

Inhibition of Bid-induced Apoptosis by Bcl-2

tBid INSERTION, Bax TRANSLOCATION, AND Bax/Bak OLIGOMERIZATION SUPPRESSED*

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Bcl-2 family proteins are important regulators of apoptosis. They can be pro-apoptotic (e.g. Bid, Bax, and Bak) or anti-apoptotic (e.g. Bcl-2 and Bcl-x_L). The current study examined Bid-induced apoptosis and its inhibition by Bcl-2. Transfection of Bid led to apoptosis in HeLa cells. In these cells, Bid was processed into active forms of truncated Bid or tBid. Following processing, tBid translocated to the membrane-bound organellar fraction. Bcl-2 co-transfection inhibited Bid-induced apoptosis but did not prevent Bid processing or tBid translocation. On the other hand, Bcl-2 blocked the release of mitochondrial cytochrome *c* in Bid-transfected cells, suggesting actions at the mitochondrial level. Alkaline treatment stripped off tBid from the membrane-bound organellar fraction of Bid plus Bcl-2-co-transfected cells, but not from cells transfected with only Bid, suggesting inhibition of tBid insertion into mitochondrial membranes by Bcl-2. Bcl-2 also prevented Bid-induced Bax translocation from cytosol to the membrane-bound organellar fraction. Finally, Bcl-2 diminished Bid-induced oligomerization of Bax and Bak within the membrane-bound organellar fraction, shown by cross-linking experiments. In conclusion, Bcl-2 inhibited Bid-induced apoptosis at the mitochondrial level by blocking cytochrome *c* release, without suppressing Bid processing or activation. Critical steps blocked by Bcl-2 included tBid insertion, Bax translocation, and Bax/Bak oligomerization in the mitochondrial membranes.

Apoptosis, also called programmed cell death, is a highly regulated process that plays an essential role in the development and maintenance of homeostasis within multicellular organisms (1, 2). Dysregulation of apoptosis has been implicated in the development of cancer, autoimmune disorder, neurodegeneration, ischemic damage, and other devastating diseases (3–6). Whereas apoptosis regulation takes place at multiple levels, Bcl-2 family proteins are of paramount importance (7–10).

Bcl-2 family proteins are defined by the presence of Bcl-2

homology (BH)¹ domains (7–11). They can be pro-apoptotic or anti-apoptotic. Specific function of individual members is determined by the presence and organization of the BH domains (8, 9). For example, anti-apoptotic members, like Bcl-2 and Bcl-x_L, contain four BH domains, whereas some pro-apoptotic molecules such as Bax and Bak contain three (BH1–3), and others contain only one, the BH3 domain (7–12).

Bid is a unique BH3-only pro-apoptotic protein (13). Unlike others, Bid activation depends on the proteolytic processing of intact Bid into truncated forms of tBid. tBid, thus generated, translocates to mitochondria and leads to disruption of the organelles and the release of apoptogenic molecules such as cytochrome *c* (14, 15). Bid processing can be conducted by several proteases (16, 17); however, caspase-8 has been shown to be the major protease responsible for Bid cleavage during death receptor-mediated apoptosis (14, 15). Caspase-8-mediated Bid processing therefore bridges the extrinsic death receptor-mediated pathway of apoptosis to the intrinsic mitochondrial pathway (14, 15, 18). This provides a mechanism to amplify the execution signal and exacerbate the pace of cell demise.

Interactions among Bcl-2 family proteins have been documented (7–12). Functionally, expression of anti-apoptotic Bcl-2 or Bcl-x_L suppresses cell death initiated or mediated by pro-apoptotic members. Recent studies (19) have further suggested a sequence of Bcl-2 family protein activation during apoptosis, where Bcl-2 is positioned to block apoptosis at two separate steps, through inhibition of Bax/Bak and BH3-only proteins including Bid. Despite these important observations, it remains unclear how Bcl-2 antagonizes the pro-apoptotic action of Bid or tBid (20, 21).

Here, by using an expression model, we have systematically analyzed Bid-induced apoptosis and its inhibition by Bcl-2. Our results show that Bcl-2 inhibited Bid-induced cytochrome *c* release and apoptosis, without suppressing Bid processing into tBid and subsequent tBid translocation to mitochondria. At the mitochondria, three critical events that were blocked by Bcl-2 have been identified. First, Bcl-2 suppressed tBid insertion into mitochondrial membranes. Second, Bcl-2 inhibited Bid-induced Bax translocation from the cytosol to mitochondria. Third, Bcl-2 diminished Bid-induced Bax/Bak oligomerization in the mitochondrial membranes. The results suggest that Bcl-2 may suppress Bid-induced apoptosis at the mitochondrial level by multiple mechanisms.

EXPERIMENTAL PROCEDURES

Materials—PcDNA-Bid was prepared as described previously (13). pBactBcl-2 was a gift from Dr. Junyin Yuan at Harvard Medical School.

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¹ The abbreviations used are: BH, Bcl-2 homology; DSP, dithio-bis(succinimidyl propionate); BMH, bismaleimido-hexane; BSO-COES, bis[2-(succinimidyl)oxycarbonyloxy]-ethyl sulfone; GFP, green fluorescent protein.

pEGFP was purchased from Clontech. Transfection reagents were purchased from Invitrogen. Chemical cross-linkers dithiobis(succinimidyl propionate) (DSP), bismaleimido-hexane (BMH), and bis[2-(succinimidyl-oxy-carbonyloxy)-ethyl] sulfone (BSOCOES) were purchased from Pierce. Antibodies used in this study were from the following sources: rabbit polyclonal anti-Bax (N-20), mouse monoclonal anti-Bcl-2 (C-2), rabbit polyclonal anti-Bcl-2 (Δ C21), and goat polyclonal anti-lamin B from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit polyclonal anti-Bak from Upstate Biotechnology, Inc. (Lake Placid, NY); mouse monoclonal antibodies against native (6H2.B4) and denatured (7H8.2C12) cytochrome *c* from Pharmingen; mouse monoclonal 20E8 anti-cytochrome oxidase IV from Molecular Probes (Eugene, OR); rabbit polyclonal anti-murine Bid was prepared as described previously (13). All secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA).

Transfection—HeLa cells were maintained in minimum essential medium supplemented with 10% fetal bovine serum, 0.1 mM non-essential amino acids, 2 mM L-glutamine, and 1% antibiotics. The cells were plated at 3.0×10^5 /35-mm dish to reach ~50% confluence by the next day for transfection. Cells in each dish were transfected with 0.25 μ g of empty pcDNA vectors, 0.25 μ g of pcDNA-Bid, or 0.25 μ g of pcDNA-Bid along with 0.25 μ g of p β actBcl-2. To identify the transfectants, the same dishes were co-transfected with pEGFP-C1, which led to the expression of green fluorescence protein in transfected cells. Transfection was facilitated with LipofectAMINE PLUS (Invitrogen), according to the manufacturer's instructions. Transfection efficacy was usually over 70%. After transfection, cells were incubated in serum-free medium for 4–5 h and then transferred to full growth medium. Morphological and biochemical analyses were conducted at ~17 h post-transfection.

Analysis of Apoptosis—Apoptotic cells were identified by their morphology as described in our previous studies (22). Typical apoptotic morphology evaluated included cellular shrinkage and the formation of apoptotic bodies. To verify apoptosis, nucleus was stained with Hoechst 33342 to reveal nuclear condensation and fragmentation. For each condition, apoptosis was monitored in five fields with ~60 cells per field. The experiments were repeated for at least four times with duplicate dishes for each condition in every experiment.

Subcellular Fractionation—To analyze the distributions of various proteins, cells were fractionated into cytosolic and membrane-bound organellar fractions with low concentrations of digitonin (23–25). Selective permeabilization of plasma membranes was monitored by microscopy. Digitonin permeabilization has been used to study protein redistributions within cells during apoptosis (24–27). Briefly, cells were exposed to 0.05% digitonin in isotonic sucrose buffer (in mM: 250 sucrose, 10 Hepes, 10 KCl, 1.5 MgCl₂, 1 EDTA, and 1 EGTA; pH 7.1) for 2 min at room temperature to collect the soluble fraction as cytosolic extracts. Digitonin-insoluble fraction was washed with isotonic sucrose buffer and further dissolved in 2% SDS buffer to collect the membrane-bound organellar fraction. Because apoptotic redistribution of cytochrome *c* and Bcl-2 family proteins including Bid and Bax mainly takes place between the cytosol and mitochondria, immunoblot analysis of the membrane-bound part is expected to reveal mitochondrial content of the molecules.

Protein Cross-linking—Cross-linking was conducted by procedures modified from our previous work (25). All cross-linking chemicals (DSP, BMH, and BSOCOES) were dissolved in Me₂SO at concentrations of 100 mM prior to experiments and further diluted in phosphate-buffered saline to 10 mM before using. DSP was added to the cells for 30 min of incubation at room temperature under constant mixing. The cells were subsequently fractionated by digitonin as described above. For BMH and BSOCOES, cells were first fractionated to collect the membrane fraction for cross-linking. Cross-linked samples were subjected to electrophoresis under non-reducing conditions for immunoblot analysis.

Immunofluorescence Analysis of Cytochrome *c*—Cytochrome *c* immunofluorescence was examined as described in our previous publications (23, 24). Briefly, cells were grown on collagen-coated glass coverslips and subjected to transfection. The cells were fixed in a modified Zamboni's fixative containing 4% paraformaldehyde and 0.19% picric acid and permeabilized with 0.1% SDS prior to blocking and primary antibody (mouse anti-native cytochrome *c*) exposure. Finally, antigenic sites within the cells were revealed by staining with CY-3-conjugated goat anti-mouse antibodies. To examine the nucleus, Hoechst 33342 (10 μ g/ml) was added to cells in phosphate-buffered saline and stained for 5 min at room temperature.

Alkaline Treatment—After digitonin permeabilization, the membrane-bound organellar fraction was collected and washed once with phosphate-buffered saline. The fraction was incubated on ice in 0.1 M Na₂CO₃ at pH 11.5 for 30 min and then subjected to 1 h of centrifuga-

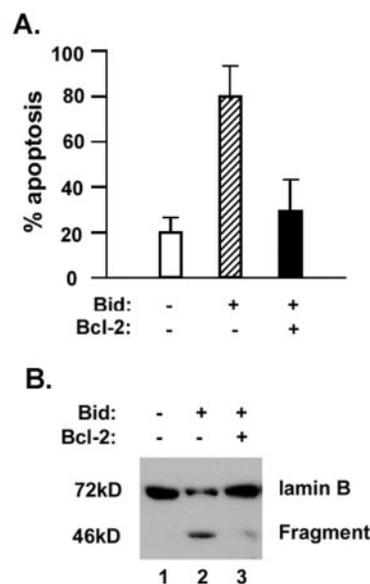


FIG. 1. Inhibition of Bid-induced apoptosis by Bcl-2. HeLa cells were transfected with Bid, Bid + Bcl-2, or empty vectors (mock), along with pEGFP. Apoptosis of transfected cells was identified by cell morphology, as described under "Experimental Procedures," and calculated as a percentage of transfectants (A). Data are expressed as the mean \pm S.D. ($n = 4$). To analyze lamin B breakdown, whole cell lysates were collected for immunoblot analysis (B). The results show that Bid transfection led to the development of apoptotic morphology and apoptotic cleavage of lamin B. Bcl-2 co-transfection inhibited Bid-induced apoptosis.

tion at $100,000 \times g$ at 4 $^{\circ}$ C to collect the supernatant and pellet. The supernatant contained the proteins released during alkaline incubation, whereas the pellet contained the proteins that were resistant to the treatment (28).

Immunoprecipitation—Cells were extracted directly with RIPA buffer to collect whole cell lysate, or subjected to sequential extractions with 0.05% digitonin and RIPA buffer to collect cytosolic and membrane-bound organellar extracts, respectively. Isotonic digitonin buffer was described above under "Subcellular Fractionation." The composition of RIPA buffer was (in mM): 150 NaCl, 1 MgCl₂, 1 EGTA, 10 β -mercaptoethanol, 15 Tris-HCl, pH 7.4, containing 0.5% sodium deoxycholate, 0.2% SDS, and 1% Triton X-100. Immunoprecipitation was conducted according to our previous work (24, 25). Extracts (whole cell extracts by RIPA, cytosolic extracts by digitonin, post-digitonin extracts by RIPA) from $\sim 1 \times 10^6$ cells were pre-cleared by incubation with 1 μ g of normal serum and 30 μ l of agarose protein A/G (Santa Cruz Biotechnology). The pre-cleared lysates were subsequently incubated for 2 h with 2 μ g of immunoprecipitation antibody and 30 μ l of agarose protein A/G. Immunoprecipitates were collected by centrifugation and dissolved in SDS sample buffer for immunoblot analysis. To detect Bcl-2 and Bid/tBid interaction, a mouse monoclonal anti-Bcl-2 was used for immunoprecipitation, and the resultant precipitates were subjected to immunoblot analysis of Bid/tBid.

RESULTS

Inhibition of Bid-induced Apoptosis by Bcl-2—We initially determined whether transfection of full-length Bid induced apoptosis in HeLa cells and whether Bcl-2 co-transfection was able to suppress it. To identify the transfected cells, a vector containing green fluorescence protein (GFP) was co-transfected. The results are shown in Fig. 1A. The control group that was transfected with empty vectors had an apoptosis rate of 22.8%. Bid transfection led to a dramatic increase in apoptosis, to 81.3%. Bid-induced apoptosis was blocked by Bcl-2 co-transfection. As a result, cells co-transfected with Bid + Bcl-2 showed an apoptosis rate of 33.7% (Fig. 1A). The morphological observations were confirmed by biochemical analyses. As shown in Fig. 1B, Bid transfection led to the cleavage of lamin B into an apoptosis-indicative fragment of 46 kDa (lane 2). Co-transfection of Bcl-2 blocked lamin B cleavage (lane 3). Data

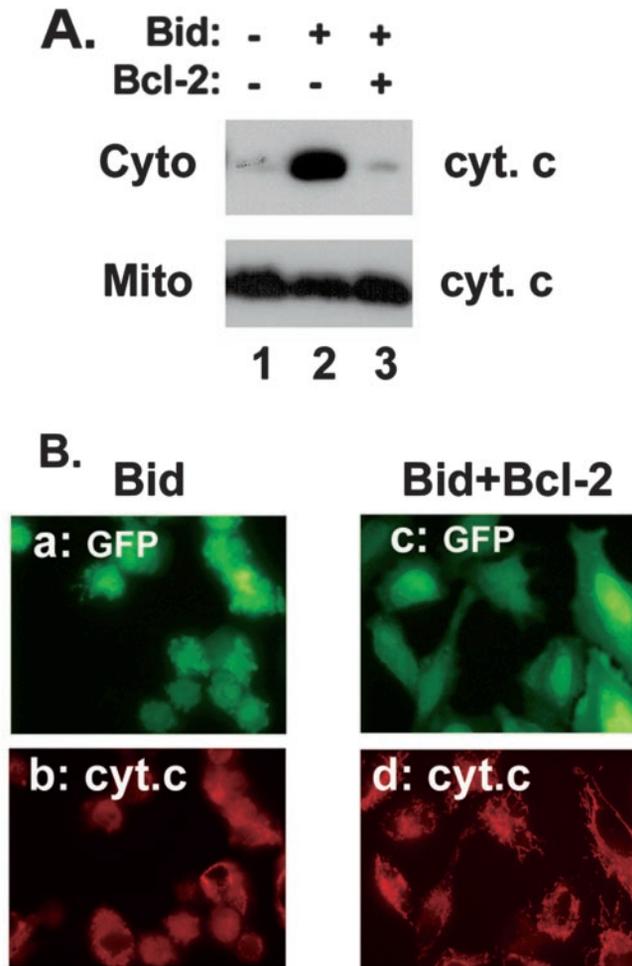


FIG. 2. Bcl-2 blocks Bid-induced cytochrome *c* release from mitochondria. *A*, immunoblot analysis. HeLa cells transfected with Bid, Bid + Bcl-2, or empty vectors were fractionated into cytosolic and membrane-bound organellar fractions and analyzed for cytochrome *c* (cyt. *c*) by immunoblotting. In mock transfection with empty vectors, the majority of cytochrome *c* was in the organellar fraction (lane 1). After Bid transfection, significant amounts of cytochrome *c* appeared in the cytosol (Cyto) (lane 2). Bcl-2 co-transfection blocked Bid-induced release of cytochrome *c* (lane 3). *Mito*, mitochondria. *B*, immunofluorescence analysis. HeLa cells were transfected with Bid (*a* and *b*) or Bid + Bcl-2 (*c* and *d*). pEGFP was co-transfected to identify the transfectants. The cells were processed for cytochrome *c* immunofluorescence as described under "Experimental Procedures." The same fields were examined for cytochrome *c* (red, *b* and *d*) and transfected cells containing green fluorescence protein (green, *a* and *c*). Bid transfection led to apoptosis, showing cellular shrinkage and the formation of apoptotic bodies (*a*). In these cells, cytochrome *c* leaked into the cytosol, resulting in diffuse cytosolic staining (*b*). Bcl-2 co-transfection prevented apoptosis (*c*) as well as the leakage of mitochondrial cytochrome *c* (*d*). As a result, cytochrome *c* in Bcl-2-co-transfected cells was preserved in the mitochondria, exhibiting perinuclear organellar staining (*d*).

not shown also demonstrated apoptotic cleavage of poly(ADP-ribose) polymerase in Bid-only transfected cells, which was again suppressed by Bcl-2 co-transfection. Together, these experiments have demonstrated Bcl-2 inhibition of Bid-induced apoptosis in HeLa cells.

Bcl-2 Inhibits Bid-induced Cytochrome *c* Release from Mitochondria—A major cellular site targeted by Bcl-2 family proteins is the mitochondrion (7–12). Disruption of this organelle results in the release of apoptogenic molecules including cytochrome *c* and may therefore underlie the pro-apoptotic actions of Bid (20, 21). Thus, to pursue the mechanisms responsible for Bcl-2 inhibition of Bid-induced apoptosis, we examined cellular distributions of cytochrome *c*. The results are shown in Fig. 2.

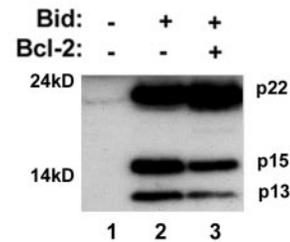


FIG. 3. Bcl-2 does not prevent Bid processing into active forms of tBid. HeLa cells transfected with Bid, Bid + Bcl-2, or empty vectors. Whole cell lysates were extracted with Laemmli buffer containing 2% SDS for immunoblot analysis of Bid/tBid. Clearly, Bid of 22 kDa (p22) was processed into active forms of tBid, which showed apparent molecular sizes of 15 and 13 kDa (lane 2, p15 and p13). Of significance, Bid processing was not inhibited by Bcl-2 co-transfection (lane 3).

By immunoblot analyses, the majority of cytochrome *c* was detected in the mitochondrial fraction of the control cells (Fig. 2*A*, lane 1). Bid transfection led to an increase of cytochrome *c* in the cytosol, accompanied by loss of the molecule from the membrane-bound organellar fraction (lane 2), indicating cytochrome *c* release from mitochondria in these cells. Significantly, Bcl-2 co-transfection blocked Bid-induced cytochrome *c* release (lane 3). The immunoblot results were confirmed by immunofluorescence staining of cytochrome *c* within these cells. As shown in Fig. 2*B*, Bid-transfected cells (*a*, green) exhibited typical morphology of apoptosis, assuming a round-up and fragmented configuration. In the same cells, cytochrome *c* (Fig. 2*B*, *b*, red) was released from mitochondria, resulting in whole cell staining. In sharp contrast, cells co-transfected with Bid + Bcl-2 displayed a much healthier morphology (Fig. 2*B*, *c*). These cells maintained cytochrome *c* in the mitochondria, showing a punctate perinuclear staining (Fig. 2*B*, *d*). The results suggest that Bcl-2 inhibited Bid-induced apoptosis at the mitochondrial level by blocking cytochrome *c* leakage from mitochondria.

Bid Processing Is Not Prevented by Bcl-2—The pro-apoptotic activity of Bid depends on its processing into the active forms of tBid (14, 15). Thus, to identify further the mechanisms responsible for Bcl-2 inhibition, we examined Bid processing in transfected cells and the effects of Bcl-2 co-transfection by using whole cell lysates. The results are shown in Fig. 3. In Bid-transfected cells, intact Bid of 22 kDa was expressed at high levels (lane 2), compared with control transfection (lane 1). Moreover, tBid of 15 and 13 kDa was detected, indicating Bid processing in these cells (lane 2). Bcl-2 co-transfection did not attenuate either Bid expression or its processing into tBid (lane 3). The results suggest that Bcl-2 inhibited Bid-induced apoptosis without significantly suppressing Bid expression or processing.

tBid Translocation to Mitochondria Is Not Prevented by Bcl-2—An important event for Bid activation and toxicity is the targeting of mitochondria by tBid (14, 15). Thus, our subsequent experiments tested the possibility that Bcl-2 might inhibit Bid-induced apoptosis by blocking tBid translocation. For this purpose, transfected cells were fractionated into cytosolic and membrane-bound organellar fractions for immunoblot analysis of Bid/tBid. The results are shown in Fig. 4. In Bid-only transfected cells (lanes 1 and 3), intact Bid of 22 kDa was detected mainly in the cytosol, whereas tBid of 15 kDa showed both cytosolic and organellar distributions, and p13 was detected only in the membrane-bound organellar fraction. Bcl-2 co-transfection did not change the cellular localization of these molecules (Fig. 4, lanes 2 and 4). The results, together with those shown in Fig. 3, indicate that Bcl-2 inhibited Bid-induced cytochrome *c* leakage and apoptosis without blocking Bid processing and translocation.

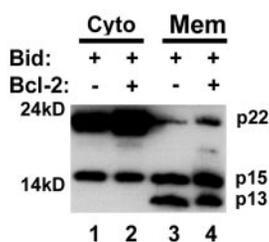


FIG. 4. Bcl-2 does not prevent tBid translocation to the organellar fraction. HeLa cells transfected with Bid or Bid + Bcl-2 were fractionated into cytosolic (Cyto) and membrane-bound (Mem) organellar fractions and analyzed for Bid/tBid by immunoblotting. The majority of full-length Bid (p22) was detected in the cytosol (lane 1), and tBid of p15/p13 translocated to the organellar fraction (lane 3). Bcl-2 co-transfection did not prevent tBid accumulation in the organellar fraction with mitochondria (lane 4).

Inhibition of Membranous Insertion of tBid by Bcl-2—Bcl-2 did not affect Bid processing into tBid or tBid translocation (Figs. 3 and 4). However, it inhibited Bid-induced mitochondrial disruption including the release of cytochrome *c* (Fig. 2). These observations promoted us to examine the status of tBid association with mitochondrial membranes. Specifically, we asked the following: does Bcl-2 prevent tBid integration or insertion into the membranes? To address this question, we utilized a classical method of alkaline treatment to examine the integration status of tBid (28). Alkaline incubation of cellular membranes at pH 11.5 leads to the dissociation of loosely attached proteins, whereas integrated proteins remain. This approach was successfully employed to demonstrate Bax insertion into mitochondria (29, 30). In our experiments, cells were transfected with Bid alone or Bid + Bcl-2. Membrane-bound organellar fractions were collected for incubation with 0.1 M NaHCO₃ at pH 11.5. Proteins stripped into the incubation solution were collected and analyzed for Bid/tBid, along with the proteins that were resistant to alkaline incubation. The results are shown in Fig. 5. In Bid-only transfected cells, alkaline incubation stripped off intact Bid of 22 kDa from the organellar membranes into the supernatant (lane S1). However, tBid of 15 and 13 kDa was rather resistant to the treatment and thus remained associated with the membranes after alkaline exposure (lane P1), suggesting that tBid and not intact Bid had integrated into the mitochondrial membranes in these cells. In sharp contrast, for Bid + Bcl-2-co-transfected cells, alkaline treatment led to the release of not only intact Bid but also a significant portion of 15-kDa tBid (lane S2). To estimate the percentage of 15-kDa tBid that was sensitive to alkaline treatment, blots from 4 separate experiments were analyzed by densitometry (Fig. 5B). In cells co-transfected with Bcl-2, over 30% of 15-kDa tBid was released during alkaline incubation; by sharp contrast, less than 2% was released from cells transfected with Bid only. As a control, cytochrome oxidase IV, an integral mitochondrial membrane protein, was not stripped off from the membranes under these experimental conditions, regardless of the presence or absence of Bcl-2 (Fig. 5A, lower panel). The results suggest that Bcl-2 may suppress Bid-induced mitochondrial disruption partly by prevention of tBid insertion into the organellar membranes.

Co-immunoprecipitation of Bcl-2 with Bid but Not tBid—Our results suggested that Bcl-2 inhibited tBid insertion into mitochondrial membranes (Fig. 5). However, the underlying mechanism was unclear. One hypothesis was that Bcl-2 might directly interact with tBid, resulting in conformational changes in this molecule that prevented its integration into organellar membranes. To test this possibility, we examined the interactions between Bcl-2 and Bid/tBid by co-immunoprecipitation. In this experiment, cells were extracted either directly with

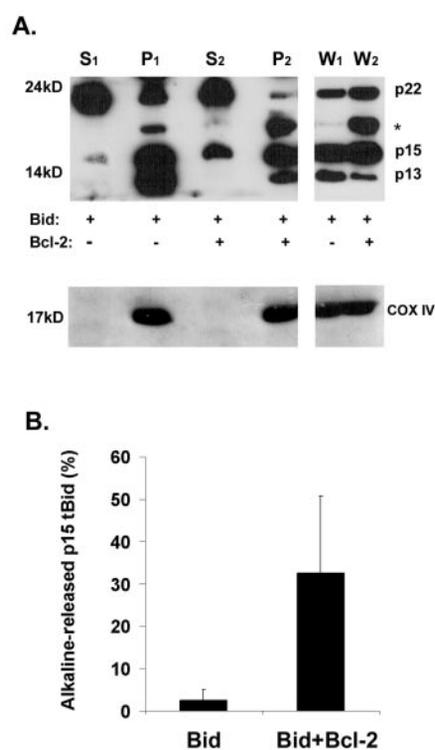


FIG. 5. Bcl-2 prevents membranous insertion of tBid. HeLa cells were transfected with Bid only or Bid + Bcl-2. Membrane-bound organellar fractions enriched with mitochondria were collected from these two groups of cells and subjected to alkaline treatment with 0.1 M NaHCO₃, pH 11.5. Alkaline incubation strips off the proteins that are not integrated into the membranes, whereas integral proteins remain (28). **A.** Whole membrane fractions (lanes W1 and W2), alkaline-released proteins (lanes S1 and S2), and alkaline-resistant proteins (lanes P1 and P2) were analyzed for Bid/tBid (upper panel) and cytochrome oxidase IV (lower panel). Alkaline treatment released a significant portion of p15 tBid from the membrane fraction of Bid + Bcl-2-co-transfected cells (lane S2); much less p15 tBid was released from Bid-only transfected cells (lane S1). Cytochrome oxidase IV, an integral mitochondrial protein, was not released during alkaline treatment, regardless of the presence or absence of Bcl-2 (lanes S2 and S1). *, a Bid antibody-reactive band that was occasionally detected in Bcl-2-transfected cell lysates. **B.** Immunoblot signals from four separate experiments were quantified by densitometry, and the p15 tBid released during alkaline incubation was expressed as a percentage of total (mean ± S.D.; n = 4). The results suggest that membranous integration of tBid was suppressed by Bcl-2.

RIPA buffer to collect whole cell lysate or sequentially with digitonin and RIPA buffer to collect the cytosolic fraction and the membrane-bound organellar fraction including mitochondria. The extracts were subjected to immunoprecipitation with Bcl-2 antibodies. The immunoprecipitates were analyzed for the presence of Bid and tBid. As shown in Fig. 6A, intact Bid was detected in all Bcl-2 immunoprecipitates, irrespective of the extracts utilized for immunoprecipitation. On the contrary, tBid was not shown in any of the Bcl-2 immunoprecipitates (Fig. 6A). To demonstrate that the extracts prior to immunoprecipitation did contain tBid, we analyzed the extracts directly by immunoblotting (Fig. 6B). Clearly, significant amounts of 15-kDa tBid were present in the whole cell lysates and in the membrane fraction (Fig. 6B, lanes 4 and 6). Of note, whereas tBid of 13 and 15 kDa was detected in cell lysates extracted with Laemmli buffer containing 2% SDS (Figs. 3 and 4), only 15-kDa tBid was extracted by the RIPA buffer in these experiments (Fig. 6B, lanes 4 and 6). Presumably, this was caused by the limited extraction capacity of the RIPA buffer, which had only 0.2% SDS. Nevertheless, the results suggest that Bcl-2 did not co-immunoprecipitate 15-kDa tBid under the

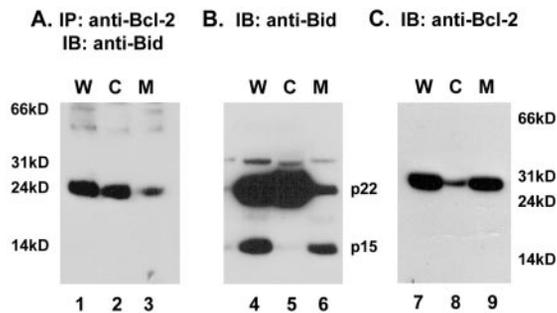


FIG. 6. Co-immunoprecipitation analysis of Bcl-2 and Bid/tBid. HeLa cells were transfected with Bid + Bcl-2. One group of cells was directly extracted with RIPA buffer to collect whole cell lysate (W), and the other group was sequentially extracted with digitonin and RIPA buffer to collect the cytosolic fraction (C) and the membrane-bound organellar fraction (M). The extracts were directly (IB), or subjected to immunoprecipitation (IP) with a monoclonal Bcl-2 antibody followed by detection of Bid/tBid in the precipitates by immunoblotting (A). Intact Bid was detected in Bcl-2 immunoprecipitates from whole cell lysates, cytosolic fractions, as well as the membrane fractions; however, tBid was not shown in any of the precipitates (lanes 1–3). The absence of tBid was not due to the incapability of RIPA buffer to extract the molecules. As shown in lanes 4 and 6, p15 tBid was detected in RIPA extracts prior to immunoprecipitation. The results suggest that Bcl-2 did not co-immunoprecipitate p15 tBid, although membranous insert of this molecule was suppressed by Bcl-2 (Fig. 5). The results in C indicate that, although Bcl-2 transfection led to its expression mainly in the membrane-bound fraction, there were limited amounts of Bcl-2 in the cytosol.

experimental conditions, although membranous insert of this molecule was suppressed by Bcl-2. Also included in this figure was a Bcl-2 immunoblot, showing Bcl-2 expression mainly in membrane-bound fraction, with limited amounts in the cytosol (Fig. 6C).

Bid-induced Bax Translocation and Its Inhibition by Bcl-2—Recent studies (19, 29, 31, 32) have documented Bid/tBid-induced alterations of Bax and Bak, resulting in mitochondrial insertion and oligomerization of Bax and Bak in the organelles. These observations are very important, because they suggest a sequential cascade for the activation of Bcl-2 family proteins (32). To examine whether Bcl-2 inhibited Bid-induced mitochondrial damage through its actions on Bax/Bak, we first analyzed cellular distribution of these two proteins by fractionation of the cells into the cytosolic fraction and the membrane-bound organellar fraction including mitochondria. The results are shown in Fig. 7. In control cells without transfection, the majority of Bax was detected in the cytosol (lane 1), with much weaker signals in the membrane-bound organellar fraction (lane 4). Bid transfection led to the loss of cytosolic Bax (lane 2), accompanied by increases in organellar Bax (lane 5), indicating translocation of this molecule. Bcl-2 co-transfection significantly prevented the translocation of Bax. As a result, in these cells Bax was detected mainly in the cytosol (lane 3), with weaker signals in the organellar fraction (lane 6). For Bak, a membrane-bound organellar location was always detected in control cells, cells transfected with Bid, and cells co-transfected with Bid + Bcl-2 (Fig. 7, Bak blots in the lower panel).

Bid-induced Bax/Bak Oligomerization and Its Inhibition by Bcl-2—A critical event for mitochondrial disruption by proapoptotic Bcl-2 family proteins seems to be the oligomerization of these molecules within the outer membranes (19, 32–34). Moreover, recent studies (25, 33) including ours showed that Bax oligomerization in the outer membrane of mitochondria was abolished by Bcl-2. Thus, following the experiments demonstrating inhibition of Bax translocation by Bcl-2, we went on to examine the oligomerization status of Bax and Bak in the membrane-bound organellar fraction. For this purpose, cells were subjected to cross-linking treatment to preserve protein-

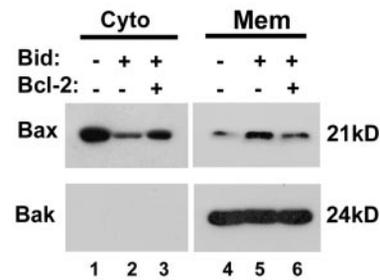


FIG. 7. Suppression of Bid-induced Bax translocation by Bcl-2. Control cells, cells transfected with Bid, and cells transfected with Bid + Bcl-2 were fractionated into cytosolic (Cyto) and membrane-bound organellar fractions (Mem) for immunoblot analysis of Bax and Bak. In control cells without gene transfection, the majority of Bax was detected in the cytosol (lane 1), with weak signal in the membrane-bound organellar fraction (lane 4). Bid transfection led to the loss of cytosolic Bax (lane 2), accompanied by increases in organellar Bax (lane 5). Bcl-2 co-transfection suppressed the translocation of Bax. In these cells, Bax was detected mainly in the cytosol (lane 3), with weak signal in the organellar fraction (lane 6). Bak was always detected in the membrane-bound organellar fractions, regardless of the transfection of Bid or Bid + Bcl-2 (blots in the lower panel).

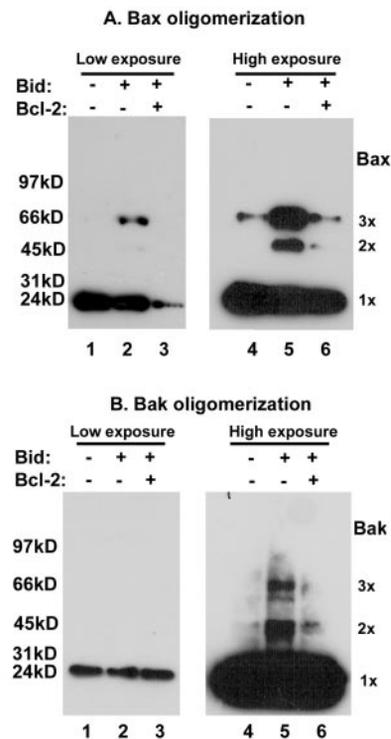


FIG. 8. Inhibition of Bid-induced Bak and Bax oligomerization by Bcl-2. Cells were transfected with Bid, Bid + Bcl-2, or empty vectors. The cells were subjected to cross-linking by DSP, followed by fractionation to collect membrane-bound organellar fractions including mitochondria. Proteins in the fractions were resolved by electrophoresis under non-reducing conditions for immunoblot analysis of Bax (A) and Bak (B). Images in the left and right panels were obtained by short and long film exposures, respectively. Bid transfection led to oligomerization of Bax and Bak; Bcl-2 co-transfection diminished the oligomerizations.

protein interactions, and the membrane-bound organellar fractions were extracted for immunoblot analysis of Bax and Bak. The results are shown in Fig. 8. The blots in the left panel were obtained by regular exposure, and the blots in the right panel were subjected to overexposure to reveal Bax/Bak oligomers. In control cells, Bax oligomerization was minimal, even after overexposure (Fig. 8A, lanes 1 and 4). Bid transfection led to the formation of Bax oligomers, accompanied by a decrease in Bax

monomers (Fig. 8A, lanes 2 and 5). Importantly, Bcl-2 co-transfection diminished Bid-induced Bax oligomerization, as shown in Fig. 8A, lanes 3 and 6. Similar observations were obtained for Bak (Fig. 8B). Bak existed as monomers in control cells (lanes 1 and 4) and oligomerized after Bid transfection (lanes 2 and 5). Again, Bak oligomerization induced by Bid was attenuated by Bcl-2 co-transfection (Fig. 8B, lanes 3 and 6). Together, these results suggest that, in addition to preventing tBid insertion and Bax translocation, Bcl-2 may antagonize Bid-induced apoptosis by blocking Bax/Bak oligomerization in the mitochondrial membranes.

DISCUSSION

This study has examined inhibition of Bid-induced apoptosis by Bcl-2 and the underlying mechanisms. In Bid-transfected cells, the pro-apoptotic molecule was processed into active forms of tBid. tBid translocated or accumulated into the mitochondria, leading to disruption of the organelles, releasing cytochrome *c*. Co-transfection of Bcl-2 inhibited cytochrome *c* leakage and associated apoptosis in Bid-transfected cells. On the other hand, Bcl-2 did not suppress Bid processing or tBid translocation. Three important events that were suppressed by Bcl-2 have been identified. First, Bcl-2 suppressed organellar insertion of tBid. Second, Bcl-2 attenuated Bax translocation from the cytosol to mitochondria. Third, Bcl-2 blocked Bid-induced oligomerizations of Bax and Bak in the mitochondrial membranes.

Recent studies have significantly advanced our understanding of the pro-apoptotic actions of Bid. It has been shown that Bid activation depends on its proteolytic processing into tBid and tBid translocation to mitochondria (14, 15). In the mitochondria, tBid may form oligomers by itself and induces oligomerization of Bax and Bak (19, 29, 32, 34). Although it remains unclear how tBid triggers such oligomerizations, strong evidence has been provided to suggest that Bid-induced apoptosis depends on the presence of Bax and Bak (19, 35). Consistent with these observations, our experiments have demonstrated Bid processing and tBid translocation to the membrane-bound organellar fraction including mitochondria, which was accompanied by cytochrome *c* leakage from the organelles. Significantly, Bcl-2 did not prevent either Bid processing or tBid movement to mitochondria but blocked cytochrome *c* leakage. Similar observations have been shown for Bcl-x_L in a model of tumor necrosis factor-induced apoptosis (36). Therefore, tBid accumulation to mitochondria is an essential but not a sufficient event leading to mitochondrial disruption for cytochrome *c* leakage.

Our results suggest that, after mitochondrial association, tBid integrates into the membranes to exert its toxicity. An important action of Bcl-2 was shown to suppress membranous insertion of tBid, which was accompanied by the preservation of mitochondrial integrity. Insertion of pro-apoptotic molecules into mitochondrial membranes has been investigated for Bax. In a series of carefully controlled experiments, Bax was shown to insert into mitochondrial membranes upon apoptotic stimulation (30). Bax insertion depends on the unfolding of the amino-terminal domain and the consequent exposure of the transmembrane domain at the carboxyl terminus. Little has been learned, however, about membrane insertion of other pro- or anti-apoptotic Bcl-2 proteins including Bid/tBid. Apparently, tBid insertion might take place in a way that can be quite different from that of Bax. After all, tBid does not contain a specific transmembrane domain. Nevertheless, Bcl-2 family proteins share a conserved overall structure and conformation. In addition, a mitochondrial targeting domain has been identified in Bid/tBid. Proteolytic removal of the amino-terminal fragment may increase the hydrophobicity of tBid for mem-

brane integration and unveil the targeting domain, leading to its translocation to mitochondria (20, 21). Results of the current study indicate that Bcl-2 does not inhibit Bid processing or tBid translocation to mitochondria but suppresses tBid insertion, suggesting that tBid translocation and membrane integration are separate events regulated by different mechanisms.

It remains unclear how Bcl-2 suppressed tBid insertion. In the mitochondria, Bcl-2 appeared to interact with Bid but not tBid, although the latter was the predominant form associated with the organelles (Fig. 6). The results suggest that direct interaction between Bcl-2 and tBid may not be responsible for the expelling effects of Bcl-2. On the other hand, tBid insertion may depend on the availability of specific anchoring sites, and Bcl-2 might compete for these targets. Alternatively, Bcl-2 expression may change the microenvironment or composition of mitochondrial membranes and thereby reduce the compatibility for tBid integration and the formation of supramolecular openings in mitochondria (37). Of interest, recent studies (38) have demonstrated Bid binding to cellular membranous lipids at high affinities. All these possibilities remain to be tested by further investigations.

Although our results showed co-immunoprecipitation of Bcl-2 with intact Bid and not tBid, it remains unclear whether or not Bcl-2 interacts with tBid *in situ* within the cells. Recent results from Korsmeyer and co-workers (32) demonstrated binding of tBid with Bcl-2 by co-immunoprecipitation of prior cross-linked samples. In those experiments, mitochondria were isolated from Bcl-2 expressing cells, incubated with recombinant tBid, and then cross-linked with irreversible homobifunctional cross-linkers. The mitochondria were finally extracted with RIPA buffer to collect soluble fractions for immunoprecipitation, showing a Bcl-2-tBid complex. Similar complexes were detected in extracts from cells undergoing tumor necrosis factor- α -induced apoptosis (32). In our experiments, cells were not cross-linked before extraction. Thus, it is possible that Bcl-2 and tBid indeed bound each other, and their interaction was disrupted during extraction and sample preparation. Although this possibility needs to be tested by further experiments, our results showed that the interaction between Bcl-2 and intact Bid was not disrupted during sample preparation under the experimental conditions (Fig. 6A).

In normal cells without transfection or overexpression, Bcl-2 associates with organellar membranes. Thus, the interaction of Bcl-2 with intact Bid in the cytosol shown in Fig. 6A was most likely a result of simultaneous overexpression of the proteins. Indeed, portions of Bcl-2 were detected in the cytosol of the Bcl-2-transfected cells, although the majority of the protein was shown in the membrane fraction (Fig. 6C).

Interestingly, earlier studies (36) implied that Bcl-x_L, another important anti-apoptotic molecule, did not affect tBid insertion into mitochondrial membranes. Discrepancy between those results and our observations might be caused by specific features of experimental models. Alternatively, Bcl-2 and Bcl-x_L may not antagonize apoptosis in exactly the same way, despite close homology (39). Of note, in our experiments, Bcl-2 did not completely diminish tBid insertion. Significant amounts of tBid stayed integrated in mitochondrial membranes, after Bcl-2 co-transfection (Fig. 5). Nevertheless, cytochrome *c* leakage as well as apoptosis in these cells was reduced close to control levels (Figs. 1 and 2). These observations suggest that prevention of tBid insertion is an important but not the single event responsible for the mitochondrial protective effects of Bcl-2.

The current study has identified Bax translocation as the second action site for Bcl-2. Consistent with previous studies (29), the majority of Bax in control cells was detected in the

cytosol, which translocated to mitochondria upon Bid stimulation. Significantly, our results further demonstrated that Bid-induced Bax translocation was evidently suppressed by Bcl-2 co-transfection (Fig. 7). The signal directing Bax translocation from the cytosol to mitochondria remains to be clarified (7, 9). Structurally, Bax has a hydrophobic transmembrane domain at the carboxyl terminus. In normal non-apoptotic cells, this domain is buried by its interaction with the amino terminus of the protein. Deletion of the amino terminus results in mitochondrial localization of Bax even in the absence of apoptotic stimuli (30). However, removal of the amino terminus by proteolysis is not considered as a common mechanism for the exposure of the transmembrane domain *in vivo*, because Bax remains intact during apoptosis regardless of its location within the cells. There are at least two hypotheses on the regulation of Bax movement; each is currently supported by significant evidence. In the first hypothesis, Bax is proposed to interact with a regulatory protein. Modifications of the interaction may lead to conformational changes in Bax and the exposure of the transmembrane domain at the carboxyl terminus. A potential Bax interacting protein might be Bid. During apoptosis, Bid interacts with Bax and induces conformational changes to expose the carboxyl terminus for integration of Bax into mitochondrial membranes (29, 31, 32). Another protein regulating Bax might be 14-3-3, as suggested by recent studies (40). In living cells, specific isoforms of the 14-3-3 protein bind Bax. Upon apoptotic stimulation, Bax is liberated from 14-3-3 and translocates to mitochondria. The second hypothesis on the mechanisms of Bax translocation emphasizes a role for alterations of the cytosolic environment. In particular, pH changes in the cytosol might be critical. A shift of intracellular pH toward either alkalization or acidification has been linked to conformational changes in Bax, followed by insertion of the molecule into mitochondrial membranes (41, 42). Apparently, these two hypotheses are not mutually exclusive. For example, cytoplasmic alkalization as well as acidification may decrease the interactions between Bax and its partnering proteins (40). All these possibilities need to be tested in future investigations to address the following questions: how did Bid trigger Bax translocation and why was the translocation suppressed by Bcl-2?

This study has further identified Bax/Bak oligomerization in the mitochondria as the third action site for Bcl-2. Oligomerization of Bax/Bak has been demonstrated in the mitochondria during apoptosis (19, 29, 31, 32). Recent studies (25, 33, 43) have further suggested an essential role for the oligomerizations in the development of mitochondrial pathology. Results of the current study showed that, in mitochondria of control cells, Bax and Bak existed as monomers. Bid transfection led to oligomerization of these molecules. Significantly, such oligomerizations were abolished by Bcl-2 expression (Fig. 8). These results provide strong support for the scenario of sequential activation of Bcl-2 family proteins (19, 32). In this model, Bcl-2/Bcl-x_L was proposed to sequester BH3 domain-only proteins and prevent the formation of Bax/Bak oligomers in mitochondria, resulting in the preservation of mitochondrial integrity.

Despite a recognized role for Bax/Bak oligomerization in apoptosis, the mechanisms regulating such oligomerization are largely unknown. In the present study, Bax/Bak oligomerization took place in mitochondria and was triggered by Bid/tBid. Thus, membranous integration of tBid might be a prerequisite for its Bax/Bak oligomerizing activity. Should this be the case, Bcl-2 might attenuate Bax/Bak oligomerization as least in part by preventing tBid insertion. On the other hand, tBid insertion was not completely diminished by Bcl-2 (Fig. 5), and yet Bax/

Bak oligomerization was (Fig. 8). The results suggest that, in addition to preventing tBid insertion, Bcl-2 may also disrupt the formation of Bax/Bak oligomers in the mitochondrial membranes by more direct mechanisms. These considerations are supported by recent studies (44) showing Bax/Bak oligomerization and its prevention by Bcl-2 in the absence of Bid activation.

One mechanism whereby Bcl-2 may directly disrupt Bax/Bak oligomerization is through heterodimerization. Such interaction has been reported in various kinds of experimental models (45). Although some of the heterodimerizations might be caused by the presence of detergents during sample preparation (46), interactions between pro- and anti-apoptotic proteins have been demonstrated by detergent-free approaches such as yeast two-hybrid systems (47, 48). Moreover, the lipid-rich cellular membranes including that of mitochondria may readily provide a "detergent"-like microenvironment that favors physical interactions between pro-apoptotic and anti-apoptotic Bcl-2 family proteins. Physical associations between Bcl-2 and Bax were shown in mitochondria within intact cells by the technique of fluorescence resonance energy transfer (49).

In conclusion, this study has examined Bcl-2 inhibition of Bid-induced apoptosis in an expression model. Bcl-2 inhibited Bid-induced cytochrome *c* leakage from mitochondria and the associated apoptosis, without ameliorating Bid processing or tBid translocation to mitochondria. Three critical events that were attenuated by Bcl-2 have been identified. First, Bcl-2 suppressed tBid insertion into mitochondrial membranes. Second, Bcl-2 inhibited Bax translocation to mitochondria. Third, Bcl-2 diminished Bax and Bak oligomerization in the mitochondrial membranes. By preventing tBid integration, Bax translocation, and Bax/Bak oligomerization, Bcl-2 may preserve the integrity of mitochondria and abolish apoptosis in the experimental model.

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