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Prediction of a Putative Functional Region in the Human Bax Protein by Computational Analysis

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Abstract: Structural domains are relevant elements for interactions between Bcl-2 family members to regulate apoptosis and cell survival. Although BH1-BH4 domains can be identified by their sequence and selective occurrence in Bcl-2-family members, structural regions have not been entirely determined yet. The functional residues of Bax, the most representative pro-apoptotic protein of the human Bcl-2 family, remain almost unknown. Here, we identified the human Bax homologues through PSI-Blast analysis. By phylogenetic study, protein sequence multialignment and three-dimensional mapping, we detected the most conserved amino acids in these proteins. Based on these results, we predicted that the human Bax protein has a putative functional region formed by ten relevant residues in BH1 (G103, N106, G108, R109, V111 and A112) and BH2 domains (W151, G157 and W158), as well as outside of them (V95). Interestingly, the structural analysis of this functional region showed that these residues are closely located inside the protein, forming a putative active site. Moreover, this site seems to be protected by the C-terminal end α9 helix that could act as a regulating gate for the access to this region. In addition, the hydrophobic feature of this helix suggests that it could be involved in the insertion into the mitochondrial membrane that is thought to be important for Bcl-2 family members dimerization and activation.

Key words: Bcl-2 family, bioinformatics, structure, apoptosis

INTRODUCTION

Disruption of the balance between pro- and anti-apoptotic signals leads to diseases related to uncontrolled cell proliferation or degeneration. [1] Bcl-2 family members form a set of proteins classified according to their overexpression effects. Thus, Bcl-2-family is an interesting prototype of a protein-interaction network that induces or inhibits apoptosis. Typically, Bcl-2 proteins have been involved in embryogenesis, tissue repair and immune response. [2, 3] They all possess at least one of the four Bcl-2 homology domains (BH1 to BH4). For example, pro-apoptotic proteins have the central short BH3 domain, whereas most anti-apoptotic members contain at least BH1 and BH2. [4, 5] Interestingly, proteins that are the most closely related to Bcl-2, including Bcl-2, Bcl-XL and Bcl-W, have the four BH domains. [6] Mutagenesis assays have demonstrated that BH1, BH2 and BH3 domains play a relevant role in the homo- and heterodimerization of various Bcl-2 family members. [7] Regarding the three-dimensional protein structure, the α-helices in BH1, BH2 and BH3 domains form a long hydrophobic cleft to which the amphipathic BH3 domain of another monomer can bind. [8] On the other hand, BH4 is thought to be important for the structure stabilization of the hydrophobic groove. [9] Particularly, it has been suggested an structural explanation for the reported mutations in BH1 (G145A and G145E) and BH2 (W188A) domains of Bcl-2, which eliminate its ability to regulate apoptosis. [10] However, structural conservation related to specific residues has not been completely described in the entire family.

Bax is a cytoplasmic protein that belongs to the Bcl-2 family. In early steps of apoptosis, Bax is detected in the external membrane of the mitochondria. [6] Additionally, the homodimerization and heterodimerization with Bcl-2 was mapped in residues 63-73, in the BH3 domain. [8] Moreover, it has been shown that the interaction of a Bax dimer with the mitochondria induces cell toxicity, suggesting that Bax forms a channel. The three-dimensional structure of a complete Bax monomer has been recently determined. Bax consists in a central hydrophobic helix surrounded by eight amphipatic helices with varying lengths. Furthermore, α-helices 4, 5, 6, 7 and 8 form a groove that is covered by the α9-helix. [11, 12]

In the present work, we took advantage of this data to perform a functional characterization of the human Bax protein through a computer-structure approach. By comparison with homologous proteins, we predicted a region with conserved amino acids that are closely located inside the protein. We proposed that this functional region corresponding to BH1 and BH2 domains, could be related to an evolutionary selection

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ruled by interactions between Bcl-2 family members. However, the BH3 domain that is important for dimerization[8] was detected in a less conserved region. Besides, our results suggest that the mobility of the hydrophobic α9–helix could be necessary for its integration into the mitochondrial membrane and the active site exposure.

Table 1: Identification of human Bax homologues through PSI-Blast analysis.

Code name	Accession number[a]	Gene name	Identity (%)	E-value
BAXA_BOVIN	O02703	Bax	97	e-101
BAXA_MOUSE	Q07813	Bax	92	8e-95
BAXA_RAT	Q63690	Bax	93	1e-91
BAXB_HUMAN	Q07814	Bax	98	6e-87
BAXD_HUMAN	P55269	Bax	74	4e-68
AR1_XENLA	Q91827	Xr1	29	8e-11
Bcl-2_RAT	P49950	Bcl-2	31	4e-10
Bcl-2_MOUSE	P10417	Bcl-2	31	4e-10
Bcl-2_CHICK	Q00709	Bcl-2	36	5e-10
AR11_XENLA	Q91828	Xr11	30	7e-10
Bcl-2_CRIGR	Q9JJV8	Bcl-2	28	9e-10
Bcl-2_HUMAN	P10415	Bcl-2	30	2e-09
BOK_CHICK	Q9I8I2	Bok	30	2e-09
BCLX_HUMAN	Q07817	Bcl-X	28	5e-09
BCLX_MOUSE	Q64373	Bcl-X	28	5e-09
BCLX_PIG	O77737	Bcl-X	28	1e-08
BCLX_CHICK	Q07816	Bcl-X	34	3e-08
MCL1_FELCA	Q7YRZ9	Mcl-1	31	4e-08
BOK_HUMAN	Q9UMX3	Bok	28	6e-08
MCL1_HUMAN	Q07820	Mcl-1	35	6e-08
MCL1_MOUSE	P97287	Mcl-1	30	7e-08
MCL1_CANFA	Q8HYS5	Mcl-1	31	7e-08
Bcl-2_BOVIN	O02718	Bcl-2	26	8e-08
BAK2_HUMAN	Q13014	Bak	26	1e-07
MCL1_RAT	Q9Z1P3	Mcl-1	30	1e-07
BOK_MOUSE	O35425	Bok	28	2e-07
BCLW_MOUSE	P70345	Bcl-W	28	3e-07
BAK_HUMAN	Q16611	Bak	26	3e-07
BCLW_HUMAN	Q92843	Bcl-W	28	3e-07
B2LA1_BOVIN	Q3C2I0	B2LA1	28	4e-07
B2LA1_HUMAN	Q16548	B2LA1	26	2e-06
BOK_BRARE	Q7T381	Bok	27	7e-06
BAK_MOUSE	O08734	Bak	23	8e-06
B2LA1_MOUSE	Q07440	B2LA1	29	1e-04
NR13_COTJA	Q90343	NR13	27	4e-04

[a]Swiss-Prot database.

METHODS

In order to identify functional regions in the human Bax protein, we used the ConSurf protocol[13], which involves the following steps: i) the amino acid sequence of the human Bax protein was obtained from the PDB 1F16 file[11] and used to screen the SWISS-PROT database for homologous proteins through PSI-BLAST analysis with E-value cutoff of 0.001[14]; ii) predicted amino acids sequences were aligned by ClustalW algorithm with default parameters[15]; iii) the phylogenetic tree was constructed using the Neighbor-Joining method[16]; iv) conservation scores for each specific position were calculated using an empirical Bayesian algorithm[17]; and v) they were clustered within a nine grades scale for three-dimensional visualization purpose. To determine the most conserved amino acids by ClustalW alignment, Blosum 62 scoring matrix was used. To model the amino acid substitutions the ConSurf server used the JTT model, which computed the probability that an amino acid *i* will be substituted by amino acid *j* along a branch of length *t*[18]. BioEdit biological sequences alignment editor (Version 7.0.5.3) and Molecular Evolutionary Genetics Analysis MEGA software (Version 3.1) were employed to visualize alignment and phylogenetic tree, respectively. The three-dimensional structure of Bax protein was visualized by the Jmol program as implemented in the ConSurf Server, except in Figure 3B where the predicted active site was illustrated by Pymol program (v0.99, DeLano Scientific LLC). The same program was also used to determine the distance between Cα atoms of residues forming this site. The hydrophobic profile was calculated by the Kyte and Doolittle algorithm[19] as implemented in the ProtScale server. [20]

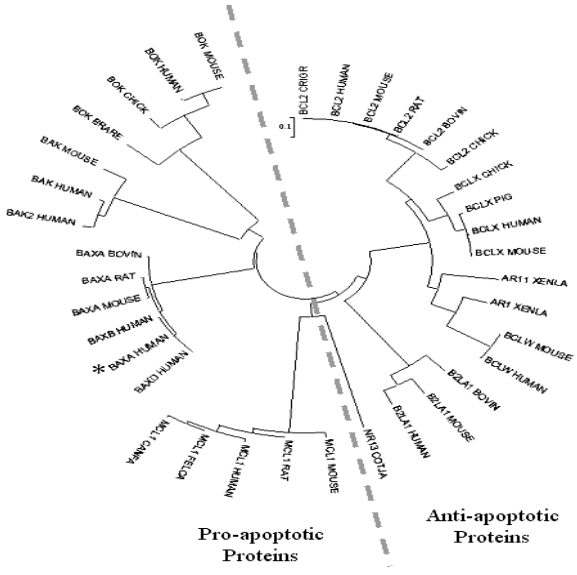

Fig 1: Phylogenetic analysis of human Bax homologues. Complete amino acid sequences were analyzed by the Neighbor Joining method and the resulting phylogenetic tree was visualized by the MEGA software.

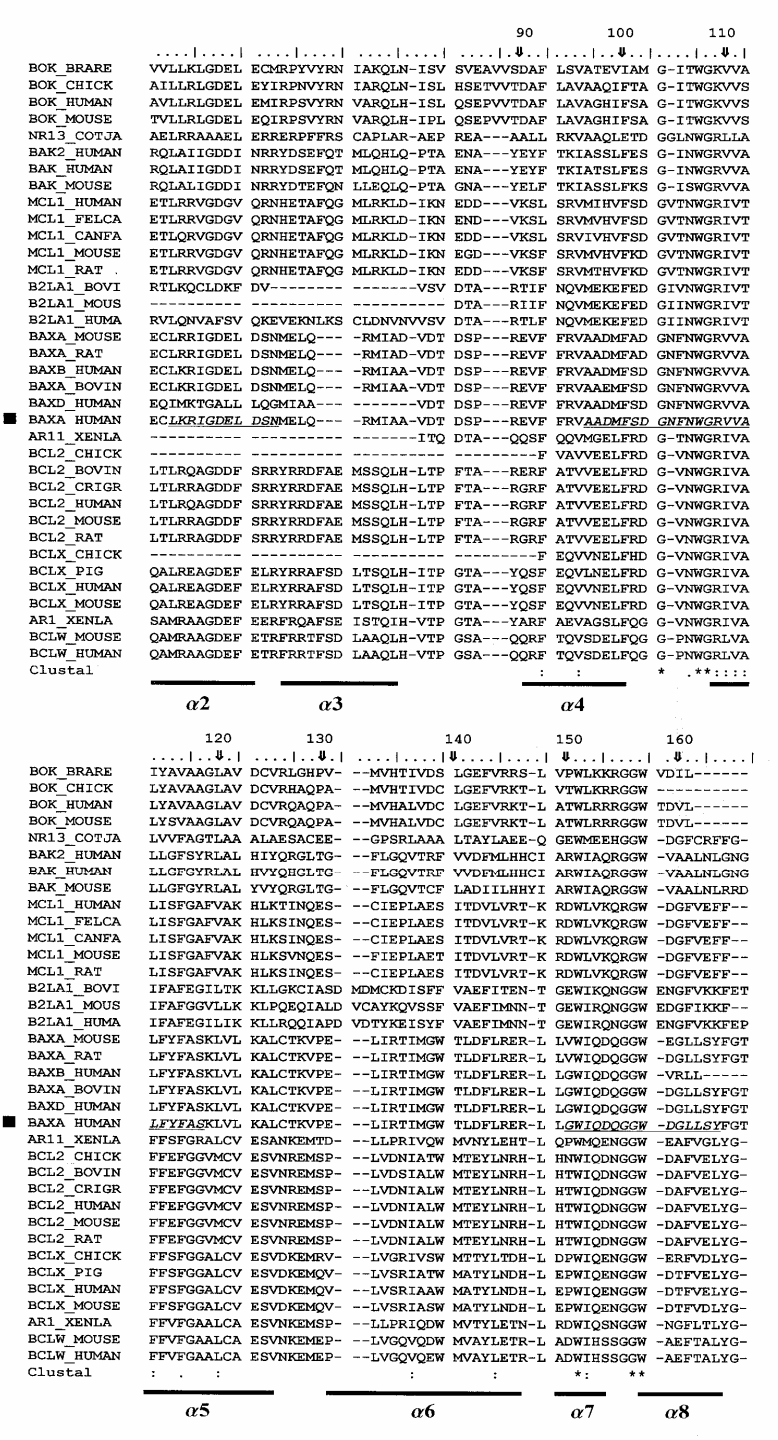

Fig 2: Multialignment of human Bax homologues using the ClustalW program and the substitution BLOSUM62 matrix. Numbers at the top represent the amino acid position related to the first methionine in the human Bax protein indicated by a black square. BH domains are underlined and predicted α-helices are shown by a continuous line. Conserved (star) and substituted (point) residues are indicated.

RESULTS

PSI-BLAST analysis against all Swiss-Prot database led to the detection of 35 homologous proteins that showed low e-values (4e-44 to e-101) and high identity (23-97%) with the human Bax protein. These proteins are from various eukaryotic organisms through evolutionary scale, including mammals (*Homo sapiens, Mus musculus, Rattus norvegicus, Bos taurus, Canis familiaris, Felis silvestris cattus, Sus scrofa* and *Cricetulus griseus*), fishes (*Brachydanio rerio*), amphibians (*Xenopus laevis*) and birds (*Gallus gallus* and *Coturnix coturnix japonica*). All of them are Bcl-2 family members; several proteins belong to the subgroup of pro-apoptotic proteins (Bok, Bak, Bax and MCL1) whereas others are Bcl-2-like survival factors (Bcl-2, BclX, BclW, NR13, Xr1, Xr11 and B2LA1) (Table 1).

The unrooted phylogenetic tree of protein sequences listed in Table 1, including the human Bax protein, was constructed by applying the Neighbor-Joining method that is a simplified version of the minimum evolution method and is considered as the adequate algorithm for the characterization of a small set of sequences. Phylogenetic inference of human Bax homologues showed a clear separation between pro- (Bok, Bak, Bax and MCL1) and anti-apoptotic (Bcl-2, BclX, BclW, NR13, Xr1, Xr11 and B2LA1) factors (Fig 1). The human Bax protein was evolutionary related to bovine, mouse and rat homologues that all emerged from a common Operational Taxonomic Unit (OTU). Moreover, the Bax OTU was as compact as the Bcl-2 OTU, exhibiting the shortest branches of the phylogenetic tree. A similar observation could be made for MCL1 proteins that form a compact UTO closely related to the Bax cluster. However, the other members of the Blc-2 family, including Bok, Bak, B2LA1, BclW and BclX, emerged from longer branches, suggesting that they are more divergent (Fig 1).

In order to carry out an extensive comparative study of the amino acids sequence of the human Bax protein and its homologues, we aligned the whole data set using the ClustalW algorithm. Visualization of the multialignment allowed the identification of the two conserved BH1 and BH2 domains in regions that were predicted as α-helices in the human Bax protein[21]: i) BH1 domain (α4 and α5 helices): (F/L)-X_2-(V/I)-X_7-G-X_{1-2}-(N/S/T)-W-G-(R/K)-(L/I/V)-(L/V)-(A/T/S)-(F/I/L)-X_2-(F/V)-X_3-(L/M/V), and ii) BH2 domain (α7 and α8 helices): W-(I/M/L)-X_4-GW, where X is any amino acid. Additionally, we identified a conserved region in the α6 helix: (V/I/L)-X_7-(L/I/M/V). A detailed examination of identical and substituted amino acids in the multialignment showed that conserved residues in all these proteins were Glycine (G103, G108 and G157) and Tryptophan (W107, W151 and W158), which are both non polar amino acids (the amino acid position is related to the human Bax protein) (Fig 2). Interestingly, the majority of substituted residues also involved non polar amino acids, such as: (F/L)92, (V/I)95, (L/I/V)110, (L/V)111, (F/I/L)113, (F/V)116, (L/M/V)120, (V/I/L)136, (L/I/M/V)144 and (I/M/L)152. In contrast, the (N/S/T) substitution at position 106 between α4 and α5 helices and the (R/K) substitution at position 109 in the amino terminus of the α5 helix involve polar residues, whereas (A/T/S)112 substitution in the BH1 domain involves residues with distinct biochemical properties. On the other hand, the BH3 domain and other regions without known function appeared as more variable domains, since no conserved residue was identified using ClustalW (Fig 2).

The evaluation of amino acid changes in Bax homologues was also studied through the ConSurf protocol expressing the conservation score for each residue in a nine grades scale. Results showed that ten amino acids were in the ninth grade, indicating that they are the most conserved residues. Three of them were conserved in all the proteins (G108, W151 and W158) whereas the others were substituted residues including (I/V)95, (G/K)103, (E/N/S/T)106, (K/P/R)109, (I/V)111, (A/S/T)112 and (G/Y)157 (the amino acid position is related to the human Bax protein). Notably, only two substitutions involved non-polar amino acids (Table 2). In accordance with results shown in Figure 2, all these residues are located in conserved BH1 and BH2 domains. In contrast, the BH3 domain and other regions with unknown function did not present any conserved residue.

Table 2: Detection of conserved and substituted amino acids in human Bax homologues through ConSurf analysis

Identical Residue	Substituted Residue
G108	(I / V) 95*
W151	(G / K) 103
W158	(E / N / S / T) 106
	(K / P / R) 109
	(I / V) 111*
	(A / S / T) 112
	(G / Y) 157

Numbers are relative to the amino acid position in the human Bax protein.
*Non polar residue.

To gain insights into the relative position of conserved residues, we analyzed the three-dimensional structure of the human Bax protein using a nine grades colors scale to represent the conservation scores of each amino acid as implemented in ConSurf server. We

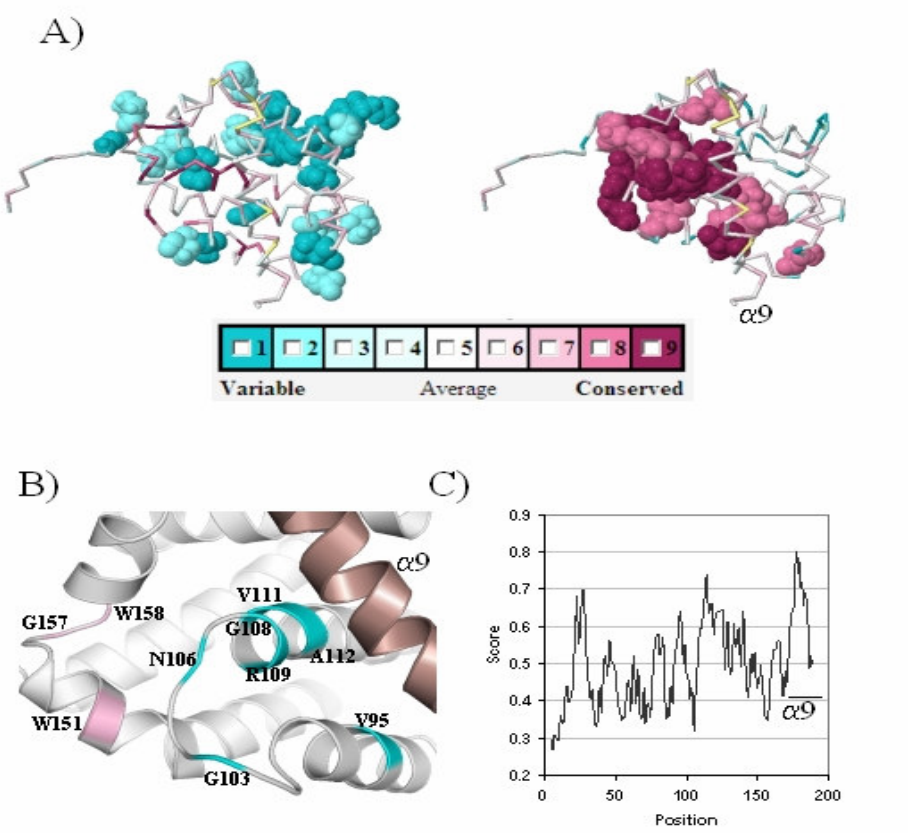

Fig 3: Three-dimensional mapping of the putative active site of the human Bax protein. A) Localization of the conserved amino acids. The human Bax structure was obtained from the PDB file (PDB:1F16)[9] and the most and less conserved residues were indicated according to the nine grade colored scale shown at the bottom. B) Magnification of the putative functional region of the human Bax protein. C) Hydrophobic profile of the human Bax protein.

focused on the most (eighth and ninth grades) and less (first and second grades) conserved positions (Fig 3A). The most conserved amino acids, including the ten residues described above (Table 2), are spatially close in the central part of the protein. Remarkably, this conserved region seems to be protected by the α9 helix. In contrast, the more variable positions showed a dispersed distribution at the external part of Bax. In the ConSurf methodology, it was assumed that the conservation grade of an amino acid is indicative of its evolution rate. Then, sites that are evolutionary conserved are thought to have evolved slowly indicating their structural and functional importance, while those that change more quickly over evolutionary time are more variable and represent less relevant amino acids. This categorization takes various parameters in consideration, including the phylogenetic relation of sequences, the amino acids similarity and the empirical Bayesian algorithm.[13]

To extensively examine this central region of Bax, we measured the distance between the ten most conserved amino acids. The minimal, maximal and mean distance were 3.82 Å (G108-R109), 25.77 Å (V95-G157) and 12.9 Å, respectively (Table 3). The proximity of these residues confirms that they could be involved in the formation of an active site inside the human Bax protein (Fig 3B). Finally, in order to evaluate the functional relationship between this putative active site and the α9 helix that seems to protect it (Fig 3A), hydrophobic profile of the complete Bax protein was determined (Fig 3C). Particularly, α9-helix is presented as the most hydrophobic region. This suggests that its interaction with mitochondrial membrane could regulate the active site exposure.

Table 3: Distance in Angstrom (Å) between the conserved residues forming the putative active site in the human Bax protein

	V95	G103	N106	G108	R109	V111	A112	W151	G157	W158
V95	0	15.68	16.16	13.41	10.71	14.16	10.91	20.31	**25.77**	23.92
G103	-	0	7.94	12.10	10.94	16.36	15.84	13.21	20.54	20.33
N106	-	-	0	6.17	6.48	9.92	10.87	10.70	14.91	13.75
G108	-	-	-	0	**3.82**	5.48	5.45	14.90	17.43	15.00
R109	-	-	-	-	0	5.80	5.13	12.97	16.84	14.75
V111	-	-	-	-	-	0	3.83	15.05	15.59	12.47
A112	-	-	-	-	-	-	0	16.95	18.92	16.03
W151	-	-	-	-	-	-	-	0	9.05	10.44
G157	-	-	-	-	-	-	-	-	0	3.83
W158	-	-	-	-	-	-	-	-	-	0

Numbers in bold represent the minimal and maximal distances

DISCUSSION

In silico analysis of genomes have confirmed that protein domains are fundamental units through evolution because they duplicate and mix in different ways. Besides, several domain families seem to be versatile while others are restricted to specific organisms or kingdoms.[22] The increasing number of structures resolved through X rays or nuclear and magnetic resonance (RMN) suggests that protein structures are more conserved through evolution than amino acid sequences.[23] Thus, several families are constituted by proteins sharing only 5% identity, while related members of other families exhibit 50% structure identity, mainly in the central part of the proteins.[24] Moreover, in some cases, it is difficult to predict the presence of a given domain whose size and location are not well defined in the family members.[22] Therefore, evolutionary analysis of protein families as well as the knowledge of any data about a specific gene or its product, such as genome localization, molecular organization, structure or expression will contribute to better understand the function of a specific protein. Then, this information can be used as a guide for further experimental studies and practical applications.

In the present work, we performed a functional characterization of the human Bax protein through a computer-structure approach, mainly based on the ConSurf protocol.[13, 25] As expected, the human Bax protein is closely related to other Bcl-2 family members, clustering with pro-apoptotic factors while anti-apoptotic proteins form a separated group in the phylogenetic tree. A similar observation was reported when the complete Bcl-2 family was phylogenetically characterized.[26]

Although results obtained from the ConSurf protocol were slightly different from those obtained using the ClustalW algorithm, both strategies identified the BH1 and BH2 domains as the most conserved part of Bax homologues. Moreover, they were in agreement with a previous work in which the evolutionary conservation of BH1 and BH2 domains were used to suggest that conserved residues G108 and W151 are involved in the formation of an active site in Bcl-2 structural homologues, including the human Bax protein.[10] Remarkably, *in silico* predictions and experimental results also demonstrated that G108 and W151 mutation disrupts the pore forming ability of the Bcl-2 protein[27] and completely abrogates its heterodimerization and consequently death repressor activity in IL-3 deprivation, gamma irradiation and glucocorticoid-induced apoptosis.[4, 28, 29] Recent studies by ConSurf analysis with dataset of proteins whose three-dimensional structure was known showed that regions with evolutionarily conserved residues were often functionally important.[30-33] However, since the ConSurf protocol does not allow to quantify the global conservation of a region formed by conserved residues, it is only considered as a qualitative method.[34] In the present work, we extended the list of the relevant residues in the active site of the Bax protein, demonstrating the importance of V95, G103, N106, V111, A112, G157 and W158 amino acids, in addition to G108, R109 and W151 residues previously described.[10] In fact, the mean distance between these

ten amino acids is 12.9 Å, which is close to the 10.56 Å measured between only three residues.[10] The high conservation of these amino acids in the Bcl-2 family members shows that BH1 and BH2 domains have evolved slowly due to their functional importance for Bcl-2 proteins dimerization and activation. On the other hand, BH3 domain that is also necessary for the dimerization, does not correspond to a conserved region. Finally, the hydrophobic feature of the α9-helix at the C-terminal end of Bax protein suggests that it could be a transmembranal domain. Based on our results, we proposed an hypothetical working model in which the α9-helix could be a mobile element responsible for the interaction with phospholipids of the mitochondrial membrane, allowing the exposure of the internal active site and the activation of the Bax protein.

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