Structural insights of tBid, the caspase-8 activated Bid, and its BH3 domain

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Background: The membrane-associated tBid promotes Bax membrane insertion and activation.

Results: tBid adopts an extended structure in LPPG micelles with its six helices including BH3 domain interacting with the micelles.

Conclusion: An “on the membrane” binding mode was suggested for tBid interaction with Bax.

Significance: Revealing tBid structure on the membrane is key to the understanding of tBid mediated Bax activation.

SUMMARY
The Bcl-2 family proteins regulate mitochondria mediated apoptosis through intricate molecular mechanisms. One of the pro-apoptotic proteins, tBid, can induce apoptosis by promoting Bax activation, Bax homo-oligomerization and mitochondrial outer membrane permeabilization. Association of tBid on the mitochondrial outer membrane is key to its biological function. Therefore knowing the conformation of tBid on the membrane will be the first step toward understanding its crucial role in triggering apoptosis. Here, we present the NMR characterization of the structure and dynamics of human tBid in LPPG micelles. Our data showed that tBid is monomeric with six well-defined α-helices in the micelles. Compared to the full-length Bid structure, a longer flexible loop between tBid helix α4 and α5 was observed. Helices in tBid do not pack into a compact fold but form an extended structure with a C-shape configuration in the micelles. All six tBid helices were shown to interact with LPPG micelles, with helix α6 and α5 being more embedded. Of note, the BH3-containing helix α3, which previously believed to be exposed above the membrane surface, is also membrane associated, suggesting an “on the membrane” binding mode for tBid interaction with Bax. Our data provided structural details on membrane-associated state of tBid and the functional implications of its membrane-associated BH3 domain.

INTRODUCTION
Apoptosis (programmed cell death) plays important role in tissue development and maintenance (1). A group of apoptosis regulator proteins, the Bcl-2 (B-cell lymphoma 2) family proteins, were discovered to work coordinately to modulate mitochondrial outer membrane permeabilization (MOMP) (2-4). Bcl-2 family members share at least one of four BH (Bcl-2 homology) domains and many of them possess a trans-membrane (TM) domain at the C-terminus. Based on their apoptotic function, Bcl-2 family proteins are classified into two groups: pro-apoptotic and anti-apoptotic proteins. A subgroup of the pro-apoptotic proteins, containing only the BH3 domain (e.g. Bid, Bim and PUMA), can directly interact with pro-apoptotic proteins Bax/Bak and promote their homo-oligomerization on the membrane and lead to MOMP (5-7). This represents the major process of the direct activation model for apoptosis initiation. Another competing model, the indirect activation (or displacement) model, suggests anti-apoptotic proteins (e.g. Bcl-2, Bcl-xL, Bcl-w, Mcl-1) can heterodimerize with Bax/Bak to suppress their pro-apoptotic activity (8). Within this model, a sensitizing role was implied for the BH3-only proteins (e.g. Bad), which can bind anti-apoptotic proteins.
proteins, displace Bax/Bak and indirectly promote Bax/Bak oligomerization and MOMP (9-16). Cumulative evidences imply mitochondrial membrane insertion of many Bcl-2 family proteins is critical for their apoptotic functions and the translocation from cytosol to mitochondrial membrane could account for totally different inter-protein interactions among Bcl-2 family members (13,17,18). To accommodate such effects in the context of membrane association, the “embedded together” model was proposed with the recognition that final steps of Bax/Bak activation and MOMP take place on the membrane (13). Currently, little structural information is available on the membrane-associated state of Bcl-2 family proteins compared to their well-studied cytosolic forms (19).

BH3-only protein, Bid, was shown to bridge the crosstalk between extrinsic and intrinsic pathways of apoptosis through its cleavage by caspase-8 (20-22), a downstream mediator of Fas or tumor necrosis factor (TNF) death receptor signaling pathway (23,24). The proteolytic cleavage of cytosolic Bid by activated caspase-8 results in two fragments: p7 (7 kDa) and p15 (15 kDa) (21,25). After the cleavage, the C-terminal fragment (p15), also known as tBid (truncated Bid), translocates to mitochondrial outer membrane (21). The membrane association of tBid was shown to be important for the efficient recruitment of cytosolic Bax to the mitochondrial outer membrane (18,26). Through the interaction with tBid BH3 domain, Bax was suggested to undergo conformational changes, membrane insertion and homo-oligomerization at the mitochondrial outer membrane, mediating the MOMP and cytochrome c release (17,18,26-28). However, studies also suggested tBid could bypass Bax/Bak and directly promote the mitochondrial permeabilization by tBid alone with the induction of the negative membrane curvature (29-31).

In the cytosol, both human and mouse Bid contain eight α-helices with two hydrophobic helices (helix α4 and α5) forming the core of the protein (32,33). After caspase-8 cleavage, Bid N-terminal p7 fragment retains tight association with tBid through primarily hydrophobic interactions (32). Earlier studies suggested that tBid dissociated from the p7 fragment upon contact with the hydrophobic membrane and exposed its hydrophobic p7 binding pocket, promoting tBid to undergo conformational changes on the membrane (34-36). In vitro, isolated tBid was shown to bind to membranes quickly and is competent to activate Bax-mediated membrane permeabilization (18). Previous solid-state NMR study suggested that tBid associated with the membrane with an orientation parallel to the membrane surface and without membrane-traversing helix insertion (37). Additionally, membrane-associated tBid showed helical propensity at Bid helices α6,8 region with helix α6 tilted into the membrane based on the EPR studies (34). However, these studies didn’t provide specific information on tBid helical boundaries, the tertiary fold of tBid and its overall interaction with the membrane. Currently, a proposed model indicated that tBid helices α6,8 are membrane associated, leaving the tBid BH3 domain fully exposed above the membrane surface for potential interactions with other proteins (35). This model is useful to explain some of the observed tBid functional data in solution but provided limited information regarding tBid function on the membrane (35). The exposed conformation of tBid BH3 domain above the membrane surface is energetically unfavorable unless stabilized by other proteins. It is inconsistent with the finding that tBid BH3 domain peptide was membrane associated and was fully competent to activate Bax (38). Thus, it is still not clear whether tBid BH3 domain forms a helix on the membrane and whether its hydrophobic residues that were believed to be involved in the protein interactions in solution behave differently on the membrane by interacting with the membrane instead (20).

In order to address these questions with more structural details, we chose to determine the full-length tBid structure on the membrane along with the characterization of tBid regions that are interacting with the membrane. Here, we used solution NMR methods to characterize human tBid in a membrane mimic, LPPG micelles. Our data showed tBid preserved all six Bid helices (α1 to α6), including the BH3-containing helix α5. Comparing to full-length Bid, the biggest difference was observed for tBid helix α5, which is
a shorter helix with a longer flexible loop connecting it to helix α₄. tBid possessed a C-shape conformation with a close but no direct contact between N- and C-termini of tBid in LPPG micelles as evidenced by paramagnetic relaxation enhancement (PRE) measurements. Furthermore, extensive interactions with LPPG micelles were observed for all six tBid helices. Specifically, the BH₂-containing helix α₃, which previously believed to be exposed outside the membrane, is also membrane associated. The protein-micelle interaction and protein backbone dynamics data suggested helices α₆ and α₂ are likely to be more embedded into the micelles than the rest of the helices. Our findings provided detailed structural information of membrane-associated tBid and suggested the membrane-associated feature of tBid BH₂ domain favors an “on the membrane” binding mode with Bax.

**EXPERIMENTAL PROCEDURES**

**Cloning and Sample Preparation**

The pET15b-Bid\(^{FL}\) vector carrying human Bid cDNA was used to produce full length Bid protein. In order to produce tBid in a more efficient manner without caspase-8 cleavage, we replaced the caspase-8 cleavage site with a TEV protease cleavage site by mutating pET15b-Bid\(^{FL}\) vector (\(^{53}\)YDELQTDG to \(^{53}\)DENLYFQG). Thus the modified full-length Bid can be cleaved by TEV protease and the resulting cleavage product tBid will be the same as the native tBid. The modified plasmid (pET15b-Bid\(^{TEV}\)) was transformed into *Escherichia coli* BL21 (DE3) cells to express recombinant protein. Isotopically (\(^{15}\)N or \(^{13}\)N, \(^{13}\)C) labeled proteins were produced in isotope enriched M9 minimal media. The perdeuterated tBid sample was grown in 99% D₂O minimal media. The *E. coli* cells were grown at 37°C, induced with 1 mM IPTG at OD\(_{600}\) ~ 0.8, and cell growth was continued at 18°C overnight. The cells were harvested by centrifugation, resuspended in lysis buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 5 mM imidazole, 1 mM PMSF) and sonicated. The lysate was centrifuged at 34,000g for 30 mins at 4 °C and the supernatant was loaded to Ni\(^{2+}\) affinity chromatography. The His₆-tagged full-length Bid\(^{TEV}\) was eluted using elution buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 250 mM imidazole) and dialyzed into buffer A (20mM Tris-HCl pH 8.0) before it was further purified by an ion-exchange chromatography on HiTrap 1ml Q column (GE Healthcare) with a linear salt gradient. The purified full-length Bid\(^{TEV}\) was pooled and mixed with 200 units AcTEV protease (Invitrogen) for cleavage by dialyzing against 1 liter TEV cleavage buffer (50 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 1.0 mM DTT) at room temperature overnight. The cleaved protein mixture was loaded on to the Ni\(^{2+}\) affinity chromatography and tBid was separated by washing with lysis buffer with 1.2% (w/v) β-OG (n-Octyl-β-D-Glucoside). The fraction containing purified tBid was collected and dialyzed against buffer (20mM Tris-HCl, 100mM NaCl, pH 8.0) to remove detergent β-OG. After the dialysis, tBid precipitated out of solution and was collected by centrifugation. The NMR sample of tBid with a concentration 0.5-1.0 mM was prepared by dissolving the protein pellet into NMR buffer (50mM Potassium Phosphate, pH 6.6) supplemented with 8% (160 mM) LPPG (1-palmitoyl-2-hydroxy-sn-glycero-3-[phospho-RAC-(1-glycerol)]), 10% D₂O and 0.02% sodium azide. The tBid samples in LPPG micelles were kept at 45 °C at all times for better sample stability.

**NMR Spectroscopy**

All NMR measurements were carried out on a Bruker Avance 600 MHz spectrometer with a room temperature probe, a Bruker Avance 800 MHz or a Bruker Avance 900 MHz spectrometer with cryogenic probes. The following experiments were used for backbone resonances assignments:

- 3D HNCA (39), CBCA(CO)NH (40), HNCACB (41), HBHA(CO)NH (42) and HNCO (43).
- 3D \(^{15}\)N-edited NOESY-HSQC (\(t_{mix}=70\)ms) (42), and 4D \(^{13}\)C/\(^{15}\)N-edited NOESY (\(t_{mix}=70\)ms) (44) experiments were acquired for inter-proton NOE distance restraints.

The backbone \(^{15}\)N \(T_1\) measurement at 800 MHz spectrometer was recorded at various delays: 8, 120, 320, 600, 880, 1200, 1600 and 2240 ms (45). For 600 MHz, \(T_2\) delays used are 8.0, 128, 384, 560, 800, 1040, 1280 and 1600 ms. The \(^{15}\)N \(T_2\) measurement at 800 MHz spectrometer was carried out using CPMG pulse sequence at various total delays: 4.0, 12, 24, 36, 48, 68, 88 and 128 ms (45). For 600 MHz, \(T_2\) delays used were 8.0, 24, 40, 56, 72, 100, 132 and
sulfonate (Toronto Research Chemicals Inc.) at room temperature overnight. The stock of both MTSL and dMTSL reagents had been previously dissolved at 20 mg/ml in acetonitrile. After the reactions, the excess spin label was removed by dialysis against a buffer (20mM Tris-HCl, 100mM NaCl, pH 8.0). Both the paramagnetic and diamagnetic protein samples were further purified in 1.2% β-OG as described above. Experimental errors for the PRE were estimated as previously described (51). The labeling of the tBid samples was confirmed by liquid chromatography/mass spectrometry. 2D $^1$H-$^{15}$N HSQC spectrum of each paramagnetic tBid sample was recorded and no obvious chemical shift changes were observed, indicating spin labeling did not disrupt tBid structure. Solvent PRE experiments were performed by stepwise addition of the watersoluble spin label reagent gadodiamide (aqua[5,8-bid(carboxymethyl)-11-[2-(methylamino)-2-oxoethyl]-3-oxo-2,5,8,11-tetraazatridecan-13-oato (3-)-N$^8$,N$^{11}$,O$^2$,O$^8$, O$^5$,O$^{13}$] gadolinium hydrate; trade name Omniscan, GE Healthcare) into the NMR sample to arrive at final concentrations of 0.25, 0.5, 1.0, 2.0 and 5.0mM. A reference spectrum was recorded prior to the addition of spin label reagent.

**Structure Calculation**

Cross peak intensities from NOESY experiments were fitted and translated into a continuous distribution of proton–proton distances. Based on the assigned tBid backbone chemical shifts, the TALOS program (52) was used to predict $\phi$ and $\psi$ dihedral angles. The statistically significant dihedral angles in regular α-helical secondary structure were used as structural restraints. Generic hydrogen bond distance restraints were imposed for residues located at well-defined α-helical regions. Additionally, PRE relaxation rates were calibrated over residue pairs within one single helix and converted into distances for structure calculation. The backbone $T_1/T_2$ relaxation ratios were also implemented into structure calculation as previously described (53). Planar energy restraints for tBid residues with strong micelle-interactions were also applied by mimicking a close distance with the membrane interface (54). tBid structures were calculated using simulated annealing protocol by the program
RESULTS
Secondary Structure of tBid in LPPG Micelles

The overall secondary structural composition is similar to the previously reported NMR structure of cytosolic full-length human Bid (Fig. 1), but there are some obvious differences in the helical boundaries between tBid and Bid. The most significant difference comes from helix $\alpha_5$. Compared to Bid, tBid helix $\alpha_5$ is a shorter helix and residues S119-D126 are in loop conformation instead of $\alpha$-helical conformation. Of note, weak helical inter-proton NOE distance ($H\alpha(i)$ to $H\beta(i+3)$) were observed for these residues (S119-D126), suggesting a residual $\alpha$-helical character (Fig. 1). This particular feature was also observed for tBid residues adjacent to the N-terminal end of helix $\alpha_3$.

Backbone Dynamics of tBid in LPPG Micelles

The backbone $^{15}$N $T_1$ and $T_2$ relaxations were measured for tBid at both 800 MHz and 600 MHz proton resonance frequencies. The backbone $^{15}$N $T_2$ relaxation data indicated that all tBid helical regions are rigid compared to the non-helical regions (Fig. 2B), and show similar diffusive behavior. This implies that these rigid helices tumble together with the micelle. In contrast, the loop between helices $\alpha_2$ and $\alpha_3$ (N115-D126) are highly flexible with high $T_2$ and low $T_1$ values (Fig. 2), similar to flexible N- and C-termini. The average $T_1$ and $T_2$ relaxation times for the rigid helical regions at 800 MHz frequency are $1.55 \pm 0.21$ s and $38.2 \pm 4.1$ ms, respectively. The calculated $T_1/T_2$ ratios (data not shown) gave an estimate of rotational correlation time ($\tau_c$) of 16.0 ns, which is roughly equivalent to a globular protein with a molecular weight of 43 kDa in solution. The variations in $T_1/T_2$ ratios (data not shown) for the helices in tBid indicate that the molecule is quite anisotropic in LPPG micelles. It is also worth noting that tBid helices $\alpha_5$ and $\alpha_7$ have shorter $^{15}$N $T_2$ values ($33.3 \pm 1.1$ ms) and higher $T_1/T_2$ ratios (55.0 $\pm 2.9$) at 800 MHz frequency than the rest of the helices were observed (Fig. 2). The similar $T_1/T_2$ ratios also suggested helices $\alpha_6$ and $\alpha_7$ have comparable effective correlation times and orient similarly to each other with respect to the rotational diffusion axis. In order to identify residues that experience large amplitude fast motion, residue specific order parameters ($S^2$) were calculated by fitting the relaxation data obtained at the two magnetic fields (Fig. 2C). As expected the exposed loops between helices $\alpha_2$-$\alpha_4$, $\alpha_2$-$\alpha_5$, and $\alpha_3$-$\alpha_6$ have low $S^2$, in addition to the termini. All helices show no evidence of large amplitude fast motion. Initial probe for possible conformational exchange was done by evaluating the ratio of $T_2$ values obtained...
at 600 MHz versus 800 MHz (data not shown). Residues whose $T_2$ ratio lies outside the 1.5 times the standard deviation from the average value are N85, I86, L149, A156, G193, M194, and D195. Residues N85 and I86 are in helix $\alpha_3$, however their $T_2$ ratios are not significantly outside the cutoff. While residues L149 and A156 are in $\alpha_5$, while G193, M194, and D195 are at the C-terminal all show significant deviation. To identify additional residues that might be undergoing conformational exchange as well as to confirm those identified by the $T_2$ ratio at the two magnetic fields, relaxation dispersion experiments were carried out. The relaxation dispersion data (Fig. 2D) suggested a few residues (N124, E145, A156, V159, T173 and I178) undergo $\mu$s-ms time scale exchange. They are identified by choosing a cutoff of $\Delta R_1$ beyond 1.5 times standard deviation. Residues E145, A156 and V159 suggest tBid helix $\alpha_6$ is likely to experience some conformational exchange, which is consistent with the $T_2$ ratio data. Of note, residues N124 at loop between helices $\alpha_4$-$\alpha_5$ and T173 and I178 at helix $\alpha_7$ are also shown to undergo conformational exchange.

**tBid Adopts a C-shape Conformation in LPPG Micelles**

The near complete assignments of the inter-proton cross peak NOE dataset yielded 931 NOEs. Analysis of these inter-proton NOEs did not produce any long-range contacts (greater than 5 residues apart). The absence of long-range NOEs renders the determination of tBid helix $\alpha_6$ to possible. Therefore, longer distance oriented paramagnetic relaxation enhancement (PRE) experiments were performed. Paramagnetic spin labels were incorporated at three tBid sites with cysteine mutation (S78C, Q136C, and Q180C). The backbone $^{1}H_N$-$\Gamma_2$ rate measurements were carried out and the results showed residues close to the paramagnetic center experienced obvious enhancement (Fig. 3). For S78C sample, weak enhancements were shown for residues at tBid C-terminus, suggesting a close conformation but with no NOE contact (<5Å) between tBid N-terminus and C-terminus (Fig. 3). Consistently, weaker PRE effects were shown for tBid N-terminal residues to C-terminal spin label at residue 180 (Q180C sample). For Q136C, weak PRE effects were observed for residues in helices $\alpha_4$ and $\alpha_5$ as well as some residues in the N and C-termini (Fig. 3). Another possible contribution to the observed PRE effects is due to intermolecular contacts. In order to address whether tBid homooligomerizes in LPPG micelles, PRE measurements by mixing $^{15}N$-labeled wild type tBid with spin labeled $^{15}N$-tBid at equal molar ratio were carried out and no obvious enhancement was observed for both S78C and Q136C samples (data not shown). This implied no inter-molecular contact between tBid monomers and therefore tBid does not homooligomerize and is monomeric in LPPG micelles.

**All tBid Helices Are Involved in the Interaction with LPPG Micelles**

In order to understand the molecular interaction between tBid and LPPG micelles, a perdeuterated $^{15}N$-labeled tBid was prepared in LPPG micelles. The 3D $^{15}N$-edited NOESY-HSQC experiment was carried out to detect direct NOE interactions between the protein backbone amide protons and protons from LPPG micelles. The LPPG molecule possesses a long acyl chain with abundant methylene protons, which give a dominant $^1$H NMR signal at chemical shift of 1.26 ppm (Fig. 4A). Extensive analysis of the NOE cross-peaks at this chemical shift yielded a residue specific protein-micelle interaction profile. Due to overlaps with the signals from some tBid Thr H$_{\gamma_2}$ or Ile H$_{\gamma_1}$ protons because of incomplete deuteration, we simply omitted those residues for further consideration of possible protein-micelle interaction. In the end, 28 tBid residues with obvious protein-micelle interactions were identified (Fig. 4B). These tBid residues are mostly hydrophobic residues and are scattered over all six tBid helices: $\alpha_6$ (I86, A87, L90, V93, G94), $\alpha_4$ (V106, L109, A110, L113), $\alpha_5$ (L127, L131, L135), Y138, $\alpha_6$ (L149, L153, A156, K157, V159, A160), S161, $\alpha_7$ (L167, R168, F171, H172, V175, N179), $\alpha_8$ (L182, V186). This strongly suggested those rigid tBid helices are embedded in LPPG micelles through extensive hydrophobic interactions. Among those identified, residues in helix $\alpha_6$ and helix $\alpha_8$ showed noticeable higher relative NOE intensities, suggesting both helices are more embedded in LPPG micelles than other tBid helices (Fig. 4B). This is consistent with previous EPR studies that showed tBid helices $\alpha_{6,8}$
are membrane-associated (34). Of note, three positive charged residues (K157, R168 and H172) in these two helices also showed strong micelle-interactions. Surprisingly, the BH3 domain containing helix α3, which was previously proposed to be exposed and outside the membrane surface, also show extensive micelle-interactions (Fig. 4A). Taken together, tBid helices α3,4 and α5 showed consistent micelle-interaction pattern for hydrophobic residues located at one side of the helix, suggesting tBid helices are embedded parallel to the membrane surface. In contrast, residues (S76, E77, S78, Q79, R118, S119, E120, E121, N124 and G193) in tBid flexible regions including the long loop (N115-D126) between helix α3 and helix α4 clearly did not show any obvious interaction with the micelles. This observation was further corroborated with the strong protein-water interaction for this flexible region (data not shown). In addition, the solvent PRE experiment were carried out and showed consistent limited water accessibility, that is weak solvent PRE effect, for residues that are lipid embedded for all tBid helices and strong PRE effect for water exposed residues (Fig. 4C).

Structure of tBid in LPPG micelles
Structure calculation of tBid were carried out using the following NMR restraints: NOE-derived distances, TALOS-derived dihedral angles, generic hydrogen bonds, PRE-derived distances, T1/T2 derived relaxation restraints (Table 1). With the assumption of tBid helices are embedded parallel to the membrane surface, tBid residues that showed strong micelle-interaction were also implemented into structure calculation with residue-specific planar energy restraints. The calculated 20 lowest energy tBid structures show well-defined individual helices (α3 to α6) with backbone r.m.s.d. of 0.45 ± 0.16 Å, 0.47 ± 0.13 Å, 0.29 ± 0.09 Å, 0.38 ± 0.15 Å, 0.32 ± 0.10 Å and 0.41 ± 0.11 Å, respectively. As previously noted the absence of long-range NOE restraints left the tBid global conformation to be mainly defined by long-range PRE restraints. This resulted in calculated tBid structures with a backbone r.m.s.d. of 3.64 ± 1.37 Å when superimposing all six tBid helices (Fig. 5A). The calculated tBid structures adopted an extended conformation with N-terminal end being close but without direct atomic contact to the C-terminus, thus forming a C-shape structure (Fig. 5A&B). Relative to the membrane interface, tBid helices formed two faces that showed dramatic differences in electrostatic surface potentials (Fig. 5C). One side of the tBid structure that faces the membrane shows dominant hydrophobic patches, which correspond to hydrophobic tBid residues that showed strong interactions to the LPPG micelles (Fig. 5D). Distinct electrostatic surfaces were shown on the other side of the tBid structure with many charged surfaces including the BH3 domain helix α3 (Fig. 5E). With micelle-interacting residues (I86, A87, L90, V93 and G94) in helix α3 facing towards the membrane surface, charged or polar residues (D81, R84, R88, Q92 and D95) are left facing away from the membrane surface for potential electrostatic interaction with other proteins (Fig. 5B&C). Of note, strong positive charged surface areas were formed along tBid helices α5-α6 (Fig. 5E), which could potentially form electrostatic interactions with the negative charged LPPG micelle head groups. This electrostatic interaction would prevent these helices to be completely buried inside the micelles. This is consistent with previous suggested tBid conformation on the membrane by solid-state NMR studies (37). Overall, these structural features displayed by tBid in LPPG micelles are consistent with the fact that most tBid helices are amphipathic.

DISCUSSION
The cleavage of full-length Bid by caspase-8 forms the activated tBid, which can be translocated to the mitochondrial outer membrane (21). The association of tBid to membrane is critical for the promotion of apoptosis (18). Upon the interaction with the hydrophobic environment in vitro, tBid undergoes conformational changes for favorable lipid interactions (34). Similar to full-length Bid, tBid was shown to be highly helical in various membrane mimics (37,58,59). Previously, membrane associated tBid was shown to be parallel to the membrane surface with no trans-membrane helix and the region bound to the membrane was suggested to be located at helices α5-α6 (34,37). Those studies did not provide details on tBid helical boundaries and the membrane-associated full-length tBid structure at atomic level. Especially, no structural information
on the functionally important BH$_3$ domain is yet available. This renders the understanding of the precise apoptotic function of tBid and its molecular interactions with Bax/Bak or Bcl-xL on the membrane impossible. In order to fill this gap, we determined the solution structure of full-length human tBid in a lipid membrane mimic, LPPG micelles by NMR.

Secondary structure characterization by NMR demonstrated that tBid has six well-defined helices, which are mostly preserved from the full-length Bid (Fig. 1). However, some obvious differences were observed between full-length Bid and tBid in LPPG micelles. For instance, tBid residues (S119-D126) close to the N-terminal end of helix $\alpha_5$ did not form a rigid helix (Fig. 1). Comparing to full-length Bid structure, the loop between helices $\alpha_4$ and $\alpha_5$ is much shorter (32) than the loop in tBid. This extended tBid loop between helices $\alpha_4$ and $\alpha_5$ was shown to be highly flexible and did not show any interaction with the micelles, contrasting to the rigid helical conformation in Bid (Fig. 1 & 4). The highly flexible behavior of this loop is likely due to the presence of many charged residues (R118, E120, E121, D122, R123, R125 and D126), which could potentiate electrostatic interactions of tBid with other protein(s). Recent peptide screening proposed an important role for this flexible tBid region (60), through which tBid can directly interact with protein MTCH2 (Mitochondrial Carrier Homologue 2) (36,61). The exposure of this flexible tBid loop enables a scenario where the interaction between tBid and MTCH2 is likely to occur at the mitochondria outer membrane.

The analysis of tBid NOE data did not produce any long-range NOEs, indicating that six well-defined tBid helices do not form a compact structure (Fig. 5). Long-distance oriented PRE measurements, however showed some distances between tBid N- and C-termini within 20-25 Å (Fig. 3). These distances impose a C-shape conformation for tBid on LPPG micelles surface. A rather similar result was also seen previously for the micelle bound $\alpha$-synuclein (62) with a contrast that tBid is more complex with 6 helices. The size of the LPPG micelle was estimated to have a diameter of ~ 68 Å (63). At the longest circumference, it is considerably too large for tBid to wrap around it and form a C-shape configuration as described (Fig. 5). Based on our experimental data, however, we believe the C-shape conformation of tBid is likely due to its characteristic interaction with the curved LPPG micelle membrane within one constrained area on the micelle hemisphere. This is consistent with the previous findings that tBid could colocalize with cardiolipin, a mitochondrial lipid that was previously suggested to induce mitochondrial membrane curvatures \cite{5}, at the mitochondrial outer membrane contact sites with “curvature stress” \cite{64}. Therefore, tBid could be attracted to the contact sites with membrane curvatures on the mitochondria outer membrane, hence sensitizing the mitochondrial membrane for Bax-induced membrane permeabilization.

The tBid $\alpha_6$ helix contains a stretch of hydrophobic residues (L$^{148}$MLVLALLLA$^{156}$) and were predicted to be membrane-associated. Our data indeed showed strong micelle-interactions for hydrophobic residues in tBid helix $\alpha_6$ and helix $\alpha_7$ as well (Fig. 4). The actual tBid membrane interaction site was suggested to be at the mitochondrial contact site with clustered negative charged lipid cardiolipin (64-72). The negatively charged LPPG was chosen not only to provide the best working sample, but as a relevant mimic to the cardiolipin-rich mitochondrial membrane as well. Positive charged lysine residues (K157 and K158) in tBid helix $\alpha_6$ were suggested to be critical for the specific electrostatic interaction with the mitochondrial outer membrane (71,72). Our data confirmed this hypothesis by showing strong micelle-interaction for tBid residue lysine K157 as well as charged residues R168 and H172 in helix $\alpha_7$ (Fig. 4). Consistently, the electrostatic surface map of tBid structure further showed continuous positive charged surfaces along helices $\alpha_{6,8}$ (Fig. 5), which might form electrostatic interactions with negatively charged lipid cardiolipin head groups on the membrane. Furthermore, this additional electrostatic interaction may account for our findings that both helix $\alpha_6$ and helix $\alpha_7$ are more membrane-embedded than the rest of tBid helices (Fig. 2 & 4) and implicate a membrane recognition and docking role for these two helices. The recently
reported fluorescence data showed tBid regions corresponding to Bid helix α1,7 are membrane embedded and also suggested a conformational equilibrium between two major states of tBid (36). Our structure, which show all tBid helices are membrane associated, is consistent with their findings. Furthermore, to be in equilibrium of multiple conformations, tBid structure must have a considerable degree of flexibility. Our structure and dynamic data support this possibility. We showed that tBid adopted an extended membrane-associated structure that contains flexible loops connecting helices α2-α4, α4-α5, and α5-α6. In addition, we also showed that some residues in helix α6 (L149, A156 and V159) and at its N-terminal (E145), the site of membrane targeting, undergo conformational exchange. This result seems to be supporting the conformational equilibrium observation by the fluorescence measurement.

Previous model proposed that tBid BH3 domain was fully exposed and located above the membrane surface, poised for protein interactions with Bax/Bak or Bcl-xL (35). However, our studies revealed that the important BH3 domain containing helix α5 was membrane associated with hydrophobic residues facing toward the membrane surface (Fig. 4 & 5E). In contrast to the latest suggested solvent-exposed BH3 domain (36), our data presented an energetically more favorable conformation for tBid BH3 domain that is membrane-associated due to its residue composition. Therefore, our structure supports a scenario that the interaction between membrane-associated helical BH3 domain and Bax happens on the membrane surface. This “on the membrane” interaction is consistent with the previous observations that membrane-targeted tBid BH3 peptides can still effectively interact with and activate Bax to permeabilize mitochondrial membrane (38,73). Previous studies also suggested that Bax helix α1 interacts with tBid BH3 domain as the first-step in its tBid-induced activation (28,74). Upon the contact with the membrane surface, Bax was suggested to experience a reversible conformational change and its N-terminus can get exposed and recognized by the conformation-specific antibody 6A7 (75). The subsequent membrane insertion and activation of Bax was shown to involve the interaction with membrane associated tBid (18). It is possible that tBid BH3 domain interacts with the transiently exposed Bax helix α1 on the membrane surface and this specific interaction can shift the reversible conformational change of membrane encountered Bax into a membrane-associated state. In fact, a specific electrostatic interaction between tBid BH3 domain residue R84 and Bax helix α1 residue D33 was reported to be important for tBid-induced Bax activation in a previous study (74). Therefore, our results of tBid structure in LPPG micelles in combination with the above reported findings lead to the possibility that charged residues in the tBid BH3 domain (D81, R84, R88, and D95) that were shown to face away from the membrane could potentially form electrostatic interactions with charged residues (D33 and R34) in Bax helix α1 on the membrane surface. If the conventional BH3 binding mode to Bax is required (19,76-78), then the interaction has to occur in such a way to allow the hydrophobic tBid BH3 residues to be available to bind Bax. One possible scenario would be the electrostatic interactions between tBid BH3 domain and Bax helix α1 could promote the displacement of the tBid BH3 residues from the lipid membrane and exposing them for binding to Bax BH3 binding pocket.

For Bcl-xL, it is difficult to discuss its interaction with tBid on the membrane with very limited information on membrane-associated Bcl-xL. However, it is likely that a similar interaction between tBid and Bcl-xL on the mitochondrial membrane was adopted as in the tBid-Bax interaction given the structural similarity between Bax and Bcl-xL.

Overall, Our data provided novel molecular information regarding the structure and dynamics of tBid in LPPG micelles, which leads to a first-step in understanding the tBid apoptotic function on the mitochondrial membrane. Although our findings did not directly address the favorability of a direct or indirect activation model, the membrane-embedded tBid BH3 domain suggested an “on the membrane” binding mode of tBid with Bax, thus leaning towards the previously proposed embedded together model (13). The recently solved X-ray structure of Bax dimer promoted by tBid BH3 peptide and detergent provided valuable
information on the tBid induced Bax activation (79). The reported binding mode with tBid BH$_3$ peptide sitting inside the hydrophobic BH$_3$–binding cleft of Bax is essentially similar to inter-protein interactions among Bcl-2 family members in solution (19,76,77). Nevertheless, how tBid BH$_3$ domain shown in our study to be associated with the micelles can go into the BH$_3$ binding pocket of Bax still remains unclear. Further elucidation of tBid-Bax complex structure on the membrane will provide more molecular details on the mechanism of tBid-mediated Bax activation.

REFERENCES


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FOOTNOTES

This work was supported by the Intramural Research Program of the National Institutes of Health, NHLBI to N. T.
The atomic coordinates and structure factors (code 2M51) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).
The chemical shifts can be accessed through the Biological Magnetic Resonance Bank (BMRB) under BMRB accession number 19054.

FIGURE LEGENDS:

FIGURE 1. Secondary structures of human tBid in LPPG micelles. The secondary chemical shift index ($^{13}$C$_\alpha$, $^{13}$C$_\beta$, $^1$H$_\alpha$ and $^1$CO) defined the presence of six $\alpha$-helices. The chemical shift differences were calculated by subtracting the average random coil chemical shift from the assigned tBid chemical shift values. The consecutive large positive bars in $^{13}$C$_\alpha$, $^{13}$CO and negative bars in $^{13}$C$_\beta$, $^1$H$_\alpha$ suggest the presence of the $\alpha$-helical conformation. The defined $\alpha$-helices were also indicated by the characteristic medium range inter-proton NOE connectivities of H$_\alpha$ to H$_{\beta i+3}$ and strong NOE connectivities of H$_N$ to H$_{i+1}$. The thickness of bars used to show NOE connectivities corresponds to the relative NOE intensity.
The missing NOEs within the α-helical regions are due to overlapped peaks. The secondary structures of human Bid reported previously were also shown for comparison (32). Residue numbering is kept the same as the full length Bid.

**FIGURE 2.** Backbone $^{15}$N relaxation dynamics of human tBid in LPPG micelles. tBid $^{15}$N $T_1$(A), $T_2$(B) relaxation times at two magnetic fields of 600 MHz (red) and 800 MHz (black) and the fitted backbone order parameter $S^2$ using measured $T_1$ and $T_2$ data at both magnetic fields (C) are plotted as a function of residue number, indicating all six ($\alpha_{3,8}$) helical regions were shown to be rigid. The shorter $T_2$ values (B) for both helices $\alpha_3$ and $\alpha_7$ suggested they are likely more embedded in the micelle. Error bar for $^{15}$N-$T_1$ and $T_2$ relaxation data were estimated as previously described (50). (D) The residues undergoing μs-ms conformational exchange identified using the backbone $^{15}$N $R_2$-CPMG rate differences $\Delta R_2$, the difference between measurements at two different $\tau_{cp}$ values ($\Delta R_2=R_2(6\text{ms})- R_2(0.3\text{ms})$). The cutoff line corresponds to the $\Delta R_2$ threshold of 1.5 times the standard of deviation. The missing values are due to resonance peak overlap. Secondary structure elements of tBid are indicated at the top for reference.

**FIGURE 3.** Measured paramagnetic relaxation enhancement (PRE) effects for human tBid cysteine mutants in LPPG micelles at three different sites. The experimental $^1H_N-T_2$ values for S78C (A), Q136C (B) and Q180C (C) were plotted as a function of residue number. Residues experiencing significant PRE effects are mainly localized to residues that are close to the paramagnetic center. For S78C sample (A), weak PRE effects for residues in C-terminus including helix $\alpha_6$. (C) Albeit even weaker, similar weak PRE effects were also observed between tBid N-terminus and C-terminus. For Q136C sample (B), weak PRE effects were observed for residues in helices $\alpha_3$ and $\alpha_4$ as well as some residues in flexible N and C-termini. Error bars were estimated as previously described (51). Secondary structure elements of tBid are indicated at the top for reference.

**FIGURE 4.** Interactions between human tBid and LPPG micelles. (A) Illustration of inter-proton NOEs between tBid BH3 domain and LPPG micelles. A selection of the $^{15}$N-edited 3D NOESY-HSQC strips of the tBid BH3 domain, containing helix $\alpha_3$ residues, showing the intermolecular NOEs to the LPPG acyl chain $^1H$ at chemical shift of 1.26 ppm. Among these residues, I86, A87, L90, V93 and G94 showed obvious NOE cross peaks to LPPG micelles. Conversely, these residues did not show obvious cross peaks signal to water (4.61 ppm). (B) Relative peak intensities are calculated by taking the ratios of measured NOE intensities between protein backbone amides ($^1H_N$) and the LPPG acyl chain methylene (CH2) groups at 1.26 ppm against the diagonal peak intensities. These relative peak intensity ratios are used to quantify the strength of interaction between various sites in tBid and the micelles. Residues showing obvious protein-lipid interactions are from all six tBid helices ($\alpha_{3,8}$). (C) The experimental solvent PRE measurement for tBid backbone amide protons. The measured $^1H_N-T_2$ PRE values in the presence of water-soluble paramagnetic probe showed obvious relaxation enhancement for residues that are water exposed and weak enhancement for residues that are embedded in the lipid. Secondary structure elements of tBid are indicated at the top for reference.

**FIGURE 5.** Calculated human tBid structure shows a C-shape conformation. (A) Cartoon representation of the 20 lowest energy structures of human tBid in LPPG micelles. The calculated tBid structures show six well-defined helices $\alpha_{3,8}$ and the superposition to the mean structure on tBid helical regions results in a backbone r.m.s.d. of 3.64 Å. Both N-terminal (G61-S78) and C-terminal (A190-D195) tails are unstructured and are omitted for clarity. Overall, (B) tBid adopts a C-shape structure with both N- and C-termini are close in conformation. (C) tBid helices ($\alpha_{3,8}$) are parallel to the putative membrane surface with helices $\alpha_6$ and $\alpha_7$ are more embedded. For the membrane-associated tBid helix $\alpha_3$, charged or polar residues (D81, R84, R88, Q92 and D95) are facing away from the membrane surface and are exposed for potential interactions with other proteins. (C) On the opposite side of helix $\alpha_3$, residues (I86, A87, L90, V93 and G94) with strong micelle-interactions are facing toward the membrane surface. The flexible N-
terminus (G61-S78) was omitted for clarity. (D) Electrostatic potential surface view of the side of tBid structure that are facing the membrane shows dominant hydrophobic patches along the ordered tBid helices. (E) The opposite side of the tBid surface shows strong charged areas along tBid helices with a continuous positive charged surface along the helices $\alpha_6$-$\alpha_8$. (D & E) The flexible loop with highly charged surface between helix $\alpha_4$ and helix $\alpha_5$ contains a number of charged residues.
Table 1. Structural statistics of the human tBid in LPPG micelles. Statistics are given for the 20 lowest energy structures out of 200 calculated NMR structures.

**Distance restraints (Å)**

<table>
<thead>
<tr>
<th>Restraint</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOE (930)</td>
<td>0.021 ± 0.003</td>
</tr>
<tr>
<td>Intraresidual NOE (180)</td>
<td>0.007 ± 0.007</td>
</tr>
<tr>
<td>Sequential NOE (</td>
<td>i-j</td>
</tr>
<tr>
<td>Medium range (2 ≤</td>
<td>i-j</td>
</tr>
<tr>
<td>H-Bonds (80)</td>
<td>0.028 ± 0.004</td>
</tr>
<tr>
<td>Paramagnetic Relaxation Enhancement (PRE) (137)</td>
<td>0.017 ± 0.006</td>
</tr>
</tbody>
</table>

**Dihedral angle restraints (°)**

<table>
<thead>
<tr>
<th>Dihedral angle</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>φ (72)</td>
<td>0.157 ± 0.054</td>
</tr>
<tr>
<td>ψ (72)</td>
<td>0.241 ± 0.070</td>
</tr>
</tbody>
</table>

**Other restraints**

<table>
<thead>
<tr>
<th>Restraint</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plane energy (Å) (28)</td>
<td>0.75 ± 0.17</td>
</tr>
<tr>
<td>T₁/T₂ ratio (59)</td>
<td>25.48 ± 3.62</td>
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**Coordinate precision r.m.s.d.**

<table>
<thead>
<tr>
<th>Restraint</th>
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<tbody>
<tr>
<td>Backbone (Å)</td>
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<td>Heavy atoms (Å)</td>
<td>4.24 ± 1.36</td>
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**Ramachandran plot**

<table>
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<th>Region</th>
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</thead>
<tbody>
<tr>
<td>Most favored regions</td>
<td>79.0 ± 2.3%</td>
</tr>
<tr>
<td>Allowed regions</td>
<td>14.1 ± 2.4%</td>
</tr>
<tr>
<td>Generously allowed regions</td>
<td>4.0 ± 2.0%</td>
</tr>
<tr>
<td>Disallowed regions</td>
<td>2.9 ± 1.5%</td>
</tr>
</tbody>
</table>

*Number in a parenthesis corresponds to the total number of restraints. The r.m.s.d. values were calculated by superimposing all six α-helices.*
Figure 2

A

B

C

D

Residue Number

$\alpha_3$ $\alpha_4$ $\alpha_5$ $\alpha_6$ $\alpha_7$ $\alpha_8$

$T_1$ (s)

$T_2$ (s)

$S^2$

$\Delta R_e$ (Hz)

N124 E145 A156 V159 T173 I178
Figure 4
Structural insights of tBid, the caspase-8 activated Bid, and its BH3 domain
Yu Wang and Nico Tjandra

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