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Preparing samples for crystallization of Bcl-2 family complexes

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Summary:

High-resolution protein structures determined by X-ray crystallography or NMR have proven invaluable for deciphering the molecular mechanisms underlying the function of a vast range of proteins. Here we describe methods to generate complexes of proteins belonging to the Bcl-2 family of proteins with either biological ligands or small molecule antagonists.

1. Introduction:

Members of the Bcl-2 family of proteins fall into two opposing factions, the pro-survival group and the pro-apoptotic group (Figure 1). The interplay between members of these rival family factions ultimately determines cellular fate, and structural insights into these interactions have led to a wealth of information into Bcl-2 mediated signalling and its role in disease (see e.g. [1]). Despite substantial unresolved challenges in the preparation of complexes of full-length Bcl-2 constructs, mechanisms of action governing the biology of these proteins are increasingly well understood. These advances have relied heavily on the structural analysis of protein complexes of the various family members bound to relevant partners.

The first structural analysis of a Bcl-2 family protein complex was achieved using NMR, and revealed in detail the interactions between Bcl-x_L and a short 16 mer peptide spanning the BH3 domain of the pro-apoptotic executioner molecule Bak [2] (Table 1, Figure 2). The interaction was mediated through hydrophobic interactions between the amphipathic BH3 helix and a groove on the surface of the pro-survival protein, a salt bridge between a conserved Aspartate on the BH3 peptide and a conserved Arginine on the pro-survival protein was also observed. Subsequent structural analyses were informed by the realization that 26-mer peptides of BH3 domains of pro-apoptotic BH3-only proteins faithfully recapitulate key aspects of these interactions [3]. This work also provided the first insights into the specificity of interactions occurring between different family members (Figure 1). Structures for a large number of various complexes have now been solved (Table 1).

A number of complexes have also now been solved for pro-survival proteins in complex with peptides corresponding to the BH3 regions of the Bax-like executioner

proteins (Table 1). However, the absence of a structure of a full-length mammalian pro-survival Bcl-2 protein bound to a Bax-like protein is hampering a complete understanding of the intricacies of pro-survival Bcl-2 mediated regulation of Bax and Bak. Nonetheless, the structure determination of a full-length complex of CED9 bound to CED4, two key regulators of intrinsic apoptosis in the worm *C. elegans* [4], suggests that these challenges are not insurmountable. More recently structures have also been solved for complexes of Bax bound to activating BH3-only proteins, providing insight into the initiation of Bax conformational change, and of Bax and Bak dimers, providing insight into the ensuing oligomerisation of these proteins (Table 1).

Here we will focus on methods and strategies related to the analysis of Bcl-2 family protein complexes with crystallography. However, it should be noted that other structural and biophysical techniques have contributed greatly to our understanding of Bcl-2 family protein structure, function and drug discovery including NMR (eg [2,5]), Fluorescence Resonance Energy Transfer (FRET)(eg [6]), Double Electron-Electron Resonance spectroscopy (DEER)(eg [7]) and chemical cross linking (eg [8]).

Preparation of pro-survival protein:peptide complexes for crystallization

Preparation of complexes of pro-survival proteins bound to peptides of their pro-apoptotic counterparts has led to important insights into Bcl-2 mediated signalling and its role in disease. In this example we demonstrate how to prepare a complex of vaccinia virus F1L with the human Bim BH3 domain peptide. This method has been successfully used to prepare complexes for crystallisation trials of pro-survival Bcl-2 proteins bound to BH3 domain peptides with affinities ranging from the 1 nM to 7 μ M [9,10]. Final concentrations for crystallization experiments may vary depending on the sample.

2 Materials

Recombinant pro-survival protein (eg.. vaccinia virus F1L protein, Bcl-x_L, etc) purified to homogeneity in final sample buffer (e.g. 25 mM Hepes pH 7.5, 150 mM NaCl). Synthetic BH3 domain peptide (e.g. Bim BH3, Uniprot accession code O43521-3, residues 51-77, Genscript) dissolved in H₂O. Centrifugal concentrator (MWCO 10 kDa, Merck Millipore).

3 Methods

1. Wash a 5 mL centrifugal concentrator with 5 mL of final sample buffer by centrifugation.
2. Add 1 mg of pro-survival protein in final sample buffer, and top up with additional buffer to a final volume of 4 mL.
3. Aspirate a 1.25 molar excess of BH3 domain peptide.
4. Slowly add peptide to centrifugal concentrator whilst stirring with pipette to avoid local precipitation of sample.
5. Concentrate sample to a final concentration of 5 mg/mL of pro-survival protein.
6. Top up sample with additional buffer to a final volume of 4 mL.
7. Concentrate sample to a final concentration of 5 mg/mL of pro-survival protein. Final concentrations for crystallization experiments may vary with each sample.

4. Notes

4.1 Crystallization of Bcl-2:peptide complexes expressed as single-chain constructs with cleavable linkers

An alternative method of producing complexes of pro-survival protein bound to BH3 domain peptides is to express both as a single chain construct with a protease cleavable linker [11]. It has been found in some cases that this aids the expression of the pro-survival protein and ensures complete saturation of all available binding sites. The constructs consisted of a C-terminally truncated form of the pro-survival protein linked to human Bim_s BH3 peptide via a (GS) linker. This enables the Bcl-2 hydrophobic groove to be fully occupied with the native ligand. The final expression construct thus consists of: 6His-x-Bcl-2ΔC-x-(GS)₉-x-Bim-BH3 (where -x- represents a TEV cleavage site ENLYFQGS). Following initial affinity purification TEV-cleavable linkers are cleaved via incubation with TEV protease, followed by re-application of cleaved sample to affinity resin to remove uncleaved protein and purification tag. The final sample can then be concentrated for crystallization.

4.2 Crystallization of Bcl-2 family members with organic molecules

Preparation of complexes of pro-survival proteins with small molecules for crystallization can often be achieved using similar methods to those described above for pro-survival: BH3 domain peptide complexes (Table 2). However, an added difficulty with small molecules is that the ligands are usually dissolved in DMSO which can sometimes hinder crystallization. Furthermore, small molecules often have significantly reduced affinity for their target proteins as compared to wild type BH3-only proteins. In the preparation of such samples DMSO is most efficiently removed from sample mixtures of protein and ligand through buffer exchange, but for low affinity targets this might also result in loss of compound. One approach to minimize such loss is to add a molar excess of compound to protein at high concentrations in small volumes and then to dilute these samples to a final DMSO concentration of 1% (or lower), followed by concentration using low molecular weight centrifugal filters back to the desired final molarity. Using this strategy the solubility of the compound in solution is reduced during the dilution step thereby minimising the rate of ligand dissociation during the purification step.

4.3 Enhancing crystallization of Bcl-2 family proteins

As with all attempts at protein crystallization there are a variety of different strategies to obtain diffracting crystals of target proteins [12]. Routinely initial crystallization trials are performed with a desired construct in a large number of crystallization conditions, and sometimes at a range of protein concentrations, in order to find conditions in which the protein is enticed towards formation of a crystal rather than precipitation. However, often crystallization conditions for target constructs are not forthcoming despite extensive screening and in these situations alternative construct strategies are often tried. In the case of the Bcl-2 family of proteins a range of different construct design strategies have been successful as follows.

4.4 Constructs from different species

A common strategy for obtaining diffracting crystals of difficult targets is to attempt to crystallize the protein of interest from different species. Structures of Bcl-2 family proteins from a variety of different species have been crystallized (Tables 1 and 2) and in some cases chimeric constructs consisting of sequence from two different species have proved useful ([13]). Naturally for drug discovery programs it is usually desirable to use human constructs and so for these projects alternative strategies for enabling crystallization may be pursued.

4.5 Fusion Tags

One method by which crystallization can be enhanced is through the use of protein fusion partners. These can act to both aid with protein solubility and may also provide extra opportunities for the formation of crystal contacts upon which a crystal lattice can build. One recent notable success has been achieved with a Maltose Binding Protein (MBP) fusion with Mcl-1 [14]. This construct provided the first crystal structure for apo Mcl-1 and enabled ligand bound Mcl-1 structures to be obtained through both soaking of compounds into the apo crystals and through co-crystallization of compound and protein. Fusion partners have also enabled the crystallization of truncated constructs of Bax and Bak that reveal details for the initial steps of dimerization. For example, it was recently discovered that one of the conformational changes occurring to these proteins upon activation includes separation into “core” ($\alpha 2$ - $\alpha 5$ and possibly including 1) and “latch” ($\alpha 6$ - $\alpha 7$) domains [15,16]. Fusion of GFP to the “core” domains of these proteins [17] enabled their expression and crystallization and revealed the atomic details of the dimer units upon which the larger Bax and Bak oligomers build [18,8].

4.6 Alternative constructs

Often it proves useful to make truncations or modifications to constructs in order to enable proteins to be expressed, purified and/or crystallized. The vast majority of Bcl-2 constructs used for structural studies have lacked the C-terminal transmembrane domain ($\alpha 9$ helix), primarily because it is difficult to produce sufficient quantities of soluble protein containing this highly hydrophobic region. Bax, however, is a notable exception as it can be expressed as a full-length protein in relatively high quantities [19]. Expression and purification of full length constructs for Bak [20], Bcl-x_L [21] and Bcl-w [22] have also been reported, however, these have not been used in structural studies. Another region of the Bcl-2 family fold that is often modified is the loop between the $\alpha 1$ and $\alpha 2$ helices. This segment is large and unstructured in most family members and is thus often either shortened (eg Bcl-x_L $\Delta 45$ -84 [23]), or replaced with the shorter loop from another family member (eg the Bcl-2 loop being replaced with sequence from Bcl-x_L [24]). A particularly useful construct for crystallization has been Bcl-x_L in which the $\alpha 1$ - $\alpha 2$ loop is dramatically shortened (lacking residues 27 to 82) such that the $\alpha 1$ cannot fold correctly with the remainder of the protein. Instead this construct forms a domain swap dimer, with the $\alpha 1$ of one monomer folding into its neighbor to complete the Bcl-2 fold [25,26]. These dimers readily produce crystals in a number of different crystal forms and thus have proven extremely fruitful for drug discovery (eg [27-30]). Similarly, a domain swapped dimer version of Bax, in which the $\alpha 6$ - $\alpha 8$ “latch” region swaps with a

neighbor, has been useful for solving structures of Bax bound to activator BH3 domains (Figure 2)[16,31]. One possible reason for enhanced crystallization of these dimer constructs is that the dimerization interface provides a point of symmetry on which the crystal can build. In a similar manner, in the first structure solved of Bcl-x_L bound to a compound within the benzoylurea series (Bcl-x_L:1HI from PDB code 3ZK6 – [28]), the compound itself dimerizes between 2 proteins across a 2-fold axis within the crystal, this may have similarly enhanced the crystallization of this low affinity inhibitor complex. Notably, however, the compound did not dimerize Bcl-x_L in gel filtration experiments and so may only act within the crystal or at the high concentrations of protein found within the crystallization drop.

4.7 Concluding remarks:

Table 1 demonstrates that an enormous collection of structures of Bcl-2 family protein complexes has now been accumulated. These structures have informed our understanding of the molecular mechanisms controlling apoptosis and guided the development of inhibitors targeting these proteins. However, the family portrait is by no means complete. We are yet to determine a structure of a pro-survival protein in complex with a full-length Bax-like executioner protein and there are a large number of viral derived family members for which structures have not yet been solved. Such structures are likely to offer further insights into the molecular interactions governing these pathways and may provide new strategies for targeting them for novel therapeutic outcomes.

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Figure 1: Bcl-2 family members and interactions. (A) The Bcl-2 protein family consists of two opposing groups, the pro-survival proteins and the pro-apoptotic proteins. The pro-apoptotic members can be further subdivided into the BH3-only proteins, whose role is to initiate death signalling, and the executioner proteins Bax and Bak (and possibly Bok) that are responsible for mitochondrial outer membrane permeabilisation (MOMP), a point of no return in the death signalling pathway. (B) Apoptotic signalling is initiated through the upregulation of BH3-only proteins. These inhibit the activity of the pro-survival proteins and can directly interact with and activate the executioner proteins. Pro-survival proteins inhibit activated executioners by binding to their BH3 domains, and possibly other regions, to prevent oligomerisation. An excess of BH3-only proteins competes for this interaction, releasing activated Bax-like proteins so that they can oligomerise and initiate MOMP. (C) Some BH3-only proteins, such as Bim, Puma and Bid, interact with the full suite of pro-survival proteins whereas others, such as Bad and Noxa, interact with only a subset [32,3,33]. (D) Bak is primarily inhibited by Bcl-x_L, Mcl-1 and A1 [34], Bax is most likely inhibited by the full range of pro-survival proteins [35].

Figure 2: a) Structure of Bcl-x_L with Bim [36]. B) Structure of BHRF1 with Bak BH3 [37]. D) Crystal structure of ABT-737 bound to Bcl-x_L [38]. D) Structure of Bid BH3 bound to Bax [16].

Table 1: PDB entries of Bcl-2 family member complexes.

Protein complex	Species	PDB code	Method	Reference
Bcl-x _L :Bak BH3	Human	1BXL	NMR	[39]
Bcl-x _L :Bad BH3	Human	1G5J	NMR	[40]
Bcl-x _L :Bim	Mouse	1PQ1	X-ray	[36]
CED9:EGL-1 BH3	<i>C. elegans</i>	1TY4	X-ray	[41]
CED4:CED9	<i>C. elegans</i>	2A5Y	X-ray	[4]
Bcl-w:Bid BH3	Human	1ZY3	NMR	[42]
Bcl-x _L :Beclin-1 BH3	Human	2P1L	X-ray	[25]
M11L:Bak BH3	Myxoma virus/Human	2JBY	X-ray	[43]
Mcl-1:Bim BH3	Human/Rat	2NL9	X-ray	[13]
Mcl-1:mNoxaB BH3	Human/Rat	2NLA	X-ray	[13]
Mcl-1:mNoxaB BH3	Mouse	2JM6	NMR	[13]
Bax:BimSAHB	Human	2K7W	NMR	[44]
Bcl-x _L :Beclin-1 BH3	Human	2PON	NMR	[45]
A1/Bfl-1:Bim BH3	Human	2VM6	X-ray	[46]
Bcl-x _L :Bad BH3	Mouse	2BZW	X-ray	[47]
M11:Beclin-1 BH3	myHV68/Mouse	3BL2	X-ray	[47]
A1/Bfl-1:Puma BH3	Mouse	2VOF	X-ray	[48]
A1/Bfl-1:Bmf BH3	Mouse	2VOG	X-ray	[48]
A1/Bfl-1:Bak BH3	Mouse	2VOH	X-ray	[48]
A1/Bfl-1:Bid BH3	Mouse	2VOI	X-ray	[48]
Mcl-1:Puma BH3	Mouse	2ROC	NMR	[49]
Mcl-1:mNoxaA BH3	Mouse	2ROD	NMR	[49]
Mcl-1:mutBim BH3	Human/Mouse	3D7V	X-ray	[50]
BHRF1:Bim BH3	EBV/Human	2V6Q	X-ray	[37]
BHRF1:Bak BH3	EBV/Human	2XPX	X-ray	[37]
M11:Beclin-1 BH3	myHV68/Mouse	3DVU	X-ray	[51]
Bcl-x _L :Foldamer	Human	3FDM	X-ray	[52]
Bcl-x _L :BimBH3	Human	3FDL	x-ray	[52]
Bcl-x _L :BimBH3L12F	Human	3IO8	X-ray	[27]
Mcl-1:BimL12Y	Human/Rat	3IO9	X-ray	[27]
Mcl-1:Bim BH3	Human	2PQK	X-ray	[53]
Mcl-1:Bim BH3	Human	2PQK	X-ray	[53]
Mcl-1:Bim12dY BH3	Human	3KJ0	X-ray	[53]
Mcl-1:Bim12dA BH3	Human	3KJ1	X-ray	[53]
Mcl-1:BimF4aE BH3	Human	3KJ2	X-ray	[53]
Mcl-1:Bid BH3	Human	2KBW	NMR	[54]
Mcl-1:Mcl-1 BH3	Human	3MK8	X-ray	[55]
Mcl-1:MB7	Human	3KZ0	X-ray	[56]
Bcl-2:Bak BH3	Human	2XA0	X-ray	[57]
Mcl-1:Bax BH3	Human	3PK1	X-ray	[58]
Bcl-x _L :Bax BH3	Human	3PL7	X-ray	[58]
sJA:Bak BH3	Schistosome/Human	3QBR	X-ray	[59]
Bcl-x _L :Soul BH3	Human	3R85	X-ray	[60]
Bcl-x _L :Puma Foldamer	Human	2YJ1	X-ray	[61]
Bax:vMIA	Human/CMV	2LR1	NMR	[62]
Bcl-x _L :αβ foldamer 4C	Human	4A1W	X-ray	[63]
Bcl-x _L :αβ foldamer 2C	Human	4A1U	X-ray	[63]
Bcl-b:Bim BH3	Human	4B4S	X-ray	[11]
A1/Bfl-1:Bid BH3	Human	4ZEQ	X-ray	Not published
A1/Bfl-1:Bak BH3	Human	3I1H	X-ray	Not published
A1/Bfl-1:Noxa BH3	Human	3MQP	X-ray	Not published
Mcl-1:αβPuma2	Human	4BPI	X-ray	[64]
Mcl-1:αβPuma3	Human	4BPJ	X-ray	[64]
Mcl-1:αβPuma5	Human	4BPK	X-ray	[64]
Bcl-x _L :BimLOCK BH3	Human	2YQ7	X-ray	[65]
Bcl-x _L :BimSAHB BH3	Human	2YQ6	X-ray	[65]

Bcl-x _L :Puma BH3	Human	4HNJ	X-ray	[66]
Bcl-x _L :Puma BH3	Human	2M04	NMR	[66]
Bax:Bid BH3	Human	4BD2	X-ray	[16]
Bax:Bax BH3	Human	4BD6	X-ray	[16]
Bax BH3-inGroove dimer	Human	4BDU	X-ray	[16]
Bak:Bid SAHB BH3	Human	2M5B	NMR	[67]
Mcl-1:Mcl-1BH3	Human	4HW4	X-ray	[68]
Bcl-w:Bcl-w BH3	Human	4CIM	X-ray	[69]
Bcl-x _L :Bcl-xL BH3	Human	4CIN	X-ray	[69]
BHRF1:BINDI	EBV	4OYD	X-ray	[70]
Bak BH3-in-Groove dimer	Human	4U2V	X-ray	[15]
F1L:Bim BH3	Vaccinia virus/Human	4D2M	X-ray	[10]
F1L:Bak BH3	Vaccinia virus/Human	4D2L	X-ray	[10]
Bcl-x _L :p53	Human	2MEJ	NMR	[71]
DPV022:Bim BH3	Deerpoxvirus/Human	4UF3	X-ray	[9]
DPV022:Bak BH3	Deerpoxvirus/Human	4UF1	X-ray	[9]
DPV022:Bax BH3	Deerpoxvirus/Human	4UF2	X-ray	[9]
F1L:Bid BH3	Variola virus/Human	5AJJ	X-ray	[72]
F1L:Bak BH3	Variola virus/Human	5AJK	X-ray	[72]
Bcl-x _L :Bid BH3	Human	4QVE	X-ray	[73]
Bcl-x _L :Bim BH3	Human	4QVF	X-ray	[73]
Bax:Bim BH3mini	Human	4ZIF	X-ray	[31]
Bax:Bim BH3mini	Human	4ZIH	X-ray	[31]
Bax:Bim BH3	Human	4ZIE	X-ray	[31]
Bax:Bid BH3mini	Human	4ZIG	X-ray	[31]
BaxI66A:Bid BH3	Human	4ZII	X-ray	[31]
Bcl-x _L :BimBH3 with AKT site	Human	4YJ4	X-ray	[74]

Table 2: PDB entries of Bcl-2 family members in complex with compounds. Three letter codes from the PDB entries are used to describe ligands unless a specific name for the compound has been published.

Protein:Drug complex	Species	PDB code	Method	Reference
Bcl-x _L :N3B	Human	1YSI	NMR	[5]
Bcl-x _L :4FC/TN1	Human	1YSG	NMR	[5]
Bcl-x _L :43B	Human	1YSN	NMR	[5]
Bcl-2:43B	Human	1YSW	NMR	[5]
Bcl-x _L :43B	Human	2O1Y	NMR	[75]
Bcl-2:43B	Human	2O21	NMR	[75]
Bcl-2:LIU	Human	2O22	NMR	[75]
Bcl-2:LI0	Human	2O2F	NMR	[75]
Bcl-x _L :LI0	Human	2O2M	NMR	[75]
Bcl-x _L :LIW	Human	2O2N	NMR	[75]
Bcl-x _L :ABT-737	Human	2YXJ	X-ray	[38]
Bcl-2:DRO	Human	2W3L	X-ray	[76]
Bcl-x _L :W1191542	Human	3INQ	X-ray	[27]
Bcl-x _L :HI0	Human	3QKD	X-ray	[77]
Bcl-2:398	Human	4AQ3	X-ray	[78]
Bcl-x _L :0Q5	Human	4EHR	X-ray	[79]
Bcl-x _L :B50	Human	3SPF	X-ray	[80]
Bcl-x _L :03B	Human	3SP7	X-ray	[80]
Bcl-x _L :33B	Human	2LP8	NMR	[81]
Mcl-1:PRD_000921	Human	4G35	X-ray	[82]
Bcl-x _L :WEHI-539	Human	3ZLR	X-ray	[28]
Bcl-x _L :1HI	Human	3ZK6	X-ray	[28]
Bcl-x _L :H0Y	Human	3ZLN	X-ray	[28]
Bcl-x _L :X8U	Human	3ZLO	X-ray	[28]
Bcl-2:1E9	Human	4IEH	X-ray	[83]
Mcl-1:19H	Human	4HW2	X-ray	[68]
Mcl-1:19G	Human	4HW3	X-ray	[68]
Bcl-2:ABT-263	Human	4LVT	X-ray	[84]
Bcl-2:1XV	Human	4LXD	X-ray	[84]
Bcl-2:1Y1	Human	4MAN	X-ray	[84]
Mcl-1:LC3	Human	3WIX	X-ray	[85]
Mcl-1:LC6	Human	3WIY	X-ray	[85]

Bcl-x _L :LC6	Human	3WIZ	X-ray	[85]
Bcl-x _L :XOR	Human	4C5D	X-ray	[29]
Bcl-x _L :X0D	Human	4C52	X-ray	[29]
Mcl-1:2UU	Human	4OQ5	X-ray	[86]
Mcl-1:2UV	Human	4OQ6	X-ray	[86]
Bcl-w:013_D12	Bos Taurus	4K5A	X-ray	[87]
Bcl-w:UNP	Bos Taurus	4K5B	X-ray	[87]
Bcl-x _L :38H	Human	4TUH	X-ray	[88]
Mcl-1:3M6	Human	4WGI	X-ray	[89]
Bcl-x _L :3CQ	Human	4QVX	X-ray	[30]
Mcl-1:4M7	Human	4ZBF	X-ray	[90]
Mcl-1:4M6	Human	4ZBI	X-ray	[90]
Mcl-1:BRDI1	Human	4WMR	X-ray	[14]
Mcl-1:865	Human	4WMT	X-ray	[14]
Mcl-1:19H	Human	4WMU	X-ray	[14]
Mcl-1:BRDI3	Human	4WMV	X-ray	[14]
Mcl-1:BRDI4	Human	4WMW	X-ray	[14]
Mcl-1:BRDI5	Human	4WMX	X-ray	[14]
Mcl-1:BRDI6	Human	4WMY	X-ray	[14]

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Figure 1

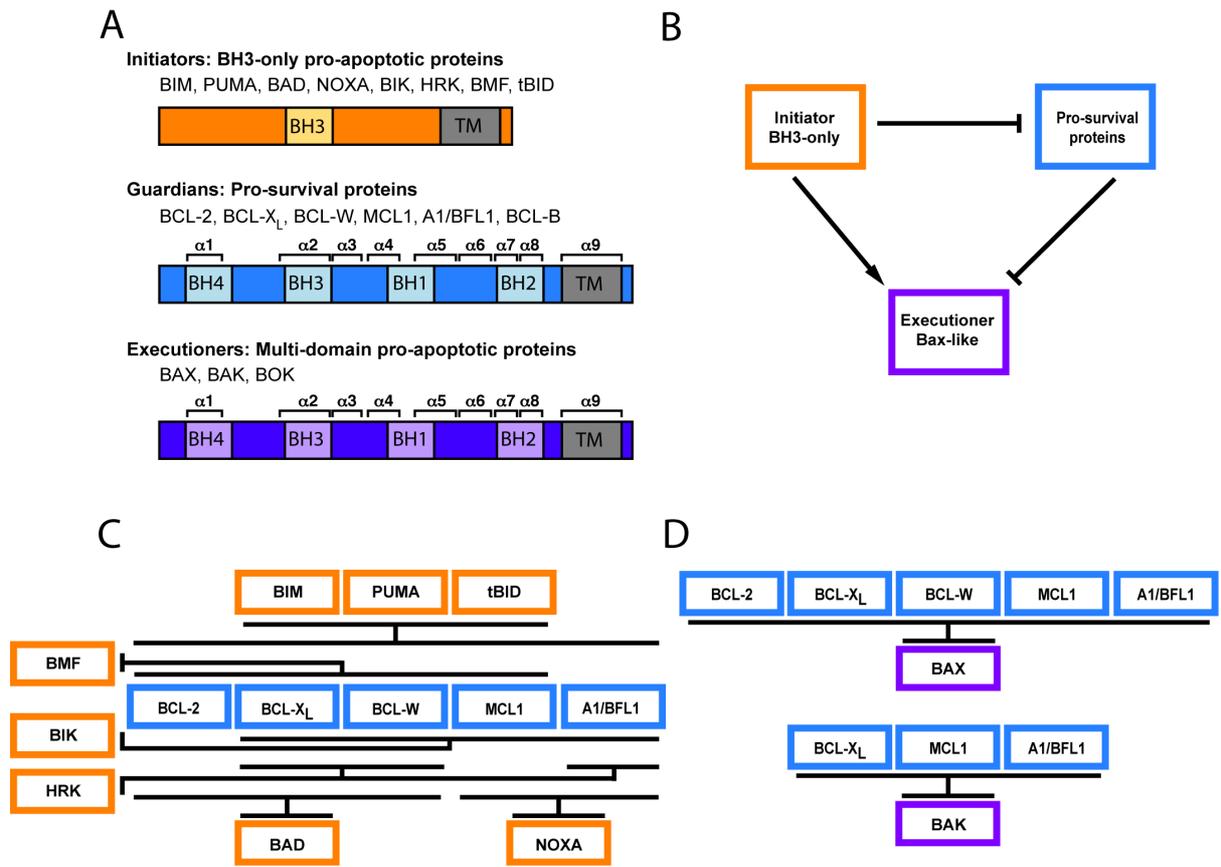


Figure 2

