Calphostin C-mediated translocation and integration of Bax into mitochondria induces cytochrome *c* release before mitochondrial dysfunction

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Abstract

Calphostin C-mediated apoptosis in glioma cells was reported previously to be associated with down-regulation of Bcl-2 and Bcl-x_L. In this study, we report that 100 nM calphostin C also induces translocation and integration of monomeric Bax into mitochondrial membrane, followed by cytochrome *c* release into cytosol and subsequent decrease of mitochondrial inner membrane potential ($\Delta\Psi$ m) before activation of caspase-3. The integration of monomeric Bax was associated with acquirement of alkali-resistance. The translocated monomeric Bax was partly homodimerized after cytochrome *c* release and decrease of $\Delta\Psi$ m. The translocation and homodimerization of Bax, cytochrome *c* release, and decrease of $\Delta\Psi$ m were not blocked by 100 μ M z-VAD.fmk, a pan-caspase inhibitor, but the homodimerization of Bax and decrease of $\Delta\Psi$ m were inhibited by 10 μ M oligomycin, a mitochondrial F₀F₁-ATPase inhibitor. Therefore, it would be assumed that mitochondrial release of cytochrome *c* results from translocation and integration of Bax and is independent of permeability transition of mitochondria and caspase activation, representing a critical step in calphostin C-induced cell death. *Cell Death and Differentiation* (2000) 7, 511–520.

Keywords: calphostin C; Bax; cytochrome *c*; mitochondria

Abbreviations: Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp aldehyde; Ac-DEVD-MCA, acetyl-Asp-Glu-Val-Asp α -(4-methylcoumarin-7-yl)-7-amide; DTT, dithiothreitol; $\Delta\Psi$ m, mitochondrial inner membrane potential; DiOC₆(3), 3,3'-dihexyloxacarbocyanine; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene-glycol-bis(β -amino-ethylether)N,N'-tetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HM, heavy membrane; LH, light membrane; mCICCP, carbonyl cyanide *m*-chlorophenylhydrazone; NP-40, Nonidet P-40; PARP, poly(ADP-ribose) polymerase; PMSF, phenylmethylsulfonyl fluoride; PTPC, permeability transition pore complex; P1, low speed pellet; SDS, sodium dodecylsulfate; z-VAD.fmk, z-Val-Ala-Asn fluoromethylketone

Introduction

A large body of evidence supports the idea that the apoptotic features are mediated by the activation of several cytosolic proteases, the caspases, which then orchestrate apoptosis via the cleavage of key substrates.^{1–4} Recent studies have disclosed one key mechanism responsible for initiating the execution phase of apoptosis. Early in the process, release of mitochondrial cytochrome *c*,⁵ which upon entry into the cytosol forms as complex with Apaf-1^{6,7} and inactive form of caspase-9.⁸ In the presence of dATP or ATP, this complex activates caspase-9, which in turn can trigger a cascade processing and activating the effector caspases-3, -6, and -7.^{8,9} This then cleaves key substrates and coordinates the process of apoptotic cell death.

Calphostin C-mediated cell death of human glioma cells has been reported previously to be associated with a marked and rapid down-regulation of Bcl-2 and Bcl-x_L proteins and subsequent activation of caspase-3.^{10,11} Calphostin C is a microbial compound isolated from *Cladosporium cladosporioides* and a highly potent and specific inhibitor of protein kinase C, interacting not with the catalytic domain, but with the regulatory domain.¹² Bcl-2 family proteins can have profound effects on apoptosis and play a pivotal role in controlling life and death. Pro-apoptotic Bax plays a crucial role in the apoptotic process via a number of different mechanism,^{13–16} and the genetic approach using knockout mice has recently indicated that Bax can clearly promote apoptosis in the absence of Bcl-2.¹⁷ The pretreatment with a pan-caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD.fmk), inhibited calphostin C-induced activation of caspase-3 as well as apoptotic nuclear damages (chromatin condensation and DNA fragmentation) and cell shrinkage but did not block calphostin C-induced surface blebbing and cell death,¹¹ suggesting the involvement of Bax in calphostin C-induced apoptosis as indicated by Xiang *et al.*¹⁸ Bax has been shown to have both cytosolic and membrane-associated location. Death signals such as IL-3 withdrawal, staurosporine, dexamethasone, and γ -irradiation induce the translocation of Bax protein from cytosol to mitochondrial membrane^{15,19,20–22} and subsequent all mitochondrial hallmarks of apoptosis. For example, Bax induces directly cytochrome *c* release from mitochondria and caspase activation *in vivo*^{23,24} and *in vitro*.^{24–26} Anti-apoptotic Bcl-2 and Bcl-x_L have been shown to be capable of blocking spontaneous cytochrome *c* release in cell-free extracts and in cells treated with apoptosis-inducing agents.^{5,24,27–29} The cytochrome *c* release has been also reportedly dependent upon induction of the mitochondrial permeability transition which is associated with disruption of $\Delta\Psi$ m^{30,31} and has been

implicated in a variety of apoptotic phenomena.^{32–35} However, this release can occur in the absence of a decrease in $\Delta\Psi_m$ in both cell-free systems and in cells undergoing apoptosis.^{24,27,28,36} Therefore, we examine whether Bax is involved in calphostin C-mediated cell death through the translocation to mitochondria as a result of down-regulation of Bcl-2 and Bcl-x_L, and evaluate the relationships between the translocation of Bax, release of cytochrome c, disruption of $\Delta\Psi_m$, and activation of caspase-3.

Results

Bax redistributes and homodimerizes in mitochondria

Bcl-2 was shown as a single 26 kDa peptide by immunoblot analysis and Bcl-x_L as a doublet of peptides with an apparent molecular mass of 29–31 kDa. Bcl-2 and Bcl-x_L were down-regulated nearly synchronously as early as 1 h after treatment with 100 nM calphostin C in both U-87MG and T98G cells (Figure 1) and were barely detected as early as 4 h in U-87MG cells and 8 h in T98G cells after calphostin C treatment.¹¹ In addition, 29 kDa Bcl-x_L isoform was denser than 31 kDa one before calphostin C treatment whereas both Bcl-x_L isoforms became similar in density with each other as early as 2 h after 100 nM calphostin C treatment, indicating a mobility shift of Bcl-x_L.

The present study of Bax distribution demonstrates that Bax is found substantially in the soluble S100 cytosolic fraction and moderately in the mitochondria-enriched HM fraction as documented by the mitochondrial marker such as cytochrome c oxidase (Figure 2A). In contrast, Bcl-2 was shown predominantly in mitochondria-rich HM as well as endoplasmic reticulum- and plasma membrane-rich LM fractions and moderately in P1 fraction, which is comprised

of residual whole cells, nuclei and some mitochondria (Figure 2A). Finally, Bcl-x_L was found mainly in HM fraction and moderately to slightly in other three fractions (Figure 2A). These results indicate that Bax, Bcl-2, and Bcl-x_L are differentially compartmentalized with Bax being predominantly soluble, Bcl-2 being exclusively membrane-bound, and Bcl-x_L being present in both soluble and membrane-bound forms.

Although immunoreactive levels of Bax were not changed during treatment with 100 nM calphostin C in both U-87MG and T98G cells,¹¹ most of Bax moved from the cytosol mainly to the mitochondrial HM fraction and partly to P1 fraction (Figure 2A) following the exposure to 100 nM calphostin C. In contrast, Bcl-2 and Bcl-x_L in each compartment were markedly reduced following the exposure to 100 nM calphostin C probably as a result of their down-regulation. Constitutive Bcl-2 in HM fraction was resistant to extraction at alkaline pH as a result of its

Figure 1 Immunoblot analysis showing Bcl-2 protein as a single band (thick arrows) and Bcl-x_L protein as a doublet (thin arrows). Glioma cells (U-87MG and T98G) were exposed to 100 nM calphostin C for 0.5–3 h, and steady-state level of each protein was monitored by immunoblot analysis. Results are representative of three separate experiments with comparable outcomes

Figure 2 Translocation and integration of Bax into mitochondria. **(A)** Cytosol-to-membrane distribution of Bax and down-regulation of Bcl-2 and Bcl-x_L in T98G cells following treatment with 100 nM calphostin C for 6 h are shown. Control and treated cells were suspended in isotonic buffer, homogenized and separated into soluble S100 fraction (S), light membrane fraction (LM), heavy membrane fraction (HM), and low speed pellet (P1) by differential centrifugation. The P1 pellet contains residual whole cells, nuclei and mitochondria. The HM fraction is enriched for intact mitochondria. The LM fraction contains the endoplasmic reticulum and plasma membrane, and the S fraction represents the cytosol. The fractions were analyzed by immunoblot with anti-Bax, anti-Bcl-2, anti-Bcl-x, and anti-cytochrome c oxidase subunit II (Cyt Oxi) antibodies. **(B)** Monomeric Bax in HM fraction of T98G cells was alkali-sensitive before calphostin C treatment and became alkali-resistant after calphostin C treatment for 6 h, indicative of integration of Bax into mitochondrial membrane after calphostin C treatment. Bcl-2 in HM fraction of T98G cells was alkali-resistant before and after calphostin C treatment for 6 h. In addition, the resistance of Bax and Bcl-2 in HM fraction to alkali extraction was not reduced by 100 μ M z-VAD.fmk. Bax and Bcl-2 in HM fraction prepared from cells treated without or with calphostin C in the absence or presence of z-VAD.fmk were analyzed either directly (lanes 1–4) or after extraction with 0.1 M Na₂CO₃, pH 11.5 (lanes 5–8), as described in **A**. Similar results were achieved in three separate experiments in **A** and **B**, respectively

integration into the membrane lipid bilayer as suggested by Nguyen *et al.*^{37,38} but the majority of constitutive monomeric Bax was liberated from HM fraction under the same conditions before the exposure to calphostin C (Figure 2B), indicative of a peripheral association of monomeric Bax with the organelle as suggested by Fujiki *et al.*³⁹ After the exposure to 100 nM calphostin C for 6 h, however, this situation was reversed and most of the monomeric Bax in HM fraction now acquired resistance to alkaline extraction (Figure 2B), indicative of an integral membrane position as suggested by Goping *et al.*²⁰ Furthermore, the translocation and integration of monomeric Bax into mitochondrial membrane in response to calphostin C was not blocked by 100 μ M z-VAD.fmk and 10 μ M oligomycin, a pharmacological inhibitor of proton pump, as shown in Figures 2B and 3A, respectively. We conclude, therefore, that monomeric Bax demonstrates a specific response to the exposure to calphostin C *in vivo*, moving from the cytosol to the mitochondria and from a membrane-peripheral to a membrane-integrated position in the mitochondria. The increase of monomeric Bax levels in mitochondria, namely the translocation of monomeric Bax, was detected as early as 1 h after treatment with 100 nM calphostin C and then gradually enhanced in both U-87MG and T98G cells (Figure 3B).

In the absence of nonionic detergent Bax fails to form homodimers or heterodimers with Bcl-x_L and nonionic detergent is capable to enable the homodimerization of Bax.⁴⁰ Consequently, we utilized membrane-impermeable BS³ non-cleavable primary amine cross-linker to assess the conformation of mitochondrial-associated Bax. Before the exposure to calphostin C, Bax could be barely cross-linked as a 42 kDa presumptive homodimer with BS³ (Figure 3A,B). However, BS³-cross-linkable Bax homodimers were formed as early as 4–6 h (Figure 3A,B) and markedly increased as early as 8 h (Figure 3B) after the exposure to 100 nM calphostin C in both U-87MG and T98G cells. The levels of translocated monomeric Bax were not decreased after Bax homodimers were formed but rather increased at similar pace to that before Bax homodimerization (Figure 3B), suggesting that the translocated monomeric Bax is partly homodimerized. The homodimerization of Bax was not affected by 100 μ M z-VAD.fmk but moderately inhibited by 10 μ M oligomycin. Bcl-2 in mitochondria was decreased, on the other hand, as early as 1 h and barely detectable as early as 8 h after exposure to 100 nM calphostin C in both U-87 MG and T98G cells (Figure 3B), suggesting its down-regulation.

Cytochrome *c* release and caspase-3 activation

Bcl-2 and Bcl-x_L are reported to inhibit cytochrome *c* release from mitochondria,^{5,27,28} whereas Bax induces cytochrome *c* release.^{23–26} Therefore, we examined by immunoblot analysis whether cytochrome *c* is released from mitochondria into cytosol in calphostin C-induced apoptosis. Immunoreactive cytochrome *c* in S100 cytosolic fractions increased slightly as early as 2 h in U-87MG cells and 1–2 h in T98G cells after treatment with 100 nM calphostin C, namely concurrent with or just after the translocation of Bax to

Figure 3 A marked increase in Bax homodimers and monomers following treatment with 100 nM calphostin C (A) Heavy membranes prepared from T98G cells treated without (lanes 1–2) or with calphostin C for 6 h (lanes 3–5) in the absence or presence of 100 μ M z-VAD.fmk or 10 μ M oligomycin were incubated with 10 mM membrane-impermeable BS³ (lanes 2–5) cross-linker, or with DMSO as a control (lane 1). After incubation, membranes were quenched and lysed, Bax was immunoprecipitated with anti-Bax monoclonal antibody (4F11), size-fractionated by polyacrylamide gel electrophoresis and analyzed by immunoblot with anti-Bax polyclonal antibody. The 4F11 monoclonal antibody was run as a control (lane 6). (B) Heavy membranes prepared from U-87MG and T98G cells exposed to calphostin C for 1–8 h were treated with 10 mM membrane-impermeable BS³ cross-linker. After treatment, membranes were lysed and analyzed by immunoblot with anti-Bax polyclonal antibody. The membranes were reprobed with anti-Bcl-2 antibody (6C8), showing down-regulation of Bcl-2. Results are representative of three independent experiments with comparable outcome

mitochondria, and then enhanced maximally at 6–8 h in U-87MG cells and at 4 h in T98G cells (Figure 4A), when Bax homodimers were formed and ATP levels were reduced to within about 75% of control levels (data not shown). In addition, cytochrome *c* release into the cytosol was not inhibited by 100 μ M z-VAD.fmk or 10 μ M oligomycin in both U-87MG and T98G cells (Figure 4B).

Previous reports demonstrated that the exposure to 100 nM calphostin C induced activation of caspase-3 but not caspase-1 in both U-87MG and T98G cells.¹¹ Immunoreactive 32 kDa procaspase-3 was degraded into active 12 kDa caspase-3 fragment as early as 6 h in U-87MG cells and 4 h in T98G cells after treatment with 100 nM calphostin C, when cytochrome *c* was released maximally from mitochondria. The generation of a COOH-terminal 85 kDa PARP apoptosis fragment was shown to

be associated with the production of active 12 kDa caspase-3 fragment (Figure 5). These data are consistent with caspase-3 activation in calphostin C-treated gliomas. However, the membrane-associated caspase-3 is reported to be not activated in the presence of exogenous cytochrome *c* and suppresses by expression of Bcl-2, while the cytoplasmic caspase-3 is robustly activated by cytochrome *c*.⁴¹ Therefore, the inhibition of the membrane-associated caspase-3 by Bcl-2 could be reduced by the down-regulation of Bcl-2 induced by calphostin C. However, the activation of caspase-3 was not detected before the cytochrome *c* release in calphostin C-induced apoptosis, probably because the amount of membrane-associated caspase activity is small compared with that in the cytoplasmic caspase.⁴¹

Figure 4 Immunoblot analysis of cytochrome *c* in S100 cytosolic fractions of U-87MG and T98G cells treated with 100 nM calphostin C alone for the indicated times (**A**) and for 4 h in the absence or presence of 100 μM z-VAD.fmk or 10 μM oligomycin (**B**). Cytochrome *c* (Cyto *c*) in S100 cytosolic fractions was immunologically probed with anti-cytochrome *c* monoclonal antibody (7H8.2C12). The results are from a representative study performed three times with comparable outcomes

Figure 5 Immunoblot analysis showing activation of caspase-3 associated with cleavage of PARP. Formation of active 12 kDa caspase-3 fragment from 32 kDa procaspase-3 in both U-87MG and T98G cells, indicating activation of caspase-3. Cleavage of 116 kDa PARP into an 85 kDa apoptotic fragment was monitored by immunoblot analysis. Similar results were achieved in three separate experiments

Dissipation of mitochondrial $\Delta\Psi_m$

In many systems, apoptosis is associated with a loss of mitochondrial $\Delta\Psi_m$. Therefore, we examined $\Delta\Psi_m$ in calphostin C-induced apoptosis using DiOC₆(3), a fluorochrome. DiOC₆(3)-based measurement of $\Delta\Psi_m$ demonstrated a marked dissipation as early as 3 h in U-87MG cells and 2 h in T98G cells after treatment with 100 nM calphostin C, namely after the translocation of Bax and the release of cytochrome *c* (Figure 6A). Acute exposure of cells to the protonophore mCICCP confirmed that the DiOC₆(3) staining was dependent on the inner mitochondrial membrane potential, thus verifying the specificity of this assay. In addition, the decrease in $\Delta\Psi_m$ induced by 100 nM calphostin C was unaffected by 100 μM z-VAD.fmk (Figure 6B,C), showing no decrease in the number of cells displaying the low $\Delta\Psi_m$.

Oligomycin inhibits calphostin C-induced apoptosis and activation of caspases

Oligomycin binds to the F₀ portion of the mammalian F₀F₁-ATPases and prevents the proton pump from transporting H⁺ ions, thus effectively shutting it off.⁴² Since oligomycin is toxic in mammalian cells, the experiments were performed before oligomycin caused cell death (>90% trypan blue dye exclusion in control cultures). As shown in Figure 7A, oligomycin reduced the percentage of apoptotic cells in dose-dependent manner, with 10 μM oligomycin preventing approximately more than half of the calphostin C-induced cell death. DiOC₆(3)-based measurement of $\Delta\Psi_m$ demonstrated that 10 μM oligomycin resulted in hyperpolarization of mitochondria in glioma cells (Figure 7B), consistent with a block of the proton pump causing accumulation of H⁺ ions in the intermembrane space of mitochondria. In contrast to oligomycin, the respiratory complex III inhibitor antimycin A did not impair calphostin C-induced apoptosis (Figure 7A), but did reduce mitochondrial $\Delta\Psi_m$ (Figure 7B).

Since gene transfer-mediated overexpression of Bax has been shown to induce activation of caspases that can cleave the substrate peptide DEVD,^{25,43} we measured the effects of oligomycin treatment on calphostin C-induced activation of DEVD-cleaving caspases using lysates from glioma cells. As shown in Figure 7C, calphostin C-treated glioma cells contained markedly elevated levels of caspase activity compared to control cells. Addition of 10 μM oligomycin to the cultures had no effect on cytochrome *c* release (Figure 4) but substantially reduced the amount of calphostin C-induced caspase activity (Figure 7C). Following the treatment with 100 nM calphostin C for 6 h, glioma cells showed apoptotic morphological features (Figure 7D): cell shrinkage and surface blebbing by phase contrast images as well as nuclear condensation and fragmentation by Hoechst 33258 stain. However, the addition of 10 μM oligomycin induced marked decrease in cell shrinkage as well as chromatin condensation and fragmentation, suggesting the inactivation of caspase-3.

Figure 6 Calphostin C inducing reduction of the mitochondrial $\Delta\Psi_m$. **(A)** U-87MG and T98G cells were treated with 100 nM calphostin C for the indicated times and incubated with DiOC₆(3), followed by FACS analysis. Data represent log fluorescence versus relative cell number. As a control for specificity of DiOC₆(3) labeling, an aliquot of the control untreated cells was exposed to the protonophore mCICCP for 15 min prior to incubation with DiOC₆(3). **(B)** Time course analysis of mitochondrial permeability transition after U-87MG and T98G cells were treated with or without 100 μ M z-VAD.fmk and then with 100 nM calphostin C. Samples were analyzed at the indicated time points for mitochondrial depolarization as described in **A**. open squares, no treatment; open circles, treatment with calphostin C; crosses, treatment with z-VAD.fmk plus calphostin C. **(C)** Histograms show DiOC₆(3) staining of T98G cells induced by 100 nM calphostin C for 6 h in the presence or absence of 100 μ M z-VAD.fmk. mCICCP that completely disrupt the $\Delta\Psi_m$, was used as a positive control. The numbers in parenthesis represent the percentage of $\Delta\Psi_m^{\text{low}}$ cells. Similar results were obtained in three separate experiments in **A**, **B**, and **C**, respectively

Discussion

In the present study, we have shown that calphostin C induces down-regulation of anti-apoptotic Bcl-2 and Bcl-x_L as well as concurrent translocation of pro-apoptotic monomeric Bax to mitochondria, followed by release of cytochrome *c*, dissipation of $\Delta\Psi_m$, and activation of caspase-3. The previous study demonstrated that caspase inhibitors did not block calphostin C-induced cell death.¹¹ In addition, the genetic approach using knockout mice indicated that Bax promoted apoptosis in the absence of Bcl-2.¹⁷ These observations, taken together, suggest the involvement of Bax in calphostin C-mediated apoptosis. In addition, 10 μ M oligomycin inhibits calphostin C-induced apoptosis and activation of caspase-3, probably because intact F₀F₁-ATPase in the inner mitochondrial membrane is necessary for optimal function of Bax as shown by Matsuyama *et al.*⁴⁴ Bax, although present in many cell types, does not trigger apoptosis on its own but rather promotes apoptosis induced by removal of growth factors and other stimuli,^{13,15,45,46} and in some cases, ectopic expression of Bax can induce apoptosis.^{18,24,31,47,48} Therefore, despite no evidence of any alterations of Bax levels in calphostin C-induced apoptosis,¹¹ the translocation of pro-apoptotic monomeric Bax concurrent with the down-regulation of anti-apoptotic Bcl-2 and Bcl-x_L seems to leave glioma cells prone to apoptosis. The signals and mechanisms responsible for the change in Bax distribution

are not known. Bcl-2 blocked the translocation of monomeric Bax in response to a death signal.^{15,21,49}

The present study demonstrates that the translocation of Bax is associated with the acquirement of resistance to alkali extraction. Bax in HM fraction before calphostin C treatment did not exhibit any resistance to alkali extraction. A recent report has indicated that, during apoptosis, the association of Bax with mitochondrial membranes changes from a weak to a strong insertion as suggested by an acquirement of resistance to alkali extraction, a process which is regulated by the NH₂-terminal domain of Bax.²⁰ Altogether, these results suggest that Bax undergoes a conformational change after translocation to mitochondria induced by calphostin C. The Bax-conformational changes in calphostin C-induced apoptosis were not blocked by 100 μ M z-VAD.fmk or 10 μ M oligomycin. No inhibition of Bax-conformation by the latter suggests that Bax-conformation is not prevented by loss of the proton pump in the inner mitochondrial membrane. As a working hypothesis, we propose that Bax remains weakly attached to mitochondria in the absence of apoptotic stimulus, and that Bax which is translocated to mitochondria by exposure to calphostin C modifies the structure of Bax leading to its insertion into mitochondrial membranes.

How does calphostin C induce the release of cytochrome *c*? The cytochrome *c* release was shown before the activation of caspase-3 and not inhibited by 100 μ M z-VAD.fmk in calphostin C-mediated apoptosis, suggesting

Figure 7 Oligomycin suppresses calphostin C-induced apoptosis and caspase activation in T98G cells. **(A)** Cells were treated with 100 nM calphostin C for 8 h in the absence or presence of 0.1, 1, or 10 μ M oligomycin or 10 μ M antimycin A. The percentage of nonviable cells was determined by trypan blue dye exclusion method. **(B)** Cells were treated with 10 μ M oligomycin alone for 4 h or with 100 nM calphostin C for 4 h in the absence or presence of 0.1, 1, or 10 μ M oligomycin, 10 μ M antimycin A, or 100 μ M z-VAD.fmk, and were incubated with DiOC₆(3), followed by FACS analysis. Data represent log fluorescence versus relative cell number. As a control for specificity of DiOC₆(3) labeling, an aliquot of the control untreated cells was exposed to the protonophore mClCCP for 15 min prior to incubation with DiOC₆(3). **(C)** Lysate derived from cells treated with 100 nM calphostin C for the indicated times in the absence or presence of 10 μ M oligomycin were prepared and normalized for total protein content, and caspase-3 activity was measured based on hydrolysis of DEVD-MCA: open circles, treatment with calphostin C; crosses, treatment with oligomycin; closed circles, treatment with calphostin C plus oligomycin. **(D)** Representative photographs of phase contrast images and Hoechst 33258 stain of T98G treated with 100 nM calphostin C for 6 h in the absence or presence of 10 μ M oligomycin. Phase contrast $\times$ 200; Hoechst 33258 stain $\times$ 200. The results of **A**, **B**, **C** and **D** are from representative study performed in three experiments with comparable outcomes

that it occurs independently of caspase-3 activation, as reported by Bossy-Wetzel *et al*³⁶ in staurosporine- and UVB irradiation-induced apoptosis and by Finucane *et al*²⁴ in Bax-transfected apoptosis. In addition, the down-regulation of Bcl-2 and Bcl-x_L in calphostin C-induced apoptosis suggests an incapability of preventing cytochrome *c* release. Therefore, the cytochrome *c* release from the intermembrane space of mitochondria in calphostin C-induced apoptosis is a direct effect of Bax on the outer mitochondrial membrane as reported in other apoptosis by Rossé *et al*,²³ Jürgensmeier *et al*²⁵ and Finucane *et al*.²⁴ Although Bax-induced cytochrome *c* release was reported to be inhibited by 10 μ M oligomycin in isolated mitochondria,⁵⁰ calphostin C-induced cytochrome *c* release was not blocked by oligomycin when cytochrome *c* in cytosolic S100 fraction was examined. Thus, the treatment with oligomycin did not induce any alterations in monomeric Bax

levels in mitochondria and cytochrome *c* release from mitochondria. Since cytochrome *c* release was induced following the translocation of monomeric Bax in calphostin C-mediated apoptosis, the nature of the translocation signal of monomeric Bax leading to induction of cytochrome *c* release potentially takes on significance as a major apoptotic signaling pathway. It has been suggested also that the loss of $\Delta\Psi_m$ is responsible for the cytochrome *c* release,³⁰ but the cytochrome *c* release was shown before the decrease in $\Delta\Psi_m$ in calphostin C-induced apoptosis as reported by Yang *et al*²⁷ and Kluck *et al*²⁸ in other apoptosis, suggesting that the loss of $\Delta\Psi_m$ is not required for the cytochrome *c* release. Recently, Vander Heiden *et al*⁵¹ provided evidence that during apoptosis, a hyperpolarization of the mitochondrial inner membrane causes a swelling that might act to puncture the outer membrane without necessarily disrupting $\Delta\Psi_m$ in the short term, but it

remains to be determined whether mitochondrial swelling occurs in calphostin C-mediated apoptosis.

The decrease in $\Delta\Psi_m$ induced by 100 nM calphostin C was detectable following the translocation of Bax to mitochondria. Finucane *et al*²⁴ have reported that any decrease in $\Delta\Psi_m$ is not detected in isolated mitochondria treated with Bax despite the release of cytochrome *c*, but Bax protein has been shown to induce alterations in $\Delta\Psi_m$ *in vivo*.^{18,24,31,52} Bax protein binds to PTPC, a composite proteaceous channel that is involved in the regulation of mitochondrial membrane permeability, and Bax and adenine nucleotide translocator, a constitutive mitochondrial protein, cooperate within the PTPC to increase mitochondrial membrane permeability.⁵² The decrease in $\Delta\Psi_m$ induced by 100 nM calphostin C was ameliorated by 10 μ M oligomycin. Since cellular ATP levels were higher in cells treated with calphostin C and oligomycin than those treated with calphostin C alone (data not shown), it is conceivable that Bax integrated into mitochondria in calphostin C-mediated apoptosis renders the outer membrane more porous, causing a faster dissipation of the proton gradient than usual through leakage of H^+ ions into the cytosol. Consequently, the F_0F_1 -ATPase proton pump would run in reverse direction, thus consuming ATP and alkalinizing the matrix by extruding protons to cause opening of the mitochondrial PTPC.^{53,54} The decrease in $\Delta\Psi_m$ in calphostin C-induced apoptosis was unaffected by 100 μ M z-VAD.fmk, consistent with the observations of Xiang *et al*¹⁸ in Bax-induced cell death and by Marzo *et al*⁵² in Bax microinjection, but Finucane *et al*²⁴ have found that the addition of 100 μ M z-VAD.fmk substantially decreases the number of cells displaying the loss of $\Delta\Psi_m$, suggesting requirement of cytosolic participation for the decrease of $\Delta\Psi_m$. Calphostin C triggers a rapid caspase-dependent apoptosis, but even if caspase activity is inhibited, a nonapoptotic cell death proceeds.¹¹ In calphostin C-induced cell death, the release of cytochrome *c* might be expected to result in disruption of electron transport, inducing the decrease in ATP which is not so prominent in the early stage (data not shown) but may ultimately cause cell death even in the absence of caspase function. The translocation of monomeric Bax from the cytosol to mitochondria is accompanied by the integration of monomeric Bax into mitochondria and is followed by cytochrome *c* release and the dissipation of $\Delta\Psi_m$ before the homodimerization of Bax in calphostin C-induced apoptosis. Bcl-2 inhibits the homodimerization of Bax in response to death signal,^{15,21,49} and enforced dimerization of FKBP-Bax by the bivalent ligand FK1012 overrode the anti-apoptotic protection of Bcl- x_L .²¹ Since Bcl-2 and Bcl- x_L were down-regulated in calphostin C-induced cell death, Bax could be homodimerized without any inhibition. Although the homodimerization of Bax and the pore formation of Bax are shown to be required for the proapoptotic effect of Bax,^{21,52} the present study suggests that translocated monomeric Bax is associated with Bax-conformational change and capable of inducing mitochondrial hallmarks of apoptosis, and that the Bax homodimerization is rather associated with the later maximum release of cytochrome *c* and the marked decrease in ATP levels

(less than 30% of control; data not shown). Although the BH3 domain of the pro-apoptotic family members is crucial for their death-promoting and protein binding functions, BH3-independent mechanism of cell killing has been obtained through experiments involving BH3 domain mutants of Bax.^{55,56} In addition, Bax mutant mIII-1 which replaces hydrophobic face of BH3 with alanines, fails to form homodimers or heterodimers in classic binding assays but still inserts into mitochondrial membranes as a cross-linkable pair to induce apoptosis.⁵⁷ The homodimerization of Bax in mitochondria was decreased in the presence of 10 μ M oligomycin, suggesting that the F_0F_1 -ATPase activity in the inner mitochondrial membrane is involved in the homodimerization of Bax.

The important question which remains to be understood is why the cells, despite showing significant levels of mitochondria-associated Bax, do not spontaneously undergo apoptosis. Does Bax remain inactive because of possible association with anti-apoptotic proteins such as Bcl-2 or Bcl- x_L ?¹³ which would interfere with the availability and translocation of Bax from the cytoplasm to the mitochondria, or because of an inert configuration? The data presented here provide that the alkali-resistant integration of Bax into mitochondria is possible explanation to this question. In addition, despite the vast literature dealing with Bax, little is known about how integrated membrane Bax kill the cells. Some clues were provided by the observations that Bax, Bcl-2 and Bcl- x_L form distinct ion-conductive pores.^{58–61} Bax is reported to have redundant mechanisms for inducing apoptosis, based on its ability to form $\alpha 5$ and $\alpha 6$ helices-dependent channels in membranes and to dimerize with and antagonize anti-apoptotic proteins such as Bcl-2,⁶² and may regulate an electrochemical gradient, alter mitochondrial volume homeostasis, or redistribute critical substrates or products residing in the intermembrane space of mitochondria. Bcl-2 and Bcl- x_L inhibit the redistribution and homodimerization of Bax in response to a number of apoptosis-inducing stimuli, probably by formation of heterodimers between Bcl-2/Bcl- x_L and Bax or by counteracting ion channels at the level of the mitochondria.^{52,63,64}

Materials and Methods

Glioma cell culture

Two human glioma cell lines, wild-type p53-positive U-87 MG and mutant p53-positive T98G cells, were obtained from American Type Culture Collection (Rockville, MD, USA) and were maintained in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% fetal calf serum and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin) in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. Test exposures to 100 nM calphostin C were conducted in the continuous presence of light to promote photoactivation of this compound.

Immunoblot analysis

Cells were lysed in TNES buffer (50 mM Tris-HCl at pH 7.5, 100 mM NaCl, 1% NP-40, 2 mM EDTA, 20 μ g/ml leupeptin, 20 μ g/ml aprotinin,

1 mM PMSF) and centrifuged at $15\,000\times g$ for 10 min. Equivalent amounts of cell lysates were heated in $2\times$ loading buffer at 95°C for 5 min, and then electrophoresed in 12% SDS-polyacrylamide gel, except for immunoblot of PARP in which 8% SDS-polyacrylamide gel was used, and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 for 3 h, and were incubated with primary antibody against Bcl-2 (6C8: Pharmingen, San Diego, CA, USA), Bcl-x (Transduction Laboratories, Lexington, KY, USA), p12 subunit of caspase-3 (Santa Cruz, Santa Cruz, CA, USA), and PARP (a kind gift from Dr. Guy G Poirier, CHUL Research Center, Quebec, Canada), washed, and blotted with species-specific biotinylated secondary antibodies (Vector, Burlingame, CA, USA) for 1 h and then with horseradish peroxidase-streptavidin (Vector) for 1 h. The membrane was then developed in ECL reagent (Amersham, Arlington Heights, IL, USA).

Preparation of subcellular fractions

Cells were washed once in PBS, resuspended in isotonic buffer A (10 mM HEPES at pH 7.5, 200 mM mannitol, 70 mM sucrose, 1 mM EGTA) supplemented with protease inhibitors (1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ pepstatin A, 10 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor and 10 $\mu\text{g}/\text{ml}$ aprotinin), and homogenized with 25 strokes of a Teflon homogenizer. Nuclei and unbroken cells were separated at $120\times g$ for 5 min as P1 pellet. This supernatant was centrifuged at $10\,000\times g$ for 10 min to collect HM pellet. This supernatant was centrifuged at $10\,000\times g$ for 30 min to yield LM pellet and final S 100 fraction. Hypotonic lysis was performed by resuspending the cells in hypotonic buffer (10 mM Tris at pH 7.5, 5 mM MgCl_2 , 1 mM EGTA, 1 mM DTT) and homogenized with a Dounce homogenizer. For alkali extraction, the HM fraction was resuspended (0.25 mg protein/ml) in freshly prepared 0.1 M Na_2CO_3 , pH 11.5, and incubated for 30 min on ice. The membranes were then centrifuged at $100\,000\text{ g}$ for 45 min. Bax, Bcl-2, Bcl-x_L and cytochrome c oxidase in each fraction and cytochrome c in cytosolic S 100 fraction were immunologically probed with antibodies against Bax (DAKO, Kyoto, Japan), Bcl-2 (Pharmingen), Bcl-x_L (Transduction Laboratories), cytochrome c oxidase (Molecular Probes, Eugene, OR, USA) and cytochrome c (7H8.2C12: Pharmingen), respectively.

Cross-linking

The HM fraction was resuspended in buffer A, and membrane-impermeable bis(sulfosuccinimidyl) suberate (BS^3 ; Pierce, Rockford, IL, USA) in 5 mM sodium citrate buffer, pH 5.0, was added from a 10 fold stock solution to a final concentration of 10 mM.⁶⁵ After incubation for 30 min at room temperature, the cross-linker was quenched by addition of 1 M Tris-HCl, pH 7.5, to a final concentration of 20 mM. After quenching, membranes were lysed in RIPA buffer (10 mM HEPES at pH 7.5, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 1 mM EDTA, 10 $\mu\text{g}/\text{ml}$ aprotinin) and cleared by centrifugation at $12\,000\times g$. Part of this lysate was immunoprecipitated with anti-Bax monoclonal antibody (4F11: MBL, Nagoya, Japan), then size-fractionated by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblot with anti-Bax polyclonal antibody (DAKO). The remainder was analyzed directly by SDS-polyacrylamide gel electrophoresis and immunoblot with anti-Bax polyclonal antibody (DAKO).

Measurement of Mitochondrial $\Delta\Psi\text{m}$

Cells were incubated with 50 nM DiOC₆(3) (Molecular Probes) for 15 min in cultured medium at 37°C for measurement of $\Delta\Psi\text{m}$. As a positive control for $\Delta\Psi\text{m}$ loss, cells were incubated at the same time with the uncoupling agent mCICCP (50 μM), a protonophore, which disrupts $\Delta\Psi\text{m}$. Cells were washed once with PBS, collected by centrifugation at $1000\times g$, and stored in the dark at 4°C prior to analysis by flow cytometry using excitation of a single 488 nm argon laser. Data acquisition and analysis were performed using the CellFit software. Forward scatter, perpendicular scatter and DiOC₆(3) fluorescence were measured with a 530/15 nm band pass filter. In all cases, cells were gated to exclude cellular debris associated with necrosis. At least 10 000 events were collected per sample, with DiOC₆(3) fluorescence recorded as logarithmic amplified data.

Caspases-3 activity assay

Cells were lysed in extraction buffer [50 mM piperazine-N,N'-bis(2-ethanesulfonic acid) at pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM MgCl_2 , 1 mM DTT, 20 μM cytochalasin B, 1 mM PMSF, 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ pepstatin A, 50 $\mu\text{g}/\text{ml}$ antipain, 10 $\mu\text{g}/\text{ml}$ chymopain]. Crude extracts were obtained by centrifugation at $10\,000\times g$ for 12 min at 4°C , then diluted with caspase standard buffer (100 mM HEPES, pH 7.5, 10% sucrose, 0.1% 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid, 10 mM DTT, 0.1 mg/ml ovalbumin), and incubated at 37°C for 30 min with 1 μM fluorescent substrates, Ac-DEVD-MCA, to determine specific activities of caspases-3. Ac-DEVD-CHO was added to the reaction mixture at a concentration of 5 μM . Levels of released 7-amino-4-methylcoumarin were measured using a spectrofluorometer with excitation at 380 nm and emission at 460 nm. Excitation and emission slit width were adjusted to 10 and 20 nm, respectively. The fluorescent substrates were custom-synthesized at the Peptides Institute, Osaka, Japan.

Microscopic analysis

Cells were fixed with 4% paraformaldehyde for 30 min after washing with ice-cold PBS and then stained with 2.5 $\mu\text{g}/\text{ml}$ Hoechst 33258. The number of apoptotic cells was assessed by nuclei staining, and nuclei that were fragmented or condensed were scored as apoptotic. The viability of the cells was confirmed by trypan blue dye exclusion method.

Note added in proof

Since this manuscript was submitted for publication, Shimizu *et al* [Shimizu S, Narita M and Tsujimoto Y (1999) Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. *Nature* **399**: 483–487] have shown that Bax and Bak stimulate opening of the voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane, a mitochondrial channel through which cytochrome c permeates, whereas the anti-apoptotic proteins Bcl-x_L closes VDAC by binding to it directly.

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