

Bax transmembrane domain interacts with prosurvival Bcl-2 proteins in biological membranes

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The Bcl-2 (B-cell lymphoma 2) protein Bax (Bcl-2 associated X, apoptosis regulator) can commit cells to apoptosis via outer mitochondrial membrane permeabilization. Bax activity is controlled in healthy cells by prosurvival Bcl-2 proteins. C-terminal Bax transmembrane domain interactions were implicated recently in Bax pore formation. Here, we show that the isolated transmembrane domains of Bax, Bcl-x_L (B-cell lymphoma-extra large), and Bcl-2 can mediate interactions between Bax and prosurvival proteins inside the membrane in the absence of apoptotic stimuli. Bcl-2 protein transmembrane domains specifically homooligomerize and heterooligomerize in bacterial and mitochondrial membranes. Their interactions participate in the regulation of Bcl-2 proteins, thus modulating apoptotic activity. Our results suggest that interactions between the transmembrane domains of Bax and antiapoptotic Bcl-2 proteins represent a previously unappreciated level of apoptosis regulation.

apoptosis | Bcl-2 | mitochondria | oligomerization | transmembrane

The mitochondrial apoptosis program can activate the proapoptotic Bcl-2 (B-cell lymphoma 2) protein Bax (Bcl-2 associated X, apoptosis regulator) in response to stress, resulting in outer mitochondrial membrane (OMM) permeabilization and the release of cytochrome *c* (cyt *c*) and other proteins of the intermembrane space into the cytosol. The Bcl-2 family controls Bax activity and thus the integrity of the OMM (1–3). Prosurvival Bcl-2 proteins harbor four Bcl-2 homology domains [BH1–4, as represented by Bcl-2, Bcl-x_L (B-cell lymphoma-extra large), or Mcl-1] and counteract proapoptotic Bcl-2 proteins with three BH domains (BH1–3; e.g., Bax or Bak). The diverse group of BH3-only proteins regulates both prosurvival and proapoptotic Bcl-2 proteins. Prosurvival Bcl-2 proteins either inhibit Bax via direct interaction or by sequestering “activator” BH3-only proteins, thus preventing their interaction with Bax (4–9).

In healthy cells, newly synthesized Bax initially translocates to the OMM, but efficiently retrotranslocates to the cytoplasm, depending on prosurvival Bcl-2 proteins (10, 11). Bax shuttling thus establishes an equilibrium between cytosolic and mitochondrially anchored molecules (10, 12), determining the cellular response to apoptotic stress (13). Upon the induction of apoptosis, Bax and Bak interact and at least partially insert into the OMM (14–16). Regulatory interactions between Bax and other Bcl-2 proteins can only be observed in the presence of the OMM or liposomes (17, 18). Recent studies suggest that Bax is inserted in mitochondrial membranes as a monomer that oligomerizes once inserted (19–21). These studies also have shown that Bcl-x_L inhibits Bax by dissociating Bax oligomers. However, the contribution of the different protein domains to oligomer formation and apoptosis modulation within the membrane is still unclear.

Transmembrane domains (TMDs) can mediate protein–protein interactions within membranes and be involved in signal transduction

across bilayers via changes in the oligomeric state or protein conformation (22–24). The Bax TMD targets fusion proteins to the OMM; its deletion results in cytosolic Bax localization and impaired Bax activity (25). Analysis of the active Bax membrane topology suggests that the TMD could play a central role in Bax oligomerization (26). Förster resonance energy transfer studies have shown that Bax forms homooligomers in the mitochondria through TMD interactions (27). Bcl-x_L-mediated Bax retrotranslocation into the cytosol depends on the Bcl-x_L TMD, suggesting the involvement of TMD interactions in Bax inhibition (13). In addition, distance mapping of cysteine-labeled Bax variants in large unilamellar vesicles suggests a role of the Bax TMD in the formation of potential Bax pore structures (28). Thus, TMD interactions could be involved in Bax regulation, oligomerization, and pore formation.

Here, we report the self-association and interaction of the TMDs of Bax, Bcl-2, and Bcl-x_L in the biological membranes of living cells in the absence and presence of apoptosis induction. The TMDs mediate homooligomerization and heterooligomerization between proapoptotic Bax, and prosurvival Bcl-2 and Bcl-x_L members independent of extramembrane protein regions, modulating the response to apoptosis signaling.

Significance

Bcl-2 (B-cell lymphoma 2) proteins are key regulators of apoptosis. The recruitment of the predominantly cytosolic Bcl-2 protein Bax (Bcl-2 associated X, apoptosis regulator) to the mitochondria is associated with mitochondrial outer membrane permeabilization and apoptosis. We report specific interactions between the transmembrane domains (TMDs) of Bax and the prosurvival Bcl-x_L (B-cell lymphoma-extra large) and Bcl-2 proteins. Our results demonstrate that these interactions occur in nonapoptotic human cells and participate in the regulation of Bcl-2 proteins, introducing the concept of modulation mitochondrial apoptosis signaling by TMD-mediated Bcl-2 protein interactions.

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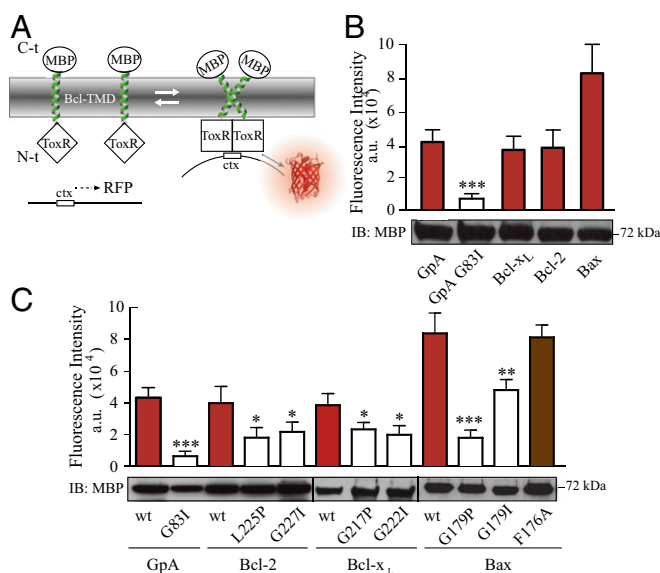
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Results and Discussion

Bax, Bcl-x_L, and Bcl-2 TMDs Homooligomerize in *E. coli* Membranes.

Interactions between Bcl-2 proteins require membranes (29, 30). Therefore, the potential homooligomerization of Bax, Bcl-2, and Bcl-x_L TMDs was analyzed in living cells by using the ToxRed system (31). In this assay, TMDs were inserted between the transcriptional activation domain (ToxR) and maltose-binding protein (MBP), targeted to the periplasm (32). Transcriptional activation of the cholera toxin promoter strictly depends on ToxR oligomerization (33). Consequently, TMD oligomerization resulted in red fluorescent protein (RFP) expression (Fig. 1A). The level of fluorescence is proportional to the analyzed oligomerization. Fusion protein functionality, orientation, and membrane insertion were controlled by the growth of mutant *Escherichia coli* on maltose as the sole carbon source dependent on MBP activity in the periplasm (Fig. S1). The TMDs of Bcl-x_L and Bcl-2 induced as much RFP fluorescence and therefore oligomerization as the positive control, glycophorin A (GpA) TMD (Fig. 1B). Similar levels of Bax TMD generated the highest fluorescence levels (Fig. 1B). The emission spectra of ToxRed chimeras corroborate the formation of Bcl-2 protein homooligomers (Fig. S2). Interestingly, the amino acid sequences of the Bax, Bcl-x_L, and Bcl-2 TMDs (Table 1) revealed central glycine residues as potential sites for strong helix-helix interactions through ridge-into-groove arrangements, as observed for other interacting TMDs (24, 34–37). These glycine residues are evolutionarily conserved (Fig. S3). Structural studies have demonstrated



that conserved glycine residues rarely face lipids, and many of them participate in close helix-helix packing (38). Accordingly, Fig. 1C shows disruptive effects for mutations Bcl-2 G227I, Bcl-x_L G222I, and, to a lesser extent, for Bax G179I (similar to the GpA G83I mutant) (39). The helix-breaking G179P mutation [similar to GpA (40)] resulted in an even-more-prominent disruption of Bax TMD homooligomerization, whereas mutation of the conserved Bax F176 had no effect. In all cases, expression levels, as determined by Western blotting, were comparable (Fig. 1B and C). Furthermore, appropriate membrane insertion of chimeric proteins was confirmed (Fig. S4). Therefore, the TMDs of Bax, Bcl-2, and Bcl-x_L mediate specific and efficient homooligomerization in a biological membrane.

Table 1. Sequences of the TMDs of human Bax, Bcl-x_L, and Bcl-2 proteins

Protein	Accession no.	Amino acid sequence
Bcl-x _L	NP_612815	²¹⁰ FNRWFLTGMTVAGVVLLGSLFSR ²³²
Bcl-2	NP_000624	²¹⁴ WLSLKTLLSLALVGCITLGAYL ²³⁶
Bax	Q07812	¹⁶⁹ TWQVTVI FAGVLTASLTW ¹⁸⁸

Central glycine residues are shown in bold, and mutated Phe¹⁷⁶ from Bax is in italics.

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Bax, Bcl-x_L, and Bcl-2 TMDs Form Oligomers in Mitochondrial Membranes.

Next, we analyzed the self-association capacity of Bax, Bcl-x_L, and Bcl-2 TMDs in nonapoptotic human cells using bimolecular fluorescence complementation (BiFC) assays (41). TMDs were fused with two nonfluorescent fragments (VN: 1–155 amino acids, I152L; and VC: 155–238 amino acids, A206K) of the venus fluorescent protein to assess whether TMD interactions would reconstitute fluorescence of venus (Fig. 2A) (42). VN and VC constructs fused to the same TMD were cotransfected in HCT116 colon carcinoma cells. This process, indeed, resulted in Bax, Bcl-2, and Bcl-x_L TMD homooligomerization in the absence of apoptotic stimuli (Fig. 2B). The nonoligomerizing TMD of the abundant mitochondrial Tom20 protein was used as a negative control for overcrowding. Bcl-x_L oligomerization is still controversial; some cross-linking experiments suggest oligomer formation, whereas other studies using detergents or structural data in nanodiscs have revealed monomers (43). However, extraction of transmembrane proteins with detergents could dissociate them, and nanodisc studies are highly dependent on the lipid composition (44). A striking advantage of the BiFC assays is the observation of interactions in eukaryotic membranes. The Western blot analysis confirmed comparable expression levels of the VN and VC constructs. Therefore, the observed fluorescence values indicate different association levels and agree with the results obtained in the ToxRed system (Fig. 1B and Fig. S5), where self-association of the proapoptotic Bax TMD caused the highest fluorescence signal. The analysis of the intracellular distribution of TMD fusion proteins by confocal microscopy revealed mitochondrial localization (Fig. 2C).

Next, single amino-acid substitutions were introduced into the TMDs to analyze the sequence specificity of TMD homooligomerization (Fig. 2D). The subcellular distribution of chimeric constructs was also corroborated by cellular fractionation (Fig. 2E). In agreement with ToxRed homooligomerization experiments, strong interference by Bax G179P (see also Fig. 2C, bottom row), Bcl-2 G227I, and Bcl-x_L G222I was observed, whereas Bax F176A again exhibited no difference from the wild-type construct. The Bcl-2 L225P and Bcl-x_L G217P mutants also exhibited strong interference with TMD oligomerization. Therefore, Bax, Bcl-2, and Bcl-x_L TMDs form specific homooligomers within the OMM.

Bax TMD Interacts with Prosurvival Bcl-2 Protein TMDs. Because of the specific homooligomerization of the Bax, Bcl-2, and Bcl-x_L TMDs in nonapoptotic cells, the possibility of heterooligomerization and regulatory interactions between these TMDs was tested.

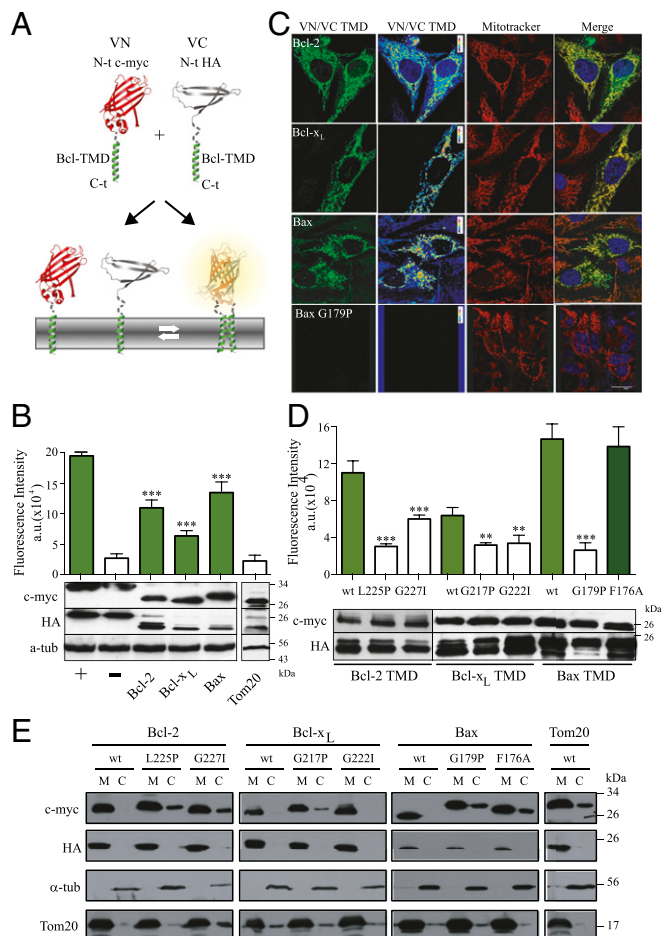


Fig. 2. Bax, Bcl-2, and Bcl- x_L homooligomerize in the OMM. (A) BiFC assay analyzing TMD homooligomerization by reconstitution of Venus fluorescence from separate N and C termini fragments fused to TMD segments. (B) Bax, Bcl-2, and Bcl- x_L TMD homooligomerization measured by BiFC in HCT116 cells. Fusions of b-Fos and b-Jun protein domains were used as positive controls (+), and the Δ b-Fos/b-Jun pair served as a negative control (-); $n = 3$. Significant increases compared with negative control were analyzed by using Dunnett's multiple comparison test (95% confidence interval). Chimeric protein expression of VN (c-myc) and VC (HA) constructs is compared in B, Lower; α -tubulin was used as loading control. (C) Confocal images of HCT116 cells transfected with VC and VN constructs of the Bcl-2 and Bcl- x_L , Bax, and Bax G197P TMDs. Formation of homooligomers (green and rainbow scale, first and second column, respectively) and mitochondria (red, third column), colocalized (yellow, fourth column). (Scale bar, 20 μ m.) (D) Self-association assays of wild-type and single amino acid substitution variants of the Bax, Bcl-2, and Bcl- x_L TMDs measured by BiFC in HCT116 cells. Error bars represent the mean \pm SD, $n \geq 3$. P values according to Dunnett's test are displayed. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (E) Subcellular fractionation of HCT116 cells transfected with TMD constructs was controlled by using Tom20 (mitochondrial fraction; M) and α -tubulin (cytosol; C).

To this end, we analyzed the competition between homomerization of ToxR fusion proteins and their heterooligomerization with fusion proteins with the disabled ToxR DNA binding domain (ToxR*; Fig. 3A). Disabled ToxR* fusion proteins can interact with ToxR proteins in a dominant-negative fashion; the resulting decrease in RFP expression thus indicates the ratio between homooligomerization and heterooligomerization. In this assay, competition between homooligomer and heterooligomer formation with similar affinities results in a 50% decrease in fluorescence (31).

The Bax TMD has a strong tendency to self-associate (Figs. 1B and 3B). As expected, this signal was attenuated by coexpressing

ToxR*/BaxTMD, whereas the ToxR*/GpATMD did not interfere with RFP expression (Fig. 3B). Interestingly, both TMDs derived from prosurvival Bcl-2 or Bcl- x_L proteins interfered with Bax TMD homooligomerization to at least the extent of the Bax TMD. These results suggest a strong capability of the TMDs of prosurvival Bcl-2 proteins to heterooligomerize with the Bax TMD. Accordingly, the Bax TMD reduced Bcl- x_L and Bcl-2 homooligomerization (Fig. 3C and D). Strikingly, the Bcl-2 and Bcl- x_L TMDs did not interfere with each other's homooligomerization, suggesting an absence of heterooligomerization between both prosurvival Bcl-2 TMDs (Fig. 3C and D). These results suggest that the interactions of prosurvival Bcl-2 protein TMDs enable heterooligomerization with Bax. Interactions between the Bax TMD and TMDs of prosurvival Bcl-2 proteins could participate in Bax regulation, because the membrane-bound form of Bcl- x_L has been described to insert only its TMD into the membrane (43). Therefore, the influence of TMD interactions on Bax regulation was tested by analyzing isolated mitochondria permeabilization in HCT116 Bak knockout (KO) cells in the presence or absence of corresponding Bax, Bcl- x_L , Bcl-2, and Fis1 TMD segments. The presence of high Bcl- x_L or Bcl-2 TMD concentrations inhibited the release of Smac from mitochondria (Fig. 3E). These results show that heterooligomerization between Bax TMD and prosurvival Bcl-2 protein

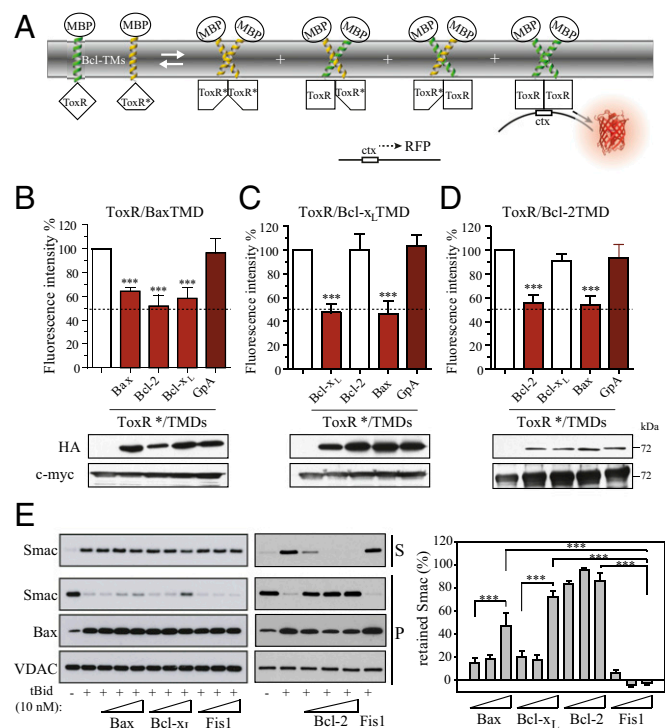


Fig. 3. The Bax TMD interacts with antiapoptotic Bcl- x_L and Bcl-2 TMDs. (A) Dominant-negative ToxR assay to analyze Bcl-2 protein TMD heterooligomerization. Coexpression of TMD constructs fused to wild-type ToxR (green) and TMD constructs fused to an inactive ToxR* mutant (yellow) in *E. coli* can result in heterooligomerization, leading to reduced RFP synthesis. (B) Effect of the Bax, Bcl-2, and Bcl- x_L TMDs on ToxR/Bax TMD homooligomerization. The GpA TMD served as a control. (C) Effect of the Bax, Bcl-2, and Bcl- x_L TMDs on Bcl- x_L TMD homooligomerization as in B. (D) Effect of the Bax, Bcl-2, and Bcl- x_L TMDs on Bcl-2 TMD homooligomerization as in B. The GpA TMD served as a control. (E) Smac release by endogenous Bax with and without tBid in the absence and presence of the Bax, Bcl- x_L , Bcl-2, or Fis1 TMD peptides from purified HCT116 Bak KO mitochondria. Smac was monitored in the supernatant (S) and pellet (P) by Western blot. Bax and VDAC served as controls. Error bars represent the mean \pm SD, $n \geq 3$. P values according to Dunnett's test are displayed. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

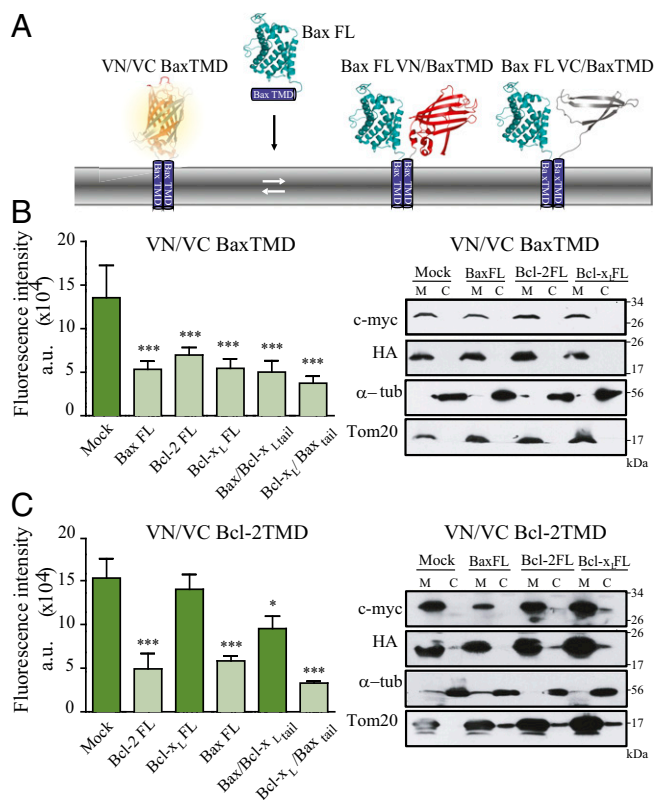


Fig. 4. Bcl-2 protein TMDs interact with full-length Bcl-2 proteins. (A) Heterooligomerization studies with the BifC system using venus fluorescent protein (VFP) reconstituted from VN/VC BaxTMD chimeras and full-length Bax (BaxFL). In this assay, a decrease in fluorescence indicates the formation of heterooligomers between venus-derived chimeras and full-length Bcl-2 proteins. (B) Oligomerization analysis of the Bax TMD in the presence of full-length Bax, Bcl-2, Bcl-x_L, Bax-Bcl-x_L tail, and Bcl-x_L-Bax tail. VFP reconstitution by VN/VC BaxTMD chimeras was challenged with the indicated full-length proteins in HCT116 cells (B, Left). Subcellular fractionation showed mitochondrial (M) localization of VN/Bax and VC/Bax in the absence or presence of full-length proteins. C indicates the cytosolic fraction. (C) Analysis of Bcl-2 TMD oligomerization in the presence of full-length Bax, Bcl-2, Bcl-x_L, Bax-Bcl-x_L tail, and Bcl-x_L-Bax tail proteins in HCT116 cells. Subcellular fractionation showed Bcl-2 TMD-derived constructs localization in the absence or the presence of full-length proteins. Tom20 and α -tubulin served as controls. * $P < 0.05$; *** $P < 0.001$.

TMDs interferes with Bax-induced OMM permeabilization. However, other mechanisms, such as competition for a common binding site or interference in the interaction with other OMM components, could also account for the observed effects. Bcl-x_L TMD-dependent inhibition of OMM permeabilization is not complete, because cyt *c* is still released (Fig. S6). Lack of inhibition by the Bax TMD is particularly interesting, because the peptide concentration exceeded the native Bax protein concentrations. Therefore, symmetric Bax, and perhaps Bak oligomers, as recently suggested (27, 45), could tolerate the association of multiple TMDs. Alternatively, other oligomeric Bax structures could be more prominent in OMM permeabilization.

Bax TMD Interacts with Full-Length Proteins. Next, we tested the potential of Bax, Bcl-2, and Bcl-x_L TMDs to interact with full-length proteins. Self-associating VN/VC-BaxTMD or VN/VC-Bcl-2TMD chimeras that reconstitute the venus fluorescent protein were coexpressed with antiapoptotic and proapoptotic full-length proteins in HCT116 cells (Fig. 4A). BaxTMD homooligomerization was disturbed in the presence of full-length Bax, Bcl-2, and Bcl-x_L (Fig. 4B and Fig. S7A). Therefore, full-length proteins bind to the Bax TMD-derived chimeras, corroborating the interactions

between the isolated TMD segments (Fig. 3 B–D). Interestingly, Bax and Bcl-2 proteins interfered with Bcl-2 TMD homooligomerization, but Bcl-x_L protein did not (Fig. 4C and Fig. S7B). The mitochondrial location of overexpressed TMD constructs was corroborated by subcellular fractionation experiments (Fig. 4B and C, Right). The specificity and TMD dependence of these interactions were tested by replacing the Bax TMD with the corresponding Bcl-x_L TMD segment in the full-length Bax protein (Bax/Bcl-x_Ltail). Bax/Bcl-x_Ltail interfered with Bax, but slightly altered Bcl-2 TMD homooligomerization (Fig. 4B and C). On the other hand, the reciprocal chimera harboring the BaxTMD in full-length Bcl-x_L (Bcl-x_L/Bax_{tail}) bound to Bax and Bcl-2 (Fig. 4B and C). Then, the interaction between the TMDs of Bax and Bcl-2 and full-length proteins is specific and depends on the TMD of the Bcl-2 protein. These results were also corroborated in Bax/Bak double-KO (DKO) cells (Figs. S8 and S9).

Bax TMD Modulates Interactions with Endogenous Proteins and Activates Apoptosis. The Bcl-x_L TMD is involved in Bax retrotranslocation, and we thus focused our studies on the interactions between both TMDs (13). A fusion of the Bax TMD to the C terminus of the biotin ligase BirA (BirA/BaxTMD) biotinylated endogenous Bax and Bcl-x_L in nonapoptotic HCT116 cells (Fig. 5A and B). These results imply binding between Bax TMD and endogenous Bax and Bcl-x_L proteins in the absence of apoptotic stimuli and independent of cytosolic (extramembranous) domain

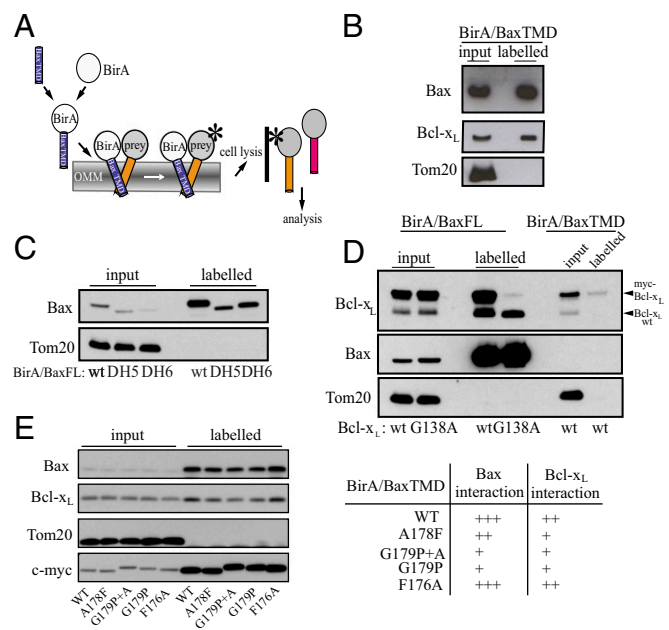


Fig. 5. Bax TMD interacts with endogenous Bax and Bcl-x_L. (A) The BirA in situ proximal biotinylation assay was used to identify interactions between the Bax TMD and endogenous Bax and Bcl-x_L proteins in human cells. After the expression of BirA/BaxTMD, proteins interacting with Bax TMD (blue) were labeled with biotin by BirA. Crude cell extract was subsequently applied to a biotin affinity matrix (solid line). Bound (labeled) proteins were analyzed by SDS/PAGE and Western blot. (B) Input and labeled proteins of the biotin affinity matrix from HCT116 cells expressing BirA/BaxTMD fusion were analyzed by Western blot. Tom20 served as a control. (C) BirA fusion assay analyzing BirA/BaxFL-interactions with wild-type Bax compared with Bax Δ H5 and Bax Δ H6 present in the labeled fraction (L) compared with the input (I) by Western blot. $n = 3$. (D) BirA fusion assay comparing BirA/BaxFL and BirA/BaxTMD interactions with either endogenous Bcl-x_L (Bcl-x_L WT) or ectopically expressed wild-type myc-Bcl-x_L or the myc-Bcl-x_L G138A variant by Western blot. Tom20 serves as control. $n = 3$. (E) The BirA fusion assay was used to identify interactions between Bax TMD mutants and endogenous Bax and Bcl-x_L proteins in human cells.

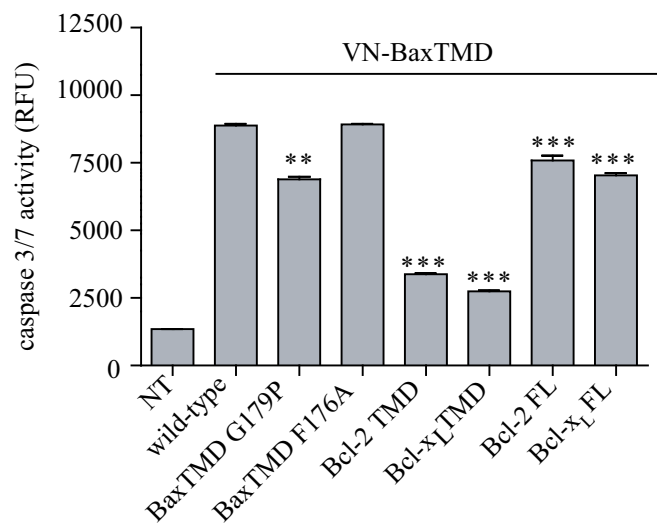


Fig. 6. TMD interactions modulate apoptotic response. Effect of VN/BaxTMD variants, VN/Bcl-2TMD and VN/Bcl-x_LTMD chimeric proteins, or Bcl-2 and Bcl-x_L full-length proteins on caspase-3/7 activity induced by VN-BaxTMD in HCT116 cells is shown. Caspase 3/7 activity was analyzed in cytosolic extracts 24 h after transfection. Error bars represent the mean \pm SD, $n = 3$. P values according to Dunnett's test are displayed. ** $P < 0.01$; *** $P < 0.001$.

interactions. Our results are supported by Bak (and Bax) TMD interfaces mediating homooligomerization in the absence of BH3-dependent interactions (45). Mutant Bax proteins lacking either alpha 5 ($\Delta 5$) or alpha 6 ($\Delta 6$) helices, which are potentially relevant for membrane insertion and pore formation (19, 26, 28, 46), retained the capacity for Bax association in mitochondrial membranes (Fig. 5C), emphasizing the role of the TMDs in early protein interactions. Both BirA/BaxFL and BirA/BaxTMD bound to wild-type Bcl-x_L (Fig. 5D). This binding was strongly reduced when cells were cotransfected with the nonfunctional Bcl-x_L G138A mutant (47). Strikingly, some capacity for heterooligomer formation was retained (Fig. 5D) and matches the Bax TMD capability of oligomer formation, corroborating the contribution of Bax TMD interactions with prosurvival Bcl-x_L. Mutations in the BirA/BaxTMD construct that decrease Bax homooligomerization, such as G179P, provoked a similar decrease in Bcl-x_L biotinylation (Fig. 5E), suggesting at least a partial common interface for heterooligomerization and homooligomerization.

Together, our results demonstrate that interactions between the TMDs of Bax and Bcl-x_L occur in the OMM of human cells before the induction of apoptosis. The analysis of a potential role of TMD interactions in apoptosis induction revealed that the ectopic expression of the BaxTMD in HCT116 cells induces caspase-3/7 activation (Fig. 6). BaxTMD G179P, but not BaxTMD F176A, interferes with this activation, in good agreement with the impact of these mutants on oligomerization in Fig. 2D. Furthermore, cotransfection with antiapoptotic Bcl-2- and Bcl-x_L TMD-derived constructs or with full-length proteins significantly reduced apoptosis activation (Fig. 6). Therefore, Bcl-2 protein TMD interactions are involved in mitochondrial apoptosis signaling.

Conclusion

Antiapoptotic and proapoptotic Bcl-2 proteins regulate mitochondrial apoptosis signaling, and thus the cell fate, by dynamic interactions. Interplay between the BH3 domains and hydrophobic grooves of the respective interaction partner have been characterized (48). In

the present study, we discovered interactions between the TMDs of Bax, Bcl-2, and Bcl-x_L that occur in nonapoptotic cells and modulate mitochondrial apoptosis signaling. The consistent picture that emerges from these studies is that Bcl-2 and Bcl-x_L TMDs could have the ability to regulate Bax pore-forming activity by means of direct competition, leading to the formation of heterooligomers that abate Bax homooligomer formation and OMM permeabilization. The existence of Bax TMD interactions has been proposed based on cross-linking experiments (49, 50) and 3D models (28) and has been suggested to contribute to the enlargement of the Bax pore (51). Although not as tight as hydrophobic groove and BH3 domain interactions, TMD-TMD interactions are sufficient for heterooligomerization and homooligomerization of Bcl-2 proteins. Therefore, Bax dimers and oligomers could facilitate lateral sorting in the OMM or the formation of Bcl-2 protein-containing complexes (52). TMD-TMD interactions are consistent with models of Bax activation, suggesting separation of helices $\alpha 5$ and $\alpha 6$ (27, 53) and the concerted insertion of both helices into the OMM (51, 54). Conversely, Bax activation according to the clamp model would require a sequential mechanism to allow formation of antiparallel TMD interactions (28). The observation of TMD-TMD interactions between Bcl-2 proteins in proliferating cells further emphasizes the necessity to assess the protein conformation of Bax and other Bcl-2 proteins. These new surfaces of protein-protein interaction among proapoptotic and prosurvival members could represent attractive targets for selective drug design.

Methods

Methods are fully described in *SI Methods*. ToxRed chimeric constructs were generated by specific primer annealing of Bcl-2 protein TMDs in the HindIII/XhoI restriction sites of ToxRed vectors (Table 1 and Table S1). The maltose complementation assay was performed as described (39, 55).

ToxRed Oligomerization Assays. ToxR-Bcl-2 TMD constructs (Table S1) were transformed into the *malE* mutant *E. coli* MM39 strain. For RFP measurements, 24-well plates were adjusted to equivalent growth (OD₆₀₀ 0.6–0.8), and the RFP emission spectra were collected by a Wallac 1420 Workstation (λ_{exc} 560 and λ_{em} 595 nm).

BiFC-TMD Assays. BiFC assays were performed as described (56). An improved BiFC assay with a high signal-to-noise ratio was selected to avoid background interference (57, 58). The system was adapted to clone Bax, Bcl-x_L, and Bcl-2 TMDs at the C terminus of venus protein fragments, according to their natural topology in full-length proteins.

BirA Interaction Partner Identification. HCT116 Bax/Bak DKO cells were transfected with pcDNA3-mycBioID-Bax plasmid, resulting in the expression of myc-tagged BirA/Bax fusion. The cell lysate was incubated with streptavidin agarose beads (Thermo) at 4 °C overnight. Input and bead samples were resolved on a 10% (wt/vol) SDS/PAGE and analyzed by Western blot for the indicated proteins.

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