Conformational Changes in BID, a Pro-Apoptotic
BCL-2 Family Member, Upon Membrane-Binding:
A Site-Directed Spin Labeling Study

Kyoung Joon Oh\(^1\), Scott Barbuto\(^1\), Natalie Meyer\(^1\), Ryung-Suk Kim\(^2\), R. John Collier\(^3\)
and Stanley J. Korsmeyer\(^1\).†

\(^1\)Howard Hughes Medical Institute, Department of Pathology and Medicine, Harvard Medical School, Dana-Farber Cancer Institute, Boston, MA 02115

\(^2\)Department of Biostatistics, Harvard School of Public Health, Boston, MA 02115

\(^3\)Department of Microbiology & Molecular Genetics, Harvard Medical School, Boston, MA 02115

†Corresponding author: Howard Hughes Medical Institute, Department of Pathology and Medicine, Harvard Medical School, Dana-Farber Cancer Institute, Boston, MA 02115

Telephone: (617) 632-6402; Fax: (617) 632-6401;
e-mail:Stanley_korsmeyer@dfci.harvard.edu

Running title: Conformation of tBID in membranes

Copyright 2004 by The American Society for Biochemistry and Molecular Biology, Inc.
SUMMARY

BCL-2 family proteins constitute a critical control point in apoptosis. BCL-2 family proteins display structural homology to channel forming bacterial toxins, such as colicins, transmembrane domain of diphtheria toxin, and the N-terminal domain of δ-endotoxin. By analogy, it has been hypothesized the BCL-2 family proteins would unfold and insert into the lipid bilayer upon membrane association. We applied the site-directed spin labeling method of electron paramagnetic resonance spectroscopy to the pro-apoptotic member BID. Here we show that helices 6-8 maintain an α-helical conformation in membranes with a lipid composition resembling mitochondrial outer membrane contact sites. However, unlike colicins and the transmembrane domain of diphtheria toxin, these helices of BID are bound to the lipid bilayer without adopting a transmembrane orientation. Our study presents a more detailed model for the reorganization of the structure of tBID on membranes.
INTRODUCTION

Programmed cell death or apoptosis is a normal physiological form of cell death essential for successful embryonic development and the maintenance of cellular homeostasis (1-3). The BCL-2 family is comprised of pro- as well as anti-apoptotic proteins and constitutes critical points in the apoptosis pathway. BID is a pro-apoptotic member of the “BH3-only” subset of the BCL-2 family proteins (4) that interconnects extrinsic pathway TNFR1 and Fas death signals to the mitochondrial amplification of the intrinsic pathway. Engagement of TNFR1 and Fas activates Caspase-8 which cleaves p22 BID within an unstructured loop generating an N-terminal 7 KDa fragment and a C-terminal 15 KDa fragment (5-7). Cleaved BID then undergoes post-translational modification by myristoylation at a newly generated N-terminal glycine of the p15 KDa fragment (p15 BID, or tBID) (8). Myristoylation of the BID complex p7/myr-p15 serves as a molecular switch facilitating the targeting of BID to the mitochondrion. tBID localizes to mitochondrial contact sites where cardiolipin has been reported to exist (9-11). Targeted p15 BID triggers the homo-oligomerization of multidomain pro-apoptotic BAX or BAK in the mitochondrial outer membrane (MOM1) (12-15). This results in the permeabilization of the MOM and release of intermembrane space proteins, including cytochrome c which forms an apoptosome complex with Apaf-1 and Caspase-9 to activate effector caspases (2,3,16,17). In addition to the role of tBID in activation of the critical gateway proteins BAX and BAK, targeted p15 BID also triggers remodeling of the inner membrane and the cristae structure of the mitochondria in a BAX, BAK-independent manner, resulting in the mobilization of the majority of cytochrome c within the cristae which is stored in the intermembrane space of the organelle (18). The actions
of tBID are antagonized by anti-apoptotic proteins BCL-2 or BCL-X\textsubscript{L} (5,7,19). Recently, it has been reported that BID and p15 BID are capable of binding distinct lipids, which led to the hypothesis that BID might play a role in the transport and re-distribution of cardiolipin between the endoplasmic reticulum, cytosol and mitochondria (20-23). However, the precise mechanism of BID insertion into the mitochondrial membrane and its effect on function at that organelle are not completely understood at the structural level.

BCL-2 proteins (24-37) share some structural similarity with certain membrane translocating bacterial toxin domains (24) such as the channel forming domain of colicins (38), the transmembrane domain of diphtheria toxin (39), and the N-terminal domain of \(\delta\)-endotoxin (40). These domains consist of \(\alpha\)-helices in which two contiguous hydrophobic \(\alpha\)-helices form a helical hairpin structure that is surrounded by other helices. The helical hairpin inserts into the membrane in a transmembrane orientation when the toxin molecules bind to the lipid bilayer (41-47). In BID, helices H6 and H7 correspond to the ‘helical hairpin’ region of membrane translocating bacterial toxins and a corresponding region proposed for BCL-X\textsubscript{L}. However, it is not known whether these BID helices insert into the membrane in the same fashion as the membrane translocating toxins.

In order to better understand the mechanism of BID effector function when inserted in the membrane, we applied the site-directed spin labeling (SDSL) method of electron paramagnetic resonance (EPR) spectroscopy (48-51). We generated 37 single-cysteine mutants between residues 147 and 192 of mouse BID in order to scan nearly the entire sequence of the helices H6-H8 with SDSL (Fig. 1). Unexpectedly, we find for full
length BID (p22 BID) in solution that helix 8 is tightly folded against the protein, unlike the NMR model of mouse BID (28). Distance measurements show that for mouse BID the C-terminus of helix H8 is very close to helix H3. We also reconstituted spin-labeled p15 BID in membranes resembling the lipid composition of mitochondrial contact sites (52). Our data demonstrate that p15 BID upon targeting membranes partially unfolds and H6-H8 insert into the bilayer, yet maintaining an α-helical conformation. Surprisingly, the helices do not adopt a transmembrane orientation, but are parallel to the membrane surface with small tilting angles. These results indicate that BID, a Bcl-2 family protein, interacts with the lipid bilayer in a mechanism distinct from membrane translocating bacterial toxins despite their structural similarity.
EXPERIMENTAL PROCEDURES

Preparation of Lipid Vesicles - 

1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (POPG), beef liver phosphatidylinositol (PI), beef heart cardiolipin, beef heart monolysocardiolipin (MCL), cholesterol, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphotempocholine (PC tempo), 1-palmitoyl-2-stearoyl(5-doxyl)-sn-glycero-3-phosphocholine (5-doxyl PC), 1-palmitoyl-2-stearoyl(7-doxyl)-sn-glycero-3-phosphocholine (7-doxyl PC), and 1-palmitoyl-2-stearoyl(10-doxyl)-sn-glycero-3-phosphocholine (10-doxyl PC) were purchased from Avanti Polar Lipids, Inc. N-tempoylpalmitamide was synthesized as described (53). Mixtures of lipids were prepared in chloroform, divided in 50 mg aliquots and dried as thin films in glass test tubes under nitrogen gas. These were further dried under vacuum for 16 hours and resuspended in 20 mM Hepes, 150 mM KCl, pH 7.0 (buffer A, hereafter). The lipid suspensions were freeze-thawed three times and extruded 15 times through two sheets of polycarbonate membrane with a pore size of 100 nm (Avestin) using an extruder (Avanti Polar Lipids), resulting in large unilamellar vesicles (LUVs) (54). Vesicles resembling the lipid composition of the mitochondrial outer-membrane contact sites, designated as OMCT vesicles, were made with a mixture of POPC, POPE, PI, cholesterol and cardiolipin in the weight ratio of 41:22:9:8:20 (9,52). Vesicles resembling the mitochondrial outer-membrane, designated as OM vesicles, were made without cardiolipin by mixing POPC, POPE, PI, and cholesterol in the weight ratio of 61:22:9:8. Vesicles with 20 % POPG, or 15 % MCL and 5 % cardiolipin, designated as OMPG (or OMCL) vesicles, were also made using the same ratio of lipids as in the
OMCT except of cardiolipin. For the immersion-depth measurements, OMCT vesicles containing trace amount (1/1000 by weight) of PC tempo, N-tempoylpalmitamide, 5-, 7-, or 10-doxy1 PC were also prepared in buffer A. The phosphate contents of the vesicles were determined as described (55).

*Expression and Purification of Recombinant Proteins* - Full-length mouse Bid gene was cloned into a modified pET22b vector, which allows the expression of p22 BID as a fusion protein with an amino-terminal hexahistidine tag (56). The complete sequence of the amino-terminal tag is ‘MGSSHHHHHH SSGLVPRGSH’ in a single letter code. The two native cysteine residues C30 and C126 were mutated to serines using Quick Change Site-Directed Mutagenesis Kit (Stratagene). In this cysteine-less clone, designated as p22BID30S126S, cysteine mutations were introduced. The nucleotide sequences were verified by sequencing the entire gene. The p22 BID proteins were expressed and purified according to the pET system manual (Novagen). Proteins were purified from the periplasmic extracts by Ni\(^{2+}\)-affinity and anion-exchange chromatography using an AKTA FPLC system (Amersham-Pharmacia). Proteins were stored at -80 °C in 20 mM Tris buffer (pH 7.5) containing 16 % (v/v) glycerol, 10 mM DTT, and approximately 200 mM NaCl. The p22BID proteins with a C-terminal histidine tag (p22BID-Chis) were also produced using the pET22b expression system. The complete sequence of the C-terminal tag is ‘LEHHHHHH’. A cysteine-less clone, p22BID30S126S-Chis, and its derivative with a G to E substitution mutation at residue 94, p22BID30S126S94E-Chis, were prepared similarly by above procedures. A glutathione S-transferase fusion protein of BCL-X\(_L\) (residues 1-212) lacking the C-terminal transmembrane domain, designated as GST-BCL-X\(_L\)ΔC, was expressed in *E. coli*. 
coli BL21 using pGEX2T (Pharmaci Biotech Inc) and purified by affinity chromatography with glutathione-agarose beads from Sigma. Caspase-8 was purified as described (57). Full-length human BAX protein was purified as described (30). Protein concentrations were determined by Bradford assay with Coomassie Brilliant Blue G-250 dye (Bio-Rad) using bovine serum albumin as a standard (58).

**Peptide synthesis** - Peptides corresponding to the BH3 domains of wild type and mutant human BID, and human BAD were synthesized at the Tufts Peptide Synthesis Core facility. They were purified by reverse-phase HPLC and masses were confirmed by mass spectrometry. The amino acid sequences for BH3 peptides for the wild type BID, Mutant BID and BAD are EDIIRNIARHLAQVGSMDR; EDIIRNIAHAAQVGA$\text{SMDR}_1$; and NLWAAQRYGRELRRMSDEFVD$\text{SFKK}_2$, respectively. The peptides are modified with acetyl and amino groups at the N- and C-terminus, respectively.

**In Vitro Cytochrome c Release Assay** - The biological activity of BID proteins were assessed by the cytochrome c release assay (5). First, recombinant p22 BID proteins were cleaved with Caspase-8. Caspase-8 was mixed with p22 BID (1-2 mg/ml) at 1:100 weight ratio in 20 mM Tris buffer (pH 7.5) containing ~200 mM NaCl and 10 mM DTT. The reaction mixture was incubated for 16 hours at room temperature, resulting in p7/p15 BID complex. Cleavage was verified by polyacrylamide gel electrophoresis. All the protein samples were diluted to a final concentration of 17.6 μg/ml. Next, mouse liver mitochondria were isolated at 4 °C according to the procedures in the literature with minor modifications (5,59). Briefly, the liver was removed from a mouse after sacrifice, minced with scissors, and homogenized in a glass homogenizer in
20 ml ice-cold mitochondria isolation buffer (buffer B, hereafter) containing 250 mM sucrose, 0.5 mM EGTA, and 5 mM 10 mM Tris-HCl. Intact cells and nuclei were pelleted by centrifugation at 600 g for 10 min. The supernatants were further centrifuged at 7,000 g for 10 min to pellet the mitochondria. The mitochondrial pellets were gently resuspended in 10 ml buffer B and centrifuged at 7,000 g for 10 min. The mitochondria were then resuspended gently in 0.3 ml buffer B. The protein concentration of the mitochondrial preparation was determined after solubilizing the mitochondria in 0.1 % deoxycholic acid in phosphate buffered saline (PBS) solution. Mitochondria were then diluted to a final concentration of 0.5 mg mitochondrial protein per ml in buffer C (10 mM Tris-Mops, pH 7.4, 1 mM KH2PO4, 10 μM Tris-EGTA, 5 mM glutamate, 2 mM malate, and 125 mM KCl). Finally, 2 μl of p7/p15 BID was added to 100 μl of the diluted mitochondria, resulting in the BID/mitochondrial protein ratio of 32 pmol/mg. The reaction mixture was incubated at room temperature for 40 min. The reaction mixture was then centrifuged at 12,000 g for 5 min at 4°C to pellet the mitochondria. The supernatants were quickly removed and the pellets were resuspended in the same volume of phosphate buffered saline containing 0.5 % Triton X-100. The amount of cytochrome c in the supernatants or in the pellet fractions was determined in duplicate by an ELISA assay (Quantikine M kit, R&D Systems, Minneapolis, MN).

Spin Labeling p22 BID Cysteine Mutants - Cysteine mutant proteins of p22 BID (1-2 mg) were reacted with 10-20 fold excess of (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)methanethiosulfonate spin label (MTSL) to result in R1 side chain (Fig. 1C) immediately after removing DTT by gel filtration chromatography in buffer A (20 mM Hepes, 150 mM KCl, pH 7.0). Unreacted spin label was removed by gel filtration
chromatography in buffer A. Proteins were concentrated with centrifugal concentrators with the molecular weight cutoff of 5 kDa (Millipore).

Spin Labeling p15 BIDs and their Reconstitution in the Membranes - In order to spin label p15 BID, p22 BID single-cysteine mutants (~5 mg) were first cleaved by incubation with Caspase-8 in the presence of 10 mM DTT as described above. DTT was then removed by passing the reaction mixture through a PD-10 desalting column (Amersham-Pharmacia) after pre-equilibrating it with buffer A. To the eluted protein solution, MTSL was added at 10-fold excess and reacted for 40-60 minutes. Unreacted spin label was removed by passing the reaction mixture through a second PD-10 column pre-equilibrated with buffer A. The spin labeled p7/p15 BID was then loaded onto a column containing 0.5 ml of Ni²⁺-NTA agarose beads (Qiagen) and washed with 10 ml of buffer A. Spin-labeled p15 BID was eluted with 40 ml buffer A containing 2 % n-octyl-β-D-glucopyranoside (OG, Calbiochem). The eluted spin-labeled p15 BID solution was then concentrated using a centrifugal concentrator (MWCO of 5 kDa, Millipore) to a final volume of ~2.5 ml. Protein concentration was determined using bicinchoninic acid protein assay kit (BCA, Pierce) using bovine serum albumin as a standard (60). Large unilamellar vesicles prepared as described above were then added to the concentrated tBID solution at the lipid to protein ratio of 1:10 (wt/wt). The protein/lipid mixture was placed in a dialysis cassette with a 10 kDa molecular weight cut off (Pierce) and dialyzed for 16-24 hours at 4 °C against 1 L buffer A four times to remove the detergent. Protein aggregates that did not incorporate into the membranes were removed by sucrose-step gradient. Briefly, sucrose stock solutions of 2.5, 5.0, 7.5, 10.0, 12.5, and 15 % (wt/vol) were prepared in buffer A containing 0.05 % NaN₃. A volume of one ml of the sucrose
stock solutions was successively layered in an ultracentrifuge tube and the reconstituted p15 BID vesicles were placed on top. This was subjected to centrifugation at 210,000 g for 7-16 hours at 4 °C. Fractions containing reconstituted p15 BID were collected from 5-7.5 % sucrose bands. These fractions were shown to contain large and round vesicles by electron microscopy while fractions in 10-12.5 % sucrose layers contained protein/lipid aggregates. Collected fractions were further centrifuged to pellet the vesicles at 150,000 g for approximately 5 hours after diluting the sample in buffer A in a 5 ml ultracentrifuge tube. For immersion-depth measurements, p15 BID prepared with BID30S126S was reconstituted in OMCT vesicles containing trace amount of spin labeled fatty acid or lipids as described above. After reconstitution, the phosphate content of the vesicles and the protein concentrations were determined as described (55).

**EPR Spectroscopy** - EPR spectra were obtained on a Bruker EMX spectrometer using a Bruker High Sensitivity resonator at room temperature. All spectra were recorded at 2 mW incident microwave power using a field modulation of 1.0-1.5 G at 100 kHz. For power saturation experiments, NiEDDA was synthesized as described (61,62). In order to measure the accessibility parameters, Π, of O₂ and NiEDDA, power saturation experiments were carried out with a loop-gap resonator (JAGMAR, Krakow, Poland) (62-66). The source of oxygen gas was air supplied in house and the concentration of NiEDDA was 5 mM. N₂ gas was used to purge O₂ when necessary. In order to measure the immersion-depths of membrane-inserted R1 residues, air O₂ and 50 mM NiEDDA were used as collision reagents. The range of the incident microwave power was 0.2 to 80 mW for power saturation experiments. Power saturation data were analyzed using the R program (version 1.5.1) (67). Depth calibration curve was determined using the p15
BID-bound vesicles containing spin labeled lipids (61,65). The depth standards used were PC tempo, N-tempoylpalmitamide, 5-doxyl PC, 7-doxyl PC and 10-doxyl PC. Briefly, power saturation experiments were carried out in triplicate for the depth standards to determine the accessibility parameters Π(O₂) and Π(50 mM NiEDDA) with air oxygen and 50 mM NiEDDA, respectively. Φ values defined as log [Π(O₂)/ Π(50 mM NiEDDA)]) were calculated. These values were plotted against the known depths of the standards, which were –5.0, 0.0, 8.1, 10.5, and 14.0 Å for PC tempo, N-tempoylpalmitamide, 5-doxyl PC, 7-doxyl PC and 10-doxyl PC, respectively (68). The data were fitted by the least-square fit. In order to determine the number of spin labels attached to proteins, EPR spectra were taken after liberating the spin labels from the protein molecules by incubating the labeled proteins with 50 μM tris-(2-carboxyethyl)phosphine (Molecular Probes, Inc.). The amount of spin label was calculated by double integration of the EPR spectra using 3-carboxy-proxyl (Sigma-Aldrich) as a standard. The distance between two R1 residues were estimated by a deconvolution method as described (69,70). Briefly, the spectra of singly labeled samples and the spectrum of the doubly labeled samples were obtained for a pair of R1 residues. EPR signals were normalized to the same area by double integration after base line corrections. Depending on the magnitude of the line broadening, various scan widths such as 100, 150 or 250 Gauss were used in order to avoid the base line distortions of the spectra. Using the normalized spectrum of the doubly labeled sample and the sum of the two normalized spectra of the singly labeled samples, the distance distributions between the paired nitroxide residues were calculated by the method developed by Rabenstein et al. (69, 70).
**Fluorescence Dequenching Assay** - We adopted the fluorescence dequenching assay by Terrones et al. (71) in order to assess the biological activity of p15 BID prepared as described above. Briefly, concentrated p15 BID in buffer A containing OG was passed through the PD-10 column that was pre-equilibrated in buffer A containing 2 % OG. FITC-labeled dextran 10 (10 kDa, 100 mg/ml) was entrapped in LUVs with the lipid composition of the OMCT vesicles (50 mg/ml) in buffer A, by 20 cycles of freeze/thaw and 15 extrusions through the two polycarbonate membranes of 0.2 µm pore size (Nuclepore, San Diego, CA). Untrapped FITC-labeled dextran 10 was removed by gel filtration in Sephacryl S-300 HR columns (Amersham). The phosphate content of the vesicles was determined as described (55). The release of the FITC-labeled dextran 10 from the LUVs was monitored in a fluorometer (ISS Inc., Champaign, IL), in a thermostatted 1 cm path length quartz cuvette with constant stirring at 37 °C. Excitation and emission wavelengths were 488 and 525 nm, respectively (slits, 2 nm). The extent of marker release was quantified on a percentage basis according to the equation: \([(F_t - F_0)/(F_{100} - F_0) \times 100]\), where \(F_t\) is the measured fluorescence of reagents-treated LUV at time \(t\), \(F_0\) is the average fluorescence of the LUV suspension for the initial 2 min before reagents addition, and \(F_{100}\) is the average fluorescence value of the final 2 min after complete disruption of LUVs by addition of Triton X-100 (final concentration, 0.66 mM). Lipid concentration was 6.7 µg/ml unless otherwise stated.

**BCL-X<sub>L</sub> Binding assays** – (a) Binding assays with p15 BID: p15 BID prepared with p22 BID 30S126S mutant was reconstituted in OMCT vesicles as described above. In 60 µl of buffer A, GST-BCL-X<sub>L</sub>ΔC (7.8 µM) was allowed to bind to OMCT vesicles or the p15 BID (6.4 µM) reconstituted in OMCT for 2 hrs at room temperature. In the
reaction mixture, the concentration of lipids was 2.2 μg/μl. At the end of the incubation, 60 μl of buffer A was added to the reaction mixture, and the vesicles were spun down by centrifugation in the Beckman A-110 airfuge rotor for 2 hrs at 104,000 rpm. The supernatants were saved, and the vesicle pellets were resuspended in 120 μl Buffer A. The vesicles were pelleted again by centrifugation for 2 hrs. The pellets were resuspended in 120 μl of buffer A. The supernatants (20 μl) from the first centrifugation and the pellets (20 μl) were analyzed by gel electrophoresis using pre-cast NuPage (Invitrogen) gels. Proteins were transferred to the immobilon-P membrane (Millipore).

In order to detect GST-BCL-X_1ΔC, a mouse anti-glutathione S-transferase antibody (BD Pharmingen) and an anti-mouse Ig G coupled to horse raddish peroxidase (Amersham) were used for primary and secondary antibodies, respectively. For the BAD BH3 peptide competition experiments (72), the BAD BH3 peptide stock solution (0.42 mM in dimethylsulfoxide, 1μl) was diluted to a final concentration of 6 μM in GST-BCL-X_1ΔC (5.8 μM) solution in an initial reaction volume of 70 μl for 40 min. Then 50 μl of the p15BID reconstituted in OMCT vesicles were added to the reaction mixture and further incubated for 1 hr. The final concentration of OMCT vesicles was 1.1 μg/μl. The final concentrations of GST-BCL-X_1ΔC, BAD BH3 peptide and p15 BID, were 3.4, 3.5 and 3.2 μM, respectively. The final concentration of dimethylsulfoxide (DMSO) was 0.83 % (v/v). The samples were then subjected to the same procedures for centrifugation, gel-electrophoresis and western blotting as described above.

(b) Binding assays with p15 BID with C-terminal hexahistidine tag (p15 BID-Chis): p15 BID-Chis was reconstituted in OMCT vesicles separately. Briefly, p22 BID 30S126S-Chis, or p22 BID 30S126S94E-Chis was first cleaved by incubation with
Caspase-8 in the presence of 10 mM DTT. After removing DTT with a PD-10 desalting column (Amersham-Pharmacia) pre-equilibrated with buffer A, the sample was loaded onto a column containing 0.5 ml Ni\(^{2+}\)-NTA agarose beads from (Qiagen) and washed with 10 ml of buffer A. The p7 BID was removed by washing with 40 ml of buffer A containing 2 % OG, and the p15 BID-Chis was eluted with 20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, 1 M imidazole. The eluted sample was further desalted using a PD-10 column pre-equilibrated with buffer A containing 2 % OG. This desalted p15BID-Chis sample was used for reconstitution in OMCT vesicles as described above by detergent dialysis followed by ultracentrifugation in sucrose-step gradient. The phosphate content of the vesicles and the protein concentrations were determined as described (55). For binding experiments, GST-BCL-XL\(_{\Delta C}\) (7.26 \(\mu\)M) was first incubated with BAD BH3 (10 \(\mu\)M) or dimethylsulfoxide as a control in 50 \(\mu\)l of buffer A for 1 hr. Vesicles in the same volume were then added and further incubated for 2 hrs. p15 BID molecules were at a final concentration of 3.6 \(\mu\)M. The lipids were at 2.3, 2.3 and 1.5 mg/ml for OMCT, OMCT reconstituted with p15BID, and OMCT reconstituted with p15BIDG94E, respectively. At the end of the incubation, the samples were then subjected to the same procedures for centrifugation, gel-electrophoresis and western blotting as described above. The p15 BID was detected by Western-blotting against the C-terminal histidine tag with a mouse monoclonal antibody from Novagen.

**Circular Dichroism** - Protein samples with or without spin labels were prepared in 10 mM KH\(_2\)PO\(_4\), 50 mM KCl, pH 7. Protein concentrations were determined by the absorbance measurements at 280 nm with the extinction coefficient 8,370 cm\(^{-1}\)M\(^{-1}\) for full-length BID. The concentrations were also verified by Bradford assay with bovine...
serum albumin as a standard (58). All protein samples were diluted to 0.2 mg/ml for CD measurements. CD spectra were obtained in a quartz cell with a 0.5 mm path length using an Aviv model 215 CD spectrometer at 25 °C. Band width of 1 nm was used for all measurements.
RESULTS

Recombinant Single-Cysteine Mutant BID Proteins Release Cytochrome c from Isolated Mouse Liver Mitochondria - To assess whether purified recombinant mutant BID proteins were correctly folded and functional, we performed a cytochrome c release assay (Fig. 2). A cysteine-less BID had C30S/C126S substitutions (referred to here out as the BID30S126S mutant) and demonstrated comparable release of cytochrome c from the isolated mouse liver mitochondria as wt BID, which tested as p7/p15 BID complex targeted to mitochondria. All subsequent single-cysteine mutant sites added to this “cysteine-less backbone” also showed comparable to only slightly reduced activity when compared to wt BID. These results confirm that the introduced mutations did not cause any significant deleterious effect on the structure and function of the proteins.

EPR Data of p22 BID in Solution Reveal the C-terminal Helix H8 Makes Tight Tertiary Contacts at Room Temperature - In solution, the EPR spectra and accessibility parameters of the R1 residues (Figures 3, 4, and 5) were overall consistent with the NMR structural models of mouse BID by McDonnell et al. (28), except for 186R1 and 190R1. The 2-dimensional plot of the accessibility parameters of oxygen and NiEDDA [Π(O2) and Π(NiEDDA)] for the R1 residues of p22 BID in solution (Figure 4) clearly demonstrate that collision reagents oxygen and NiEDDA have varying degrees of accessibility to the R1 chains depending on their topological location. Sites buried within the protein show very limited exposure to the collision reagents, while sites on helical surfaces or in loops show much larger exposures. The mobility of the R1 residues, measured by the inverse of the central linewidth values of the EPR spectra, demonstrates this most clearly (Fig. 5A). Strikingly, the pattern of the mobility as a function of the
residue number (Fig. 5A) is in exact agreement with the accessibility values vs. the residue number (Fig. 5B). Buried residues such as 149R1-155R1 in H6 (residues 142-162) show immobile populations in the line shapes (Fig 3), increased central linewidth (small 1/\Delta H) (Fig 5A) and very limited exposure to oxygen and NiEDDA (Figs. 4 and 5B). Exposed residues such as 148R1 and 158R1 show increased mobility and accessibility values. Residue 163R1, located at the C-terminus of H6, also has high mobility and accessibility values. These data are consistent with the NMR structure (28). However, residue 156R1, which was presumed to be buried, displayed unexpectedly large mobility and accessibility values (Fig 5A and B). The EPR spectrum of 156R1 shows that it is a composite spectrum of multiple components, a very mobile spin population ‘\text{m}’ and immobile populations ‘\text{im}’ (Fig. 3). This indicates that spin-labeling of this position caused significant alterations in the overall structure, resulting in a highly mobile unfolded structure(s) at residue 156. The very mobile component dominated the central line of the EPR spectrum, resulting in apparently increased mobility and accessibility values (Fig 5). This accounts for its characteristics of a helix surface residue instead of a buried residue in Fig. 4. The overall structure of BID30S126S156C, however, appears to be intact as assessed by the CD spectrum (Fig 6A). Moreover, the CD spectrum of the spin-labeled BID30S126S156R1 did not display any significant changes (Fig 6B). The numbers of spin labels per protein molecule was 0.25 (±0.02) for BID30S126S156R1. The lack of any significant differences in the CD spectrum between the labeled and unlabeled BID30S126S156C could reflect this low labeling efficiency or a lack of sensitivity of CD to the structural changes caused by spin labeling.
Residues 170R1-192R1, which encompass nearly the entire helices H7 (residues 167-181) and H8 (residues 183-192), show periodic patterns in mobility and accessibility values (Fig 5). The maxima (or minima) of inverse linewidth, $\Pi(O_2)$ and $\Pi(NiEDDA)$ occur at every 4th residue, indicating that this polypeptide region is on a protein surface in a helical conformation. Interestingly, 186R1 and 190R1 have relatively small mobility and accessibility values that correspond to tertiary contact site residues (Figs. 4 and 5). In Fig. 3, residues 186R1 and 190R1 show EPR spectra for a strongly immobilized and somewhat immobilized side chain, respectively. Introduction of a cysteine residue, or a R1 chain at this position did not appear to cause any significant structural changes based on the CD spectra (Fig. 6A and B). These results are in sharp contrast with the NMR model of mouse BID in solution, in which the C-terminal helix H8 is not in close contact with any other part of the molecule (Fig 7A) (28). We noted that in the NMR model of human BID H8 is folded back close to H3 in a hydrophobic pocket formed by helices 1, 3 and 6 (Fig 7B) (27). In this model, residues 188 and 192, which correspond to residues 186 and 190 in mouse BID, respectively, are indeed in tertiary contacts (Fig 7B). Noting that the NMR data for the mouse BID were obtained at 45 °C (28), we reasoned that at room temperature the C-terminal helix H8 of mouse BID would have a folded conformation similar to the human BID. In human BID, residue 193 on H8 is in close proximity to residues 84, 88, and 92 on helix H3 (Fig. 7B), all within 10 Å between the $C\beta$ carbons. In order to test whether mouse BID has such a structure in solution, we measured distances between the corresponding residues in mouse BID using spin-spin interactions at room temperature (Fig 7A and 7C) (69). When two nitrooxide residues are in close proximity (<25 Å), their EPR spectra become broadened while peak heights...
become reduced due to the interactions of the magnetic dipoles of the unpaired electrons in the nitroxides. The line broadening and peak height reduction can be measured by comparing the spectrum of the doubly labeled protein with the spectral sum of the individually labeled proteins (69, 70). The distance between the two nitroxides can be estimated using these two spectra by the deconvolution method developed by Rabenstein et al. (70). In Fig 7C, the EPR spectra of the doubly labeled p22 BID proteins are superimposed onto the spectral sum of the singly labeled proteins for the three pairs, 191R1/82R1, 191R1/86R1, and 191R1/90R1, respectively. The EPR spectrum of the doubly labeled mutant (red trace), 191R1/82R1 was significantly broadened with much reduced peak heights compared to the sum of 191R1 and 82R1 (black trace, Fig 7C). The estimated distance between the two R1 residues is approximately 6-10 Å. This shows that residue 191 is indeed very close to residue 82 as shown in the NMR structure (Fig 7A). The other pair, 191R1/86R1, also showed significant line broadening and peak height attenuation when compared to the spectral sum of 191R1 and 86R1. The nitroxide pairs are within the distance of approximately 15-20 Å. For the pair 191R1 and 90R1, the line broadening and signal reduction was observable but was less significant. The estimated distance between 191R1 and 90R1 is approximately 20-25 Å. The close distance between 191R1 and 82R1 suggests that H8 is indeed folded back close to H3 in mouse BID at room temperature. The discrepancy between the measured distances in mouse BID and the predicted Cβ- Cβ distances in human BID (Fig 7B) for the latter two pairs suggests that the orientation of H3 with respect to H8 might be different in mouse and human BID. Alternatively, the spin labels might have taken conformations that would make the spin-spin interactions weaker, resulting in longer distances than expected.
We predict that H7 is connected to H8 by a very short loop, involving residues 181-183, similar to human BID in which the side chains of these residues are in tertiary contacts (27). This would result in reduced mobility and accessibility values for the R1 residues at these sites compared to more exposed adjacent ones, 180 and 184, that exist on the termini of the two connected helices H7 and H8, respectively (Fig 5). The mobility and accessibility values of 181R1 and 183R1 are certainly smaller than those of 180R1 and 184R1 (Fig 5). Taken together, our EPR data for residues 170R1-192R1 are consistent with two surface-exposed α-helices (H7 and H8) that are connected by a short loop (residues 181-183) in tertiary contact.

In conclusion, our EPR data are consistent with a structural model in which helix H6 is mostly buried in the protein while helices H7 and H8 are exposed on the surface of the protein connected by a tight loop. Our data also supports H8 being folded rather closely to H3.

*p15 BID Displays High Reconstitution Efficiency in Cardiolipin Containing OMCT Membranes* - We reconstituted spin-labeled tBID in membranes with 4 different lipid compositions; OMCT, OMCL, OMPG, and OM as described in the EXPERIMENTAL PROCEDURES section. In reconstitution experiments using 1 mg p15 BID and 10 mg lipids in a final volume of 2.5 ml buffer A, the amount of p15 BID bound to 1 mg OMCT, OMCL, OMPG and OM vesicles were 105.1 (±5.2) μg, 82.9 (±3.5) μg, 40.9 (±1.2) μg, and 10.9 (±0.7) μg, respectively. OMCL, OMPG, and OM vesicles bound approximately 80 %, 40 %, and 10% of p15 BID compared to OMCT ones. Therefore, OMCT vesicles containing 20 % cardiolipin had the highest reconstitution efficiency among all the vesicle systems, consistent with Lutter et al.(9).
Interestingly, OM vesicles that contained neither cardiolipin nor POPG had the lowest efficiency while the OMPG systems with 20 % POPG had slightly increased efficiency. The OMCT and OMPG vesicles contain approximately the same amount of negatively charged head groups, yet the OMCT vesicles are more efficient in p15 BID reconstitution.

*p15 BID induces pore formation by BAX in OMCT membranes* – In order to determine whether the p15 BID solubilized in OG as described above was functional in activating BAX to form pores, we used the fluorescence dequenching assay reported by Terrones et al. (71). In the presence of BAX, p15 BID induced dequenching of fluorescence of the FITC-labeled dextran 10 molecules (10 kDa) encapsulated in the LUVs made of OMCT membranes, implying leakage of the dextran molecules through the pore formed by BAX (red trace, Fig 8A). BAX alone did not cause any fluorescence dequenching (Fig 8A). OG did not cause BAX to form pores at a concentration comparable to what is present in the p15 BID preparation (Fig 8A). Neither p15 BID nor OG alone induced significant release of dextran (Fig 8B). These results suggest that pores were formed by the concerted action of BAX and p15 BID, releasing the macromolecules as shown by Terrones et al. (71). In order to determine whether the pore formation by BAX and p15 BID was dependent on the BH3 domain of BID, the LUVs were pre-incubated with GST-BCL-X<sub>L</sub>ΔC before addition of BAX and p15 BID (Fig 8A). Interestingly, the pore formation was inhibited by GST-BCL-X<sub>L</sub>ΔC (Fig 8A). The pore formation was no longer inhibited by GST-BCL-X<sub>L</sub>ΔC when BID BH3 peptide with wild type sequence was present (Fig 8A). Mutant BID BH3 peptide, in which two amino acids critical for binding to BCL-X<sub>L</sub>ΔC were substituted with alanine residues as shown
in EXPERIMENTAL PROCEDURES, was not effective in blocking the action of BCL-X₁ΔC (Fig 8A). These results support that BCL-X₁ΔC binds BH3 domain of p15 BID, inhibiting BAX activation by p15 BID. Note that the LUVs remained intact after addition of BID BH3 peptide with wild type or mutant sequence (Fig 8B). They also remained intact by BID BH3 peptide treatment in combination with p15 BID or BCL-X₁ΔC (Fig 8C). These results strongly suggest that the anti-apoptotic BCL-X₁ΔC intercepted the p15 BID-mediated activation of BAX by binding the BH3 domain of p15 BID. A BAD BH3 peptide, which binds to BCL-X₁ΔC more avidly than the BID BH3 peptide (72), also effectively inhibited the action of BCL-X₁ΔC (Fig 8A). The BAD BH3 peptide alone did not permeabilize the membrane (Fig 8B). These results further support the importance of the BH3 domain of p15 BID in BAX activation. In summary, our results show that the p15 BID preparation is functional in activating BAX to form pores in OMCT membranes in a BH3-dependent manner.

_H6-8 of BID, Residues 147-192, Still Maintain their α-Helical Conformation in Membrane_ – In order to determine the conformation of the putative membrane-associated sequences of p15 BID, we generated single cysteine mutants of BID covering nearly the entire helices H6-H8 (Fig 1A and B). The SDSL study was carried with these p15 BID mutants reconstituted in OMCT vesicles. The EPR spectra and the inverse linewidths of their central lines are summarized in Figures 3 and 5A, respectively. In most of the cases, large spectral changes were observed, indicating that conformational changes have occurred involving most of the residues when the proteins were reconstituted in the membrane. This was even more evident from the two-dimensional plot of the Π(O₂) and Π(NiEDDA) (open circles, Fig 4). Many residues distribute near the ordinate axis with
increased $\Pi(O_2)$ and sharply decreased $\Pi(NiEDDA)$ values (Fig. 4), compared to those for solution BID (filled symbols). These data indicate that many R1 residues are now located in environments where the concentration of the nonpolar oxygen molecule is high but the concentration of the polar NiEDDA is low, suggesting insertion of these residues into the hydrophobic compartment of the lipid bilayer. The plot of $\Pi(O_2)$ and $\Pi(NiEDDA)$ vs. residue number (Fig 9A) reveals that the R1 side chains have periodic accessibility to oxygen and NiEDDA when in the membrane-bound state. The maxima (or minima) of $\Pi(O_2)$ and $\Pi(NiEDDA)$ occur at every 3rd or 4th residue and the two curves are opposite in phase (except for residue 180), suggesting that these residues are in an $\alpha$-helical conformation. In Fig 9C, the positions of $\Pi(O_2)$ maxima and $\Pi(NiEDDA)$ maxima are indicated on helical wheel diagrams in circles and squares, respectively. For residues in H6, residues 149, 152, 156, 160 and 163 (circled residues) cluster on one side of the helix while residues 147, 150, 154, 158 and 161 (squared residues) are clustered on the opposite side. Clearly, these data show that the polypeptide sequences are located at an interface between a polar and non-polar environment in an $\alpha$-helical conformation. Similar patterns are also observed from the helical wheel diagrams of H7 and H8 (Fig 9C). $\Pi(O_2)$ maxima occur at residues 170, 174 and 177 (Fig 9A) and they are clustered on one side of the helix H7 (Fig 9C). $\Pi(NiEDDA)$ maxima occur at residues 172 and nearby 176. In H8, $\Pi(O_2)$ maxima occur at residues 183, 186 and 190 and they are clustered on one side (Fig 9A and 9C). $\Pi(NiEDDA)$ maxima occur at nearby residues 184, 188 and 191. These data indicate that H7 and H8 are also located at the interface between a polar and non-polar environment in an $\alpha$-helical conformation. Note that residue 180R1 shows local maxima for both $\Pi(O_2)$ and $\Pi(NiEDDA)$ in the membrane.
(Fig 9A), where the two curves behave in the same phase. This indicates that the two helices H7 and H8 are divided at this position in the membrane. Also note that 180R1 has the second largest \( \Pi(\text{NiEDDA}) \) value and an intermediate \( \Pi(O_2) \) value among all the R1 residues in the membrane (Fig 9A). In the 2-D plot of \( \Pi(O_2) \) and \( \Pi(\text{NiEDDA}) \) (Fig 4), 180R1 is located near the overlapping regions of helix surface and loop sites in solution BID, implying that 180R1 forms a highly water-exposed loop. It is not likely that H7 and H8 form a single stretch of helix located at the water-membrane interface. In that case, a group of membrane-exposed residues 170, 174 and 177 would be on the opposite side of another group of membrane-exposed residues 183, 186 and 190 in a single helix (superimpose the two helical wheel diagrams for H7 and H8 in Fig 9C). Furthermore, the water-exposed residues (squared residues) would interdigitate on or between the membrane-exposed residues. Such a pattern would not be compatible with a single \( \alpha \)-helix lying at the water-membrane interface in any orientation. Therefore, the data are consistent with the existence of two helices H7 and H8.

In conclusion, EPR data show that the three \( \alpha \)-helices H6-8 of p22 BID noted in solution still maintain their \( \alpha \)-helical conformation in the membrane but they are located at the water-membrane interface.

_Helices H6 – 8 of p15 BID are Associated with the Lipid Bilayer, but at Shallow Depths that do not Span the Lipid Bilayer_ - In order to determine the orientation of the membrane-associated helices, we measured the immersion-depth of the membrane-associated R1 residues (Fig 9B and 9C). First, we obtained an immersion-depth calibration curve as described in the EXPERIMENTAL PROCEDURES. The \( \Phi \) values (= \( \log [\Pi(O_2) / \Pi(50 \text{ mM NiEDDA})] \)) for the depth standards, 10-doxy1 PC, 7-doxy1 PC,
5-doxyl PC, N-tempoylpalmitamide and PC tempo were 2.44(±0.09), 1.86(±0.05), 1.45(±0.07), 0.12 (±0.06) and 0.02 (±0.17), respectively in OMCT membranes with p15 BID reconstituted. In the Φ versus depth plot, the least-square fit for the depth range 0 – 14 Å gave a straight line; immersion-depth (Å) = 6.0 Φ – 0.8 (coefficient of determination, \( R^2 = 0.9999 \)). Depth for the headgroup region (-5 – 0 Å) was extrapolated with this curve due to the large variations of the Φ values for the headgroup label, PC tempo. The Φ values were also obtained for the R1 residues, and the immersion-depths were calculated using the calibration curve. The results summarized in Fig 9B and 9C show that H6-8 are overall inserted at depths less than 10 Å in the membrane. In H6, residues 149R1, 152R1, 156R1, 160R1 and 163R1 were in the acyl chain compartment of the bilayer (Fig 9B). Residues 148R1, 153R1 and 159R1 appear to be in the headgroup region. In H7, only one residue 174R1 was buried in the hydrophobic region of the membrane while three residues 170R1, 177R1 and 178R1 were in the headgroup region. In H8, residues 183R1, 186R1 and 190R1 were buried in the acyl chain region at shallow depths and residues 185R1, 189R1 and 192R1 were in or above the headgroup region. Immersion depths of all the R1 residues mentioned above were far less than 20 - 25 Å, an immersion-depth expected for residues residing in or near the core of a lipid bilayer approximately 50 Å thick. Interestingly, the residues buried in the membrane are all clustered on one-side of the helix in each case (Fig 9C). These sites are clustered on the opposite side of the sites of Π(NiEDDA) maxima (Fig 9C, residues in squares). Here it should be noted that the depth measurements were hampered for the rest of the R1 residues mentioned above including those with Π(NiEDDA) maxima. In the presence of 50 mM NiEDDA, the EPR signal of these residues did not saturate within the range of

26
the microwave power in the power saturation experiments, indicating that these residues are indeed highly exposed to water-phase. Taken together, our data clearly show that none of helices H6-8 are deeply buried enough to penetrate to the other side of the membrane. The data are consistent with helices in which only one-side is buried in the membrane at shallow depths while the opposite side exposed to water (Fig 9).

**Helices H6 might be tilted in the membrane surface** - In order to determine the orientation of the helices more precisely, we attempted to fit the immersion-depths of the R1 residues to helical models with various tilting angles and rotational orientations with respect to the membrane. In data fitting, we assumed that the R1 chains extend perpendicularly from the helical axis in an \( \alpha \)-helix as a radial vector (74) (Fig. 10A). We used coordinate transformations to describe the depths of this vector in the lipid bilayers as follows (see the Appendix);

\[
\text{Depth} (N, N_0, r, \theta_0, \omega, p) =
\{(N - N_0) p/3.6\} \sin (\pi \omega/180) - r \cos (\pi \omega/180) \cos \{5\pi (N - N_0)/9 + \pi \theta_0/180\}
\]  

(Eq. 1)

where ‘N’ represents the amino acid residue number, ‘\( N_0 \)’ represents the residue at which the helical axis intercepts the lipid bilayer surface that is the interface between the lipid head groups and the hydrocarbon chains, ‘r’ represents the length of the nitroxide arm, ‘\( \theta_0 \)’ represents the rotational orientation angle in degrees of the residue \( N_0 \) vector with respect to the membrane normal, ‘\( \omega \)’ represents the helix tilting angle in degrees, and ‘p’ represents the pitch of an \( \alpha \)-helix with 5.41 Å for a turn that has 3.6 residues in it. By varying these variables, we obtained fits for depth values of H6, of which a representative one is shown in Fig 9B (solid trace). Although residues 159R1 and 163R1 deviated from the theoretical curve, other values fit fairly well (Fig 9B). For this, we used the following
parameters; \( r = 7.5 \, \text{Å} \) (\( \pm 0.5 \, \text{Å} \)), \( \omega = 25^\circ (\pm 5^\circ) \), \( \theta_0 = 185^\circ (\pm 5^\circ) \), and \( N_0 = 156 (\pm 0.1) \). X-ray crystallographic structures of R1 chains show that the length of the R1 chain is 7-10 Å from the helical axis (73). The 7.5 Å (\( \pm 0.5 \, \text{Å} \)) for ‘r’ is certainly in this range. The model of H6 accounting for these fitted parameters is described in Fig 10B, where H6 helical axis intercepts the membrane surface at residue 156 (\( \pm 0.1 \) (=\( N_0 \)) with a tilting angle (\( \omega \)) of approximately 25° (\( \pm 5^\circ \)) between the helical axis and the membrane surface. The rotational orientation angle (\( \theta_0 \)) of 185° (\( \pm 5^\circ \)) indicates that residue 156 is 5° off from the ‘vector of the greatest depth’ (Fig 10B right panel, dotted arrow), facing down toward the hydrophobic compartment of the bilayer. Taken together, our data suggests that H6 is tilted into the membrane going toward the C-terminus.

The orientations of H7 and H8 could not be determined definitely due to the limited number of reliable depth values (positive ones, Fig 9B). However, it is certain that these two helices are also associated with the membrane at only shallow depths. The EPR spectra of 172R1 and 175R1 show strongly immobile components in the membrane-bound state (Fig 3), indicating tertiary contacts at these sites. Interestingly, these two sites are closely located on one side of H7 (underlined residues in Fig 9C, center panel). This suggests that H7 is in tertiary contact at these sites in the membrane. Residues 172 and 175 are at the helix-helix interface and helix surface, respectively, in the solution structure. In conclusion, our data show that all of the helices H6-8 are associated with the lipid bilayer at shallow depths without spanning the bilayer.

*Helix 3, the BID BH3 domain, moves away from Helix 8 in the membrane* - The conformation of BID H6-H8 shows that large structural rearrangements occur in BID upon reconstitution in the membrane. These structural rearrangements would inevitably
lead to disruption of interactions between BH3 domain (H3) and other parts of BID. We tested if this is the case by measuring the distances between residues on H8 and H3 in the membrane. Three pairs of residues were chosen involving 191R1 on H8 and 82R1, 86R1 and 90R1 on H3 (Fig 7A). The Spectra of doubly labeled samples and the sum of singly labeled samples, normalized to the same area, were superimposable (Fig 7D). These results indicate that the distances between the paired residues are all greater than 25 Å, distances at which the spin-spin interaction is not readily detectable with this method (69, 70). Note that the distances between 82R1 and 191R1, 86R1 and 191R1, and 90R1 and 191R1 were 6-10 Å, 15-20 Å, and 20-25 Å, respectively in p22 BID in solution (Fig 7C). Therefore, H3 is clearly further apart to H8 in the membrane, compared to solution structure (Fig 7C).

The Helix3, the BH3 domain, of p15 BID reconstituted in OMCT Membrane is available for BCL-X_L binding – In order to test whether the disruption of the interactions between Helix 3 and other parts of the p15 BID makes the Helix 3, the BH3 domain, available for interactions with other BCL-2 family proteins, we measured the binding of GST-tagged BCL-X_LΔC (GST-BCL-X_LΔC) to p15 BID reconstituted in the OMCT membrane. As shown in Fig 11A, GST-BCL-X_LΔC bound to the vesicles only when p15 BID was present (see lanes 2, 4 and 6 in Fig 11A; also lane 3 in Fig 11B). The binding of GST- BCL-X_LΔC to the vesicles with reconstituted p15 BID could be inhibited by a BH3 peptide derived from BAD as shown in Fig 11B (lane 5). This indicates that the binding pocket of BCL-X_LΔC was occupied by the BAD BH3 peptide, inhibiting the binding of BCL-X_LΔC to p15 BID. Taken together, these results suggest that the BH3 domain of the p15 BID reconstituted in the OMCT membrane was available for binding to BCL-
X₁ΔC binding pocket. In order to test this, we used a mutant p15 BID that had a glycine to glutamate substitution at residue 94 in the BH3 domain, which abolishes binding to other anti-apoptotic BCL-2 proteins (4) (Fig 11C). As shown in Fig 11C, this single substitution in BID completely abolished the binding of GST-BCL-X₁ΔC to the vesicles (lane 6, top panel). In contrast, p15 BID with a wild type sequence in the BH3 domain showed high binding capacity for GST-BCL-X₁ΔC (lane 4, top panel in Fig 11C). Note that the amounts of p15 BID proteins were comparable in both experiments (lanes 4 and 6, lower panel in Fig 11C). Also note that the BAD BH3 peptide inhibited the binding of GST-BCL-X₁ΔC to p15 BID in OMCT vesicles almost completely (lane 8, top panel in Fig 11C). Combination of G94E substitution and BAD BH3 peptide further reduced the binding (lane 10, top panel in Fig 11C). These results prove that the binding of GST-BCL-X₁ΔC to the vesicles was indeed mediated by the BH3 domain (Helix 3) of reconstituted p15 BID available on the surface of the vesicles.
DISCUSSION

We reconstituted p15 BID in a lipid bilayer with the lipid composition of MOM. We demonstrated that p15 BID used in the reconstitution experiments was functional in activating BAX to form pores (Fig 8). We also demonstrated that the reconstituted p15 BID was able to interact with BCL-XLΔC in a BH3-dependent manner (Fig 11). After demonstrating that our reconstituted system recapitulates some aspects of apoptotic processes, we were able to determine the structural changes of p15 BID upon binding to the lipid bilayer using SDSL method of EPR. Based on the results described above, BID, a globular protein consisting of primarily α-helices in solution, becomes unfolded upon interaction with the membrane. The helices H6 and H7, which form the core of the protein in solution, become exposed to the membrane and adopt non-transmembrane orientations on or near the membrane surface maintaining α-helical conformations upon membrane binding. H6 was tilted into the membrane toward the C-terminus. This may reflect the presence of negatively charged residues at the N-terminus of the helix. Aspartate residues at position 141 and 145 will overlap with residues 159 and 163, respectively, in the helical wheel diagram (Fig 10B). These negatively charged residues would energetically disfavor the insertion of the N-terminus of the helix into the hydrophobic compartment of the membrane, contributing to the tilting of the helix. In the case of H7, only one residue (174R1) reached the hydrophobic compartment while other neighboring residues (170R1, 177R1, and 178R1) were in the head group region. In the solution structure, there is a large kink in H7 around residue 174, where this residue protrudes from the helical axis considerably more than the other residues (28). This conformational irregularity may be preserved in the membrane as well, resulting in the
insertion of 174R1 deeper than the other neighboring residues. Alternatively, it could reflect an artifact generated by SDSL.

We found that our isolated p15 BID mutants still bound to membranes in reconstitution experiments. Lutter et al. (9) and Kim et al. (11) reported helices H4-H6 of p15BID as the minimal domain for mitochondrial targeting. Recently, Hu et al. (75) reported that helices H6 and H7, but not H6 alone, confer the targeting of BID to mitochondria. These results endorse H6 as crucial for membrane targeting and binding. In agreement with these reports, our data show that H6 is inserted into the membrane. Interestingly, there are two lysines in H6 at positions 157 and 158, which would be located near the head group region in the membrane-associated state (Figs 9B and Fig 10B). The positively charged lysine side chains may have electrostatic interactions with the negatively charged head groups of the phospholipids, favoring the association of the helix in the membrane. In addition, the hydrophobic interactions between the helix and the membranes appear to be important. In H6, residues L149, T152, A156, A160, and A163 are inserted into the hydrophobic compartment of the bilayer (Figs. 9B and 9C). Except T152, all the residues have nonpolar side chains. It remains to be determined whether these nonpolar residues and the two lysine residues in H6 have any significant roles in the targeting of BID to the mitochondrial membrane. More specifically, it will be interesting to see if any of these residues play a role in cardiolipin recognition in the membrane.

Despite the limitations to depth and orientation measurements of helices, neither H6, H7, nor H8 are deeply buried enough to penetrate to the other side of the membrane. This is in sharp contrast to the conformation of the membrane translocating bacterial
toxins, in which the helical hairpins insert into the membrane and span the bilayer in a transmembrane orientation (41-47). Recently, based on one-dimensional $^{15}$N chemical shift solid-state NMR spectra of uniformly $^{15}$N-labeled BCL-X$_L$ in oriented lipid bilayers, Franzin et al. (76) proposed that the helical hairpin region of BCL-X$_L$ including helices 5 and 6 inserts into the membrane with the helices tilted at an approximately 40° angle away from the membrane. In this study, however, the NMR peaks were not assigned to the individual residues in helices 5 and 6 and it is uncertain whether helices 5 and 6 were in a transmembrane orientation. If helices 5 and 6 of Bcl-X$_L$ span the membrane as suggested, the mode of interaction of Bcl-X$_L$ clearly differs from BID. Consistent with our findings here, Gong et al., based on CD and NMR experiments, (77) recently proposed that tBID associates with the DOPC/DOPG (60:40) membrane with its helices nearly parallel to the membrane surface and without trans-membrane helix insertion. In our study using SDSL, we clearly demonstrated that helices 6-8 still maintain helical conformations in the membrane and determined their rotational orientations, tilting angles and immersion-depths in the membrane in greater detail. Here one cannot rule out the possibility that the spin-labeling reaction might have interfered with the membrane binding of p15 BID in some cases. Cytochrome c release assays with the spin-labeled p7/p15 BID mutants were difficult to carry out due to the technical difficulties in preparing spin-labeled p7/p15 BID. The reducing agent DTT necessary for caspase-8 digestion of the p22 BID released the spin labels from the pre-labeled p22 BID proteins. Stoichiometric labeling of the cysteine residues in the p7/p15 BID mutants was also difficult to achieve. The accessibility data and depth data, however, appear to be overall
self-consistent with a model in which the helices are associated with the membrane at shallow depths, which is also consistent with the reports by Gong et al. (77).

The structural changes that accompany the insertion of BID H6-H8 into membranes provide an important clue as to how the BH3 domain (helix H3) might be presented on the surface of the membrane. We were able to confirm the folding of H8 close to H3 in solution by the accessibility parameters (Fig 5) and by spin-spin interactions (Figure 7). In the solution structure, H3 also makes contacts with helices H1, H6 and H7 (28). Upon membrane binding, H8 appears to be freed in the membrane since the EPR spectra show high mobility for all the residues 184R1-192R1 (Fig 3). Furthermore, the hydrophobic interactions at the interface between H6 and H7 are disrupted and the interfacial residues become exposed to lipids. This involves the rotation of the two helices, H6 and H7, with respect to each other. These large structural rearrangements would naturally lead to the disruption of interactions between BH3 domain (H3) and other helices (H6, H7 and H8) of BID. Distance measurements using spin-spin interaction clearly demonstrate that H3 moves away from H8 in membrane (Fig 7), which would make BH3 domain available to BAX or BAK, leading to their activation. Alternatively, the exposed BH3 domain could be intercepted by anti-apoptotic BCL-2 or BCL-XL, to sequester BID and protect cells. Our finding that BCL-XL bound to the BH3 domain of the reconstituted p15 BID strongly supports this (Fig 8).

Scorrano et al. (18) reported that p15 BID also triggers remodeling of the inner membrane and the cristae structure of the mitochondria in a BAX-, BAK-independent manner. The topology of p15 BID elucidated by our SDSL study indicates that H6-H8 do not span the lipid bilayer. Furthermore, other helices H3-H5 are amphipathic and
none of them are long enough to span the bilayer. These findings warrant the search for a mediator that would relay the signal from the outer membrane where BID is targeted to the inner membrane that constitutes the cristae.

There have been reports of channel-formation or membrane destabilization by BID (78,79). Schendel et al. (78) described channel-forming activity of Caspase-8 cleaved BID, or BID lacking 55 amino-terminal residues in planar lipid bilayers at acidic and neutral pH. Kudla et al. (79) reported that p15 BID permeabilizes liposomes at physiological pH, which is inhibited by the amino-terminal p7 fragment. Recently, Terrones et al. (71) reported that tBID alone does not form pores in the membrane, but it activates BAX to form large lipic pores in membranes permeable to dextran molecules of 10-70 kDa. This report contradicts previous reports on tBID’s channel forming activity. Based on the topology of helices H6-H8, it is uncertain how these helices might be involved in channel formation or membrane destabilization. Further studies are necessary to understand these phenomena.

In summary, using a SDSL approach we were able to determine the conformation of helices H6-H8 both in solution and of p15 BID reconstituted in membranes. This study reiterates the power of the SDSL method in elucidating structures of biopolymers including proteins. Our results also provide important insights as to how BID functions as a pro-apoptotic cell molecule.

*Acknowledgements* - This work was supported in part by NIH grant PO1 CA68484 and the Dana-Farber Cancer Institute Multidisciplinary High-Tech Fund. The authors are grateful to Drs. Christian Altenbach, Yeon-Kyun Shin and Melanie Ohi for helpful
discussions. The authors thank Dr. Olga Gursky at the Boston University for CD measurements. The authors also thank Dr. Emily Cheng and Savita Dandapani for their helps.

Appendix – The coordinates of a position on a helix with a radius ‘r’ and a pitch ‘p’ can be described using a cylindrical coordinate system as shown in Fig 11A. Assume that a radial vector with length ‘r’ rooted at the helical axis glides on the Y-axis to trace the helix. The position \((x, y, z)\) of a point representing a residue \(N\) on the helix can then be expressed as follows;

\[
(\mathbf{x}, \mathbf{y}, \mathbf{z}) = (r \sin (\pi \theta/180), (\theta-\theta_0)p/360, r \cos (\pi \theta/180)) \quad \text{(i)}
\]

We assume that the X-axis remains in the surface of the bilayer, defined as the plane between the headgroups and the acyl chains of the lipids in the membrane. If the helix is now rotated clockwise through X-axis by \(\phi\) degrees, the new position of the residue \(N\) can be described by a coordinate transformation as follows;

\[
(\mathbf{x'}, \mathbf{y}, \mathbf{z'}) = \\
(x, y \cos (\pi \phi/180)+z \sin (\pi \phi/180), -y \sin (\pi \phi/180)+z \cos (\pi \phi/180)) \quad \text{(ii)}
\]

Now, we find that the ‘immersion depth’ of the position for residue \(N\) is equal to \(-z'\), which corresponds to the distance from the XY plane (i.e., the membrane surface) to the residue.

Immersion depth = \(-z'\) = \(y \sin (\pi \phi/180) - z \cos (\pi \phi/180)\) \quad \text{(iii)}

In an \(\alpha\)-helix, there are 3.6 amino acid residues per turn, i.e., per 360° gliding, which means 100° gliding per residue. Therefore we have the following relationship;

\[
(\theta - \theta_0) = 100 (N - N_0) \quad \text{-------------------------(iv)}
\]
and \[ \theta = 100 (N - N_0) + \theta_0 \] \hfill (v)

where \( N_0 \) is the position of the amino acid at which the helical axis intercepts the membrane surface (XY-plane) and \( \theta_0 \) represents the initial angle of the radial vector from the YZ-plane.

Substituting \( \theta \) in eq (i) with eq (v), we get the following:

\[
y = (\theta-\theta_0)p/360 = (N - N_0) p/3.6 \hfill (vi)
\]

\[
z = r \cos (\pi\theta/180) = r \cos \{5\pi (N - N_0)/9 + \pi\theta_0/180\} \hfill (vii)
\]

Substituting \( y \) and \( z \) in eq (iii) with eqs (vi) and (vii), we get

\[
\text{Immersion depth} = \\
\{(N - N_0) p/3.6\} \sin (\pi\omega/180) - r \cos (\pi\omega/180) \cos \{5\pi (N - N_0)/9 + \pi\theta_0/180\} \hfill (viii)
\]

This is Eq 1.
REFERENCES


FOOTNOTES

The abbreviations used are: MOM, mitochondrial outer membrane; SDSL, site-directed spin labeling; EPR, electron paramagnetic resonance; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)]; PI, phosphatidylinositol; MCL, monolysocardiolipin; PC tempo, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphotemepocholine; n-doxyl PC, 1-palmitoyl-2-stearoyl(n-doxyl)-sn-glycero-3-phosphocholine where n = 5, 7, or 10; OMCT, mitochondrial outer-membrane contact site; OM, mitochondrial outer-membrane; IPTG, isopropyl-β-D-thiogalactopyranoside; FPLC, fast performance liquid chromatography; DTT, dithiothreitol; OG, n-octyl-β-D-glucopyranoside; MTSL, (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)methanethiosulfonate spin label; MWCO, molecular weight cut-off; NiEDDA, nickel(II)ethylenediamine diacetate; CD, circular dichroism;
FIGURE LEGENDS

Fig. 1. (A) Amino acid sequence alignment of mouse (m) and human (h) BID proteins of known structures in solution (27,28). The human BID is numbered according to Chou et al (27), in which two amino acids are added at the N-terminus for recombinant protein production. Identical sequences are shaded in grey. The underlined sequences, also shaded in red, represent α-helices from the solution structures. (B) Sites of site-directed spin labeling. Sites of single-cysteine mutagenesis are shown in yellow spheres in the ribbon diagram of a mouse BID model (28). Residues 147-161, 163, 170, 172-181, and 183-192 were mutated to cysteines as described in the EXPERIMENTAL PROCEDURES. H1-H8 represent α-helices. (C) Spin labeling reaction. Spin label (I) reacts with the thiol group of a cysteine residue to form the R1 side chain.

Fig. 2. Cytochrome c release assay. The percent of cytochrome c released from the isolated mouse liver mitochondria are shown for wild type BID (wt BID), cyteine-less BID (BID30S126S) and all the single-cysteine mutants. p7/p15 BID, generated by cleavage of p22 BID by Caspase-8, was added to the mitochondria at 32 pmol/mg ratio. The percent of cytochrome c released was determined by Elisa after 40 min incubation. Error bars represent the range of data for duplicate experiments.

Fig. 3. EPR spectra of the R1 side chains. Black traces represent spectra for R1 residues of p22 BID in solution. Thin Red traces represent spectra for p15 BID reconstituted in vesicles resembling the lipid composition of the mitochondrial outermembrane contact sites (OMCT). Characteristic features of a highly immobile
spectrum (p22 BID I150R1) and a highly mobile one (p22 BID Q180R1) are indicated by arrows as an example. In A156R1 of p22 BID, highly mobile and immobile components are indicated by ‘m’ and ‘im’, respectively. 100 Gauss scan.

Fig. 4. **2-Dimensional plot of the accessibility parameters $\Pi(O_2)$ and $\Pi(\text{NiEDDA})$ for the R1 side chains.** Filled symbols are for p22 BID in solution. Open circles are for p15 BID in OMCT vesicles. The numbers denote the residue numbers for the data points. Italicized numbers are for p15 BID in OMCT vesicles. The locations of the residues in solution mouse BID models (28) are grouped in 4 categories; ‘buried’, ‘tertiary contact’, ‘helix surface’, and ‘loop’. Residue numbers 156, 186 and 190 are underlined where the mouse BID NMR model and the EPR data are not compatible. Note that the ordinate and the abscissa are in two different scales divided at the value of 0.2 in order to resolve the data points clearly. Air oxygen and 5 mM NiEDDA were used to measure the accessibility parameters.

Fig. 5. (A) Inverse of the central line width of the R1 side chains. Solid circles denote p22 BID samples in solution. Open circles denote p15 BID samples reconstituted in OMCT vesicles. (B) Accessibility parameters $\Pi(O_2)$ and $\Pi(\text{NiEDDA})$ for R1 residues in p22 BID in solution. Solid circles and triangles represent $\Pi(O_2)$ and $\Pi(\text{NiEDDA})$ for R1 residues of p22 BID in solution, respectively.

Fig. 6. **Circular dichroism (CD) spectra of BID proteins.** (A) CD spectra of BID 30S126S, BID30S126S156C, BID30S126S186C, and BID30S126S190C. (B) CD
spectra of BID30S126S, BID30S126S156R1, BID30S126S186R1, and BID30S126S190R1. In B, the numbers of spin labels per protein molecule were 0.25 (±0.02), 0.83 (±0.07), and 0.82 (±0.04) for BID30S126S156R1, BID30S126S186R1, and BID30S126S190R1, respectively. The numbers in parentheses are the range of data for duplicate experiments. Protein concentrations were 0.2 mg/ml for all the samples.

Fig. 7. **Distances between helices H8 and H3 of p22 BID in solution and p15 BID in the membrane measured by spin-spin interactions.** (A) Cβ-Cβ distances of 3 pairs of residues in one mouse BID model (28). (B) The Cβ-Cβ distances of corresponding pairs of human BID (27). (C) The EPR spectra for the 3 pairs of R1 residues 82+191, 86+191 and 90+191 in mouse BID. Black traces represent the spectral sum of singly labeled proteins. Red traces represent the spectra of doubly labeled proteins. Scan widths were 250 G for 82+191, and 150 G for 86+191 and 90+191, respectively. Spectra were normalized to the same area. The estimated inter-spin distances for the R1 pairs are shown in Å. (D) The EPR spectra for the 3 pairs of R1 residues 82+191, 86+191 and 90+191 in mouse p15 BID reconstituted in OMCT vesicles. Black traces represent the spectral sum of singly labeled proteins. Red traces represent the spectra of doubly labeled proteins. Scan widths were 100 G for all pairs. Spectra were normalized to the same area. The estimated inter-spin distances for the R1 pairs are shown in Å.

Fig 8. p15 BID activates BAX to form large pores in the membrane. The release of FITC-labeled dextran 10 (10 kDa) was measured by fluorescence dequenching assay as described in the Experimental Procedures. (A) LUVs of OMCT membrane encapsulating
FITC-dextran 10 molecules were brought to 37 °C before the measurements started. BAX was added to a final concentration of 50 nM at 120 sec as indicated by the arrow. p15 BID (126S mutant) (100 nM, red trace) or other reagents (0.03 % n-octyl-β-D-glucopyranoside, pink trace; 1 μM BID BH3, black trace) were added at 420 sec as shown. For the other measurements, the vesicles were pre-incubated with 400 nM BCL-XlΔC in the absence or presence of 1 μM BH3 peptides as indicated before the measurements started. BAX and p15 BID were added at the indicated times as shown. Triton X-100 was added to a final concentration of 0.66 mM at 2700 sec (not shown). Measurements were terminated at 3000 sec. Also see the captions in the box for the traces arranged from top to bottom. (B) LUVs of OMCT membrane encapsulating FITC-dextran 10 molecules were brought to 37 °C before the measurements started. p15 BID (100 nM, black trace), n-octyl-β-D-glucopyranoside (0.003 %, pale blue trace), or various BH3 peptides (1 μM, red trace for BAD BH3; dark blue for BID BH3; green for mutant BH3) was added to the vesicles at the time indicated by an arrow. Triton X-100 was added to a final concentration of 0.66 mM to disrupt the vesicles at 800 sec (not shown). Measurements were terminated at t = 950 sec. (C) LUVs of OMCT membrane encapsulating FITC-dextran 10 molecules were brought to 37 °C in the presence of 1 μM BID BH3 peptide before the measurements started. p15 BID (100 nM, black trace), or BCL-XlΔC (400 nM, red trace) was added as indicated at 120 sec (arrow). Triton X-100 was added to a final concentration of 0.66 mM to disrupt the vesicles at 800 sec (not shown). Measurements were terminated at t = 950 sec.
Fig. 9. (A) Accessibility parameters $\Pi(O_2)$ and $\Pi(\text{NiEDDA})$ for R1 residues in p15 BID reconstituted in OMCT vesicles as a function of residue number. (B) Immersion-depth of the lipid-facing residues of p15 BID reconstituted in OMCT vesicles (C) Helical wheel diagrams for $\alpha$-helices H6, H7 and H8. In (A), air oxygen and 5 mM NiEDDA were used to measure the accessibility parameters. The positions of $\Pi(O_2)$ maxima are marked with vertical dotted lines. In (B), a depth calibration curve, immersion-depth ($\text{Å}$) = 6.0 $\Phi$ – 0.8 was used to determine the immersion depths of the R1 residues. Average of two measurements are reported with error range. Approximate immersion-depth range for the headgroup and the acyl chain regions are indicated. In (C), the residue numbers of $\Pi(O_2)$ maxima or near maxima in Fig 9A are in circles. The residue numbers of $\Pi(O_2)$ minima (or $\Pi(\text{NiEDDA}, 5 \text{ mM})$ maxima) are in squares. The average depths of R1 chains are also shown in Å.

Fig. 10. (A) Description of positions of a R1 side chain in cylindrical coordinates. ‘N’ represents the amino acid residue number, ‘$N_0$’, the residue at which the helical axis intercepts the surface of the lipid bilayers that is the interface between the lipid head group and the hydrocarbon chain, ‘r’, the length of the nitroxide arm, ‘$\theta_0$’, the rotational orientation angle of the residue $N_0$ vector with respect to the membrane normal, ‘$\omega$’, the helix tilting angle, and ‘p’, the helical pitch, 5.41Å, for 3.6 residues rise for a turn in an $\alpha$-helix. (B) Tilting angle of helix H6 (left panel) and a helical wheel for helices H6 (right panel). In the left panel, the large arrow denotes the helical axis drawn from N- to C-terminus, which is 25 ° ($\pm 0.5$ °) (= $\omega$) tilted from the membrane surface. The small arrow denotes the radial vector of R1 residue with the length of 7.5 Å ($\pm 0.5$ Å) (= r) at
residue 156 (±0.5) (= N₀), at which the helical axis crosses the headgroup/acyl chain (shaded area) interface. In the right panel, the helical wheel is labeled with the residues in H6. The dotted vertical arrow represents the direction of the greatest depth of the helix viewed through the helical axis. The arrow crossing the residue 156 represents the R1 radial vector at 185 ° (±5 °) (= θ₀) away from the plane perpendicular to the membrane surface.

Fig. 11 (A) Binding of glutathione S-transferase fusion protein of BCL-X_L with C-terminal truncation (GST-BCL-X_LΔC) to OMCT vesicles or OMCT vesicles with p15 BID reconstituted (OMCT/p15 BID). (B) Inhibition of GST-BCL-X_LΔC binding to p15 BID reconstituted in OMCT vesicles by BAD BH3 peptide. (C) Binding of GST-BCL-X_LΔC to OMCT vesicles or OMCT vesicles with p15 BID mutants with C-terminal his tag (p15BID-Chis). The binding experiments were carried out as described in the EXPERIMENTAL PROCEDURES. In (A), (B) and the top panel of (C), bands of BCL-X_L protein fused with glutathione S-transferase are shown, detected by western-blotting with an antibody against the glutathione S-transferase (α-GST). In (C), the bands in the lower panel represents p15BID-Chis proteins detected by an antibody against hexahistidine tag (α-His tag). ‘S’ represents the supernatant solution fraction after pelleting the vesicles by centrifugation. ‘P’ represents the pellet fractions containing vesicles sedimented by two centrifugation runs. ‘C’ represents loading control. The ‘+’ and ‘−’ signs represent the presence or absence of the reagents indicated. OMCT represents OMCT vesicles. OMCT/p15 BID represents OMCT vesicles with p15 BID reconstituted. In (A) and (B), p15BID was prepared from the N-terminally his-tagged...
p22BID. In (C), p15BID was derived from p22BID with a C-terminal his-tag (see EXPERIMENTAL PROCEDURES). All the proteins had 30S126S substitutions. p15BIDG94E had an additional glycine to glutamate mutation at residue 94 in the BH3 domain, which abolishes the BCL-\textit{X}_{L} binding.
A

mBID: --MDSEVSNGSGLGAKHITDLLVFGLQSGCT----ROELEVGLRELPVQ-A-YWBEADLE
hBID: GSMDCEVNNSSIRLDECTNLLVFGLQSS--CSDNSFRRLEDALGHELPVLAPQWE--Y

mBID: DELQTDGSQAARSFSNQGRIEPSESDESEEIIHNIARHLAQIGDEMDHNIQPTLVRQLAQOF
hBID: DELQTDGRSSHS-RLGRIEADSESQEDIRNIARHLAQVGDSMDRSIPGLVNGLALQL

mBID: MNGLDSEEDKRNCALKALDEVKTAFFPRDMENDKAMLIMTLMLAKKVASHAPSSLRDFVHT
hBID: RNTSRSEDNRDLATLEVLLQLQYPRDMEKETMLVALLAKKVASHTPSLLRDFVHT

mBID: TVNFNFQNLFSYVRNLVRNEMD
hBID: TVNFNFQNLRTYVRSLARMGMD

B

C

side-chain R1

Oh, et al. Figure 1
A

$[\theta] \times 10^{-6} \text{ (deg cm}^2 \text{ dmol}^{-1})$

BID30S126S

BID30S126S156C

BID30S126S186C

BID30S126S190C

wavelength (nm)

B

$[\theta] \times 10^{-6} \text{ (deg cm}^2 \text{ dmol}^{-1})$

BID30S126S

BID30S126S156R1

BID30S126S186R1

BID30S126S190R1

wavelength (nm)
Double Sum of singles

250 Gauss scan

150 Gauss scan

p22 BID in solution

> 25 Å

100 Gauss scan

> 25 Å

100 Gauss scan

p15 BID in membrane

Oh, et al. Figure 7
Figure 8

(A) % release of FITC-dextran 10 % release of FITC-dextran 10 % release of FITC-dextran 10

- p15BID + BCL-X_{L}\Delta C + BAD BH3
- p15BID + BCL-X_{L}\Delta C + BID BH3
- p15BID
- p15BID + BCL-X_{L}\Delta C + mtBID BH3
- p15BID + BCL-X_{L}\Delta C
- Octyl glucoside
- BID BH3

(B) % release of FITC-dextran 10

- p15 BID
- BAD BH3
- BID BH3
- Octyl glucoside
- mtBID BH3

(C) % release of FITC-dextran 10

- BID BH3 + p15 BID
- BID BH3 + BCL-X_{L}\Delta C

Oh, et al. Figure 8
Oh, et al. Figure 9
Clockwise rotation by $\omega$ degrees results in helical tilt.

\[ r = 7.5 \AA \pm 0.5 \]
\[ N_0 = 156 \pm 0.1 \]
\[ \omega = 25^\circ \pm 5 \]
Conformational changes in BID, a pro-apoptotic BCL-2 family member, upon membrane-binding: A site-directed spin labeling study
Kyoung Joon Oh, Scott Barbuto, Natalie Meyer, Ryung-Suk Kim, R. John Collier and Stanley J. Korsmeyer

J. Biol. Chem. published online October 21, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M405428200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2004/10/21/jbc.M405428200.citation.full.html#ref-list-1