantial potential after BH3 exposure (Fig. 6). Analysis of cytochrome *c* distribution by enzyme-linked immunosorbent assay revealed that ~90% of the cytochrome *c* was lost from the mitochondrial fraction during the course of the incubation (data not shown). To test whether the small reduction in $\Delta\Psi$ that was observed after BH3 peptide exposure was due to loss of cytochrome *c*, BH3 peptide was added to GT1–7 mitochondria in the presence of 10 μM exogenous cytochrome *c*. The drop in membrane potential due to BH3 peptide addition was largely abrogated by the presence of exogenous cytochrome *c* (Fig. 6), indicating that the decline in $\Delta\Psi$ was due to a decrease

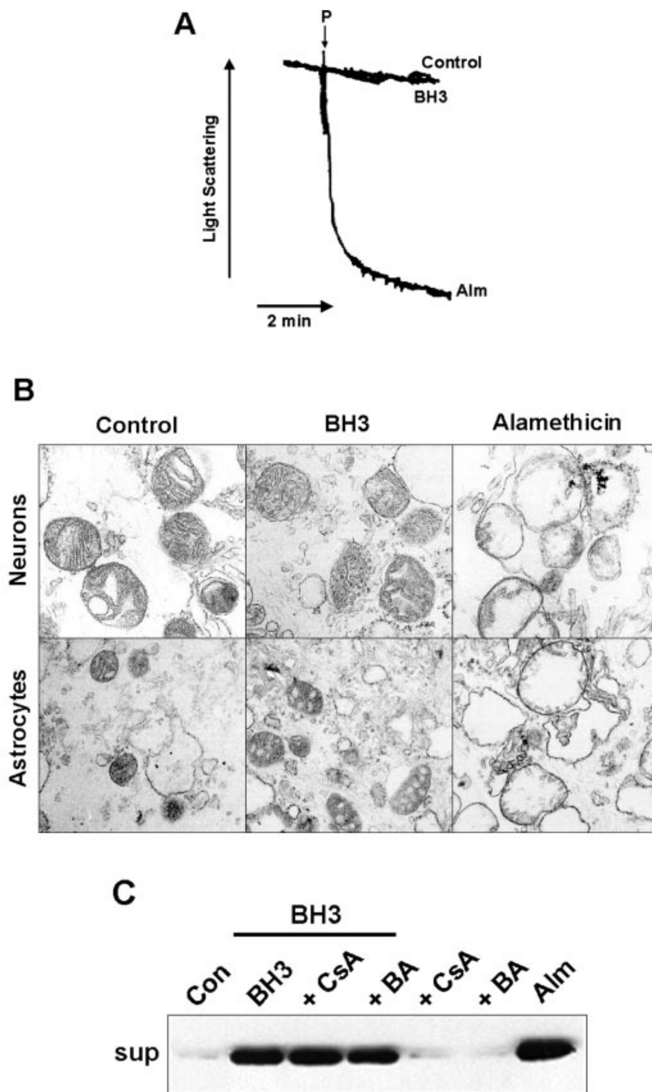

FIG. 5. **BH3 peptide releases cytochrome *c* in the absence of mitochondrial swelling and the presence of permeability transition pore inhibition.** Swelling was assessed by monitoring light scattering by isolated GT1–7 *puro* mitochondria in A (a decrease in light scattering corresponds to an increase in volume) and by morphological examination of mitochondria within permeabilized cerebellar granule neurons (*upper panel*, 20×) or cortical astrocytes (*lower panel*, 5×) in *B*. In both *A* and *B*, 60 μM BH3 peptide was used, and a large amount of cytochrome *c* release occurred (see Fig. 3). The *arrows* designate timing of additions, and *P* denotes peptide or vehicle control. In *C*, GT1–7 *puro* mitochondria (0.25 mg/ml) were incubated at 30 °C in KCl media with 5 mM succinate and 2 μM rotenone for 2 min and exposed to BH3 peptide (1.5 μM) in the presence or absence of cyclosporin A (1 μM) or bongkrekic acid (5 μM). Cytochrome *c* release was assayed in the supernatant (*sup*) fraction after the incubation and centrifugation described in Fig. 1. *Con, control; Alm*, alamethicin; *CsA*, cyclosporin A; *BA*, bongkrekic acid.

in accessibility of cytochrome *c* to the respiratory chain rather than an increase in inner membrane permeability.

Mitochondria Maintain Membrane Potential in the Absence of Respiration after BH3 Peptide-induced Cytochrome c Release by Reversal of F_1F_0 ATPase Function—To determine whether access of released cytochrome *c* to the mitochondrial electron transport chain was responsible for the ability of GT1–7 mitochondria to maintain substantial membrane potential after BH3-induced cytochrome *c* efflux, mitochondrial respiration was measured by directly monitoring oxygen consumption. The addition of BH3 peptide resulted in a greater than 7-fold inhibition in the consumption of oxygen (a reduction from 101 nmol of O_2/mg of mitochondrial protein/min to 14 nmol of O_2/mg/min,

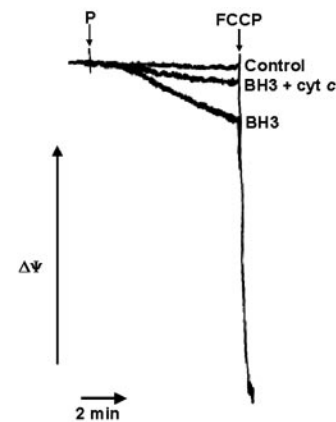

FIG. 6. **Mitochondrial depolarization initiated by BH3 peptide is inhibited by exogenous cytochrome *c* (*cyt c*).** GT1–7 *puro* mitochondria were incubated under the conditions described in Fig. 1 in the presence of the potential-sensitive fluorescent indicator safranine-O (5 μM). Excitation and emission wavelengths were 485 and 586 nm, respectively. BH3 peptide (60 μM) and cytochrome *c* (10 μM) were present where indicated. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (*FCCP*) (1 μM) was added after 8 min of total incubation to completely dissipate mitochondrial membrane potential ($\Delta\Psi$). *Arrows* designate the timing of additions, and *P* denotes peptide or vehicle control.

Fig. 7*A*). Respiration was almost completely restored by adding 10 μM exogenous cytochrome *c* (89 nmol O_2/mg/min), indicating that the inhibition of respiration was indeed due to the release of cytochrome *c* rather than impairment of other electron transport chain components.

The ability of GT1–7 mitochondria to maintain $\Delta\Psi$ despite almost complete respiratory inhibition could be explained if the F_1F_0 ATPase was operating in reverse, hydrolyzing ATP in the media to pump protons across the mitochondrial inner membrane and establish an electrochemical gradient. To test this hypothesis, oligomycin was added to mitochondria after 4.5 min of incubation with BH3 peptide. The inhibition of the F_1F_0 ATPase by oligomycin resulted in the swift dissipation of membrane potential to an extent similar to that achieved with the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (Fig. 7*B*). The addition of oligomycin to mitochondria in the absence of BH3 peptide resulted in no decline in $\Delta\Psi$ (data not shown). Thus, GT1–7 mitochondria appear to maintain membrane potential subsequent to BH3-induced cytochrome *c* release by reversing the function of the ATP synthase.

DISCUSSION

The mechanism by which mitochondria release cytochrome *c* during apoptosis is highly controversial, and the mechanism of release may differ depending on cell type, the cellular environment, and the apoptotic trigger. The release of cytochrome *c* by BH3 death domain-containing proteins appears to be particularly important in neurons undergoing apoptosis, either during normal development or after an acute or chronic neurodegenerative insult (42). The present study therefore utilized a synthetic BH3 peptide corresponding to amino acids 53–86 of Bax to resolve the question of whether a mitochondrial permeability transition is required for cytochrome *c* release in response to this minimal death domain.

The synthetic BH3 peptide derived from amino acids 53–86 of Bax was found to release cytochrome *c* from mitochondria in a cell-type selective manner (Fig. 3). A BH3 peptide with glycine 67 replaced by arginine was ineffective at inducing cytochrome *c* efflux. Several lines of evidence presented here preclude outer membrane disruption by an inner membrane permeability transition-dependent mechanism as a plausible explanation for the redistribution of cytochrome *c* by BH3 do-

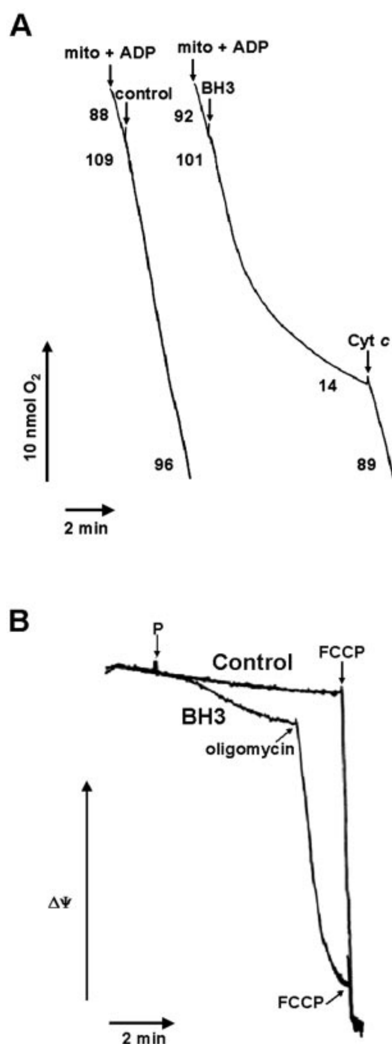

FIG. 7. **Mitochondria maintain membrane potential after BH3 peptide addition in the absence of respiration by F_1F_0 ATPase reversal.** In *A*, GT1–7 *puro* mitochondria (0.25 mg/ml) were added to the Clark-type oxygen electrode chamber at 30 °C under the conditions described in Fig. 1, and oxygen consumption was stimulated by 0.8 mM ADP. *Arrows* denote BH3 peptide (30 μM), vehicle control, or cytochrome *c* (*Cyt c*, 10 μM) addition. Respiration rates are expressed in nmol O_2/mg of protein/min adjacent to each trace. In *B*, GT1–7 *puro* mitochondrial membrane potential changes were followed by monitoring safranine-O fluorescence as described in Fig. 5. Oligomycin (10 μM) was added after 4.5 min of incubation with BH3 peptide (60 μM) where indicated, and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (*FCCP*) was added after 8 min of total incubation. *P* denotes peptide or vehicle control

main peptide. First, GT1–7 neural cell mitochondria and mitochondria within primary cultures of neurons and astrocytes released cytochrome *c* without apparent swelling that would be required for outer membrane rupture (Fig. 5, *A* and *B*). Second, mitochondria retained their membrane potential after cytochrome *c* release in the presence of exogenously added cytochrome *c*, signifying an intact inner membrane (Fig. 6). Third, two potent PTP inhibitors, cyclosporin A and bongkrekic acid, were incapable of modulating the cytochrome *c* release induced by BH3 peptide (Fig. 5*C*).

A previous study has provided evidence for cytochrome *c* release by BH3 peptides that is accompanied by swelling and a loss of $\Delta\Psi$ (26). The release of cytochrome *c* in their system was blocked by the Ca^{2+} chelator EGTA, however, raising the possibility that contaminating Ca^{2+} and not the BH3 peptide was responsible for permeability transition-mediated cytochrome *c* release. The current study therefore examined cytochrome *c* release by BH3 peptide under conditions designed to exclude Ca^{2+}-activated permeability transition to determine whether BH3 peptide has the ability to release cytochrome *c* independent of Ca^{2+}-mediated effects. The BH3 peptide was unable to mediate cytochrome *c* efflux from adult rat liver mitochondria in the presence of EGTA, as was reported (3, 7), or from adult rat forebrain mitochondria (Fig. 3). However, under the same conditions, robust cytochrome *c* release was observed from GT1–7 and PC12S neural cell mitochondria in response to BH3 peptide, and release from GT1–7 mitochondria was inhibited by overexpression of Bcl-2 (Figs. 1 and 3). This cytochrome *c* release was not dependent on the permeablity transition pore, as indicated by the lack of swelling and lack of effect of EGTA, cyclosporin A, bongkrekic acid, ATP, and Mg^{2+}, all known inhibitors of the PTP (Fig. 5). Additionally, the release of cytochrome *c* by a PTP-independent mechanism was not limited to mitochondria from immortalized cells, as it also occurred in permeabilized primary cerebellar granule neurons and cortical astrocytes (Fig. 3). Although a PTP-dependent mechanism for BH3-mediated cytochrome *c* efflux cannot be excluded for all types of mitochondria under different conditions, this study demonstrates that the minimal BH3 death domain possesses the capacity to release cytochrome *c* without an inner membrane permeability transition.

Mitochondria have now been found to contain and release many proteins involved in the apoptotic process, including procaspases, apoptosis-inducing factor, and the recently characterized Smac/DIABLO. It has previously been suggested that a mitochondrial permeability transition leading to outer membrane rupture would be required for the release of such relatively large proteins (>25 kDa) from the intermembrane space (5, 43). Within cells undergoing apoptosis, however, proteins in excess of 50 kDa appear to redistribute from the mitochondria to the cytosol without gross changes in mitochondrial morphology (44). Adenylate kinase is an intermembrane space protein twice the size of cytochrome *c* in molecular weight. In some cases, cytochrome *c* efflux without adenylate kinase release has been observed in response to particular stimuli (45, 46). Although these observations are consistent with an outer membrane-specific selective conductance pathway for cytochrome *c*, the release of important apoptotic factors that are greater than cytochrome *c* in size remains unaccounted for. The present study now demonstrates that a substantially larger protein than cytochrome *c* can be released directly from isolated mitochondria by a BH3 domain peptide without an increase in inner membrane permeability (Fig. 2). This is consistent with the release of adenylate kinase in Bax- and Bid-mediated apoptosis that has been reported in intact cells (44).

This laboratory has previously reported that Bcl-2 was able to inhibit Ca^{2+}-activated permeability transition by maintenance of a reduced pyridine nucleotide redox state (41). However, the ability of Bcl-2 to inhibit BH3 peptide-induced cytochrome *c* release is not related to its effect on mitochondrial redox state (47). In the "rheostat" model of apoptosis, the extent of homo- *versus* heterodimerization among Bcl-2 family members is critical for regulating Bax function and determining cell survival (48). It has been shown that a BH3 peptide derived from Bak, a highly homologous protein to Bax, can bind directly to Bcl-X_L and Bcl-2 (49). Therefore, Bcl-2 may inhibit BH3-induced cytochrome *c* release either by heterodimerizing with Bax and impairing its function or by directly sequestering BH3 peptide and preventing an interaction with Bax or an alternative target protein.

Cytochrome *c* release has been reported to precede the loss of mitochondrial membrane potential during apoptosis in some

cell types (10–12). Because the loss of cytochrome *c* itself would be expected to dissipate $\Delta\Psi$ by interrupting electron flow down the electron transport chain, the present study investigated the mechanism by which membrane potential is maintained after the release of cytochrome *c*. In contrast to reports indicating that cytochrome *c* can still maintain mitochondrial membrane potential through electron transport after its release into the cytosol of cells undergoing apoptosis (50–52), a profound inhibition of respiration and a reduction in $\Delta\Psi$ were observed when cytochrome *c* was released from isolated GT1–7 mitochondria exposed to BH3 peptide (Fig. 6 and 7*A*). These effects were due to the loss of cytochrome *c*, as oxygen consumption and $\Delta\Psi$ were restored by the addition of a high concentration of exogenous cytochrome *c*. Mitochondrial membrane potential was partially supported in the absence of respiration by the electrogenic pumping of protons across the inner membrane by the F_1F_0 ATPase at the expense of ATP, since inhibition of the ATPase by oligomycin resulted in the swift dissipation of $\Delta\Psi$ (Fig. 7*B*). Thus, the respiratory inhibition coinciding with the loss of cytochrome *c* that was observed in the present study is consistent with reported respiratory inhibition in Fas-mediated apoptosis (53), where cytochrome *c* is released by truncated Bid (54). It is, however, possible that in some apoptotic cells where cytochrome *c* is released, the intracellular concentration of cytochrome *c* is still sufficient to maintain respiration-dependent mitochondrial membrane potential.

The observations that cytochrome *c* release in response to BH3 peptide occurs from some types of mitochondria but not others and that Bcl-2 is able to inhibit release strongly suggest that cytochrome *c* efflux occurs by a relatively specific mechanism that may be related to the ability of BH3 domain-containing proteins to release cytochrome *c in vitro* and *in vivo* (Figs. 1 and 3). Additional support for a specific mechanism of action comes from the inability of a BH3 peptide with a single amino acid substitution at glycine 67 to promote cytochrome *c* efflux (Fig. 1*C*). The same glycine-to-arginine alteration of a BH3 peptide at this site was previously found to impair interactions with Bcl-2 family proteins (16), and mutation of the homologous glycine in the BH3 domain of Bid was found to prevent Bax membrane insertion release and cytochrome *c* release (40).

The finding that BH3 peptide releases cytochrome *c* from neural cell mitochondria but not isolated liver or brain mitochondria raises the interesting possibility that a cell-selective mitochondrial protein may be required for its action. It has recently been reported that the "BH3 only" protein Bid is able to promote cytochrome *c* redistribution through an interaction with Bax or Bak at the outer mitochondrial membrane (17). Truncated Bid did not promote cytochrome *c* efflux or cell death in cells cultured from Bax, Bak double knockout mice, indicating a requirement for Bax or Bak expression for cytochrome *c* release by this BH3-only protein (40, 55, 56). Bax is expressed at high levels in the newborn rat, but decreases rapidly during postnatal development and is absent from most regions of the adult central nervous system (57). One region where Bax expression is maintained in the central nervous system, however, is the hypothalamus, from which the immortalized GT1–7 cell line is derived (58). An immunoblot was performed to determine whether Bax was present in the mitochondria of GT1–7 and PC12S cells or in mitochondria isolated from the liver or forebrain of the adult rat. Bax was detected in both GT1–7 and PC12S mitochondria but was absent in adult rat forebrain and liver mitochondria (Fig. 4*A*). Bax was also present in cerebellar granule neuron and cortical astrocyte cell lysates (data not shown). The presence of endogenous Bax protein therefore correlates with the ability of BH3 peptide to release cytochrome *c*. Because liver mitochondria were reported to contain Bak as an integral membrane protein and release cytochrome *c* in response to tBid (56), the ability of the BH3 peptide to induce cytochrome *c* release could potentially be due to an interaction with Bak. However, although isolated rat liver and forebrain mitochondria possess Bak (Fig. 4*A*), they did not respond to BH3 peptide treatment (Fig. 3), suggesting that either BH3 peptide does not interact strongly with Bak or that a BH3-Bak interaction is insufficient to release cytochrome *c*. Although adult liver and brain mitochondria did not contain detectable levels of Bcl-2 and Bcl-X_L (Fig. 4*A*), these are unlikely candidates as mediators of BH3-induced cytochrome *c* efflux because these proteins are reported to be anti-apoptotic in cells, and overexpressed Bcl-2 levels in GT1–7 mitochondria inhibited cytochrome *c* release by BH3 peptide. Further support for a specific interaction of the BH3 peptide with Bax may come from future experiments measuring the relative binding affinity of BH3 peptide for various Bcl-2 family members.

To further examine whether Bax is involved in the mechanism of cytochrome *c* redistribution promoted by BH3 peptide, mitochondrial pellets were subjected to alkali extraction after BH3 peptide addition. Consistent with the hypothesis that BH3 peptide acts like the BH3-only protein Bid to mediate cytochrome *c* efflux by the oligomerization and insertion of Bax, endogenous Bax became integrally inserted into the mitochondrial membrane after BH3 peptide treatment (Fig. 4*B*). Experiments are in progress to determine if BH3 peptide is capable of releasing cytochrome *c* from mitochondria devoid of endogenous Bax, *i.e.* those from adult rat liver and brain, when exogenous full-length Bax is present.

If the BH3 peptide indeed mediates cytochrome *c* release through an interaction with Bax, there are profound implications not only for developmental neuronal death, where in some cell types Bax is required (59, 60), but also for neuronal death occurring during cerebral ischemia/reperfusion brain injury, amyotrophic lateral sclerosis, Alzheimer's disease, and Parkinson's disease, where Bax levels are up-regulated (60–65). The presence or absence of Bax in various brain regions could potentially provide at least a partial explanation for the selective cell death that occurs in various neurodegenerative disorders. The apparent sensitivity to BH3 peptide of only mitochondria that possess associated Bax may also provide a means to target tumor cells with dysregulated Bax expression for selective destruction.

In summary, results of this study support the hypothesis that cytochrome *c* release can occur in response to a BH3 death domain peptide without a mitochondrial permeability transition and suggest that endogenous Bax protein may be required. Additionally, an analysis of mitochondrial function after cytochrome *c* release by BH3 peptide indicates that although respiration was inhibited, mitochondria are able to maintain respiration by ATP synthase reversal, consistent with observations reported in some cells undergoing apoptosis *in vivo*.

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