

Bim, Bad and Bmf: intrinsically unstructured BH3-only proteins that undergo a localized conformational change upon binding to prosurvival Bcl-2 targets

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All BH3-only proteins, key initiators of programmed cell death, interact tightly with multiple binding partners and have sequences of low complexity, properties that are the hallmark of intrinsically unstructured proteins (IUPs). We show, using spectroscopic methods, that the BH3-only proteins Bim, Bad and Bmf are unstructured in the absence of binding partners. Detailed sequence analyses are consistent with this observation and suggest that most BH3-only proteins are unstructured. When Bim binds and inactivates prosurvival proteins, most residues remain disordered, only the BH3 element becomes structured, and the short α -helical molecular recognition element can be considered to behave as a 'bead on a string'. Coupled folding and binding is typical of many IUPs that have important signaling roles, such as BH3-only proteins, as the inherent structural plasticity favors interaction with multiple targets. This understanding offers promise for the development of BH3 mimetics, as multiple modes of binding are tolerated.

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Bcl-2 family proteins are important regulators of cell fate. The opposing factions of this family integrate diverse death and survival signals, and regulate initiation of apoptosis in response to various stimuli.¹ Family members that promote cell survival (Bcl-2, Bcl-x_L, Bcl-w, A1 and Mcl-1) contain up to four Bcl-2 homology (BH) domains in addition to a C-terminal hydrophobic sequence that targets them to the cytoplasmic face of intracellular membranes, particularly the mitochondria. The proapoptotic family members are more diverse. Those with multiple BH domains such as Bax and Bak (often referred to as Bax-like) have a critical nonredundant role that results in mitochondrial membrane disruption, whereas those with a single BH domain, referred to as BH3-only molecules, appear to function as the key initiators of death. In a healthy cell, the BH3-only molecules are either repressed (e.g. Noxa, Puma) or are present in an inactive state (e.g. Bim, Bmf, Bad and Bid).² Upon receipt of a death stimulus, BH3-only molecules are activated, allowing them to engage and inactivate prosurvival Bcl-2 proteins.³ Interaction of BH3-only proteins with their prosurvival partners ultimately leads to oligomerization of Bax and Bak, and release of apoptogenic factors from the mitochondria.

Initiation of apoptosis is thus intimately associated with the ability of BH3-only proteins to bind their prosurvival counterparts. This interaction depends on the integrity of both the BH3 domain of BH3-only proteins and their binding site, a surface exposed hydrophobic groove on prosurvival proteins.³ Structures of prosurvival proteins (CED-9 and Bcl-x_L) from *Caenorhabditis elegans* and mammals bound to BH3

domain peptides from proapoptotic proteins, have revealed the conserved nature of this interaction despite only a low level of shared sequence identity.^{4–7} Notably, the conserved leucine and aspartic acid residues of the LXXXGDE motif, which defines BH3-only proteins, make critical contacts with conserved residues in the hydrophobic binding groove.⁴ Comparison of the CED-9 and Bcl-x_L structures, in the presence and absence of ligand, also suggests that flexibility is important for binding as complex formation is accompanied by local rearrangements within the binding groove, but not by appreciable conformational change of the prosurvival Bcl-2 protein core.^{4–7}

Rearrangements of the ligand associated with binding have not been fully characterized. Most studies have focused on short peptides that span the BH3 domain-binding motif. When bound to their cognate targets, these peptides adopt an α -helical conformation,^{4–7} although in isolation they are mainly random coil in conformation,^{5,8} suggesting that a structural transition is associated with binding. However, Petros *et al.*⁵ showed that in the case of Bad BH3 peptides, there appeared to be a correlation between helical propensity and binding affinity for Bcl-x_L. Other studies have focused on the BH3-only proteins Bid, which presents a complex picture, as full-length Bid has an α -helical structure similar to that of the prosurvival Bcl-2 proteins, and the BH3 domain is well defined.^{9,10} Activation of Bid differs from other BH3-only proteins in that it requires proteolysis followed by myristoylation.² These events may trigger rearrangement and exposure of the BH3 domain, allowing Bid to bind and inactivate prosurvival

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molecules. However, the sequence and functional diversity shown by the BH3-only proteins make Bid a poor model.^{2,11}

Many molecules with key regulatory roles in cells belong to the class of proteins referred to as ‘intrinsically unstructured proteins (IUPs)’.¹² These proteins do not have a well-defined tertiary structure and commonly undergo a structural transition on binding their cognate partners.¹³ Such conformational plasticity is consistent with the observed properties of the BH3 domain peptides from BH3-only proteins but it is not known, if like Bid, these peptides have a defined structure when in the context of the intact protein. Using a combined bioinformatic and experimental approach, we have characterized both the solution structure of the BH3-only protein Bim and the nature of the conformational change associated with binding. Our data suggest that Bim is a member of the intrinsically unstructured class of proteins, and that it undergoes a localized conformational change when it binds prosurvival molecules. At least some other BH3-only proteins, including Bmf and Bad, also appear to be largely unstructured, suggesting that the model developed for Bim reflects other BH3-only proteins. Unstructured regions of proteins have been previously reported to promote binding diversity,¹³ and it seems likely that the unstructured nature of BH3-only molecules contributes to their ability to bind multiple partners.⁸

Results

Sequence analysis suggests that Bim is a disordered protein. Alternative splicing gives rise to numerous *bim* mRNA transcripts,¹⁴ although the three main splice variants in cells are Bim_S, Bim_L and Bim_{EL}.¹⁵ The distinct isoforms differ in size and have different apoptotic activity owing to the presence or absence of specific exons (Figure 1a).¹⁶ All three main splice variants contain the BH3 domain encoded by exon 8 that is required for binding to prosurvival proteins, and a predicted hydrophobic transmembrane (TM) region (exon 11).¹⁵ Exon 4, present in both Bim_L and Bim_{EL}, but not in Bim_S, encodes the dynein light chain (DLC)-binding motif required for interaction with DLC1.¹⁷ Bim_S, which is not negatively regulated by interaction with DLC1, is a more potent inducer of death than isoforms that contain exon 4. Sequences encoded by exon 3 are phosphorylated and

targets Bim_{EL} for proteosomal degradation.¹⁶ The function of exon 2, present in all Bim splice forms,¹⁴ is unknown, but it contains a conserved lysine and potential ubiquitylation site.¹⁸

The structural data available for Bim is limited to peptide fragments bound to its targets. Liu *et al.*⁶ showed that a 33-residue peptide encoding the BH3 domain of Bim formed an α -helix when bound to the hydrophobic groove of Bcl-x_L. In contrast, a nine-residue peptide containing the short motif

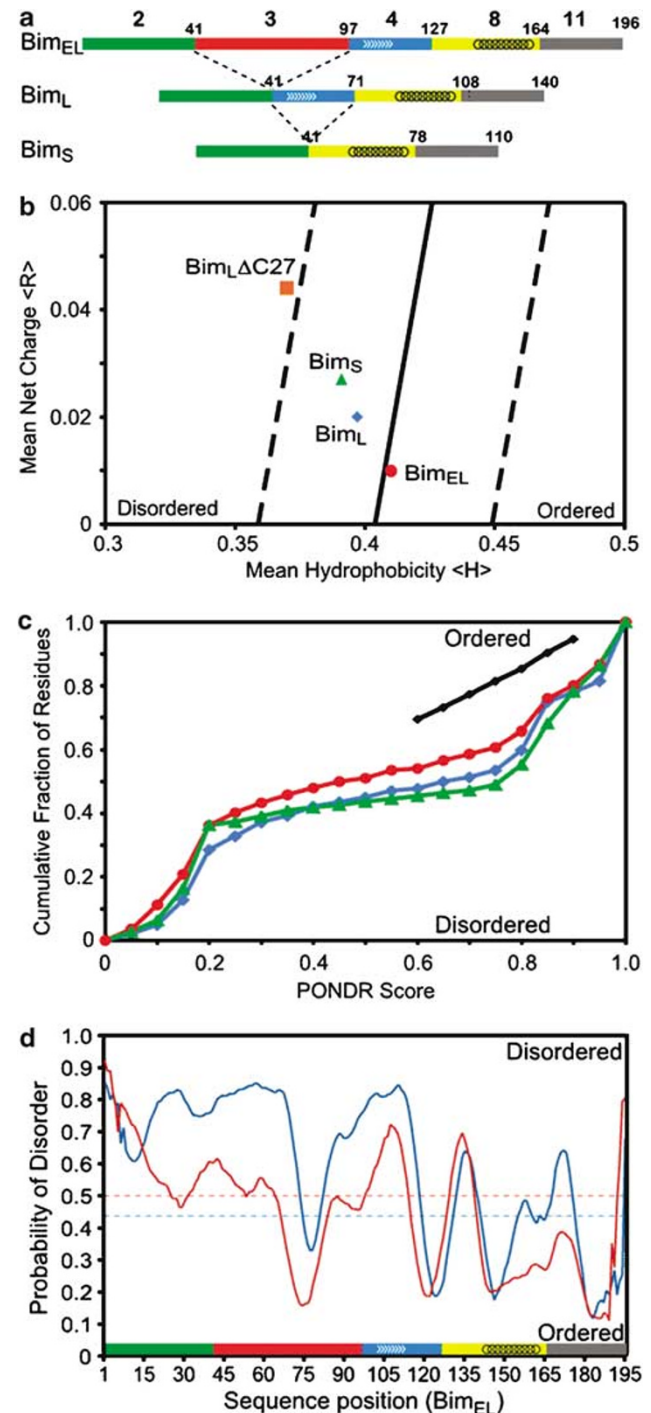


Figure 1 Sequence analysis of Bim predicts that it is largely unstructured. (a) The exonic structure of the three main expressed Bim protein isoforms is shown (exons are color coded and their number is indicated above). The relative position of the α -helix and β -strand identified in complexes with Bcl-x_L and DLC1 are indicated. Sequence positions shown above the line refer to mouse Bim. (b) Net charge/hydrophobicity plot of the three main Bim splice variants and the Bim protein used in subsequent experiments (Bim_L Δ C27). The charge/hydrophobicity diagram is divided into two regions by a line (–), which corresponds to the equation $\langle R \rangle = 2.743 \langle H \rangle - 1.109$. Proteins on the left side of the diagram are predicted to be disordered, whereas those on the right are predicted to be ordered.^{20,21} The positions of the 0.045 boundaries are shown (---). (c) CDF plots for Bim_S (green), Bim_L (blue) and Bim_{EL} (red). The order–disorder boundary (black) is as reported previously.²¹ (d) DisEMBL predicts structural disorder for Bim_{EL}. The blue curve is the REMARK465 prediction and the red curve is the COILS prediction.²³ The horizontal blue and red lines correspond to the random expectation level for each predictor.²³

required for binding to DLC forms a β -strand when bound to DLC1.¹⁹ In isolation, both peptides adopt a random coil conformation suggesting that a conformational change is associated with binding (MG Hinds and CL Day, unpublished).⁸ To determine whether the peptide studies reflect the structure of the equivalent regions within the context of full-length Bim and to obtain a molecular understanding of protein conformations that tolerate extensive alternative splicing, a bioinformatic and structural analysis of full-length Bim was undertaken.

Initial analysis of the sequences of the three main Bim splice variants suggests that they have features typical of IUPs (Figure 1). When analyzed by the method of Uversky *et al.*,²⁰ which assesses intrinsic disorder based on the ratio between mean hydrophobicity and mean net charge, Bim_S and Bim_L are predicted to be disordered, whereas Bim_{EL} lies on the boundary between the two states (Figure 1b). Cumulative distribution frequency plots (CDF) predict, with a high degree of confidence, that all three Bim splice variants are disordered (Figure 1c). In CDF plots, the predictors of natural disordered regions (PONDR) scores for each residue are represented as a histogram, allowing estimates of whole protein disorder to be made. Plots that fall below the seven-point boundary line between PONDR scores of 0.6 and 0.9 are likely to be disordered, as the majority of residues in these proteins have high PONDR scores.²¹ Analysis of 71 *Arabidopsis thaliana* proteins by both PONDR VL-XT and by nuclear magnetic resonance (NMR) (a ¹H-¹⁵N-HSQC, was recorded for each protein) revealed an 84% accuracy of predicting protein disorder.²² To identify any localized regions of structure, Bim_{EL} was also analyzed using DisEMBL.²³ The DisEMBL predictor, which uses artificial neural networks that have been trained using data sets obtained from analysis of the protein databank, predicts that the N-terminal region including exons 2 and 3, and most of exon 4 is largely unstructured. Regions of order are predicted in the C-terminal exons, which contain the BH3 domain and the predicted TM region (Figure 1d), both of which are predicted to adopt an α -helical conformation in certain conditions. Taken together, the sequence analyses suggest that when free in solution, multiple isoforms of Bim lack a well-defined structure, and that Bim is a member of the intrinsically unstructured class of proteins. IUPs commonly have an increased prevalence of phosphorylation sites²⁴ and PEST (proline, glutamic acid, serine and threonine) sequences,²⁵ and consistent with this Bim is phosphorylated and targeted for degradation by the proteasome in certain cell types.¹⁶

Bim lacks stable structure in solution. In order to directly investigate the structural properties of Bim, a number of expression constructs were generated. To obtain soluble protein, it was necessary to delete the 27 C-terminal hydrophobic residues (Δ C27), which comprise most of exon 11 and are predicted to form a TM helix.¹⁵ Even with this deletion, only Bim_L could be produced and purified in significant quantities. During purification, Bim_L Δ C27 was observed to be highly protease sensitive and behaved as a larger protein, migrating in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as an approximately 16kDa protein and eluting from gel filtration

columns with an apparent molecular weight of \sim 25 kDa (see Figure 3a). However, when analyzed by sedimentation velocity centrifugation and evaluated in terms of a single class of noninteracting solute, a molecular mass (M_r) of 13.2 kDa was obtained (Figure 2a). This is within 1% of 13.1 kDa, the calculated mass based on the amino-acid sequence, and indicates that Bim_L Δ C27 exists as a monomer under the experimental conditions employed in the analytical ultracentrifuge. The slow migration on SDS-PAGE may be correlated with the high acidic content (16%) of Bim_L Δ C27 relative to globular proteins (11.8%), as high negative charge is associated with both a larger Stokes radius for the protein detergent complex and a reduction in detergent binding.²⁶ Elution as a larger protein when analyzed by size exclusion chromatography, and sensitivity to proteases, are both properties commonly observed for IUPs and suggest that Bim_L Δ C27 may be disordered in solution.

Circular dichroism (CD) and NMR spectroscopy were used to directly assess the secondary structure of Bim_L Δ C27. Unstructured proteins give rise to CD spectra with minima near 200 nm and some weak features (positive or negative) between 220 and 230 nm. The presence of significant α -helical structure is associated with minima at 222 and 208 nm, and a positive signal at 200 nm. The CD spectra obtained for Bim_L Δ C27 over the range 5–65°C is characteristic of an unstructured protein, although the slight inflection near 222 nm may indicate some residual α -helical structure, but this is not enhanced at lower temperatures as seen for some proteins (Figure 2b).²⁷ Analysis of NMR spectra for Bim_L Δ C27 support this conclusion (Figure 2c). In the ¹H-¹⁵N-HSQC spectrum (Figure 2c), the backbone amide resonances fall between 8.0 and 8.5 p.p.m., and there is little dispersion in the aliphatic region in the 1D ¹H NMR spectrum (data not shown). The sharp resonances and lack of chemical shift dispersion reflect the intrinsic mobility and lack of long-range order in Bim_L Δ C27. The absence of stable tertiary structure is supported by the complete absence of any HN–HN cross peaks in both proton 2D NOESY and a three-dimensional (3D) ¹⁵N-NOESY spectra (data not shown). In heteronuclear {¹H}¹⁵N, NOEs spectra, most of the resonances were of negative intensity (data not shown), indicating mobility on the ps–ns time scale, features typical of IUPs.²⁸ Bim_L Δ C27 contains a large number of proline residues (14 of 118 residues), and *cis*–*trans* isomerization about the proline amide bond, tolerated by unstructured proteins, probably accounts for the heterogeneity seen in the HSQC spectrum. Together, the CD and NMR data indicate that bacterially expressed Bim_L Δ C27 is largely unstructured. Bim_L purified from insect Sf-9 cells was also susceptible to proteolysis, suggesting that this protein was unfolded.⁶ In both cases, the C-terminal putative TM region of Bim has been deleted, but it seems unlikely that the presence of the C-terminal residues would result in a compact globular structure, instead, as predicted based on sequence analysis (Figure 1), the major Bim isoforms are likely to be unstructured in solution.

Bim undergoes a limited structural transition in the presence of prosurvival molecules. Many IUPs undergo a structural transition upon binding to interacting molecules.^{12,13} Bim binds tightly to all prosurvival proteins⁹

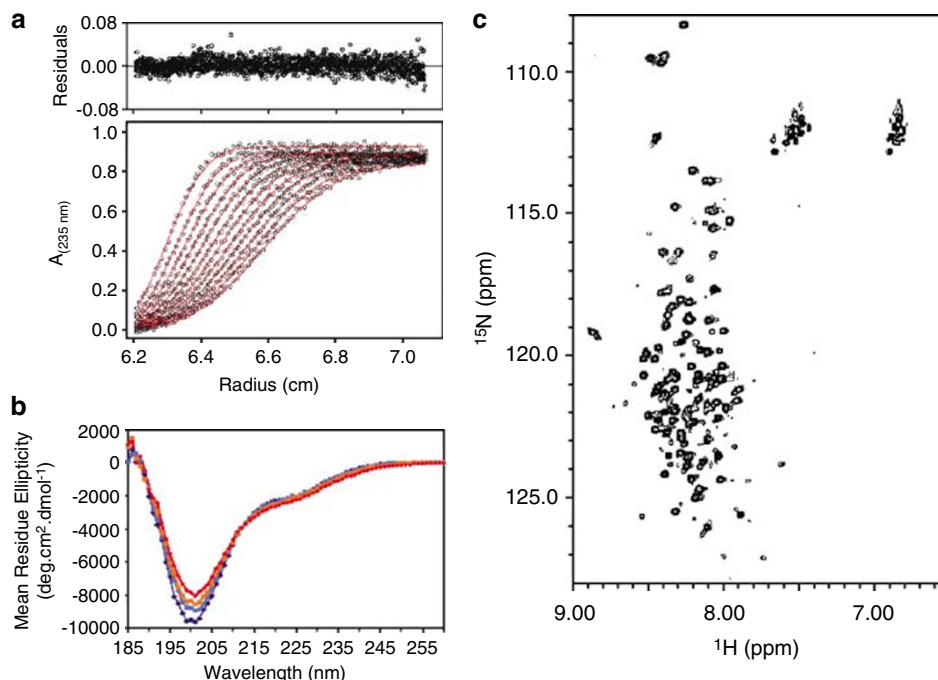


Figure 2 Bim_LΔC27 is unstructured. (a) Analytical ultracentrifugation analysis of Bim_LΔC27. Data acquisition and analyses were carried out as described. The circles represent the radial scans used in the analysis (15–50). For clarity, every third data set is shown. The continuous red lines represent the best fit of the data to the Lamm equation (rmsd = 0.010). The values of the parameters recovered from the analysis were $c = 0.94$, $M_r = 13\,200$ (12\,600–14\,000) and $S = 1.60$ (1.56–1.63), where the numbers in parentheses represent 95% confidence limits. The distribution of unweighted residuals is shown above the main figure. (b) Far-UV CD spectrum of recombinant Bim_LΔC27 at 5, 25, 45 and 65 °C recorded in 20 mM sodium phosphate pH 6.7. (c) ¹H-¹⁵N-HSQC spectra of ¹⁵N-labeled Bim_LΔC27 recorded at 25 °C

and residues 85–113 (mouse Bim_L numbering), which include the BH3 domain, have been shown to form an α -helix when bound to Bcl-x_L.⁶ As Bim_LΔC27 is largely unstructured in solution, a structural transition of at least the BH3 domain was expected upon binding prosurvival molecules. Gel filtration chromatography was used to characterize the complex formed between Bcl-w and Bim (Figure 3a). When separated on a Superdex 200 column, Bim_LΔC27 behaved as a large protein as commonly observed for IUPs, and had an apparent molecular mass of 26.7 kDa (calculated mass of 13.1 kDa), whereas the apparent mass for Bcl-wΔC10 was 17.1 kDa (calculated mass of 18.3 kDa). When equimolar amounts of the two proteins were mixed before chromatography, they eluted together with an apparent molecular mass of 44.4 kDa (Figure 3a, lower panel), close to the calculated M_r of 43.8 kDa based on the apparent masses of the individual proteins, but larger than the predicted heterodimer molecular weight of 31.4 kDa. This suggested that, like Bim alone, the complex did not have a compact structure.

Partial proteolysis experiments were used to further evaluate the conformational changes associated with complex formation. When treated with trypsin, Bim_LΔC27 was rapidly degraded to small peptides (Figure 3b, lanes 3 and 4), whereas Bcl-w was relatively resistant although after 60 min digestion, several proteolytic fragments appeared (Figure 3b, lanes 6 and 7). When Bcl-wΔC10 and Bim_LΔC27 were mixed before trypsin digestion, a different proteolytic profile was obtained (Figure 3b, lanes 8 and 9). Mass spectrometry analysis of the peptide mixtures revealed changes consistent

with complex formation. Notably, a peptide arising from proteolysis after R59 (helix 2–3 loop) and R78 (N-terminal of helix 4) in Bcl-w was no longer apparent, although a new peptide of 4621 kDa was detected. The mass of the new peptide corresponds to that expected for a peptide derived from the C-terminus of Bim_LΔC27 (sequence shown at the bottom of Figure 3b). This peptide includes the BH3 domain and suggests that interaction with the hydrophobic binding groove protects the BH3 domain and the hydrophobic groove of Bcl-w from proteolysis. The Bim BH3 peptide was not detected when Bim_LΔC27 was digested alone, but a similar peptide was found in the crystals of Bcl-x_L/Bim where the Bim protein had undergone nonspecific proteolysis.⁶ Proteolysis of the N-terminus of Bim was not affected by the presence of Bcl-w. Together, these results suggest that Bim remains largely unstructured in the presence of prosurvival proteins and that only a limited conformational change of just the BH3 domain is associated with binding.

To further investigate the nature of the structural transition associated with binding of Bim to Bcl-w, CD and NMR methods were used. A localized conformational change is supported by comparison of the ¹H-¹⁵N-HSQC spectra for ¹⁵N-labeled Bim_LΔC27 in the presence and absence of Bcl-w (Figure 3c). Notably, although addition of Bcl-w results in the appearance of a number of new peaks, the majority of peaks have the same position in both spectra suggesting that the structure of many residues in Bim_LΔC27 is not altered by the presence of Bcl-w. The new peaks in the ¹⁵N-Bim_LΔC27/Bcl-wΔC10 complex spectra primarily arise from interaction of the BH3 domain with Bcl-w as addition of Bcl-wΔC10 to

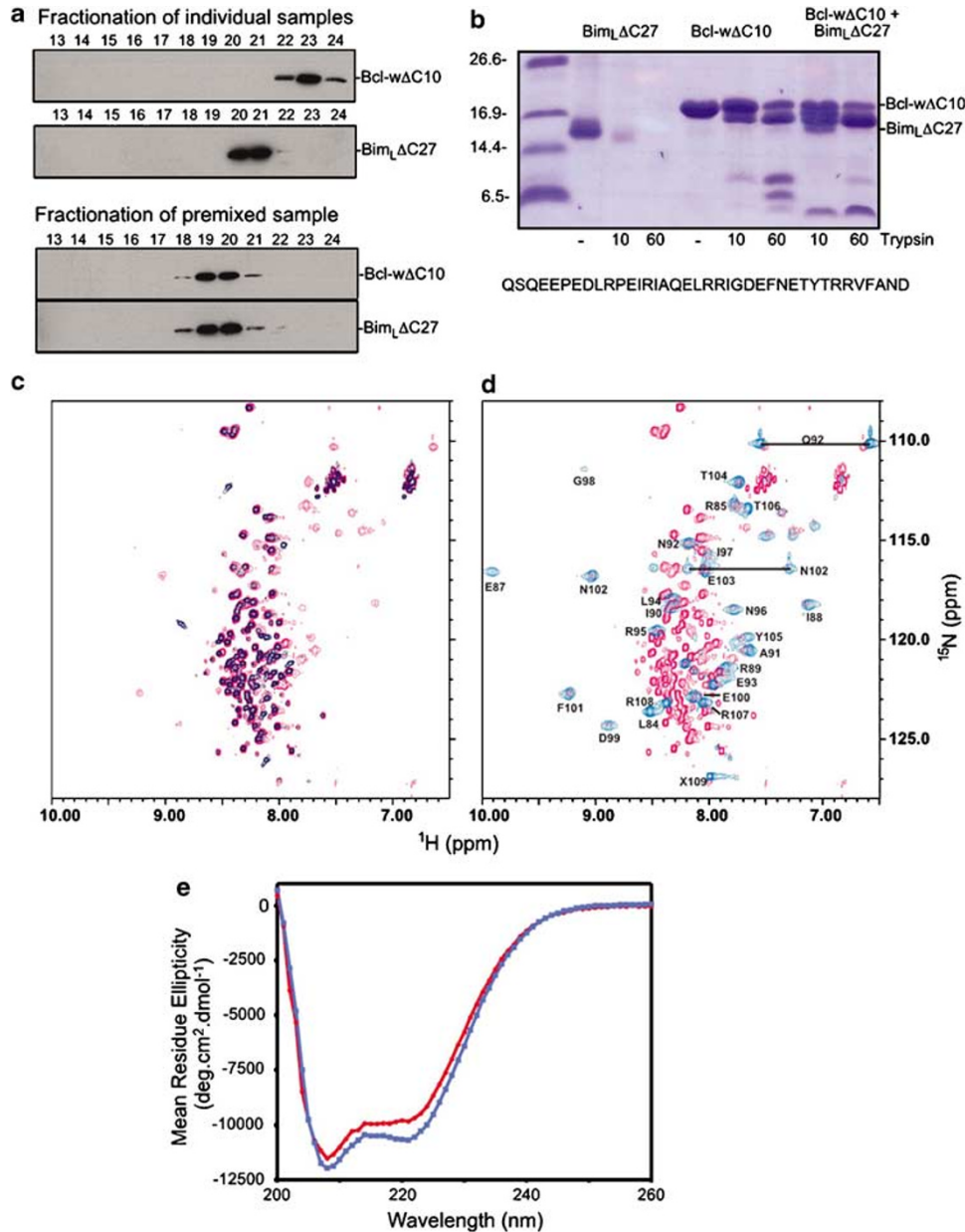


Figure 3 Interaction of Bim_LΔC27 with prosurvival Bcl-2 molecules is associated with a localized conformational change. **(a)** Bim_LΔC27 and Bcl-wΔC10 copurify when analyzed by gel filtration chromatography. Bim_LΔC27 and Bcl-wΔC10 were individually separated on an S200 column, and then an approximately equimolar mixture of the two proteins was separated. The indicated fractions were separated by SDS-PAGE and the proteins detected by Western blotting. **(b)** Purified Bim_LΔC27 and Bcl-wΔC10 were treated with trypsin for the indicated times, either alone (lanes 2, 3, 5 and 6) or following mixing (lanes 7 and 8). The sequence of the Bim peptide identified in lane 8 but not in lane 3 is shown below. **(c)** ¹H-¹⁵N-HSQC spectra of ¹⁵N-labelled Bim_LΔC27 in the absence (blue) and presence (pink) of unlabelled Bcl-wΔC10. **(d)** ¹H-¹⁵N-HSQC spectra of ¹⁵N-labelled BimBH3 (blue) and ¹⁵N-labelled Bim_LΔC27 (pink) both in the presence of excess unlabelled Bcl-wΔC10. The assignments for the BimBH3 peptide are indicated. **(e)** Equimolar amounts of Bim_LΔC27 and Bcl-wΔC27 and Bcl-wΔC37 were analyzed in a split mixing cuvette by CD spectroscopy. The signals before mixing (red) and after mixing (blue) are shown

¹⁵N-labelled BimBH3 (a peptide including residues 83–108 of mouse Bim_L) results in similar shifts (Figure 3d). The coincidence of the shifted peaks in the two complex spectra suggests that the BimBH3 peptide contains the full binding site found in Bim_LΔC27. The few differences seen are likely due to end effects. The comparable binding constants observed for Bcl-w binding to Bim_LΔC27 protein (32 nM) and the BimBH3 peptide (22 nM)²⁹ supports this conclusion.

When the BH3 domain of Bim binds to Bcl-x_L, it adopts a helical conformation with contacts to residues in the hydrophobic groove.⁶ The BH3 domain of Bim_LΔC27 is likely to adopt a similar conformation when it binds to Bcl-w, as a small increase in helicity occurs after mixing equimolar amounts of Bcl-w and Bim_LΔC27 (Figure 3e). Bcl-wΔC37 was used in this experiment as the C-terminal residues of Bcl-w, become unstructured upon complex formation³⁰ and mask the CD

signal owing to helix formation of the ligand. Deletion of the C-terminal residues does not affect binding as they are disordered and do not contact the peptide as evidenced by the absence of NOEs between the Bim BH3 domain and the C-terminal residues of Bcl-w Δ C10 (data not shown). Consistent with these results Denisov *et al.*³¹ recently reported a similar structure for the C-terminal residues of Bcl-w when a Bid BH3 peptide is bound.

Collectively, these data suggest that Bim is a member of the intrinsically unstructured class of proteins that undergo a localized conformational change, involving helix formation of the contact residues in the BH3 domain, upon binding to its partner protein. Residues outside the BH3 domain contribute little to binding and have the same conformation in the presence and absence of prosurvival proteins.

Bad and Bmf are also largely unstructured in solution.

Comparable BH3 domain peptides from all BH3-only molecules have been shown to be unstructured when studied in isolation.^{5,8} In the case of Bid, these same residues are ordered and have a distinct structure within the context of the full-length protein.^{9,10} In contrast, we have shown that Bim does not have a well-defined structure and that the Bim BH3 domain is unstructured. Our data strongly suggest that coupled folding and binding occur when the BH3 domain of Bim interacts with prosurvival proteins. To determine which model best represents other BH3-only proteins, we analyzed the sequence of all mouse BH3-only proteins using several predictors of disorder. Analysis based on mean hydrophobicity and mean net charge predicts that some (e.g. Bad and Noxa) are unstructured like Bim, whereas others are predicted to have a well-defined structure (e.g. Bik), like Bid (Figure 4a). For comparison, the prosurvival proteins, which all have a well-defined structure except for the residues that connect helices 1 and 2 in Bcl-x_L and Bcl-2, were analyzed. CDF analysis was also used to discriminate between BH3-only proteins that are likely to be ordered and disordered in solution (Figure 4b). Using the seven-point boundary for PONDR scores between 0.6 and 0.9 as the discriminator,²¹ all BH3-only proteins, except Bid and Bik, are predicted to be unstructured when free in solution.

To evaluate these predictive methods, we attempted to express and purify all mouse BH3-only proteins. However, many of the proteins proved difficult to express and purify. Only Bmf and Bad, minus their C-terminal 25 (Bmf Δ C25) and 22 (Bad Δ C22) residues respectively, could be purified to homogeneity. As observed for Bim Δ C27, both Bmf and Bad proteins were susceptible to proteolysis during purification and eluted from sizing columns with a larger molecular mass than expected, suggestive of a disordered conformation (data not shown). In agreement with predictive methods, CD analysis of Bad and Bmf revealed a largely random coil conformation, with minima near 200 nm, similar to that observed for Bim Δ C27 (Figure 4c). As predicted, based on sequence analysis and the available NMR structures,^{9,10} the CD spectrum for Bid was that of a primarily α -helical protein (Figure 4c).

The presence of residual secondary structure, based on analysis of the CD spectra of Bim, Bad and Bmf, was

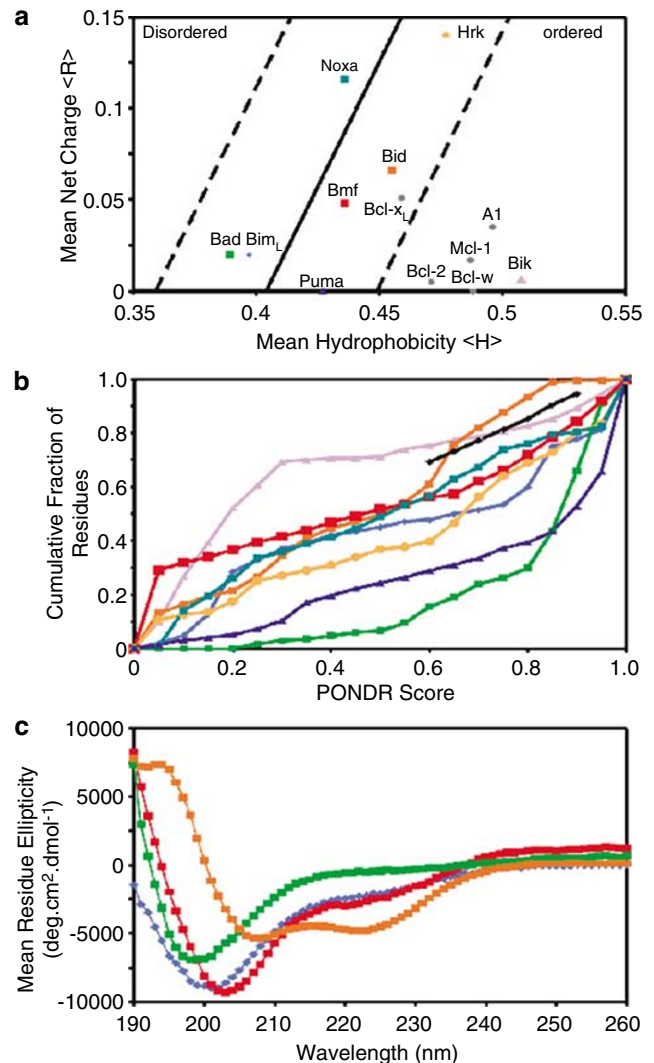


Figure 4 Some other BH3-only proteins are also unstructured. (a) Net charge hydrophobicity plot of all the mouse BH3-only proteins, and for reference the prosurvival Bcl-2 proteins (in gray). In the case of Mcl-1, only the BH domain was analyzed. The loop that connects α 1 and α 2 was included in the analysis and accounts for the relative disorder seen for Bcl-x_L. (b) CDF plots for Bim_L (light blue), Bmf (red), Puma (dark blue), Bid (orange), Bad (green), Bik (purple), Hrk (yellow) and Noxa (teal). The order-disorder boundary (black) is as reported.²¹ (c) Far UV CD spectra of Bid (orange), Bim Δ C27 (light blue), Bmf Δ C25 (red) and Bad Δ C22 (green)

assessed using several algorithms as implemented at the DICHROWEB server.³² All three proteins were calculated to have a primarily random coil conformation with no significant α -helical regions, although some residual β -structure was predicted. However, the fits were poor and sequence analysis does not indicate significant β -structure. Instead, sequence analysis of Bad and Bmf reveals regions with α -helical propensity that are likely to be stabilized upon interaction with partner proteins such as Bcl-w, as seen for Bim.

Secondary structure characterization, based on CD spectra of four BH3-only proteins, therefore supports the CDF-ordered/disordered classification of BH3-only proteins (Figure 4b). Preliminary analysis of Noxa and Puma also

suggests that these proteins are disordered when free in solution as they are both highly susceptible to proteolysis. In contrast, it seems highly likely that Bik, like Bid, will have a well-defined 3D structure in solution.

Discussion

IUPs have become the focus of many studies since it was recognized that a defined 3D structure is not a prerequisite for biological activity, indeed some proteins require the absence of 3D structure to carry out their functions.¹² Such proteins are remarkably prevalent, with up to 28% of proteins encoded for by the mouse genome predicted to be intrinsically unstructured,²¹ and frequently involved in cell regulatory functions.¹² Significantly, a recent study predicts a high level of structural disorder in human cancer-associated proteins.³³ We now report that many proapoptotic BH3-only proteins, which are critical regulators of apoptosis, do not have a well-defined 3D structure under physiological conditions and that they belong to the IUP family.

Protein sequence analysis predicted that Bim contained extensive regions of disorder (Figure 1). The absence of a well-defined structure was confirmed by CD and NMR spectroscopy (Figure 2). Bmf and Bad also behave as unstructured proteins under physiological conditions and have CD spectra typical of IUPs (Figure 4). A more general protein sequence analysis of the eight mammalian BH3-only proteins suggests that, with the exception of Bid and probably Bik, these proteins are largely unstructured (Figure 4). This classification of Bik as a structured protein is in agreement with the phylogenetic analysis of Aouacheria *et al.*,¹¹ where they suggested that Bik is more closely related to Bak and therefore would be expected to have a folded structure similar to that of Bax.³⁰

Frequently, IUPs undergo a structural transition when they bind to their targets.^{12,13} Comparison of the spectra for both Bim₁ΔC27 and the isolated BH3 domain, in complex with Bcl-w, indicates that a localized conformational change accompanies interaction with prosurvival proteins (Figure 3). However, consistent with their continued sensitivity to proteolysis, the majority of residues in Bim remain unstructured in the presence of prosurvival proteins. The term molecular recognition element (MoRE) has been coined for a short disordered element of a protein that becomes ordered upon specific binding to a partner protein.³⁴ Structural studies on peptides spanning the BH3-domain of Bim,⁶ Bad⁵ and Egl-1⁷ show that they all form α -helices when bound to prosurvival proteins, suggesting that the BH3 domain is an alpha helical MoRE (α -MoRE). The increased helicity (Figure 3e) and shifts in the presence of Bcl-w (Figure 3c) indicates that within the context of Bim₁ΔC27, the BH3-domain also has a helical conformation similar to that seen in the Bcl-x₁/BimBH3 peptide complex.⁶ Thus, the BH3 domain of Bim behaves as an α -MoRE. Bim also binds a component of the dynein motor complex, DLC1,¹⁷ but only a short region of nine residues is involved in binding.^{19,35} Like the interaction of Bim with prosurvival Bcl-2 proteins, there is no evidence to suggest conformational changes in Bim outside the region of direct contact with DLC1 (MG Hinds and CL Day, unpublished), rather a short β -strand is formed that extends a β -sheet within the DLC, fitting the

description of a β -MoRE.³⁴ Together, these observations suggest that Bim contains a series of linear sequence motifs, which have independent structure and function, analogous to 'beads on a string'.

It has been estimated that each α -MoRE, such as the induced α -helix of a BH3-only protein, requires fewer than 10, and commonly only five residues to interact with its partner.³⁴ BH3 domains of BH3-only proteins such as Bim, Bad and Bmf, are only weakly conserved motifs that in addition to three hydrophobic residues include a LXXXGDE sequence.¹¹ As predicted for an α -MoRE, when bound to prosurvival proteins, five residues of the BH3 domain, including the four conserved hydrophobic residues found on one face of an amphipathic helix, and the invariant aspartate that forms a conserved salt bridge, are the key binding determinants. Proteins containing α -MoRE motifs are more frequently found localized to the cellular cortex and cytoskeleton³⁴ and again, fitting this prediction, in healthy cells Bim, Bmf and Bad, are located on the microtubule-based dynein motor complex,¹⁷ Actin-based Myosin V motor complex and bound to 14-3-3 proteins, respectively.²

What triggers folding of BH3 domains? It seems likely that small helical segments will make the first contacts with the prosurvival protein and then additional contacts will follow resulting in propagation of structure and stable complex formation as shown by others.¹³ The increased α -helicity of BH3-domain peptides observed on addition of trifluoroethanol, a helix inducer, shows that the BH3 domains behave as nascent helices.⁸ However, the low abundance of hydrophobic residues in Bim, and other BH3-only proteins, means that while helical structure is favored by the BH3 domain, it is not stabilized by additional contacts and does not persist in the absence of a partner protein. Folding is therefore coupled to binding.

The functional advantages of being unstructured include decoupling of specificity from binding strength, increasing the speed of interaction and enabling proteins to interact with multiple partners.³⁴ The eight major BH3-only proteins all bind at least two, and in the case of Bim and Puma interact tightly (nM affinity) with all five, mammalian prosurvival proteins.⁸ The hydrophobic groove to which BH3-only proteins bind is not highly conserved and the plasticity afforded by being unstructured undoubtedly contributes to their tight binding to multiple partners. When bound, approximately 1000 Å² of the BH3 domain is buried.^{6,7} As observed for other unstructured proteins, small proteins/motifs can have a large interaction interface if they are disordered. If a preformed structure mediated binding, a conserved region 2–3 times larger would be expected.³⁶ Such a short unstructured binding motif suggests that BH3-only proteins will be relatively tolerant of mutations, as only mutations that specifically disrupt the binding site are likely to have a deleterious role. Consistent with this prediction, inactivating mutations of Bad have been detected in the BH3 domain.³⁷ In addition, when Egl-1, the *C. elegans* BH3-only protein was cloned, loss-of-function mutations occurred at a much lower frequency than predicted.³⁸ The absence of a tertiary structure may account for the low frequency of deleterious mutations as only mutations that disrupt the BH3 domain or cause a frameshift error would have disrupted Egl-1 function. Typically, many loss-of-

function mutations arise owing to disruption of the tertiary structure, but in IUPs, there is little structure to be disrupted.

BH3-only proteins are tightly controlled and a number of mechanisms keep these proteins in check, and as seen for other proteins a disordered structure offers advantages.¹⁶ For example, unstructured proteins would appear well suited to tolerate extensive splicing as seen in Bim, as fewer structural constraints are placed on the ability to insert or delete exons that modulate biological function. Phosphorylation of Bim, Bad and Bmf plays an important role in their regulation. Kinases typically bind with low affinity but high specificity, and it has been suggested that coupled folding and binding, possible when proteins are disordered, contributes to this.²⁴ Proteosomal degradation, which regulates the levels of several BH3-only proteins, has also been shown to be more efficient for proteins with unstructured initiation sites.³⁹ Combined, these factors point to the importance of the unstructured state in regulating the levels of the key cell killers, Bim, Bad and Bmf.

IUPs play important roles in many signaling pathways.¹² Predictive methods, based on sequence analysis suggest that many of the proapoptotic BH3-only proteins belong to this class of protein and experimental evidence supports this conclusion in the case of Bim, Bad and Bmf. Further studies are required to determine the extent to which other members of this family are disordered, although both promiscuous binding and multifaceted regulation, properties of these proteins, indicate that a disordered state would be advantageous. Elucidating the nature of the structural changes associated with binding will undoubtedly contribute to our understanding of BH3-only protein function, as the apparent structural simplicity of the BH3-only proteins belies their complex behavior. These studies also offer promise for the further development of small molecule inhibitors that target Bcl-2-like prosurvival proteins, as the 'induced-fit' mechanism described here suggests that different modes of binding will be accommodated.

Materials and methods

Sequence analysis. Global analysis of sequences was carried out as described previously.²⁰ Briefly, the mean net charge of a protein was determined as the absolute value of the difference between the numbers of positively and negatively charged residues divided by the total number of residues. The mean hydrophobicity was defined as the sum of the normalized Kyte-Doolittle hydrophobicities, divided by the total number of residues minus four residues (to take account of fringe effects). These values were then plotted together with disorder-order boundary, which was defined as $\langle \text{charge} \rangle = 2.743$ $\langle \text{hydrophobicity} \rangle - 1.109$, and the 0.045 boundaries.^{20,21} Disordered proteins are accurately (95%) predicted if outside the 0.045 boundary whereas 76% of disordered proteins are accurately predicted with no boundary.²¹ Sequences were also analyzed using a series of neural network predictors, which use amino-acid sequence data to predict disorder, as implemented in PONDR VL-XT⁴³ (<http://www.pondr.com>). The optimal boundary was used as the discriminator for ordered and disordered proteins.²¹ The probability of sequences having regions of localized order and disorder was determined using DisEMBL, as implemented via the web interface (<http://dis.embl.de>).²³ PEST sequences were found using PESTfind (<https://emb1.bcc.univie.ac.at/toolbox/pestfind/pestfind-analysis-webtool.htm>).

Overexpression and purification of proteins. Mouse Bmf (accession number: AAK38747), Bad (NP_031548), Bim_L (AAC40030), truncated at the C-terminus by 25, 22 and 27 residues, respectively; full-length mouse Bid (AAC71064) and human Bcl-w (Q92843), either truncated by 10 or 37 residues, were expressed as glutathione-S-transferase (GST) fusion proteins in *E. coli* BL21(DE3) and purified as described previously.^{30,35,40} Following release from GST using PreScission

protease, all purified proteins have five additional N-terminal residues, GPLGS, as a result of cloning. Isotopically, labeled proteins were expressed as described previously.^{30,40} The BH3 domain of Bim (BimBH3) was made using the pET-31b expression vector (Novagen). Briefly, oligonucleotides that encoded the peptide (sequence DLRPEIRIAQELRRIGDEFNETYTRR) were annealed and cloned into *AlwN1* digested and phosphatased vector, a recombinant plasmid that contained one copy of the oligonucleotide sequence was selected and used for expression. The fusion protein was expressed and the peptide purified as described by the suppliers (Novagen) except that following cyanogen bromide digestion and dialysis, the soluble peptide was purified from the histidine tag using reverse phase chromatography on a 3 ml Resource RPC column (acetonitrile gradient from 28 to 37% for peptide elution). Any remaining histidine-tagged material was removed by passage through another Ni²⁺ column. Mass spectrometry was used to confirm peptide homogeneity.

Analytical centrifugation. Sedimentation velocity data were acquired using an Optima XL-A analytical ultracentrifuge (Beckman Coulter) equipped with an An-Ti60 rotor. The sample and reference sectors were filled with 380 μ l Bim_L Δ C27 (0.8 mg/ml in phosphate-buffered saline (PBS) pH 7.4) and 400 μ l PBS pH 7.4, respectively. Centrifugation was carried out at 20°C and 50 000 r.p.m. The absorbance of the sample at 235 nm (relative to the reference) was recorded at 0.001 cm increments across the cell at 5 min intervals for 5.7 h, yielding a data set of 70 radial scans.

The sedimentation of Bim_L Δ C27 was modeled as a single class of non-interacting solute under ideal solution conditions. Data were analyzed by nonlinear regression using the program Sedfit.⁴¹ Scans 15-50 were included in the analysis, as they exhibited unequivocal evidence of a solute boundary and plateau. The buffer density (ρ) and partial specific volume of Bim_L Δ C27 (\bar{V}) at 20°C were calculated as 1.0045 g/ml and 0.7095 ml/g, respectively, using the program Sednterp. During the fit, the loading absorbance (c), relative molecular mass (M_r) and sedimentation coefficient (S) of Bim_L Δ C27 were optimized for best fit to the Lamm equation. In addition, time-independent noise (analogous to the baseline) was iteratively separated from the data.

CD spectroscopy. CD experiments were recorded on an Olis spectropolarimeter equipped with a Peltier type temperature controller. All scans were recorded at 25°C with protein concentrations in the range of 10–50 μ M in 5 mM sodium phosphate pH 6.7 except where noted. Spectra were recorded in either 1 or 2 mm pathlength cuvettes, using a 1 nm step resolution and 3 s integration time. Three scans were averaged for each run and appropriate baselines were subtracted. For analysis of the conformational change associated with Bim/Bcl-w complex formation, a 10 mm path length split cuvette was used. Spectra were acquired, before and after mixing by inversion, using the same conditions as above. All CD signals were converted to mean residue ellipticity and have the units, deg \cdot cm²/dmol.

Size exclusion chromatography and immunoblotting. Purified proteins were separated on a 20 ml Superdex S200 column (GE Healthcare) that had been equilibrated in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4, 0.02% NaN₃). Fractions, 500 μ l in volume, were collected and then analyzed by SDS-PAGE followed by immunoblotting. Proteins were detected using rat monoclonal anti-Bim (3C5) or anti-Bcl-w (13F9) and HRP-conjugated goat anti-rat (SouthernBiotech). Protein sizes were estimated by comparison with a range of standard proteins that had been separated under the same conditions.

NMR spectroscopy. ¹H-¹⁵N-HSQC spectra were recorded at 25°C on a Bruker DRX-600 spectrometer equipped with triple resonance probes and pulsed field gradients. Samples used for NMR contained ~0.5 mM protein in 50 mM sodium phosphate (pH 6.7), 70 mM NaCl, 2 mM TCEP and 0.04% sodium azide in H₂O:²H₂O (95:5). For analysis of the complexes, excess unlabeled Bcl-w Δ C10 (A128E)³⁰ was added to ¹⁵N-labeled Bim_L Δ C27 and BimBH3 samples.

Limited proteolysis and mass spectrometry. Purified Bim_L Δ C27 alone (~26 μ g), Bcl-w Δ C10 alone (40 μ g) and mixtures of the two proteins were subjected to digestion with trypsin (70 ng) in a total volume of 130 μ l. Digests were incubated at room temperature. Samples taken at the indicated time points were treated with formic acid and rapidly heat inactivated. The products were evaluated by gel electrophoresis. Before mass spectrometry, peptides were purified using a Sep-pak reverse phase cartridge.

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