NMR approach to characterize Bcl-xl structure and interaction mechanism in membrane state

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**NMR APPROACH TO CHARACTERIZE BCL-X_L
STRUCTURE AND INTERACTION MECHANISM IN
MEMBRANE STATE**

LIU WEI

SCHOOL OF BIOLOGICAL SCIENCE

2013

NMR to Study Bcl-X_L Structure and Interaction in Membrane

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NMR Approach to Characterize Bcl-x_l Structure and Interaction Mechanism in Membrane State

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Abbreviations

aa	amino acid
Bcl-2	B cell lymphoma 2
BH	Bcl-2 homolog
DISC	death-inducing signaling complex
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	ethylene glycol-bis (β -aminoethylether)-N, N' tetraacetic
FADD	Fas-associated protein with death domain
FPLC	fast protein liquid chromatography
HSQC	heteronuclear single quantum coherence
ICAD	Inhibitor of Caspase Activated DNase
IPSL	(Iodoacetamido)proxyl spin label
IPTG	Isopropyl β -D-1-Thiogalactopyranoside
kDa	kilo Daltons
LB	Luria-Bertani
MOMP	mitochondrial outer membrane permeabilization

Abbreviations

MPT	mitochondrial permeability transition
MS	mass spectrometry
Ni-NTA	Nickel-nitrilotriacetic acid resin
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
OD	optical density
OMM	outer mitochondrial membrane
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
ppm	parts per million
rpm	revolutions per minute
RMSD	root mean square deviation
SDS	sodium dodecyl sulfate
TCEP	tris(2-carboxyethyl)phosphine
TNF	tumor necrosis factor
TROSY	transverse relaxation optimized spectroscopy

Abstract

Bcl-2 family proteins play central roles in the apoptotic regulation at the mitochondrial membrane. Bcl-X_L belongs to the anti-apoptotic subfamily of the Bcl-2 family. It interacts with pro-apoptotic subfamilies to inhibit them from forming pores on the mitochondrial membrane. Our current understanding on the function of Bcl-X_L is primarily based upon its structural features and molecular characteristics in solution. Currently the membrane structure of Bcl-X_L and its molecular mechanism in membrane environment remain poorly understood. Understanding the mode of binding is essential to unravel the biological function of proteins. In this thesis research we have characterized Bcl-X_L in a detergent micelle which mimics membrane environment by employing biophysical methods including NMR spectroscopy. We first assigned NMR resonances of Bcl-X_L and determined structure of Bcl-X_L in DPC micelle. Furthermore, the binding mechanism with other Bcl-2 family members was also examined in detergent micelle by employing NMR spectroscopy. The molecular binding analysis demonstrated that the BH3 peptides fail to bind with Bcl-X_L in micelle, indicating that Bcl-X_L undergoes structural transition when Bcl-X_L anchors to the micelle. Even though truncated Bid maintains the binding ability to Bcl-X_L in micelle, NMR cross-saturation data indicates that the binding interface is significantly different from that in solution phase. Based on the results, we proposed an interaction model of Bcl-X_L in apoptosis regulation.

Chapter 1 Background

1.1. Apoptosis

Apoptosis, together with necrosis/necroptosis and autophagy, are the three major ways of cell death. When apoptosis is committed, the chromatin in the nucleus degrades, cell shrinks and then breaks into small membrane-wrapped fragments. These fragments, also called apoptotic bodies, will subsequently be engulfed and digested by macrophages. In general, apoptosis is considered to be a controlled and programmed process where cells commit suicide in an energy-dependent mode.

In healthy condition, apoptosis occurs during development, aging and also to maintain cell populations in a homeostatic mechanism (Jacobson et al., 1997). Also it is induced as a cellular defense mechanism to immunological reactions or when cells are impaired upon virus infection or toxic agents (Norbury and Hickson, 2001). Disruption of apoptosis is associated with various human diseases, such as cancer, autoimmunity and neurodegenerative disorders (Reed, 2000; Thompson, 1995).

1.2. Apoptotic Pathway

The apoptotic mechanisms are highly intricate, consisting of a series of energy-dependent molecular events (Figure 1.1). Apoptosis is classified into two main pathways: the death receptor-mediated extrinsic pathway and the mitochondrial-mediated intrinsic pathway. Furthermore, there is an additional perforin-granzyme-

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dependent pathway in which cells are killed by cytotoxic T-cells. Although there are notable differences between these three pathways, they are not mutually exclusive. Instead, it is reported that they are linked and one pathway can be regulated by molecules from the other (Igney and Krammer, 2002). All the pathways merge on the same terminal, the execution pathway, which is initiated by the activation of caspase-3 and subsequently results in degradation of nuclear proteins, cytoskeletal reorganization, DNA fragmentation, formation of apoptotic bodies and finally engulfment by phagocytic cells.

Figure 1.1 Schematic overview of all the apoptotic pathways. (Figure is adapted from Elmore, 2007)

1.2.1. Extrinsic Pathway

The extrinsic signaling pathways recognize death activators outside the cell and initiate apoptosis through the ligand-death receptor interactions. These death receptors

involved in the molecular recognition are members of the tumor necrosis factor (TNF) receptor superfamily, which contain extracellular domains with cysteine-rich sequence and also have a cytoplasmic domain called the “death domain” (Ashkenazi and Dixit, 1998; Locksley et al., 2001). This death domain plays a critical role in apoptotic signal transduction from the cell surface to the intracellular signaling pathways. Fas-associated protein with death domain (FADD) is recruited by this signal, and makes the N-terminal effector domain available to dimerize with procaspase-8 (Hsu et al., 1995; Wajant, 2002), forming a death-inducing signaling complex (DISC), which results in the auto-activation of procaspase-8 and consequently releases caspase-8 (Kischkel et al., 1995). Once activated, caspase-8 cleaves and activates caspase-3 to trigger the execution phase of apoptosis.

1.2.2. Intrinsic pathway

The intrinsic signaling pathways start with intracellular signals created by a diverse collection of non-receptor-mediated stimuli that act directly on targets in the cell. The stimuli that initiate the intrinsic pathway may proceed in either a positive way like damaging DNA or in a negative way like the lack of definite growth factors. All of these stimuli lead to changes in the outer mitochondrial membrane (OMM) that marks in an opening of the mitochondrial permeability transition (MPT) hole, loss of the mitochondrial transmembrane potential and release normally sequestered pro-apoptotic proteins such as cytochrome c into the cytosol (Saelens et al., 2004). An apoptosome is formed when released cytochrome c binds with APAF1, and once formed it can recruit and activate the inactive procaspase-9 (Chinnaiyan, 1999; Hill et

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al., 2004). Once activated, caspase-9 cleaves and activates caspase-3 to triggers the execution phase.

1.2.3. Perforin/granzyme Pathway

In this pathway, T-cells exert their cytotoxic effects on cancer or virus-infected cells through a novel pathway which involves secretion of perforin to form transmembrane pore that allows injection of cytoplasmic granules through the pore into the target cell (Trapani and Smyth, 2002). Granzyme A and granzyme B are the most important component within the granules.

Once in target cell, Granzyme B cleaves proteins at aspartate residues and consequently activates procaspase-10 and digests cellular enzymes, such as Inhibitor of Caspase Activated DNase (ICAD) (Sakahira et al., 1998). Caspase-3 can be activated by caspase-10 or directly by granzyme B to induce of the execution phase of apoptosis. Granzyme A also plays an important role in cytotoxic T cell induced apoptosis which cleaves the SET complex to NM23-H1, leading to apoptotic DNA degradation (Fan et al., 2003; Lieberman and Fan, 2003).

1.2.4. Execution pathway

The extrinsic, intrinsic and perforin/granzyme pathways all lead to the point of the execution phase, which is the final step of apoptosis. Caspase-3 is considered to be the key executioner caspase which can be activated by anyone of the initiator

caspases (caspase-8, caspase-9, or caspase-10). Caspase-3 activates the endonuclease CAD to degrade DNA within the nuclei and consequently causes chromatin condensation; also it activates caspase-6 and caspase-7. Caspase-3, caspase-6, and caspase-7 function as effector or “executioner” caspases, cleaving various substrates and eventually cause cell death.

1.3. Bcl-2 family

B cell lymphoma 2 (Bcl-2) family is a family of proteins which share at least one of the four sequence homology regions with Bcl-2 (BH1–4) (Huang et al., 1998; Yin et al., 1994; Zha et al., 1996) (Figure 1.2). The major function of the Bcl-2 family is to provide a regulatory role on the mitochondrial homeostasis. However, it is reported that one of its member (Bid) can be activated by caspase-8 or granzyme (Barry and Bleackley, 2002; Li et al., 1998; Russell and Ley, 2002). Hence, this family also serves as a liaison between intrinsic with extrinsic or perforin/granzyme pathway.

Based on the number of homologous regions and also their functions, Bcl-2 family members can be further divided into three groups. Anti-apoptotic proteins possess all four BH motifs, like Bcl-2 and Bcl-X_L (Cheng et al., 2001). Most Pro-apoptotic proteins have BH1, BH2 and BH3 motif, like Bax and Bak (Lindsten et al., 2000). The rest called BH3 only proteins, such as Bad, Bid and Bim, will function as activators or sensitizers (Letai et al., 2002). BH3 domain is the only conserved region they share with other family members.

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Bcl-2 members localize to different sub-cellular compartments in healthy cells. Anti-apoptotic members are membrane-associated proteins with a C-terminal transmembrane domain, anchored in the mitochondria, endoplasmic reticulum (ER), or nuclear membrane (Lithgow et al., 1994). In contrast, a considerable portion of pro-apoptotic members localize to cytosol or cytoskeleton before induced by apoptotic signal. For example, Bax mostly are founded in cytoplasm with its hydrophobic C-terminal membrane anchor sequestered in its BH3-binding pocket (Hsu et al., 1997). Another pro-apoptotic protein, Bak, already resides on the OMM prior to death signals, where it has been reported to be bound to Mcl-1 (Cuconati et al., 2003) and to Bcl-X_L (Willis et al., 2005).

Figure 1.2 Sequence alignment of Bcl-2 family proteins. The family is defined by the possession of up to four conserved BH motifs, all of which include α -helical segments.

1.4. Mitochondrion

Mitochondrion is the major place where the regulation of the Bcl-2 family to apoptosis takes place. This organelle contains an outer and an inner membrane which

consist of different phospholipids and proteins. The protein-to-phospholipid weight ratio of the outer membrane is about 1:1, which is similar to that of the eukaryotic plasma membrane. A large number of the integral proteins on the outer membrane are called porins. These porins can form membrane channels, so that small molecules can diffuse freely.

The protein-to-phospholipid ratio of inner membrane is about three times that of outer membrane. A unique phospholipid, cardiolipin, exclusively exists in the inner membrane in eukaryotic cells. Unlike the outer membrane, the inner membrane doesn't contain any porins, and is highly impermeable to all molecules.

1.5. Regulation Mechanism of Bcl-2 family

Because of noticeable structure similarity to the pore-forming domains of several bacterial toxins, early research was focused on the characteristic differences of the ion channel formed by pro-apoptotic or anti-apoptotic proteins (Minn et al., 1997; Schlesinger et al., 1997). Now it is commonly believed that Bcl-2 family proteins primarily function on the regulation of mitochondrial outer membrane permeabilization (MOMP) to release cytochrome c. It has been a paradox that pro-apoptotic and anti-apoptotic proteins have very similar structures, yet proposed models for their function suggest that they behave contradictorily. The most acceptable explanation is that they function in a homologous manner and compete with each other at most steps of the process but with the major difference being that anti-apoptotic proteins are defective for MOMP. Thus, for most functions anti-

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apoptotic proteins show a behavior similar to a dominant-negative pro-apoptotic protein.

Several models were proposed to explain the underlying molecular mechanism of apoptotic regulation (Figure 1.3). The most popular one is the direct activation model: activation of pro-apoptotic proteins requires direct interaction with at least one of the BH3-only activators which is constitutively sequestered by anti-apoptotic proteins. In contrast, activated sensitizer BH3-only proteins bind anti-apoptotic proteins to release the BH3-only activator proteins from anti-apoptotic proteins. Another possible model to explain the regulation of apoptosis by Bcl-2 family is derepression (or indirect activation): the main feature is that the pro-apoptotic proteins remain in active and must be continuously bound and inhibited by anti-apoptotic proteins for cells to survive. When induced by death signal, BH3-only proteins bind to anti-apoptotic proteins to replace and release pro-apoptotic proteins.

However, MOMP does not occur until multi-BH motifs containing pro-apoptotic proteins such as Bax and Bak insert into the OMM lipid bilayer. Therefore, the final and essential stage for the MOMP should be regulated in the bilayer. Furthermore, large conformational change is inevitable during insertion into membranes. Hence, it is claimed that characterization of interactions of Bcl-2 proteins must base on the membrane environment which is relevant to the final regulated stage of MOMP (Leber et al., 2007). Unfortunately, the structural basis for the pores formed by Bcl-2 family proteins remains unknown, since the structures of this family determined so far are all solution structures.

Figure 1.3 Regulation models of apoptosis by Bcl-2 family proteins (Figure is adapted from Leber et al., 2010). This diagram neglects the role of the membrane to facilitate the comparison of the embedded together model with other two models. There is no role for membrane in direct activation and derepression models, while in embedded together model the regulations take place in membrane environment.

1.6. Objectives of the research project

Although the Bcl-2 family proteins have been intensively studied for more than ten years, major questions about their structure, interaction and function still remain unclear. Especially, the structures of the active membrane-associated or -inserted conformations remain poorly characterized. Consequently, the molecular basis of the

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regulatory mechanism of the Bcl-2 family proteins in membrane stage is not clearly elucidated.

To answer these questions, we focused on a key anti-apoptotic protein Bcl-X_L to (i) determine its structure in membrane environment; (ii) characterization of the interaction of Bcl-X_L with other Bcl-2 family members in membrane.

1.7. Scope of the research project

The main scope of the research project is outlined below:

- a. Structure studies of Bcl-X_L in membrane, in particular
 - Screening of detergents
 - Backbone NMR resonance assignments
 - Secondary structure prediction
 - Backbone relaxation studies
 - DSA titration
 - Paramagnetic spin labeling studies
 - Structure calculation
- b. Binding test of BH3 peptides with Bcl-X_L in DPC environment, in particular
 - BH3 peptide titrations into Bcl-X_L in DPC
 - DPC titrations into Bcl-X_L/BH3 complex
- c. tBid binding test with Bcl-X_L in DPC micelle, in particular
 - tBid titration with Bcl-X_L in DPC

- Identification of binding site in Bcl-X_L using NMR cross-saturation experiment

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Chapter 2 Structure determination of Bcl-X_L in membrane

2.1. Introduction

2.1.1. Solution structure of Bcl-X_L

The solution structure of Bcl-X_L was determined in 1996 (Muchmore et al., 1996). Generally the protein consists of eight α -helices with various lengths and linked only by loops (Figure 2.1). Two central helices ($\alpha 5$ and $\alpha 6$) form the hydrophobic core and are flanked by $\alpha 1$ and $\alpha 2$ on one side, and $\alpha 3$ and $\alpha 4$ on the other.

Between $\alpha 1$ and $\alpha 2$, there is a long unstructured loop indicated by lacking electron density or medium- and long-range NOEs. Previous data showed that loop deletion mutant maintains the ability to inhibit programmed cell death, suggesting that the loop may not directly contribute to the anti-apoptotic function of Bcl-X_L (Muchmore et al., 1996). Hence, the loop deletion mutant was commonly used to replace wild type.

However, it has been shown that Bcl-2, which is close homologue of Bcl-X_L, possesses a similar long loop, abolishes its anti-apoptotic activity when Ser 70 in the loop was phosphorylated (Srivastava et al., 1999). Furthermore, Bcl-2 can even be converted to a pro-apoptotic protein when the loop was cleaved by caspase-3 at Asp34 (Cheng et al., 1997). Hence, the role of the long loop of Bcl-X_L in anti-apoptotic activity might be underestimated.

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Figure 2.1 Protein sequence and solution structure of Bcl-XL.(A) Protein sequence of Bcl-XL and its secondary structure in solution. Residues with red line are the deleted residues in the loop truncated version. (B) Structure overlay of two constructs of Bcl-XL, greencyan solid line indicates the long unstructured loop between $\alpha 1$ and $\alpha 2$ (pdb code 1LXL); red dashed line indicates the residual after the loop was truncated (pdb code 1G5J).

2.1.2. Membrane insertion

The essential role of the two central helices, $\alpha 5$ - and $\alpha 6$ -helices, in the mitochondria pore formation by Bcl-2 family proteins has been supported by deletion, site-directed or exchange mutagenesis experiments (Heimlich et al., 2004; Matsuyama et al., 1998). Membrane insertion of $\alpha 5$, $\alpha 6$ -helices of Bcl-2 was also confirmed at cellular level (Dlugosz et al., 2006; Kim et al., 2004). By introducing two cysteine residues into Bcl-2, which can form cross-linking to prevent the insertion of $\alpha 5$ and $\alpha 6$, the anti-apoptotic activity of Bcl-2 was abrogated. Furthermore, the activity can be restored when the dicysteine bond was broken by reduction. Similarly, solid-state NMR experiment suggested that Bcl-X_L with C-terminal tail deletion can insert $\alpha 5$ - and $\alpha 6$ -helices in the membrane with other helices resting on the membrane surface (Santagostini et al., 2004).

Pro-apoptotic members of the Bcl-2 family such as Bax also go through conformational changes, in a manner similar to those of anti-apoptotic proteins (Youle and Strasser, 2008).

2.1.3. Membrane structure of Bcl-X_L

While solution structures of Bcl-X_L have been extensively characterized (Aritomi et al., 1997; Muchmore et al., 1996; Petros et al., 2000a; Sattler et al., 1997), currently structural information of the Bcl-2 family members in membrane environment remains poorly characterized. Thus, full understanding of the biological function of Bcl-2 family proteins and their regulatory mechanism at the mitochondria requires the

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structural knowledge of the Bcl-2 proteins in the membrane. . As structural studies of Bcl-X_L in detergent micelle provided limited information of Bcl-X_L in membrane environment (Losonczi et al., 2000; Malia and Wagner, 2007), in this study, attempts were made to characterize Bcl-X_L structure in membrane-mimetic detergent micelle.

2.2. Experiments

2.2.1. Expression and purification of Bcl-X_L Δ L Δ TM

Plasmid coding for Bcl-X_L protein was transformed into *E. coli* BL21 (DE3) cells, and the transformed cells were grown in LB medium containing 30 μ g/ml of Kanamycin at 37 °C. 1mM IPTG was added to induce protein expression when the cell culture reached Abs₆₀₀=0.6-1.0, and the culture was further incubated for additional 3 h at 25 °C or overnight at 16 °C. The cells were harvested by centrifugation at 8000 rpm for 10 min, re-suspended in re-suspension buffer (20 mM NaPO₄, 500 mM NaCl), and broken by sonication for 30 min on ice. The cell lysate was cleared by centrifugation at 20,000 rpm for 30 min and purified by affinity chromatography on Ni²⁺-NTA resin. The elution fractions from Ni²⁺-NTA were loaded on to Sephacryl S-75/200 gel filtration column equilibrated with 20 mM NaPO₄, 50 mM NaCl, 1 mM DTT, 0.01% NaN₃. The purified proteins were analyzed on 12% SDS-PAGE, and protein concentration was determined by Bradford dye assay kit (Bio-Rad Laboratories).

2.2.2. Preparation of double/triple labeled Bcl-X_L ΔLΔTM

Transformed cells were grown in LB medium for pre-culture, and then transferred into final M9 medium through multiple times 1:100 ratio inoculation. Protein expression and purification procedure is the same as described in 2.2.1.

2.2.3. Site-directed mutagenesis

Bcl-X_L C155S mutant was prepared by PCR (18 cycles – 95 °C 50 s, 60 °C 60 s, 68 °C 7 m) using Pfu DNA Polymerase. Primers are 5'-GGGGCACTG TCGGTGGAAAGCGTA (sense primer) and 5'-TACGCTTTCCACCGACAG TGCCCC (antisense primer).

Based on Bcl-X_L C155S mutant, fifteen double mutants were prepared by PCR (18 cycles – 95 °C 50 s, 55-58 °C 50 s, 72-70 °C 7 m) with the primers listed in Table 1.

Table 1 PCR Primers used in site-directed mutagenesis of Bcl-X_L.

Name	Sequence
S8C forward	GCA ATG TCT CAG TGC AAC CGG GAG CTG
S8C reverse	CAG CTC CCG GTT GCA CTG AGA CAT TGC
V14C forward	GC AAC CGG GAG CTG TGC GTT GAC TTT CTC
V14C reverse	GAG AAA GTC AAC GCA CAG CTC CCG GTT GC

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S22C forward	CC TAC AAG CTT TGC CAG AAA GGA TAC
S22C reverse	GTA TCC TTT CTG GCA AAG CTT GTA GG
S47C forward	GGG ACT GAA TGC GAG GCA GTA AAG
S47C reverse	CTT TAC TGC CTC GCA TTC AGT CCC
G98C forward	CTG AGG GAG GCA TGC GAC GAG TTT GAA
G98C reverse	TTC AAA CTC GTC GCA TGC CTC CCT CAG
A108C forward	CGG TAC CGG CGG TGC TTC AGT GAC CTG
A108C reverse	CAG GTC ACT GAA GCA CCG CCG GTA CCG
S110C forward	CGG CGG GCA TTC TGC GAC CTG ACA TCC
S110C reverse	GGA TGT CAG GTC GCA GAA TGC CCG CCG
S114C forward	GAC CTG ACA TGC CAG CTC CAC ATC
S114C reverse	GAT GTG GAG CTG GCA TGT CAG GTC
S126C forward	G ACA GCA TAT CAG TGC TTT GAA CAG GTA G
S126 reverse	C TAC CTG TTC AAA GCA CTG ATA TGC TGT C
G138C forward	TTC CGG GAT TGC GTA AAC TGG GGT
G138C reverse	ACC CCA GTT TAC GCA ATC CCG GAA

G142C forward	GGG GTA AAC TGG TGC CGC ATT GTG GCC
G142C reverse	GGC CAC AAT GCG GCA CCA GTT TAC CCC
S168C forward	CAG GTA TTG GTG TGC CGG ATC GCA GCT
S168C reverse	AGC TGC GAT CCG GCA CAC CAA TAC CTG
A175C forward	GCA GCT TGG ATG TGC ACT TAC CTG AAT G
A175C reverse	C ATT CAG GTA AGT GCA CAT CCA AGC TGC
G191C forward	CAG GAG AAC GGC TGC TGG GAT ACT TTT GTG
G191C reverse	CAC AAA AGT ATC CCA GCA GCC GTT CTC CTG
G200C forward	GTG GAA CTC TAT TGC AAC AAT GCA GC
G200C reverse	GC TGC ATT GTT GCA ATA GAG TTC CAC

Successful reaction mixtures consisted of 8.75 μ l H₂O, 100 ng of template DNA, 10 μ l each primer (10 μ M), 0.25 μ l dNTP mix (10 mM each), 1.25 μ l 10X Pfu buffer with MgSO₄, and 0.625 U of Pfu DNA polymerase in a final volume of 12.5 μ l. After PCR program, DpnI endonuclease (5 units) was added into PCR product to digest methylated template. After 1 hour incubation at 37 °C, the mixture was transformed into DH5 α competent cell.

After confirmed by sequencing, Bcl-xl mutants were expressed and purified in a procedure the same as wild type Bcl-X_L which described in 2.2.1.

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2.2.4. NMR spectroscopy

Except those specially mentioned, all NMR experiments were performed on a Bruker (Germany) Avance 700 MHz spectrometer, equipped with cryo-probe. The backbone resonances of Bcl-X_L in DPC were assigned from a suite of deuterium-decoupled triple resonance experiments, HNCA, HN(CO)CA, HNCO, HN(CA)CO, CBCANH, CBCA(CO)NH and HSQC on ¹⁵N, ¹³C-labeled or ¹⁵N, ¹³C, ²H-labeled protein. These spectra were acquired in TROSY (transverse relaxation optimized spectroscopy) version at 310 K on Varian 900 MHz spectrometer (Korea Institute of Science & Technology, Seoul, Korea) NMR data was processed using Topspin version 1.3 (Bruker) or NMRpipe (Delaglio et al., 1995), and processed spectra were assigned and analyzed using the program SPARKY.

2.2.7 Paramagnetic relaxation enhancement (PRE) experiments

TROSY-HSQC spectra of Bcl-X_L ΔTMΔL in titration experiment with 5/16-DSA were acquired at 310 K. Maximum 0.4 mM 5 or 16-DSA was added into 0.1 mM Bcl-X_L with 10 mM DPC separately.

Wild type and mutated Bcl-X_L in buffer of 200 mM Tris (pH 8.0), 200 mM NaCl was treated by (iodoacetamido)proxyl spin label (IPSL) in a molar ratio of 1:5 at 4 °C for overnight. After incubation, protein was buffer exchanged into 40 mM NaPO₄ (pH 7.0), 50 mM NaCl for NMR data collection. Then ascorbic acid was added

consequently with a ratio of 10:1 to reduce the nitroxide group on IPSL. The spectrum was collected again as a control.

2.3. Results

2.3.1. Expression and purification of Bcl-X_L

Bcl-X_L without the flexible loop and transmembrane domain at C-terminal was expressed and purified as mentioned in the Method section. The protein of interest was well expressed and purified with high yield in our system (Figure 2.2). Finally, the purified sample was analysis by mass spectrometry for molecular weight determination and protein ID searching (data not shown). Together, these results suggested that the Bcl-X_L was successfully cloned, expressed and purified.

Figure 2.2 Purification of Bcl-X_LΔTMΔL. (A) Bcl-X_LΔTMΔL was purified on Ni²⁺-NTA resin. Lane 1, the supernatant portion of cell lysate after centrifugation; lane 2, the flow through fraction; lane 3, the wash fraction with resuspension buffer; lane 4, the wash fraction with 30 mM imidazole; lane 5, the elution fraction. (B) The eluted

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fraction from Ni^{2+} -NTA resin was loaded on a size exclusion chromatography for further purification and imidazole elimination.

2.3.2. Screening of Detergents

For membrane-bound proteins, small micelles containing approximately 60-100 lipids and one protein provide a generally effective model membrane environment, without the damaging effects of organic solvents. Although micelles only have single layer, the smaller size and higher diameter homogeneous compared to bicelles will provide the narrowest possible line widths and the best spectra quality in membrane environment.

In this study, HSQC spectra of Bcl-X_L ΔTMΔL titrated with five kinds of widely used detergents (Table 2) were collected at 25 °C.

Dramatic spectrum perturbation induced by all these detergents indicates that Bcl-X_L reshuffled topology in hydrophobic environment (Figure 2.3). Finally, DPC was selected because of 1) higher binding affinity-less amount of detergent need, 2) better spectrum quality-more peaks and better dispersion.

Table 2 Detergents utilized to mimic membrane environment for Bcl-X_L.

Name	Structure Formula	Critical Micelle Concentration (cmc)
Sodium dodecyl sulfate (SDS)		8.3 mM
Lyso-1-myristoyl-phosphatidylcholine (LPC)		0.04-0.09 mM
Lauryldimethylamine oxide (LDAO)		2 mM
1,2-dicaproyl- <i>sn</i> -glycero-3-phosphocholin (DHPC)		15 mM
Dodecylphosphocholine (DPC)		1.1 mM

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Figure 2.3 HSQC spectra of Bcl-X_L in solution or detergent micelles. (A) Bcl-X_L in solution. (B) Bcl-X_L in SDS micelle. (C) Bcl-X_L in LPC micelle. (D) Bcl-X_L in LDAO micelle. (E) Bcl-X_L in DHPC micelle. (F) Bcl-X_L in DPC micelle.

2.3.3. Backbone NMR resonance assignments

With the help of Dr. Ye Hong, we have assigned 85.2% back bone resonance by using the data from HNCA, HN (CO) CA, HNCO, HN (CA) CO, CBCANH, CBCA (CO)

NH experiments. The unassigned residues majorly congregate in N-terminal, BH3 region and helix $\alpha 5$ in solution structure (residue 147-164) (Fig. 2.4B).

2.3.4. Secondary structure prediction

Chemical shifts of certain nuclei are tightly related to secondary structure of protein. ^{15}N , C_β and H_α chemical shifts are generally higher in extended structures and lower in helices, while the relationship is opposite for C_O and C_α shifts (Wishart and Sykes, 1994; Wishart et al., 1992). Base on this standard, the chemical shift of C_α , C_β and C_O subtracting the random coil value was plotted (Figure 2.4A, B, C). Residues which are consistent in all three standards (Figure 2.4D) are considered as in helices. Generally like the solution structure, Bcl-X_L in micelle is still composed by several α helices and loops connecting them. However, helices are shortened or extended in detergent environment (Figure 2.4E).

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Figure 2.4 Secondary structure predication of Bcl-X_L in DPC and its comparison with solution form. Chemical shift indexes of (A) C_α, (B) C_β and (C) C_O. (D) CSI plot of Bcl-X_L in DPC micelle. 1 value indicates that the corresponding residue is in β sheet form, while -1 value indicates the residue in α helix. 0 value suggests the residue is in random coil. (E) Comparison of secondary structure of Bcl-X_L in solution and in DPC micelle. The unassigned residues in our data are indicated with blue underline.

2.3.5. Circular Dichroism (CD) Experiment

The second structural prediction result of Bcl-X_L in DPC micelle is also confirmed by the circular dichroism experiment. The CD curve of Bcl-X_L in DPC micelle shows that the protein is still comprised of α-helices and loops. Furthermore, the slightly

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increase of the minima at 220 nm indicates that the overall helicity decreased when the protein inserted into DPC micelle, which is also consistent with the NMR result.

Figure 2.5 Comparison of the circular dichroism spectrum of Bcl-X_L in solution and in DPC micelle

2.3.6. Backbone Relaxation Studies

¹H-¹⁵N NOE in steady state is sensitive to motions of the protein backbone in high-frequency. Hence it is commonly used to probe protein backbone dynamics and overall molecular tumbling motions (Kay, 1998; Kay et al., 1989). The relaxation rates of heteronuclear NOEs for Bcl-X_L were measured and plotted as bar graphs in Figure 2.6. The negative values of NOEs indicate the existence of large-amplitude motions on a subnanosecond time scale. Based on this criterion, N- and C- terminal

and residue 38-48 of Bcl-X_L are suggested to form a highly flexible polypeptide chain in these regions. It is interesting that the truncated form of the long flexible loop still retains high flexibility.

Figure 2.6 Relaxation parameters for Bcl-X_L in DPC. ^1H - ^{15}N heteronuclear NOEs are plotted as a function of residue number in the protein sequence.

2.3.7. DSA titration

As an amphiphile molecular, doxyl stearic acid (DSA) will automatically insert into DPC micelle (Figure 2.6). The doxyl group, which covalently linked to the 5th carbon

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(5-DSA) or 16th carbon (16-DSA), will enhance the transverse relaxation of nearby amide protons manifested by line-broadening and intensity reduction of relative peaks in the 2D ^{15}N , ^1H -TROSY spectra (Figure 2.7A&B). Hence, those residues whose peaks were affected by adding DSA, especially 16-DSA, should be buried inside the membrane (Figure 2.7C). The highest ratio appeared at A155 which is consistent with reported result that $\alpha 5$ and $\alpha 6$ in solution will insert into membrane. However, because many residues in putative transmembrane domain are not assigned, no further information could be derived.

Figure 2.7 Titrations of DSA into Bcl-X_L/DPC. (A) Spectrum of Bcl-X_L in DPC micelle with 0.4 mM 5-DSA. (B) Spectrum of Bcl-X_L in DPC micelle with 0.4 mM 16-DSA. (C) Peak height ratios from spectra of 5-DSA to 16-DSA are plotted as a function of residue number in the protein sequence.

2.3.8. Paramagnetic Spin Labeling Studies

Long range NOE information is essential for NMR structure calculation. However in this study, only three NOEs involving two pairs of residues were identified. In this case, paramagnetic relaxation enhancement (PRE) induced by paramagnetic spin label was introduced to obtain long-range structural restraints. Unlike NOEs, which are limited to distances of up to 5 Å, the range of distances for which PREs can yield quantitative information is about 10–25 Å. Because IPSL will attach to the protein via covalent bond to a cysteine residue, a serial of mutants of Bcl-X_L have to be prepared. Based on the secondary structure information, 15 dispersive residues were selected for mutant to provide enough 3D structure information and in the mean time minimize the structure change because of mutation (Figure 2.8).

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Figure 2.8 Site-directed mutagenesis on Bcl-X_L. Blue circle indicates C155 in wild type Bcl-X_L, which was replaced by serine. Based on C155S template, red circles indicate the residues selected for double mutants.

TROSY-HSQC or HSQC spectra of mutated Bcl-X_L, which treated with IPSL in advance, were collected. Consequently, the covalently linked IPSL was reduced by ascorbic acid to remove the PRE effect. Spectra were collected again as the control. Overall, the control spectra show high similarity with that of wild type Bcl-X_L except the mutated residues and the neighbor ones, suggesting that the overall structure did not significant changed by mutation or IPSL binding. By comparing the spectral density of Bcl-X_L before and after addition of ascorbic acid, distance information can be derived by Solomon-Bloembergen (SB) equation:

$$J_{SB}(\omega) = r^{-6} \frac{\tau_c}{1 + (\omega\tau_c)^2}$$

$J_{SB}(\omega)$ is the generalized spectral density, and τ_c is the correlation time. The peak height ratios of Bcl-X_L effected or unaffected by PRE were plotted (Figure 2.10).

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Figure 2.9 Spectra overlay of spin labeled Bcl-X_L mutant before (red) and after (black) ascorbic acid was added. Mutated residues are indicated in spectra.

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Figure 2.10 Peak height ratios between Bcl-X_L before and after ascorbic acid added were plotted by residue number. Mutated residues are indicated in the plots.

2.3.9. Structure calculation

PRE was determined by peak intensity ratio from 2D ^1H - ^{15}N HSQC or TROSY-HSQC spectra without and with the addition of twelve molar equivalents of ascorbic acid. A total of 113 experimentally determined PRE distances were used as restraints in calculations (Figure 2.11). Torsion angle restraints Φ , Ψ were determined from the analysis of N, C', C $_{\alpha}$, and H $_{\alpha}$ chemical shifts using the program TALOS (Cornilescu et al., 1999). With the long-range PRE restraints, torsion angle restraints and the secondary structure predicted from the NMR chemical shift data N, C', C $_{\alpha}$, and H $_{\alpha}$, total 100 structures were calculated and the 10 structures with the lowest target function energy were further calculated with a full molecular dynamics force field, including electrostatic and Lennard-Jones potentials, and water-refined in CNS (Brunger et al., 1998).

Till now, excluding the N-terminal residues and helix $\alpha 1$ spanning Met1 - Gln23, which were not close to other helices indicated by the PRE data, the dynamic region of Asp33 - Ala92, and the C-terminal residues containing Ala203 - Glu215 and a hexa histidine-tag, the root-mean-square deviation (RMSD) of the ensemble were 7.07 ± 1.01 Å for the backbone and 7.81 ± 1.06 Å for all heavy atoms (Table 4).

In general, the NMR structure of Bcl-XL in DPC micelles is very flexible comparing to its solution structure (Figure 2.12). Two helices $\alpha 5$ and $\alpha 6$, which is the hydrophobic core of Bcl-XL in solution, insert into membranes via a hairpin; while the rest of the helices became loosely ordered but retained alpha-helical secondary structural elements. It's highly possible the roll of the micelle is to help Bcl-XL to overcome the energy barrier, so it can change the conformation to interact with its partners in membrane state.

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Figure 2.11 Distance constraints derived from PRE experiments. All the PREs are treated in the same criteria, and a uniform error of the distances was added (± 2 Å).

Table 3 NMR statistics for top 10 structures of Bcl-X_L in DPC

NMR distance and dihedral constraints	
Distance constraints	
Total NOE	0
Total PRE	113
Hydrogen bonds	43
Total dihedral angle restraints	
$\square \Phi$	95
$\square \Psi$	95
Structure statistics	
Violations	
Distance constraints ($>0.5\text{\AA}$)	22
Max. distance constraint violation ($\text{\AA}$)	11.56
Dihedral angle constraints ($>5^\circ$)	18
Max. dihedral angle violation ($^\circ$)	11.554
Deviations from idealized geometry	
Bond lengths ($\text{\AA}$)	0.01
Bond angles ($^\circ$)	0.7
Average pairwise r.m.s. deviation* ($\text{\AA}$)	
Backbone	6.89 ± 0.91
Heavy	7.60 ± 0.96
Average pairwise r.m.s. deviation** ($\text{\AA}$)	
Backbone	6.25 ± 0.73
Heavy	7.06 ± 0.79

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Ramachandran Plot (All residues)

Most favored	79.00%
Additionally allowed	14.20%
Generously allowed	4.10%
Disallowed	2.70%

Ramachandran Plot **

Most favored	97.70%
Additionally allowed	2.30%
Generously allowed	0.00%
Disallowed	0.00%

* For residue range of 92-202 by Procheck.

** For order residues: 9-65, 88-90, 101-115, 126-137, 141-146, 164-179, 183-189, 192-200 by Procheck.

Figure 2.12 NMR ensemble (A) and a representative model (B) of Bcl-X_L in DPC micelle. The putative micelle position is indicated by dash line.

2.3.10. Experiments to improve structure characterization

Since the Bcl-X_L mutants have been prepared, electron paramagnetic resonance (EPR) can be used to detect the molecular architecture. In general, this method is similar to the nuclear paramagnetic resonance as described above. The difference is that EPR monitors the spin of the unpaired electron on the nitroxide, while PRE monitors the spins of nearby protons which are affected by the unpaired electron (Longhi et al., 2011). By comparing the spectra of the free electron before and after DPC addition, the conformational change of Bcl-X_L can be characterized

Another useful source to detect the structure of Bcl-X_L in DPC micelle is the intrinsic fluorescence from the aromatic residues of the protein. Tryptophan is most commonly used because it has the strongest fluorescence and highest quantum yield of the three aromatic amino acids. When the tryptophan exposes from hydrophobic environment into solvent accessible environment, a red shift will occur to the fluorescence emission wavelength. Hence, using tryptophan or its analogs like azatryptophans as a probe, we can trace the change of nearby environment during topology reshuffle (Lepthien et al., 2008). However, there are five tryptophans in the loop deletion construct of Bcl-X_L. It is difficult to differentiate the signal of one tryptophan from that of the others, unless site-directed mutations are involved to silence those uninterested ones.

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Chapter 3 Interaction of Bcl-X_L in DPC micelle with BH3 peptides

3.1. Introduction

3.1.1. Role of BH3 in apoptosis

As the BH3 region is the most conserved sequence among all the Bcl-2 family members, it has been extensively studied to characterize its function and regulation in apoptosis signaling pathways.

In HeLa cells, a 15-mer Bak BH3 peptides was demonstrated can inhibit Bcl-X_L function and induce apoptosis through caspases activation without translocation of cytochrome c from mitochondria to cytosol (Holinger et al., 1999). A systemic study about 25-mer BH3 peptides from BH3 only protein suggested that some (from Bim and Bid) are sufficient to permeabilize liposomes in the presence of Bax, while others can function in an indirect manner by opposing Bcl-X_L (Kuwana et al., 2005).

Furthermore, small molecules mimicking BH3-only proteins are being developed for cancer therapy. ABT-737 and its orally active derivative ABT-263, which bind to anti-apoptotic proteins such as Bcl-2, Bcl-X_L and Bcl-W with high affinity, kill cells via a Bax- and Bak-dependent pathway. In preclinical studies, ABT-737 was an efficacious single agent for treating certain leukemias, lymphomas, myelomas and small cell lung cancers. (Happo et al., 2012). However, the author also claimed that ABT-737 shows limited effect on tumor development in p53-deficient mice (Grabow et al., 2012).

3.1.2. Structure basis of BH3 motif interacting with Bcl-X_L in aqueous solution

Previously, the solution structure of Bcl-X_L complexed with a 16-mer peptide from the BH3 region of Bak (Bak₇₂₋₈₇) was determined by NMR (Sattler et al., 1997). Generally, the structure of Bcl-X_L when binding with Bak₇₂₋₈₇ is similar to its free form. The structure revealed that Bak₇₂₋₈₇ binds in a hydrophobic pocket formed by the BH1, BH2, and BH3 regions of Bcl-X_L (Figure 3.1A). The loops connecting helices $\alpha 2$ to $\alpha 3$ and H3 to H4 are also involved in the interactions.

Later, the solution structure of Bcl-X_L in complex with a 25-mer peptide from the BH3 region of Bad (Bad₁₀₃₋₁₂₇) was also determined using NMR spectroscopy (Petros et al., 2000b). Generally the overall structure is similar to Bcl-X_L with Bak₇₂₋₈₇, but additional interactions are formed in the complex of Bcl-X_L with Bad₁₀₃₋₁₂₇ (Figure 3.1B). However, site-directed mutagenesis experiments suggest that greater affinity of Bad₁₀₃₋₁₂₇ peptide with Bcl-X_L is not due to additional contacts but higher propensity to form α -helix before docking.

Figure 3.1 Cartoon representations for Bcl-X_L (blue) complexed to BH3 peptides (red). (A) Bcl-X_L with a 16-mer peptide from Bak protein (pdb code 1BXL). (B) Bcl-X_L with a 25-mer peptide from Bad protein (pdb code 1G5J).

3.1.3. Interaction of BH3 region with Bcl-X_L in membrane

Despite intense research on the binding pattern of BH3 peptides with solution state Bcl-X_L, limited experimental data is available for the interaction of them in membranes. It is highly in doubt about the intactness of the hydrophobic cleft of Bcl-X_L after its dramatic conformational change during membrane insertion. To date, there is no strong evidence supporting whether the binding pattern of BH3 with Bcl-X_L in membrane remains the same as that in aqueous solution. On the contrary, a FRET-based binding assay suggested that Bak BH3-peptide cannot bind to Bcl-X_L in large unilamellar vesicles (Feng et al., 2009).

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To this end, in this chapter, the interaction of BH3 peptides with Bcl-X_L in micelle was systemically studied using NMR spectroscopy.

3.2. Experiments

3.2.1. Expression and purification of Bcl-X_LΔL ΔTM

Expression and purification procedure is the same as described in 2.2.1.

3.2.2. Expression and purification of Bcl-X_LΔTM

Bcl-X_L ΔTM can be expressed in BL21 (DE3) cells but quickly degraded. Hence the plasmid for the protein was transformed into *E. coli* HMS174 (DE3) cells. The expression and purification procedures are the same as Bcl-X_L ΔLΔTM.

3.2.3. Purification of Bcl-X_L in DPC micelle

Transformed cells were grown and induced as described before. After sonication, 0.5% DPC was added into cell lysate and all the down-stream purifications were performed in the presence of 0.5% DPC. The supernatant after centrifugation was loaded onto Ni²⁺-NTA resin, washed with 100 mM imidazole buffer, and eluted with 200 mM imidazole buffer. The concentrated elution fraction from Ni-NTA resin was subject a PD-10 column for size fractionation to eliminate imidazole. The protein concentration was determined by the absorbance at 280 nm instead of Bradford dye assay, because the latter is more sensitive to DPC.

3.2.4. BH3 peptides

The Bad₁₀₈₋₁₂₃ was obtained from SBS or GL Biochem (Shanghai) Ltd. The unlabeled peptides, Bad₁₀₃₋₁₂₇, Bak₇₂₋₈₇, Bax₃₉₋₅₄, Bid₈₁₋₉₆, Bcl-2₉₁₋₁₀₆, were obtained from GL Biochem (Shanghai) Ltd (Table 3.1). All these peptides with a purity of 95% were used without further purification.

Table 4 BH3 peptides from Bcl-2 family proteins

Source	Sequence	Pos ition
Bad 25-mer	NLWAAQRYGRELRRMSDEFVDSFKK	103-127
Bad 16-mer	QRYGRELRRMSDEFVD	108-123
Bak 16-mer	GQVGRQLAIIGDDINR	72-87
Bax 16-mer	KKLSECLKRIGDELDS	39-54
Bid 16-mer	DIIRNIARHLAQVGDS	81-96
Bcl-2 16-mer	PVVHLALRQAGDDFSR	91-106

3.2.5. NMR spectroscopy

Titration experiments of Bcl-X_L with BH3 peptides and/or DPC were monitored on heteronuclear single quantum coherence (HSQC) spectra. All NMR experiments were

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performed on a Bruker Avance 600 or 700 MHz spectrometer, equipped with cryo-probe. NMR data was processed using Topspin version 1.3 (Bruker) software, and processed spectra were assigned and analyzed using the program SPARKY.

Without specially mention, NMR sample were prepared in a buffer containing 20 mM NaPO₄ (pH 7.0), 50 mM NaCl, 1 mM DTT, 0.01% NaN₃ and 10% D₂O.

3.3. Results and Discussion

3.3.1. BH3 peptides titration with Bcl-X_L Δ L Δ TM in DPC micelle

To characterize the interaction between BH3 peptides and Bcl-X_L in DPC micelle, BH3 peptides from different Bcl-2 family members were added into Bcl-X_L/DPC complex and monitored by NMR spectroscopy.

The BH3 peptides (Bak₇₂₋₈₇, Bad₁₀₈₋₁₂₃ and Bid₈₁₋₉₆) were added into ¹⁵N-labeled Bcl-X_L containing DPC, The final ratio of Bak₇₂₋₈₇: Bcl-X_L: DPC reaches 6: 1: 100 (Figure 3.2A). No significant chemical shift change was detected, except only few residues exceeding 0.01 ppm and none of them reaches 0.02 ppm (Figure 3.2B). Hence, Bak₇₂₋₈₇ has little affinity with Bcl-X_L in DPC micelle. The final ratios for Bad₁₀₈₋₁₂₃ and Bid₈₁₋₉₆ titration experiments are 4: 1: 100 (Figure 3.2C) and 6: 1: 100 (Figure 3.2D), respectively. Again, both peptides have little affinity with Bcl-X_L in DPC micelle as indicated by the unmoved NMR spectra.

The consistency of the titration results eliminates the possibility that the failure of binding is caused by spatial conflict or charge repulsion from specific residues. All the

data suggest that there is no or little interaction between BH3 peptides and Bcl-X_L in DPC micelle. Taken together, the data prompted us to propose that Bcl-X_L structure in membrane environment is different from that in solution phase.

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Figure 3.2 Bcl-X_L ΔTMΔL in DPC micelle was titrated by BH3 peptides. (A) Spectra of Bcl-X_L ΔTMΔL in DPC micelle before (black) and after (red) titrated by Bak₇₂₋₈₇. (B) Plot of amide chemical shift of unambiguous residues in the titration, as calculated by $\Delta\delta_{\text{NH}} = [(\Delta\delta_{\text{H}})^2 + (0.154 * \Delta\delta_{\text{N}})^2]^{1/2}$. (C) Bcl-X_L ΔL ΔTM in DPC micelle was titrated by Bad₁₀₈₋₁₂₃. (D) Bcl-X_L ΔL ΔTM in DPC micelle was titrated by Bid₈₁₋₉₆.

3.3.2. Interaction Bcl-X_L ΔTM with BH3 peptides

As mentioned above, BH3 peptides failed to bind with Bcl-X_L in DPC micelle environment, which is different from what has been reported with solution Bcl-X_L. To examine whether the flexible loop connecting helix α1 and α2 is responsible for the lack of the binding, loop intact construct of Bcl-X_L (Bcl-X_L ΔTM) was also tested in BH3 titration experiment.

The BH3 peptides (Bad₁₀₈₋₁₂₃, Bad₁₀₃₋₁₂₇ and Bid₈₁₋₉₆) were added into ¹⁵N-labeled Bcl-X_L containing DPC, and the final ratio of peptides: Bcl-X_L: DPC are 2: 1: 100, 4: 1: 300 and 6: 1: 300, respectively (Figure 3.3). Our data showed that no noticeable perturbation is detected. The results are consistent with the loop deletion construct of Bcl-X_L, indicating that loop deletion is not the reason for the failure of binding between BH3 peptides and Bcl-X_L in DPC micelle.

Figure 3.3 Bcl-X_L ΔTM in DPC micelle (black) was titrated by varies BH3 peptides (red). (A) Bcl-X_L ΔTM in DPC micelle was titrated by Bad₁₀₈₋₁₂₃. (B) Bcl-X_L ΔTM in DPC micelle was titrated by Bad₁₀₃₋₁₂₇. (C) Bcl-X_L ΔTM in DPC micelle was titrated by Bid₈₁₋₉₆.

3.3.3. The hydrophobic groove of Bcl-X_L demolished in membrane state

The conclusion derived from NMR titrations also supported by our membrane structure of Bcl-X_L. When $\alpha 5$ and $\alpha 6$ insert into membrane, the topology of Bcl-X_L in DPC micelle changes the shape of hydrophobic groove and consequently affects the binding of BH3 peptides. Residues of Bcl-X_L which contribute to the binding of BH3 peptide in solution, spread in a wide range rather than congregate together.

3.3.4. DPC titration with Bcl-X_L Δ TM/BH3 peptide complex

As mentioned above, the conformation change of Bcl-X_L induced by DPC micelle prohibits its binding with BH3 peptides. However, it is remain in question whether the complex formation of Bcl-X_L with BH3 peptides can prevent structural transform induce by DPC. To test this, in this section the sequence of titration was altered. BH3 peptides were first added into Bcl-X_L, and then followed by DPC. Although BH3 peptides showed significant binding with solution Bcl-X_L, this interaction was disrupted by addition of DPC.

Bak₇₂₋₈₇ peptide was titrated into Bcl-X_L solution till saturation, about 0.25 mM (Figure 3.4A). The complex formation is confirmed by resonant perturbations, which is consistent with the complex formation report (Sattler et al., 1997). Then DPC was added till 10 mM and the final molar ratio of Bcl-X_L, Bak₇₂₋₈₇ and DPC is 1:2.5:100 (Figure 3.4B). DPC induced dramatic and all-involved spectrum perturbation indicates that the topology of Bcl-X_L was converted into membrane-state, and the

conclusion is supported by the high identity of two spectra from Bcl-X_L in DPC with or without Bak₇₂₋₈₇. Similar results were obtained with Bid₈₁₋₉₆ peptide in a final ratio of 1:4:100 (Figure 3.4C&D). For Bad₁₀₃₋₁₂₇ peptide, because of the much higher affinity DPC was added till 15 mM and the final molar ratio is 1:4:150. Eventually, DPC micelle again disrupted the binding of Bcl-X_L with peptide and induced its conformational change.

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Figure 3.4 Spectra of Bcl-X_L ΔTMΔL when BH3 peptides and DPC were added sequentially. (A) Bcl-X_L ΔTMΔL in solution (black) was titrated by Bak₇₂₋₈₇ (red). (B) Bcl-X_L/Bak₇₂₋₈₇ complex (black) was titrated by DPC (red). (C) Bcl-X_L ΔTMΔL in solution (black) was titrated by Bid₈₁₋₉₆ (red). (D) Bcl-X_L/Bid₈₁₋₉₆ complex (black) was titrated by DPC (red). (E) Bcl-X_L ΔTMΔL in solution (black) was titrated by Bad₁₀₃₋₁₂₇ (red). (F) Bcl-X_L/Bad₁₀₃₋₁₂₇ complex (black) was titrated by DPC (red).

The loop intact construct was also tested to verify the role of the flexible loop played in BH3 motifs binding. BH3 peptides (Bad₁₀₈₋₁₂₄, Bcl-2₉₁₋₁₀₆ and Bad₁₀₃₋₁₂₇) were

added into ^{15}N -labeled Bcl-X_L till saturation. Again with the addition of DPC, spectra of Bcl-X_L shifted back to the form of when Bcl-X_L alone in DPC micelle (Figure 3.5).

In summary, BH3 peptides form complex with Bcl-X_L in detergent free environment which is indicated by significant perturbations in NMR spectra. However, when DPC was added, the spectra shifted back to the form of when Bcl-X_L alone in DPC micelle. Complex formation of Bcl-X_L with BH3 peptides cannot prevent its conformation change induced by DPC.

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Figure 3.5 Spectra of Bcl-X_L ΔTM when BH3 peptides and DPC were added sequentially. (A) Bcl-X_L ΔTM in solution (black) was titrated by Bad₁₀₈₋₁₂₃ (red). (B) Bcl-X_L/Bad₁₀₈₋₁₂₃ complex (black) was titrated by DPC (red). (C) Bcl-X_L ΔTM in solution (black) was titrated by Bcl-2₉₁₋₁₀₆ (red). (D) Bcl-X_L/Bcl-2₉₁₋₁₀₆ complex (black) was titrated by DPC (red). (E) Bcl-X_L ΔTM in solution (black) was titrated by DPC (red). (F) Bcl-X_L/Bad₁₀₃₋₁₂₇ complex (black) was titrated by DPC (red).

Chapter 4 Interaction of Bcl-X_L in DPC micelle with truncated Bid

4.1. Introduction

As mentioned previously, BH3 motif is suggested no longer to be an efficient binding target for Bcl-X_L in membrane. However, truncated form of Bid (tBid) which is a BH3-only protein was reported to heterodimerize with Bcl-X_L in membrane with relatively high affinity. In this chapter, we examined the interaction of tBid with Bcl-X_L in DPC micelle environment using NMR technology.

4.1.1. Function of Bid in apoptosis

Bid is a key BH3-only protein which connecting death receptor cross-linking with intrinsic pathway events of apoptosis at mitochondria. Full length Bid is a 22 kDa protein mainly localizing in cytoplasm. Even though there are experiments indicating that pro-apoptotic activity remains in full-length Bid, in most cases activation by truncation is necessary for Bid to exert its function. Bid can be cleaved at internal aspartic acid by caspase-8, but also a possible substrate for other caspases, like caspase-3 and caspase-10 (Degli Esposti et al., 2003; Li et al., 1998; Milhas et al., 2005). This cleavage results in the generation of a major p15 fragment or two minor fragments, p13 and p11. Both p15 and p13 fragment are confirmed as active truncated Bid (tBid). Although it has been presumed that the cleavage leads to the translocation of tBid from cytoplasm to mitochondria membrane, more recently it has

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been reported that the two Bid fragments forms a stable dimer which is highly similar to the uncleaved full length Bid. A complete dissociation of the complex can be triggered by detergents, and restored by detergent removal (Bleicken et al., 2012).

Truncated Bid can activate and oligomerizes BAX/BAK to release cytochrome c, while this process can be abrogated by Bcl-X_L (Billen et al., 2008; Eskes et al., 2000; Korsmeyer et al., 2000).

Figure 4.1 Structure of Full length Bid before (A) and after (B) caspase-8 cleavage. (A) Structure of FLBid (pdb code 2BID) (Chou et al., 1999). (B) Structure model of p15-p7 complex. Fragment p7 is indicated by blue, while p15 is indicated by red.

4.1.2. Interaction of tBid with Bcl-X_L

NMR experiments suggest that the heterodimerization complex of tBid and Bcl-X_L is formed by tBid docks its BH3 region into the hydrophobic cleft of Bcl-X_L (Ji et al., 2006; Yao et al., 2009).

Using fluorescence cross-correlation spectroscopy (FCCS), it was reported that tBid binds with Bcl-X_L in membrane environment with higher affinity than that in solution. Furthermore, a BH3 peptide derived from Bid can induce dissociation of the tBid/Bcl-X_L complex in solution, but failed in membrane environment (Garcia-Saez et al., 2009).

Combining this result and summary of Chapter 3, it is highly possible that tBid binds with Bcl-X_L in a novel pattern in membrane other than BH3 engagement. Hence in this chapter, we monitored the interaction of tBid with Bcl-X_L in DPC micelle, and the binding involved residues were identified.

4.2. Experiments

4.2.1. Construction of full length Bid

DNA sequence coding human full length Bid (FLBid) was downloaded from NCBI database and optimized by GenScript. It was subcloned into vector pET16b (Novagen) by using two primers containing NdeI and XhoI restriction sites, 5'-CACTCAGCATATG GACTGTGAAGTGAAC (forward) and 5'-GCGAAGCTCGAGTCATTAGTCCATGCCATT ACGTGC (reverse). The final protein construct contains a poly-histidine tag and nine extra amino acids (MGHHHHHHHHHSSGHIEGRH-) at the N terminus.

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4.2.2. Purification of full length Bid

Plasmid coding for FLBid was transformed into *E. coli* BL21 (DE3) cells, and the transformed cells were grown in LB medium containing 100 µg/ml of carbenicillin at 37 °C. 1 mM IPTG was added to induce protein expression when the cell culture reached Abs₆₀₀=0.6-1.0, and the culture was further incubated for additional 3 h at 25 °C or overnight at 16 °C. The cells were harvested by centrifugation at 8000 rpm for 10 min, re-suspended in re-suspension buffer (20 mM NaPO₄, 500 mM NaCl), and broken by sonication for 30 min on ice. The cell lysate was cleared by centrifugation at 20,000 rpm for 30 min and purified by affinity chromatography on Ni²⁺-NTA resin. The elution fractions from Ni²⁺-NTA were loaded on to Sephacryl S-200 filtration column equilibrated with 20 mM NaPO₄, 50 mM NaCl, 1 mM DTT, 0.01% NaN₃. The purified proteins were analyzed on 12% SDS-PAGE. The protein concentration was determined by Bradford dye assay kit.

4.2.3. Full length Bid cleavage

Purified FLBid concentrated in 2.5 ml volume was loaded onto PD-10 column, and then washed out with 3.5 ml buffer containing 50 mM HEPES, 100 mM NaCl, 0.5% CHAPS, 5 mM TCEP, 1 mM EDTA and 10% glycerol. The buffer exchange was further improved by multiple times concentrate-dilute step with a concentrator.

To achieve maximum utility of the enzyme, 2 µl (>200 units) active mouse caspase 8 (its-science medical) was added into about 10 mg FLBid. After 48 h incubation at 30°C, FLBid was cleaved into a p15-p7 c omplex.

4.2.4. Purification of truncated Bid

The complex was applied to Ni-NTA column again. Truncated Bid (p15) was washed out in the buffer of 2% DPC, 2 mM TCEP and 40 mM NaPO₄, while p7 remained in the column. Attempt to decrease DPC concentration after purification will induce aggregation or even precipitation of tBid.

4.2.5. NMR spectroscopy

All NMR experiments were performed on a Bruker Avance 700 MHz spectrometer, equipped with cryo-probe and pulse field gradients. NMR data was processed using Topspin version 1.3 (Bruker) software, and processed spectra were assigned and analyzed using the program SPARKY.

4.3. Results and discussion

4.3.1. Preparation of truncated Bid

Full length Bid was expressed, purified and cleaved as mentioned in the Method section (Figure 4.2). Finally, the purified tBid was analysis by mass spectrometry for molecular weight determination and protein ID searching (data not shown). Together, these results suggested that the tBid was successfully prepared.

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Figure 4.2 Purification of FLBid and tBid. (A) FLBid was purified on Ni^{2+} -NTA resin. Lane 1, the supernatant portion of cell lysate after centrifugation; lane 2, the flow through fraction; lane 3, the wash fraction with resuspension buffer; lane 4, the wash fraction with 30 mM imidazole; lane 5, the elution fraction. (B) FLBid cleavage and tBid purification. Lane 1, FLBid with caspase-8 in 0 hour incubation; Lane 2, FLBid with caspase-8 in 48 hours incubation; Lane 3, tBid washed out from Ni^{2+} -NTA resin in 2% DPC. (C) The eluted fraction from Ni^{2+} -NTA resin was concentrated and further purified on a size exclusion chromatography (blue). Cleaved FLBid was concentrated and purified by size exclusion chromatography again to remove caspase-8 and exchange buffer (red). Bid eluted at the same volume indicating that the cleavage has no effect on the shape of Bid.

4.3.2. Interaction of tBid with Bcl-X_L in micelle

Truncated Bid in 2% DPC, which is proved necessary to maintain function, was titrated with Bcl-X_L in DPC micelle. Like what happened in the titrations of BH3 peptide into aqueous Bcl-X_L, the perturbation and disappearing is significant and numerous peaks were involved (Figure 4.3).

Figure 4.3 Comparison of the 2D [^{15}N , ^1H]-HSQC spectra titrated by tBid. 0.1 mM Bcl-X_L in 10 mM DPC (black) was titrated by about 0.1 mM tBid with 2% DPC (red).

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In the titration experiment mentioned above, the DPC concentration increased about five times at the end compare to initial condition. To eliminate the possible effect of DPC concentration increase, the overlay of TROSY spectra of 0.1 mM ^{15}N , ^2H -labeled Bcl-X_L in 8% DPC with or without 0.3 mM tBid was made. The same moving pattern confirmed that the perturbations were induced by tBid (Figure 4.4).

Figure 4.4 Chemical perturbation of Bcl-X_L by tBid in different (A) or same (B) DPC concentration. (A) 0.1 mM Bcl-X_L in 10 mM DPC (black) was titrated by about 0.1 mM tBid with 2% DPC (red). (B) Overlay of TROSY spectra of 0.1 mM Bcl-X_L in 8% DPC with (red) or without (black) 0.3 mM tBid.

4.3.3. Binding interface determination using Cross-Saturation

To probe more accurate interface and validate the binding, NMR cross-saturation technology was utilized. In this method, ^{15}N , ^2H -labeled Bcl-X_L was mix with unlabeled tBid in a 10:1 ratio. By applying RF pulses to the aliphatic proton resonances, all the aromatic and amide proton resonances in the unlabeled tBid were saturated because of spin diffusion effect. This saturation was transferred to doubly labeled Bcl-X_L by cross relaxation through the interface of the complex. The ratios of

TROSY cross-peak intensities, with and without saturation, provide an identification of the residues involved in binding interaction (Takahashi et al., 2000).

Figure 4.5 Identification of binding interface from Cross-Saturation Data.(a) Secondary structure prediction of Bcl-X_L in DPC micelle based on our NMR data (b) Intensity ratio (I_s/I_0) of residues of Bcl-X_L in cross-saturation.

Sample for cross-saturation was prepared using 0.2 mM ¹⁵N, ²H-labeled Bcl-X_L mixed with unlabeled 0.02 mM tBid in 10% H₂O/90% ²H₂O. Low concentration of tBid was applied to minimize Bcl-X_L spectra perturbations caused by binding. The

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degree of saturation of each residue was represented by the peak intensity ratio of the saturated spectrum (I_s) to the control spectrum (I_o) (unsaturated). In this study an (I_s/I_o) value of <0.75 or very close to is considered significant and saturated. Based upon these criteria, nine unambiguously assigned residues were clearly saturated in the experiment, and there are twelve more residues whose intensity ratios are less than 0.80 (Figure 4.5). Most of the involved residues locate at three regions, E11 to Q23, A123 to D137 and A175 to N189.

In contrast, in solution binding pattern which is mediated by the hydrophobic cleft from Bcl-X_L and BH3 peptides, the residues involved in start from A97 to L154, including BH1, BH3 and some residues between them (Petros et al., 2000c). The range of residues involved in membrane state interaction is much wider than that in solution. Among the nine significant binding involved residues, L12 and V14 are from BH4 motif and N189 is from BH2 motif, while these two domains are not deeply involved in the solution binding pattern. This might shed a light on how these two conserved domains serve in the apoptotic function.

4.3.4. A hypothesis of Bcl-X_L regulation mechanism on apoptosis

Base on all the experiment results presented in this study, the interaction mechanism of Bcl-X_L with other family members in DPC is significantly different from which in solution. However, there are some questions need to be answered. As demonstrated in detergent titration experiments, Bcl-X_L will automatically insert into micelles. Since it permanently resides at the mitochondrial membrane by the C-terminal hydrophobic

anchor, it seems more possible that Bcl-X_L remains in membrane state rather than solution state. We have to ask when Bcl-X_L will adopt solution form. Furthermore, it has been proved above that BH3 peptides only bind with solution Bcl-X_L. So then, how BH3 peptides or the mimetic ligands regulate the apoptosis progress which mainly takes place at the membrane of mitochondria? To answer these questions, a co-retrotranslocation model could be considered for explaining the possible molecular regulatory mechanism.

It is reported that in membrane state, Bcl-X_L will bind with Bax and retrotranslocate Bax from the OMM into the cytosol (Edlich et al., 2011). In this model, Bcl-X_L behaves as a guard at the mitochondria. In most of the time it remains in membrane state. Once Bcl-X_L detected activated Bax which also was inserted into the membrane, they will form a complex and together translocate from the mitochondria into the cytosol. In cytosolic state, the interaction of two proteins will be disrupted, and Bax is released into cytoplasm. On the other hand, Bcl-X_L is anchored to the mitochondria. It will take much less time for Bcl-X_L to insert into the membrane and get ready for the next round of retrotranslocation. With a much higher circulation speed, Bcl-X_L is capable to control the amount of Bax at OMM.

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Figure 4.6 Bax (red) and Bcl- x_L (blue) constantly translocate to the mitochondria and coretrotranslocate back into the cytosol, stabilizing cytosolic Bax in healthy cells.

As an effective ligand, BH3 peptides and the mimics will bind with solution state Bcl- x_L . The binding will tether the insertion of Bcl- x_L into membrane, which was confirmed by NMR titration results Bcl- x_L alone or binding with low affinity BH3 peptides will undergo complete conformational change when the molar ratio of DPC/protein reaches 100:1. However, when Bcl- x_L binds with the Bad 25-mer BH3 peptide (Bad₁₀₃₋₁₂₇), which has a more than 1,000 times higher affinity than the 16-mer peptides, the molar ratio has to reach 150:1 to induce the conformational change of Bcl- x_L . Even though the ligand binding may not be strong enough to prevent Bcl- x_L from inserting into the membrane, the circulation speed will slow down. Consequently, activated Bax may not be retrotranslocated into the cytosol in time. Once enough amount of Bax is accumulated at OMM, permeable pores will be formed to release a serial of fatal proteins, and the cell will experience irreversible cell death. This model renders a possibility how BH3 peptides and the mimics can

only binds to solution state Bcl-X_L, but still can regulate the progress take place in membrane.

Figure 4.7 Bcl-X_L ΔTMΔL complexed with Bad₁₀₃₋₁₂₇ in solution (green) was titrated by DPC in a ratio of 1: 4: 100 (black) and 1: 4: 150 (red).

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