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Structural conservation of residues in BH1 and BH2 domains of Bcl-2 family proteins

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Abstract **The sequence of Bcl-2 homology domains, BH1 and BH2, is known to be conserved among anti- and pro-apoptotic members of Bcl-2 family proteins. But structural conservation of these domains with respect to functionally active residues playing role in heterodimerization-mediated regulation of apoptosis has never been elucidated. Here, we have suggested the formation of an active site by structurally conserved residues in BH1 (glycine, arginine) and BH2 (tryptophan) domains of Bcl-2 family members, which also accounts for the functional effect of known mutations in BH1 (G145A, G145E) and BH2 (W188A) domains of Bcl-2.**
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Keywords: Structural conservation; Active site prediction; Heterodimerization; Docking; Mutation

1. Introduction

Bcl-2 family members play an important role in embryogenesis, tissue remodeling and immune response through their action as either inhibitors (anti-apoptotic) or promoters (pro-apoptotic) of apoptosis. Bcl-2 family members are classified as anti-apoptotic or pro-apoptotic on the basis of possessing Bcl-2 homology domains (BH) i.e., BH1, BH2, BH3 and BH4. Several anti-apoptotic members such as Bcl-2, Bcl-X_L and Bcl-W possess all four BH domains. Other anti-apoptotic members such as Mcl-1, BHRF-1, and KSHV-Bcl-2 possess homology only in BH1, BH2 and BH3 domains. The pro-apoptotic proteins of Bax subclass possess sequence homology in BH1, BH2 and BH3 domains, while the members of the BH3 subclass such as Bid have strong homology only in BH3 region. Firstly discovered member of this family, Bcl-2, is folded into eight α-helices, possess all four homology domains i.e., BH4 (10–29 amino acids), BH3 (90–107 amino acids), BH1 (133–152 amino acids), BH2 (184–199 amino acids) in addition to one X domain (192–203 amino acids) and one regulatory/flexible loop domain (30–90 amino acids). Its BH1 and BH2 domains along with BH3 domain form a solvent accessible hydrophobic receptor cleft essential for heterodimerization with pro-apoptotic proteins through their α-helical, amphipathic BH3 domain [1]. Reports have shown that BH1 and BH2 domains are essential for co-immunoprecipitation of Bcl-2 with Bax and for prolongation of cell survival in the setting of induced apoptosis on IL-3 deprivation and gamma irradiation. Substitution of G145 (in BH1 domain) with A/E and W188 (in BH2 domain) with A abrogates Bcl-2 heterodimerization potential and its death repressor activity [2,3]. Although the sequence conservation of BH1 and BH2 domains is known within the Bcl-2 family, their structural conservation with respect to amino acid residues forming an active site has never been elucidated. Functional active site of a protein is a region that performs any of the diverse set of activities including acting as an enzyme active site or being a binding region for a small molecule or a macromolecule [4]. Detailed knowledge of an active site may provide a new insight into the molecular mechanism by which Bcl-2 family members balance cellular proliferation and death. To analyze the role played by predicted active site residues in heterodimerization, we docked Bcl-2 with BH3 domain of pro-apoptotic protein Bax. The docking results were consistent with the experimentally obtained data for Bcl-X_L/Bak and Bcl-X_L/Bad complexes [5,6].

To analyze the structural conservation of residues in BH1 and BH2 domains of Bcl family proteins, we chose Bcl-2 as a model. The homology models of Bcl-2 and Bax BH3 domain

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Abbreviations: BH, Bcl-2 Homology; G, Glycine; A, Alanine; K, Lysine; E, Glutamic acid; R, Arginine; W, Tryptophan; 1MAZ, *E. coli* Bcl-X_L (GI 2098338); 1BXL, *E. coli* Bcl-X_L/Bak complex (GI 26246-21); 1PQ0, Mouse Bcl-X_L (GI 37927566); 1PQ1, Mouse Bcl-X_L/Bim complex (GI 37927568); 1WSX, Mouse Mcl-1 (GI 56966992); 1AF3, Rat Bcl-X_L (GI 2392082); 1G5M, Human Bcl-2 Isoform 1 (GI 1378-6963); 1GJH, Human Bcl-2 Isoform 2 (GI 14719780); 1LXL, Human Bcl-X_L (GI 2098333); 1R2D, Human Bcl-X_L (GI 42543462); 1G5J, Human Bcl-X_L/Bad complex (GI 13096159); 1MK3, Human Bcl-W (GI 31615587); 1F16, Human Bax-alpha (GI 11513492); 1Q59A, EB2 virus BHRF-1 protein (GI 37927821); 1K3K, Kaposi's sarcoma virus 2 (GI 20663999); 1TY4, *C. elegans* CED-9/EGL-1 complex (GI 55670-071)

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were generated at SWISS-MODEL server [7]. The model of Bcl-2 was generated because only the chimeric NMR structure of Bcl-2 is solved in which the regulatory loop domain was replaced with that of Bcl-X_L [8]. However, to identify conserved residues in Bcl-2, we needed an intact structure. The models of Bcl-2 and Bax BH3 domain were checked for their conformational accuracy in secondary and tertiary structure. As the sequence of a protein diverges, only those residues which are required for its biochemical activity, functional activity, proper folding and transport will be absolutely conserved so that the structure made of these active site residues in 3D space should also remain conserved [4]. Therefore, we identified the structural homologs of Bcl-2 in PDB database (Protein Data Bank) [9] and structurally aligned them to find the similar folded residues. This helped us to identify the commonality of local structural pattern (active site) in proteins of Bcl-2 family. The distance between Cα atoms of predicted residues, presumed to be forming an active site in each Bcl-2 structural homolog, was found out to be near the mean value that suggested their superimposition in both position and orientation. We then docked active site residues in Bcl-2 with BH3 domain of Bax to analyze their importance in Bcl-2-Bax heterodimerization. On the basis of these results, we have hypothesized the structural explanation of reported mutations in BH1 (G145A, G145E) and BH2 (W188A) domains of Bcl-2, which functions to abolish its potential as cell survival regulator.

2. Materials and methods

The Bcl-2 and Bax BH3 domain homology models were generated using SWISS-MODEL, an automated protein homology-modeling server available at www.expasy.org. The secondary structure of models was predicted by NPS@GORI [10] and PredictProtein server [11,12]. The models were analyzed for their correctness in stereochemistry, non-bonded-atomic interactions, 3D profile and protein volume using structure analysis and verification server (SAVS) [13–18]. We searched PDB database using BLAST 2.2.10 [19] to find structural homologs of Bcl-2 in BH1 and BH2 domains with known tertiary structure. All homologs were structurally aligned with Bcl-2 in SPDB viewer using its iterative fit option [20]. The distance between Cα atoms of structurally conserved residues was measured using distance-measuring tool of SPDB viewer. To show the superimposability of active site, its mean distance value was calculated. Hex 4.1 was used to dock Bax BH3 domain with Bcl-2 homology model. The parameters used were, search mode-full, post processing-backbone bumps, receptor range-45°, ligand range-90°, electrostatic calculations-enabled and the final search was done at $N = 30$ [21,22].

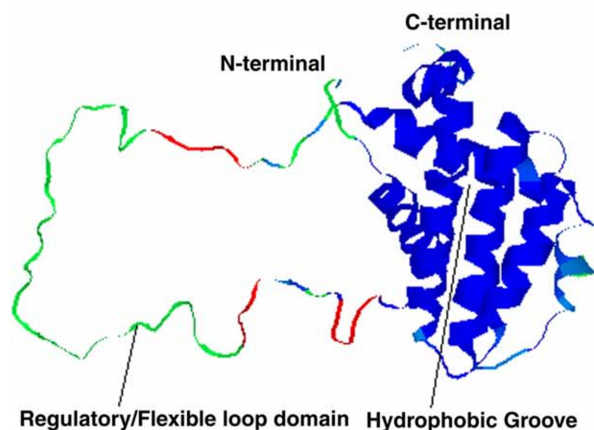
3. Results

3.1. Structure of Bcl-2 and BH3 domain of pro-apoptotic protein Bax

All Bcl-2 homology domains (BH1, BH2, BH3, and BH4), regulatory domain and X domain was modeled by SWISS-MODEL server except transmembrane domain. It was modeled from R6 to R207 out of 239 amino acids in Bcl-2 full protein. In secondary structure conformation, the model shows seven α helices, α1 (E8–Y23), α2 (V87–R102), α3 (A108–R112), α4 (A121– L132), α5 (T139–N158), α6 (P163–D186) and α7 (D191–L196) and an unstructured loop, which is regulatory/flexible loop domain. BH3 domain of Bax protein was all α-helical in secondary structure conformation, encompassing 59–73 amino acid residues (Fig. 1).

3.2. Identification of Bcl-2 Structural homologs in BH1 and BH2 domains

The Bcl-2 protein sequence was input as a query in BLAST, calculation matrix used, BLOSUM62 [24] and *E* value selected, 100. The proteins with greater than or equal to 40% structural identity in BH1 and BH2 domains were: 1MAZ (homology 57%) [8], 1BXL (homology 54%) [5], 1PQ0 (homology 57%) [6], 1PQ1 (homology 57%) [6], 1WSX (homology 52%) [25], 1AF3 (homology 57%) [26], 1G5M (homology 70%) [27], 1GJH (homology 71%) [27], 1LXL (homology 57%) [8],



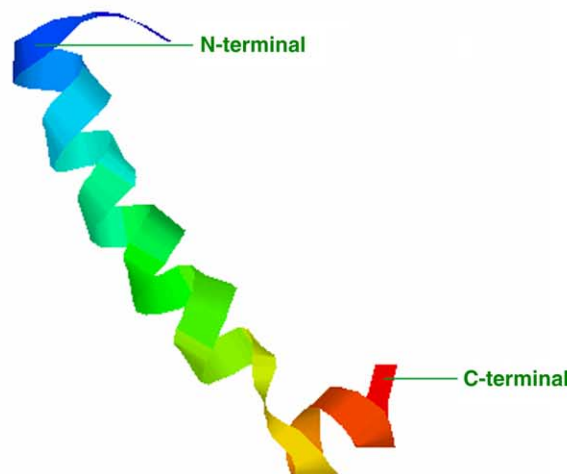


Fig. 1. Homology model of Bcl-2 is colored according to RasMol "Temperature" color scheme, which color codes each atom according to the anisotropic temperature (beta) value stored in the PDB file. Typically this gives a measure of the mobility/uncertainty of a given atom's position. High values are colored in warmer (red) colors and lower values in colder (blue) colors. Homology model of BH3 domain of Bax is colored according to RasMol "Group" color scheme, which color codes residues by their position in a macromolecular chain. Each chain is drawn as a smooth spectrum from blue through green, yellow and orange to red. The N terminus of proteins is colored blue and the C terminus of proteins is drawn in red [23].

1R2D (homology 57%) [28], 1G5J (homology 54%) [29], 1MK3 (homology 48%) [30], 1F16 (homology 56%) [31], 1Q59A (homology 43%) [32], 1K3K (homology 44%) [33] and 1TY4 (homology 40%) [34]. Except three proteins, 1K3K, 1Q59 and 1TY4, all proteins were either pro-apoptotic or anti-apoptotic members of Bcl-2 family. The proteins 1K3K and 1Q59 were from Kaposi's sarcoma virus 2 and Epstein-Barr virus 2, respectively. The 1TY4 protein is a Bcl-2 homolog, CED-9, present in *C. elegans* in complex with EGL-1 and CED-4.

3.3. Prediction of structurally conserved/active site residues

All Bcl-2 homologs were aligned structurally in SPDB viewer (Fig. 2). On alignment three residues viz. G145, R146 and W188 of Bcl-2 were found to be forming a similar fold in each protein. This prompted us to measure the distance between these three residues in all the proteins. We observed that the distance between them is almost equal and, the mean distance was also found to be nearly same (Table 1).

This shows that these residues are superimposable in position and orientation in 3D space in each structural homolog. It implies that these residues are perhaps involved in the formation of an active site in Bcl-2 structural homologs and any mutation within predicted active site is most likely to abrogate the heterodimerization of pro- and anti-apoptotic members of Bcl-2 family. This has been demonstrated experimentally for the predicted residues G145 (in BH1 domain) and W188 (in BH2 domain) in Bcl-2; their substitution with A disrupts the pore forming ability of the Bcl-2 protein [35] and completely abrogates its heterodimerization and consequently death repressor activity in IL-3 deprivation, gamma irradiation and glucocorticoid-induced apoptosis [3,36,37]. Similarly, the substitution of G138A,

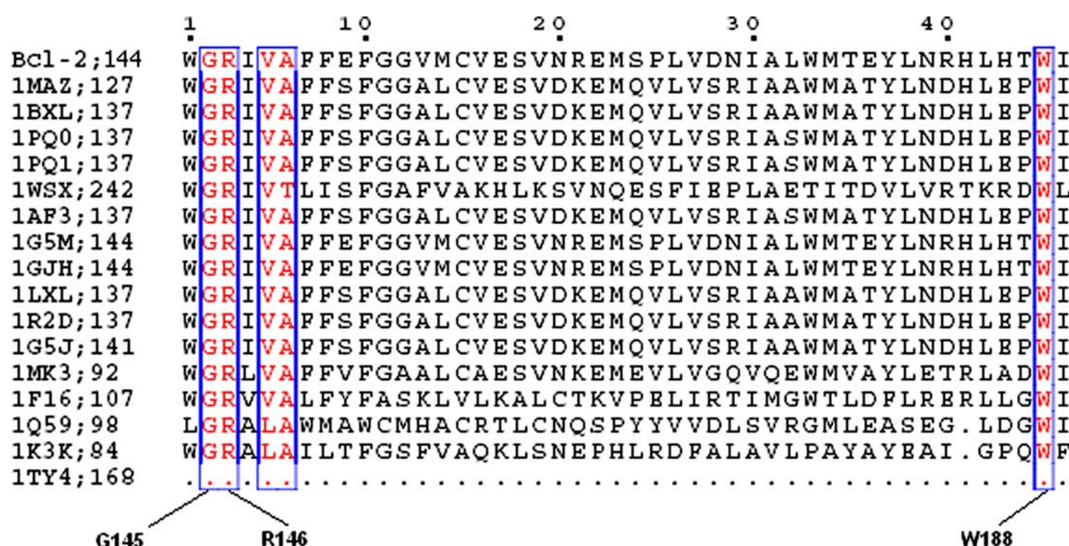


Fig. 2. Structural alignment of Bcl-2 homologs showing conservation of glycine and arginine in BH1 domain and tryptophan in BH2 domain of Bcl-2 family proteins. PDB ID of homologous proteins is followed by starting amino acid number for each sequence. Residue numbers are shown for the sequence of Bcl-2 homology model, i.e., G145, R146 and W188.

Table 1
Distance in angstrom (Å) between residue pair forming an active site in Bcl-2 and its family members

Name	PDB ID (organism)	Structurally conserved residues			Distance between active site forming residues (Å)			Mean distance (Å)
					G–R	R–W	W–G	
Homology model	BCL-2 (Human)	G145	R146	W188	3.83	11.92	12.90	9.55
Bcl-X_L	1MAZ (*E. coli*)	G138	R139	W181	3.82	11.65	12.82	9.43
Bcl-X_L/Bak complex	1BXL (*E. coli*)	G138	R139	W181	3.82	12.93	13.53	10.09
Bcl-X_L	1PQ0 (Mouse)	G138	R139	W181	3.80	11.62	12.61	9.34
Bcl-X_L/Bim complex	1PQ1 (Mouse)	G138	R139	W181	3.79	12.23	13.21	9.74
Mcl-1	1WSX (Mouse)	G243	R244	W286	3.79	12.28	13.21	9.76
Bcl-X_L	1AF3 (Rat)	G138	R139	W181	3.86	11.82	12.92	9.53
Bcl-2 Isoform 1	1G5M (Human)	G145	R146	W188	3.79	11.57	12.93	9.43
Bcl-2 Isoform 2	1GJH (Human)	G145	R146	W188	3.79	11.45	12.64	9.29
Bcl-X_L	1LXL (Human)	G138	R139	W181	3.83	11.42	12.71	9.32
Bcl-X_L	1R2D (Human)	G138	R139	W181	3.82	11.57	12.67	9.35
Bcl-X_L/Bad complex	1G5J (Human)	G142	R143	W185	3.80	12.63	13.15	9.86
Bcl-W	1MK3 (Human)	G93	R94	W136	3.80	12.41	13.89	10.03
Bax-alpha	1F16 (Human)	G108	R109	W151	3.82	12.97	14.90	10.56
BHRF-1 protein	1Q59A (EB2 virus)	G99	R100	W143	3.80	10.05	11.35	8.4
Bcl-2 homolog	1K3K (Kaposi's sarcoma virus 2)	G85	R86	W127	3.80	13.01	13.57	10.12
CED-9/EGL-1 complex	1TY4 (*C. elegans*)	G169	R170	W212	3.80	14.70	16.63	11.71

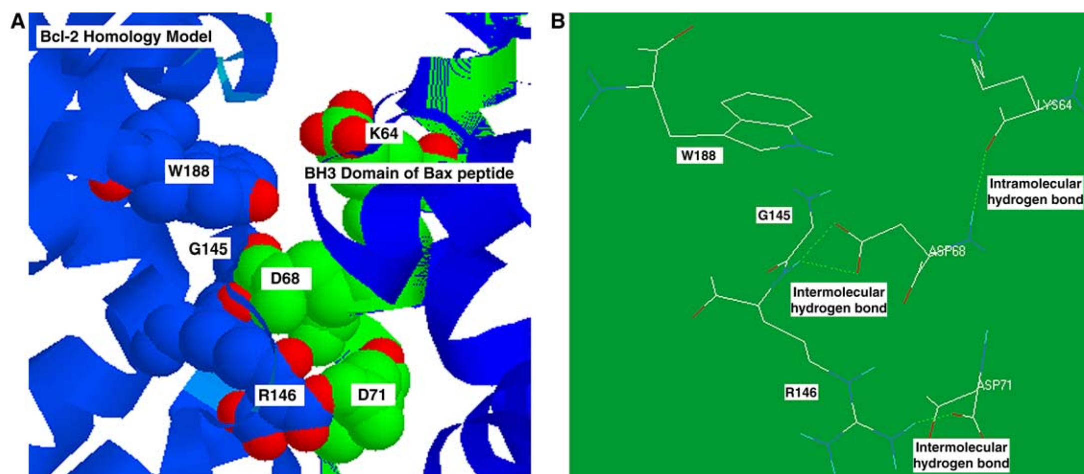


Fig. 3. (A) Bcl-2 (blue) active site residues interacting with Bax (green). The interacting residues are numbered and shown in spacefilled display in Rasmol, rest of the residues are shown in ribbon display. (B) R146 forms a double intermolecular hydrogen bond with D68 on one side and a single intermolecular hydrogen bond with D71 on the other side, which is the main docking site of BH3 domain of pro-apoptotic proteins. This interaction is shown to stabilize the dimeric complex formation. The residue K64 interacts hydrophobically with W188 in the hydrophobic receptor cleft, whereas it intramolecularly binds with D68.

R139Q in Bcl-X_L alters the accessibility, and binding properties of BH3 receptor cleft of Bcl-X_L to pro-apoptotic proteins [5].

3.4. Interaction of active site residues in Bcl-2 with BH3 domain of Bax

The Bcl-2 protein was docked to BH3 domain of Bax protein to understand the role of active site residues in Bcl-2 and Bax heterodimerization. The best docking solution was present among the first energy minimized cluster in Hex-docked complexes (Fig. 3A). The Cα RMS deviation of the best-docked structure was −1.00 with zero backbone bumps. The active site residue, R146 of Bcl-2, forms a hydrophilic hydrogen bond with D68 and D71 of Bax peptide (Fig. 3B). The importance of hydrogen bonding interaction in between R and D can be understood from the solved complex of Bcl-X_L and Bak peptide (1BXL), where interaction between D83 of Bak and R139 of Bcl-X_L stabilizes the complex formation. D83, which is completely conserved within Bcl-2 family, when substituted with A in Bak peptide markedly reduces the binding of peptide to Bcl-X_L. Moreover, conserved R139 mutation to E in Bcl-X_L inhibits its anti-apoptotic activity and binding to Bak protein [5]. Similarly, the same interaction was observed in three-dimensional NMR structure of anti-apoptotic protein Bcl-X_L complexed to a 25-residue peptide (1G5J) from the death promoting BH3 region of Bad. In this complex R139 of Bcl-X_L interacts with D156 of Bad peptide [6]. In vivo, Bax mutant D68A is shown to retain the ability to homodimerize but failed to interact with Bcl-2, as determined by yeast two-hybrid assays and co-immunoprecipitation analysis using transfected 293 mammalian cells. The co-expression of wild type Bcl-2 with Bax mutant, D68A, rescues cells from apoptosis indicating the importance of D68 in heterodimerization interaction and induction of apoptosis by inhibiting Bcl-2 cell survival potential [38].

The other active site residue, W188, was within the docking distance with K64 of Bax peptide, whereas G145 provides the space, due to its small size, required for accommodating D68 within the groove formed by G145 and R146. Therefore, it may be contemplated that any other amino acid in place of G would sterically inhibit the entry of D68 required to stably heterodimerize Bax protein with Bcl-2 (Fig. 3A). These interactions were never shown earlier, which account for the abrogation of Bcl-2 activity in which the clones expressing the Bcl-2 mutants G145A/E and W188A in FL5.12 and 2B4 cell lines were not able to heterodimerize with pro-apoptotic proteins and subsequently unable to inhibit programmed cell death [3]. Since substitution of G145A/E and W188A abrogates heterodimerization, therefore, we also measured distance between Cα atoms of G145 and W188. On substitution G145A or W188A or both, the distance between G145 and W188 remains the same, however A in place of G and W involves itself in the formation of an intramolecular hydrogen bond with A149 (with nitrogen) and L185 (with oxygen) in Bcl-2, respectively (data not shown). The formation of hydrogen bond subsequently disturbs the accessible surface of active site in which A replaces the smaller G and probably suppresses the cleft accessibility, whereas the side chain of W that may be functionally interacting with, e.g., D64 of pro-apoptotic protein Bax, is no longer available. The presence of bulky group of E in G145E substitution perhaps suppresses the stabilization of heterodimeric complex between anti- and pro-apoptotic proteins mediated through the predicted active site.

Similar types of interactions were observed in other experimentally solved complexes such as 1BXL, 1PQ1 and 1G5J. Although there is a difference in binding affinity of Bcl-2 family proteins towards pro-apoptotic Bax, Bad, Bim and Bak peptides due to difference in residues lining the hydrophobic receptor cleft [5,6,27,39], the position of predicted active site residues remains conserved sequentially as well as structurally in each of Bcl-2 structural homologs.

4. Conclusion and discussion

We have reported here the interaction of only three residues with Bax protein, however there may be other residual interactions, to verify the presence of an active site in Bcl-2 and its family members. These three residues may be providing the

basic structural skeleton onto which pro-apoptotic proteins sit, whereas the interactions in between other residues decide the specificity and efficacy of Bcl-2 family members for heterodimerization. For example, the three-dimensional structure of 1Q59 does not contain prominent hydrophobic groove that mediates binding to pro-apoptotic family members. However, it does binds to Bax, Bak and to Bad with low affinity. This binding may be attributed to the predicted active site residues which are structurally conserved in 1Q59 protein also [32].

This knowledge of structural conservation of residues in BH1 and BH2 domains of Bcl-2 family members may be potentially exploited in better understanding of hitherto unrevealed mechanism of cell death regulation.

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