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Reconstitution and Characterization of BCL-2 Family Proteins in Lipid Bilayer Nanodiscs

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Abstract

The BCL-2 family proteins are key regulators of programmed cell death or apoptosis, and represent important targets for the development of anticancer drugs. Because their functions are intimately connected with intracellular membranes, it is important to perform structural and activity studies in precisely characterized samples that include phospholipids and capture the features of the native physiological environment as closely as possible. NMR studies and activity assays based on lipid bilayer nanodiscs are ideally suited for this purpose: they enable the conformations and interactions of these proteins to be probed at atomic resolution in their membrane-associated states. Here we describe detailed protocols for generating the protein components and the reconstituted nanodisc samples suitable for NMR studies and functional assays. The protocols focus on the BCL-2 family protein BCL-XL, a dominant inhibitor of programmed cell death and a major anticancer drug target. The protocols are relatively straightforward. Provided care is taken to ensure protein integrity and sample homogeneity, BCL-XL can be readily reconstituted in nanodiscs, with its hydrophobic C-terminal tail anchored through the nanodisc lipid bilayer, and its folded N-terminal head and ligand binding pocket exposed to the aqueous solution. We anticipate that BCL-2 samples prepared with these protocols will advance structural and mechanistic studies for this important protein family.

Keywords

BCL-2; Apoptosis; Membrane; Nanodisc; Structure; NMR

1 Introduction

The BCL-2 family proteins are major regulators of the mitochondrial pathway to programmed cell death. Their functions are regulated by their interactions with a wide range of proteins, both within and outside the BCL-2 family, as well as with intracellular membranes, most notably, the mitochondrial outer membrane, where cell fate is ultimately decided. The BCL-2 family member, BCL-XL, is a dominant inhibitor of apoptosis [1]. Its gene is highly conserved in vertebrate evolution, and expressed in a wide variety of tissues and cell types, including many tumors. Because BCL-XL promotes tumor cell survival, tumor formation, and tumor resistance to chemotherapy [2], it is an important anticancer drug target and, hence, continues to be the focus of studies aimed at understanding its mechanism of cytoprotection.

Structural studies have helped shape our current understanding of BCL-2 protein function, and have been critical for the development of anticancer therapeutics [1, 3]. The molecular

structure of the water-soluble N-terminal domain [4] of BCL-XL represents the prototypical fold of the BCL-2 family [5]: six α -helices fold around a central α -helical hairpin to form a surface-exposed groove that engages the BH3 motifs of its BCL-2 protein partners. The majority of structural studies have focused on the water-soluble, cytosolic states of the proteins. BCL-XL, however, is found primarily associated with intracellular membranes, and less abundantly in the cytosol [6–9]. Its 233-residue sequence (Fig. 1) includes the four, signature BCL-2 homology (BH) motifs that define the protein family, plus a C-terminal, hydrophobic tail that is required for membrane association and cytoprotection [10]. Understanding how BCL-XL and other BCL-2 family members interact with cellular membrane is critical for gaining complete mechanistic understanding of this major protein family.

Recently, we described the preparation and structural characterization of membrane-associated BCL-XL [11,12]. Recombinant BCL-XL, including its C-terminal tail, can be reconstituted in detergent-free lipid bilayer nanodiscs for NMR structural studies and ligand binding assays. We showed that the C-terminal tail forms a transmembrane helix that anchors the protein to the membrane, while the N-terminal head domain adopts the canonical structure observed for water-soluble, tail-truncated BCL-XL, with the surface BH3-binding groove exposed and available for ligand binding. Notably, the affinity of membrane-associated BCL-XL for its BH3 ligands appears to be enhanced relative to its tail-truncated, soluble form, suggesting that membrane association can provide an additional level of BCL-2 protein regulation. Here we outline the methods for generating nanodisc samples of BCL-XL for structural and functional studies.

2 Materials

Specialized materials and equipment used for the experiments described in this chapter are listed in Table 1. They include: *E. coli* cells for recombinant gene expression; chromatography media for protein purification; detergents, lipids, and reagents for protein-nanodisc reconstitution; and isotopically labeled salts to produce ^{15}N and ^{13}C labeled proteins for NMR studies.

3 Methods

3.1 Preparation of BCL-XL Protein

3.1.1 Cloning and Gene Expression—Four DNA sequences, encoding variants of human BCL-XL (Genbank [NM_001191](#)) fused to the N-terminal His-tag MAHHHHHGSPEF, were cloned into the NcoI and HindIII restriction sites of the pET-28a plasmid. The sequences included (Fig. 1): full-length BCL-XL; BCL-XL- L where residues 45–84 in the flexible loop were deleted; BCL-XL- L C, where residues 45–84 and 213–233 were deleted; and BCL-XL- L(tev) where the Tobacco Etch Virus (TEV) protease recognition sequence (ENLYFQ) was inserted between residues K205 and G206 of BCL-XL, to enable isolation of the C-terminal tail polypeptide, BCL-XL(Ct).

The resulting plasmids were transformed in *E. coli* BL21 cells. Positive clones were grown in 5 mL of LB medium at 37 °C for 8 h, and a 100 μL volume of this culture was used to

inoculate 50 mL of minimal M9 medium for overnight growth at 37 °C. The next morning, the full 50 mL volume of overnight culture was transferred to 1 L of fresh M9 medium, and the cells were grown at 37 °C, to a density of $OD_{600} = 0.6$, before induction with 1 mM IPTG for 3–6 h, after which the cells were harvested by centrifugation ($6000 \times g$, 4 °C, 15 min), frozen, and stored at –80 °C overnight. For NMR studies, the M9 culture medium was prepared with $(15NH_4)_2SO_4$ and/or ^{13}C -glucose as the sole sources of nitrogen and carbon, to obtain proteins uniformly labeled with ^{15}N and ^{13}C .

3.1.2 Protein Purification and Analysis—Our protocol for the preparation of soluble BCL-XL- L C [13] is derived from well-established methods widely described in the literature [4]. Here we focus on describing the production of proteins with the C-terminal tail. For BCL-XL, BCL-XL- L, and BCL-XL- L(tev), the cells harvested from 1 L of culture were suspended in 30 mL of buffer A, supplemented with cOmplete Mini EDTA-free protease inhibitor cocktail, and lysed by two passes through a French press. SDS-polyacrylamide gel electrophoresis (PAGE) shows that the recombinant protein accumulates predominantly (90% of total protein) in the insoluble cell fraction and can be isolated by centrifugation ($48,000 \times g$, 30 min, 4 °C) to relatively high purity (Fig. 2a, b). For further purification, the protein was dissolved in buffer B, and then subjected to preparative size exclusion chromatography (HiPrep 16/60 Sephacryl S-200 column), followed by ion exchange purification (HiPrep 16/10 Q FF column) with a linear gradient of NaCl. Purified protein was precipitated by dialysis against water, lyophilized, and stored at –20 °C.

The soluble fraction of BCL-XL- L can also be readily purified. Note, however, that soluble protein produced in this way undergoes truncation after M218 [12], as evidenced by NMR spectroscopy and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (Fig. 2d). These tools, combined with size exclusion chromatography and SDS-PAGE, are critical for assessing the integrity and oligomeric state of the purified proteins for biophysical and biochemical experiments. In the case of BCL-XL- L, the mass spectra (Fig. 2d) show that the soluble and insoluble fractions of the protein have different masses. The mass of the soluble protein fraction (21,936.99 Da) is smaller than the theoretical mass (23,535.4 Da) expected for ^{15}N BCL-XL- L, and corresponds to the sequence truncated after M218. By contrast, the protein purified from the insoluble cell fraction has exactly the theoretical mass expected for ^{15}N BCL-XL- L, including the intact C-terminus. Retention of the His-tag in both proteins was confirmed by blotting with anti-His antibody. Interestingly, the soluble M218-truncated protein is folded, with its partial C-terminal tail bound into the surface-exposed BH3-binding groove [11].

3.2 Preparation of Nanodisc Membrane Scaffold Protein (MSP)

Lipid bilayer nanodiscs [14–16] are effective samples for solution NMR studies of membrane proteins [17]. Nanodiscs provide a detergent-free environment that retains the essential anisotropic physical and chemical properties of biological membranes. Furthermore, since both lipid bilayer leaflets are exposed to the same aqueous solution, nanodiscs are better suited for functional assays than sealed liposomes.

Nanodiscs are prepared by mixing phospholipids with a helical membrane scaffold protein (MSP), derived from the sequence of the apolipoprotein Apo-A1. Two molecules of MSP wrap around a patch of phospholipid bilayer membrane to form a nanodisc, and the nanodisc diameter can be titrated by varying the length of the MSP sequence. For BCL-XL, we used the short MSP variant, MSP1D1 H5, lacking helix 5 of ApoA1, which was developed [18] specifically to produce small, 8 nm diameter nanodiscs, suitable for solution NMR studies of membrane proteins.

Addgene offers a variety of plasmids for the production of MSP in *E. coli*. To produce recombinant MSP1D1 H5 we followed the established protocol [18]. Briefly, the MSP-encoding nucleotide was cloned into the NcoI and HindIII restriction sites of the pET-28a plasmid to drive biosynthesis of MSP1D1 H5 fused to a C-terminal His tag that is cleavable by TEV protease. After *E. coli* cell culture and lysis, the protein was isolated from the soluble cell fraction, and then purified by a first step of Ni-affinity chromatography, followed by proteolytic cleavage of the His tag and a second Ni-affinity purification to obtain pure, untagged protein.

3.3 Preparation of BCL-XL Nanodiscs

3.3.1 Nanodisc Preparation—BCL-XL, BCL-XL- L, and BCL-XL- L(tev) were incorporated in nanodiscs, prepared with the phospholipids dimyristoyl-phosphatidylcholine (DMPC) and dimyristoyl-phosphatidylglycerol (DMPG), following our published protocol [11]. Three solutions were prepared: Solution 1 contained pure lyophilized BCL-XL protein (2 mg) dissolved in 0.5 mL of nanodisc buffer, supplemented with 100 mM n-decyl-phosphocholine (DePC); Solution 2 contained a 3:1 molar mixture of the lipids DMPC (5.8 mg) and DMPG (2 mg) dissolved in 1 mL of nanodisc buffer, supplemented with Na-cholate to obtain a final 1:2 molar ratio of lipid to cholate; Solution 3 contained MSP1D1 H5 (4.3 mg) dissolved in 250 μ L of nanodisc buffer. The three solutions were combined to obtain a final volume of 1.75 mL. After incubation at room temperature for 1 h, 2 g of Biobeads, prewashed in nanodisc buffer, were added, and the mixture was further incubated at room temperature, with gentle rocking overnight. The Biobeads were removed by centrifugation and the resulting nanodiscs were washed twice with one sample volume of nanodisc buffer. The nanodisc solution was concentrated using a 10 kD cutoff Amicon concentrator and stored at 4 °C. Similar protocols carried out either without BCL-XL or without MSP yield either empty nanodiscs or BCL-XL-reconstituted liposomes.

3.3.2 Sample Analysis—BCL-XL- L nanodiscs prepared according to this protocol have one nominal BCL-XL- L molecule per nanodisc, with a molar ratio of 1:2:100 for BCL-XL- L: MSP: phospholipid. Note that the precise molecular ratio of these components needs to be worked out case by case, depending on their specific chemical structures, to obtain the highest possible homogeneity of nanodisc size and composition. Nanodisc size homogeneity is critical for solution NMR studies and can be readily assessed by size exclusion chromatography, performed directly in NMR buffer. We routinely use Superdex 75 10/300 GL or Superdex 200 10/300 GL columns, coupled with Breeze HPLC or AKTA Pure chromatography systems, for the analysis of BCL-XL nanodiscs. Further analyses of the column fractions by SDS-PAGE (Fig. 2a–c) and immunoblotting (Fig. 3) are essential for

confirming the presence of all nanodisc components in each sample. For example, size exclusion (Fig. 3a) shows that the BCL-XL- L nanodiscs have a narrow elution profile similar to that of empty nanodiscs, consistent with high size homo-geneity. The presence of both BCL-XL- L and MSP components in the major size exclusion fraction is readily confirmed by immunoblotting (Fig. 3b).

3.3.3 BCL-XL (Ct) Nanodiscs—Nanodiscs containing BCL-XL(Ct), spanning tail residues G206-K233, were prepared by first generating BCL-XL- L(tev) nano-discs, as described for BCL-XL and BCL-XL- L, and then incubating them with TEV protease to cleave off the soluble N-terminal head domain [12]. Complete proteolysis can be obtained with an eight-fold molar excess of protease relative to substrate, in TEVp buffer, for 12 h, at room temperature. The cleavage reaction was then transferred to buffer C and mixed with 4 mL of Ni-NTA resin for 2 h, to isolate BCL-XL(Ct) nanodiscs in the unbound flow-through fraction, and remove the His-tagged N-terminus, which binds to the resin. This procedure is illustrated in Fig. 2c. Analysis of the cleavage reaction and the resulting nanodiscs by SDS-PAGE (Fig. 2c), MALDI mass spectrometry (Fig. 2e), size exclusion chromatography (Fig. 3a), and immunoblotting (Fig. 3b), con-firms that the resulting polypeptide has the exact mass expected for the sequence of ¹⁵N-labeld BCL-XL(Ct), elutes associated with nanodiscs on size exclusion, and is free of the His-tagged N-terminal head.

3.4 Solution NMR Studies

3.4.1 NMR Experiments—A wide range of solution NMR experiments [19–24] can be applied to nanodisc samples. Here we describe the analysis of backbone ¹H and ¹⁵N chemical shifts resolved in ¹H/¹⁵N 2D HSQC experiments, as they provide the first view of the conformational state of the protein. These NMR experiments were performed on a Bruker AVANCE 600 MHz spectrometer equipped with a Bruker ¹H/¹⁵N/¹³C triple-resonance cryoprobe. Experiments at higher magnetic fields will only improve the spectral resolution and sensitivity, which are already quite good. The NMR data were processed and analyzed using NMRPipe [25], NMRViewJ [26] and Sparky [27]. Prior to NMR, all samples were transferred to NMR buffer.

3.4.2 NMR of BCL-XL- L Nanodiscs—The most striking result is that the N-terminal head domain of the protein adopts the canonical globular fold of the BCL-2 family and is anchored to the membrane by its C-terminal tail [11]. The NMR spectrum of BCL-XL- L in nanodiscs (Fig. 4a, red) is well resolved, and overlaps significantly with the spectrum of water-soluble BCL-XL- L C (Fig. 4a, black), such that most resonances from the N-terminal head can be assigned by direct comparison. Differences between the two spectra map to helix α8 and its structurally proximal sites (Fig. 4d), consistent with small rearrangements in conformation, and/or intermolecular interactions, associated with insertion of the C-terminal tail in the lipid bilayer membrane. Additional small differences are also observed for I114 and T115 in the loop connecting helices α3 and α4, E7 in α1, and F27 in the long loop connecting α1 and α2. The data indicate the presence of a loose interaction between the globular head and the membrane surface. The BCL-XL- L nanodiscs, however, yield appreciably broader resonance lines, consistent with the reduced rotational tumbling motion expected for a ~130 kDa protein-nanodisc particle. NMR spectra

acquired at 45 °C have narrower lines and display most of peaks from the globular head as well some additional peaks from the tail. Many signals from the membrane-embedded C-terminal tail, however, remain weak and undetected, notwithstanding the use of the smallest available nanodiscs.

3.4.3 NMR of BCL-XL(Ct) Nanodiscs—The preparation of BCL-XL(Ct) nanodiscs facilitates NMR structure characterization of the transmembrane C-terminal tail [12]. This sample yields a ¹H/¹⁵N correlation NMR spectrum (Fig. 4b, blue) with many resolved NH signals for the backbone sites. Parallel solid-state NMR and paramagnetic relaxation enhancement experiments confirmed that BCL-XL(Ct) is deeply embedded in the lipid bilayer [11, 12].

The NMR spectrum of BCL-XL(Ct) nanodiscs has broader lines than expected for a peptide of this size, even when considering the slower tumbling rate of the peptide-nanodisc assembly. More-over, the line widths are heterogeneous: narrower, strong peaks are observed for the C-terminal end of the BCL-XL(Ct) peptide; broader, weak peaks are observed for the N-terminal end; and peak doubling is observed for the W213 indole sidechain (Fig. 4b, inset). These features indicate that the tail experiences varying degrees of dynamic conformational exchange. By contrast, the W213 sidechain appears as a single signal in the spectrum of BCL-XL- L nanodiscs, suggesting that residues upstream of G206 could help stabilize the membrane-associated conformation.

Addition of 170 mM DePC to BCL-XL(Ct) nanodiscs reduces the NMR linewidths (Fig. 4c, yellow), enabling resonance assignments and structure determination [12]. The ¹H and ¹⁵N chemical shifts together with the NOE measurements indicate that the C terminal tail of BCL-XL adopts α-helical structure from W213 to K233 in mixed lipid-detergent. By contrast, signals from G206-N211 could not be detected, probably due to ¹H exchange with solvent. The lipid-DePC mixture is likely to form micelles or small bicelles and addition of DePC induced only minor chemical shift perturbations. The largest perturbations are observed for G227, and its neighboring residues, and for the W213 side chain, which adopts a unique conformation in the presence of DePC, suggesting that intermolecular interactions may play a more important role in the C-terminal end of the transmembrane tail. The data are consistent with the profile of ¹H/¹⁵N peak intensities and ¹H/²H exchange data, showing relatively uniform backbone dynamics for residues M218-S231, higher mobility and/or conformational exchange at the N-terminus, and a highly mobile C-terminus, as well as a relatively loose hydrogen bond network across the entire C-terminal tail.

3.4.4 Ligand Binding Experiments—The interaction of soluble BCL-XL N-terminal domain with the BH3 sequences of anti-apoptotic BCL-2 family members is well established and structurally characterized [4]. Helical BH3 ligands engage the surface groove of BCL-XL inducing a subtle contraction of the globular structure, akin to a hand tightening around a stick. NMR and isothermal calorimetry (ITC) experiments provide excellent molecular and quantitative probes for this binding event.

Addition of a BID_{BH3} peptide (residues 80–99 of human BID, obtained commercially from GenScript) to either soluble BCL-XL- L C, or BCL-XL- L nanodiscs, induces essentially

identical $^1\text{H}/^{15}\text{N}$ chemical shift perturbations in the respective NMR spectra (Fig. 5a). The perturbations localize to the canonical BH3-binding pocket of BCL-XL and demonstrate that membrane-tethered BCL-XL is fully capable of ligand binding. Contrary to the notion that membrane-bound BCL-XL has diminished affinity for its BH3 ligands due to a loosening of the groove structure in the membrane environment [28], the ITC measurements (Fig. 5b) show that the affinity of membrane-integrated BCL-XL- L for the BH3 peptide is 1.6 times greater than that of its isolated water-soluble head, BCL-XL- L C, a result with important implications for the design of targeted BH3 mimetics.

4 Conclusions

The functions of BCL-2 proteins are intimately connected to inter-actions with intracellular membranes. The use of nanodiscs allows us to probe the conformations and functional interactions of these important proteins in their membrane-associated states, at atomic resolution, and free of potentially disruptive additives such as detergents. NMR studies with BCL-XL embedded in nanodiscs, suspended in a detergent-free physiological buffer, show that membrane-integration does not alter the globular structure of the N-terminal domain of BCL-XL and does not abrogate its BH3 ligand binding activity. The C-terminal tail of BCL-XL forms a membrane-embedded α -helix that anchors the protein's globular head to the lipid bilayer membrane, yet retains a significant degree of conformational dynamics. Notably, the BH3 binding affinity of membrane-bound BCL-XL is not only supported, but appears to be enhanced, suggesting that further characterization of the membrane-integrated states of prosurvival BCL-2 proteins could provide useful insights for the design of BH3 mimetics, especially for more refractory BCL-2 targets. The protocols for generating the nanodisc samples are relatively straightforward, provided care is taken to ensure structural integrity and sample homogeneity.

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References

1. Oltsersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA, Bruncko M, Deckwerth TL, Dinges J, Hajduk PJ, Joseph MK, Kitada S, Korsmeyer SJ, Kunzer AR, Letai A, Li C, Mitten MJ, Nettesheim DG, Ng S, Nimmer PM, O'Connor JM, Oleksijew A, Petros AM, Reed JC, Shen W, Tahir SK, Thompson CB, Tomaselli KJ, Wang B, Wendt MD, Zhang H, Fesik SW, Rosenberg SH (2005) An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* 435:677–681 [PubMed: 15902208]
2. Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G, Thompson CB (1993) bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* 74:597–608 [PubMed: 8358789]
3. Lessene G, Czabotar PE, Sleebs BE, Zobel K, Lowes KN, Adams JM, Baell JB, Colman PM, Deshayes K, Fairbrother WJ, Flygare JA, Gibbons P, Kersten WJ, Kulasegaram S, Moss RM, Parisot JP, Smith BJ, Street IP, Yang H, Huang DC, Watson KG (2013) Structure-guided design of a selective BCL-X(L) inhibitor. *Nat Chem Biol* 9:390–397 [PubMed: 23603658]
4. Sattler M, Liang H, Nettesheim D, Meadows RP, Harlan JE, Eberstadt M, Yoon Hs, Shuker SB, Chang BS, Minn AJ, Thompson CB, Fesik SW (1997) Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. *Science* 275:983–986 [PubMed: 9020082]

5. Kvanakul M, Hinds MG (2013) Structural biology of the Bcl-2 family and its mimicry by viral proteins. *Cell Death Dis* 4:e909 [PubMed: 24201808]
6. Gonzalez-Garcia M, Perez-Ballester R, Ding L, Duan L, Boise LH, Thompson CB, Nunez G (1994) bcl-XL is the major bcl-x mRNA form expressed during murine development and its product localizes to mitochondria. *Development* 120:3033–3042 [PubMed: 7607090]
7. Fang W, Rivard JJ, Mueller DL, Behrens TW (1994) Cloning and molecular characterization of mouse bcl-x in B and T lymphocytes. *J Immunol* 153:4388–4398 [PubMed: 7963517]
8. Mizuguchi M, Sohma O, Takashima S, Ikeda K, Yamada M, Shiraiwa N, Ohta S (1996) Immunochemical and immunohisto-chemical localization of Bcl-x protein in the rat central nervous system. *Brain Res* 712:281–286 [PubMed: 8814903]
9. Wolter KG, Hsu YT, Smith CL, Nechushtan A, Xi XG, Youle RJ (1997) Movement of Bax from the cytosol to mitochondria during apoptosis. *J Cell Biol* 139:1281–1292 [PubMed: 9382873]
10. Todt F, Cakir Z, Reichenbach F, Youle RJ, Edlich F (2013) The C-terminal helix of Bcl-x (L) mediates Bax retrotranslocation from the mitochondria. *Cell Death Differ* 20:333–342 [PubMed: 23079612]
11. Yao Y, Fujimoto LM, Hirshman N, Bobkov AA, Antignani A, Youle RJ, Marassi FM (2015) Conformation of BCL-XL upon membrane integration. *J Mol Biol* 427:2262–2270 [PubMed: 25731750]
12. Yao Y, Nisan D, Fujimoto LM, Antignani A, Barnes A, Tjandra N, Youle RJ, Marassi FM (2016) Characterization of the membrane-inserted C-terminus of cytoprotective BCL-XL. *Protein Expr Purif* 122:56–63 [PubMed: 26923059]
13. Franzin CM, Choi J, Zhai D, Reed JC, Marassi FM (2004) Structural studies of apoptosis and ion transport regulatory proteins in membranes. *Magn Reson Chem* 42:172–179 [PubMed: 14745797]
14. Nath A, Atkins WM, Sligar SG (2007) Applications of phospholipid bilayer nanodiscs in the study of membranes and membrane proteins. *Biochemistry* 46:2059–2069 [PubMed: 17263563]
15. Denisov IG, Grinkova YV, Lazarides AA, Sligar SG (2004) Directed self-assembly of monodisperse phospholipid bilayer Nanodiscs with controlled size. *J Am Chem Soc* 126:3477–3487 [PubMed: 15025475]
16. Ritchie TK, Grinkova YV, Bayburt TH, Denisov IG, Zolnerciks JK, Atkins WM, Sligar SG (2009) Chapter 11 - Reconstitution of membrane proteins in phospholipid bilayer nanodiscs. *Methods Enzymol* 464:211–231 [PubMed: 19903557]
17. Viegas A, Viennet T, Etzkorn M (2016) The power, pitfalls and potential of the nanodisc system for NMR-based studies. *Biol Chem* 397:1335–1354 [PubMed: 27451995]
18. Hagn F, Etzkorn M, Raschle T, Wagner G (2013) Optimized phospholipid bilayer nanodiscs facilitate high-resolution structure determination of membrane proteins. *J Am Chem Soc* 135:1919–1925 [PubMed: 23294159]
19. Fesik SW, Zuiderweg ER (1990) Heteronuclear three-dimensional NMR spectroscopy of isotopically labelled biological macromolecules. *Q Rev Biophys* 23:97–131 [PubMed: 2188281]
20. Bax A, Grzesiek S (1993) Methodological advances in protein NMR. *Acc Chem Res* 26:131–138
21. Cavanagh J, Fairbrother WJ, Palmer AG, Skelton NJ (1996) Protein NMR spectroscopy: principles and practice. Academic Press, San Diego
22. Clore GM, Gronenborn AM (1998) NMR structure determination of proteins and protein complexes larger than 20 kDa. *Curr Opin Chem Biol* 2:564–570 [PubMed: 9818180]
23. Ferentz AE, Wagner G (2000) NMR spectroscopy: a multifaceted approach to macromolecular structure. *Q Rev Biophys* 33:29–65 [PubMed: 11075388]
24. Kay LE (2001) Nuclear magnetic resonance methods for high molecular weight proteins: a study involving a complex of maltose binding protein and beta-cyclodextrin. *Methods Enzymol* 339:174–203 [PubMed: 11462811]
25. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR* 6:277–293 [PubMed: 8520220]
26. Johnson BA, Blevins RA (1995) NMR view: a computer program for the visualization and analysis of NMR data. *J Biomol NMR* 5:603–614
27. Goddard TD, Kneller DG (2004) SPARKY 3. University of California, San Francisco

28. Denisov AY, Chen G, Sprules T, Moldoveanu T, Beauparlant P, Gehring K (2006) Structural model of the BCL-w-BID peptide complex and its interactions with phospholipid micelles. *Biochemistry* 45:2250–2256 [PubMed: 16475813]

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Fig. 1.

Sequences of recombinant BCL-XL proteins. **(a)** Full-length human BCL-XL including: additional N-terminal His-tag (green), the flexible loop (red), and the hydrophobic C-terminus (blue). **(b)** Purified recombinant proteins. BCL-XL: full-length sequence. BCL-XL- L: loop-deleted protein missing residues 44–84. BCL-XL- L C: loop-deleted and C-deleted protein missing residues 44–84 and 213–233. BCL-XL- L(tev): loop-deleted protein with the TEV protease recognition sequence (ENLYFQ) inserted before residue G206; TEV protease cleaves between Q and G). BCL-XL(Ct): C-terminal tail obtained from TEV protease cleavage of BCL-XL- L(tev). Adapted from [12]

Fig. 2.

Analysis of recombinant BCL-XL produced in *E. coli* and reconstituted in nanodiscs. (a–c) SDS-PAGE showing isolation of BCL-XL- L and BCL-XL- L(tev), and preparation of BCL-XL(Ct) nanodiscs. The 4–12% Bis-Tris gradient gels were stained with Coomassie brilliant blue. Molecular weight markers are shown in the left lane (kD). (a, b) Proteins accumulate predominantly in the insoluble (i) rather than soluble (s) cell fractions. (c) (Lane 1) Nanodiscs reconstituted with purified BCL-XL- L(tev) contain both BCL-XL- L(tev) and MSP. (Lane 2) Treatment of BCL-XL- L(tev) nanodiscs with TEV protease produces the C-terminal tail peptide, BCL-XL(Ct), and the N-terminal fragment BCL-XL(Nt). (Lane 3) Further incubation with Ni-NTA removes His-tagged BCL-XL(Nt) and yields purified BCL-XL(Ct) nanodiscs in the flow-through unbound fraction. (d, e) MALDI mass spectra. Molecular masses are given in atomic mass units (amu) derived from mass to charge (m/z) ratio. The spectra were obtained, using a Bruker Daltonics Autoflex II time of flight spectrometer. Samples for MALDI were prepared by mixing 2 μ L of protein solution (0.2 mg/mL of lyophilized protein in MALDI buffer) with MALDI matrix solution, loading the mixture onto a MALDI plate and allowing the solvents to evaporate for 15 min at room temperature. (d) ¹⁵N-labeled BCL-XL- L purified from soluble or insoluble cell fractions; asterisks denote protein truncated after M218. (e) BCL-XL(Ct) isolated from TEV protease cleavage of BCL-XL- L(tev) in nanodiscs. Adapted from [12]

Fig. 3.

Size and composition analysis of BCL-XL nanodiscs. **(a)** Size exclusion chromatography. Asterisks mark the major fractions collected for immunoblotting. Soluble BCL-XL- L C elutes with an apparent molecular weight of 21 kDa as expected from its amino acid sequence. By contrast, BCL-XL- L nanodiscs and BCL-XL(Ct) nanodiscs elute with the same apparent molecular weight of empty nanodiscs. **(b)** Immuno dot blots of the major size exclusion fractions (*). Blots were obtained by adsorbing the samples onto nitrocellulose, and probing with mouse anti-His monoclonal antibody (1/5000 dilution) to detect the His-tagged N-terminus of BCL-XL, or goat anti-ApoA1 polyclonal antibody (1/1000 dilution) to detect MSP in nanodiscs, and visualizing with antibody-conjugated alkaline phosphatase, 5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt substrate, and nitro-blue tetrazolium chloride developer

Fig.4.

Effects of membrane integration on the structure of BCL-XL. (a–c) Solution NMR $^1\text{H}/^{15}\text{N}$ HSQC spectra recorded at 45°C , for water-soluble BCL-XL- L C (black), nanodisc-integrated BCL-XL- L (red), nanodisc-integrated BCL-XL(Ct) (blue), and BCL-XL(Ct) in mixed lipid-DePC micelles (yellow). (d) Conformational model of membrane-bound BCL-XL derived from the solution and solid-state NMR data. The globular head (gray) shows residues affected by nanodisc integration (red) detected by chemical shift perturbation analysis. The dashed yellow line delineates the water-exposed BH3-binding groove. The C-terminal transmembrane helix (blue) adopts a 25° tilt relative to the membrane normal (n). The lipid bilayer is depicted in gray bounded by the membrane-water interfaces (dashed lines). Adapted from [11,12]

Fig. 5.

Membrane-tethered BCL-XL binds its canonical BID_{BH3} ligand. **(a, b)** Solution NMR ¹H/¹⁵N HSQC spectra recorded before and after addition of BID_{BH3}, for water-soluble BCL-XL- L C **(a; black, pink)** and nanodisc-integrated BCL-XL- L **(b; red, green)**. Pink circles represent peak positions for BID_{BH3}-bound BCL-XL- L C. **(c, d)** ITC titrations of BID_{BH3} into BCL-XL- L C **(black)** and BCL-XL- L nanodiscs **(red)**. Calorimetric data (top) and integrated heat (bottom) are shown as functions of BID_{BH3}/BCL-XL molar ratio. The data were corrected for nonspecific binding by subtracting control ITC titrations performed with peptide and buffer alone, or empty nanodiscs in buffer alone. Solid lines are the best fits of the binding isotherms to a single-site binding model, used to extract the values of the dissociation constant (K_d). Adapted from [11]

Table 1

Specialized materials and equipment

Materials	Source
Cell culture, isotope labeling, and protein isolation	
<i>E. coli</i> BL21(DE3) cells and pET-28a plasmid	Novagen (www.emdmillipore.com)
IPTG (isopropyl 1-thio- β -D-galactopyranoside)	Sigma (www.sigmaaldrich.com)
M9 media (prepare without (NH ₄) ₂ SO ₄ or glucose if isotope labeling is required.	Prepared in-house or available from Gibco and other vendors.
(¹⁵ NH ₄) ₂ SO ₄ and ¹³ C-glucose	Cambridge Isotope Laboratories (www.isotope.com)
Protease inhibitors (cOmplete Mini EDTA-free cocktail)	Roche (cat # 11836170001)
10 kD cutoff Amicon concentrator	Merck Millipore (cat #UFC901024)
Buffers and Solutions. Prepared with ultrapure deionized water and analytical grade reagents. Stored at room temperature, unless indicated otherwise.	
Buffer A	25 mM Tris-HCl, pH 8, 100 mM NaCl
Buffer B	20 mM Tris-HCl, pH 8, 2 mM DTT, 1 mM EDTA, 6 M urea
Buffer C	25 mM Tris-HCl pH 8, 150 mM NaCl
Nanodisc buffer	20 mM Tris-HCl, pH 7.5, 2 mM DTT, 1 mM EDTA
TEVp buffer	20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM DTT, 0.5 mM EDTA
SDS-PAGE and immunoblotting	
SDS (sodium-dodecyl-sulfate)	Sigma (www.sigmaaldrich.com)
NUPAGE 4–12% Bis-Tris	Life Technologies (cat # NP0322BOX)
Coomassie brilliant blue	Sigma (cat # 27816–25g)
Nitrocellulose	Life Technologies (cat # LG2001)
Antibody-conjugated alkaline phosphatase	Pierce (cat # AP 31337)
AP Conjugate Substrate Kit	Bio-Rad (cat # 1706432)
Anti-His monoclonal antibody (1/5000 dilution)	Qiagen Penta His (cat # 34660)
Anti-ApoA1 polyclonal antibody (1/1000 dilution)	Millipore Calbiochem (cat # 178463)
Enzymes and protein reagents	
Tobacco Etch Virus protease (TEVp)	Prepared in-house.
MSP1D1	Prepared in-house.
MSP1D1 h5	Prepared in-house.
NMR buffer	20 mM Na-phosphate pH 6.5, 2 mM DTT, 1 mM EDTA
Detergents, lipids, and reconstitution	
Na-cholate	Pierce (PI89907)
DePC (n-decyl-phosphocholine)	Anatrace (F3045 g)
DMPC (di-myristoyl-phosphocholine)	Avanti Polar Lipids (www.avantilipids.com)
DMPG (di-myristoyl-phosphoglycerol)	Avanti Polar Lipids (www.avantilipids.com)
Biobeads SM-2	Bio-Rad (cat # 1523920)
Chromatography	
Ni affinity (HisTrap FF 5 mL column)	GE Healthcare Life Sciences (www.gelifesciences.com)
Ion exchange (HiPrep 16/10 Q FF column)	GE Healthcare Life Sciences (www.gelifesciences.com)
Preparative size exclusion (HiPrep 16/60 Sephacryl S-200 column)	GE Healthcare Life Sciences (www.gelifesciences.com)

Materials	Source
Analytical size exclusion (Superdex 75 10/300 GL column)	GE Healthcare Life Sciences (www.gelifesciences.com)
AKTA Chromatography system (AKTA Pure)	GE Healthcare Life Sciences (www.gelifesciences.com)
High performance liquid chromatography sysrtem (Breeze)	Waters (www.waters.com)