Structural and Functional Complementation of an Inactive Bcl-2 Mutant by Bax Truncation*

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Interactions among proteins in the Bcl-2 family regulate the onset of programmed cell death. Previous work has shown that the death-inhibiting family members Bcl-2 and Bcl-xL form heterodimers with the death-promoting homologue Bax and that certain site-directed mutants of Bcl-2 and Bcl-xL lose both biological activity and the ability to bind Bax. To better understand the structural basis of heterodimer formation, we have used a yeast two-hybrid assay to screen for mutants of Bax that regain the ability to bind to these inactive Bcl-2(G145A) and Bcl-xL(G138A) mutants. This screen identified a series of C-terminally truncated Bax molecules that contain complete BH3 (Bcl-2 homology domain 3) domains but that have lost BH1 and BH2 sequences. These results indicate that while the Bcl-2 and Bcl-xL mutants fail to bind full-length Bax, they still retain a binding site for the critical BH3 domain. This suggests that conformational constraints in full-length Bax regulate its ability to bind to other Bcl-2 family members. Furthermore, we demonstrate that the normally inert Bcl-2(G145A) mutant effectively blocks apoptosis induced by a C-terminally truncated Bax molecule, but does not block apoptosis induced by wild-type Bax. This demonstrates that cell protection can be effected by directly binding pro-apoptotic members of the Bcl-2 family.

The bcl-2 gene family encodes proteins that regulate programmed cell death (1, 2). For example, in the nematode Caenorhabditis elegans, the bcl-2 homologue ced-9 acts to inhibit developmental cell deaths. This is evidenced by the elevated levels of cell death that characterize the embryos of loss-of-function ced-9 mutants (3, 4). Similarly, in cultured mammalian cells, apoptosis is inhibited by the expression of several family members, including Bcl-2, Bcl-xL, Bcl-w, and Mcl-1 (5–8). Furthermore, gene ablation experiments in mice have further confirmed the anti-apoptotic effects of bcl-2 and bcl-x (9, 10). In contrast to the anti-apoptotic members of the Bcl-2 family, mammalian cells also express family members that promote apoptosis. For example, Bax and Bak have been shown to kill when overexpressed in mammalian cells and can antagonize the cell-protective functions of Bcl-2 and Bcl-xL (11–14).

While the mechanisms by which Bcl-2 family members modulate cell death remain unclear, a variety of studies indicate that many of the family members can interact with each other and that these interactions are functionally important. Immuno-precipitation studies using genetically engineered cells first demonstrated that members of the Bcl-2 family can form protein-protein dimers with each other. These studies demonstrated that the Bcl-2 molecule can form a heterodimer with Bax and can also form a homodimer with another Bcl-2 molecule (11, 15). These conclusions were subsequently confirmed using several systems of yeast two-hybrid analysis (16–19). Similarly, Bcl-xL and Bax can form heterodimers, although there has been controversy concerning the ability of Bcl-xL to homodimerize (18, 20, 21).

Sequence alignments of Bcl-2 family proteins have identified several conserved domains, denoted BH1, BH2, and BH3, that are common to all homologues and a fourth domain, BH4, present in at least some of the family members (15, 22, 23). Site-directed mutagenesis has been used to assess the functional importance of these domains. Mutations in the BH1 and BH2 domains have been shown to abrogate the death-inhibiting functions of Bcl-2 and Bcl-xL. For example, mutation of the highly conserved glycine 145 to alanine in Bcl-2 (15), or the homologous G138A mutation in Bcl-xL (17), renders the respective proteins functionally inactive. These mutations also abrogate the ability of Bcl-2 and Bcl-xL to dimerize with Bax (15, 17). Based on these results and the results obtained with additional BH1 and BH2 mutants (15), it was suggested that the death-inhibiting function of Bcl-2 and Bcl-xL is dependent on their ability to form Bax heterodimers. Further mutagenesis work, however, has identified Bcl-xL mutants that do not bind Bax, but that still block apoptosis, suggesting that inhibition of cell death does not require heterodimerization (24). Nevertheless, the observation that the G145A and G138A mutations in Bcl-2 and Bcl-xL, respectively, are inactive suggests that the structure of this region of these molecules is important for biological function.

Mutagenesis studies on the death-promoting Bcl-2 family members Bak and Bax indicate that the BH3 domains of these molecules are critical both for induction of apoptosis and for binding to Bcl-xL and Bcl-2 (22, 23). The three-dimensional structure of Bcl-xL, derived by x-ray diffraction and NMR techniques, demonstrates the presence of a hydrophobic groove on the Bcl-xL surface (25) that has been shown to bind the BH3 domain of Bak (26). Glycine 138 forms part of this groove, and it was therefore further suggested that the bulkier alanine in the G138A mutant may block Bak or Bax from entering the binding pocket (25).

To obtain further information regarding the structural requirements for Bax-Bcl-2 and Bax-Bcl-xL heterodimer formation and to obtain new reagents to probe the functional importance of such dimerization, we utilized random mutagenesis and yeast two-hybrid analysis to identify novel Bax mutants that have acquired the ability to bind to the G145A mutant of Bcl-2. This approach was based on the strategy that was successfully used to identify mutants of Raf-1 that bind Ras(E37G),
a Ras mutant with which wild-type Raf-1 does not interact (27).

Our results indicate that the G145A and G138A mutants of Bcl-2 and Bcl-xL retain intact binding sites for dimerization partners, but suggest that structural constraints in full-length Bax prevent it from occupying those sites. Our results further indicate that Bcl-2 family members can inhibit cell death directly as a result of binding death-promoting family members.

**MATERIALS AND METHODS**

**Plasmid Constructs**

**Yeast Two-hybrid Plasmids—** Human Bax minus its transmembrane domain (Bax(-TM)) was PCR-amplified to add EcoRI and XhoI restriction sites and then subcloned into vector pGilda to yield pCIBax (-TM). Constructs containing human Bcl-2(-TM) and Bcl-xL(-TM) in the yeast two-hybrid vector pGilda were described previously (16). They were excised as EcoRI/XhoI fragments and ligated into the bait vector pGilda, which produces a LexA fusion protein. Bcl-2(G145A)(-TM) was PCR-amplified from plasmid M1-3 (15) and subcloned into the vectors pGilda and pJG4-5. Site-directed mutagenesis was performed on Bcl-xL (in pBlueScript (Stratagene)) to generate mutants F131V,D133A and G148E,G187A using the Muta-Gene M13 Kit (Bio-Rad). The mutants were subsequently PCR-amplified and subcloned into pGilda as EcoRI/XhoI fragments.

**Mammalian Expression Plasmids—** Bcl-2, Bcl-2(G145A), and Bax, including their transmembrane-spanning domains, were subcloned into the mammalian expression vector pCIneo (Promega) to yield pCIBcl-2, pCIBcl-2(G145A), and pCIBax, respectively. To generate an expression construct for Bax with a transmembrane domain (BaxD(TM)), two separate PCRs were performed with Bax(wt) (where wt is wild-type) in pCIneo as a template to generate two fragments of 369 and 94 base pairs, respectively. The following primers were used: 5'-ATC AGT GAA TTC ACT ATG GAC GGG GAG-3', 5'-CGC CAC AAA GAT GGT CAC GTT GAA GTT GCC GTC AGA AAA CAT GTC-3', 5'-TCT GAC GGC AAC TTC AAC GTG ACC ATC TTT CTT CCA GAT-3', and 5'-ATC GAT CTC GAG TCA GCC CAT TTG CTT CCA GAT-3'. BaxD(TM) was generated by annealing these two PCR products together (95 °C, 5 min; 65 °C, 5 min; and cooling slowly to 30 °C) and amplifying the resulting fragment. The final 463-base pair fragment was gel-isolated, digested with EcoRI and XhoI, and ligated into EcoRI/SalI-digested pCIneo.

**Bacterial Expression Plasmids—** For GST and 6-histidine fusion proteins, Bcl-2(G145A), Bcl-xL(G138A), Bcl-xL(F131V,D133A), Bcl-xL(G148E,G187A), and Bax were cloned into the bacterial expression vector pGEX-4T-1 (Pharmacia Biotech Inc.) or pET15b (Novagen) and expressed in bacteria. In each case, bound protein was quantitated by detection with an anti-GST monoclonal antibody followed by an alkaline phosphatase-conjugated secondary antibody.

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1 The abbreviations used are: PCR, polymerase chain reaction; GST, glutathione S-transferase; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.
### Bax Truncation Restores Binding to Bcl-2 and Bcl-x<sub>L</sub> Mutants

#### BHI domain

| Bax wt | TDSFREAFFRVAALMPSDGNNMGKRSVLKALKCTKVE... |
|--------|------------------------------------------|
| #5     | TDSPPRLPPFSGS*                           |
| #48    | TDSFREAPSSFREVLQICFL/TATSTGAGLSFPSTLPMNCSPRAFCRNI* |
| #11    | TDSREAPFSGS*                            |
| #31    | TDSFREAPFRVAALMPSDGNNMGLSPSTLPMNCSPRAFCRNI* |
| #38    | TDSFREAPFRVAALMPSDGNNMGLSPSTLPMNCSPRAFCRNI* |

1 bp (C) insertion
1 bp (T) deletion
1 bp (T) insertion
1 bp (G/A) change
1 bp (G) deletion

### Yeast Two-hybrid Interaction Assays

To generate a library of mutant Bax plasmids, pJG4-5-Bax was transformed into *Escherichia coli* strain XLRD (Stratagene), and DNA was purified from 1 × 10<sup>10</sup> cells. *Saccharomyces cerevisiae* strain EGY48 (*MATa* ura3 trp1 his3 LEU2::pLexAop1-3) was transformed with the plasmids: pGilda-Bcl-2(G145A), p18-34 (28), and 10 µg of pJG4-5-Bax library DNA. 500,000 transformants were screened for growth in the absence of leucine and assayed for β-galactosidase activity. The library plasmid was recovered from selected clones, and DNA sequence analysis was performed using an Applied Biosystems Model 377 sequencer.

### Protein Expression and Solid-phase Protein-Protein Binding Assays

Protein expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to bacteria transformed with the appropriate expression plasmid expressing the LexA DNA-binding domain fused to Bcl-2(wt), Bcl-2(G145A), or Bad. The transfected cells were incubated for 6 h at 37°C, and the reaction was stopped by the addition of 50 µl of 1 N NaOH. The absorbance of the reaction was measured at 420 nm.

### Yeast Two-hybrid Interaction Assays

To generate a library of mutant Bax plasmids, pJG4-5-Bax was transformed into *Escherichia coli* strain EGY191 (*MATa* ura3 trp1 his3 LEU2::pLexAop2-LEU2). Yeast cells were grown in synthetic complete medium lacking uracil, tryptophan, and histidine as necessary to select for the presence of various plasmids and containing 2% raffinose to 800 µg/ml. Cultures were induced with galactose to a final concentration of 2% for 6 h. 100 µl of the cells were lysed in 0.1 ml of Z-buffer (60 mM Na<sub>H</sub>PO<sub>4</sub>, 40 mM Na<sub>2</sub>PO<sub>4</sub>, 10 mM KCl, 1 mM MgSO<sub>4</sub>, and 50 mM 2-mercaptoethanol) and 3.5 units of lyticase (Boehringer Mannheim) for 1 h in 96-well plates. To assay β-galactosidase activity, 50 µl of o-nitrophenyl-β-D-galactopyranoside (4 mg/ml) in Z-buffer were added to the reaction, and the reaction was stopped by the addition of 50 µl of 1 N NaOH. The absorbance of the reaction was measured at 400 nm.

### Mammalian Transfection Assays

For single transfections, mammalian 293 cells (2 × 10<sup>5</sup> cells/35-mm well) were transfected either with carrier DNA or with 3.5 µg of pcIneo, or pcBax, or pcBax/A+TM and 0.7 µg of CMV-β-gal (where CMV is cytomegalovirus and β-gal is β-galactosidase). For cotransfection experiments, cells were, in addition, transfected with either pCMV-Bcl-2 or pCMV-Bcl-2(G145A). The transfected cells were incubated for 6 h at 37°C, washed twice with phosphate-buffered saline, refed with fresh complete medium, and incubated overnight. 24 h after transfection, the cells were rinsed with phosphate-buffered saline, fixed in phosphate-buffered saline containing 2% formaldehyde and 0.2% glutaraldehyde, and stained with 1 mg/ml X-gal solution. Blue cells were counted as either viable (flat with normal epithelial shape) or dead (rounded and shrunken).

### RESULTS

**Mutant Bcl-2(G145A) Can Homodimerize and Thus Contains an Active Binding Site**—Previous experiments with the BHI mutant Bcl-2(G145A) demonstrated that while it is functionally inactive and fails to bind Bax, it can bind wild-type Bcl-2 (15). We have recently demonstrated that Bcl-2 utilizes a com-
mon binding site to form either a homodimer with the BH3 domain of another Bcl-2 molecule or a heterodimer with the BH3 domain of Bax (21). Thus, the ability of Bcl-2(G145A) to bind wild-type Bcl-2 could simply reflect an intact binding site in the wild-type molecule binding to the intact BH3 domain in Bcl-2(G145A). To determine whether Bcl-2(G145A) itself contains an active binding site, we used a yeast two-hybrid assay to measure its interactions with different family members. The results confirm that wild-type Bcl-2 can dimerize with Bax, whereas Bcl-2(G145A) cannot, and that Bcl-2(G145A) can bind to wild-type Bcl-2 (Fig. 1A). More important, the results also show that Bcl-2(G145A) is capable of dimerizing with another molecule of Bcl-2(G145A) and that the strength of this interaction in this assay is similar to that seen with wild-type Bcl-2 (Fig. 1B). These data indicate that while the binding site in Bcl-2(G145A) is altered so that it no longer binds Bax, it is still capable of binding another Bcl-2(G145A) molecule.

Isolation of Bax Mutants That Bind Bcl-2(G145A)—Given the observation that Bcl-2(G145A) contains an active binding
site for at least some ligands, we were interested in probing the structural basis of the failure of this mutant to bind Bax. Our strategy was to randomly mutagenize Bax, screen for mutants that bind Bcl-2(G145A) in a yeast two-hybrid system, and then characterize the resulting mutant Bax clones (Fig. 2). Bax mutants were prepared by growing a yeast two-hybrid expression plasmid encoding a B42-Bax fusion protein in an E. coli strain deficient in DNA repair (E. coli XLRed). A library of Bax mutants was cotransformed with a LexA-Bcl-2(G145A) plasmid into S. cerevisiae strain EGY48. 500,000 mutants were screened for interaction with Bcl-2(G145A), resulting in 500 colonies that formed on selective medium. 120 of these were further analyzed for 

binding observed in the yeast two-hybrid assays was confirmed in yeast two-hybrid assays. As predicted from the nonquantitative screen, Bcl-2(G145A) bound avidly to both wild-type and truncated Bax, whereas Bcl-xL(G138A) did not bind wild-type Bax but retained substantial cell survival activity (17, 24). The failure of these mutant Bcl-xL molecules to bind wild-type Bax was confirmed in yeast two-hybrid assays (Fig. 5, A and B). However, when tested for binding to BaxΔC, all of the Bcl-xL mutants bound avidly (Fig. 5, A and B). The Bcl-xL(G138A) and Bcl-xL(F131V,D133A) mutants were further analyzed in solid-phase binding assays. As in the yeast two-hybrid assay, these Bcl-xL mutants failed to bind His6-Bax, but showed clear saturable binding to 6×His-BaxΔC (Fig. 5, D and E). Wild-type Bcl-xL bound to both wild-type and truncated Bax molecules, with an apparent affinity that was greater for truncated Bax (Fig. 5C).

**FIG. 7.** Bcl-2(G145A) inhibits cell death induced by BaxΔC+TM. 293 cells were cotransfected with the following: 1) CMV-β-gal and either pCIneo, Bcl-2(wt), or Bcl-2(G145A) (first bars); 2) CMV-β-gal, Bax(wt), and either pCIneo, Bcl-2(wt), or Bcl-2(G145A) (middle three bars); or 3) CMV-β-gal, BaxAC+TM, and either pCIneo, Bcl-2(wt), or Bcl-2(G145A) (last three bars). A minimum of 200 blue cells were counted as either viable (flat with normal epithelial shape) or dead (rounded and shrunken). Each transfection was performed in triplicate, and the values given are the means ± S.D.

**FIG. 6.** BaxΔC induces apoptosis in 293 cells. Cells were transfected with either the CMV-β-gal vector alone (--) or together with pCIneo, Bax(wt), or BaxAC+TM. A minimum of 200 blue cells were counted as either viable (flat with normal epithelial shape) or dead (rounded and shrunken). Each transfection was performed in triplicate, and the values given are the means ± S.D.

**Binding of Bcl-2 and Bcl-2(G145A) to BaxΔC**—The binding of Bcl-2 and Bcl-2(G145A) to wild-type and mutant Bax was analyzed using quantitative yeast two-hybrid assays and a quantitative solid-phase binding assay. As predicted from the results of the nonquantitative screen, Bcl-2(G145A) bound avidly to BaxΔC in the quantitative yeast two-hybrid assay. Wild-type Bcl-2 also bound to the BaxΔC mutant (Fig. 4A). To exclude the possibility that BaxΔC nonspecifically binds to LexA fusion proteins or directly activates β-galactosidase expression by itself, we tested it in the yeast two-hybrid assay against three control plasmids: an empty LexA vector, LexA-Bad, and LexA-Ced-3. No β-galactosidase reaction product was observed in any of these controls (Fig. 4A and data not shown). The binding observed in the yeast two-hybrid assays was confirmed by solid-phase assays using purified recombinant proteins.

GST fusions of both wild-type Bcl-2 and Bcl-2(G145A) demonstrated saturable binding to immobilized 6×His-BaxΔC (Fig. 4B). In contrast, only wild-type Bcl-2 showed strong binding to wild-type Bax in this assay (Fig. 4B).

**BaxΔC Binds Mutants of Bcl-xL**—Since BaxΔC was found to bind to mutant Bcl-2, we sought to determine whether this truncated Bax molecule would also bind to mutants of Bcl-xL. Site-directed mutagenesis of Bcl-xL has yielded a series of mutants that fail to bind wild-type Bax, but that differ in their functional effects on cell death (17, 24). For example, immunoprecipitation studies have shown that Bcl-xL(G138A) does not bind wild-type Bax and is functionally inactive, whereas the double mutants Bcl-xL(F131V,D133A) and Bcl-xL(G146E,G187A) do not bind wild-type Bax, but retain substantial cell survival activity (17, 24). The failure of these mutant Bcl-xL molecules to bind wild-type Bax was confirmed in yeast two-hybrid assays (Fig. 5, A and B). However, when tested for binding to BaxΔC, all of the Bcl-xL mutants bound avidly (Fig. 5, A and B). The Bcl-xL(G138A) and Bcl-xL(F131V,D133A) mutants were further analyzed in solid-phase binding assays. As in the yeast two-hybrid assay, these Bcl-xL mutants failed to bind His6-Bax, but showed clear saturable binding to 6×His-BaxΔC (Fig. 5, D and E). Wild-type Bcl-xL bound to both wild-type and truncated Bax molecules, with an apparent affinity that was greater for truncated Bax (Fig. 5C).

**BaxΔC+TM Induces Apoptosis in 293 Cells**—Since BaxΔC was found to bind more avidly to Bcl-2 and Bcl-xL than did wild-type Bax, we compared these two Bax molecules for their ability to induce apoptosis in the human embryonic kidney 293 cell line. The efficiency with which wild-type Bax induces apoptosis is dependent in part on the presence of its membrane anchor sequence, as was seen previously for certain fragments of Bak (22). Thus, to compare the death-inducing effects of truncated Bax with wild-type Bax, we constructed an expression plasmid with the Bax transmembrane domain fused to the C terminus of BaxΔC, denoted BaxAC+TM. 293 cells were transiently transfected with the relevant Bax construct or empty expression vector or no vector, together with a β-galactosidase expression construct. After 24 h, cells were stained with X-gal, and blue cells were evaluated for survival. The BaxΔC+TM construct killed as efficiently as did wild-type Bax (Fig. 6), although the expression level of the BaxΔC+TM protein was at least 20-fold lower than that of wild-type Bax by Western blot analysis (data not shown). Thus, enhanced death-

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promoting activity correlates with the enhanced binding avidity of BaxΔC.

Bcl-2(G145A) Inhibits BaxΔC+TM-induced Cell Death—Expression of wild-type Bcl-2 inhibited the cell death induced by Bax expression in 293 cells (Fig. 7). In contrast, Bcl-2(G145A), which cannot bind wild-type Bax, did not protect against Bax-induced death in 293 cells (Fig. 7). These results are in accord with earlier studies demonstrating that wild-type Bcl-2 inhibits cell death induced by interleukin-3 withdrawal in FL5.12 cells, but that Bcl-2(G145A) is inert (15). Since Bcl-2(G145A) binds BaxΔC, we tested to see if this Bcl-2 mutant would specifically inhibit BaxΔC+TM-induced apoptosis. When co-transfected with BaxΔC+TM, the normally inactive Bcl-2(G145A) protein was as effective as wild-type Bcl-2 in inhibiting cell death (Fig. 7). These results indicate that apoptosis can be suppressed by molecules that bind Bax-like proteins but that have no other intrinsic death-inhibiting activity.

DISCUSSION

We have utilized a strategy of random mutagenesis and yeast two-hybrid analysis to identify mutants of Bax that regain the ability to bind the inactive Bcl-2(G145A) protein. A similar strategy had previously been used to isolate mutants of Raf-1 that complement inactivating Ras mutations (27). These methods represent a general approach for probing the structural requirements for protein-protein interactions.

A panel of Bax mutants that bound to Bcl-2(G145A) was isolated, and they all coded for truncated Bax proteins that included the BH3 domain but lacked complete BH1 and BH2 domains. These results are in accord with previous studies indicating that the BH3 domain in Bax is responsible for the binding of Bax to Bcl-2 (23). The fact that full-length Bax fails to bind Bcl-2(G145A) but that truncated Bax does bind has interesting structural implications. The results demonstrate that the G145A mutation in Bcl-2 does not destroy the binding site for BH3 domains. This conclusion is further supported by our observation that Bcl-2(G145A) can form a homodimer (Fig. 1, A and B) since our previous work has shown that Bcl-2 homodimerization is a BH3-dependent interaction (21). Thus, the data suggest a model in which the BH3 domain in wild-type Bax is constrained in such a way that it cannot fit into the binding groove of the Bcl-2(G145A) or Bcl-xL(G138A) mutant. In this model, when conformational constraints are released by C-terminal Bax truncation, binding can occur (Fig. 8). Similarly, other mutations such as Bcl-xL(F131V,D133A) and Bcl-xL(G148E,G187A) cannot bind the constrained BH3 domain of wild-type Bax, but are capable of binding the presumably more flexible truncated molecule.

The Bax mutants isolated in our screen all contained intact BH3 domains, but lacked most or all of the BH1/BH2 region. Interestingly, two naturally occurring proteins, Bik (22, 29, 30) and Bid (31), that also contain only the BH3 domain have been described. Like Bax and BaxΔC+TM, Bik and Bid are also inducers of cell death (29–31). The existence of Bax-like molecules lacking BH1 and BH2 domains presents the possibility that these molecules may have binding properties that are distinct from those of wild-type Bax and that resemble the mutant truncated Bax molecules described here. If so, cell survival functions retained by mutants Bcl-xL(F131V,D133A) and Bcl-xL(G148E,G187A) (24) could be due in part to their ability to bind death-inducing family members that contain only BH3 domains, even though these mutants do not interact with Bax or Bak.

It has not been clearly established whether the cell-protective Bcl-2 family members or the death-inducing family members are the key molecules that interface with the downstream death effector elements in the cell. Data exist to support the hypothesis that the pro-survival members have intrinsic anti-apoptotic activity that is antagonized by the death-promoting

![Model to account for the different binding properties of Bax and BaxΔC](image-url)
members (24). Such anti-apoptotic activity may be related to the binding of death effector molecules (32–34) or to effects on ion homeostasis (35). However, other data support the idea that the death-inducing family members actively promote apoptosis and that this activity is antagonized by the pro-survival members (31, 36). Additional data can be interpreted to support the heterodimer as the active molecular species (37). We have demonstrated that the normally inactive Bcl-2(G145A) mutant can suppress cell death induced by BaxΔC+TM. Since this Bcl-2 mutant has no intrinsic anti-apoptotic activity, its observed cellular effect must be due to binding and inactivating the BaxΔC+TM molecule. Thus, a Bcl-2 family member can have cell survival effects strictly by passive sequestration of a death-promoting family member.

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