

tBid Elicits a Conformational Alteration in Membrane-bound Bcl-2 Such That It Inhibits Bax Pore Formation*

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Jun Peng[‡], Chibing Tan[‡], G. Jane Roberts[§], Olga Nikolaeva[‡], Zhi Zhang[‡], Suzanne M. Lapolla[‡], Steve Primorac[§], David W. Andrews^{§1}, and Jialing Lin^{‡2}

From the [‡]Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190 and the [§]Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario L8N 3Z5, Canada

During initiation of apoptosis, Bcl-2 family proteins regulate the permeability of mitochondrial outer membrane. BH3-only protein, tBid, activates pro-apoptotic Bax to release cytochrome *c* from mitochondria. tBid also activates anti-apoptotic Bcl-2 in the mitochondrial outer membrane, changing it from a single-spanning to a multispreading conformation that binds the active Bax and inhibits cytochrome *c* release. However, it is not known whether other mitochondrial proteins are required to elicit the tBid-induced Bcl-2 conformational alteration. To define the minimal components that are required for the functionally important Bcl-2 conformational alteration, we reconstituted the reaction using purified proteins and liposomes. We found that purified tBid was sufficient to induce a conformational alteration in the liposome-tethered, but not cytosolic Bcl-2, resulting in a multispreading form that is similar to the one found in the mitochondrial outer membrane of drug-treated cells. Mutations that abolished tBid/Bcl-2 interaction also abolished the conformational alteration, demonstrating that a direct tBid/Bcl-2 interaction at the membrane is both required and sufficient to elicit the conformational alteration. Furthermore, active Bax also elicited the Bcl-2 conformational alteration. Bcl-2 mutants that displayed increased or decreased activity in the conformational alteration assay showed corresponding activities in inhibiting pore formation by Bax *in vitro* and in preventing apoptosis *in vivo*. Thus, there is a strong correlation between the direct interaction of membrane-bound Bcl-2 and tBid with activation of Bcl-2 *in vitro* and *in vivo*.

Proteins of the Bcl-2 family are key regulators of apoptosis that function either as promoters or inhibitors and that display homology in one to four short sequences termed Bcl-2 homology (BH)³ motifs (1–3). Anti-apoptotic subfamily proteins such

as Bcl-2 and Bcl-x_L contain four BH motifs (BH1–4). Pro-apoptotic proteins are grouped into either multiple or single BH motif subfamilies. The former subfamily includes the proteins Bax and Bak that display homology in BH1–3 motifs. The latter includes proteins such as Bid, Bim, and Bad that are similar only in the limited BH3 motif. Despite the limited sequence homology, the three-dimensional structures determined for seven Bcl-2 family proteins including members from all three subfamilies are very similar, consisting of a hydrophobic core of one to three helices wrapped by five to eight amphipathic helices and their connecting loops. This structure is strikingly similar to the structure of the pore-forming domains of diphtheria toxin and *Escherichia coli* colicins. In addition there is an obvious hydrophobic groove on the surface of all of the anti-apoptotic Bcl-2 family proteins (4–6).

From decades of extensive studies, two properties are well known to be shared by most, if not all, Bcl-2 family proteins: homo- or hetero-binding and pore formation in membranes. More experiments have been done on the homo- or hetero-binding of Bcl-2 family proteins, resulting in the following major conclusions. 1) The hetero-binding between different subfamily proteins is important for their function. Most BH3-only proteins have been proposed to bind to Bcl-2-like proteins following various death signals, either neutralizing the pro-survival function or inhibiting the anti-death activity of Bcl-2-like proteins. A few BH3-only proteins have also been shown to bind Bax-like proteins and activate their pro-death function (7–9). 2) Binding between two different subfamily proteins is mediated by different motifs. The interaction of a BH3-only protein and a Bcl-2-like protein is likely accomplished by the binding of the BH3-motif helix of the former into the hydrophobic groove of the latter (10–12). A similar interface may be formed in the complex between a Bax-like protein and a Bcl-2-like protein (13). 3) The interface for homo-binding of Bcl-2 is different from that for Bcl-x_L. Whereas a Bcl-2 dimer is formed asymmetrically by an acceptor surface including the BH1–3 hydrophobic groove and a donor surface including BH4 helix, a Bcl-x_L dimer is formed symmetrically by inserting the C-terminal tail of one protomer into the hydrophobic groove of the other and vice versa (14, 15). Interestingly, in healthy cells Bcl-2 is exclusively bound to membranes in a conformation in which

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¹ Holds the Canada Research Chair in Membrane Biogenesis.

² To whom correspondence should be addressed: 940 Stanton L. Young Boulevard, BMSB 935, P.O. Box 26901, Oklahoma City, OK 73190. Tel.: 405-271-2227 (ext. 1216); Fax: 405-271-3092; E-mail: jialing-lin@ouhsc.edu.

³ The abbreviations used are: BH, Bcl-2 homology; CB, Cascade Blue; Bcl-2ΔTM, Bcl-2 lacking the C-terminal transmembrane sequence thus equivalent to the cytosolic domain; 7-doxyl-PC, 1-palmitoyl-2-stearyl-(7-doxyl)-sn-glycero-3-

phosphatidylcholine; IASD, 4-acetamido-4'-[(iodoacetyl)amino]stilbene-2,2'-disulfonic acid; MOM, mitochondrial outer membrane; NBD, 7-nitrobenz-2-oxa-1,3-diazole; PARP, poly(ADP-ribose) polymerase.

the C-terminal tail is inserted into the lipid bilayer, and therefore not available for binding to the hydrophobic groove in the cytosolic domain of other Bcl-2 proteins (16, 17). In contrast, a substantial fraction of Bcl-x_L is found either in the cytosol or loosely bound to membranes in healthy cells. The C-terminal tail of the soluble Bcl-x_L could therefore, be involved in homobinding (18). During the induction of apoptosis Bcl-x_L inserts into intracellular membranes. A recent crystallography study suggests that the membrane-bound Bcl-x_L may swap helices 6–8 to form a symmetrical homodimer (19).

Because Bax can release cytochrome *c* from purified mitochondria, pore formation by Bax has been studied extensively. The following major conclusions have been reached. 1) tBid protein or BH3 peptide from Bid or Bim was required for Bax to release cytochrome *c* from purified mitochondria, and to release dextrans up to 2 MDa from mitochondrial outer membrane (MOM) vesicles or liposomes of MOM lipids (MOM-liposomes) (9, 20). Pore formation is dependent on the mitochondrial specific lipid, cardiolipin, and can be inhibited by Bcl-x_L. 2) The putative Bax pore that can release 2-MDa dextrans should have a radius more than 273 Å, suggesting that massive Bax oligomerization may be involved (21). 3) Oligomerization of Bax is required for Bax to permeabilize the MOM, thereby releasing proapoptotic factors such as cytochrome *c* and apoptosis-inducing factor that activate caspases and nucleases that eventually dismantle the cell (20, 22–24). The oligomerization occurs after Bax is inserted into the membrane as a multispanning monomer with helices 5, 6, and 9 buried in the bilayer (25). The oligomerization of Bax-like proteins requires their BH3 motif and can be inhibited by Bcl-2-like proteins (26–29). 4) The BH3-activated Bax-like proteins can activate more Bax-like proteins to amplify the initial pro-death signal produced by BH3-only proteins (30, 31).

Only a few studies have examined pore formation by Bcl-2 and Bcl-x_L proteins, resulting in the following conclusions (19, 32–36). 1) Both Bcl-2 and Bcl-x_L form pores in anionic lipid containing liposomal membranes at extreme acidic pH. The pore-forming activity can be enhanced by a trans-negative membrane potential. 2) In planar lipid bilayers both proteins form ion-conducting channels that display a linear current-voltage relationship, following the law of Ohm. The channels have discrete conductances ranging from 20 to 90 pS for Bcl-2 and 80 to 276 pS for Bcl-x_L, suggesting that they may have different structures. Unlike the pores in liposomal membranes that are only detectable at acidic pH, the channels formed in planar bilayers can be detected at both neutral and acidic pH. But the channels open more frequently at acidic pH. The channels are cation selective. 3) Deletion of helices 5 and 6 eliminates the channel activity for Bcl-2, and replacing helices 5 and 6 of Bcl-x_L with those from Bax alters the channel property, suggesting that the two hydrophobic helices are required for channel formation by the two anti-apoptosis proteins. 4) During induction of apoptosis, Bcl-2 changes from the tail-anchored conformation to a multispanning one in which in addition to the C-terminal tail (helix 9) both helices 5 and 6 are inserted into the membrane (37). This conformational alteration can be induced in isolated mitochondria by tBid and is required for anti-Bax activity (29). Thus, only the conforma-

tionally altered Bcl-2 can form a stable complex with the active, multispanning Bax, thereby inhibiting its oligomerization and membrane permeabilization.

It is intriguing that both pro- and anti-apoptotic multi-BH motif proteins can be switched to a similar multispanning conformation by the BH3-only protein, tBid. Perhaps they form different structures in the membrane to fulfill their opposite functions. The multispanning Bax is known to form large oligomeric pores. Acidic pH induces the Bcl-2 pore formation in liposomal membranes, but the relationship between the acidic pH-induced pore formation and the tBid-induced conformational alteration remains elusive. Moreover, the correlation between the *in vitro* pore-forming activity and the *in vivo* anti-apoptotic activity of Bcl-2 has yet to be established. Finally, although tBid can induce the Bcl-2 conformational alteration in isolated mitochondria, the minimal components required for the alteration needs to be defined.

In this study we used the MOM-liposomes with encapsulated fluorescent dyes and purified cytosolic domain of Bcl-2 (Bcl-2ΔTM) to reconstitute the pore-forming process *in vitro*. We found that acidic pH induced a conformational alteration in soluble Bcl-2ΔTM, exposing a hydrophobic core helix to facilitate the membrane insertion and pore formation. In contrast, tBid did not induce the pore formation by the soluble Bcl-2ΔTM. However, when Bcl-2ΔTM was tethered to the liposome, it changed conformation and formed pores after interacting with tBid. Furthermore, the pore formed by the tBid-activated Bcl-2ΔTM is smaller than that formed by the tBid-activated Bax. Most importantly, Bcl-2ΔTM inhibited the large pore formation by the activated Bax only when it was also activated by either tBid or tBid-activated Bax. Mutations that enhanced or reduced the tBid-mediated Bcl-2ΔTM activation in liposomes also enhanced or reduced Bcl-2 anti-apoptosis function in cells, respectively, thereby correlating the *in vitro* detected conformational alteration with the *in vivo* function.

EXPERIMENTAL PROCEDURES

Materials—All phospholipids and lipid analogs were purchased from Avanti Polar Lipids. Cascade Blue (CB), CB-labeled dextrans, and rabbit anti-CB polyclonal antibody were from Molecular Probes. Peptides were synthesized by Global Peptide Services (Fort Collins, CO) and their sequences were as described (31).

Preparation of Plasmids and Proteins—Construction of pET22b(+)-based plasmid encoding His₆-tagged Bcl-2ΔTM with either Gly¹⁵⁴–Gly¹⁵⁵ replaced by two alanines or Val¹⁵⁹ replaced by an aspartate (designated G154A/G155A or V159D) was done using appropriate primers and overlapping PCR-based mutagenesis as described (38). Construction of pBabe/hygromycin-based plasmid encoding each corresponding full-length Bcl-2 mutant was done by replacing the corresponding DNA segment with the one containing the mutation. The sequence of all the plasmids was verified by DNA sequencing. Expression and purification of the His₆-tagged Bcl-2ΔTM and mutant, tBid and mutant with Met⁹⁷–Asp⁹⁸ replaced by two alanines (designated M97A/D98A), and Bax proteins were as described (14, 39).

Preparation of Liposomes—Liposomes of MOM lipid composition and with CB or CB-dextran encapsulated were prepared by the extrusion method as described (31). The plain liposomes without fluorophores were prepared similarly except that no fluorophores were in the buffer A used for resuspension of the lipids and no gel filtration chromatography was needed after the extrusion. The liposomes containing lipophilic quenchers were prepared similarly except that 30 mol % of phosphatidylcholine (PC) was replaced by a doxyl-labeled PC (1-palmitoyl-2-stearoyl-(7-doxyl)-*sn*-glycero-3-PC, 7-doxyl-PC). The Ni²⁺-chelating liposomes were prepared similarly except that 1 mM Ni²⁺-chelating lipid analog, 1,2-dioleoyl-*sn*-glycero-3-[[N(5-amino-1-carboxypentyl)iminodiacetic acid]-succinyl] (nickel salt), was added to 20 mM MOM lipids before resuspension in buffer A.

Isolation of Liposome-bound Proteins or Peptides—For Figs. 2A or 3B, 400 or 200 nM Bcl-2ΔTM protein, respectively, was incubated with liposomes of 50 μM lipids (50 μM liposomes for short from here on) in 116 μl of buffer A with certain pH values at 25 °C for 1–2 h. For Fig. 3D, 50 nM Bcl-2ΔTM, 20 nM tBid, and/or 50 nM Bax were incubated with 12.5 μM Ni²⁺-liposomes. When indicated, 5 mM EDTA or 200 mM imidazole were included either during or after the incubation. The samples were then subjected to sucrose gradient float-up centrifugation as described (39). For the sample containing EDTA or imidazole, the same concentration of corresponding chemical was also presented in the sucrose gradient. Four 250-μl fractions were drawn, starting from the top of the gradient, resulting in fractions S1–S4. The pellet was re-suspended in 250 μl of buffer A, resulting in fraction P. The Bcl-2ΔTM proteins in these fractions were analyzed by SDS-PAGE and immunoblotting with sheep anti-Bcl-2 polyclonal antibody. The distribution of the liposomes among these fractions was estimated by scintillation counting of the [¹⁴C]PC of each fraction as described (31).

Assay of CB or CB-dextran Release from Liposomes by Fluorescence Quenching—For Fig. 2C, 50 μM liposomes with CB dyes encapsulated were mixed with 6 μg/ml anti-CB antibodies in 250 μl of buffer A of certain pH values that were adjusted by varying the concentration of Na₂HPO₄ and citric acid. The initial emission intensity (*F*₀) was determined after equilibrating the sample at 25 °C for 5 min. Purified Bcl-2ΔTM protein (400 nM) was then added. For other figures, if indicated, 12.5 μM liposomes with CB dyes or CB-dextran were added, and after *F*₀ was taken, 50 nM Bcl-2ΔTM or mutant protein, 20 nM tBid or mutant protein, and/or 50 nM Bax were added. The first fluorescence intensity measurement was started exactly 20 s after the addition of the protein(s) and followed by multiple measurements in a predetermined time interval for 5 h, resulting in multiple intermediate intensities (*F*). At the end of the time course 0.1% Triton X-100 was added and the final measurement was taken, resulting in the final intensity (*F*_f). The extent of CB or CB-dextran release is proportional to the extent of fluorescence quenching that is equal to Δ*F*_{Protein}/Δ*F*_{Triton}, where Δ*F*_{Protein} = *F*₀ – *F*, and Δ*F*_{Triton} = *F*₀ – *F*_f. All fluorescence intensities were measured using the SLM-8100 fluorometer as described (31).

Collisional Quenching of Soluble NBD-labeled Bcl-2ΔTM by Iodide Ions—NBD (7-nitrobenz-2-oxa-1,3-diazole) labeling of Bcl-2ΔTM with a single Cys (Cys¹⁵⁸) was done as described (40). The NBD fluorescence emission scan was carried out at 1-nm intervals between 500 and 600 nm with excitation at 468 nm. The spectrum was integrated to determine the intensity. To determine the pH effect on iodide ion accessibility to the NBD, the initial net fluorescence intensity (*F*₀) for a sample with 100 nM NBD-Bcl-2ΔTM was determined in buffer A of the indicated pH by subtracting the signal of a control without the NBD-Bcl-2ΔTM. The sample and control were titrated with aliquots of 1 M KI and 1 mM Na₂S₂O₃. The intensities of sample and control were measured after each addition of KI/Na₂S₂O₃ and corrected for dilution to obtain the net intensity (*F*). In parallel, another pair of sample and control was titrated with 1 M KCl and 1 mM Na₂S₂O₃ for correcting the effect of ionic strength on the NBD fluorescence. In each experiment, the net change in fluorescence due to the quenching at each KI/KCl concentration was determined by the equation *F*₀/*F* = (*F*₀/*F*)_{KI}/(*F*₀/*F*)_{KCl}, which was used to plot against the KI concentration to obtain the Stern-Volmer plot as described (40).

Fluorescence Intensity of NBD-labeled Bcl-2ΔTM Inserting into Liposomal Membranes—The initial fluorescence intensity (*F*₀) was determined in the absence of liposomes with 100 nM NBD-Bcl-2ΔTM in buffer A at the indicated pH. Emission intensity (*F*) was monitored exactly 20 s after addition of 100 μM liposomes to the sample, and continuously monitored until the intensity reached a plateau. The liposomes used are either the plain liposomes or the liposomes containing the lipophilic quencher 7-doxyl-PC.

Other Fluorescence Methods—The tryptophan fluorescence intensity and anisotropy of Bcl-2ΔTM, and the anisotropy of carboxyfluorescein-labeled Bax H2 peptide were measured as described (31).

Assay Bcl-2 Mutants in Rat-1MycER^{TAM} Cell Line—Rat-1MycER^{TAM} cell culture, retroviral infection, and apoptosis induction were conducted as described (37, 41, 42). Briefly, the Rat-1 cells stably expressing Bcl-2, Bcl-2 mutant, or vector were first treated with tamoxifen that induces the *c-myc* protooncogene activity and sensitizes the cells to apoptotic signals. The cells were then treated with either 6 μM etoposide for 12 and 18 h or low serum (0.03% fetal bovine serum) for 24 and 48 h. The cleavage of poly(ADP-ribose) polymerase (PARP) by caspases was used to assess the drug-induced apoptosis as described (29). The labeling of Cys¹⁵⁸ in helix 5 by 4-acetamido-4'-[(iodoacetyl)amino]stilbene-2,2'-disulfonic acid (IASD), a bilayer-impermeant, sulfhydryl-specific reagent, was used to monitor the conformational alteration of Bcl-2 as described (29, 37).

RESULTS

Acidic pH Induces a Conformational Alteration in Soluble Bcl-2ΔTM Exposing the Hydrophobic Core yet Retaining Homo- and Heterodimerization Properties—Acidic pH has been shown to induce Bcl-2ΔTM pore formation in membranes. The mechanism is unclear but it was suggested that acidic pH could induce a conformational alteration in the homologous Bcl-x_L (43). We thus monitored the conforma-

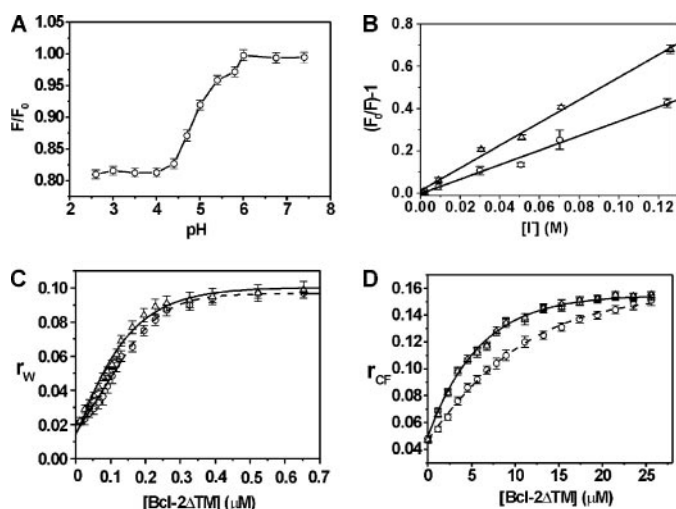


FIGURE 1. Effect of pH on Bcl-2ΔTM conformation, homo-binding, and hetero-binding with Bax peptide. *A*, the pH-dependent conformational alteration of soluble Bcl-2ΔTM was assessed by monitoring the tryptophan fluorescence intensity in solutions of various pH. Data shown are averages of relative intensities (F/F_0) from three independent measurements (\circ) with standard deviations (error bars). F_0 , the intensity measured at pH 7.4; F , the intensity measured at other pH. *B*, solvent exposure of the hydrophobic helix 5 of Bcl-2ΔTM was assessed by measuring I^- quenching of fluorescence intensity of an NBD dye attached to Cys¹⁵⁸ in helix 5. F_0 is the net fluorescence intensity prior to addition of I^- , and F the dilution-corrected fluorescence intensities after addition of indicated concentrations of I^- . Data shown are averages of fluorescence quenching (F_0/F_-) from two independent experiments conducted either at pH 7.4 (\circ) or 5.0 (Δ) with S.D. (error bars). The linear least-squares best-fit lines are also shown. *C*, Bcl-2ΔTM homo-binding in solution was monitored by measuring the tryptophan fluorescence anisotropy (r_w) at various protein concentrations. Data shown are averages from three independent titrations conducted either at pH 7.4 (\circ) or 5.0 (Δ) with S.D. (error bars) and nonlinear least-squares best-fit curves (dash or solid) that were generated using the quadratic equation as described (31). *D*, binding of Bax H2-H3 peptide to Bcl-2ΔTM at pH 5.0 was monitored by measuring the carboxyfluorescein fluorescence anisotropy (r_{CF}) of a carboxyfluorescein-labeled Bax H2 peptide after titrating Bcl-2ΔTM. Data shown are averages from three independent titrations with S.D. (error bars), conducted either in the absence (\square) or presence of an unlabeled Bax H2-H3 peptide (\circ) or its mutant (G67R) (Δ). Also shown are the nonlinear least-squares best-fit curves for the former two data sets that were generated using the cubic and quartic equations, respectively, as described (31).

tion of Bcl-2ΔTM in solutions of different pH values by measuring tryptophan fluorescence of the protein. The tryptophan fluorescence provides information about the protein conformation because it is sensitive to the environment of tryptophan residues in the protein structure. Bcl-2ΔTM contains six tryptophan residues most of which are buried in the protein core at neutral pH (44). If reducing pH changes the protein conformation and exposes the tryptophan residues to water, the tryptophan fluorescence will decrease. As shown in Fig. 1A, a pH lower than 6.0 significantly decreased the tryptophan fluorescence of Bcl-2ΔTM. About 50% maximum fluorescence reduction was observed at pH 5.0, indicating that the protein is partially unfolded at the acidic pH required for the protein to form pores in membranes (see Fig. 2C). Of note, the tryptophan fluorescence itself is not sensitive to the range of pH change used here (45).

To assess if the putative pore-forming helix 5 in the hydrophobic core of Bcl-2ΔTM is exposed to solvent by the acidic pH, we used a Bcl-2ΔTM derivative with a NBD fluorescent probe attached to helix 5 via the sulfhydryl group of Cys¹⁵⁸, the only cysteine in the protein. We monitored the quenching of the

NBD fluorescence in pH 5.0 or 7.4 buffers by iodide ions. Iodide ions are charged and hence cannot diffuse into the protein hydrophobic core to collide with the NBD and quench its fluorescence, one thus expects to see more quenching for the more solvent-exposed probe (46). As shown in Fig. 1B, more quenching was detected for the NBD-labeled Bcl-2ΔTM in pH 5.0 buffer than that in pH 7.4 buffer, indicating that the NBD at Cys¹⁵⁸ in helix 5 is more exposed to solvent at the acidic pH. The NBD as well as tryptophan fluorescence data thus suggest that the conformational alteration of Bcl-2ΔTM induced by acidification elicits an exposure of the core helix 5 to solvent.

We recently reported that at neutral pH Bcl-2ΔTM associated with other Bcl-2ΔTM and with a peptide containing the sequence of Bax helices 2 and 3 (designated H2-H3 peptide) (31). Using the fluorescence anisotropy-based binding assay as described (31), we investigated whether the partially unfolded Bcl-2ΔTM at pH 5.0 retained similar binding properties. As shown in Fig. 1C, the isotherm of Bcl-2ΔTM homo-binding determined at pH 5.0 was very similar to that determined at pH 7.4, indicating that the acidic pH-unfolded proteins interacted similarly to the folded proteins. The Bcl-2ΔTM protein also bound to the H2-H3 peptide in pH 5.0 buffer with an isotherm similar to that obtained at neutral pH (compare Fig. 1D with the Fig. 4B in Ref. 31). More importantly, a mutation in the peptide (G67R) that eliminated binding at pH 7.4 also eliminated binding at pH 5.0, suggesting that the partially unfolded protein still retains the ligand selectivity of the folded protein. These results indicate that the acidic pH-unfolded Bcl-2ΔTM protein still retains certain important properties of the folded protein.

Acidic pH Induces Insertion of Helix 5 of Bcl-2ΔTM into Liposomal Membranes Resulting in Pore Formation—Because the acidic pH induces the exposure of hydrophobic core helix 5 to water, it may enhance binding of the protein to liposomal membranes. We monitored Bcl-2ΔTM binding with MOM-liposomes after an incubation at pH 5.0 or 7.4 using a sucrose gradient float-up centrifugation to separate the membrane-bound from the soluble and aggregated proteins (39). As shown in Fig. 2A, a significant fraction of total Bcl-2ΔTM co-fractionated with the liposome after incubation at pH 5.0. In contrast, liposome-bound protein was not detected after incubation at pH 7.4.

We used the Bcl-2ΔTM protein with an NBD attached to Cys¹⁵⁸ in helix 5 to see if the helix is inserted into the liposomal membrane at the acidic pH because NBD fluorescence will increase when the fluorophore is moved from the aqueous milieu to hydrophobic bilayer (46). As shown in Fig. 2B, NBD fluorescence was increased by about 50% after the protein was incubated with the liposomes at pH 5.0. Furthermore, the fluorescence increase was quenched by a lipophilic quenching agent, 7-doxyl-PC, incorporated into the liposomal membrane, demonstrating a direct contact between the NBD and hydrophobic core of lipid bilayer. As expected neither the increase nor the quenching of NBD fluorescence were observed when the incubation was done at pH 7.4 (Fig. 2B), consistent with the fact that Bcl-2ΔTM does not bind to the liposome at this pH (Fig. 2A). Therefore the acidic pH facilitates membrane binding of Bcl-2ΔTM by partially unfolding the protein, exposing the

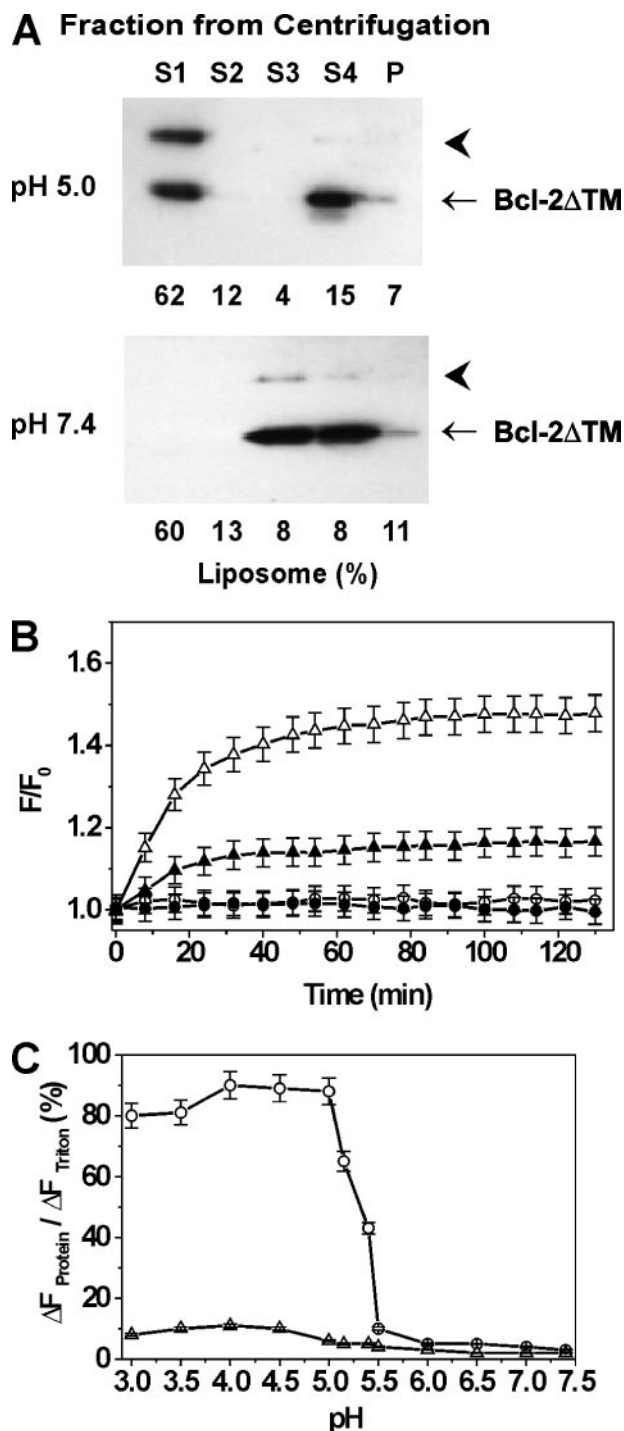


FIGURE 2. Effect of pH on Bcl-2ΔTM insertion into liposomal membranes and pore formation. *A*, data shown in the top or bottom panel are immunoblots of Bcl-2ΔTM in various fractions from a sucrose gradient float-up centrifugation after incubating the protein with liposomes at pH 5.0 or 7.4, respectively. The liposome-bound protein migrates into the upper supernatant fractions (S1–S2), whereas the soluble protein remains in the bottom fractions (S3–S4). The aggregated protein is in the pellet fraction (P). The distribution of liposomes in the gradient is indicated below each lane as the % of total liposomes. Data shown are representatives of three independent experiments. Arrowheads indicate a SDS-resistant Bcl-2ΔTM dimer. *B*, insertion of helix 5 of Bcl-2ΔTM into liposomal membranes was monitored by the change of fluorescence intensity of the NBD labeled at Cys¹⁵⁸ after mixing the protein and liposome at pH 7.4 (○) or 5.0 (Δ). The liposome contained 7-doxyl-PC that quenches the fluorescence if the NBD dye inserted into the bilayer was used in the parallel experiment at pH 7.4 (●) or 5.0 (▲). F_0 , the net intensity prior to addition of liposome; F_t , the net dilution-corrected intensities at the indicated

hydrophobic core helix 5 to the liposome and eventually this helix inserts into the liposomal membrane.

To determine whether the above Bcl-2ΔTM conformational alteration and helix 5 insertion result in pore formation in the liposomal membrane, we encapsulated fluorescent CB dyes into the MOM-liposome. Purified Bcl-2ΔTM protein was then incubated with the liposome in solutions of various pH. The Bcl-2ΔTM-dependent CB dye release from the liposome was monitored by quenching the CB fluorescence by anti-CB antibody on the outside of the liposome. Because both CB dye and anti-CB antibody do not diffuse across the lipid bilayer spontaneously, quenching occurs only if the CB dye passes through the pore formed by Bcl-2ΔTM in the bilayer. In contrast the antibody is likely too large to pass through the pore. As shown in Fig. 2C, no or very little Bcl-2ΔTM-dependent CB fluorescence quenching was observed when the protein was incubated with the liposome at pH 5.5 or higher, whereas maximal quenching was detected when the pH was lowered to 5.0 or less. Intermediate quenching was observed when the pH was between 5.0 and 5.5. Therefore pH of 5.0 or lower is required to induce a full pore-forming activity for Bcl-2ΔTM in the bilayer of MOM lipids, consistent with previous reports (32, 33, 35). Interestingly pH 5.0 also induces a conformational alteration in Bcl-2ΔTM that triggers insertion of hydrophobic core helix 5 into the membrane (Figs. 1B and 2B), a reaction that most likely elicits pore formation.

tBid Induces Pore Formation by Membrane-tethered, but Not Soluble Bcl-2ΔTM—We recently reported that tBid can switch the Bcl-2 conformation at mitochondrion from the tail-anchored one to a conformation in which helix 5 is inserted into the membrane (29). Because a similar helix 5 insertion also occurred during the acidic pH-induced Bcl-2ΔTM pore conformation in liposome, we tested if tBid induced Bcl-2ΔTM pore formation as well. However, when both tBid and Bcl-2ΔTM were incubated with the liposome with entrapped CB dyes at neutral pH, the fluorescence quenching was not increased compared with the buffer control or each protein alone (Fig. 3A). Therefore, tBid did not induce pore formation by Bcl-2ΔTM.

One obvious difference between the Bcl-2 in mitochondria and the Bcl-2ΔTM in reactions containing liposomes is that the former is bound to the membrane via the C-terminal transmembrane sequence, whereas the latter is free in solution because it lacks the transmembrane sequence. To investigate the importance of membrane tethering in the tBid/Bcl-2 interaction, we tested whether Bcl-2ΔTM could be tethered to the liposomal membrane via a C-terminal His₆ tag binding to a Ni²⁺-chelating lipid analog incorporated into the membrane. The His₆-tagged Bcl-2ΔTM was incubated with either the

times after addition of liposome. Data shown are averages from two independent experiments with S.D. (error bars). *C*, Bcl-2ΔTM pore formation in the liposomal membrane at various pH values was monitored by the Bcl-2ΔTM-induced release of CB dyes from the liposomes after a 5-h incubation. The release resulted in the quenching of CB fluorescence by the anti-CB antibody located outside of the liposome. The extent of Bcl-2ΔTM-induced release is determined by the value of $\Delta F_{\text{Protein}} / \Delta F_{\text{Triton}}$. Data shown are averages from three independent experiments using Bcl-2ΔTM protein (○) or the buffer control (Δ) with S.D. (error bars).

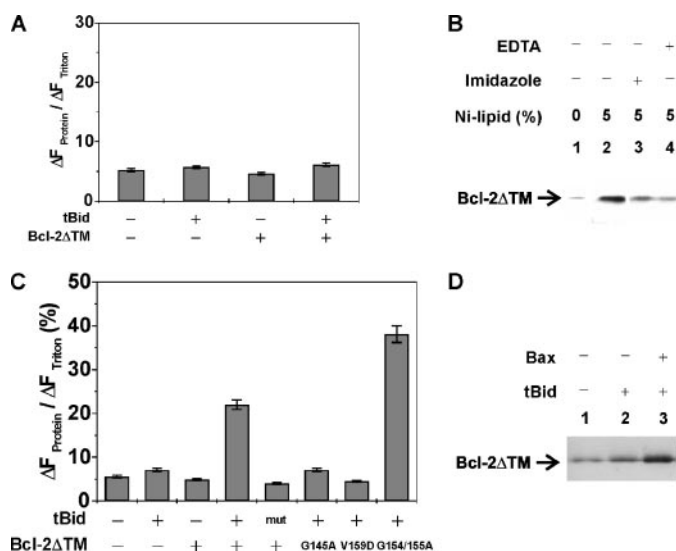


FIGURE 3. Effect of tBid on the pore formation by soluble and membrane-tethered Bcl-2ΔTM. A, extent of CB dye release by the buffer control (minus tBid and Bcl-2), the soluble Bcl-2ΔTM and/or tBid at pH 7.4 was monitored as above. Data shown are averages of two to three independent experiments with S.D. (error bars). B, binding of the C-terminal His₆-tagged Bcl-2ΔTM with regular or Ni²⁺-liposome (with 0 or 5% Ni²⁺-lipid, respectively) in the absence or presence of EDTA or imidazole was assayed by the float-up centrifugation. Data shown are immunoblots of the Bcl-2ΔTM that was recovered from the S1 fraction after centrifugation. Similar results were obtained in other two independent repeats. C, extent of CB dye release by the buffer control, tBid, or mutant (M97A/D98A, *mut*), and/or His₆-tagged Bcl-2ΔTM or mutant (G145A, V159D, or G154A/G155A) from Ni²⁺-liposome at pH 7.4 was monitored as above. Data shown are averages of two to three independent experiments with S.D. (error bars). D, insertion of Bcl-2ΔTM into the membrane was monitored by float-up centrifugation after incubating the protein with Ni²⁺-liposomes in the absence or presence of tBid and/or Bax and then treating with EDTA. Data shown are representative immunoblots of Bcl-2ΔTM bound to the liposomes in the S1 fraction of the float-up centrifugation. Similar data were obtained in other two independent experiments.

Ni²⁺-chelating or regular liposomes at neutral pH and then subjected to the float-up centrifugation. As shown in Fig. 3B, a large amount of the His₆-tagged Bcl-2ΔTM was detected in the liposome-containing top fraction when the liposome contained the Ni²⁺-chelating lipid analog. Furthermore, the amount of His₆-Bcl-2ΔTM associated with the Ni²⁺-liposome was greatly reduced if the incubation was done in the presence of imidazole or EDTA. In contrast, the residual amount of His₆-Bcl-2ΔTM bound to the regular liposomes was not sensitive to imidazole nor EDTA treatments (data not shown). These results demonstrate that His₆-Bcl-2ΔTM can be tethered to the Ni²⁺-liposome via His₆-Ni²⁺ interaction. Because the His₆ tag is at the C terminus of Bcl-2ΔTM and Ni²⁺ is at the surface of liposome, the liposome-tethered Bcl-2ΔTM topologically mimics the native mitochondrion-bound Bcl-2.

Using the liposome-tethered Bcl-2ΔTM we re-investigated whether tBid can induce the pore formation. As shown in Fig. 3C, neither Bcl-2ΔTM nor tBid alone released more CB dyes from the Ni²⁺-liposome than the buffer control. In contrast, together they caused a significant dye release and hence fluorescence quenching. A mutant tBid (M97A/D98A) that did not bind Bcl-2 also did not trigger Bcl-2ΔTM pore formation (23, 47). Similarly, a mutant Bcl-2ΔTM (G145A) that did not bind tBid also did not cause dye release in the presence of tBid (47). Moreover, the mutation V159D shown previously to inhibit

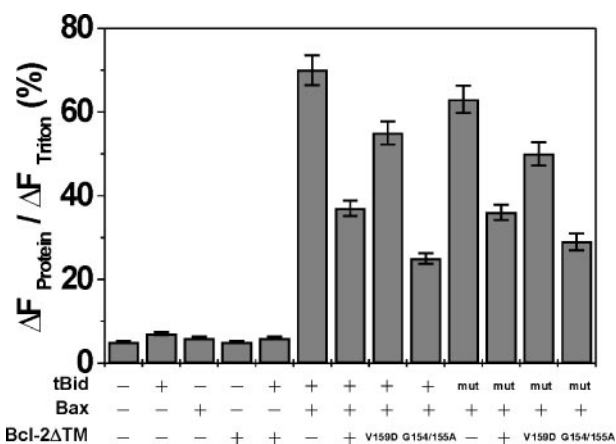


FIGURE 4. Effect of tBid, Bax, and/or membrane-tethered Bcl-2ΔTM on pore formation. Extent of CB-dextran release by the buffer control, tBid or mutant, Bax and/or His₆-Bcl-2ΔTM, or mutants from Ni²⁺-liposome was monitored. Data shown are averages of two to three independent experiments with S.D. (error bars).

insertion of Bcl-2 helix 5 into membranes showed no function in the dye release assay (29). However, the hyperfunctional mutant Bcl-2ΔTM (G154A/G155A; see Fig. 5) triggered more dye release than Bcl-2ΔTM. In the presence of tBid, more Bcl-2ΔTM became resistant to EDTA treatment that removed the membrane-tethered Bcl-2 from the membrane (Fig. 3D), suggesting that tBid caused insertion of membrane-tethered Bcl-2ΔTM into the membrane. Together these results suggest that tBid interacts with the membrane-tethered Bcl-2ΔTM, causes the insertion of Bcl-2ΔTM, and thereby elicits pores in the membrane. These results also demonstrate that tBid alone can induce the conformational alteration in the membrane-bound Bcl-2ΔTM, suggesting that no other mitochondrial proteins are required to mediate the tBid-induced Bcl-2 conformational alteration (29).

When Activated by tBid, Bcl-2ΔTM Forms Small Pores, whereas Bax Forms Large Pores—If the native mitochondrion-bound Bcl-2 forms pores in the MOM after it interacts with tBid, why do these pores not release cytochrome *c* and other apoptotic proteins from the mitochondrial intermembrane space (29)? One possibility is that Bcl-2 forms pores that are smaller than these mitochondrial proteins. To assess whether the pore formed by tBid-activated Bcl-2ΔTM is large enough to release cytochrome *c*, we incubated tBid and His₆-Bcl-2ΔTM with Ni²⁺-liposomes that entrapped 10-kDa CB-dextran, and monitored the release of CB-dextran by the fluorescence quenching assay. As shown in Fig. 4, tBid and Bcl-2ΔTM together did not release more CB-dextran than were released by the buffer control or each protein alone. Together with the data shown in Fig. 3C, we demonstrated that after it was activated by tBid, the membrane-tethered Bcl-2ΔTM formed pores that can let 0.5-kDa CB dye but not 10-kDa CB-dextran pass through. Because the size of cytochrome *c* (12 kDa) is similar to that of 10-kDa CB-dextran, the Bcl-2 pore, if it forms in the native MOM after tBid treatment, is unlikely to release cytochrome *c* or other larger apoptotic mitochondrial proteins.

In contrast, tBid activated soluble Bax and released the 10-kDa or larger CB-dextran from the liposomes (Fig. 4; data not shown), consistent with a previous report (20). Therefore,

tBid Alters Bcl-2 Conformation

tBid induced the pore-forming activity of both anti-apoptotic Bcl-2 and pro-apoptotic Bax, but the consequences were different: the former formed a pore much smaller than cytochrome *c*, whereas the latter formed a pore much larger than cytochrome *c*.

The Conformational Alteration of Membrane-tethered Bcl-2 Δ TM Can Also be Induced by Active Bax and Is Critical for Bcl-2 to Inhibit Active Bax—The Bcl-2 conformational alteration induced by tBid or another apoptotic stimulus is required for Bcl-2 to inhibit Bax-mediated cytochrome *c* release from mitochondria as we demonstrated recently (29). Because the Bcl-2 Δ TM pore formation involved a similar conformational alteration and was induced by a similar stimulus, we tested whether the pore-forming conformer of Bcl-2 Δ TM inhibits Bax pore formation. As shown in Fig. 4, the membrane-tethered Bcl-2 Δ TM significantly reduced the release of 10-kDa CB-dextran from the liposome by tBid-activated Bax. Interestingly, more EDTA-resistant Bcl-2 Δ TM proteins were detected in the liposomes in the presence of both tBid and Bax than were observed when only tBid was added to liposomes with membrane-tethered Bcl-2 Δ TM (Fig. 3D). This result suggests that tBid-activated Bax can also alter the conformation of membrane-tethered Bcl-2 Δ TM.

To distinguish whether the inhibition of tBid-activated Bax is caused by the membrane-tethered or conformationally altered Bcl-2 Δ TM (with Cys¹⁵⁸ inserted into the bilayer), we employed a Bcl-2 mutant (V159D) that binds tBid but is defective in the conformational alteration induced by tBid (29). In accordance with our previous results in cells and isolated mitochondria, membrane-tethered Bcl-2 Δ TM-V159D did not form pores in the liposome in response to added tBid (Fig. 3C). This mutant also exhibited reduced inhibition of pore formation by tBid-activated Bax (Fig. 4). In contrast, another Bcl-2 Δ TM mutant (G154A/G155A) that had increased tBid-induced pore-forming activity provided a greater inhibition of pore formation by tBid-activated Bax (Figs. 3C and 4). These results suggest that the conformational alteration of Bcl-2- Δ TM induced by tBid is critical for Bcl-2- Δ TM to inhibit membrane permeabilization by Bax.

Because tBid-activated Bax also induced a conformational alteration of Bcl-2 Δ TM (Fig. 3D), we tested whether this conformational alteration is also correlated with the Bcl-2 Δ TM inhibition of Bax. For these experiments we used the tBid mutant (M97A/D98A) because it does not bind stably to Bcl-2 and did not lead to the membrane-tethered Bcl-2 Δ TM adopting the pore conformation (Fig. 3C). However, this mutant activated Bax to permeabilize the membrane (Fig. 4), consistent with previous reports that the tBid mutant does not interact with Bcl-2 but interacts with Bax (23, 47). The membrane-tethered Bcl-2 Δ TM inhibited pore formation by the tBid mutant activated Bax (Fig. 4). Moreover, addition of the mutant tBid and Bax increased the amount of Bcl-2- Δ TM that bound to liposomes in a fashion resistant to the addition of EDTA (data not shown), confirming that Bax activated by the mutant tBid induced the conformational alteration required for Bcl-2 Δ TM to inhibit membrane permeabilization by Bax. In addition, the Bcl-2 Δ TM mutants (V159D and G154A/G155A) that showed decreased and increased activities in the conformational alter-

ation assays shown in Fig. 3C also showed decreased and increased activities in inhibiting the tBid mutant-activated Bax, respectively (Fig. 4). Therefore, the conformational alteration of membrane-tethered Bcl-2 Δ TM can be induced by either tBid or active Bax and plays a critical role in the inhibition of active Bax.

Conformational Alteration Activity in Vitro and Anti-apoptosis Activity in Vivo Are Correlated for Two Bcl-2 Mutants—To assess further the physiological significance of the Bcl-2 Δ TM conformational alteration in liposomes, we tested whether mutations in Bcl-2 that affect the tBid-induced pore formation in liposomes also affect anti-apoptosis activity in cells. Our previous study showed that the V159D mutation resulted in a hypo-functional Bcl-2 in cells (29). The V159D mutation abolished the pore-forming activity in liposomes (Fig. 3C) and inhibited the conformational alteration in both cells and isolated mitochondria (29), suggesting a positive correlation between the pore-forming activity, the conformational alteration, and the anti-apoptosis function of Bcl-2.

To further substantiate this correlation, we identified a Bcl-2 mutant, Bcl-2-G154A/G155A, which is hyper-active in forming pores in liposomes (Fig. 3C), and then assayed it for inhibition of apoptosis and the accompanying change in conformation inside the cells. To determine the anti-apoptosis activity of Bcl-2-G154A/G155A, it was expressed in Rat-1MycER^{TAM} cells and then apoptosis was induced either by treating the cells with etoposide or by growth in low serum with activated *c-myc* as described (42). The anti-apoptosis activity of Bcl-2-G154A/G155A was compared with wild-type Bcl-2 by measuring the extent of caspase-mediated PARP cleavage 12 or 18 h after the cells were treated with etoposide, a DNA-damaging agent. The effects of growth in low serum with activated *c-myc* were assayed after 24 or 48 h. Rat-1 cells expressing wild-type Bcl-2 or empty vector were used as positive or negative controls, respectively. As shown in Fig. 5A, the mutant Bcl-2 had substantially higher anti-apoptotic activity (inhibition of PARP cleavage) than the wild-type in both etoposide and low serum-activated *myc*-treated cells. We have shown elsewhere that in these cells the extent of PARP cleavage accurately reflects ongoing apoptosis measured as externalization of phosphatidylserine at the plasma membrane (annexin V labeling) and chromatin condensation (Hoescht staining) (42).

Next, we monitored the conformational alteration of Bcl-2-G154A/G155A using the IASD labeling assay as described (29, 37). As shown in Fig. 5B, more protection of Cys¹⁵⁸ in the mutant Bcl-2 from the labeling was observed in membranes isolated from the etoposide-treated cells than the untreated cells, indicating that the mutant changed conformation in response to the apoptotic stimulus as reported previously for the wild-type protein (29). The mutant displayed an even greater extent of conformational alteration (protection from labeling, indicated by ○) and elevated anti-apoptosis activity in Rat-1 cells cultured in the low serum media (Fig. 5, A and B, *low serum*), suggesting that the conformation change correlates with anti-apoptotic activity for the mutant as reported previously for wild-type Bcl-2 (29). Taken together the results from the hypo- and hyper-functional mutants suggest a very strong

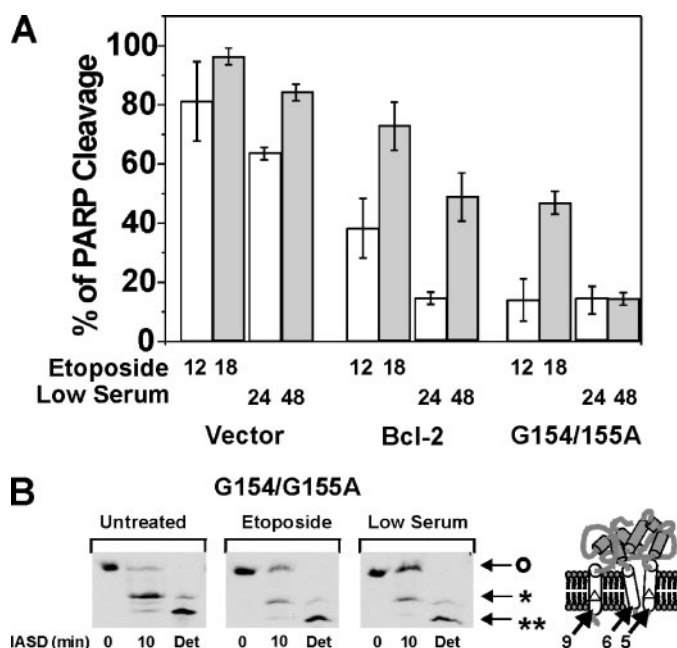


FIGURE 5. Effect of mutations in helix 5 on anti-apoptosis activity and conformational alteration of Bcl-2. A, the anti-apoptotic activity of Bcl-2 or the G154A/G155A mutant were assayed after addition of etoposide for 12 (open bar) or 18 h (shaded bar) or growth in low serum for 24 (open bar) or 48 h (shaded bar) by measuring PARP cleavage. Rat-1MycER^{TAM} cells were infected with packaged retroviral vector, or vector that expresses wild-type human Bcl-2 or Bcl-2-G154A/G155A, as indicated. Clones were selected that stably expressed equivalent amounts of the two proteins and then assayed for resistance to etoposide or serum starvation as indicated below the bars. Data shown are averages of three independent experiments with S.D. (error bars). B, the conformational alteration of Bcl-2-G154A/G155A induced by etoposide treatment or serum starvation was monitored by protection from IASD labeling assayed by isoelectric focusing followed by immunoblotting for human Bcl-2. Modification with IASD adds two negative charges at each cysteine modified and results in a discrete shift in mobility during isoelectric focusing. The cysteines available for modification in helix 5 and 9 are indicated as white triangles on the diagram to the right of the panels. The migration position before IASD labeling (IASD, 0 min) indicates the migration position of the unlabeled protein (○). The migration position after labeling with detergent added to solubilize the membrane (Det) indicates the migration position of the protein labeled at both cysteines (**). Labeling of a single cysteine (Cys¹⁵⁸ in helix 5) results in a band with intermediate migration (*; IASD, 10 min). In untreated cells Bcl-2 has not changed conformation and the major band (*) after IASD labeling represents protein in which Cys¹⁵⁸ is labeled but Cys²²⁹ in helix 9 is protected unless detergent is added (Det). After treatment with etoposide or low serum the relative intensity of the unlabeled band (○) increases indicating protection of both cysteines from IASD. Addition of detergent renders both cysteines accessible to the reagent confirming that protection from IASD was due to insertion of the cysteines into the bilayer. The predicted topology of Bcl-2 with both cysteines protected from IASD is shown to the right of the panels with helices 9, 6, and 5 indicated.

correlation between the *in vitro* conformational alteration activity and the *in vivo* anti-apoptosis function of Bcl-2.

DISCUSSION

Whether native Bcl-2 forms pores in intracellular membranes is currently unknown. However, we previously found that native Bcl-2 changed conformation during the induction of apoptosis, resulting in insertion of Cys¹⁵⁸ of helix 5 into intracellular membranes, and that tBid could trigger the Bcl-2 conformational alteration in isolated mitochondria (29, 37). Perhaps a direct interaction with tBid can trigger a conformational alteration in Bcl-2, promoting helix 5 insertion that may result

in pore formation in intracellular membranes. Our data demonstrate that acidic pH increases the exposure of the internal helix 5 of Bcl-2ΔTM thereby promoting the insertion of the helix into the membrane. Acidic pH also induces some conformational alterations in Bcl-x_LΔTM such as increasing solvent exposure of hydrophobic sites while retaining the overall secondary structure (43), similar to what we observed for Bcl-2ΔTM. Therefore, it seems likely that the conformational alteration in Bcl-2 triggered by tBid involves exposure of helix 5 to promote integration into membranes.

However, tBid did not trigger the soluble Bcl-2ΔTM to form pores in the liposomal membrane. In addition to altering the conformation, acidic pH may also promote protonation of some ionizable residues such as glutamate, aspartate, and histidine, as shown previously for the pore-forming diphtheria toxin and some membrane-inserting peptides (48–51). Notably there are four glutamates, one aspartate, and one histidine in helices 5 and 6 of Bcl-2. Protonation of these residues will reduce the free energy cost for insertion of the two putative pore-forming helices into the lipid bilayer. Thus, combination of the conformational alteration and protonation of ionizable residues may account for the effect of low pH on insertion and pore formation by Bcl-2ΔTM.

Bcl-2ΔTM is the cytosolic domain of Bcl-2, and therefore it is not anchored onto membranes in our assay system using regular liposomes. Bcl-2 that is naturally anchored to an intracellular membrane via the C-terminal transmembrane sequence responds to interaction with tBid by inserting helix 5 into the membrane (29). Therefore, the additional effect of low pH compared with interaction with tBid may serve to promote membrane binding by Bcl-2ΔTM. When Bcl-2ΔTM was tethered to a liposomal membrane we have discovered that tBid directly interacts with the Bcl-2 cytosolic domain and alters its conformation. The conformationally altered Bcl-2 inserts helix 5 into the membrane and forms small pores. Significantly, our results suggest that the major function of the constitutively transmembrane helix 9 in wild-type Bcl-2 may be only to hold the protein in close proximity to the membrane. Therefore, by using purified proteins and liposomes we have defined the minimal requirements for the Bcl-2 conformational alteration to be the membrane-bound Bcl-2 and tBid. Furthermore, because more conformationally altered Bcl-2 was generated in the presence of tBid and Bax than tBid alone, the tBid-activated Bax seems also able to induce the conformational alteration of Bcl-2.

We recently demonstrated that Bcl-2 conformational alteration was required for Bcl-2 to inhibit apoptosis in cells and active Bax in mitochondria (29), but we could not rule out the possibility that other mitochondrial proteins may be required either to induce the conformational change or for the conformationally altered Bcl-2 to inhibit Bax activity. Using the liposome system with minimal and defined components, we have shown that conformationally altered Bcl-2 is sufficient and likely required to inhibit large pore formation by active Bax.

Based on this study and other studies (20, 25, 29, 37), it is clear that tBid can independently activate both Bcl-2 and Bax, changing them to what appears to be a similar multi-spanning transmembrane conformation. In the absence of

the multispinning Bcl-2, multispinning Bax forms large pores in the MOM to release cytochrome *c* and perhaps other mitochondrial proteins. In the presence of the multispinning Bcl-2, both multispinning proteins form hetero-complexes that do not permeabilize the mitochondrion. This suggests that the critical feature of BH3 mimetics with anti-Bcl-2 activity may be to inhibit the conformation change in Bcl-2 rather than simply blocking the hydrophobic binding surface on Bcl-2 (52, 53). It is interesting to speculate what structure the multispinning Bcl-2 may form in the MOM in the absence of the multispinning Bax. Our liposome data suggest that the multispinning Bcl-2 may form small pores in the MOM and this activity may intimately link to the anti-apoptosis activity. If the multispinning Bcl-2 indeed forms small pores *in vivo*, what would be the function of these pores? Are they pre-activated Bcl-2 traps waiting for active Bax? Do they affect the MOM permeability independent of inhibiting Bax? These intriguing questions about the functionally important multispinning Bcl-2 conformer must be addressed by future experiments before one can elucidate the molecular mechanism by which Bcl-2 inhibits apoptosis and develop better anti-Bcl-2 drugs for cancer therapy.

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tBid Elicits a Conformational Alteration in Membrane-bound Bcl-2 Such That It Inhibits Bax Pore Formation

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