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STRUCTURE NOTE

The structure of Boo/Diva reveals a divergent Bcl-2 protein

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INTRODUCTION

Cells initiate apoptosis, a programmed cell suicide, in response to intracellular stress signals. Members of the B-cell lymphoma-2 (Bcl-2) family of proteins are critical to the regulation of the "intrinsic" or mitochondrial initiated cell death.[1] This family comprises about 20 proteins in mammals and consists of members that either promote apoptosis the pro-apoptotic proteins or inhibit this action, the pro-survival proteins. Protein–protein interactions between the opposing factions mediate the life/death switch and it is thought the balance between the pro-survival and pro-apoptotic actions decides cell fate.[2]

Boo (*B*cl-2 homolog of ovary, also known as Diva, Bcl-B, Bcl2L10, or Nrh) is a mouse Bcl-2 homolog, whose gene encodes a 191 residue 22 kDa protein that is highly expressed in the ovary,[3] but only weakly in other tissues.[4] Knockout of the *boo* gene does not give rise to an obvious phenotype suggesting a redundant function for this protein.[4] There is little functional data available for Boo and initial reports on its function were contradictory, one indicating it had pro-survival activity,[3] the other pro-apoptotic activity.[5]

Although the shared sequence identity between members of the Bcl-2 family is low, they harbor up to four regions of conserved sequence known as Bcl-2 homology (BH) domains, BH1–BH4.[6] The pro-survival proteins bear 2–4 of these BH domains while the pro-apoptotic proteins are divided into those that bear a single BH domain, the BH3, and are known as the BH3-only proteins (members of this group include the proteins Bim, Bad, Bmf, Bid, Bik, Puma, Noxa and Hrk) and multidomain Bax-like proteins (Bax, Bak, and Bok) that share both sequence and structural homology to the pro-survival proteins Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, and A1.[6] The presence of a BH3 domain is the minimal requirement for cell killing activity.[7] The BH3-only proteins act as cellular sentinels that are activated in response to an apoptotic stimulus and bind in a hydrophobic groove located on the surface of the pro-survival proteins, neutralizing their action. This interaction frees the pro-apoptotic Bax-like proteins, which effect apoptosis by destroying the integrity of the mitochondrial outer membrane. Factors such as cytochrome *c* are consequently released from the mitochondrial intermembrane space, a process that results in the activation of caspases, cysteine aspartyl proteases, which execute a proteolytic cascade destroying the cell from within.[1]

The BH3 domain is a 13 residue sequence motif, $\Phi_1$sxx$\Phi_2$xxΦ_3sDzΦ_4B that contains a conserved leucine at position Φ_2 and aspartic acid residue at position D.[8] This short motif interacts with a hydrophobic groove on pro-survival proteins through the hydrophobic face of an amphipathic helix formed by the four hydrophobic residues, Φ_1–Φ_4. The other positions are: x is any residue,

Abbreviations: Bcl-2, B-cell lymphoma-2; BH, Bcl-2 homology; NMR, nuclear magnetic resonance.

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s small residues (G, A, or S), z is normally an acidic residue and B a hydrogen bond acceptor.[8] The conserved aspartic acid, D, forms a salt bridge with a complimentary conserved basic residue (K or R) resident in the BH1 domain of the pro-survival protein [Fig. 1(A)]. Mutation of this conserved basic residue in BH1 ablates the ability of a pro-survival protein to bind its ligands.[9]

Aberrant regulation of apoptosis has been directly linked to many diseases and is one of the hallmarks of cancer.[10] The Bcl-2 family proteins key role in determining cell fate has led to an intensive effort to understand their mode of action with the aim of therapeutic intervention.[11] Each of the Bcl-2 proteins is unique, not only do they have distinct molecular features, but they show substantial conformational adaptability for their ligands and have unique biological activities. The protein sequence of Boo bears features, such as an altered BH1 domain and the lack of a BH3 domain, indicating that it is not functionally equivalent to other Bcl-2 proteins. We report the solution structure of Boo and show that unlike other pro-survival proteins it does not bind any of the confirmed BH3-only proteins.

MATERIALS AND METHODS

Protein expression and purification

Mouse Boo (accession number Q9Z0F3) truncated by 26 residues was expressed as a glutathione-*S*-transferase (GST) fusion protein in *Escherichia coli* BL21(DE3). The cell pellet was resuspended in Mouse Tonicity Phosphate Buffered Saline (MTPBS) (140 m*M* NaCl, 16 m*M* Na_2HPO_4, 4 m*M* NaH_2PO_4, pH 7.3). The protein was purified by affinity chromatography on glutathione Sepharose 4B (GE Healthcare) then cleaved with PreScission protease (GE Healthcare) while bound to the resin. After cleavage five additional N-terminal vector-derived residues (GPLGS) remained. Following separation on a S75 column, fractions that contained pure protein as judged by SDS–PAGE were combined and concentrated. Electrospray mass spectrometry was used to confirm protein identity and purity. The protocol for isotope labeling followed the published method.[12] *E. coli* BL21(DE3) were grown at 37°C in 2 L of Super Broth medium and upon reaching $OD_{600} \sim 0.7$, cells were pelleted at 5000*g*, then washed with 500 mL of M9 salt solution that excluded all nitrogen and carbon sources and re-centrifuged. The cell pellet was re-suspended in 500 mL of isotopically labeled M9 medium with $^{13}C_6$-glucose and/or $^{15}NH_4Cl$ (Cambridge Isotope Laboratories), and incubated to allow the recovery of growth and the clearance of unlabeled metabolites. After 1 h, protein expression was induced by the addition of Isopropyl-β-D-Thiogalactopyranoside (IPTG) to a final concentration of 1 m*M*. Cells were harvested after a 3 h incubation period at 37°C by centrifugation at 4000*g* for 5 min. Protein purification was performed as described above. Nuclear magnetic resonance (NMR) samples contained 1.5 m*M* protein in 50 m*M* sodium phosphate (pH 6.7), 50 m*M* NaCl, 5 m*M* Tris(2-Carboxyethyl) phosphine and 0.05% sodium azide in H_2O:^2H_2O 95:5. Boo mutants (S88G, Q89R, and the double mutant S88G/Q89R) were generated using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. Mutant proteins were produced, labeled, and purified as GST fusions as previously described.

Sequence alignment

Sequences were found by a BLAST[13] search of the National Center for Biotechnology (NCBI) sequence database and aligned using Clustal-W.[14] A search for structural homologs of Boo in the Protein Data Bank was performed using secondary structure matching as described.[15] Structure alignment was performed using TOP.[16] Accession numbers for the protein sequences from mouse aligned in Figure 1 are: Boo (Q9Z0F3), Bcl-2 (P10417), Bcl-w (P70345), Mcl-1 (P97287), and A1 (Q07440).

NMR spectroscopy and assignments

NMR spectra were recorded at 30°C on a Bruker DRX-600 600 MHz spectrometer equipped with a triple-resonance probe and pulsed-field gradients or AV-500 and AV-800 spectrometers equipped with cryogenically cooled probes, operating at 500 and 800 MHz, respectively. A series of heteronuclear 3-D NMR experiments were recorded by using either ^{15}N or ^{13}C, ^{15}N double-labeled Boo for ^1H, ^{15}N, and ^{13}C resonance assignment.[17] Spectra were processed with TOPSPIN (Bruker AG, Karlsruhe, Germany) and analyzed using XEASY.[18] Backbone resonance assignments were completed for 96.8% of the sequence and 89.8% of all nonexchangeable side chain resonances were assigned and residues L8, H9, E10, and R113 were unable to be assigned.

Structure calculation and analysis

Distance restraints were measured from the 120 ms mixing time 3D ^{15}N-edited NOESY and the 110 ms mixing time ^{13}C-edited NOESY. Hydrogen bond constraints were applied within α-helices at a late stage of the structure calculation. φ and ψ backbone torsion angles were derived by using TALOS[19] and are summarized in Table I. Structures were calculated using CYANA 2.1[20] and optimized to obtain low target functions. Structural statistics for the final set of 20 structures, chosen on the basis of their stereochemical energies, are presented in Table I. Procheck_NMR[21] and MOLMOL[22] were used for the assessment of structure quality. The final structures had no experimental distance violations >0.25 Å or

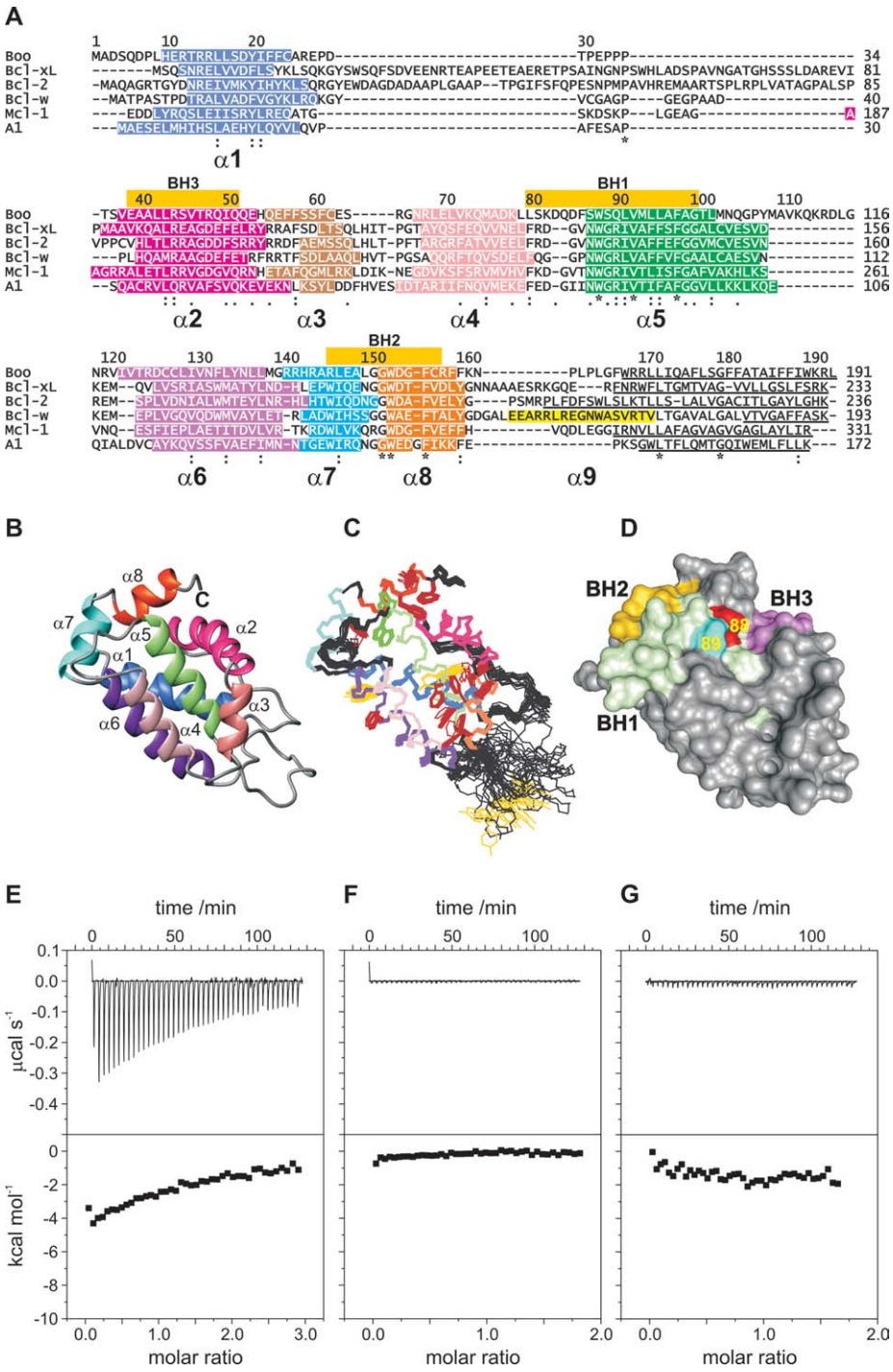

Figure 1

Sequence, structure and binding of Boo. (**A**) Structure and sequence alignment of mouse pro-survival Bcl-2 family members: Boo, Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, A1. Accession numbers are given in Materials and Methods and the unstructured N-terminal residues of Mcl-1 removed.[32] The sequence numbering is that for Boo. Helices are delineated with colored bars and named α1–α9 below the sequence and the C-terminal residues deleted in the protein constructs underlined. The extent of the BH domains is depicted by the bars above the sequence. (**B**) Ribbon diagram of Boo. Residues 7–160 are shown. Helices are labeled α1–α8 and the C-terminus (**C**). The N-terminus is hidden from view. The view selected shows the region equivalent to the binding groove in other pro-survival proteins. The helices are colored as in (A). (C) Backbone of the 20 NMR-derived structures of Boo. The view and coloring is the same as in (A) and the Phe, Tyr, and Trp side chains are colored brown, yellow, and green, respectively. (**D**) Key sequence differences mapped to a contact surface of Boo. The BH1, BH2, and BH3 domains are colored pale green, gold, and violet, respectively. The orientation is the same as the ribbon in (B). (**E–G**) ITC data for BH3 domain interaction with mutant and wild type Boo. (E) ITC plot for S88G/Q89R Boo binding mouse Bak BH3. (F and G) Wild-type Boo does not have significant affinity for Bak (F), or Bim (G) BH3 domains.

Table I
Summary of Restraints and Structural Statistics for the 20 Lowest Energy Structures of Boo at pH 6.7 and 25°C

Experimental constraints	
Total	3403
Intraresidue	670
Sequential (|i-j| = 1)	852
Short range (1 < |i-j| < 5)	730
Long range (|i-j| ≥ 5)	759
Hydrogen bonds	66
Torsion angle constraints (ϕ, 130; ψ, 130)	260
Target function (Å^2)	2.40 ± 0.19
RMSD from experimental distance restraints (Å)	0.0102 ± 0.0009
RMSD from experimental dihedral restraints (°)	0.89 ± 0.08
Measures of structural quality	
Procheck percentage residues in region of Ramachandran plot	
Most favourable	76.6
Additionally allowed	23.1
Generously allowed	0.3
Disallowed	0
Angular order: residues with S(ϕ) ≥ 0.9, S(ψ) ≥ 0.9	158, 155
Violations	
Experimental distance constraints > 0.25 Å	0
Experimental dihedral constraints > 5°	0
Coordinate precision	
Mean pairwise RMSD (Å)	C^{α}, C, N
Residues 1–165	2.66 ± 72
Regular secondary structure	0.38 ± 0.07
	All heavy atoms
Residues 1–165	3.13 ± 0.64
Regular secondary structure	1.27 ± 0.16

dihedral angle violations >5°. Structural figures were generated using MOLMOL.

Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) was performed using a VP-ITC microcalorimeter (Microcal, Amherst, MA). Experiments were performed in MTPBS at 30°C. Titrations consisted of 42 7-μL injections of peptide at 80 μ*M* into 1.34 mL of protein at a concentration of 10 μ*M*. Data were analyzed using Origin software (OriginLab, Northampton, MA). Synthetic peptides (Mimotopes, Melbourne) spanning the mouse BH3 domains of Bim, (residues: 139–152); Bad, (140–165); Bmf, (126–152); Bid, (76–109); Puma, (130–155) Blk, (52–77); Hrk, (26–51); Noxa1, (16–41); Noxa2, (68–93); Beclin, (103–128); Bak, (64–89); Bax, (51–77); and Bok (60–85).

RESULTS AND DISCUSSION

Bcl-2 family proteins control of events determining cell fate and their role in cancer has generated intense interest in understanding the molecular basis of their action.[6,11,23] Resolving the roles of these proteins in apoptosis regulation is not only important for understanding their biology but is helping to formulate new therapeutic strategies.[11] Mouse Boo has intriguing sequence differences from other Bcl-2 proteins that led us to suspect a unique binding profile for this Bcl-2 family member. Here, we examined the structure and binding of Boo.

Boo was discovered by searching databases for homologs of NR13, a chicken Bcl-2 protein.[3] Sequence analysis showed Boo resembles multidomain members of the Bcl-2 family. However, important differences are located within the critical BH regions [Fig. 1(A)] that form the binding surface for BH3-only proteins and changes to these domains are responsible for the selectivity towards BH3 ligands.[8]

The most striking difference in the Boo sequence compared with other multidomain Bcl-2 family members, including the pro-apoptotic proteins Bax, Bak, and Bok, occurs in the BH1 domain. The highly conserved sequence motif "NWGR," necessary for recognition of the BH3-only proteins[8] is replaced by the residues SWSQ in Boo [Fig. 1(A)]. When pro-survival-BH3-domain heterodimers form, the conserved arginine of the BH1 motif in pro-survival proteins makes a salt bridge contact with the conserved aspartate of the BH3-domain in BH3-only proteins.[8] In Bcl-x_L, the corresponding residue, R139, interacts with residue D99 located in the BH3 peptide of Bim[24] and the R139Q substitution results in a loss of pro-survival function and ability to associate with Bax.[9] The R139 counterpart in Boo is Q88 that cannot form such an ionic interaction.

In addition to these changes in the BH1 domain Boo, like A1, lacks the signature sequence for a canonical BH3 domain: $\Phi_1$sxx$\Phi_2$xxΦ_3sDzΦ_4B [Fig. 1(A)].[8] In pro-apoptotic proteins, as outlined above, the conserved aspartate forms the conserved salt bridge with the pro-survival protein. In Boo, R47 replaces this residue ensuring that a salt bridge with the BH1 domain is not possible. In addition, there are other substitutions in the region structurally equivalent to the BH3-domain such as T46 which is a small residue, normally Gly, in BH3-only proteins [Fig. 1(A)]. Hence, Boo lacks the conserved residues critical for heterodimerization of pro-apoptotic and pro-survival Bcl-2 family members. Biological data indicating that Boo confers resistance to induction of apoptosis and the lack of the signature motif for a BH3 domain supports its apparent pro-survival activity.[4,25]

Expression of full-length mouse Boo resulted in production of insoluble protein. However, a C-terminally truncated version of Boo (residues 1–165) with 26 C-terminal residues in the predicted transmembrane region removed yielded well behaved soluble protein. The ^{1}H, ^{15}N-Heteronuclear Single Quantum Coherence (HSQC) spectrum of Boo gave a set of well-dispersed resonances indicating the protein was folded and monomeric. Sequential resonance assignment and structural constraints were determined from a series of heteronuclear 3-D NMR experiments on uniformly ^{15}N, or ^{13}C and ^{15}N labeled protein.[17] Structures were calculated using CYANA[20] with a total of 3057 experimentally derived

distance constraints, 260 dihedral angle constraints derived using TALOS[19] and 66 hydrogen bonds as enumerated in Table I. The structures are energetically reasonable, display acceptable covalent geometry and the final set of 20 structures had no experimental distance violations >0.25 Å or dihedral angle violations >5° and all residues occurred in the allowed regions of the Ramachandran plot. The structure of Boo is a Bcl-2 fold consisting of seven amphipathic helices packed against a central solvent inaccessible hydrophobic helix, α5 [Fig. 1(A)]. The family of structures best fitting the constraints is shown in Figure 1(B). The consensus secondary structure of Boo is composed of helices α1 (residues 9–23), α2 (37–52), α3 (54–61), α4 (67–77), α5 (87–100), α6 (120–136), α7 (139–147), and α8 (150–157). The regions corresponding to the N-terminus (residues 1–5), C-terminus (158–165) and the α5–α6 loop (101–119) lack long-range NOEs and are unstructured. Two short structured loops connect α1–α2 (24–36) and α4–α5 (78–86). The surface of Boo contains a wide, shallow hydrophobic groove approximately 25 × 10 $Å^2$ that is defined by helices α2–α5 and α8 [Fig. 1(D)]. The analogous groove in pro-survival proteins corresponds to the BH3 binding site.

A search of the Protein Data Bank for structural homologs of Boo using SSM[15] indicated Mcl-1 (PDB 1WSX) and Bcl-x_L (1PQ1) were structurally closest to Boo. Structures were aligned using TOP[16] and the structure-sequence alignment is shown in Figure 1(A). The rmsd over the backbone atoms (C, O, N, Cα) of residues in the common secondary structure elements [Fig. 1(A)] of Boo and Bcl-x_L (1PQ1) and Mcl-1 (1WSX) is 1.89 and 2.08 Å respectively. Helices α3 and α4 which form the groove of Boo have a similar orientation to those of Bcl-x_L in complex with Bim and like Mcl-1 has these residues adopt a "ready to bind" orientation.

Although the overall topology of Boo is very similar to those of other multidomain Bcl-2 family proteins, there are a number of significant differences. Helix α5 is shorter in Boo compared with all other multidomain proteins and the 15 residue unstructured loop connecting helices α5 and α6 replaces a tight loop present in other Bcl-2 proteins. This unstructured loop has an eight residue insertion that differentiates Boo from other multidomain Bcl-2 proteins [Fig. 1(A)]. However, sequence differences in the BH1 domain in Boo, from the highly conserved NWGR motif to SWSQ, would appear to be the most important functionally. Q89 retains the geometry of the highly conserved BH1 arginine and its side chain projects into the binding groove in a similar orientation as the equivalent Arg in all other multidomain Bcl-2 proteins. Serine S88 replaces the glycine in the NWGR motif in Boo and its hydroxyl is exposed and projects into the usual BH3 binding site [Fig. 1(D)].

To examine the binding specificity of Boo we evaluated the ability of Boo to bind the BH3-domains of all well-characterized BH3-only proteins as well as those from Bax, Bak, and Bok using ITC. The BH3 segment of the BH3-only proteins has essentially the full binding affinity for their pro-survival targets[26] and peptides that span the BH3 domain bind with a specificity that is predictive of the biology.[27] We found no substantial affinity between Boo and any BH3 domain peptide. Significantly, Boo does not bind the most potent BH3-only protein Bim, which interacts with all other pro-survival Bcl-2 proteins with approximately equal affinity.[27] Mutation of the key SQ residues in the BH1 domain of Boo to GR as point mutations, S88G Boo or Q89R Boo did not lead to any significant affinity gain. However, a double mutation Boo S88G/Q89R gained the ability to bind BH3-only proteins and bound Bak BH3 with an affinity of 65 μ*M*, although the high affinity (nM) that characterizes the interaction between a BH3-only proteins with other pro-survival proteins was not recovered[27] (see Fig. 1). The differences in the BH1 residues are therefore only partly responsible for the absence of BH3 binding of Boo. NMR titration experiments with Bak BH3 peptide confirmed the ITC results and ^{1}H, ^{15}N HSQC chemical shift mapping of the interaction are consistent with Boo S88G/Q89R interacting with Bak BH3 domain in the groove as expected (data not shown). The observed chemical exchange was in the slow to intermediate time scale with resonances broadening until an excess of ligands was present. These findings are at variance with other observations suggesting Boo acts through Bak and Bik, but not Bax, Bad, or Bid.[3] Our findings suggest that any interaction of pro-apoptotic proteins with Boo is unlikely to occur through a BH3 domain binding in the groove. The interaction observed between Boo and Bik and Bak by Song *et al.*[3] using immunoprecipitation may reflect an alternative mode of interaction or one mediated by other proteins.

The important features that identify the interactions of Bcl-2 proteins are the presence of a BH3-domain, which is the minimum requirement for a pro-apoptotic protein[7] and a surface groove on pro-survival Bcl-2 proteins that is generated by their BH motifs.[6,28] Boo does not bear a signature BH3 domain and has a significantly altered groove compared with other pro-survival proteins. The absence of a BH3 domain makes it unlikely that Boo functions to promote cell death by binding in the groove of a pro-survival protein. On the other hand, although BH3-only proteins have a striking ability to interact with the binding grooves of multiple pro-survival proteins,[27,29,30] a feature in part due to their structural plasticity,[31,32] they do not interact with Boo because the critical BH1 residues necessary for interaction with BH3-domains are significantly different to those of other pro-survival proteins.

The human ortholog of Boo, Bcl-B, also lacks a BH3 domain, but bears a characteristic BH1 sequence motif "TWGR" and binds the BH3 domain of Bax.[33] Replacing the critical arginine (R96Q) in the Bcl-B BH1 domain with the equivalent residue in Boo (Q) abolishes Bcl-B's ability

to bind Bax and blocks Bax-induced apoptosis.[33] Peptides that span the BH3-domains of the well-characterized pro-apoptotic proteins fail to interact with Boo, although some affinity can be restored by judicious mutation in the BH1 domain. The striking structural conservation between Boo and other Bcl-2 family members implies that the binding affinity differences linked to Boo mutations are due to a combination of loss of an important ionic interaction, introduction of a potential steric clash with the side chain of S88 on binding, with an unfavorable burying of its hydrophilic hydroxyl group, and other sequence differences, rather than a modified groove geometry. These findings point to a functional divergence between mouse and other mammals, including the rat. Boo modulation of apoptotic signaling may proceed via a distinct mechanism and in the absence of a clear-cut phenotype it is possible that it has an alternative function.

Accession numbers

The protein co-ordinates have been submitted to the Protein Data Bank (PDB ID code 2KUA).

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