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14. ABSTRACT The BH3 motif of the pro-survival family of proteins, BCL, is also present in pro-apoptotic proteins like BID and BAX. Homo and hetero-oligomerization interactions of the BH3 motif are generally recognized as the critical component of their apoptotic activities. In full length BID, the putative hydrophobic binding surface of its BH3 motif is substantially occluded by intramolecular contacts, many of which are removed on BID's transformation to tBID by cleavage with caspase 8, required for tBID's proapoptotic action on mitochondria, thereby releasing cytochrome c. As a step to more complete characterization of the hetero-oligomeric complexes of BID and BCL family molecules, we report here the formation of a tight complex of the BH3 motif sequence of BID with BCL-XL. In contrast to the previously reported of BAK BH3 motif with BCL-XL, the BID BH3 peptide (PDSSESQEEIMHNIARKLAQIGDDI) forms an α -helix significantly extended to the N-terminus, as monitored by 15N {1H} nOe determination, and by 13C chemical shifts. Modeling the BID BH3 motif/ BCL-XL on the basis of the previous structure shows the extended helix well fitted to an extended cavity in BCL-XL. Mutagenesis of the peptide and of BCL-XL on the basis of this model identified the key residues in the BH3 motif, but suggests that some conformational flexibility is likely in the BCL surface.					
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Introduction: This report covers activities related to the proposal from its submission, July 1, 1998 to date. The proposal was funded from July 1, 1999. The subject of the grant is the design of interventional agents for the biological process of apoptosis based on rational analysis of structures in the family of apoptotic and anti-apoptotic regulators. The purpose of the research is the development of a better understanding of the intricate pathways of cell death and their contributions to breast cancers, and represents a first step in designing potential therapeutic agents for inducing or regulating apoptotic processes in breast cancer. The scope of this research had previously included the determination by NMR of the structure of the first pro-apoptotic BCL-2 like molecule (BID) and its analysis, identifying key structural issues associated with (a) the caspase catalyzed cleavage of part of BID (to produce a truncated form, 'tBID'), and (b) a likely structural model for the necessity of exposure of portion of one helix of BID and other homologues, in the BH3 motif. Current work focuses on efficient expression of the large quantities of BCL-2 family members for structural and biochemical assay, design and synthesis of BH3 mimics, and preliminary characterization of tBID in membrane bound form, prior to attempts to identify complexes of tBID for structural studies.

Body: *The solution structure of BID.* Programmed cell death is a highly evolutionarily conserved biological process critical for development and homeostasis in all multicellular organisms (1, 2). This process allows for the removal of redundant or damaged cells, playing a vital role in normal cellular development, tissue homeostasis, and immunological defense (3, 4). Dysregulated programmed cell death can contribute to cancer, autoimmune disease and neurodegenerative disorders (5, 6). Developmental or environmental signals can regulate signals for either cell death or cell survival. The study of complex signaling pathways of programmed cell death has led to the identification of a large number of molecules involved in regulating apoptotic death, or promoting cell survival (7, 8).

Key features of programmed cell death include a cascade of proteases tightly controlled by apoptotic signaling pathways. As part of a critical apoptotic pathway regulating a critical checkpoint in mitochondria, the Bcl-2 family of proteins comprises both anti- and pro-apoptotic regulators (5). The founding member of this family, Bcl-2, was first identified on the basis of its involvement in B-cell malignancies, where chromosomal translocations resulted in overexpression of Bcl-2 and an increased resistance to programmed cell death (9, 10). This discovery established a new class of oncogenes, for which extended cell survival and resistance to apoptosis results in increased oncogenic potential (11).

Membership in the Bcl-2 family is defined based on homology to at least one of four conserved sequence motifs known as Bcl-2 homology motifs, BH1 to BH4 (sometimes referred to as domains). Most anti-apoptosis family members have BH1 and BH2 motifs and many have all four BH motifs. In contrast, many of the pro-apoptotic family members (Bid, Bad, Bik, Bim, Blk, Hrk) only possess the conserved BH3 motif. The ratio of anti- and pro-apoptotic molecules apparently determines whether a cell will respond to a proximal apoptotic stimulus. This competition is mediated, at least in part, by competitive dimerization between anti and pro-apoptotic pairs (5).

An important sub-family within the Bcl-2 family consists of pro-apoptotic molecules with homology only in the BH3 region (12). The BH3-only class of cell death effectors are conserved components of a central death pathway. Studies of developmentally regulated cell death have revealed the BH3-only molecule Egl-1 plays a major role in controlling programmed cell death in *C. elegans* by acting as a negative regulator of the

Bcl-2-like cell death inhibitor CED-9 (13). The Bcl-2 family member Bid belongs to the BH3-only class of pro-apoptotic killers. Like other members of this class of molecules, Bid shows no recognizable sequence homology to BH1, BH2 or BH4 domains, and requires a functional BH3 domain for its dimerization and pro-apoptotic activity (14, 15, 16). Recently it has been reported that caspase-8 mediates the cleavage of an inactive 23kD cytosolic Bid to produce a truncated 15kD fragment (referred to as tBid) that translocates to the mitochondria causing cytochrome c release, activation of caspases and the final commitment to cell death (17, 18, 19). In this lab, we determined the solution structure of Bid using NMR spectroscopy. This work provides the first structure of a pro-apoptotic Bcl-2 family member (simultaneously with a paper from Wagner's Laboratory (20)) and offers insight into the structural basis of the pro-apoptotic function of the Bcl-2 family death agonists. Based on sequence/structure similarity or dissimilarity to Bid the mechanisms of other pro-apoptotic family members were proposed, illustrating a fold class including both Bid and the previously determined Bcl-X_L structure (21, 22). From the likely structure of tBid, a structural mechanism for Bid and other pro-apoptotic family members was suggested.

Model of Bid mediated apoptosis

The exposure of the BH3 domain in Bid, and the pro-apoptotic function of the molecule, is regulated through a post-translational cleavage event. Other mechanisms of post-translational modification regulate activities of other family members (16, 24). This type of regulation allows the rapid response necessary for apoptosis.

The structure of Bid sheds light on its role as a death agonist, and the requirements for pro-apoptotic action in the Bcl-2 family. What makes a Bcl-2 family member a death agonist? It appears that two criteria must be met for pro-apoptotic function. The first requirement is one of cellular localization. There is strong evidence for several Bcl-2 family members that mitochondrial translocation is a key determinant for death inducing activity. Bid (17, 18, 19) and Bax (25) are inactive as cytosolic components but induce death upon their targeting to mitochondria. The activity of Bad, on the other hand, is down-regulated by phosphorylation which sequesters the protein in the cytosol away from mitochondria targets (16). The second, and perhaps more critical, criteria for death agonism is the exposure of the BH3 surface. The cleavage of Bid by caspase-8 is likely to expose a BH3 motif that is buried in the uncleaved molecule. Based on our structure predictions for the Bcl-2 family some members of the BH3-only class appear to have their BH3 motif exposed, or at least not under the same conformational constraints as other members of the Bcl-2 family. Molecules possessing an exposed BH3 motif (Egl-1, Hrk, Bik, Bim, tBid) might be expected to be constitutively active death agonists. While other members of the family possessing a 'hidden' BH3 motif are regulated by post-translation modification events (i.e., cleavage, phosphorylation) that expose the BH3 epitope (24, 26, 27) that induce their pro-apoptotic function. The structural work then strongly supported the original hypothesis that BH3 recognition was a key event in pro-apoptotic action, and suggested that the exposure of the hydrophobic face may be key.

The expression of BCL-2 family members. The work described above involved a collaboration with Professor S. Korsmeyer, who had provided the expressed protein. He subsequently gave us *E. coli* with the plasmid expressing the GST-BID fusion protein, and the expression of the protein was tested and BID prepared in this laboratory. Since the research program seeks to investigate the biochemical interactions of BID, tBID, other BCL-2 family members, and possible binding partners, we undertook to express

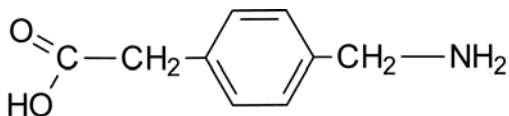
optimally several of these which are tabulated in Table 1 (Appendix). These have originally obtained from the Korsmeyer or Marie Hardwick labs.

Structure of membrane bound tBID. After caspase 8 cleavage of BID to tBID, the truncated form is very water insoluble, and is generally thought to be recruited to the mitochondrial membrane in the cell. As is widely recognized, structural characterization in the membrane or membrane-like environment presents considerable difficulties to current methods, either crystallographically or by NMR. We chose to attempt to characterize the tBID structure in membrane mimics or aqueous detergents or in organic solvents. Using sodium dodecyl sulfate micelles (previously used by us in identifying the recognition of PH domains by phospholipids (29)), CD spectra suggest that tBid is ~80% helical consistent with no overall change in secondary structure after the BID->tBID cleavage. Since this was unlikely to be controversial, we deferred further structure determination attempts until tBID complexes with mimics, or other BCL-2 family members could be prepared, and this will be continued with the non-competitive budget extension, to which the PI commits to report on again in this format.

In the last year of the project, we investigated structural determinants of the Bid BH3 interaction with anti-apoptotic Bcl-x_L. We developed highly selective binding assays which allowed us to compare various peptidic mimics of Bid BH3 motif binding to Bcl-x_L. We showed that rigidization of the Bid BH3 peptide by introduction the covalent linker does not change its binding affinity towards Bcl-x_L. N-terminal extension of Bid BH3 domain led to one order of magnitude increase in Bid BH3:Bcl-x_L binding affinity. We describe the lengthened and altered specificity of the Bid BH3 binding to Bcl-x_L using site-directed mutagenesis and heteronuclear NMR..

BH3 mimics. We designed and tested a conformationally constrained α helix mimicking the full BH3 motif of BID and other proapoptotic members of the BCL-2 family. We implemented the strategy of using *i,i*+7 side chain links for the sequence NIAKHLAQIGDEMD, with the K...D linkage being obtained by intermediate protection with allyl-K and Alloc-E (see Fig 1). Side chain deprotection was obtained by hydrogenation with Pd, and cyclization by use of the procedure of Phelan (28). The product was characterized by ESI-MS to the nominally correct mass. While α helicity is apparent in the CD spectrum (data not shown), the magnitude of the 222 nm minimum is less than the expected from the Bid solution structure helical content (~68%), and may reflect time averaging of the structure between an α helix and more random structures. The affinity of this and related mimics is discussed below.

Figure 1. Chemical structure of linker for constrained mimics.



Effect of helix propensity of Bid BH3 peptide on its binding affinity to Bcl-x_L. To understand how Bid interacts with Bcl-x_L and inhibits the ability of Bcl-x_L to promote cell survival, we measured the binding affinity of Bcl-x_L to peptides corresponding to the BH3 region of Bid

protein (Bid1ls) by using a Fluorescence Polarization (FP) binding assay (Table 1). The Bcl-x_L used for the binding assay lacks the putative C-terminal transmembrane region. The Bid 16-mer peptide was synthesized in which Met97 and Glu96 were changed to those found in the Bak peptide. His89, which is not conserved in Bcl-2 family members and shown by complex structure of Bcl-x_L /Bak peptide (22) that it is located on the surface of the complex and therefore does not contribute to peptide /protein interactions, was mutated to unusual amino acid Dap for the synthesis of the cyclic peptide. A decrease in binding affinity by a factor of 10 compared with Bak peptide was observed for the peptide derived from BH3 region of Bid, even though it contains most of the key residues in Bak that interact with Bcl-x_L (Table below. Each peptide was fluorescein labeled, X== 2,3 diamino propionic acid, stereoisomer equivalent to L-amino acid)

We concluded that the longer Bid based peptide either forming a more effective helix for binding or had additional contacts. Cyclization produced only a modest apparent affinity increment.

CD spectra were acquired for the peptides in 30% TFE. From the mean residue ellipticity at 222nm, the percentage of α -helix was derived and compared to the affinity of each peptide for Bcl-x_L (Table 1). As hypothesized, the cyclic peptide possesses a greater helix propensity compared to the linear equivalents, but did not show dramatically higher affinities. We conclude that the general surface interaction of these rigidized peptides are all similar to the original packing observed in the Bcl-x_L /Bak peptide complex (22)

Table 1. Binding affinities of different BH3 mimics towards Bcl-X_L.

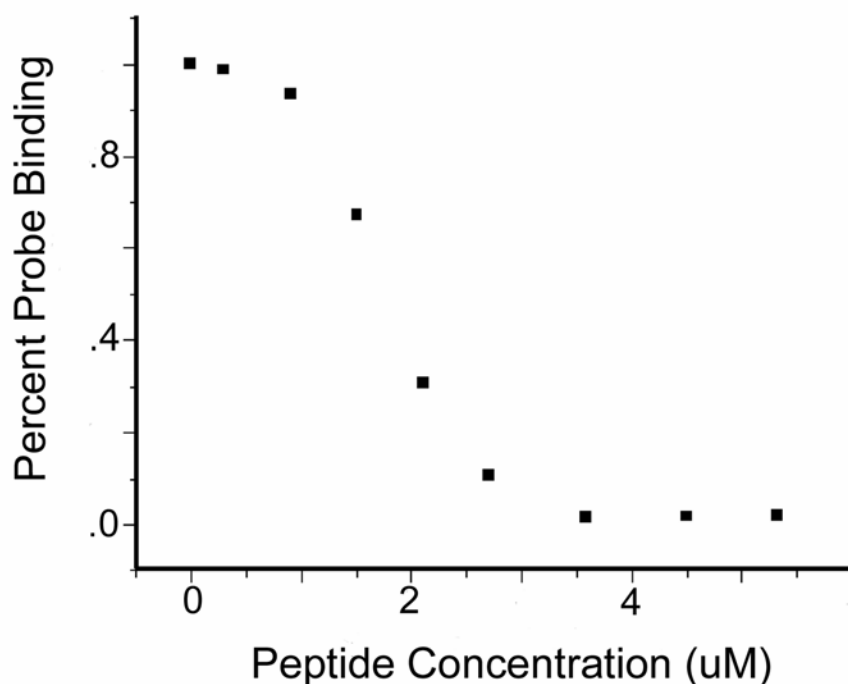
	Peptide	Affinity (nM) (SD)	% change FP
Bak	GQVGRQLAIIGDDINR	390(20)	175
Bid11	PDSESQEEIMHNIARXLAQIGDDIDH	250 (8)	250
Bid1ls	HNIARXLAQIGDDIDH	3,600(200)	88
Bid1cls	HNIARXLAQIGDDIDH (cyclized X-D)	1,600(90)	80

To explain the tight binding to Bcl-x_L, we considered that Bid peptide might form additional interactions with Bcl-x_L. Therefore, the longer peptide with 10-residue extension of Bid 16-mer at N-termini was synthesized (Bid11, table above). This longer peptide containing 26 amino acids, were found to bind tightly to Bcl-x_L with a K_d of 250nM, and has a higher helix propensity than the 16-mer peptide.

Development of Binding affinity measurements using Fluorescence Polarization. To obtain binding affinities for various Bid peptides to Bcl-x_L, the fluorescein derivative was chosen to provide a fluorophore for a fluorescence polarization binding assay. Fluorescence polarization is based on changes in polarization of light emitted by excited fluorescent molecules as they undergo changes in its rate of rotation in solution. When the fluorescent probe binds to a larger molecule, the rotation rate of the fluorescent probe is reduced. The

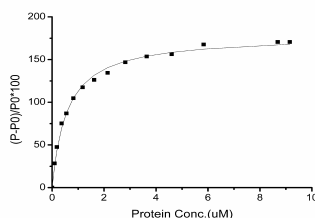
fluorophore was formed by an N-terminally or both N-terminally and Lys side chain linked 5-carbamide fluorescein moiety. The labeled peptides were first tested in a saturation binding experiment against Bcl-x_L (Figure 2, Table 1).

Figure 2. Saturation binding Assay for Bid1le peptide using Fluor-Bid1l probe . Buffer: 20mM Phosphate pH 7.2, 50mM NaCl, 2mM DTT, 1mM EDTA 0.01% BGG



The result show that, despite the addition of an N-terminal fluorescein, the affinity measured for Bak peptide is in good agreement with previously published results (22). The second FP experiment for measuring binding affinity is competition assay, which requires careful selection of the probe characteristics. The results in Table 1 show that the Bid1l has higher polarization change upon binding to Bcl-x_L, which is of importance primarily because it delimits the signal-to-noise ratio of the assay. Based on K_d and polarization values, we selected probe Fluor-Bid1l for measuring the binding affinities for other peptides (Fig 3).

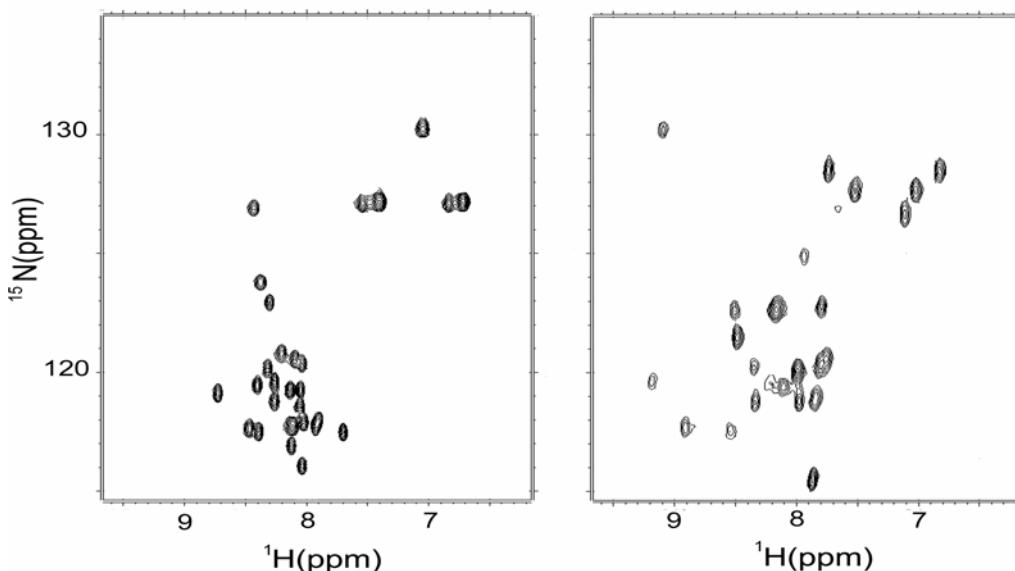
Figure 3. Competition Assay for Bid1le peptide using Fluor-Bid1l probe . Buffer: 20mM Phosphate pH 7.2, 50mM NaCl, 2mM DTT, 1mM EDTA 0.01% BGG.



NMR assignments of Bid peptide complexed to Bcl-x_L. To test whether the increase in

binding affinity observed for Bid 26-mer compared to the Bid 16-mer results from higher helix propensity or from the additional interactions between Bcl-x_L and N-terminal residues of Bid peptide, we use heteronuclear NMR to characterize the Bcl/Bid peptide interaction. The deletion mutant of Bcl-x_L used in the NMR studies is the same as that used for binding assay, which lacks the putative C-terminal transmembrane region and residues 45 to 84 which constitute a flexible loop previously shown to be dispensable for the antiapoptotic activity (21). The Bid 26-mer peptide corresponding to the BH3 region of Bid protein with ten residue extension at the N-terminus (Bid1le), in which Met97 was mutated to Leu to avoid a cyanogen cleavage site in the middle of the peptide. We determined a K_d of 250nM for Bid1IE/Bcl-x_L binding from FP competition assay. Furthermore, the K_d of the 40 amino acid peptide including the BH3 region and N-terminal residues extended to Asp59, the caspase cleavage site in the full length Bid protein, is the same as the 26 residue peptide. In the ¹H{¹⁵N} HSQC spectra of free and complexed [U-¹⁵N, ¹³C] Bid1le (Fig. 4) all the amide positions undergo significant chemical shift changes upon binding, which is in accord with the high-affinity binding measured from the fluorescence studies, and the expectation that the free peptide was either wholly or predominantly unstructured.

Fig. 4 Comparison of ¹H{¹⁵N} HSQC spectra of ligand peptide Bid 1le complexed with Bcl-x_L (right panel) and free in buffer (left panel).



Backbone resonance assignments using a [U-¹⁵N, ¹³C] Bid1IE/Bcl-x_L sample. Sequential assignments were achieved using the HNCA/CBCA (CO) NH pair of experiments so that connectivities could be traced through two independent, through-bond pathways. A ¹H{¹⁵N} NOESY-HSQC experiment allowed tracing of some amide-amide connectivities through the α-helical structures. Overall, all amide backbone resonances except Ile 83 were sequentially assigned by using this sample. The amide backbone resonance of Ile 83 was assigned using a specific [¹⁵N, ¹³C-Ile] Bid1le/Bcl-x_L sample.

The secondary structure of the Bid1IE complexed with Bcl-x_L was identified through the analysis of ¹³C^α and ¹³C^β. Segments of the protein experience a downfield shift with C^α resonances and up field shift with C^β carbons when located in helices and C^α carbons experience an up field and C^β shifts downfield when located in β-strands. The BH3 region

derived from Bid protein shows a condense group of positive C^α and C^β differences compared with the empirical values, which indicates that it adopts an α -helix when complexed to Bcl-x_L. Interestingly, the α -helix is N-extended to residue Gln79 located beyond the BH3 region which suggests that the peptide forms additional interactions with Bcl-x_L.

To further identify the boundaries of the folded peptide structure, we performed a steady-state $^{15}\text{N}\{^1\text{H}\}$ NOE experiment. The protein segments that do not participate in the folded structure have negative nOe values due to their high degree of local flexibility and motions in the sub-nanosecond to nanosecond time scale. As seen from figure 5, N-terminal residue 1-5 have negative $^{15}\text{N}\{^1\text{H}\}$ nOe values and experience essentially minimal restrictions from the rest of the complex. This boundary of well-defined tertiary structure of the Bid1le/Bcl-x_L complex is consistent with the secondary structural part determined from Chemical-Shift Index.

Figure 5 A. Chemical shifts of C^α and C^β carbons normalized to random coil value. Large positive differences indicate likely α -helical regions.

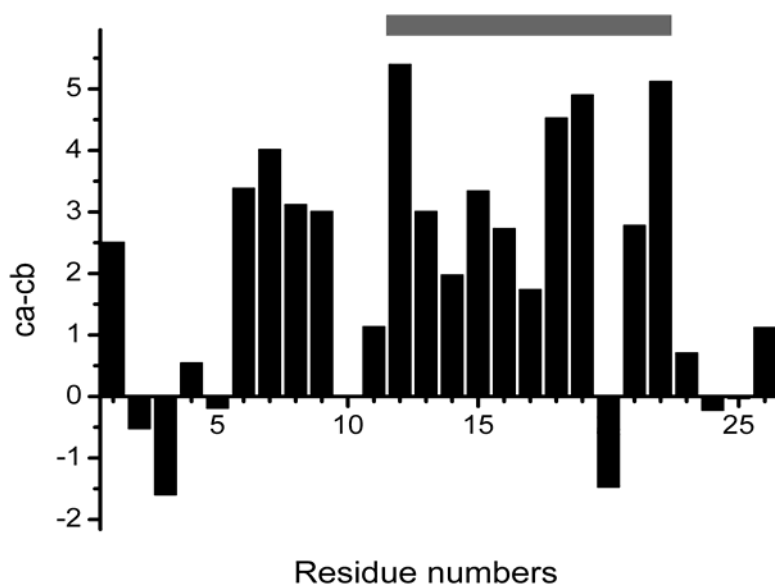
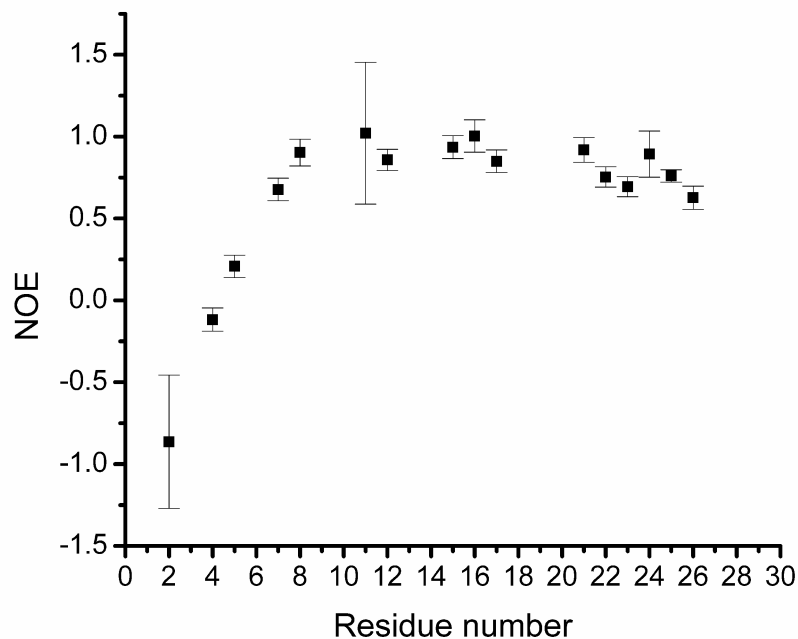


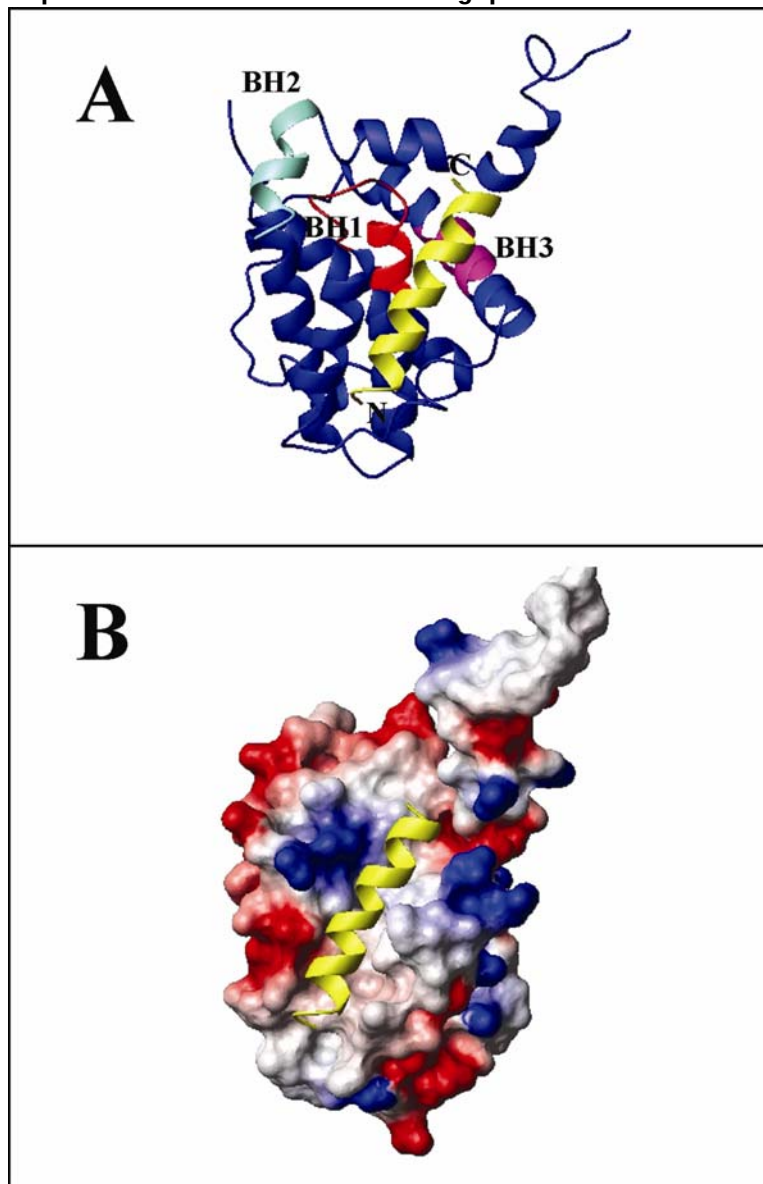
Figure 5. B $^{15}\text{N}\{^1\text{H}\}$ nOe for Bid 1le bound to Bcl-x_L.



Mutagenesis of Bid peptide and Bcl-x_L protein. To determine what additional interactions between Bcl-x_L and the N-terminal residues of the Bid peptide are responsible for the increase in binding affinity observed for the Bid 26-mer compared the Bid 16-mer, the N-terminal residue 79-83 was added to the NMR structure of Bcl-x_L/Bak complex and modeled and energy minimized using Insight (MSI) (Fig. 7 and 8). Additional helical segment of Bid BH3 fits well in the hydrophobic groove of Bcl-x_L introducing no steric clashes (Figure 7) and presenting hydrophobic Ile83 and polar Gln79 for direct interaction with the groove.

In the model proposed the residue Thr109 and Val17 located in the helix 4 of Bcl-x_L involve in the hydrophobic interactions with N-terminal residues Gln79 and Ile83 of Bid peptide.

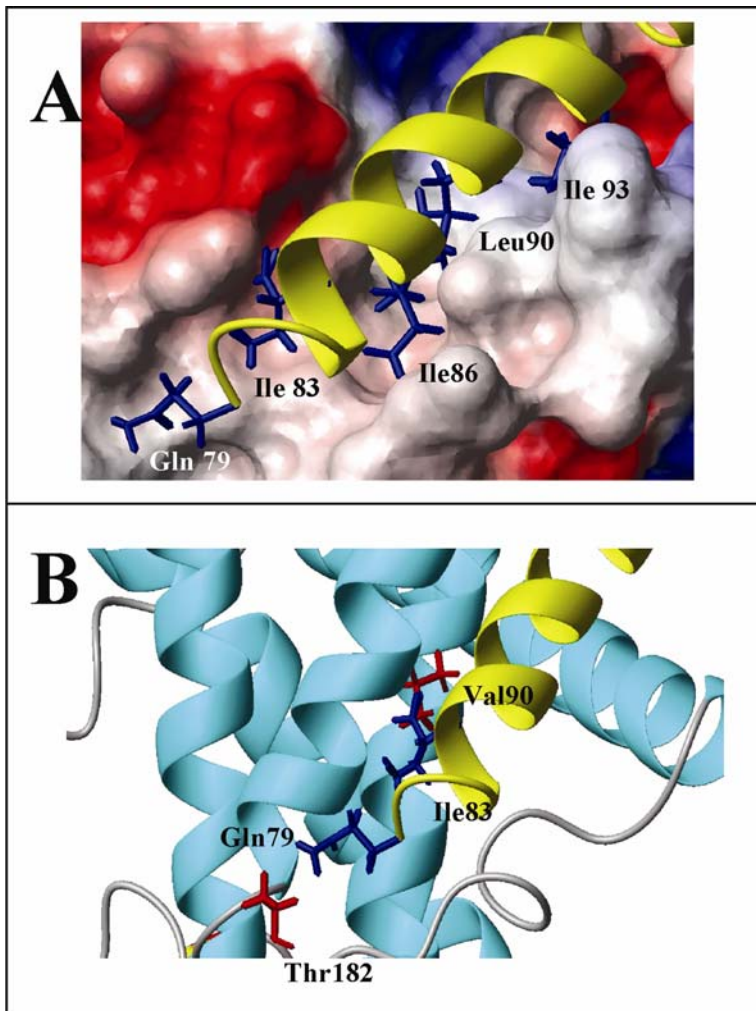
Fig 6. (A) Ribbons depiction of Bcl-xL/Bak complex. (B) Surface representation of the binding pocket of Bcl-xL bound to the Bak peptide



We performed site-directed mutagenesis of protein and peptide based on this mapping result to explore the extra binding sites. Individual mutations of two residues located at the N-termini of Bid peptide, Q79A and I83A, resulted in 2.8 fold and 2.3 fold decrease of binding affinity to Bcl-xL ($\Delta 45-84$) respectively. Mutation T109A does not affect binding affinity and V117L located in the helix 4 of Bcl-xL resulted in a 2.3 fold decrease of binding affinity to Bid peptide. However, the binding affinity of Bid1Ile I83A peptide to mutant protein Bcl-xL ($\Delta 45-84$) V117L has 4.6 fold decrease compared with Bid1Ile to Bcl-xL ($\Delta 45-84$), which is an additive of titration's of two complementary mutants. The result indicates that Gln79 and Ile 83 of Bid peptide do significantly contribute to overall ten times increase in binding affinity of extended Bid BH3 towards Bcl-xL. Val17 of Bcl-xL which lies outside of the Bak peptide:Bcl-xL interaction surface also make a contact with Bid BH3 but may not interact directly with Ile83. This result highlights the necessity of further high resolution experimental work on extended Bid BH3:Bcl-xL complex. Mutation T109A may not be

dramatic enough to introduce significant change in Bid BH3:Bcl-xL binding affinity.

Figure 7 Graphical representation of site directed mutagenesis of the Bcl-xL protein and Bid peptide and the effects to their interactions.



Our discovery of the extended and altered specificity of the Bid BH3 motif towards anti-apoptotic Bcl-xL highlights the uniqueness of homo- and heterodimerization processes of different BCL-2 family members and may be important for the development of highly specific mimics of BH3 motif to control apoptotic signals. Many of these processes are interrelated, and the final elucidation of detailed mechanisms may require multiple approaches to their full solution. None the less, we can be confident that the BH3 motif has a central role in the Bcl-related family and that the recognition motif's variance, in its own sequence, in the formation of the related receptor surfaces, and in relation to other conformational changes induced in binding and signal transduction in the apoptotic processes involved.

MATERIALS AND METHODS

Expression and purification of Bcl-xL and mutants. The deletion mutants of Bcl-xL and mutants containing single-residue mutations were constructed from the expression vector for Bcl-xL (residues 1 to 209) (1) by PCR using primers containing a BamHI upstream restriction site and a downstream EcoRI restriction site. The amplified genes were cloned in frame into the expression vector PGEX-2T (Pharmacia). The protein was expressed in E. coli BL21 (DE3) using the T7 expression system. Cell, freshly transformed with plasmid, was grown to late log phase. Protein expression was induced by addition of 0.5mM isopropyl thio- β -D-galactoside (IPTG). After growth for another 3h at 37°C, cells were harvested by centrifugation. The cell pellet was resuspended in lysis solution (1% triton, 5mM EDTA, PBS, 1mM DTT, and protease inhibitors [Aprotinin, PMSF] and sonicated for 10 minute periods on ice. The lysate was clarified by centrifugation at 4,800g for 20min. at 4°C and the supernatant was then incubated with glutathione-agarose beads (5ml beads/ 1L culture) for 2hours at 4°C with Nutator mixing. The suspension was centrifuged at 2,000 for 5min and the pellet was resuspended and washed twice with 10ml of high salt buffer (sonication buffer with additional 100mM NaCl) at 4°C. GST was cleaved from the fusion protein with human thrombin(150units thrombin in 50mM Tris-HCl pH 7.5, 100mM NaCl, 2.5mM CaCl₂, 2mM DTT) for 12 hours at 4°C. The digestion was quenched with 1mM AEBSF. The protein solution was then dialyzed against NMR buffer (20mM phosphate buffer pH 7.2, 50mM NaCl, 5mM EDTA, 1mM DTT).

Bid peptide expression and purification. Bid peptide was constructed from the expression vector for full length Bid protein by PCR. The amplified gene was subsequently cloned into the HindIII-BamHI sites of a phagemid-T7 expression vector, PTMHa(5). In the PTMHa vector, the desired sequences are expressed as chimeric proteins containing a modified form of the TrpLE leader sequence, in which a N-terminal (His)₉ tag has been added which can be removed upon cleavage by cyanogen bromide (CNBr). The peptide was expressed in E.coli BL21(DE3) using the T7 expression system. Peptide expression was induced by addition of 0.5mM IPTG. After growth for another 5h at 37°C, the bacteria were harvested by centrifugation and the pellets were stored frozen. Inclusion bodies of the cell lysate were isolated by sonicating in sucrose (50mM Tris, 25% sucrose, 1mM EDTA, pH 8) and Triton buffer (20mM Tris, 1% Triton x-100, 1mM EDTA, pH8) separately (6). The resulting pellet was treated with CNBr (0.03g/ml, 70% formic acid, 2h). After removal of CNBr, the protein was dialyzed against 5% acetic acid and lyophilized. The proteins were solubilized in 6M Gdmcl, 0.1M sodium phosphate, and 10mM Tris 9pH8.0). The solution was passed over a nickel-chelating column to remove the leader sequence containing the His tag and any uncleaved chimeric protein. Protein was purified to homogeneity by reverse-phase HPLC, using a Vydac C-18 preparative column and a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid. The DNA sequence was verified by DNA sequencing. The protein identity was confirmed by mass spectroscopy. Uniformly ¹⁵N-, ¹³C-labeled peptide was prepared by growing the E.coli strain BL21 (DE3) overexpressing Bid1le on a minimal medium containing ¹⁵NH₄Cl and ¹³C-glucose.

Synthesis of peptide for cyclization. Using Fmoc (9-fluorenyl) methoxycarbonyl chemistry (7) carried out solid-phase synthesis. Appropriate Fmoc amino acids were used with Fmoc removal by piperidine-DMF (20%), and standard BOP/HOBT/NMM activation. Synthesis of cyclic peptide use N^α-Fmoc, N^ε-Dde Lys derivate and N^α-Fmoc, O^β-Alloc Asp derivate as building blocks, side chain was assembled after removal of Dde with Hydrazine hydrate-DMF (2%) and removal of Alloc with Pd (Ph₃P)₄/CH₃Cl/AcOH/NMM separately. The rigid linker was introduced using Fmoc-p- (aminoethyl) phenylacetic acid as a building block. The synthesized peptides were characterized by mass spectra.

Fluorescence titration. Fluorescence Polarization experiments were performed at 18°C on ISA Fluorolog III (JY Horiba) spectrofluorometer. Fluorescein-containing probes were read with standard cut off filters for the fluorescein fluorophore ($\lambda_{\text{ex}}=485\text{nm}$, $\lambda_{\text{em}}=520\text{nm}$). Saturation experiments were performed using fixed concentrations of probe (20nM) titrated against increasing concentration of Bcl-xL proteins. Data were analyzed using the program Origin (Microsoft, Inc.) to fit the equation $[\text{probe bound}]/[\text{total probe}] = [\text{free protein}]/(K_d + [\text{free protein}])$ making the assumption that $[\text{free protein}] \approx [\text{total protein}]$, which holds for our experimental conditions. Competitive binding experiments were performed using fixed concentrations of probe (20nM) complexed with proteins titrated against increasing concentration of the second unlabeled peptide. Standard buffer conditions were PBS buffer, pH 7.2, 2mM DTT, 1mM EDTA plus 0.01% bovine gamma-globulin (BGG).

NMR experiments. NMR spectra were acquired at 30°C on a Bruker DMX500 or DMX600 NMR spectrometer. 700uM $[\text{U-}^{15}\text{N}, ^{13}\text{C}]$ Bid1le sample and 1mM $[\text{U-}^{15}\text{N}, ^{13}\text{C}]$ Bid1le/Bcl-xL were in NMR buffer (20mM phosphate buffer pH 7.2, 50mM NaCl, 5mM EDTA, 1mM DTT, 0.02% NaN_3). The HNCA, CBCA(CO)NH, HNHA, ^{15}N -edited NOESY and $^{15}\text{N}\{^1\text{H}\}$ steady-state experiments were collected using previously described protocols (23,29).

KEY RESEARCH ACCOMPLISHMENTS:

- 1) Solution structure of BID
- 2) Development of hypothesis for the sequence basis of anti- & pro-apoptotic BCL-2 family members, and the role of post translation modification in their conversions.
- 3) Design and synthesis of stabilized BH3 mimic.
- 4) Experimentally determined structure of the tBID BH3 motif bound to BCL-XI
- 5) Modeled structure of the interaction of tBID BH4 and BCL-XI, and analysis of the pro-apoptotic complex
- 6) Development of a specific fluorescence depolarization assay for the pro-apoptotic interaction of the BH3 motif of tBID and BCL-XI.

REPORTABLE OUTCOMES:

Manuscripts, abstracts, and presentations:

1. Presentation, Jan 1, 1999, James McDonnell, University of Cambridge, UK, Solution structure of the proapoptotic molecule BID: a structural basis for apoptotic agonists and antagonists.
2. Presentation, Mar, 1999. JM, Cell Death Club, NY, Solution structure of the proapoptotic molecule BID: a structural basis for apoptotic agonists and antagonists.
3. Presentation, July 14, 1999, David Cowburn, New York Structural Biology Discussion Group, Cold Spring Harbor. Including - Solution structure of the proapoptotic molecule BID: a structural basis for apoptotic agonists and antagonists.
4. Presentation, October, 1999. JM. Johns Hopkins School of Medicine, Dept. Pharmacology, Solution structure of the proapoptotic molecule BID: a structural basis for apoptotic agonists and antagonists.
5. Presentation, October, 1999. JM. Columbia University Coll. P&S., Dept. Pharmacology, Solution structure of the proapoptotic molecule BID: a structural basis for apoptotic agonists and antagonists.
6. Presentation, Jan 2000, DC. City College of New York Structural Biology Symp.
7. Presentation, Jul 2002, DC, PNL Structural Genomics Meeting

8. DC, Aug 2000, International Conference on Magnetic Resonance in Biological Systems, Florence, Italy, Structural biology of multidomain systems.
9. DC, Nov 2000, 39th Japanese NMR Symposium, Tokyo, Japan, Dynamic properties of multidomain proteins DC, Jan 2001, Frontiers of NMR in Molecular Biology VII, Big Sky, MN, Approaches to multidomain structures
11. DC, Feb 2001, Lecture in Molecular Biophysics, U. Oxford, UK, Domains in context: NMR doing something useful
12. DC, Mar 2001, St. Jude's Medical Center, Memphis, TN, Solution structures in signal transduction.
13. DC, May 2001, Mid Atlantic Meeting of the American Chemical Soc., Towson, MD, New methods for structure determination.
14. DC, Feb 2002, University of Cambridge, New methods in NMR structural biology
15. DC, Apr 2002, Sigma Xi lecture, Wake Forest. New frontiers in structural biology.
16. DC, Apr 2002, Washington Area NMR group. New approaches to structural biology.
17. DC May 2002, Medicinal Chemistry Conference, San Diego, Segmental labeling in NMR

18. Publication. Attached are proofs of a paper reporting this work to be published immediately in the journal Magnetic Resonance in Chemistry.

Degrees granted. None.

Development of cell lines, tissue or serum depositories. None.

Informatics such as database and animal models, etc. None.

Funding applied for based on work supported by this award. None.

Employment or research opportunities applied for and/or received on experience/training supported by this award. Dr. James McDonnell, supported by the award, became Lecturer in Molecular Biophysics, University of Oxford, UK, and Fellow of Somerville College, in September 2000.

Dr. Hong Ji supported subsequently by the award, became a Research Associate in the HHMI lab of Dr. Jennifer Doudna, University of California, Berkeley, November 2001.

Conclusions. The structural basis of Bid action by specific interactions with its mitochondrial receptor remains unknown. However, the setting of the 'regulator' between the pro- and anti-apoptotic points – a subject of continuing debate, and possible clinical application – is illuminated by the observation that the BH3 motif recognition is key to Bid's interaction with Bcl-xL, and that the recognition element for pro-apoptotic action is subtly different from that of others. This may contribute a possible route to reexamination of small molecule interactions for pro-apoptotic activity against the Bcl family as an additional approach to increasing apoptosis in tumors with high levels of Bcl family members acting as immortalisers.

References.

1. M. C. Raff, *Nature* 356, 397-400 (1992).
2. R. E. Ellis, J. Y. Yuan, H. R. Horvitz, *Annu Rev Cell Biol* 7, 663-98 (1991).
3. A. Strasser, D. C. Huang, D. L. Vaux, *Biochim Biophys Acta* 1333, F151-78 (1997).
4. C. B. Thompson, *Science* 267, 1456-62 (1995).

5. D. T. Chao, S. J. Korsmeyer, *Annu Rev Immunol* 16, 395-419 (1998).
6. J. C. Reed, *Adv Pharmacol* 41, 501-32 (1997).
7. S. Cory, *Annu Rev Immunol* 13, 513-43 (1995).
8. E. Yang, S. J. Korsmeyer, *Blood* 88, 386-401 (1996).
9. A. Bakhshi, et al., *Cell* 41, 899-906 (1985).
10. Y. Tsujimoto, J. Cossman, E. Jaffe, C. M. Croce, *Science* 228, 1440-3 (1985).
11. D. L. Vaux, S. Cory, J. M. Adams, *Nature* 335, 440-2 (1988).
12. A. Kelekar, C. B. Thompson, *Trends Cell Biol* 8, 324-30 (1998).
13. B. Conradt, H. R. Horvitz, *Cell* 93, 519-29 (1998).
14. K. Wang, X. M. Yin, D. T. Chao, C. L. Milliman, S. J. Korsmeyer, *Genes Dev* 10, 2859-69 (1996).
15. A. Kelekar, B. S. Chang, J. E. Harlan, S. W. Fesik, C. B. Thompson, *Mol Cell Biol* 17, 7040-6 (1997).
16. J. Zha, H. Harada, E. Yang, J. Jockel, S. J. Korsmeyer, *Cell* 87, 619-28 (1996).
17. H. Li, H. Zhu, C. J. Xu, J. Yuan, *Cell* 94, 491-501 (1998).
18. X. Luo, I. Budihardjo, H. Zou, C. Slaughter, X. Wang, *Cell* 94, 481-90 (1998).
19. A. Gross, et al., *J Biol Chem* 274, 1156-63 (1999).
20. J. J. Chou, H. Li, G. S. Salvesen, J. Yuan, G. Wagner, *Cell* 96, 615-24 (1999).
21. S. W. Muchmore, et al., *Nature* 381, 335-41 (1996).
22. M. Sattler, et al., *Science* 275, 983-6 (1997).
23. J. M. McDonnell, D. Fushman, C. L. Milliman, S. J. Korsmeyer, D. Cowburn, *Cell* 96, 625-34 (1999).
24. E. H. Cheng, et al., *Science* 278, 1966-8 (1997).
25. I. S. Goping, et al., *J Cell Biol* 143, 207-15 (1998).
26. L. C. Lee, J. J. Hunter, A. Mujeeb, C. Turck, T. G. Parslow, *J Biol Chem* 271, 23284-8 (1996).
27. J. J. Hunter, T. G. Parslow, *J Biol Chem* 271, 8521-4 (1996).
28. J. C. Phelan, N. J. Skelton, A. C. Braisted, R. S. McDowell, *J. Am. Chem. Soc.* 119, 455-460 (1997).
29. J. Zheng, et al., *J. Mol. Biol.* 255, 14-21 (1996).

NMR determination that an extended BH3 motif of pro-apoptotic BID is specifically bound to BCL-x_L[†]

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The BH3 motif of the pro-survival family of proteins, BCL, is also present in pro-apoptotic proteins like BID and BAX. Homo- and hetero-oligomerization interactions of the BH3 motif are generally recognized as the critical component of their apoptotic activities. In full-length BID, the putative hydrophobic binding surface of its BH3 motif is substantially occluded by intramolecular contacts, many of which are removed on BID's transformation to tBID by cleavage with caspase 8, required for tBID's pro-apoptotic action on mitochondria, thereby releasing cytochrome c. Copyright © 2006 John Wiley & Sons, Ltd.

KEYWORDS: NMR; ¹H; ¹³C; ¹⁵N; BID; BCL-x_L; apoptosis; BH3 motif; death agonist; helical propensity

As a step toward more complete characterization of the hetero-oligomeric complexes of the •BID and •BCL family of molecules, we report here the formation of a tight complex of the BH3 motif sequence of BID with BCL-x_L. In contrast to the previous report of BAK BH3 motif with BCL-x_L, the BID BH3 peptide (PDSSESQEEIMHNIARKLAQIGDDI) forms an α-helix significantly extended to the N-terminus, as monitored by ¹⁵N{¹H} nOe determination and by ¹³C chemical shifts. Modeling of the BID BH3 motif/BCL-x_L based on the previous structure showed that the extended helix fitted well into an extended cavity in BCL-x_L. Mutagenesis of the peptide and BCL-x_L based on this model identified the key residues in the BH3 motif, but suggested that some conformational flexibility is likely in the BCL surface.

The extended recognition of BID's BH3 motif indicates that the range of specificity for binding partners to the BCL family is highly complex, that specificity may incorporate conformational changes from the free proteins, and that these may be of significance in the specific targeting of these complexes to membrane and other targets.

Programmed cell death is a highly evolutionarily conserved biological process critical for development and homeostasis in multicellular organisms.^{1,2} This process allows the removal of redundant or damaged cells, playing a vital role in normal cellular development, tissue homeostasis, and

immunological defense.^{3,4} Deregulation of this pathway can contribute to cancer, autoimmune disease, and neurodegenerative disorders.^{5,6} Developmental or environmental signals can regulate signals for either cell death or cell survival. The study of complex signaling pathways of programmed cell death (apoptosis) has led to the identification of a large number of molecules involved in regulating apoptotic death, or promoting cell survival.^{7,8} As part of a critical apoptotic pathway regulating a checkpoint in mitochondria, the BCL-2 family of proteins comprises both anti- and pro-apoptotic regulators.⁹ While the continuing research in apoptosis indicates that several mechanisms are in effect, or may yet be uncovered, nonetheless the BCL-related family of anti- and pro-apoptotic agents is a major group providing checkpoints and activators of apoptosis.

Membership in the BCL-2 family is defined on the basis of homology to at least one of four conserved sequence motifs known as BCL-2 homology motifs, BH1 to BH4 (sometimes referred to as domains). Most anti-apoptotic family members, such as BCL-2 and BCL-x_L, have BH1 and BH2 motifs and many have all four BH motifs. In contrast, many of the pro-apoptotic family members (BID, BAD, BIK, BIM, BLK, HRK) possess only the conserved BH3 motif.¹⁰ The ratio of anti- and pro-apoptotic molecules apparently determines whether a cell will respond to a proximal apoptotic stimulus. This competition is mediated, at least in part, by competitive dimerization between anti- and pro-apoptotic pairs.⁵ However, other roles including self association into ion transporters in membranes, and specific association with membrane-associated receptors (protein or lipid) in organelle membranes have also been considered.¹¹

The BCL-2 family member BID belongs to the BH3-only class of pro-apoptotic proteins. Like other members of this class of molecules, BID shows no recognizable sequence homology to BH1, BH2, or BH4 domains, and requires a functional BH3 domain for its dimerization and

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pro-apoptotic activity.^{12–14} Caspase-8 mediates the cleavage of an inactive 23 kDa cytosolic BID to produce a truncated 15 kDa fragment (referred to as *tBID*) that translocates to the mitochondria causing cytochrome *c* release, activation of caspases, and the final commitment to cell death.^{15–17}

The structure of BID sheds light on its role as a death agonist, and the requirements for pro-apoptotic action in the BCL-2 family. What makes a BCL-2 family member a death agonist? It appears that two criteria must be met for pro-apoptotic function. The first requirement is cellular localization. There is strong evidence for several BCL-2 family members that mitochondrial translocation is a key determinant for death-inducing activity. BID^{15–17} and BAX¹⁸ are inactive as cytosolic components but induce death upon their targeting to mitochondria. The activity of BAD, on the other hand, is downregulated by phosphorylation, which sequesters the protein in the cytosol away from mitochondria targets.¹⁴ The second, and perhaps more critical, criteria for death agonism is the exposure of the BH3 surface. The cleavage of BID by caspase-8 is likely to expose a BH3 motif that is buried in the uncleaved molecule. On the basis of our structure predictions for the BCL-2 family, some members of the BH3-only class appear to have their BH3 motif exposed, or at least not under the same conformational constraints as other members of the BCL-2 family.¹⁰ Molecules possessing an exposed BH3 motif (Egl-1, Hrk, Bik, Bim, *tBID*) might be expected to be constitutively active death agonists, while other members of the family possessing a 'hidden' BH3 motif are regulated by post-translation modification events (i.e. cleavage, phosphorylation) that expose the BH3 epitope^{19–21} which induce their pro-apoptotic function. The structure of BID and related molecules then strongly supported the original hypothesis that BH3 recognition, and potentially exposure of the hydrophobic face of the BH3, was a key event in pro-apoptotic action. Recent structural studies of BAX, a pro-apoptotic 'multidomain' member of BCL-2 family, showed that BH3 domain interaction surface of full-length BAX is occupied by C-terminal hydrophobic helix of BAX suggesting possible control over its mitochondrial targeting and dimer formation. A similar control mechanism of BH3 domain-induced oligomerization was proposed for other 'multidomain' BCL-2 family members.²²

The conservation of sequential motifs in the BCL family led to detailed investigations of their interactions, and the identification of the BH3 motif as an interaction site in BAK's binding to BCL-2 and BCL-x_L.²³ In elegant structural studies,²⁴ the Fesik laboratory showed that this was the result of the BH3 forming a helix analogous to

its fold in full-length analogs, producing complexes with a BH1 and BH2 face of BCL-x_L through hydrophobic and electrostatic contacts. Various screening methods including chemical-shift perturbation/NMR were used to identify small molecule mimics of the BAK BH3 motif²⁵ that are able to bind to BCL-x_L. The general surface area of BCL-x_L perturbed by the small molecules was similar to that perturbed by the BAK BH3 peptide. More recently, Walensky *et al.*²⁶ elegantly demonstrated that a hydrocarbon-stapled BH3 analog of the BID BH3 could provide activation of apoptosis *in vivo*.

In this paper we investigate structural determinants of the BID BH3 interaction with anti-apoptotic BCL-x_L. We developed selective binding assays that allowed us to quantitatively compare various alternate peptidic mimics of BID BH3 motif binding to BCL-x_L. Amino-terminal extension of BID BH3 domain led to an increase in BID BH3: BCL-x_L binding affinity by 1 order of magnitude. We describe the lengthened and altered specificity of the BID BH3 binding to BCL-x_L using site-directed mutagenesis and heteronuclear NMR.

RESULTS AND DISCUSSIONS

To understand how BID interacts with BCL-x_L and inhibits the ability of BCL-x_L to promote cell survival, we measured the binding affinity of BCL-x_L to a peptide corresponding to the BH3 region of BID protein (BID11s) by using a fluorescence polarization (FP) binding assay (Table 1). The BCL-x_L used for the binding assay lacks the putative C-terminal transmembrane region. A BID 16-mer peptide was synthesized in which Glu97 and Met97 were changed to those found in the BAK peptide–Glu-Met → Asp-Leu. His89, which is not conserved in BCL-2 family members and is located on the surface of the BCL-x_L/BAK peptide complex,²⁴ was mutated to the unnatural amino acid Dap (X in Table 1) for the synthesis of the cyclic peptide. A decrease in binding affinity by a factor of 10 compared with the BAK peptide was observed for the peptide derived from the BH3 region of BID, even though it contains most of the key residues in BAK that interact with BCL-x_L. Each peptide was fluorescein labeled (in table, X == 2,3 diamino propionic acid, stereoisomer-equivalent to L-amino acid). We concluded that the longer BID-based peptide either formed a more effective helix for binding or had additional contacts.

BH3 mimics

We designed and tested a conformationally constrained α -helix mimicking the full BH3 motif of BID and other

Table 1. Binding affinities of different BH3 mimics towards BCL-x_L. Assays were saturation titration of the fluorescein-derivatized peptides using fluorescence depolarization measurements

	Peptide	Affinity (nM) (SD)	% change FP
BAK	GQVGRQLAIIGDDINR	390(20)	175
BID11	PDSESQEEMIHNIARXLAQIGDDIDH	250 (8)	250
BID11s	HNIARXLAQIGDDIDH	3600(200)	88
BID1cls	HNIARXLAQIGDDIDH (cyclized X-D)	1600(90)	80

X = K or K cyclized.

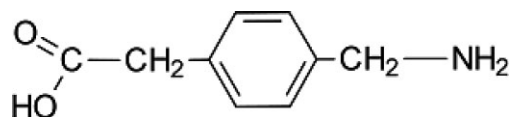


Figure 1. Chemical structure of the rigid linker of BID1cls.

pro-apoptotic members of the BCL-2 family. We implemented the strategy of using *i, i + 7* side-chain links for the sequence NIAKHLAQIGDEMD, with the K...D linkage being obtained by intermediate protection with allyl-K and Aloc-E (see Fig. 1). While α -helicity is apparent in the CD spectrum (data not shown), the magnitude of the 222 nm minimum is less than that expected from the formation of helix concordant with the BID solution structure helical content (~68%) (see Fig. 3 of Ref. 10) and may reflect time averaging of the structure between an α -helix and more random structures. Cyclization by exo-peptidic linkages produced only a modest apparent affinity increment in comparison to the optimized hydrocarbon-linked sequences of others.²⁶

CD spectra were acquired for the peptides in 30% •TFE. From the mean residue ellipticity at 222 nm, the percentage of α -helix was derived and compared to the affinity of each peptide for BCL-x_L (Table 1). As hypothesized, the cyclic peptide possesses a greater helix propensity compared to the linear equivalents but did not show dramatically higher affinities. We conclude that the general surface interaction of all these rigidized peptides is similar to the original packing observed in the BCL-x_L/BAK peptide complex.²⁴

To explain the tight binding of the extended BID BH3 peptide to BCL-x_L, we considered that the BID peptide might form additional interactions with BCL-x_L. Therefore, the longer peptide with a 10-residue extension of the BID 16-mer at the *N*-terminus was synthesized (BID11, Table 1). This longer peptide containing 26 amino acids was found to bind tightly to BCL-x_L with a *K_d* of 250 nM, and has a higher helix propensity than the 16-mer peptide.

Binding affinity measurements using fluorescence polarization

To obtain binding affinities for various BID peptides to BCL-x_L, the fluorescein derivative was chosen to provide a fluorophore for FP binding assay. The fluorophore was formed by an *N*-terminally or both *N*-terminally and Lys side-chain linked 5-carbamide fluorescein moiety. The labeled peptides were first tested in a saturation binding experiment against BCL-x_L (Fig. 2, Table 1).

The results show that, despite the addition of an *N*-terminal fluorescein, the affinity measured for the BAK peptide is in good agreement with previously published results.²⁴ A second FP experiment for measuring binding affinity was designed as a competition assay, which requires careful selection of the probe characteristics. The results in Table 1 show that BID11 has higher polarization change upon binding to BCL-x_L, which is of importance primarily because it delimits the signal-to-noise ratio of the assay. On the basis of *K_d* and polarization values, we selected the probe Fluor-BID11 for measuring the binding affinities for other peptides.

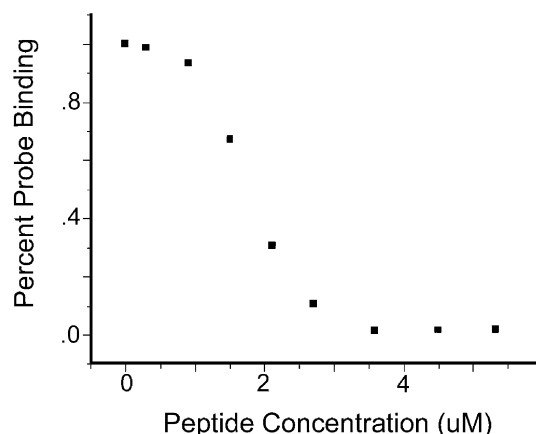


Figure 2. Saturation binding assay for fluor-BID11e with BCL-x_L. Buffer: 20 mM phosphate, pH 7.2; 50 mM NaCl; 2 mM DTT; 1 mM EDTA 0.01% BGG.

NMR assignments of the BID peptide complexed to BCL-x_L

To test whether the increase in binding affinity observed for BID 26-mer compared to the BID 16-mer results from higher helix propensity, or from the additional interactions between BCL-x_L and *N*-terminal residues of the BID peptide, we used heteronuclear NMR to characterize the BCL/BID peptide interaction. The deletion mutant of BCL-x_L used in the NMR studies is the same as that used for binding assay, which lacks the putative C-terminal transmembrane region and residues 45 to 84 which constitute a flexible loop previously shown to be dispensable for the anti-apoptotic activity.²⁷ The BID 26-mer peptide corresponding to the BH3 region of BID protein with ten residue extension at the *N*-terminus (BID11e), in which Met97 was mutated to Leu to avoid a cyanogen cleavage site in the middle of the peptide. We determined a *K_d* of 250 nM for BID11e/BCL-x_L binding from the FP competition assay. Furthermore, the *K_d* of the 40-amino acid peptide including the BH3 region and *N*-terminal residues extended to Asp59, the caspase cleavage site in the full-length BID protein, is the same as for the 26-residue peptide. In the ¹H{¹⁵N} HSQC spectra of free and complexed [¹⁵N, ¹³C] BID11e (Fig. 3), all the amide positions undergo significant chemical-shift changes upon binding, which is in accord with the change to a new structure on high-affinity binding, consistent with the fluorescence studies and with the expectation that the free peptide was either wholly or predominantly unstructured.

Backbone resonance assignments using a [¹⁵N, ¹³C] BID11e/BCL-x_L sample

Sequential assignments were achieved using the HNCACB/CBCA (CO) NH²⁸ pair of experiments so that connectivities could be traced through two independent, through-bond pathways. A ¹H{¹⁵N} NOESY-HSQC experiment allowed tracing of some amide–amide connectivities through the α -helical structures. Overall, all amide backbone resonances except Ile 83 were sequentially assigned by using this sample. The amide backbone resonance of Ile 83 was assigned using a specific [¹⁵N, ¹³C-Ile] BID11e/BCL-x_L sample.

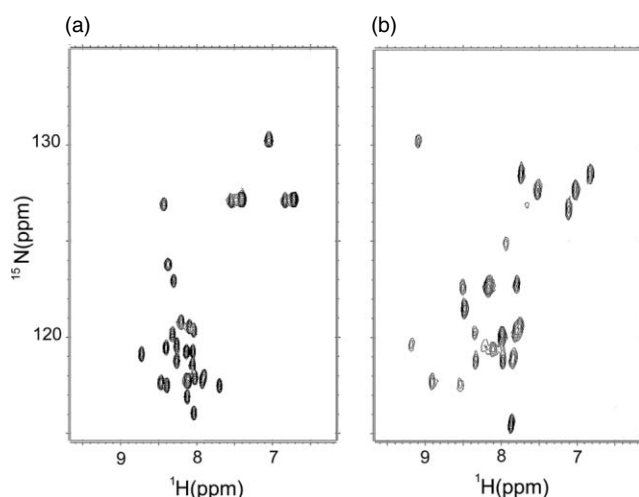


Figure 3. $^1\text{H}\{^{15}\text{N}\}$ HSQC Map free (a) and bound (b) of $[\text{U } ^{15}\text{N}, ^{13}\text{C}]$ BID1le to BCL- x_L .

The secondary structure of the BID1IE complexed with BCL- x_L was identified through the analysis of $^{13}\text{C}^\alpha$ and $^{13}\text{C}^\beta$ shifts.²⁹ Segments of the protein experience a downfield shift with C^α resonances and upfield shift with C^β carbons when located in helices, and C^α carbons experience an upfield and C^β , a downfield shift when located in the β -strands. The BH3 region derived from the BID protein shows a condensed group of positive C^α and C^β differences compared with the empirical values, which indicates that it adopts an α -helix when complexed to BCL- x_L (Fig. 4). Interestingly, the α -helix is N -terminally extended to the residue Gln79 located beyond the BH3 region, which suggests that the peptide might form additional interactions with BCL- x_L .

To further identify the boundaries of the folded peptide structure, we performed a steady-state $^{15}\text{N}\{^1\text{H}\}$ nOe experiment. The protein segments that do not participate in the folded structure have negative nOe values because of their high degree of local flexibility and motions in the sub-nanosecond to nanosecond timescale. As seen from Fig. 5,

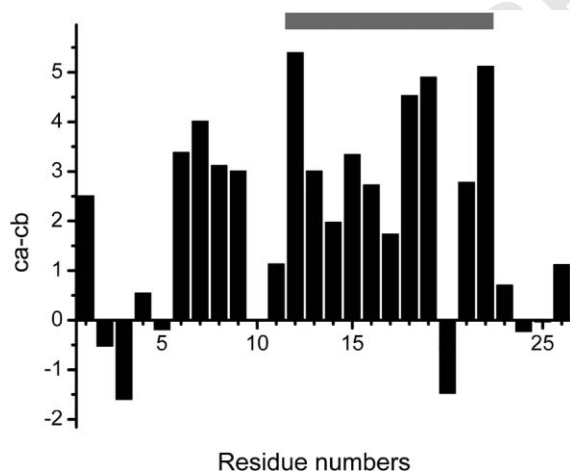


Figure 4. Apparent helicity (ordinate $\Delta^{13}\text{C}^\alpha - \Delta^{13}\text{C}^\beta$ chemical shifts in ppm) for the bound form of BID1le peptide. The horizontal line indicates the 'accepted' BH3 recognition in the BAK peptide interaction,²⁴ residues Gln77-Asp86.

N -terminal residues 1–4 have negative $^{15}\text{N}\{^1\text{H}\}$ nOe values and experience essentially minimal restrictions from the rest of the complex. This boundary of well-defined tertiary structure of the BID1le/BCL- x_L complex is consistent with the secondary structural part determined from Chemical-Shift Index.

Mutagenesis of the BID peptide and BCL- x_L protein

To determine what additional interactions between BCL- x_L and the N -terminal residues of the BID peptide are responsible for the increase in binding affinity observed for the BID 26-mer compared the BID 16-mer, the N -terminal residues 79–83 were added to the NMR structure of BCL- x_L /BAK complex, modeled, and energy-minimized using Insight (MSI) (Figs 6 and 7). An additional helical segment of BID BH3 fits well in the hydrophobic groove of BCL- x_L introducing no steric clashes (Fig. 6) and presenting hydrophobic Ile83 and polar Gln79 for direct interaction with the groove.

In the model proposed, the residues Thr109 and Val17 located in the helix 4 of BCL- x_L are involved in the hydrophobic interactions with N -terminal residues Gln79 and Ile83 of the BID peptide. We performed site-directed mutagenesis of the protein and the peptide on the basis of this mapping result to explore the extra binding sites. Individual mutations of two residues located at the N -termini of BID peptide, Q79A and I83A, resulted in 2.8-fold and 2.3-fold decrease of binding affinity to BCL- x_L ($\Delta 45$ –84), respectively. Mutation T109A does not affect the binding affinity, and V117L located in the helix 4 of BCL- x_L results in a 2.3-fold decrease of the binding affinity to BID peptide. However, the binding affinity of BID1le I83A peptide to mutant protein BCL- x_L ($\Delta 45$ –84) V117L has a 4.6-fold decrease compared to BID1le to BCL- x_L ($\Delta 45$ –84), which is additive of the titrations of two complementary mutants. The result indicates that Gln79 and Ile 83 of the BID peptide do significantly contribute to an overall 10 times increase in binding affinity of extended BID BH3 towards BCL- x_L . Val17 of BCL- x_L , which lies outside of the BAK peptide: BCL- x_L interaction surface, also makes a contact with BID BH3 but

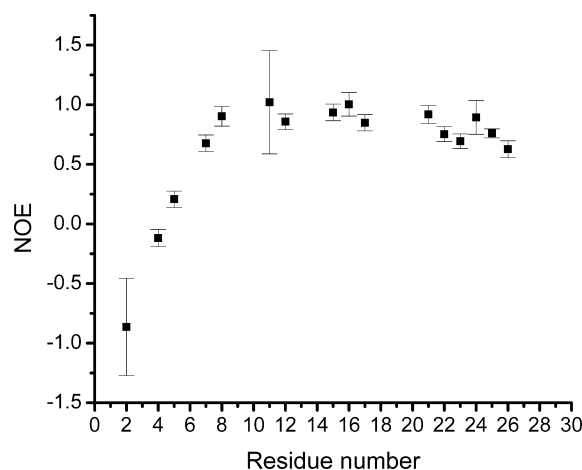


Figure 5. $^{15}\text{N}\{^1\text{H}\}$ nOe for BID 1le binding to BCL- x_L . The peptide corresponds to the BID sequence Pro74-His99 (NP_031 570).

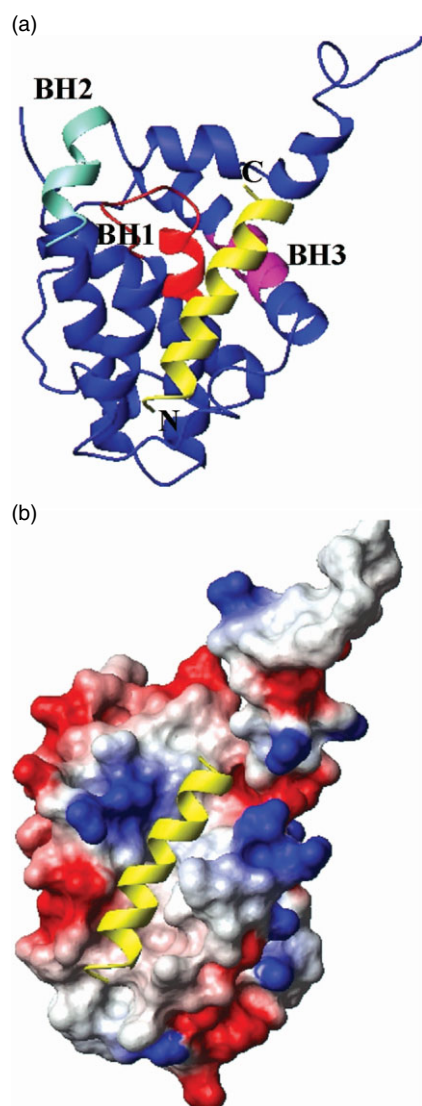


Figure 6. (a) Ribbon depiction of the BCL-x_L/BAK complex.²⁴ (b) Surface representation of the binding pocket of BCL-x_L bound to the BAK peptide.²⁴ This structure provides a model for anchoring the model of BID BH3 in Fig. 8.

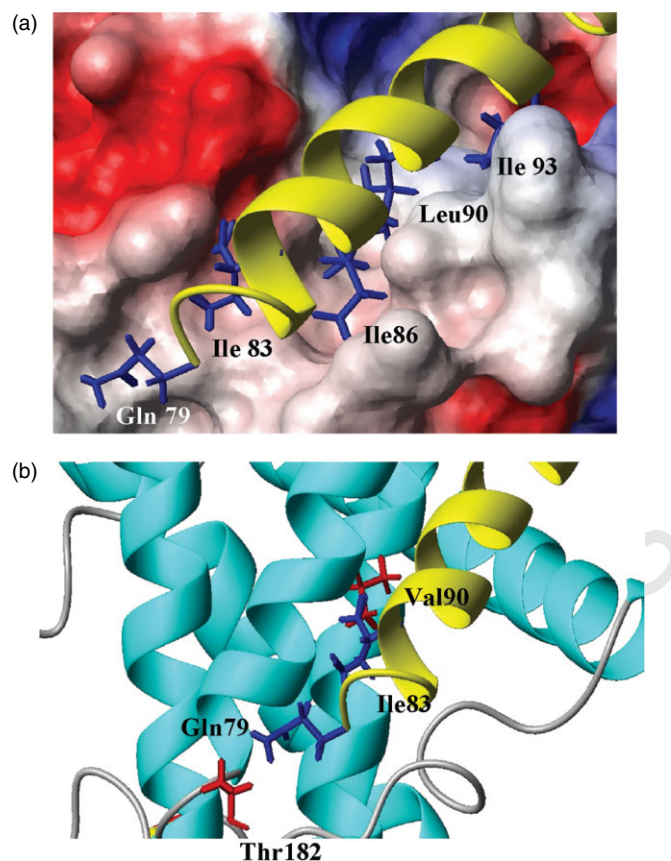


Figure 7. Graphical representation of site-directed mutagenesis of the BCL-x_L protein and the BID peptide and the effects of their interactions.

primers containing a BamHI upstream restriction site and a downstream EcoRI restriction site. The amplified genes were cloned in frame into the expression vector PGEX-2T (Pharmacia). The protein was expressed in *Escherichia coli* BL21 (DE3) using the T7 expression system. Cells, freshly transformed with plasmid, were grown to late log phase. Protein expression was induced by addition of 0.5 mM isopropyl thio- β -D-galactoside (IPTG). After growth for another 3 h at 37 °C, cells were harvested by centrifugation. The cell pellet was re-suspended in lysis solution (1% Triton X-100, 5 mM EDTA, 1 mM DTT, and protease inhibitors (Aprotinin, PMSF) and sonicated for 10 min periods on ice. The lysate was clarified by centrifugation at 4800 g for 20 min at 4 °C, and the supernatant was then incubated with glutathione-agarose beads (5 ml beads/1 l culture) for 2 h at 4 °C with Nutator mixing. The suspension was centrifuged at 2000 rpm for 5 min and the pellet was re-suspended and washed twice with 10 ml of high-salt buffer (sonication buffer with additional 100 mM NaCl) at 4 °C. GST was cleaved from the fusion protein with human thrombin (150 units) in 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 2.5 mM CaCl₂, 2 mM DTT) for 12 hours at 4 °C. The digestion was quenched with 1 mM AEBSE. The protein solution was then dialyzed against NMR buffer (20 mM phosphate buffer pH 7.2; 50 mM NaCl; 5 mM EDTA; 1 mM DTT).

MATERIALS AND METHODS

Expression and purification of BCL-x_L and mutants

The deletion mutants of BCL-x_L and mutants containing single-residue mutations were constructed from the expression vector for BCL-x_L (residues 1 to 209) by PCR using

BID peptide expression and purification

BID peptide was constructed from the expression vector for full-length BID protein by PCR. The amplified gene was subsequently cloned into the HindIII-BamHI sites of a phagemid-T7 expression vector, PTMHa.³⁰ Residue Met 97 was mutated to Leu using the megaprimer method.³¹ The DNA sequence was verified by DNA sequencing. In the PTMHa vector, the desired sequences are expressed as chimeric proteins containing a modified form of the TrpLE leader sequence, in which a N-terminal (His)₉ tag has been added, which can be removed upon cleavage by cyanogen bromide (CNBr). The peptide was expressed in *E. coli* BL21(DE3) using the T7 expression system. Peptide expression was induced by addition of 0.5 mM IPTG. After growth for another 5 h at 37 °C, the bacteria were harvested by centrifugation and the pellets were stored frozen. Inclusion bodies of the cell lysate were isolated by sonicating in sucrose (50 mM Tris, 25% sucrose, 1 mM EDTA, pH 8) and Triton X buffer (20 mM Tris, 1% Triton X-100, 1 mM EDTA, pH 8) separately.³² The resulting pellet was treated with CNBr (0.03 g/ml, 70% formic acid, 2 h). After removal of CNBr, the protein was dialyzed against 5% acetic acid and lyophilized. The protein was solubilized in 6 M GdmCl, 0.1 M sodium phosphate, and 10 mM Tris pH 8.0). The solution was passed over a nickel-chelating column to remove the leader sequence containing the His tag and any uncleaved chimeric protein. The protein was purified to homogeneity by reverse-phase HPLC, using a Vydac C-18 preparative column and a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid. The protein identity was confirmed by mass spectroscopy. Uniformly ¹⁵N-, ¹³C-labeled peptide was prepared by growing the *E. coli* strain BL21 (DE3) over-expressing BID1le on a minimal medium containing ¹⁵NH₄Cl and ¹³C-glucose.

Synthesis of peptide for cyclization

We used Fmoc (9-fluorenyl) methoxycarbonyl chemistry³³ to carry out solid-phase synthesis. Fmoc removal was achieved by piperidine-DMF (20%) cleavage, and standard BOP/HOBT/NMM activation. To synthesize cyclic peptide, we used N^α-Fmoc, N^ε-Dde Lys derivate and N^α-Fmoc, O^β-Alloc Asp derivate as building blocks; side chains were assembled after removal of Dde with hydrazine hydrate-DMF (2%) and removal of Alloc with Pd (Ph₃P)₄/CH₃Cl/AcOH/NMM, respectively. A rigid linker was introduced using Fmoc-*p*-(aminoethyl) phenylacetic acid (Fig. 1) as a building block. Side-chain deprotection was obtained by hydrogenation with Pd, and cyclization by use of the procedure of Phelan.³⁴ The synthesized peptides were characterized by ESI mass spectroscopy.

Fluorescence titration

FP experiments were performed at 18 °C on an ISA Fluorolog III (JY Horiba) spectrofluorometer. Fluorescein-containing probes were read with standard cut-off filters for the fluorescein fluorophore ($\lambda_{\text{ex}} = 485 \text{ nm}$, $\lambda_{\text{em}} = 520 \text{ nm}$). Saturation experiments were performed using fixed concentrations of the probe (20 nM) titrated against increasing concentration of BCL-X_L proteins. Data were analyzed using Origin

(Microsoft, Inc.) to fit the equation (probe bound)/(total probe) = (free protein)/(K_d + [free protein]) making the assumption that (free protein) $\cong$ (total protein), which holds for our experimental conditions. Competitive binding experiments were performed using fixed concentrations of the probe (20 nM) complexed with proteins titrated against increasing concentration of the second unlabeled peptide. Standard buffer conditions were PBS buffer, pH 7.2; 2 mM DTT; 1 mM EDTA plus 0.01% bovine gamma-globulin (BGG).

NMR experiments

NMR spectra were acquired at 30 °C on a Bruker DMX500 or DMX600 NMR spectrometer. 700 μM [U-¹⁵N, ¹³C] BID1le sample and 1 mM [U-¹⁵N, ¹³C] BID1le/BCL-X_L were in NMR buffer (20 mM phosphate buffer pH 7.2; 50 mM NaCl; 5 mM EDTA; 1 mM DTT, 0.02% NaN₃). The HNCA, CBCA(CO)NH, HNHA, ¹⁵N-edited NOESY and ¹⁵N{¹H} steady-state experiments were collected using previously described protocols.^{35,36} All spectra were processed using NmrPipe³⁷ and assignments were made with in-house software.

REFERENCES

- Raff MC. *Nature* 1992; **356**(6368): 397.
- Ellis RE, Yuan JY, Horvitz HR. *Annu. Rev. Cell Biol.* 1991; **7**: 663.
- Strasser A, Huang DC, Vaux DL. *Biochim. Biophys. Acta* 1997; **1333**(2): F151.
- Thompson CB. *Science* 1995; **267**(5203): 1456.
- Chao DT, Korsmeyer SJ. *Annu. Rev. Immunol.* 1998; **16**: 395.
- Reed JC. *Adv. Pharmacol.* 1997; **41**: 501.
- Cory S. *Annu. Rev. Immunol.* 1995; **13**: 513.
- Yang E, Korsmeyer SJ. *Blood* 1996; **88**(2): 386.
- Korsmeyer SJ. *Harvey Lect.* 1999; **95**: 21.
- McDonnell JM, Fushman D, Milliman CL, Korsmeyer SJ, Cowburn D. *Cell* 1999; **96**(5): 625.
- Kuwana T, Newmeyer DD. *Curr. Opin. Cell Biol.* 2003; **15**(6): 691.
- Wang K, Yin XM, Chao DT, Milliman CL, Korsmeyer SJ. *Genes Dev.* 1996; **10**(22): 2859.
- Kelekar A, Chang BS, Harlan JE, Fesik SW, Thompson CB. *Mol. Cell. Biol.* 1997; **17**(12): 7040.
- Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. *Cell* 1996; **87**(4): 619.
- Li H, Zhu H, Xu CJ, Yuan J. *Cell* 1998; **94**(4): 491.
- Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. *Cell* 1998; **94**(4): 481.
- Gross A, Yin XM, Wang K, Wei MC, Jockel J, Milliman C, Erdjument-Bromage H, Tempst P, Korsmeyer SJ. *J. Biol. Chem.* 1999; **274**(2): 1156.
- Goping IS, Gross A, Lavoie JN, Nguyen M, Jemmerson R, Roth K, Korsmeyer SJ, Shore GC. *J. Cell Biol.* 1998; **143**(1): 207.
- Cheng EH, Kirsch DG, Clem RJ, Ravi R, Kastan MB, Bedi A, Ueno K, Hardwick JM. *Science* 1997; **278**(5345): 1966.
- Lee LC, Hunter JJ, Mujeib A, Turck C, Parslow TG. *J. Biol. Chem.* 1996; **271**(38): 23 284.
- Hunter JJ, Parslow TG. *J. Biol. Chem.* 1996; **271**(15): 8521.
- Suzuki M, Youle RJ, Tjandra N. *Cell* 2000; **103**(4): 645.
- Chittenden T, Flemington C, Houghton AB, Ebb RG, Gallo GJ, Elangovan B, Chinnadurai G, Lutz RJ. *EMBO J.* 1995; **14**(22): 5589.
- Sattler M, Liang H, Nettesheim D, Meadows RP, Harlan JE, Eberstadt M, Yoon HS, Shuker SB, Chang BS, Minn AJ, Thompson CB, Fesik SW. *Science* 1997; **275**(5302): 983.
- Degtarev A, Lugovskoy A, Cardone M, Mulley B, Wagner G, Mitchison T, Yuan J. *Nat. Cell Biol.* 2001; **3**(2): 173.

1	26. Walensky LD, Kung AL, Escher I, Malia TJ, Barbuto S,	32. Lu M, Blacklow SC, Kim PS. <i>Nat. Struct. Biol.</i> 1995; 2 (12): 1075.	61
2	Wright RD, Wagner G, Verdine GL, Korsmeyer SJ. <i>Science</i> 2004;	33. Albericio F. <i>Curr. Opin. Chem Biol</i> 2004; 8 (3): 211.	62
3	305 (5689): 1466.	34. Phelan JC, Skelton NJ, Braisted AC, McDowell RS. <i>J. Am. Chem.</i>	63
4	27. Muchmore SW, Sattler M, Liang H, Meadows RP, Harlan JE,	<i>Soc.</i> 1997; 119 : 455.	64
5	Yoon HS, Nettesheim D, Chang BS, Thompson CB, Wong SL,	35. Ghose R, Shekhtman A, Goger MJ, Ji H, Cowburn D. <i>Nat. Struct.</i>	65
6	Ng SL, Fesik SW. <i>Nature</i> 1996; 381 (6580): 335.	<i>Biol.</i> 2001; 8 (11): 998.	66
7	28. Grzesiek S, Bax A. <i>J. Am. Chem. Soc.</i> 1992; 114 : 6291.	36. Gosser YQ, Zheng J, Overduin M, Mayer BJ, Cowburn D.	67
8	29. Wishart DS, Sykes BD. <i>J. Biomol. NMR</i> 1994; 4 : 171.	<i>Structure</i> 1995; 3 (10): 1075.	68
9	30. Staley JP, Kim PS. <i>Protein Sci.</i> 1994; 3 (10): 1822.	37. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A.	69
10	31. Barik S, Megaprimer PCR. <i>Methods Mol. Biol.</i> 2002; 192 : 189.	<i>J. Biomol. NMR</i> 1995; 6 : 277.	70
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