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The Structure of a Bcl-x_L/Bim Fragment Complex: Implications for Bim Function

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Summary

After antigen-driven expansion, the majority of T cells involved in an immune response die rapidly by apoptosis dependent on the Bcl-2 related proteins, Bim and Bax or Bak. The details of how these proteins are activated and interact are still unclear. The crystal structure of mouse Bcl-x_L bound to a long helical fragment of Bim indicates that the structure of Bim is very different from proteins with a Bcl-2-like fold and may leave the BH3 region of Bim constitutively exposed. Based on the structural homology between Bcl-x_L and Bax, we predicted that binding of Bim to Bax would require displacement of the Bax penultimate α helix. Consistent with this prediction, truncation of this short helix was required for Bim/Bax interaction and led to spontaneous activation of Bax. Our results suggest a way in which both Bim and Bax/Bak might be required for activated T cell apoptosis.

Introduction

The majority of T cells involved in an immune response die rapidly by apoptosis following a period of rapid expansion driven by engagement of the $\alpha\beta$TCR and CD28 (Kawabe and Ochi, 1991; Kearney et al., 1994; Vella et al., 1995; Webb et al., 1992). Although T cells die in several ways, their rapid death following acute antigen activation in vivo appears to be mediated primarily by interactions among the evolutionarily conserved Bcl-2 family of proteins via their effect on the viability of mitochondria (Akbar et al., 1993; Bouillet et al., 2002; Davey et al., 2002; Hildeman et al., 2002; Noel et al., 1996; Petschner et al., 1998).

Some members of the Bcl-2 family of proteins inhibit apoptosis, whereas others promote cell death (Adams and Cory, 1998; Gross et al., 1999). Originally, these proteins were assigned to three groups based on homology within certain regions of their sequences. The antiapoptotic proteins were related to each other in four regions, called BH1-4. One set of proapoptotic proteins, which includes Bax and Bak, contained three of these regions, BH1-3, but lacked a region homologous to BH4. Finally, a second set of proapoptotic proteins, which includes Bid and Bim, had only one of the homology regions, BH3. This region is essential for the death-dealing activities of all the proapoptotic proteins and probably acts by binding to grooves in the antiapoptotic Bcl-2-like proteins and/or the proapoptotic BH1-3 proteins (Petros et al., 2000; Reed et al., 1996; Wang et al., 1996; Zha et al., 1997). Despite these classifications, the NMR and crystal structures of members of these groups (Bcl-2, Bcl-x_L, Bcl-w, Bax, Bid) have so far revealed very similar folds (Aritomi et al., 1997; Hinds et al., 2003; McDonnell et al., 1999; Muchmore et al., 1996; Petros et al., 2001; Suzuki et al., 2000).

A number of studies have investigated the functions of these proteins; however, their precise roles in cell death are still argued. Bax and/or Bak appear to be involved proximally in the death of the cell apparently via their aggregation and disruption of the mitochrondrial potential (Jurgensmeier et al., 1998; Lindsten et al., 2000; Rathmell et al., 2002; Wei et al., 2001). However, whether their aggregated products act directly on the mitochrondrial membranes (Antonsson et al., 1997; Epand et al., 2002) or via other proteins (Belzacq et al., 2003; Brenner et al., 2000; Narita et al., 1998; Shimizu et al., 1999) remains unresolved. Also unresolved is whether the activity of Bax/Bak requires their binding to BH3-only proapoptotic proteins such as truncated Bid (Korsmeyer et al., 2000; Kuwana et al., 2002; Roucou et al., 2002), or whether the BH3-only proteins act via antiapoptotic proteins or even some other route (Sugiyama et al., 2002). It is also thought that the antiapoptotic proteins, Bcl-2 and Bcl-x_L, act by inhibiting the consequences of the proapoptotic proteins, although agreement has not yet been reached on exactly how this inhibition is effected (Antonsson et al., 1997; Bouillet and Strasser, 2002; Mikhailov et al., 2001; O'Connor et al., 1998).

In T cells, the major antiapoptotic proteins are Bcl-2 and Bcl-x_L (Noel et al., 1996; Petschner et al., 1998), while Bak, Bax, and Bim appear to be the major proapoptotic agents (Bouillet et al., 2002; Davey et al., 2002; Hildeman et al., 2002; Lindsten et al., 2000; Wei et al., 2001). Since all of these proteins preexist in healthy T cells, there must be some change in expression levels, functional state, and/or intracellular location to account for the eventual death of the T cell following $\alpha\beta$TCR activation.

In the present study, we have concentrated on the properties of Bim and its interactions with Bcl-x_L and Bax in an attempt to understand the role of this protein in apoptosis induction in activated T cells. We have solved at high resolution the crystal structure of mouse Bcl-x_L and of a large fragment of Bim bound to Bcl-x_L. The latter structure reveals extensive contact between Bcl-x_L and a previously unappreciated unique, long Bim α helix containing a BH3 homology region that may be constitutively accessible.

Exposure of the α9 TM helix of Bax is required for translocation of this protein to mitochondria (Goping et al., 1998; Nechushtan et al., 1999). However, our struc-

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Table 1. Data Collection and Refinement Statistics

Data Collection		
Data set	Bcl-x_L	Bcl-x_L/Bim
Space group	$P4_12_12$	$P2_1$
Unit cell dimensions (Å)	a = b = 63.61	a = 31.90
	c = 109.60	b = 47.34
		c = 49.28
Unit cell angles (°)		β = 110.10
Number of molecules in AU	1	1
Resolution limits (Å)	30.0-2.20 (2.34-2.20)[a]	20.0-1.65 (1.75-1.65)
Unique reflections	11386 (968)	19877 (1765)
Completeness (%)	95.7 (88.4)	97.2 (90.9)
Average redundancy	4.1 (3.5)	4.8 (4.4)
Average I/σ	9.4 (3.5)	8.6 (2.7)
R_{merge}(%)[b]	8.7 (28.5)	6.2 (21.8)
Refinement		
Data set	Bcl-x_L	Bcl-x_L/Bim
Resolution (Å)	30.0-2.20	20.0-1.65
I/σ cut off	2.0	2.0
Total reflections	11227 (864)	19462 (1621)
Reflections used for R_{free}	525 (74)	923 (140)
Rworking (%)	21.7 (24.3)	21.7 (27.0)
R_{free} (%)[c]	24.3 (27.0)	23.7 (31.3)
Average B factors (Å^2)	37.2	23.9
Ramachandran data (% of residues[d] in:)		
Favored regions	91.4	93.8
Allowed regions	7.8	6.2
Generously allowed regions	0.8	0.0
Disallowed regions	0.0	0.0
Rmsd		
Bonds (Å)	0.0061	0.0052
Angles (°)	1.09	1.11
B factor main chain (Å^2)	1.02	0.55
B factor side chain (Å^2)	1.51	0.73
Cross-validated coor. error (Å)	0.4	0.4

[a] All data (outer shell)
[b] Rmerge = Σ (|I − <I>|)/Σ (I)
[c] Rworking/free = Σ||Fo| − |Fc||/Σ|Fo|
[d] Excluding glycine and proline

ture and the extensive structural homology between Bcl-x_L and Bax suggested that the binding of Bim to Bax would require displacement of the short α8 helix of Bax as well. We confirmed this prediction in a series of binding studies using Bim and truncated versions of Bax. Our experiments offer an explanation for the fact that the presence of Bax or Bak on the mitochondrial surface is insufficient for their full functional activation and suggest a mechanism for the induction and stabilization of the final active form of these proteins by Bim.

Results

The Structure of Free Mouse Bcl-x_L

We crystallized free mouse Bcl-x_L produced in insect cells with baculovirus. The protein crystallized readily and the structure was solved to 2.2 Å resolution by molecular replacement using the rat Bcl-x_L structure (Aritomi et al., 1997) as the model (Table 1). For clarity, the helices which had previously been called α6′ and α7 in Bcl-x_L (Muchmore et al., 1996; Petros et al., 2000) are named α7 and α8, respectively, in this paper in accordance with the nomenclature of Bax (Figure 1A). Not surprisingly, the structure of Bcl-x_L reported here is nearly identical to those previously seen with rat and human Bcl-x_L (Aritomi et al., 1997; Muchmore et al., 1996). The greatest variation is seen in the region of Ala104 at the beginning of the α3 helix. This may be a consequence of flexibility of this region (see below). Notably, in all three structures a large loop (aa 27–aa 81) connecting helices α1 and α2 appears largely disordered. The exact function of this loop is not known. Though some studies have suggested it is a negative regulatory domain (Chang et al., 1997), others have shown that much of it can be deleted without compromising either the rest of the structure or function of Bcl-x_L (Muchmore et al., 1996).

Interactions among Bim, Bcl-x_L, and LC8

T cells express two major splice variants of Bim, Bim_{EL}, and Bim_L (Bouillet et al., 2002). The RNA message for Bim_{EL} includes an exon that adds 56 amino acids to the middle of the molecule. These are not present in Bim_L. However, Bim_{EL} and Bim_L have similar activities. Both cause cell death in transfection/transduction assays; therefore, for simplicity we used Bim_L in our studies.

Mouse Bim_L, lacking the C-terminal putative membrane association sequence, was expressed in insect

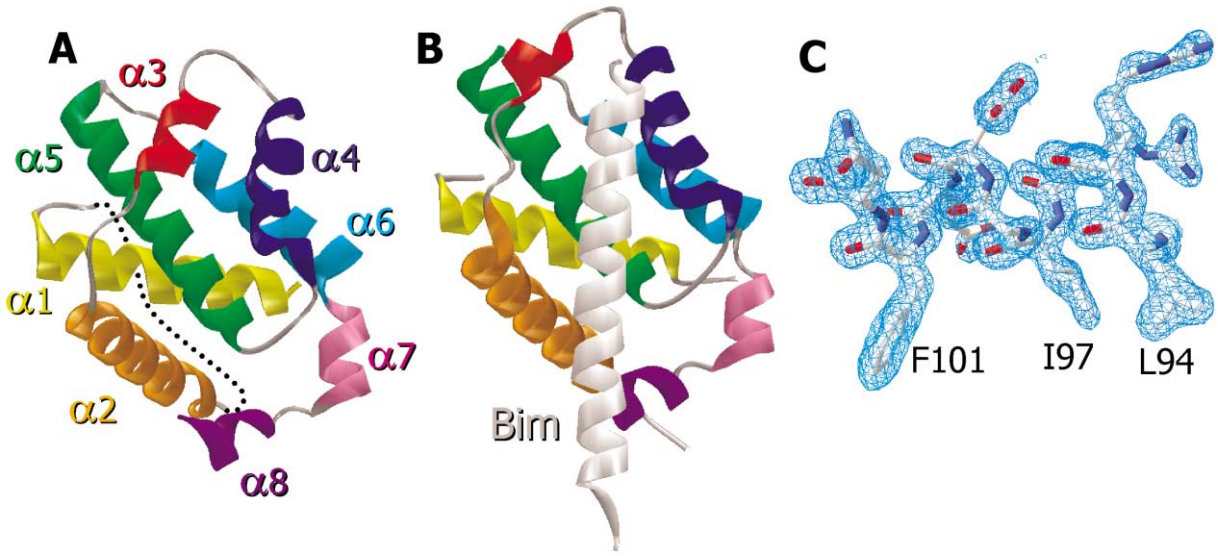

Figure 1. The Structure of Mouse Bcl-x_L and Bcl-x_L/Bim

(A) A ribbon structure of mouse Bcl-x_L with the eight α helices colored sequentially. Yellow, α1; orange, α2; red, α3; purple, α4; green, α5; blue, α6; pink, α7; cyan, α8. The loop connecting α1 to α2 was disordered and indicated with a dotted line.

(B) A ribbon structure of a large helical fragment of Bim_L (aa 83–115) bound to mouse Bcl-x_L. The eight Bcl-x_L α helices are colored as in (A).

(C) 2Fo-Fc electronic density (1.5σ cutoff) of the BH3 region of Bim in the Bcl-x_L/Bim structure. Three conserved BH3 hydrophobic amino acids, L94, I97, and F101 are labeled. (A) and (B) produced with WebLab Viewer Pro (Accelrys Inc, San Diego) and (C) produced with Swiss PDB viewer (Guex and Peitsch, 1997).

cells via baculovirus infection. The protein was soluble in dilute detergent but could not be concentrated without degradation and aggregation (data not shown). Therefore, we attempted to stabilize Bim with other potential protein binding partners. Bim has been reported to associate in vivo with the dynein motor light chain, LC8 (Puthalakath et al., 1999) and with Bcl-x_L (O'Connor et al., 1998). Using coinfection with baculoviruses expressing various combinations of the mouse versions of these proteins, we were able to purify a soluble, stable stoichiometric complex of Bim, LC8, and Bcl-x_L. While SDS-PAGE analysis of this complex indicated that it contained an equimolar amount of each component, the apparent molecular weight of this complex was about 100 kDa as judged by Superdex-200 size exclusion chromatography, suggesting a ratio of 2:2:2 of Bim, LC8, and Bcl-x_L. This was consistent with the dimerization property of LC8 (Fan et al., 2001).

Bcl-x_L Crystallizes with a Long α Helix of Bim

We attempted to crystallize the complex of LC8/Bim/Bcl-x_L. Crystals formed only after many months of incubation in the crystallization drop. Gel analysis showed that extensive degradation of Bim and LC8 but not Bcl-x_L had taken place (data not shown). Nevertheless, the crystal diffracted extremely well and a high-resolution data set was collected. The Bcl-x_L portion of the structure was readily identified using molecular replacement with free mouse Bcl-x_L as the model. Additional electron density was seen clearly in the structure occupying in part a groove of Bcl-x_L that had previously been shown to bind BH3 peptides from Bak and Bad (Petros et al., 2000; Sattler et al., 1997). Eventually, this density was attributed to a large helical fragment of Bim containing its BH3 region. The overall structure was refined to a resolution of 1.65 Å (Table 1, Figures 1B and 1C).

The most dramatic changes that take place in Bcl-x_L upon binding of Bim involve its α3 and α4 helices. Engagement of the Bim peptide causes the α3 helix of Bcl-x_L to lose some of its helical quality and also displaces it by about 4 Å away from the Bim peptide. Conversely, engagement of Bim causes the α4 of Bcl-x_L to move about the same distance toward the bound Bim (Figure 2A). Two other structures of Bcl-x_L bound to BH3 peptides, from the proapoptotic proteins Bak and Bad, have been reported (Petros et al., 2000; Sattler et al., 1997). Similar, but not identical, alterations of the Bcl-x_L α3 and α4 helices occur upon binding of these peptides with even greater loss of the α helical quality of the α3 amino acids (Figures 2B and 2C). Overall, the Bcl-x_L backbone appears to be flexible at Ala104 at the beginning of the α3 helix, and this point serves as a fulcrum for the movements observed. Taken together, these structures point out the great flexibility of this end of the binding groove. This flexibility may account for the ability of Bcl-x_L to bind to many different types of proapoptotic proteins.

Details of the Interaction between Bcl-x_L and Bim

The BH3-containing helix of Bim in this complex is considerably longer than seen in any Bcl-2 family member thus far (Figure 3). This longer helix provides a large surface for interaction between Bim and an extended groove on the surface of Bcl-x_L involving parts of the α2, α3, α4, α5, and α8 helices as well as the connecting loops between α2 and α3 and between α4 and α5 (Figure 4A). Viewed face on, the left side of the binding groove is dominated by interactions among hydrophobic amino

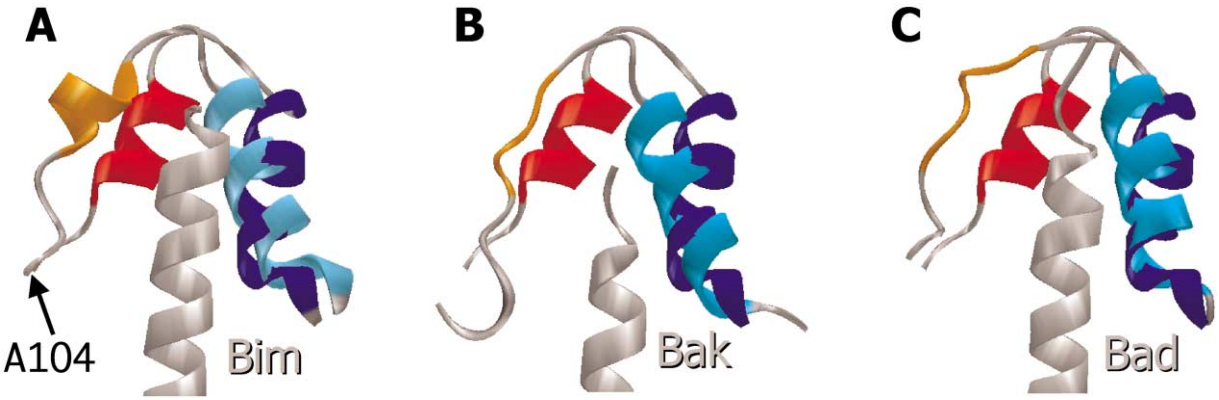

Figure 2. Changes in Bcl-x_L Upon Binding Bim, Bak, or Bad BH3 Peptides

Superimposed ribbon representations of the α3/α4 helices and connecting loop of Bcl-x_L are shown before and after binding of BH3 containing peptides from proapoptotic proteins.

(A) Mouse Bim fragment (aa 83–115) bound to mouse Bcl-x_L. The position alanine 104 is shown with an arrow. Red, α3 of Bcl-x_L; blue, α4 of Bcl-x_L; orange, α3 of Bcl-x_L/Bim; cyan, α4 of Bcl-x_L/Bim; gray, Bim peptide.

(B) Human Bak peptide (aa 572–587) bound to human Bcl-x_L. Corresponding part colored as in (A).

(C) Human Bad peptide (aa 140–164) bound to human Bcl-x_L. Corresponding part colored as in (A). Figures produced with WebLab Viewer Pro.

acids that include the four conserved hydrophobic residues of the BH3 region (Figures 3 and 4B). Amino acids at the corresponding positions in peptides derived from Bak and Bad are similarly involved in binding to Bcl-x_L. Water is excluded from this interface (Petros et al., 2000; Sattler et al., 1997). On the other hand, the right side of the binding groove involves primarily hydrophilic interactions, especially salt bridges (Figures 3 and 4C). Several well-defined water molecules are found in the interface (data not shown). Due to the length of the Bim helix, there is also considerable interaction of its C-terminal end with the α8 helix of Bcl-x_L (Figures 3 and 4D). Interactions involving Asn102, Tyr105, and Val109 from Bim and Leu194, Tyr195 from Bcl-x_L may stabilize the Bim binding to Bcl-x_L at this end of the helix. The Bim α helix ends just before the proposed C-terminal membrane interaction region of Bim. Likewise, the α8 helix of Bcl-x_L is followed in the natural protein by the predicted transmembrane of the molecule. Thus, the orientation of Bcl-x_L and Bim in this complex is appropriate for their interaction while both are membrane associated. However, we cannot predict whether or not the re-addition of these membrane-interacting regions to the proteins might alter the interactions we see in our structures of their C termini. Another point worth mentioning is that the binding of a Bim BH3 containing peptide truncated at residue 104, nevertheless binds to Bcl-2. Therefore, interaction of the C-terminal portion of the Bim helix with Bcl-2 or Bcl-x_L may improve the affinity of interaction but not be essential for binding.

Despite the proteolytic degradation of Bim_L during crystallization, the fact that the Bim fragment was generated from a preformed, stable complex of intact Bim_L with Bcl-x_L and LC8 argues for a similar mode of binding of Bcl-x_L to the full-length Bim_L. We cannot tell whether, in a manner similar to Bax and Bak, Bim might have a different conformation in an unbound state. Bax and Bak require an induced conformational change in order to bind Bcl-2 or Bcl-x_L. However, we have seen no differences in the ability of Bim_{EL} or Bim_L versus a peptide fragment corresponding to the Bim fragment in the structure presented here to form stable complexes with Bcl-x_L spontaneously during coexpression (data not shown).

Potential Interaction between Bim and Bax

Bim is required for the apoptosis that occurs in T cells after in vivo antigen-induced expansion (Bouillet et al., 2002; Davey et al., 2002; Hildeman et al., 2002). However, other experiments have pointed out that either Bax or Bak is also required for induction of this type of apoptosis (Bouillet and Strasser, 2002; Lindsten et al., 2000; Rathmell et al., 2002; Wei et al., 2001). Evidence indicates that these latter two proteins may act by forming a lethal pore in the mitochondrial membrane (Antonsson et al., 1997; Belzacq et al., 2003; Brenner et al., 2000; Epand et al., 2002; Narita et al., 1998; Shimizu et al., 1999). Formation of these pores may involve BH3-only proteins as well, as has been demonstrated with truncated Bid (Korsmeyer et al., 2000; Kuwana et al., 2002; Roucou et al., 2002). This leads to the possibility that the function of Bim, like that of truncated Bid, may be to activate or stabilize the "killer" forms of Bax and Bak. To do this, Bim must bind Bax. Since the three-dimensional structures of Bax and Bcl-x_L are very similar (Aritomi et al., 1997; Muchmore et al., 1996; Petros et al., 2001; Suzuki et al., 2000), we reasoned that Bim might bind to Bax in the same way as it binds to Bcl-x_L. Therefore, we performed some experiments to find out whether this is so.

Analysis of Bax/Bim Interactions by Computer Modeling

We superimposed our Bim/Bcl-x_L structure on that of Bax to see how well Bim might bind to Bax. In the solved structure of monomeric Bax, the presumptive BH3 binding site is occupied by the α9 transmembrane region of Bax. The modeled structure of the Bim peptide bound to Bax shows that the Bim peptide would occupy almost exactly the same position as the α9 helix of Bax (Figure

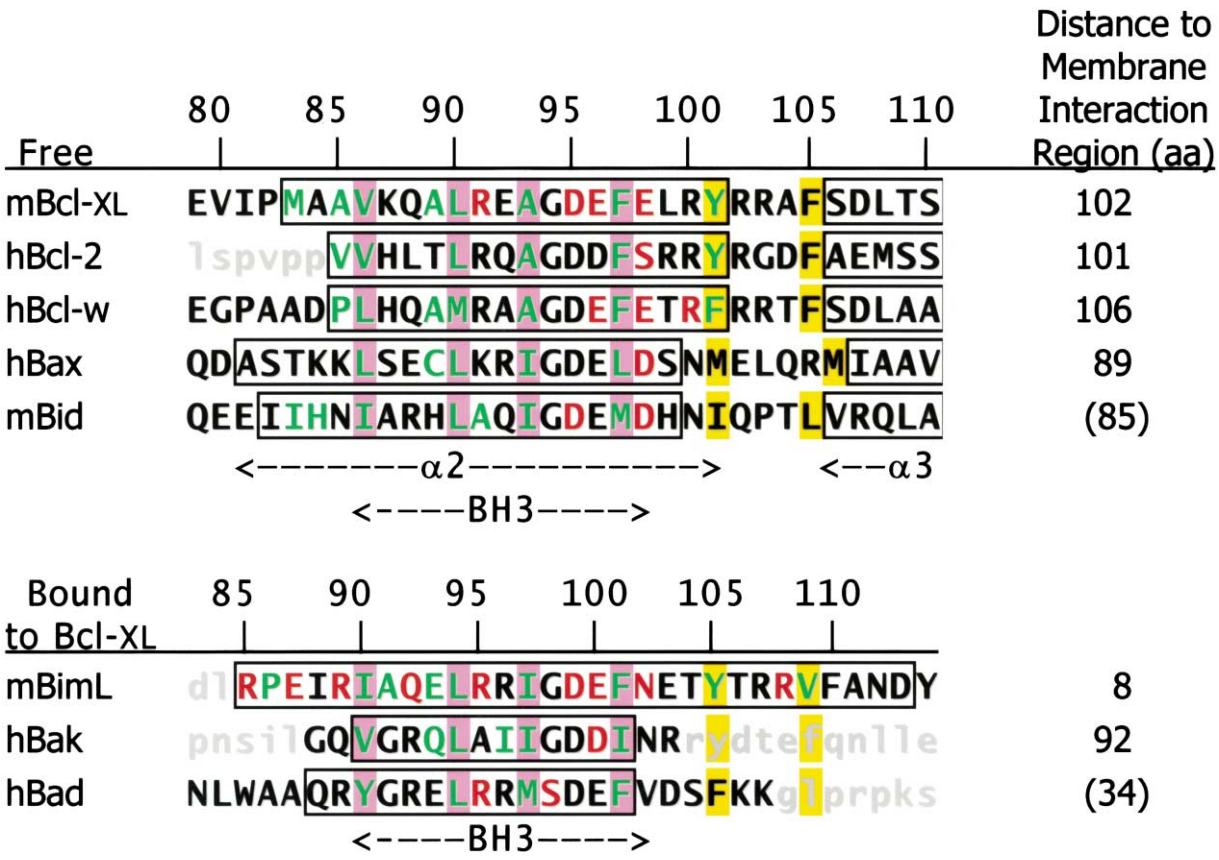

Figure 3. Comparison of the BH3 Regions of Bcl-2 Family Members

(Upper Panel) Sections of sequences containing the BH3 regions of mouse Bcl-x_L, human Bcl-2 (Petros et al., 2001), human Bcl-w (Hinds et al., 2003), human Bax (Suzuki et al., 2000), and mouse Bid (McDonnell et al., 1999) are aligned via their BH3 regions based on the structures of the monomeric proteins. Amino acid numbering is based on mouse Bcl-x_L. Upper case letters represent amino acids whose structures were solved. Lower case gray letters represent amino acids missing from the structure.

(Lower Panel) Sections of sequences of mouse Bim_L, human Bak (Sattler et al., 1997), and human Bad (Petros et al., 2000) are also aligned via their BH3 regions based on the structures of peptides fragments bound to Bcl-x_L. Upper case letters represent amino acids of the peptides. Lower case gray letters represent amino acids of the protein not present or not found in the peptide structures. Boxes enclose amino acids in α helices. Amino acids in the BH3 containing helices whose side chains are involved in hydrophobic (green) or hydrophilic (red) interactions are indicated. Conserved BH3 hydrophobic amino acids have a magenta background. Two other potentially important conserved hydrophobic amino acids have a yellow background. Also listed are the numbers of amino acids separating the shown amino acids from the putative membrane interaction region of the protein or, in parentheses (for those proteins without a identified membrane interaction region), the number of amino acids from the C-terminal end of the protein.

5A), so clearly Bim cannot bind to monomeric, soluble Bax in this way. However, when Bax translocates to mitochondria, Bax α9 moves to integrate into the mitochondrial membrane (Ruffolo et al., 2000). With α9 out of the way, the binding groove of Bax is very similar to that of Bcl-x_L, with many hydrophobic amino acids on one side and hydrophilic amino acids on the other (Figure 5B). Thus Bim could bind to Bax via Bax α3, α4, α5, and α6 similarly to the way it binds to Bcl-x_L. However, in our model the α8 of Bax clashes with the C-terminal end of the superimposed Bim α helix (Figure 6C). For example, Y105 and R108 of the Bim peptide collide with F165 and T167, respectively, of Bax. This collision does not occur when the Bim peptide binds to Bcl-x_L, perhaps because the binding groove of Bcl-x_L is some what wider than that of Bax, which allows more adjustment when Bim binds. More importantly, when either the free or Bim bound structure of Bcl-x_L is compared to that of Bax, α8 sits somewhat deeper in the binding groove where it contributes positively rather than negatively to the binding of Bim (Figure 1B).

These results suggested to us that displacement of the α9 helix and its insertion in the mitochondrial membrane may not be sufficient to activate Bax completely, but that the displacement of the α8 helix may be required as well. This displacement might be augmented or stabilized by interaction with Bim after translocation of Bax to the mitochondrial membrane and account for their synergy in apoptosis.

α8 of Bax Must Be Displaced in Order for the Protein to Be Fully Activated

To examine the role of Bax α8, we expressed in baculovirus three forms of Bax: the complete protein; a variant in which the protein was truncated at Thr167, removing the α9 transmembrane region (BaxΔTM); and a variant in which the protein was truncated at Gly157 so that both α8 and the transmembrane were lost (Bax$\Delta\alpha$8TM).

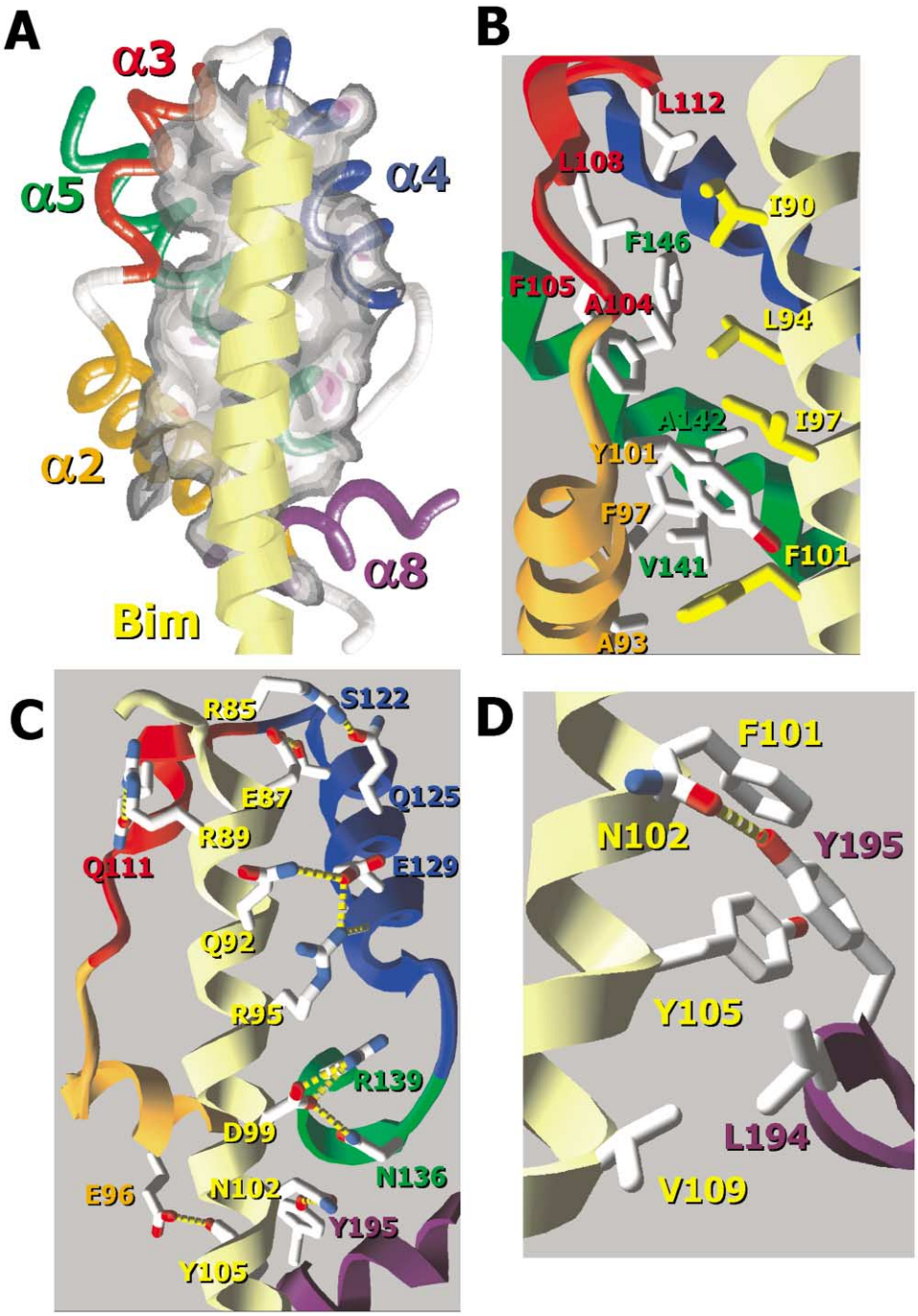

Figure 4. Details of the Interaction of Bcl-x_L with Bim

(A) The interaction surface of Bim on Bcl-x_L was calculated using Protein Explorer (Martz, 2001). The surface is colored by its distance from Bim: magenta, closest; white, intermediate; and gray, farthest.

(B) The hydrophobic interactions between Bcl-x_L and Bim. The side chains of the conserved four BH3 hydrophobic residues from Bim are shown in yellow. The side chains of the Bcl-x_L hydrophobic residues that interact with Bim are shown in CPK coloring (C, white; O, red).

(C) Hydrophilic interactions between Bcl-x_L and Bim. Side chains of Bcl-x_L and Bim involved in H bonds and salt bridges (yellow dotted lines) are shown in CPK coloring (C, white; O, red; N, blue).

(D) Interactions between α8 of Bcl-x_L (purple ribbon) and the C-terminal end of the Bim fragment (yellow ribbon). Side chains of the interacting residues are shown in CPK coloring. (B), (C), and (D) produced with Swiss PBD Viewer.

In addition, six histidine (6His) residues were added to the C-terminal end of each of these proteins. Insect cells were infected with viruses expressing each of the Bax-6His forms together with a virus expressing Bim. The infected cells were lysed by sonication in the absence or presence of 0.5% NP-40 and Bax isolated with Ni-

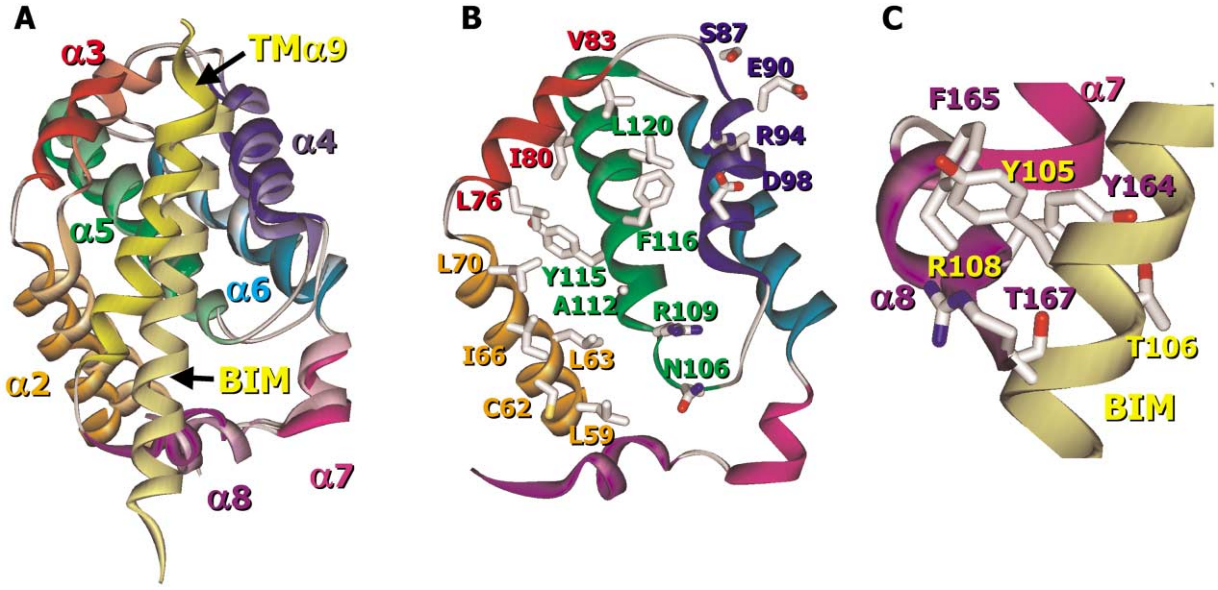

Figure 5. Postulated Interaction between Bax and Bim

(A) The structures of Bax and Bcl-x_L/Bim were superimposed by Swiss-PdbViewer based on minimal rmsd of the Cα backbone. Ribbon structures of the two proteins are shown with helices colored as in Figure 1A, except that the Bax ribbons are a darker shade of the colors than are the Bcl-x_L/Bim ribbons. For clarity, the α1 helix has been omitted from both structures.

(B) Ribbon structure of Bax omitting the α1 and α9 transmembrane helix. Ribbons are colored as in (A). Side chains of amino acids that are similar to those of Bcl-x_L interacting with Bim are shown in CPK coloring.

(C) Potential clash between the side chains of the α8 helix of Bax (purple ribbon) and the C-terminal end of the Bim fragment (yellow ribbon) docked as in (A).

NTA beads. The isolates were then run on SDS-PAGE and Western blotted for Bax and Bim protein.

Bim coisolated with all forms of Bax from lysates made in the presence of NP-40 (Figure 6A). Strong nonionic detergents such as NP-40 have been shown to induce the fully active conformation of Bax (Hsu and Youle, 1997, 1998), so that these results indicate that the Bim can bind to activated Bax and that this binding does not require the presence of the Bax TM or α8 helix.

Very different results were seen with lysates prepared in the absence of NP-40. As expected, since the Bax transmembrane region would be expected to block the BH3 binding groove, full-length Bax did not bind to Bim. However, BaxΔTM also failed to bind to Bim despite the fact that the BH3 binding groove of Bax should now be empty (Figure 6A). This result is in accord with the idea that the extended helix of Bim would clash with α8 of Bax (Figure 5C). Such a conclusion was further supported by the fact that Bim bound well to Bax once both the transmembrane and α8 regions of Bax had been removed (Figure 6A).

Unlike NP-40, the zwitterionic detergent, CHAPS, does not activate Bax (Hsu and Youle, 1998). In line with the hypothesis proposed above, Bax and Bim prepared from insect cells lysed in the presence of CHAPS behaved similarly to those prepared in the absence of detergent (data not shown).

In addition to translocation from the cytosol to mitochondria, full activation of Bax is accompanied by a number of conformational changes. These include exposure of its BH3 domain for binding to other Bcl-2 family members (Zha et al., 1996) and the appearance of a previously hidden epitope near the N terminus of the protein. This epitope is detected by the antibody 6A7 (Hsu and Youle, 1998). In addition, fully activated Bax aggregates into large multimers that may be the functional apoptotic unit (Mikhailov et al., 2003). To find out whether displacement of Bax α8 is required for these changes, we tested the various forms of Bax for these conformational alterations.

To test for exposure of the BH3 region of Bax, we coexpressed Bcl-x_L with each of the Bax-6His forms in insect cells, isolated the proteins in the presence or absence of NP-40, and purified Bax with Ni-NTA columns. The Bax isolates were run on SDS-PAGE and the gels Western blotted for Bax and Bcl-x_L. Addition of NP-40 to the lysates resulted in Bcl-x_L binding to all three Bax forms. In lysates made without NP-40, however, Bcl-x_L did not coisolate with Bax unless both the transmembrane and α8 helix of Bax had been removed (Figure 6B).

To find out what conditions were required for exposure of the 13–19 aa N-terminal epitope of Bax, lysates were prepared, with or without NP-40, from insect cells infected with viruses expressing the various forms of Bax. Bax was purified from these lysates and tested by ELISA for exposure of the N-terminal epitope. The N-terminal epitope was exposed on all three forms of Bax isolated with NP-40. In the absence of NP-40, however, the N-terminal epitope was only detectable if both the Bax C-terminal transmembrane and α8 region had been deleted (Figure 6C).

Finally, Superdex-200 size exclusion chromatography showed that, in the absence of NP-40, full-length Bax

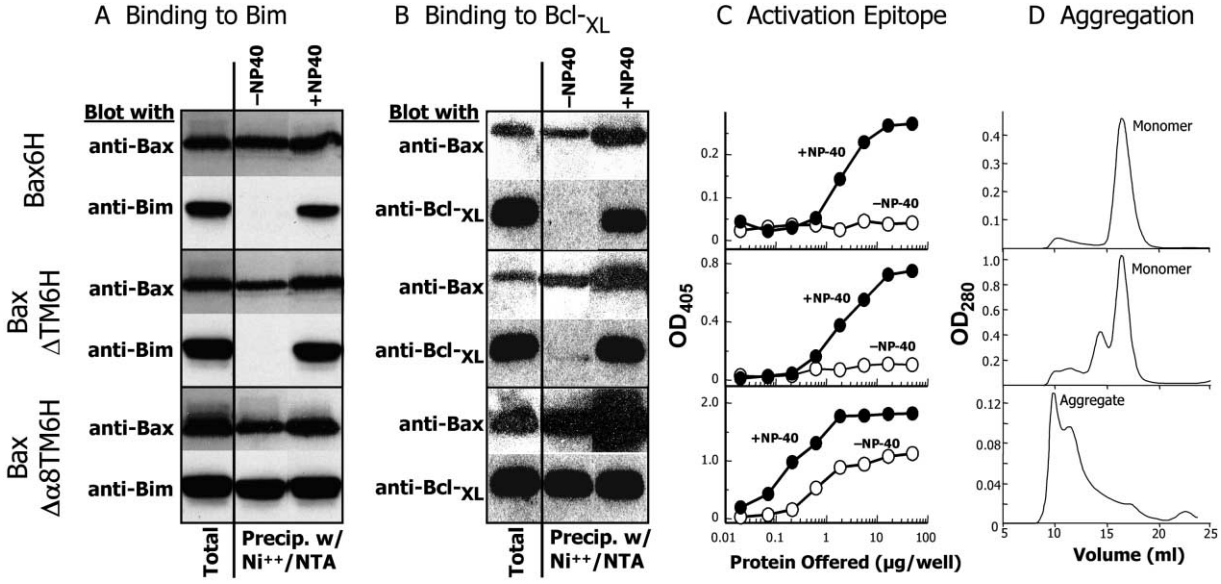

Figure 6. The Importance of the α8 Helix in the Behavior of Bax

Three 6His-tagged versions of Bax were constructed in baculovirus as described in the Experimental Procedures: upper panels, full-length Bax; middle panels, Bax truncated to remove the α9 transmembrane; lower panels, Bax truncated to remove both α8 and α9.

(A) Insect cells were coinfected with viruses expressing each of the 6His-tagged Bax variants and Bim_L. The expressed Bax and Bim were analyzed by Western blot. Right column, analysis of total cell contents. Analysis of eluates from Ni-NTA columns incubated with infected cell lysates prepared without (middle column) or with (right column) NP-40.

(B) Same as (A), but virus and antibody for Bcl-x_L, instead of Bim_L, was used.

(C) The various Bax versions were isolated by Ni-NTA chromatography from lysates of infected cells prepared with (filled circle) or without (open circle) NP-40. Various concentrations of the protein were immobilized and detected by ELISA with the Mab specific for the Bax activation epitope, 6A7.

(D) Spontaneous aggregation of the proteins isolated in (C) from cell lysates lacking NP-40, analyzed using Superdex-200 size exclusion chromatography.

was mostly monomeric. It remained monomeric after removal of its transmembrane region, but aggregated to a high molecular weight form when both its transmembrane and α8 region were deleted (Figure 6D).

These results suggest that Bax is not fully active until two events have occurred: first, removal of the transmembrane region from its BH3 binding groove; and second, a major structural reorganization of the protein which includes exposure of the N-terminal epitope detected by antibody 6A7 and displacement of the α8 helix. Such a multistep process for full activation of Bax has been suggested in the past by others (Hsu and Youle, 1997; Nechushtan et al., 1999).

Discussion

Despite the fact that they have different functions and contain different numbers of the BH homology regions, the structural cores of Bcl-2 family members solved thus far (Bcl-2, Bcl-x_L, Bcl-w, Bak, and Bid) have very similar folds consisting of compact bundles of α helices. Numbering the helices as in Figure 1, the BH3 region lies within α2 in all these structures (Figure 3). In every case, the orientation of this helix is such that a major conformational change is required to expose the conserved hydrophobic amino acids of BH3 important for interaction with other Bcl-2 family members. The BH1 and BH2 homology regions have been found in all of these proteins except BID. The BH1 region covers the C-terminal end of α4 through the first half of α5. The BH2 region covers α7 and part of α8.

There are as yet no structures of complexes between full-length pairs of Bcl-2 family members. However, the structures of two complexes have been reported of Bcl-x_L bound to helical synthetic peptides containing a BH3 region: one from Bak (Sattler et al., 1997) and one from Bad (Petros et al., 2000). The main peptide amino acids contributing to binding are again the conserved BH3 hydrophobic amino acids (Figures 2 and 3). In these two cases, the helices bind in a groove formed by α3 and α4 (Figure 2) as well as the BH1 region and the C-terminal end of α2. The extent of interaction of these peptides with BH2 and α8 is hard to evaluate in these structures because the bound peptide lacks secondary structure at its C terminus. However, the proximity of the BH1 and BH2 regions to the BH3 binding groove could be interpreted to indicate that Bcl-2 family members that have these two homology regions are capable of binding to exposed BH3 regions, whereas those that do not, such as BID, cannot, despite a similar overall fold.

Based on sequence alone, one could predict that the structure of Bim should be very different from those of these other Bcl-2 family members. There is only a short stretch of amino acids between the end of the BH3 region and the beginning of what is thought to be a membrane interacting region at the C terminus, certainly not long enough to contain core structural helices such

as the α4-α8 helices. Furthermore, the crystal structure of Bim bound to Bcl-x_L reported here reveals that nearly this entire region forms an extension of the BH3-containing helix (Figure 3). Clearly, in an unbound state, the binding interface of the BH3 region of Bim cannot be sequestered in the same way that it is in these other proteins, raising the possibility that it is always exposed and accessible to BH3 binding proteins. Hrk is the only other BH3-only protein in which the BH3 domain is immediately adjacent to the putative membrane interaction region. Perhaps these two proteins have different modes of action from other BH3-only proteins due to differences in the structure and accessibility of their BH3 regions.

Our discovery that Bim binds to Bcl-x_L via this long α helix is in line with other experiments that show amino acids surrounding canonical BH3 regions can contribute to the engagement of BH3-bearing proteins by other members of the Bcl-2 family of proteins (Petros et al., 2000). In the case of Bim, hydrophobic interactions via Tyr105 and Val109 mediate the contact of the helix extension with Bcl-x_L (Figure 4D). Alignment of the BH3 region of Bim with those of other Bcl-2 family members reveals hydrophobic amino acids at similar positions in many of these other proteins downstream of the canonical BH3 region (Figure 3). Perhaps this region can also form an extension of the BH3 helix during interactions among some of these proteins. In the case of those proteins with a Bcl-2-like fold, this region covers the connecting peptide between α2 and α3. Since α2 must undergo a major conformational change to expose its BH3, it is tempting to suggest that extension of the BH3 helix might be a part of this change.

The sequence of events that lead to activation of Bax and Bak remains a mystery, although it is clear that activation involves major structural changes (Griffiths et al., 1999; Hsu and Youle, 1998; Mikhailov et al., 2003). In the case of Bax, the process also involves movement of the protein from the cytoplasm to the mitochondria and insertion of its C-terminal transmembrane region into the mitochondrial membrane (Goping et al., 1998; Ruffolo et al., 2000). Bak, however, is constitutively bound to mitochondria as an integral membrane protein (Wei et al., 2000) even in healthy cells. Thus the mere presence of the executioner proteins Bax and Bak on the mitochondrial membranes is not sufficient for these proteins to drive apoptosis; a structural reorganization of the proteins must occur too.

The experiments described here suggest how this second reorganization may occur. Mere displacement of the transmembrane region of Bax from its BH3 binding groove is not sufficient to expose the N-terminal epitope or aggregate the protein. Our data indicate that the α8 helix must be moved as well to spring the molecule into its fully active configuration. How this occurs in the intact protein is not known. Our experiments show that simply coexpressing soluble versions of Bim and BaxΔTM is insufficient for Bim induction of Bax α8 displacement. However, they do suggest that engagement of an extended BH3 domain from another Bcl-2 family protein, such as Bim, will stabilize structures of Bax and Bak in which the α8 helix has moved. These BH3 proteins may catalyze the process more efficiently when both proteins are adjacent anchored in the same membrane. By analogy to Bax, one might predict based on the structure of the antiapoptotic protein, Bcl-w, that it too may require displacement of both its transmembrane and α8 in order to reveal a complete binding site for Bim (Hinds et al., 2003).

In a recent study on Bax/Bak activation in isolated mitochondria, a Bim peptide which is shorter than that described here had effects on direct cytochrome c release (Letai et al., 2002). Perhaps this occurred because the shorter peptide could achieve partially the effects suggested here. Alternatively, since the experiments involved intact mitochondria, perhaps the effects were due to competitive release by the short peptides of intact proapoptotic proteins bound to Bcl-2/Bcl-x_L, which in turn activated Bax/Bak. In any case, it would be interesting to find out if the longer Bim peptide was even more potent in such assays.

Despite the implication of Bim in Bax/Bak activation (Bouillet et al., 2002; Davey et al., 2002; Hildeman et al., 2002), we have not yet been able to coprecipitate this complex from apoptotic T cells (Y.Z., unpublished data). So far, Bid is the only member of the BH3-only family of proapoptotic proteins that has been shown to interact with Bax directly (Desagher et al., 1999; Eskes et al., 2000). Yet even Bid has not been found in the active Bax aggregated unit in intact cells (Sundararajan and White, 2001). So the matter remains in doubt. One possible explanation is that these interactions are transient and unstable in vivo, a so-called hit-and-run mechanism suggested by Sundararajan and White (2001).

Experimental Procedures

Preparation of Bcl-x_L and a Bim-6His/Bcl-x_L/LC8 Complex

A cDNA fragment encoding amino acids 1–196 of mouse Bcl-x_L with a C-terminal 6His tag was cloned under control of the polyhedrin promoter into a previously described baculovirus transfer plasmid (Kozono et al., 1994). Recombinant virus was made by cotransfection into SF9 insect cells (Invitrogen) of the plasmid and BacVector 3000 baculovirus DNA (Novagen) using the calcium phosphate coprecipitation method. High titer virus stock was prepared by infection of SF9 insect cells. Bcl-x_L protein was produced by infection of High Five insect cells (Invitrogen) at high multiplicity of infection. Four days later, the cells were lysed by sonication in Ni-NTA binding buffer (50 mM Na_2HPO_4, 300 mM NaCl, 5 mM imidazole [pH 8.0]). In some cases, 0.5% NP-40 was added to the sonication buffer. Lysates were cleared by centrifugation (100,000 g, 60 min) and proteins were purified from the supernatant using Ni-NTA columns (Qiagen). The protein was eluted from the column with 500 mM imidazole and further purified by size exclusion chromatography using Superdex-200 and ion exchange chromatography using Mono-Q (Pharmacia). Purified protein was concentrated to 15 mg/ml for crystallization.

In order to prepare a complex containing mouse Bim_L, Bcl-x_L, and LC8, the three proteins were coexpressed in insect cells as follows. Baculoviruses coding for Bcl-x_L 1–196 without a 6His tag, Bim_L-6His lacking the hypothetical transmembrane region (1–122), and LC8 were constructed. High Five insect cells were coinfected at high multiplicity of infection with a mixture the three active viruses. Because the 6His tag was only on the Bim_L and the expression levels of Bcl-x_L and LC8 were much higher than that of Bim_L, most of protein eluted from Ni-NTA column was a complex of Bim_L-6His/Bcl-x_L/LC8. The triple complex was further purified by Superdex-200 size exclusion chromatography in PBSA buffer (50 mM NaH_2PO_4-Na_2HPO_4, 150 mM NaCl, 5 mM NaN_3 [pH 7.5]).

Crystallization and Data Collection

Free Bcl-x_L was crystallized by the hanging drop vapor diffusion method using 16% $(NH_4)_2SO_4$, 0.1 M MES pH 6.5, and 5% 1,4-dioxane

at room temperature. Crystals appeared in hanging drops containing the Bim_L-6His/Bcl-x_L/LC8 complex in 0.4 M $NH_4H_2PO_4$ after sitting for several months at 4°C. SDS-gel analysis showed considerable degradation of Bim_L and LC8 but not Bcl-x_L.

The diffraction data for the Bcl-x_L crystals were collected at 100°K on Beamline 5.0.2 at the Advanced Light Source of the Lawrence Berkeley National Laboratory. The data were processed to 2.2 Å resolution with HKL2000 (Otwinowski and Minor, 1997). Diffraction data for the crystals obtained with the Bim_L-6His/Bcl-x_L/LC8 complex were collected at 100°K in the National Jewish X-ray Facility using a Rigaku generator/RAXIS IV system. The data were processed to 1.65 Å using the HKL2000 package. The data processing results are summarized in Table 1.

Structure Determination and Refinement

Both of the structures were determined by the molecular replacement method. The space group and cell parameters of the mouse Bcl-x_L crystal were very similar to those of human and rat Bcl-x_L, indicating the similar packing pattern in these crystals. The rat Bcl-x_L crystal structure (access code 1AF3) was used to determine the initial phases of mouse Bcl-x_L. The AMoRe program in the CCP4 package (CCP4, 1994; Navaza, 2001) was used for estimating the initial position of the molecules within the unit cell. The CNS suite (Brunger et al., 1998) was then used for further refinement to 2.2 Å resolution and 108 water molecules were added to the final structure. The refinement statistics are summarized in Table 1.

The mouse Bcl-x_L structure was used as a model for finding Bcl-x_L in the data obtained with the crystal that grew from the Bim_L-6His/Bcl-x_L/LC8 complex. Initial density maps showed poor density for the α2 and α3 helices, so that these were deleted from the model and then rebuilt based on 2Fo-Fc and Fo-Fc electronic density maps during several rounds of refinement. As refinement proceeded, density appeared for a long α helix in the putative BH3 binding groove of Bcl-x_L that could be attributed to the portion of Bim containing its BH3 region. Eventually, a 33 amino acid stretch was built and refined, 29 amino acids of which were in a continuous α helix corresponding to amino acids 83–115 of Bim_L. No ordered density could be found attributable to the rest of Bim_L or to the LC8 chain; however, disordered density was seen at the N-terminal end of the Bim helix, suggesting that the degradation had left some of the polypeptide intact prior to amino acid 83 but that this portion was not ordered. The complex was refined to a resolution of 1.65 Å and 169 water molecules were added to the final structure. The refinement statistics are summarized in Table 1.

Coprecipitation of Bax with Bim_L or Bcl-x_L

Mouse Bax-6His (1–192), BaxΔTM-6His (1–167), and Bax$\Delta\alpha$8TM-6His (1–157) were constructed and expressed in baculovirus as described above. For coprecipitation experiments, High Five cells were coinfected with viruses encoding either Bim or Bcl-x_L and any of the three versions of Bax-6His. Lysates were prepared from the infected cells with or without 0.5% NP-40. Bax was precipitated from the lysates using Ni-NTA columns and the eluates were analyzed by Western blot for Bax and either Bim or Bcl-x_L using rabbit polyclonal antibodies from Santa Cruz Biotech. As a control for expression levels, total cell lysates were also analyzed prior to precipitation.

ELISA Evaluation of Bax Activation

Bax-6His (Bax 1–192), Bax-ΔTM-6His (Bax 1–167), and Bax-$\Delta\alpha$8TM-6His (1–157) were purified using Ni-NTA columns. Proteins eluted from column were adjusted to 0.5 mg/ml according to OD280 and SDS-PAGE. Wells of a microtiter plate were coated with various concentrations of the proteins and the bound protein detected with the Bax activation specific monoclonal antibody, 6A7 (Oncogene), using alkaline-phosphatase conjugated goat anti-mouse antibody and o-phenylphosphate (Jackson ImmunoResearch Lab) as developing reagents.

Evaluation of Bax Aggregation

Purified Bax proteins were analyzed by size exclusion chromatography using Superdex-200 in PBSA buffer (50 mM NaH_2PO_4-Na_2HPO_4, 150 mM NaCl, 5 mM NaN_3 [pH 7.5]). Elution positions were compared to those of protein standards.

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Accession Numbers

Atomic coordinates of the free Bcl-x_L and Bcl-x_L/Bim complex have been deposited in the Protein Data Bank (Accession numbers 1PQ0 and 1PQ1, respectively).