Apoptotic Cytosol Facilitates Bax Translocation to Mitochondria That Involves Cytosolic Factor Regulated by Bcl-2

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ABSTRACT

Proapoptotic members of the Bcl-2 family, including Bax, Bak, and Bid, directly trigger the mitochondrial release of apoptogenic cytochrome c and apoptosis-inducing factor into the cytoplast. One of the crucial steps before Bax can exert its proapoptotic activity is translocation from the cytoplasm to the mitochondria, but the molecular mechanism of this translocation is not understood. To investigate the mechanism of apoptosis-associated Bax translocation, we used an in vitro system comprising isolated mitochondria and cytosol. We found that both endogenous and exogenous added recombinant Bax translocated to the mitochondria more efficiently in the presence of cytosol from cells with VP16-induced apoptosis than with cytoplasm from normal cells. This apoptosis-dependent promotion of Bax translocation was not seen with cytosol that was prepared from VP16-treated cells expressing Bcl-2. Cytosol from cells with VP16-induced apoptosis, but not that from normal cells or Bcl-2-expressing cells, induced cytochrome c release from isolated mitochondria, which, as assessed by immunodepletion experiments, was mainly mediated by Bax. These results suggest that Bcl-2 exerts its antiapoptotic activity partly by inhibiting the translocation of Bax through the modification of cytosolic factors that are involved in such translocation during apoptosis.

INTRODUCTION

Regulation of programmed cell death, or apoptosis, is essential for normal development and for the maintenance of homeostasis. Various apoptotic signals are transmitted to an executional phase through activation of a family of cysteine proteases called caspases, consisting mainly of initiator caspases and effector caspases, that constitute protease cascades. The Bcl-2 family of proteins are the best-characterized regulators of apoptosis (1–3). Some members of this family, such as Bcl-2 and Bcl-xL, suppress apoptosis, whereas others, such as Bax and Bak, promote apoptosis (3).

In apoptotic signal transduction, the mitochondria play an essential role (4–6) by releasing apoptogenic factors such as cytochrome c (7) and AIF (8) from the intermembrane space into the cytoplasm. After its release, cytochrome c binds to Apaf-1, a mammalian homologue of Ced-4, thus recruiting and activating initiator caspase-9, which subsequently activates effector caspase-3 (9, 10). AIF is released after loss of the mitochondrial membrane potential (11). It occurs through an as yet unidentified mechanism, and it induces apoptotic changes of the nucleus (8). Two mechanisms have been suggested for the antiapoptotic activity of Bcl-2 and Bcl-xL: proteins (a) the sequestration of a caspase activator, Apaf-1 (2); and (b) the prevention of apoptotic mitochondrial changes (6, 11), thus avoiding the release of cytochrome c and AIF. We have recently shown that Bax and Bak directly target the mitochondrial outer membrane channel, VDAC, and allow cytochrome c to pass through this channel and that Bcl-2/Bcl-xL prevents cytochrome c release by closing the VDAC (12). Although the proapoptotic Bcl-2 family members such as Bax, Bak, and Bid play a crucial role in apoptotic mitochondrial changes, the precise mechanism by which these proteins are activated has not yet been fully elucidated. It has been shown that cytoplasmic Bid is cleaved by caspases and that the truncated Bid protein translocates to the mitochondria to induce cytochrome c release (13, 14). Bax has also been shown to translocate to the mitochondria during apoptosis (15–18), and the translocation process seems to involve its dimerization and/or a conformational change (17, 18). It has recently been shown that Bax translocation to the mitochondria is dependent on caspases and cytochrome c (17), raising the possibility that Bax translocation to the mitochondria might represent an amplification loop of apoptotic signal transduction. Because Bax induces both Δψ loss, leading to AIF release, and cytochrome c release in isolated mitochondria (19–21), the mitochondrial translocation of Bax seems to be one of the critical steps for its apoptotic activity.

In this study, we investigated the mechanism of Bax translocation and found that cytosol from apoptotic cells promoted the translocation of endogenous and exogenous Bax to the mitochondria. This activity was suppressed in cytosol from Bcl-2-expressing cells, suggesting that Bcl-2 may regulate a cytoplasmic protein that is involved in the translocation of Bax.

MATERIALS AND METHODS

Chemicals. Anti-human Bax monoclonal antibody (N-20) specific for NH2-terminal amino acids 11–30 and anti-human Bax polyclonal antibody (I-19) specific for 19 amino acids at the COOH terminus were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); for the most part, antibody N-20 was used, unless otherwise indicated. Anti-Bid polyclonal antibody (C-20) and anti-human Bax monoclonal antibody (4F11) were obtained from Santa Cruz Biotechnology and Medical and Biological Laboratories, Co. (Nagoya, Japan), respectively. Anti-human Bcl-2 monoclonal antibody and anti-Xpress antibody were purchased from DAKO (Tokyo, Japan) and Invitrogen (Carlsbad, CA), respectively. Antipigeon cytochrome c monoclonal antibodies (7H2.2C12 and 6H2.84), both of which cross-react with mammalian cytochrome c, were obtained from PharMingen (San Diego, CA). Anti-human VDAC (porin) monoclonal antibody (3HL), which cross-reacts with rat VDAC, was purchased from Calbiochem (La Jolla, CA). Caspase inhibitor zVAD-fmk and Ac-DEVD-MCA were purchased from Peptide Inc. (Minoh, Japan). Bcl-2 transgenic mice were kindly provided by Drs. A. Mignon and V. Lacronique (Institut National de la Sante, Paris, France). The other chemicals used were obtained from Wako Biochemicals (Osaka, Japan).

Protein Purification. Human Bax was expressed as a His-tagged protein (rHis-Bax) in Escherichia coli (strain XL1-Blue) using the Xpress System (Invitrogen) and purified on a nickel-nitrilotriacetate-agarose (Qiagen) column according to the supplier’s protocol. The purified protein was dissolved in a lysis buffer containing 0.5 M NaCl, 10 mM Tris, pH 8.0, 0.5% NP-40, 0.5% Tween-20, and precipitated with 10% (w/v) polyethylene glycol 8000 and 1.0 M NaCl. The precipitate was dissolved in 0.2 M Tris, pH 7.5, 10 mM EDTA, 1% Triton X-100, and 25% glycerol.

Cell Lines. HeLa cells, a human cervical carcinoma-derived cell line, were maintained in RPMI 1640 as described previously (22). A stable transfectant of HeLa cells expressing human Bcl-2 (designated as HeLa-Bcl-2) was ob-
tained by transfection of the pUC-CAGGS vector bearing human bcl-2 DNA using electroporation (23). Cells transduced with the empty vector were used as a control (designated as HeLa-V).

**Analysis of Cell Death and Mitochondrial Membrane Potential.** Cell death was induced by the addition of 200 μM VP16 to the medium and assessed by nuclear morphology after staining with Hoechst 33342 (10 μM) as described previously (22). The mitochondrial membrane potential was quantified by rhodamine 123 staining using flow cytometry (FACScan; Becton Dickinson; Ref. 24). In the experiment using a caspase inhibitor (zVAD-fmk), it was added at 2 h before the addition of VP16.

**Subcellular Fractionation.** Cell fractionation was performed using digitonin as described previously (25). Briefly, after washing twice with PBS, cells were collected and treated with 10 μM digitonin for 5 min at 37°C. Centrifugation was used to separate the cytosolic and organellar fractions, followed by lysis with RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP40, 0.5% deoxycholate, and 0.1% SDS). Under these conditions, more than 84% of the cytosolic fraction was collected into a cytosolic fraction that showed less than 8% contamination by the mitochondrial fraction, as assessed by measuring cytosolic LDH and mitochondrial aspartate aminotransferase activity as described elsewhere (25). Aliquots of each fraction were subjected to Western blot analysis. Another fractionation method was also used as follows. Cells were washed twice in PBS, resuspended at 2 × 10^6 cells/ml in hypotonic buffer [10 mM potassium HEPES (pH 7.4), 10 mM MgCl2, and 42 mM KCl] on ice for 10 min, and then homogenized in a Dounce homogenizer. Nuclei and contaminating unbroken cells were separated into the crude nuclear fraction by centrifugation at 600 × g for 10 min. Half of the crude nuclear fraction was washed with 0.5% NP40 and then centrifuged to collect a nuclear fraction without the outer nuclear membrane. Next, the post-crate supernatant was centrifuged at 10,000 × g for 10 min to collect the heavy membrane fraction. This supernatant was subsequently centrifuged at 100,000 × g for 60 min to yield the light membrane fraction and the cytosolic fraction. The nuclear fraction, heavy membrane fraction, and light membrane fraction were resuspended in a volume of RIPA buffer equal to that of the final supernatant. Samples of the fractions were measured for LDH activity before the addition of RIPA buffer.

**Translocation of Bax into Isolated Mitochondria.** The cytosolic fraction of HeLa cells with or without VP16 (200 μM) treatment was prepared as follows. Cells were washed twice in PBS, resuspended in isotonic buffer [20 mM potassium HEPES (pH 7.4), 1.5 mM MgCl2, 10 mM KCl, and 250 mM sucrose] on ice for 10 min, and homogenized in a Dounce homogenizer. The cytosol was then separated by centrifugation at 100,000 × g for 60 min. All cytosols were concentrated to a volume of 10 μl by filtration using an Ultrafree-MC Centrifugal Filter Unit (Millipore) and resuspended in 400 μl of buffer A [0.3 mM mannitol, 10 mM potassium HEPES (pH 7.4), and 0.1% fatty acid-free BSA]. Some of these cytosolic fractions were subjected to immunodepletion of Bax, Bid, and cytochrome c. To do this, 100 μg of cytosol were incubated with 5 μg of anti-Bax, anti-Bid, and anti-cytochrome c (6H2, B4) antibodies and immunoprecipitated with protein G-Sepharose. After centrifugation, the supernatant was concentrated by filtration and suspended in buffer A. Aliquots of the immunodepleted cytosols were incubated with mitochondria or subjected to Western blot analysis. Control immunodepleted cytosol was made using 5 μg of normal rabbit, goat, or mouse IgG instead of anti-Bax, anti-Bid, or anti-cytochrome c antibody, respectively.

Mitochondria were prepared from the livers of male Donryu rats, Bcl-2 transgenic mice, which express human Bcl-2 in the liver, and nontransgenic littermates (26) as described previously (11). Mitochondria were finally suspended in buffer A.

Cytosolic fractions (50 μg) were added to isolated mitochondria (100 μg) at 25°C in 100 μl of buffer B (buffer A plus 100 μM potassium phosphate and 4.3 mM succinate) for 3 min and centrifuged to collect the mitochondria. Under these conditions, Bax translocation reached saturation levels within 3 min. In the experiments using rHis-Bax, the cytosolic fraction (50 μg) was preincubated with rHis-Bax (4 μg) at 25°C for 5 min and then added to the mitochondria (100 μg) at 25°C for 3 min in 100 μl of buffer B. The mixture was then centrifuged to collect the mitochondria. Aliquots of the collected mitochondria were resuspended in RIPA buffer and analyzed by Western blotting using anti-Bax antibody or anti-Xpress antibody. The Bax content was quantified by densitometric analysis using a Bio-Rad densitometer.

**Activity of Caspase-3-like Protease.** Caspase-3-like activity was measured as described elsewhere (23). Briefly, the cytosol was suspended in 50 mM Tris-CI (pH 7.4), 1 mM EDTA, and 10 mM EGTA and incubated at 37°C with 10 μM of the enzyme substrate Ac-DEVD-MCA. The release of 7-aminocoumarin was measured using a spectrophotometer (Hitachi F-3000) with excitation at 380 nm and emission at 460 nm.

**Cytochrome c Release from Mitochondria.** Cytosolic fractions (50 μg) were incubated with isolated mitochondria (100 μg) at 25°C for 30 min in 100 μl of buffer B, and the supernatants were separated by centrifugation. The aliquots were then subjected to Western blotting using anti-cytochrome c antibody to determine the cytochrome c release from the mitochondria.

**RESULTS**

**Bcl-2 Inhibits Bax Translocation from Cytosol to Organelles.** Bax translocation in cells during apoptosis was assessed using cytosolic and organellar fractions collected from cells treated with digitonin to increase cytoplasmic membrane permeability as described in “Materials and Methods.” Successful separation of each fraction was confirmed by the detection of most VDAC (a mitochondrial outer membrane protein) in the organellar fraction and most lactate dehydrogenase (a cytosolic enzyme) in the cytosolic fraction (Fig. 1A). As shown in Fig. 1A, organelar Bax was increased, and cytosolic Bax was decreased in vector-transfected HeLa cells (designated HeLa-V cells) during apoptosis induced by VP16, a cancer chemotherapeutic, and these changes preceded the loss of mitochondrial membrane potential (Fig. 1B) and the onset of apoptosis defined by nuclear morphology (Fig. 1C), cell membrane disruption, and annexin V staining (data not shown). Because the total Bax content remained unchanged (Fig. 1A), the increase of organellar Bax should reflect translocation from the cytosol to the organelles, confirming previous observations (15–18). Although an anti-Bax antibody (N-20) specific for the NH2-terminal region was used in the experiment shown in Fig. 1A, virtually identical results were obtained using a different anti-Bax antibody (I-19) specific for the COOH-terminal region of Bax (data not shown). In contrast, Bax translocation was markedly inhibited in Bcl-2-overexpressing HeLa cells (designated HeLa-Bcl-2 cells), as shown in Fig. 1A. Because the addition of a caspase inhibitor (zVAD-fmk) prevented apoptosis (Fig. 1C) but not the translocation of Bax (Fig. 1A), inhibition of Bax translocation by Bcl-2 occurred before the inhibition of caspase activation and the inhibition of apoptosis. Inhibition of Bax translocation by Bcl-2 was also confirmed by subcellular fractionation of cells by differential centrifugation, which was carried out as described in “Materials and Methods.” As shown in Fig. 1D, in live HeLa-V cells, most Bax was in the cytosol, and only a small amount of Bax was in the nuclear envelope (the difference between N− and N+) and the heavy membrane fraction, whereas treatment with VP16 for 12 h caused translocation of a considerable amount of Bax from the cytosol to the heavy membrane (HM) fraction containing most mitochondria that was inhibited in HeLa-Bcl-2 cells (Fig. 1D). The larger amount of Bax in the heavy membrane fraction of live HeLa-Bcl-2 cells than in live HeLa-V cells (Fig. 1D) might be due to the heterodimerization of Bax with organellar Bcl-2. Although it has been reported that Bax is translocated into the nucleus during apoptosis (27, 28), we did not detect a significant amount of Bax in the nucleus (N+) even after the induction of apoptosis (Fig. 1D). Translocation of Bax to the mitochondria and inhibition by Bcl-2 were also observed when HeLa cells were treated with H2O2 and anti-Fas antibody and when PC12 cells (a rat pheochromocytoma cell line) were treated with a nitric oxide donor, H2O2, and VP16 (data not shown). All of these results indicated that Bcl-2 prevented the translocation of Bax from the cytosol to the mitochondria during apoptosis.
In vitro Enhancement of Mitochondrial Bax Translocation by Apoptotic Cytosol and Inhibition by Cytosol from Bcl-2-expressing Cells. The inhibition of Bax translocation by Bcl-2 can be explained in two ways: (a) Bcl-2 acts on the mitochondria to inhibit the translocation of Bax; or (b) Bcl-2 acts on a factor that is involved in Bax translocation. To test the first possibility, rHis-Bax was incubated with mitochondria isolated from the livers of bcl-2 transgenic mice, in which human Bcl-2 expression is restricted to the liver (Bcl-2 mitochondria), and with mitochondria from nontransgenic littermates (nontransgenic mitochondria). The presence of human Bcl-2 in the Bcl-2 mitochondria but not in nontransgenic mitochondria was verified by Western blot analysis (Fig. 2A). Mouse Bcl-2 was not detected in either mitochondria with antimouse Bcl-2 antibody (data not shown). As shown in Fig. 2B, a slightly larger amount of rHis-Bax was translocated to the Bcl-2 mitochondria than to the nontransgenic mitochondria, excluding the first possibility. More efficient translocation to Bcl-2 mitochondria might be due to the heterodimerization between Bcl-2 and Bax.

We therefore tested the second possibility. Cytosolic fractions of HeLa-V and HeLa-Bcl-2 cells with or without VP16 treatment were added to rat liver mitochondria, and Bax translocation was assessed. Using HeLa-V cells, the Bax content of the cytosol from apoptotic cells was lower than that of the cytosol from nonapoptotic cells (Fig. 3A), consistent with the results shown in Fig. 1A. In addition, the amount of Bax translocating to the mitochondria in the presence of cytosol from apoptotic HeLa-V cells was about 4-fold higher than that seen in the presence of cytosol from nonapoptotic HeLa-V cells (Fig. 3A), indicating that Bax showed more efficient translocation in the presence of apoptotic cytosol, which is consistent with the previous report (17). In the presence of HeLa-Bcl-2 cytosol, Bax translocation to the mitochondria was unchanged, regardless of treatment with VP16 (Fig. 3A), indicating that translocation of Bax was suppressed by the HeLa-Bcl-2 cytosol. This inhibitory effect of HeLa-Bcl-2 cytosol was not caused directly by Bcl-2, because none of the cytosolic fractions contained a detectable level of Bcl-2 (Fig. 3B). As shown in Fig. 4A, exogenous rHis-Bax also translocated to the mito-
chondria more efficiently in the presence of cytosol from VP16-treated HeLa-V cells than cytosols from nontreated HeLa-V cells and cytosols from HeLa-Bcl-2 cells. These results indicated that apoptotic cytosol was able to facilitate Bax translocation to the mitochondria, whereas this activity was suppressed in nonapoptotic cells and Bcl-2-expressing cells. As suggested previously (18), one possibility was that rBax underwent dimerization with endogenous Bax in the apoptotic cytosol and was thereby translocated efficiently to the mitochondria. To test this possibility, Bax was immunodepleted using the antibody specific for the COOH-terminal region of Bax (C-terminal Bax antibody) from apoptotic cytosol and was thereby translocated efficiently to the mitochondria. As shown in Fig. 4, exogenous Bax still translocated into the mitochondria in the presence of immunodepleted cytosol from VP16-treated cells but not in the presence of cytosol from Bcl-2-expressing cells. A similar result was also obtained by immunodepleting Bax with a different anti-Bax antibody specific for the NH2-terminal region (data not shown). These results indicated that for exogenous Bax to translocate, dimerization with endogenous Bax was not necessary. The findings also suggested that apoptotic cytosols contained a cytoplasmic factor(s) that facilitated the translocation of Bax to the mitochondria and that this factor was kept inactivated by Bcl-2. The factor...
could be a protein involved in the modification of Bax or a protein involved directly in the translocation process, although other possibilities were not formally excluded, as described in the “Discussion.”

In keeping with the suggestion that Bax translocation occurs in a caspase- and cytochrome c-dependent manner (17), we examined the effect of caspase inhibitor and cytochrome c depletion on Bax translocation in our system. The addition of caspase inhibitor zVAD-fmk to cytosol from VP16-treated HeLa-V cells completely inhibited the caspase activity (Fig. 5A), but not the Bax translocation (Fig. 5B), consistent with the results obtained using whole cells (Fig. 1A). These results indicated that caspase was not involved in Bax translocation in our system. Immunodepletion of cytochrome c from cytosols (Fig. 5C) did not affect the translocation of rHis-Bax to the mitochondria (Fig. 5D), also excluding the possibility of cytochrome c involvement in Bax translocation in our in vitro system.

Major Role of Bax in Cytochrome c Release Induced by Apoptotic Cytosol from VP16-treated Cells. The addition of rBax to isolated mitochondria in the absence of cytosol causes the release of apoptogenic cytochrome c, which is inhibited by recombinant Bcl-2 recombinant Bcl-xL (12, 19, 20) through the antagonistic effects of Bcl-2/Bcl-xL and Bax/Bak on VDAC (12). Because we have shown that mitochondrial translocation of Bax was inhibited by cytosol from Bcl-2-expressing cells (although the cytosol did not contain detectable levels of Bcl-2), this action of Bcl-2, which is distinct from any effect of Bcl-2 on the mitochondria, should also contribute to its antiapoptotic activity. To test this possibility, we examined cytochrome c release from isolated mitochondria in the presence of various cytosols. When isolated mitochondria were incubated with apoptotic HeLa-V cytosol, cytochrome c release was extensive (Fig. 6A). In contrast, cytosol from VP16-treated HeLa-Bcl-2 induced little cytochrome c release (Fig. 6A), indicating that this cytosol contained less cytochrome c-releasing factor(s) than cytosol from apoptotic cells. To examine whether Bax played a role in cytochrome c release by cytosol from VP16-treated HeLa-V cells, Bax was immunodepleted from the cytosol, and cytochrome c releasing activity was then measured. Bax depletion of apoptotic HeLa-V cytosol markedly reduced its ability to induce cytochrome c release (Fig. 6B), indicating that Bax played a major role in cytochrome c release by cytosol from VP16-treated HeLa-V cells. Cytosol from VP16-treated HeLa-Bcl-2 cells before and after Bax depletion equally induced little release of cytochrome c, consistent with the observation that translocation of Bax to the mitochondria was inhibited by cytosol from HeLa-Bcl-2 cells. Immunodepletion of Bid, another proapoptotic Bcl-2 family protein that has been shown to induce cytochrome c release, did not alter the ability of cytosol to induce cytochrome c release (Fig. 6C), suggesting that Bid did not contribute to this action of the cytosol from VP16-treated HeLa-V cells. Similar results were also observed when cytosol from X-ray-irradiated thymocytes was used (data not shown). Thus, Bcl-2 prevents apoptotic cytochrome c release partly by inhibiting Bax translocation to the mitochondria.

DISCUSSION

Bax is involved in many forms of apoptosis, including apoptosis induced by γ-irradiation, trophic factor deprivation, and anticancer drugs (3, 29, 30). Although Bax was previously suggested to be localized to organelle membranes by its COOH-terminal hydrophobic domain (31), recent studies using fluorescence microscopy have indicated that Bax is largely localized within the cytosol of healthy cells (16, 17, 32). More recently, it has been shown that there is extensive

![Figure 5](https://example.com/cytosol.png)

Fig. 5. Nonessential role of caspase and cytochrome c in Bax translocation. A, inhibition of caspase activity by zVAD-fmk. Cytosol (50 μg) from VP16-treated HeLa-V cells was incubated with (+zVAD) or without (−zVAD) zVAD-fmk (200 μM) for 3 min, and we measured the caspase-3-like protease activity using Ac-DEVD-MCA for the indicated periods. B, no effect of caspase inhibitor on Bax translocation. rHis-Bax (2 μg) was incubated at 25°C for 5 min in the presence (+zVAD) or absence (−zVAD) of zVAD-fmk (200 μM) together with cytosol (50 μg) from HeLa-V cells treated with 200 μM VP16 for 12 h. Then mixtures were added to the mitochondria (100 μg) at 25°C for 3 min and centrifuged. The collected mitochondria and 2 μg of rHis-Bax (Total) were resuspended in RIPA buffer and analyzed by Western blotting with anti-Xpress antibody. C, immunodepletion of cytochrome c from cytosol. Cytosol (150 μg) from HeLa-V and HeLa-Bcl-2 cells treated with 200 μM VP16 for 12 h was immunodepleted with 2.5 μg of normal mouse IgG (NMI) or 2.5 μg of anti-cytochrome c monoclonal antibody (6H2, B4; α-cyt.c), and aliquots were subjected to Western blotting with anti-cytochrome c antibody. D, no effect of cytochrome c on rBax translocation to mitochondria. rHis-Bax (2 μg) was incubated at 25°C for 5 min with cytosol (50 μg) immunodepleted with anti-cytochrome c antibody or normal mouse IgG (NMI) as described in C. The mixtures were then added to the mitochondria (100 μg) at 25°C for 3 min and centrifuged. Bax translocation to the mitochondria was assessed by Western blotting with anti-Xpress antibody. Total represents 2 μg of rHis-Bax.
translocation of Bax from cytosol to organelles, especially to the mitochondria in the early phase of apoptosis (15–18). We confirmed this in the present study using a different model of apoptosis. Of note, we consistently found more Bax in the mitochondria of Bcl-2-expressing live cells than in control live cells, and this was probably due to the heterodimerization of Bax to mitochondrial Bcl-2. We and others have recently shown that the addition of Bax to isolated mitochondria induces apoptotic changes including \( \Delta \psi \) loss and cytochrome \( c \) release (19–21). All of these findings indicate that one of the critical steps for Bax to exert proapoptotic activity is translocation into the mitochondria.

Molecular Mechanism of Bax Translocation to the Mitochondria. We showed that both endogenous and exogenous Bax were translocated to the mitochondria more efficiently in the presence of cytosol from apoptotic cells than in the presence of cytosol from nonapoptotic cells. Although these observations were consistent with a recent report (17), there are substantial differences: Goping et al. (17) showed that Bax translocation was dependent on cytochrome \( c \) and active caspases and suggested that the Bax translocation observed in their system represented an amplification loop of the death signaling pathway. In contrast, the Bax translocation described in this study depended on neither cytochrome \( c \) nor caspases, suggesting that the Bax translocation observed in our system reflected an initial stage of apoptotic signal transduction to the mitochondria in cells. Because we excluded the possibility that exogenous Bax underwent dimerization with endogenous Bax in apoptotic cytosol and was then translocated to the mitochondria, our data suggested that apoptotic cytosol contained a factor that facilitated Bax translocation or that nonapoptotic cytosol contained a factor that inhibited translocation. The putative factor might modify Bax to regulate its translocation into the mitochondria, although posttranslational modification of Bax has not been reported. Alternatively, the factor might be a protein directly involved in the translocation process, which is activated in the apoptotic cytosol. Because Bax translocation was inhibited by cytosol from Bcl-2-expressing cells treated with several apoptotic stimuli, and this inhibition was not due to the Bcl-2 protein itself, a factor that facilitates Bax translocation was absent or inactive in Bcl-2-expressing cells. Bcl-2 has been shown to bind to several proteins, including Raf-1 kinase and calcineurin phosphatase (32, 33), any of which might be involved in regulating apoptosis-associated mitochondrial translocation of Bax.

**Involvement of Bax Translocation in Cytochrome \( c \) Release.** We and others have recently demonstrated that recombinant Bax induces apoptotic mitochondrial changes, including \( \Delta \psi \) loss and cytochrome \( c \) release, in isolated mitochondria (20, 34). Here we showed that cytosol from VP16-treated HeLa-V cells caused release of cytochrome \( c \) that was mainly mediated by endogenous Bax, because immunodepletion of Bax from the cytosol almost completely abolished cytochrome \( c \) release. Consistent with the reduced translocation of Bax seen with cytosol from the HeLa-Bcl-2 cells, this cytosol induced very little release of cytochrome \( c \). These results suggested that translocated Bax is the main contributor to the induction of apoptotic mitochondrial changes in HeLa cells treated with VP16. Because similar results were also obtained using other cells (such as PC12 cells) and other apoptotic stimuli (such as \( \gamma \)-irradiation and \( H_2O_2 \)), Bax seems to play a major role in inducing cytochrome \( c \) release during various forms of apoptosis. Although Bid has been implicated in cytochrome \( c \) release particularly during Fas-mediated apoptosis involving mitochondrial signal transduction (13, 14), we showed that Bid did not play a significant role in cytochrome \( c \) release during the several forms of apoptosis studied here.

**Antia apoptotic Mechanism of Bcl-2.** Bcl-2 and Bcl-x\(_L\) have been suggested to exert antia apoptotic activity by one of two mechanisms: (a) sequestration of the proforms of two major initiator caspases, pro-caspase-9 (through binding to Apaf-1) and pro-caspase-8 (through unidentified molecules; Ref 2); and (b) inhibition of apoptogenic mitochondrial changes, including cytochrome \( c \) release and \( \Delta \psi \) loss resulting in AIF release, as demonstrated using isolated mitochondria bearing endogenous Bcl-2/Bcl-x\(_L\) and recombinant forms of these proteins (6). The present study suggested a third action of Bcl-2 in

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**Fig. 6.** Major role of Bax in cytochrome \( c \) release induced by apoptotic cytosol. A, induction of cytochrome \( c \) release by cytosol from VP16-treated HeLa-V cells, but not cytosol from HeLa-Bcl-2 cells. Cytosol (50 \( \mu \)g) from HeLa-V and HeLa-Bcl-2 cells treated with (+VP16) or without 200 \( \mu \)g VP16 for 12 h was incubated with (+Mt) or without mitochondria (100 \( \mu \)g) at 25°C for 30 min. Mitochondria were also incubated without cytosol ([Cytosol (−)]). The supernatants were obtained by centrifugation, and cytochrome \( c \) was estimated by Western blotting using anti-cytochrome \( c \) antibody (cyt. \( c \)). Total represents an equivalent aliquot of mitochondria. B and C, reduction of cytochrome \( c \) release by immunodepletion of Bax but not Bid. Cytosol from VP16-treated HeLa-V and HeLa-Bcl-2 cells (50 \( \mu \)g) was immunodepleted using anti-Bax (aBax; B) or anti-Bid (aBid; C) polyclonal antibody and normal rabbit IgG (NRI; B) or normal goat IgG (NG; C). Bax-immunodepleted cytosol (B) or Bid-immunodepleted cytosol (C) was incubated with (+Mt) or without isolated mitochondria (100 \( \mu \)g) at 25°C for 30 min. Mitochondria were also incubated without cytosol ([Cytosol (−)]). The supernatants were obtained by centrifugation, and cytochrome \( c \) was estimated by Western blotting using anti-cytochrome \( c \) antibody (cyt. \( c \)).
preventing apoptosis, i.e., inhibition of the translocation of Bax to the mitochondria by affecting a cytosolic factor. Thus, Bcl-2 could potentially block three distinct steps in the apoptotic pathway.

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