Structure-function analysis of a mitochondrial cell death protein, Bcl-2/E1B 19K interacting protein 3 (BNip3)

By

Reena Ray

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba
in partial fulfilment of the requirement for the degree of

Doctor of Philosophy

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Abstract

BNip3 resembles Bcl-2-related proteins, based on sequence similarity to the Bcl-2 homology 3 (BH3) domain and COOH-terminal transmembrane (TM) domain. Characteristically, the 'BH3-only' class of Bcl-2 proteins heterodimerize with Bcl-2/Bcl-x_L and induce cell death through their BH3 domain. BNip3 and its *C. elegans* orthologue, CeBNip3 interact with cell death repressors Bcl-2, Bcl-x_L and CED-9 in yeast and mammalian expression systems. Unlike other BH3-only agonists, deletion mapping of BNip3 excluded its putative BH3 domain and identified the NH_2-terminus and TM domain critical for Bcl-2 heterodimerization, and either region sufficient for Bcl-x_L interaction. Similar mapping studies of CeBNip3 demonstrated its association with CED-9 and Bcl-x_L exclusively through its TM domain. Both BNip3 and CeBNip3 induce cell death in several cell lines. Yet, the removal of the BH3-like domain in either protein does not diminish its cytotoxicity.

Some Bcl-2 homologues form homodimers through regions of shared homology to regulate their function. Following *in vitro* transcription/translation, BNip3 was expressed primarily as a 60 kDa protein along with a 30 kDa product. Interaction of BNip3 monomers was observed in the yeast two-hybrid system and deletion mapping localized the region mediating homodimerization to the TM domain. The BNip3 TM domain is also critical for mitochondrial targeting and cell death function. Its removal results in cytosolic expression and eliminates both its homologous interaction and cytotoxicity. Several BNip3 TM domain mutations were found to disrupt SDS-resistant BNip3 homodimerization, but did not interfere with its mitochondrial localization or killing activity. The substitution of the BNip3 TM domain with cytochrome b_5 targeting sequence directed protein expression to nonmitochondrial sites, promoting both cell death and Bcl-2/Bcl-x_L heterodimerization. Therefore, the function of BNip3 is independent of homodimerization, but the TM domain is necessary for mitochondrial targeting and cell death function.

In conclusion, BNip3 is a mitochondrial cell death-inducing protein, which although structurally related to the Bcl-2 family appears to heterodimerize and to induce cell death in the absence of its BH3-like domain. Thus, BNip3-related proteins define a new class of cell death proteins, which interact with Bcl-2, Bcl-x_L and CED-9 and kill independent of a BH3 domain.

In memory of my father

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3AT	3-amino-1,2,4-triazole
α-MEM	α-minimal essential medium
β-ME	β-mercaptoethanol
AD	activating domain
AIF	apoptosis inducing factor
ATP	adenosine 5'-triphosphate
BD	binding domain
BH	Bcl-2 homology
bp	base pair
BSA	bovine serum albumin
CARD	caspase recruitment domain
cDNA	complementary deoxyribonucleic acid
Ci	curie
CIP	calf intestinal alkaline phosphatase
Cy3	cyanine fluorochrome
ddH_2O	distilled deionized water
DED	death effector domain
DMEM	Dulbecco's Modified Eagle Media
DMF	N,N-dimethylformamide
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
EtBr	ethidium bromide
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
g	gravity
g	gram
GST	glutathione-S-transferase
HCl	hydrochoric acid
HeBs	HEPES-buffered saline
HSP60	heat shock protein 60
IgH	immunoglobulin heavy chain
$K_3Fe(CN)_6$	potassium hexacyanoferrate (III)
$K_4Fe(CN)_6•3H2O$	potassium hexacyanoferrate (II)
kb	kilobase
KCl	potassium chloride
kDa	kiloDalton
LB	Luria Bertani
LiAc	lithium acetate, LiOOCCH3•2H2O
M	molar; moles L^{-1}
mA	milliampere
mAb	monoclonal antibody
$MgCl_2$	magnesium chloride
$MgSO_4•7H2O$	magnesium sulfate
µl	microlitre
µM	micromolar
ml	millilitre

mm	millimetre
mM	millimolar
M_r	molecular weight
Na_2CO_3	sodium carbonate
Na_2HPO_4	sodium hydrogen phosphate
NaCl	sodium chloride
NaH_2PO_4	disodium phosphate
NaOH	sodium hydroxide
$NaOOCCH_3$	sodium acetate
$(NH_4)_2SO_4$	ammonium sulphate
ND	not determined
ng	nanogram
nm	nanometre
NMR	nuclear magnetic resonance
NP-40	Nonidet-P40
OD_{260}	optical density at 260 nm
OD_{280}	optical density at 280 nm
ONPG	ortho-nitrophenyl β-D-galactopyranoside
pAb	polyclonal antibody
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pmole	piccomole
PT	permeability transition
RE	restriction endonuclease
rpm	revolutions per minute
^{35}[S]	radioisotope of sulphur
SC	synthetic complete
SC-	synthetic complete minus
SDS	sodium dodecyl sulfate
TAE	tris/acetate (buffer)
Taq	*Themus aquaticus* DNA (polymerase)
TE	Tris/EDTA (buffer)
TEMED	N,N,N',N'- tetramethylethylene diamine
TM	transmembrane
T_m	melting temperature; thermal denaturation
Tris	Tris (hydroxymethyl) aminomethane
Tris-HCl	Tris hydrochloride
Tween-20	Polyoxyethylenesorbitan
UV	ultraviolet
V	volts
v/v	volume/volume
w/v	weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D- galactopyranoside
YPAD	yeast extract-peptone-dextrose medium plus adenine
$ZnCl_2$	zinc chloride

1. Introduction

The development of multicellular organisms and the maintenance and renewal of differentiated cell types relies on the balance between two highly regulated processes: cell proliferation and cell death. While the complexity of regulating cell proliferation is well established, the complexity of regulating cell death is rapidly emerging. In multicellular organisms, differentiated cells all appear to have the ability to carry out their own death through a genetically encoded cell death program (Raff 1992). When activated, this intrinsic program of cell death initiates a characteristic form of cellular demise called apoptosis that occurs in a predictable and reproducible manner (Kerr *et al.*, 1972; Wyllie *et al.*, 1980; Thornberry and Lazebnik 1998). The term apoptosis taken from the ancient Greek, meaning, "falling off" of leaves or petals was adopted by Kerr *et al.*, (1972) to describe a morphologically distinct form of cell death. Apoptosis results in the loss of cell volume, membrane blebbing, chromatin condensation and DNA fragmentation. The dying cell is fragmented into apoptotic bodies that are removed by neighbouring cells (Cohen 1993). Specifically, this type of regulation allows for the elimination of: (1) cells that are produced in excess (2) cells that have developed improperly or (3) cells that have sustained genetic damage. Therefore, disruptions in the cell death pathway contribute to the pathology of various human diseases. For instance, mutations that lead to the suppression of cell death have been associated with cancer and viral infections as well as autoimmune disorders such as lupus erythematosus and rheumatoid arthritis. On the other hand, loss of control over apoptosis can lead to excessive cell death apparent in acquired immunodeficiency induced by HIV (human immunodeficiency virus), neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's disease and ischemic injury such as stroke or myocardial infarction (Thompson 1995; Rudin and Thompson 1997). Both genetic and biochemical studies

from nematodes to mammals reveal the components of the apoptotic pathway are highly conserved through metazoan evolution (Bargmann and Horvitz 1991; Ellis *et al.*, 1991; Steller 1995). Herein, particular emphasis will be placed on the Bcl-2 family of proteins which constitutes one of the most biologically relevant classes of cell death regulators, particularly highlighting the structural elements that contribute to their role in inhibiting or promoting cell death.

1. 1. The genetic and molecular framework of the apoptotic pathway in the nematode: *Caenorhabditis elegans*

Early studies in the nematode *Caenorhabditis elegans* established the genetic framework for the apoptotic pathway and enabled further biochemical characterization of critical components. Horvitz and colleagues recognized that the apparent cell deaths in *C. elegans* were a normal, genetically determined part of development. They went on to identify the genes required either to inhibit or to execute programmed cell death and ordered the genes into a specific pathway using cell death mutant strains of *C. elegans* (Horvitz 1999).

The *C. elegans* hermaphrodite is amenable for studying cell death because it is small (1 mm length) and transparent. It also has a very short generation time of 3 days. During development, 1090 somatic nuclei are generated from the fertilized egg. Specifically of the 131 (12%) cells that undergo programmed cell death, 113 occur during embryogenesis and the remainder occurs during larval development, leaving 959 somatic cells in the mature adult nematode. The cell division occurs in a predictable pattern, allowing each cell lineage to be described and mutations affecting the pattern of programmed cell death to be identified. For example, some mutations cause cells that normally survive to die or conversely, those that normally die to survive. Other mutations led to no cell death or resulted in the abnormal appearance of dying cells. Through

detailed mutational analysis, the function of 13 genes have been defined and ordered into four stages that comprise the apoptotic pathway (Desnoyers and Hengartner 1997; Horvitz 1999).

In the first stage, following exposure to an intracellular or extracellular death signal, each cell type initiates an appropriate response. If the cell is committed to die, the signals are transmitted along the cell death pathway activating genes involved in the execution phase and irreversibly compromising the integrity of the cell. This is followed by the engulfment of the cell corpses and finally degradation of the cellular debris. The latter three steps (execution, engulfment and degradation) are common to apoptosis in all cell types (Desnoyers and Hengartner 1997; Horvitz 1999).

Genetic analysis in *C. elegans* revealed the core components of the execution phase are encoded by the genes: CED-3, CED-4, CED-9 (ced, cell death abnormal) and EGL-1 (egg laying abnormal) (Figure 1). These four genes control all 131 developmental programmed cell deaths in *C. elegans* (Hengartner and Horvitz 1994a and 1994b; Conradt and Horvitz 1998). The expression of CED-3 and CED-4 is required, as loss-of-function mutants of either gene prevents the death of nearly all cells that are normally programmed to die (Ellis and Horvitz, 1986). Homozygous EGL-1 loss-of-function mutants, not only prevented the cell death of two specific neurons in *C. elegans*, but most of the 131 cells that are normally eliminated during programmed cell death (Conradt and Horvitz 1998). CED-9 functions to protect cells from programmed cell death. Mutations which decrease or eliminate CED-9 expression cause cells that normally survive to instead die, suggesting the absence of CED-9 increases the killing efficiency of CED-3 and CED-4. Moreover, mutations in CED-3 and CED-4 are able to block ectopic cell deaths and lethality in mutants lacking CED-9 (Hengartner *et al.*, 1992). In this context, mutations in EGL-1 will

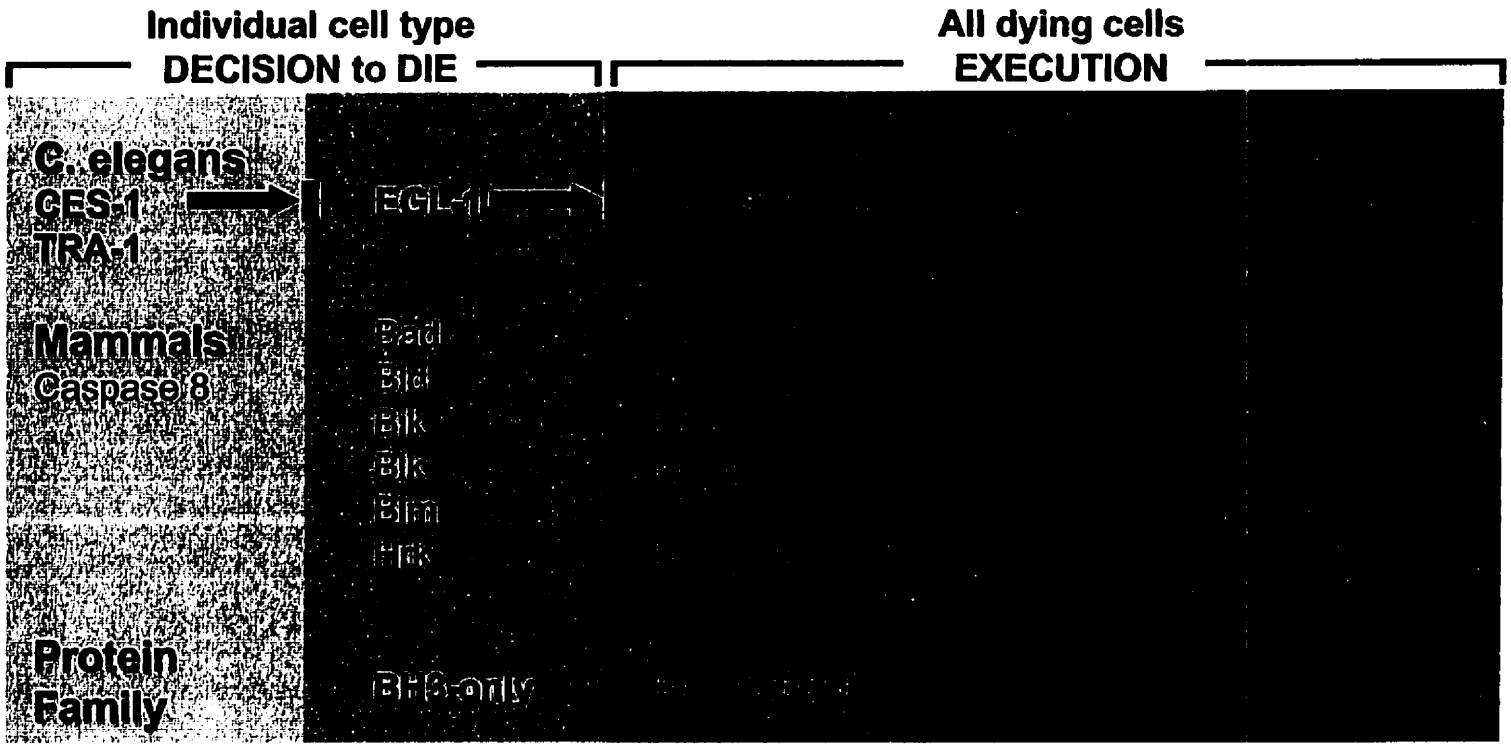

Figure 1. Apoptotic regulatory genes conserved through evolution. Genes encoding the apoptotic pathway are evolutionarily conserved from *C. elegans* to mammals. The pathway is divided into four stages. Following the reception of a death signal, the cell must decide whether to orchestrate its own demise. Once committed to initiating the apoptotic pathway, the execution stage is activated, followed by engulfment and degradation to remove cellular debris. *(Adapted from Nagata 1999a)*

neither block ectopic cell deaths or suppress the lethal phenotype of CED-9 loss of function mutants (Conradt and Horvitz 1998). Taken together, the model that emerges is as follows: (1) EGL-1 is placed upstream of CED-9 because it acts to inhibit its function (Conradt and Horvitz 1998). (2) CED-9 acts as an inhibitor of CED-4 by preventing it from activating the death inducing properties of CED-3 (Shaham and Horvitz, 1996) (3) CED-4 activates CED-3 and the execution stage of the apoptotic pathway (Xue *et al.*, 1996).

The model of programmed cell death from genetic analysis in *C. elegans* is complemented with data from biochemical studies. By co-immunoprecipitation and yeast two-hybrid assays, CED-9 was found to interact with CED-4 (Chinnaiyan *et al.*, 1997; James *et al.*, 1997; Spector *et al.*, 1997; Wu *et al.*, 1997a and1997b) and EGL-1 (Conradt and Horvitz 1998). CED-4 was observed to interact homologously as well as with the inactive form of CED-3 (Hu *et al.*, 1998b; Srinivasula *et al*, 1998; Yang *et al.*, 1998b). Although, CED-9 was not observed to interact with the latent form of CED-3, it was found in complex with inactive CED-3 and CED-4 (Chinnaiyan *et al.*, 1997). Thus, following an apoptotic signal and commitment to activating the cell death pathway, EGL-1 associates with CED-9 releasing CED-4. Then, the free CED-4 is able to oligomerize and facilitate proteolytic processing of latent CED-3 to its active form (Hu *et al.*, 1998b; Srinivasula *et al*, 1998; Yang *et al.*, 1998b). Once activated, CED-3 a cysteine protease brings about the disassembly of the cell by: (1) activating proteins that function to kill cells, (2) inactivating proteins that function to protect cells and/or (3) inactivating proteins involved in cellular homeostasis (Horvitz 1999).

During development of *C. elegans*, CED-9 expression is detectable from the embryonic stage, diminishing near the time of hatching and undetectable at the larval and adult stages. CED-4 is detectable before the first programmed cell deaths and

persists throughout embryogenesis, but is not apparent in the larval and adult stages (Chen *et al.*, 2000). Subcellular staining for either CED-9 or CED-4 reveals a pattern consistent with mitochondrial localization (Wu *et al.*, 1997b; Chen *et al.*, 2000). The loss-of-function of proteins required for developmental cell death, CED-3, CED-4 and EGL-1 did not disrupt the expression pattern or mitochondrial localization of CED-9. Similarly, the expression and localization of CED-4 remained unaffected by loss-of-function mutants in CED-3 and CED-4. However, the absence of CED-9 expression, alters the localization of CED-4 shifting from the mitochondria to nuclear membranes, suggesting a signal that induces programmed cell death would promote redistribution of CED-4 (Chen *et al.*, 2000).

In vertebrates, entire gene families have evolved to resemble the *C. elegans* death genes (Figure 1). The mammalian counterpart of CED-3 is the family of cysteine aspartyl proteases, commonly referred to as caspases (Alnemri *et al.*, 1996). The apoptotic protease activating factor 1, Apaf-1 (Zou *et al.*, 1997) and the recently identified Nod1/CARD4 (Bertin *et al.*, 1999; Inohara *et al.*, 1999) found in mammalian cells share homology with CED-4. The Bcl-2 family of genes originally identified as repressors of cell death share structural and functional similarity to CED-9 (Hengartner and Horovitz 1994a and 1994b; Adams and Cory 1998; Chao and Korsemeyer 1998; Minn *et al.*, 1998; Reed 1998). A branch of the Bcl-2 family that promotes apoptosis shares structural and functional similarity to EGL-1 (Conradt and Horvitz 1998; Kelekar and Thompson 1998). Among the three families of cell death regulators, the nomenclature for newly characterized proteins belonging to the CED-9 or CED-4 class of proteins remains somewhat arbitrary. Currently, the acronyms originate from the laboratory where the protein is first characterized, often reflecting either function, heterodimerizing partner(s)

and/or shared homology to other family members. Therefore, for brevity, only selected acronyms are explained in text.

1.2. The mammalian apoptotic pathway

The path to apoptotic cell death is embarked upon when the cell senses a death signal. Some of these signals include ligation of cell surface receptors such as Fas and TNFR1 that belong to the Tumour Necrosis Factor Receptor family (TNFR), others include growth factor withdrawal, viral infection, inappropriate oncogene activation, cytotoxic T-cell killing, drug treatments and other events that perturb normal cellular function (Ashkenazi and Dixit 1998; Nagata 1999b). Depending on the specific trigger and particular cell type, one of several signalling pathways is activated in the initiation phase. One of the major checkpoints these pathways converge on is the ratio of Bcl-2 family members that determines the apoptotic threshold (Oltvai *et al.*, 1993). Downstream of this checkpoint, the apoptotic pathway proceeds through two execution programs: the cascade of caspase activation or mitochondrial dysfunction. Both of these pathways and their interplay have been extensively studied in detail (Ashkenazi and Dixit 1998; Nagata 1999b). Although Bcl-2-related proteins reside upstream of the irreversible cellular damage caused by caspases and/or mitochondrial dysfunction, the Bcl-2 homologues also exert their effects at the level of the mitochondria preventing the cell from initiating its own demise or orchestrating an apoptotic response (Figure 2).

1.3. CED-3 orthologues: the caspase family

Two findings firmly implicated cysteine proteases as critical components of the cell death machinery. First, the discovery that CED-3 plays a role in promoting developmental cell death in *C. elegans* and secondly, its shared sequence homology (~29%) with the mammalian cysteine protease, interleukin-1β-converting enzyme (ICE/caspase 1) (Yuan

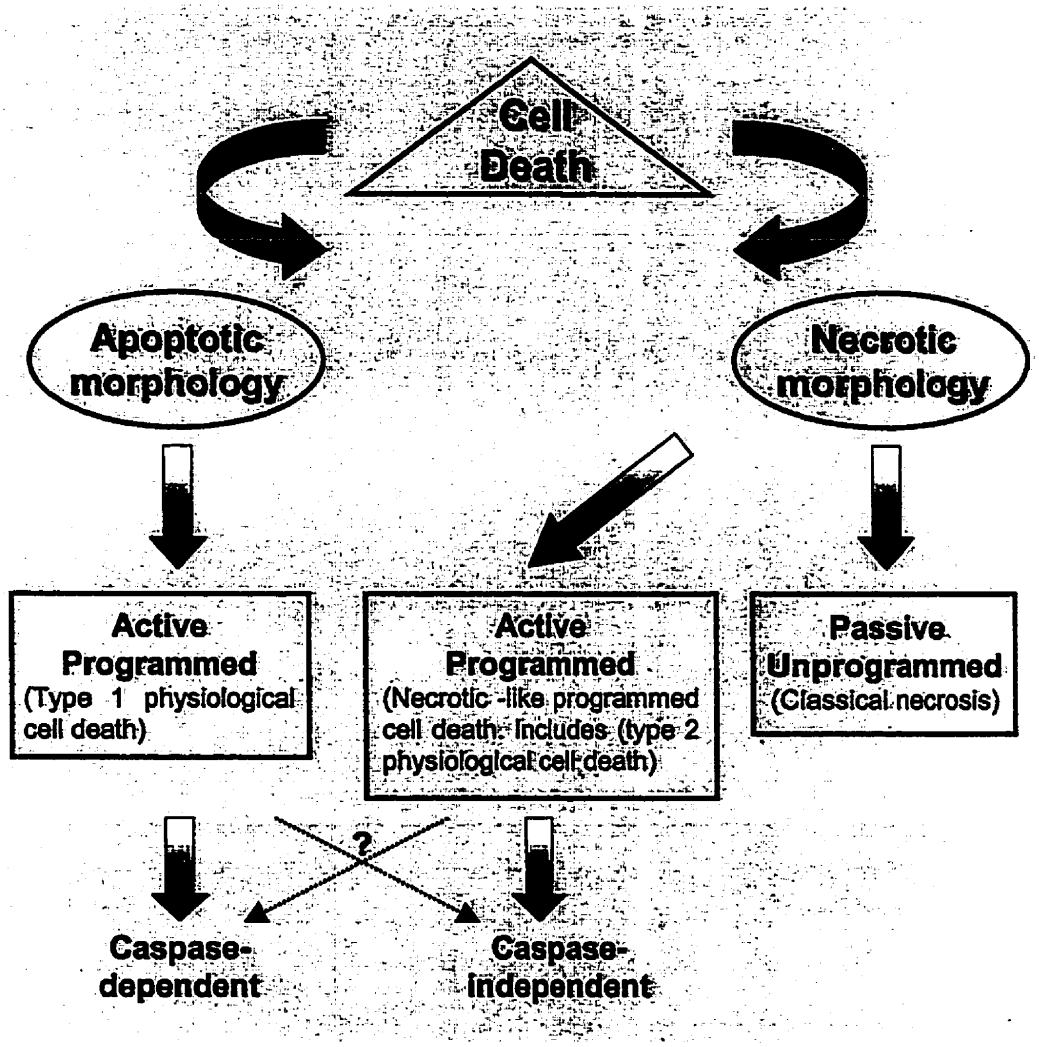

Figure 4. The morphological and functional classification of cell death. The cell deaths evident during development and in maintaining cellular homeostasis are broadly categorized into two morphological groups, 'apoptosis' and 'necrosis'. These processes are further classified into 'programmed' and 'unprogrammed' according to the presence or absence of encoded regulatory mechanisms. Insight from biochemical studies of cell death subdivides 'programmed' cell death depending on the role of caspases in disassembly of the cell. *(Adapted from Kitanaka and Kuchino 1999)*

et al., 1993). Several lines of evidence indicate that caspases play an important role in the apoptotic pathway. The ectopic expression of caspases in mammalian cells induces apoptosis. Moreover, the proteolytic processing and activation of caspases correlates with the onset of apoptosis and inhibition using peptides to block the active site attenuates the process (Bump *et al.*, 1995; Tewari *et al.*, 1995; Nicholson and Thornberry 1997; Cohen 1997). Although the role of individual caspases in apoptosis is difficult to determine due to the existence of multiple caspases, further insight is evident from the phenotype of mice deficient in particular caspases.

The targeted disruption of caspase 1 and 11 leads to defective interleukin-1β-production, but normal development and minimal apoptotic defects (Kuida *et al.*, 1995; Wang *et al.*, 1998b). Similarly, caspase 2 deficient mice exhibit normal development, but cells from these mice demonstrate diminished or enhanced apoptosis depending upon their tissue of origin (Bergeron *et al.*, 1998). Animals deficient in caspase 3, 8 or 9 die perinatally because of profound defects in developmental programmed cell death, specifically caspase 3 or 9 deficiency results in abnormal brain development and caspase 8 deficiency impairs development of heart muscle (Hakem *et al.*, 1998; Kuida *et al.*, 1998; Varfolomeev *et al.*, 1998; Woo *et al.*, 1998). The early death of these caspase deficient mice hampers the analysis of the role of caspase 3, 8 and 9 in adult tissues. Nevertheless, what emerges from studying the phenotype of mice lacking specific caspases is their role in mammalian apoptosis is complex. Multiple caspases are likely to be co-expressed in the same tissue having redundant function and/or acting in concert to execute the apoptotic process in a cell-specific and signal-dependent manner (Bergeron *et al.*, 1998; Hakem *et al.*, 1998; Woo *et al.*, 1998).

Although caspase 1 has since been found not to have an obvious role in cell death, these observations led to the identification of an expanding family of caspases that play distinct roles in inflammation and apoptosis (Earnshaw *et al.*, 1999; Nunez *et al.*, 1998; Thornberry and Lazebnik 1998; Wolf and Green 1999) (Figure 3). To date, 14 mammalian caspases (caspase 1 to caspase 14) have been cloned and partially characterized. The caspase 1 subfamily includes caspases 1, 4, 5 and 13 which are predominantly involved in the control of inflammation (Thornberry and Lazebnik 1998; Earnshaw *et al.*, 1999). The second subfamily includes caspases 3, 6, 7, 8, 9 and 10 which are involved primarily in apoptosis (Thornberry and Lazebnik 1998; Earnshaw *et al.*, 1999). Caspase 2 bears some resemblance to the caspase 1 subfamily and is involved in certain types of apoptosis, thus possibly defining a third subfamily (Bergeron *et al.*, 1998). The genes encoding human caspases span the entire genome, mapping to chromosomes 1, 2, 4, 10 and 11 (Online Mendelian Inheritance in Man available at http://www.ncbi.nlm.nih.gov/omim/). Caspase orthologues have also been identified and cloned in *Drosophila melanogaster* (Fraser *et al.*, 1997; Song *et al.*, 1997) and the lepidopteran *Spodoptera frugiperda* (Ahmad *et al.*, 1997).Members of the caspase family share similarities both in sequence and structure. The caspases are expressed as an inactive polypeptide ranging in molecular mass from 30 kDa to 50 kDa. The protein is divided into four distinct domains: (1) the NH_2-terminal region or prodomain (2) a large subunit (~20 kDa) containing the active site cysteine with conserved QACXG motif, (3) a small subunit (~10 kDa) and (4) a linker region between the large and small subunits flanked by aspartic acid residues (Figure 3). The activation of caspases involves proteolytic processing between the two subunits to remove the prodomain and linker regions allowing for the large and small subunits to heterodimerize. The crystal structure of two active caspases (caspases 1 and 3) reveal the small and large

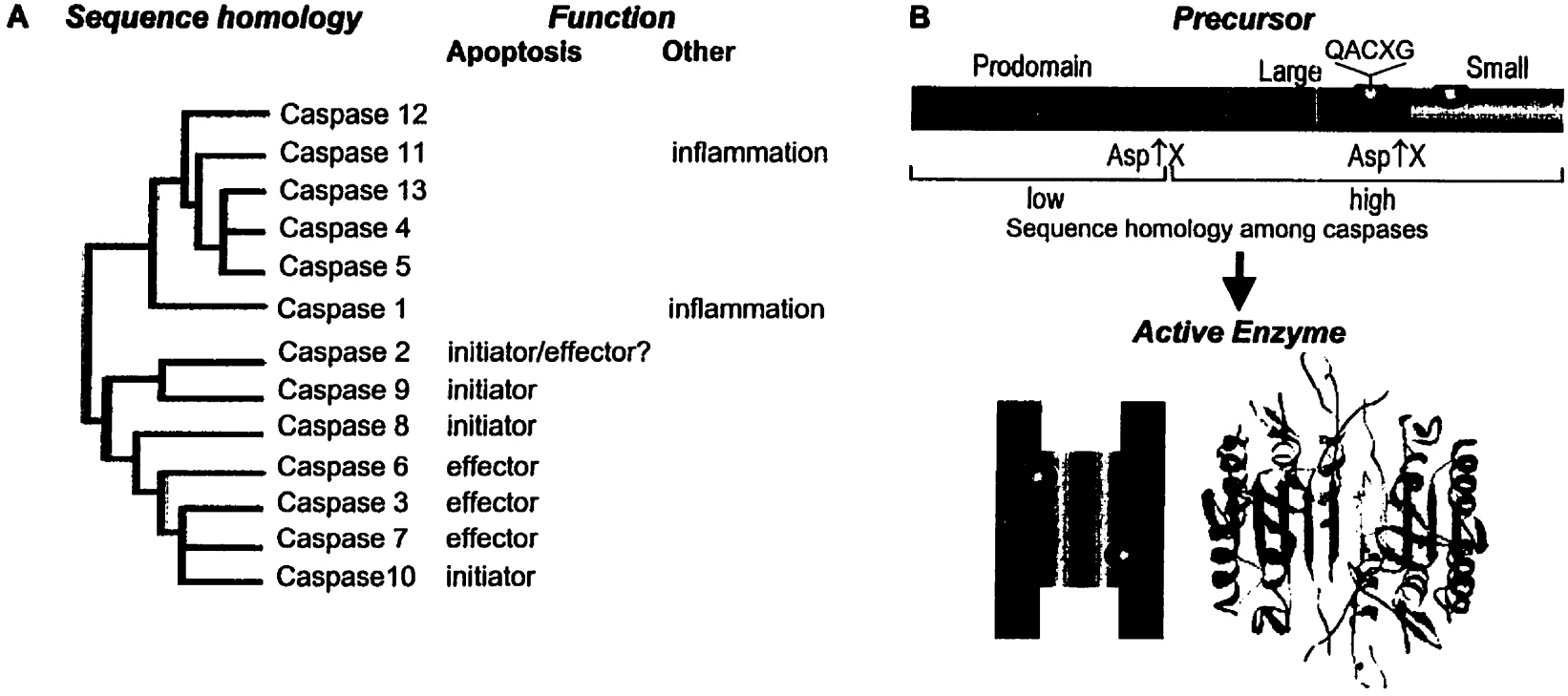

Figure 3. The caspase family: structure and function. A, Caspases have been cloned and characterized from *C. elegans* to mammals. To date, the family contains 14 members (caspase 1 to caspase 14) that play distinct roles in apoptosis and inflammation. Murine caspases 11, 12 and 14 have no known human counterparts. In apoptosis, upstream regulatory events initiate the proteolytic cleavage of latent initiator caspases leading to the systematic disassembly of the cell by effector caspases. B, Caspases are synthesized as precursors that undergo proteolytic processing at conserved aspartic acid residues occurring between the prodomain and the large subunit and between the large subunit and small subunit. The NH_2-terminal prodomain shares low sequence homology and is highly variable in length (23 to 216 amino acid residues). The region acts as a regulatory domain for initiator and effector function of caspases. The activated caspase consists of the large subunit and the small subunit, each contributing amino acid residues to the active site. The crystal structure of caspase 3 complexed to a tetrapeptide aldehyde inhibitor shows two large and small subunit heterodimers complexed together to form a tetrameter with two independent active sites. *(Adapted from Thornberry and Lazebnik 1998)*

subunit heterodimer associates together to form a tetrameter with two catalytic sites. More specifically, the large and small subunits within each heterodimer interdigitate to form a core composed of a six-stranded β-sheet flanked by α-helices such that both heterodimers contribute residues necessary for substrate binding and catalysis (Walker *et al.*, 1994; Wilson *et al.*, 1994; Mittl *et al.*, 1997; Rotanda *et al.*, 1996) (Figure 3).

Two structural features of the caspase precursor molecule are critical for its mechanism of activation. First, caspases can be classified into two groups based on sequence homology of the NH_2-terminal prodomain. The prodomain of nematode CED-3 and mammalian caspases 1, 2, 4, 5, 9, 11 and 12 contain a region termed the caspase recruitment domain (CARD), whereas caspases, 8 and 10 contain two tandem repeats of a motif referred to as the death effector domain (DED). Although the sequence between CARDs and DEDs are unrelated, NMR (nuclear magnetic resonance) structural analysis reveals the two motifs share similar tertiary structure composed of highly packed bundles of six α-helices. Therefore, either hydrophilic CARD or hydrophobic DED facilitates interactions of caspases with each other and a wide range of regulatory and adapter proteins (Huang *et al.*, 1996; Chou *et al.*, 1998; Eberstadt *et al.*, 1998). Secondly, the caspase precursors can be subdivided based on the length of their prodomain and role in executing the apoptotic pathway. For example, caspase 2, 8, 9 and 10 having longer prodomains, act as initiators of the apoptotic pathway, upstream of the small prodomain effector caspases 3, 6, 7 and 14 (Earnshaw *et al.*, 1999; Thornberry and Lazebnik 1998).

The caspases are very specific proteases, having an absolute requirement for substrate cleavage at the COOH-terminal side of aspartic acid residues. Further specificity is conferred by four amino acids NH_2-terminal to the cleavage site. The preferred tetrapeptide recognition motif differs significantly among caspases, correlating with their

function as initiators, executioners or cytokine processors. Although a substrate may contain the optimal tetrapeptide sequence, not all are cleaved implying that its topology also influences overall substrate recognition (Thornberry *et al.*, 1997; Thornberry and Lazebnik 1998). Once activated, caspases cleave a select set of proteins in a co-ordinated manner that translates into morphological changes such as cytoplasmic shrinkage, plasma membrane blebbing and vesiculation, chromatin condensation and DNA fragmentation. *In vivo*, this process culminates with the engulfment of the apoptotic bodies by surrounding cells to prevent release of intracellular contents. These changes occur in a predictable and reproducible manner within 30 to 60 minutes following an apoptotic trigger (Thornberry and Lazebnik 1998). Therefore, the identification of potential caspase substrates offers insight into how the activated caspases orchestrate significant alterations within the cell leading to its demise. The number of caspase substrates identified is increasing steadily. Many have been broadly categorized into five groups based on their putative function within the cell (Earnshaw *et al.*, 1999; Wolf and Green 1999).

After receiving a death signal, the first targets within the cell are latent caspases and Bcl-2 homologues. Proteolytic processing of these proteins either promotes signal amplification or inactivates their normal function. As the apoptotic signal permeates through the cell, caspases cleave other components of the apoptotic machinery that have a direct impact on the apoptotic phenotype. For example, the protein ICAD is an inhibitor of the nuclease CAD (caspase activated deoxyribonuclease) that is cleaved by caspases 3 and 7. The inactivation of ICAD by caspases, permits the released CAD to promote DNA fragmentation and chromatin condensation (Enari *et al.*, 1998; Liu *et al* ., 1997).

Next, caspases contribute to apoptosis by disassembling structural components of the nucleus and cytoskeleton. The nuclear lamins form a rigid structure underlying the

nuclear membrane and are involved in chromatin organization. Cleavage by caspases causes the nuclear lamina to collapse, thereby leading to chromatin condensation (Lazebnik *et al.*, 1995; Orth *et al.*, 1996; Rao *et al.*, 1996). One of the most abundant cytoskeletal proteins, actin and several of its regulators, fodrin and spectrin are cleaved by caspases during apoptosis (Mashima *et al.*, 1997; Brocksted *et al.*, 1998; Cryns *et al.*, 1996; Janicke *et al.*, 1998; Nath *et al.*, 1996; Wang *et al.*, 1998b). Gelsolin, a protein that regulates actin dynamics is activated by caspases promoting both cytoplasmic and nuclear apoptosis (Kothakota *et al.*, 1997; Kamada *et al.*, 1998). The cleavage of β-catenin (Brancolini *et al.*, 1997; Herren *et al.*, 1998) and focal adhesion kinase (FAK) may disrupt cell-cell contacts and cell-matrix focal adhesions (Crouch *et al.*, 1996; Wen *et al.*, 1997). Overall, the proteolysis of this class of substrates likely promotes cellular packaging and subsequent engulfment by neighbouring phagocytes (Wolf and Green 1999).

The fourth class of substrates encompasses those involved in maintaining cellular homeostasis. These include kinases important for cell growth and survival. Caspase-dependent proteolysis of kinases promotes degradation of Akt-1 and Raf-1 (Widmann *et al.*, 1998). There are numerous reports implicating several components of the DNA replication and repair machinery that are targeted by activated caspases. These include poly(ADP-ribose) polymerase (PARP), the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) and DNA replication factor protein RF-C140. Although, the cleavage of theses substrates in relation to cell death has not been fully elucidated, it is likely the proteolysis of proteins critical for homeostatic and repair functions leads to cellular disassembly (Earnshaw *et al.*, 1999; Wolf and Green 1999).

The final class of substrates include proteins implicated in inherited neurodegenerative diseases such as huntingtin (Huntington's disease, Goldberg *et al.*,

1996; Wellington *et al.*, 1998), presenilins and amyloid precursor protein (Alzheimer's disease, Loetscher *et al.*, 1997; Kim *et al.*, 1997; Grunberg *et al.*, 1997; Vito *et al.*, 1997; Barnes *et al.*, 1998), atrophin-1 (dentatorubropallidoluysian atrophy, Miyashita *et al.*, 1997; Wellington *et al.*, 1998). Other potential substrates include the androgen receptor implicated in spinal muscular atrophy and ataxin-3 leading to spinocerebellar atrophy type 3 (Wellington *et al.*, 1998). Some of these proteins are cleaved during apoptosis, but may also be subject to early cleavage by low endogenous activity of latent caspases, thus generating toxic protein fragments that act as precursors to the progression of neurodegenerative disease (Wellington *et al.*, 1998).

The fact that caspase precursors are constituitively expressed in living cells, but can rapidly induce apoptosis, implies these proteases are efficiently regulated. Different initiator caspases mediate distinct sets of signals. For example, caspase 8 is associated with apoptosis involving death receptors expressed at the cell surface (Ashkenazi and Dixit 1998; Nagata 1999b) and caspase 9 is involved in death induced by cytotoxic agents (Hakam *et al.*, 1998; Kuida *et al.*, 1998). Once activated caspases 8 and 9 can cleave and activate an overlapping set of effector caspases including caspase 3, 6 and 7 thereby enhancing protease activity within the cell (Takanashi *et al.*, 1996; Fajeiro *et al.*, 1997; Li *et al.*, 1997a; Stennicke *et al.*, 1999). The presence or absence of particular caspases as well as their level of expression and cellular distribution vary from cell type to cell type. In healthy cells, latent caspase localize primarily to the cytosol such as 7 and 8, yet others such as caspase 2, 3 and 9 have been found in both the cytosol and intermitochondrial space (Susin *et al.*, 1999a; Zhivotovsky *et al.*, 1999). The compartmentalization of caspases and their co-factors is critical to regulating their activation. With the onset of apoptosis, some of the activated caspases redistribute throughout the cell to their site of action such as the plasma membrane or mitochondria. The activation of caspase

precursors proceeds by three mechanisms: (1) autoactivation (2) transactivation and/or (3) proteolysis by other proteinases (Thornberry and Lazebnik 1998; Earnshaw *et al.,* 1999; Wolf and Green 1999).

Since all caspases have the same cleavage specificity, the most efficient and simplest way to activate latent caspases is via previously activated caspases, creating the opportunity for cascade amplification and positive feedback pathways (Wolf and Green, 1999). Therefore, initiator caspases could be further activated by effector caspases. Recent evidence suggests that caspase precursors have a low, but detectable level of proteolytic activity. Under appropriate conditions, these caspases have the potential to activate other latent caspases (Yamin *et al.* 1996; Muzio *et al.* 1998). *In vivo* studies have found that unusually high concentrations of caspase precursors promote autoactivation. Moreover, the overexpression of wild type caspases and not catalytically inactive mutants leads to caspase processing and activation (Orth *et al.* 1996b). Additionally, the forced oligomerization of caspases 8 and 9 as well as CED-3 promotes proteolytic processing and apoptosis (Martin *et al.,* 1998; Muzio *et al.,* 1998; Srinivasala *et al.,* 1998; Yang *et al.,* 1998a and 1998b). Thus, the propagation of the caspase cascade depends on which caspases are expressed, their relative concentrations within the cells as well as their kinetic efficiency of proteolytic processing. Other factors include the tertiary structure of latent caspases and the relative concentration and availability of cofactors (Thornberry and Lazebnik 1998; Earnshaw *et al.,* 1999).

Adapter molecules link the death receptors and mitochondria to latent caspases. The ligation of the death receptors Fas/TNFR1 at the plasma membrane recruits proteins to the death-inducing signalling complex or DISC (Medema *et al.* 1997). The activation of caspase 8 requires association with its co-factor, FADD (Fas-associated protein with death

domain) through its DED motif (Boldin *et al.* 1996; Muzio *et al.*, 1996). The aggregation of caspase 8 precursors at the DISC promotes auto-processing and activation (Muzio *et al.* 1998; MacCorkle *et al.*, 1998; Martin *et al.*, 1998; Yang *et al.* 1998a). Latent forms of caspase 9 become activated by forming a complex with co-factor Apaf-1 through their respective CARD motif (Li *et al.*, 1997b). Apaf-1 normally exists in a closed conformation. The binding of cytochrome *c* and dATP/ATP induces a conformational change that exposes its CARD motif for interaction. The subsequent recruitment and oligomerization of latent caspase 9 facilitates its autoactivation (Li *et al*, 1997b). Thus, *in vivo*, adapter proteins serve to recruit caspase precursors by restricting their mobility thereby increasing their local concentrations (Thornberry and Lazebnik 1998; Earnshaw *et al.*, 1999).

Some non-caspase proteases have been identified to promote proteolysis of caspase precursors leading to cell death. The most well known example is the protease granzyme B, a serine protease that specifically cleaves after aspartic acid residues. Cytotoxic T lymphocytes and natural killer cells destroy their target cells through granule exocytosis whereby the pore-forming protein, perforin inserts into the plasma membrane of the target cell allowing granzymes A and B to enter (Shi *et al.*, 1996). Once in the cell, granzyme B specifically can activate several caspases, such as effector caspase 3 and 7 inducing cell death (Darmon *et al.*, 1995; Shresta *et al.*, 1995; Greenberg 1996; Simon *et al.*, 1997; Zhou and Salvesen 1997).

Other potential mechanisms to control activation of latent initiator caspases involve inhibiting the interaction between caspase precursors and their activators. For example, viruses have evolved mechanisms to counter the effects of activated caspases. Four distinct classes of viral caspase inhibitors have been identified that attenuate apoptosis thereby preventing the host's normal response to infection. Several herpes viruses encode

a protein termed v-FLIP (viral FADD-like inhibiting protein) (Irmler *et al.*, 1997; Shu *et al.*, 1997). Similar to its mammalian cellular orthologue, cFLIP, these inhibitors share sequence homology with the caspase 8 precursor, but, lack the essential catalytic residues. Therefore, FLIPs may compete with inactive caspase 8 for binding to its cofactor FADD, thus limiting caspase activation (Hu *et al.*, 1997; Inohara *et al.*, 1997b; Srinivasula *et al.*, 1997; Goltsev *et al.* 1997). The product of the cowpox virus, CrmA binds and inactivates caspases, most notably caspase 1 and 8 (Ray *et al.*, 1992; Zhou *et al.*, 1997). CrmA acts as a pseudosubstrate that binds active caspases to form an inhibitory complex and releases cleaved CrmA polypeptides. Another pseudosubstrate, the baculovirus p35 protein is cleaved by activated caspases. Although the selectivity of p35 for caspases is not well defined, the cleaved p35 subunit remains in an inhibitory complex with activated caspases (Bump *et al.*, 1995; Xue and Horvitz 1995; Bertin *et al.*, 1996). Another class of inhibitors is the family of IAP (inhibitors of apoptosis) proteins originally identified in the baculovirus, of which several mammalian members have been characterized (Deveraux and Reed 1999). The precise targets of IAPs as well as their mechanism of inactivation remain elusive, though selective inhibition has been observed *in vitro* for caspase 3 and 7 (Deveraux *et al.*, 1997; Deveraux and Reed 1999).

1.3.1. Caspase-independent cell death pathways

The increasing interest towards the biochemical characterization of apoptosis has quickly uncovered that not all 'cell deaths' are identical (Fiers *et al.*, 1999; Kitanaka and Kuchino 1999). Early morphological studies by electron microscopy identified three pathways for programmed cell death (Figure 4). The typical features of apoptosis include nuclear and cytoplasmic condensation. Further characterization showed these changes were also accompanied by membrane blebbing, DNA fragmentation and formation of apoptotic bodies. The second type of cell death referred to as necrosis is defined by

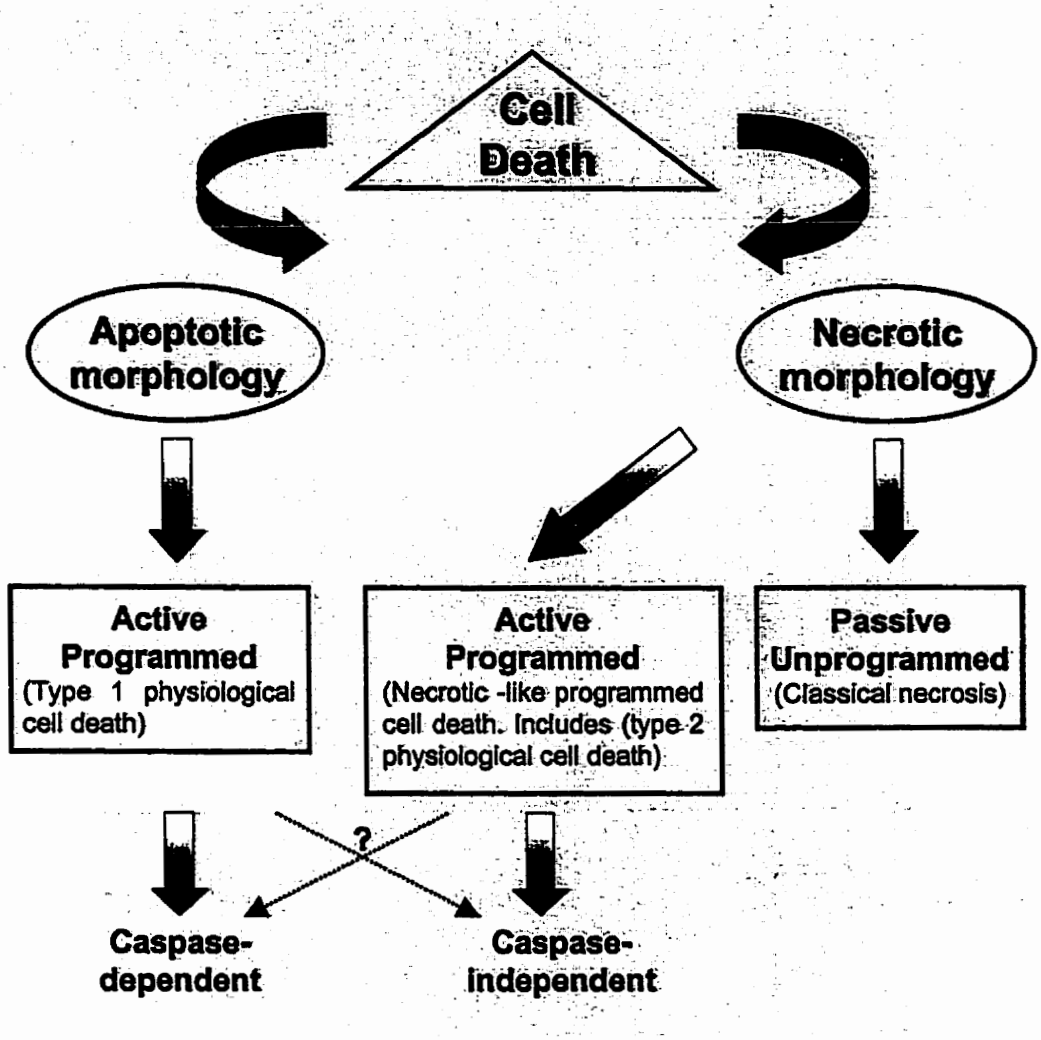

Figure 4. The morphological and functional classification of cell death. The cell deaths evident during development and in maintaining cellular homeostasis are broadly categorized into two morphological groups, 'apoptosis' and 'necrosis'. These processes are further classified into 'programmed' and 'unprogrammed' according to the presence or absence of encoded regulatory mechanisms. Insight from biochemical studies of cell death subdivides 'programmed' cell death depending on the role of caspases in disassembly of the cell. *(Adapted from Kitanaka and Kuchino 1999)*

abundant autophagic vacuoles and minimal nuclear damage. Other changes include loss of cell membrane permeability resulting in extensive cellular swelling and ultimately cellular lysis. The third type of cell death is a much rarer form of necrosis. Apart from the morphological differences between 'apoptosis' and 'necrosis', differences are also apparent in mitochondrial function and caspase activation. In apoptosis, mitochondrial dysfunction acts as an amplification step, whereby the release of intermitochondrial proteins such as cytochrome *c* activates the caspase cascade (Liu *et al.*, 1996) and apoptosis inducing factor (AIF) triggers chromatin condensation and fragmentation (Susin *et al.*, 1999b). Necrosis is accompanied by mitochondrial swelling that leads to an energy catastrophe, but no apparent caspase activation. Further mitochondrial dysfunction in both apoptosis and necrosis include opening of membrane permeability transition (PT) pores, changes in membrane potential ($\Delta\Psi m$) and production of reactive oxygen species (ROS) (Figures 4 and 5) (Fiers *et al.*, 1999; Kitanaka and Kuchino 1999).

The activation of caspases is a critical step to cellular disassembly that cleave cytoplasmic and nuclear substrates that give rise to the described apoptotic morphology. However, an increasing number of examples are being described in which the inhibition of caspase activity blocks the progression of apoptotic morphology, but not cell death itself. In mammalian cells, this phenomenon was first described by expression of Bax, a cell death promoting Bcl-2 homologue. The presence of caspase inhibitors prevented the apoptotic morphology, yet the cells died exhibiting a 'necrotic-like' phenotype characterized by cytoplasmic vacuolation and partial chromatin condensation (Xiang *et al.*, 1996). A closer examination of Bax-induced cell death revealed that it can exert its cytotoxic effect on cells through either caspase-dependent and/or independent pathways (Miller *et al.*, 1997; Kitanaka *et al.*, 1997).

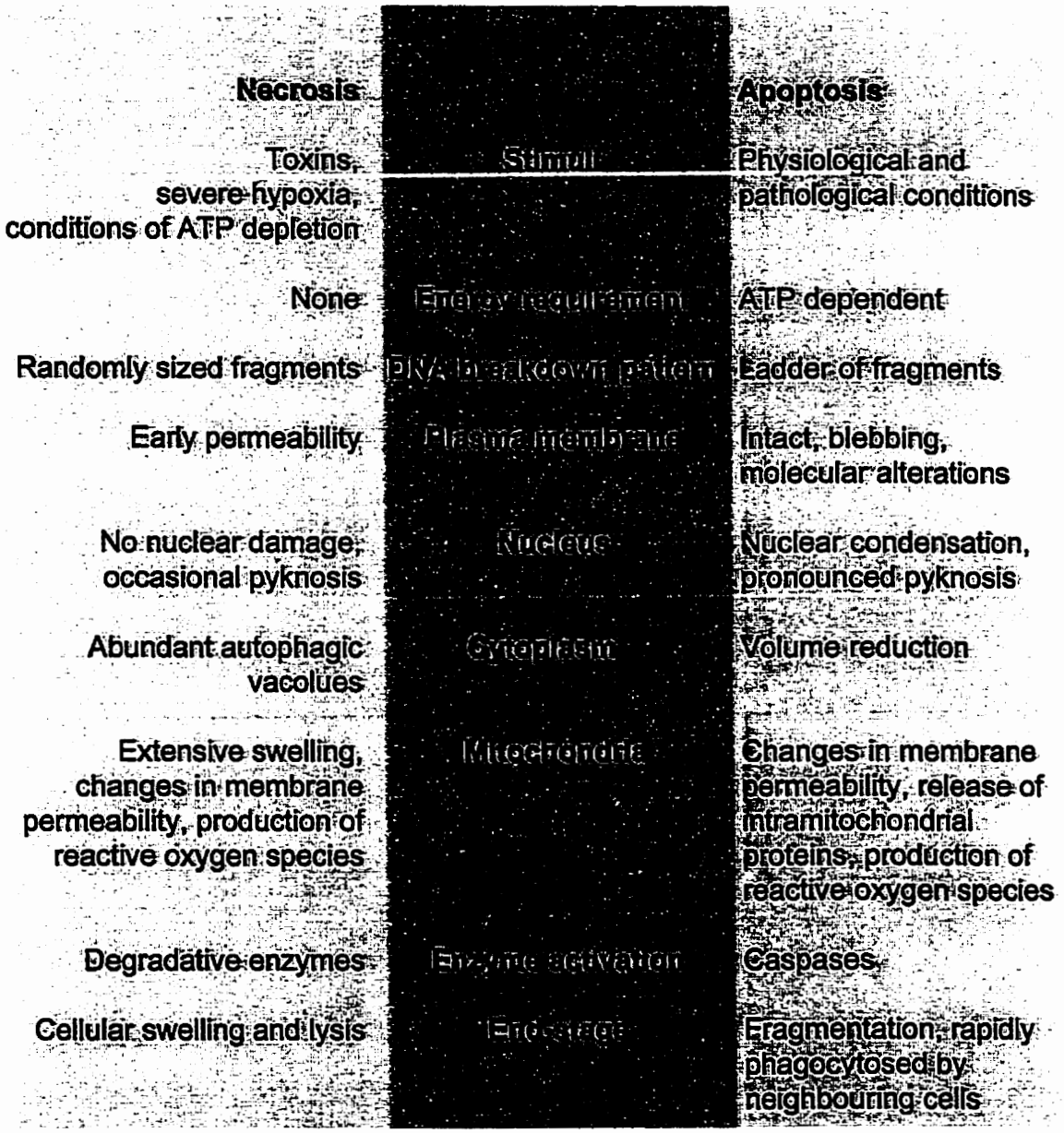

Figure 5. The cardinal features of apoptosis and necrosis. *(Adapted from Hetts 1998; Kitanaka and Kuchino 1999)*

The ability to trigger a "non-apoptotic" cell death is not only unique to Bax, other examples include the Bcl-2 homologue, Bak (McCarthy *et al.* 1997) and Mtd/Bok (Inohara *et al.*, 1998b), oncogene products such as Myc (McCarthy *et al.*, 1997) and Ras (Chi *et al.*, 1999) and the adenoviral protein E4ORF4 (Lavoie *et al.*, 1998). Treatment of cells with chemotherapeutic drugs such as dexamethasone (Brunet *et al.*, 1998), staurosporine (Amarante-Mendes *et al.*, 1998) or etoposide (Hirsch *et al.* 1997) also leads to cell death characterized by vacuolization and a lack of pronounced chromatin condensation. The treatment of cells with antibodies such as anti-CD2 (Deas *et al.*, 1998) or anti-Fas (Vercammen *et al.*, 1998) in the presence of caspase inhibitors suppressed the apoptotic morphology, but not cell death. Similarly, enforced oligomerization of the adaptor protein, FADD involved in the Fas/TNFR1 death receptor pathway induced cell death with necrotic morphology (Kawahara *et al.*, 1998). In the presence of perforin and granzyme A, target cells died despite the treatment with caspase inhibitors (Beresford *et al.*, 1999).

The examples listed describe cell death morphology under *in vitro* conditions in which the caspases were artificially inhibited. Some examples of cell death that assume a necrotic morphology in a cell-type specific manner include tumour-necrosis factor (TNF)-induced cytotoxicity (Schulze-Osthoff *et al.*, 1994) and expression of the cell growth and tumour suppressor gene PML (promyelocytic leukaemia) (Le *et al.* 1998; Quignon *et al.*, 1998). The observations from *in vitro* experiments support the existence of cell death pathways that by-pass caspase activation leading to 'necrotic-like' cell death *in vivo*. The functional role of the proteins identified to date in regulating necrotic cell death *in vivo* either alone or in concert with the caspase-dependent cell death program remains to be determined. It is conceivable that apoptosis and necrosis represent two ends of the spectrum of programmed cell death. Although genes

regulating apoptosis are continually being defined, those regulating necrosis presently remain elusive, but will be forthcoming (Kitanaka and Kuchino 1999).

1.4. Orthologues of CED-4

In *C. elegans*, the ability of CED-4 to regulate the proteolytic activation of CED-3 and to synergize with CED-3 to induce apoptosis (Chinnaiyan *et al.*, 1997; Seshagiri and Miller 1997) strongly indicates that similar mammalian CED-4 orthologues must exist to mediate caspase activation. Structurally, the CED-4 protein contains a CARD motif and putative ATP-P-loop (Chinnaiyan *et al.*, 1997; Irmler *et al.*, 1997). It oligomerizes via its nucleotide-binding domain, a process that mediates proximity and activation of latent CED-3 (Yang *et al.*, 1998b).

The prediction that other CED-4 homologues must exist was confirmed by biochemical purification of a 130 kDa protein referred to as Apaf-1 that contains a stretch of 320 amino acids homologous to CED-4, sharing overall 22% identity and 48% similarity. The alignment between Apaf-1 and CED-4 includes Walker's A and B box consensus sequences for nucleotide-binding (Walker *et al.*, 1982; Zou *et al.*, 1997). The 85 amino acids at the NH_2-terminus share 21% identity and 53% similarity to the prodomain of CED-3. Specifically, this region contains the CARD motif, which is a common element of several cell death proteins. The COOH-terminal region of Apaf-1 is comprised of 12 conserved repeating units ending in Trp (W) and Asp (D), commonly referred to as WD repeats, which are proposed to mediate protein-protein interaction (Neer *at el.*, 1994; Zou *et al.*, 1997).

When transfected into mammalian cells, Apaf-1 does not induce cell death, rather, it lies in a dormant state. Diverse death stimuli trigger mitochondrial damage and release of cytochrome *c* into the cytosol. The released cytochrome *c* binds to Apaf-1 in

the presence of dATP/ATP inducing a conformational change to expose its NH_2-terminus CARD. The Apaf-1 sequesters latent caspase 9 through homophilic CARD-CARD interaction that results in caspase oligomerization and activation (Li *et al.*, 1997b; Srinivasula *et al.*, 1998). The WD repeat region is the site of cytochrome *c* engagement as its removal leads to constituitively active Apaf-1 *in vitro* independent of cytochrome *c* and dATP/ATP. The COOH-terminally truncated Apaf-1 interacts with itself by co-immunoprecipitation and yeast two-hybrid assays. The self-association of Apaf-1 via the nucleotide binding domain is an important step that promotes proximity and activation of latent caspase 9, similar to CED-4 regulation of CED-3 (Hu *et al.*, 1998b; Srinivasula *et al.*, 1998).

Taken together, these findings support the model whereby Apaf-1 must undergo conformational changes in dying cells to activate latent caspase 9. The hydrolysis of dATP/ATP by interaction with the nucleotide-binding domain, accompanied by cytochrome *c* binding to the WD-repeat region promotes multimerization of Apaf-1. Once the multimeric complex is formed, caspase 9 precursor molecules are recruited to the complex in a 1:1 ratio to Apaf-1. The close proximity of latent caspase 9 promotes its proteolytic processing. The activated caspase 9 is released from the complex, free to activate downstream caspases, thus allowing additional caspase 9 precursors to associate with the Apaf-1-cytochrome *c* complex (Zou *et al.*, 1997).

Orthologues to the *C.elegans* CED-4 have not been forthcoming particularly in mammals as well as other organisms. Unlike *C. elegans*, mice and humans have multiple caspases, suggesting that mammalian genomes must contain additional caspase activators other than Apaf-1. The deletion of Apaf-1 gene leads to an excessive number of neurons in the brain, defects in facial features and delayed recession of

interdigital webbing (Cecconi *et al.*, 1998; Yoshida *et al.*, 1998). Apaf-1 deficient mice lack abnormalities in tissues such as the thymus (Cecconi *et al.*, 1998; Yoshida *et al.*, 1998) where appropriate cellular development depends on apoptosis (Surh and Sprent 1994). Indeed, the observations from targeted gene disruption studies of Apaf-1 indicate the existence of additional Apaf-1-like molecules and/or alternative apoptotic pathways that are independent of Apaf-1 homologues (Nunez *et al.*, 1998).

Two groups have characterized Nod1/CARD4 that is proposed to belong to the CED-4/Apaf-1 class of proteins. Similar to Apaf-1, Nod1/CARD4 consists of an NH_2-terminal CARD sequence, followed by a nucleotide-binding domain. It lacks the WD repeats at the COOH-terminus, instead the region contains a leucine rich domain. Like CED-4 and Apaf-1, Nod1/CARD4 self associates and sequesters caspase precursors via their respective CARDs. Nod1/CARD4 favours interaction with caspases containing long prodomains, particularly augmenting the activation of latent caspase 9. Similar to Apaf-1 and CED-4, the deletion of both the CARD domain and nucleotide-binding domain diminishes its function. Nod1/CARD4 has another function whereby it induces NF-κB activity, thus, implying the protein acts in regulating caspase activation for cell death and regulating transcriptional activation for cell survival (Bertin *et al.*, 1999; Inohara *et al.*, 1999).

In the fruit fly, *Drosophila melanogaster*, two Apaf-1/CED-4-related caspase activators have been reported, Dark/Dapaf-1L which bears greater resemblance to Apaf-1 (Kanuka *et al.*, 1999; Rodriguez *et al.*, 1999) and HAC-1/Dapaf-1S which is more similar to CED-4 (Kanuka *et al.*, 1999; Zhou *et al.*, 1999a). In fact, HAC-1/Dapaf-1S is a splicing variant of the gene encoding dapaf-1 (Kanuka *et al.*, 1999). Structurally, the NH_2-terminus of Dark/Dapaf-1L contains a CARD-like motif that mediates interaction

with latent fly caspases, Dredd or Dronc similar to Apaf-1-caspase 9 interaction leading to activation of downstream caspases. Dark/Dapaf-1L shares homology with CED-4 over 320 amino acids to include the nucleotide-binding region, the Walker's A and B boxes thought to mediate essential dATP/ATP driven functions. At the COOH-terminus, there are two stretches of WD-repeats separated by 200 amino acids (Kanuka *et al.*, 1999; Rodriguez *et al.*, 1999). Dapaf-1 activates specific caspases in a cytochrome *c* dependent manner. The lack of Dapaf-1 leads to defects in cytochrome *c* dependent caspase activation. The Dark/Dapaf-1L mutant phenotype bears resemblance to Apaf-1 deficient mice. In both cases, the central nervous system appears to be preferentially affected and a decrease in apoptosis leads to hyperplasia of this tissue. Apart from reduced viability in loss-of-function Dark/Dapaf-1L mutants, other abnormalities include defects of the wings and/or bristles and ectopic melanotic tumours (Kanuka *et al.*, 1999; Rodriguez *et al.*, 1999), thought to arise from abnormalities in hematopoietic blood cells during larval growth or autoimmune defects (Watson *et al.*, 1991). Dapaf-1S is similar to CED-4 containing an NH_2-terminal CARD motif, followed by a nucleotide-binding domain (Kanuka *et al.*, 1999; Zhou *et al.*, 1999a). Dapaf-1S is active without cytochrome *c*, specifically binding to and activating another fly caspase, drICE, similar to CED-4-CED-3 interaction (Kanuka *et al.*, 1999).

The CARD motif is critical for CED-4 and its orthologues to sequester apical caspases and facilitate their proteolytic processing. CARD interactions occur between proteins involved in apoptosis regulation such as initiator caspase precursors, adaptor proteins and cell death inhibitors (Hofman *et al.*, 1997). *In vitro* binding assays reveal that although, proteins containing the CARD motif share the same tertiary fold, the preferred binding partners are highly specific. For example, the Apaf-1 CARD interacts

only with caspase 9 CARD, but not with other CARD-containing caspases (Zhou *et al.*, 1999b).

Several studies elucidated the three dimensional structure of the Apaf-1 CARD motif (residues 1-97) using combined X-ray crystallography and NMR methods (Day *et al.*, 1999; Qin *et al.*, 1999; Vaughn *et al.*, 1999; Zhou *et al.*, 1999b). The Apaf-1 CARD is composed of seven short α–helices, with the core six helices arranged in an anti-parallel manner. The region shows some variation from other previously characterized CARD motifs in number, length and orientation of the helices as well as the spacing between each α-helix. When the Apaf-1 CARD is complexed to caspase 9 CARD (residues 1-112), the two regions share striking overall topology, despite low sequence identity (~20%). The acidic surface of Apaf-1 CARD formed by second and third helices is directly involved in recognition and interaction with the basic surface of caspase 9, helices one and four. These two region are not only opposite in charge distribution, but complementary in shape whereby the concave surface of caspase 9 CARD fits the convex surface of Apaf-1 CARD. The initial interaction is predicted to be electrostatic. Then, the complex is stabilized through hydrophobic interactions at the interface between the two molecules. Mutations of critical interface residues in Apaf-1 and caspase 9 precursor demonstrate reduced or diminished interaction between the two proteins. Analysis of the *C. elegans* orthologues, CED-4 and CED-3 suggests, that the recruitment of CED-3 by CED-4 is mediated by the same set of conserved structural motifs (Qin *et al.*, 1999).

CED-4, Apaf-1 and Dark/Dapaf-1L each associate with and activate apical caspases. Yet, the orthologues identified to date in nematodes, mammals and *Drosophila* do not all share identical structural elements. Overall, Apaf-1 shares approximately 20% identity with both CED-4 and Dark/Dapaf-1L (Rodriguez *et al.*,

1999). The structural similarities tend to cluster to functional domains such as the CARD sequence, nucleotide-binding site and WD-repeats. These molecules have likely evolved cell-type specific mechanisms to regulate their role in caspase activation. All the molecules characterized demonstrate functional homology by sequestering latent caspases and promoting their catalytic processing in a dATP/ATP and/or cytochrome *c* dependent manner.

The presence of WD-repeats at the COOH-terminus is the most striking difference between the CED-4 orthologues. Generally, proteins containing WD-repeats are predicted to possess regulatory function, but not enzymatic activity (Neer *et al.*, 1994). Yet, both Apaf-1 and Dark/Dapaf-1L hydrolyze dATP/ATP to activate their function (Kanuka *et al.*, 1999; Rodriguez *et al.*, 1999). The WD-repeats in Apaf-1 and its fly counterpart represent the site of engagement for mitochondrial signals such as cytochrome *c*. These two proteins share homologous function by engaging cytochrome *c*. Furthermore, the apoptosis-inducing activity of cytochrome *c*, through Apaf-1/Dark/Dapaf-1L-like molecules, may be broadly conserved. The WD-region could be a recent acquisition or, alternatively this domain may have been lost from CED-4 and Dapaf-1S (Rodriguez *et al.*, 1999). Thus, Apaf-1 and Dark/Dapaf-1L, appear to share the most common ancestry and perhaps, truer orthologues of CED-4 are yet to be found in mammals.

1.5. The Bcl-2 family

The Bcl-2-related proteins constitute one of the most biologically relevant classes of cell death regulatory proteins. Originally, the Bcl-2 (B cell lymphoma/leukemia-2) gene was identified at the intrachromosomal breakpoint t(14;18) in human follicular B cell lymphoma (Bakhshi *et al.*, 1985; Cleary *et al.*, 1985; Tsujiuoto *et al.* 1985). Interestingly,

the expression of Bcl-2 did not provide a proliferative advantage to the cells. Rather, it contributed to neoplastic cell expansion by enhancing survival capacity under suboptimal conditions. Early studies demonstrated that transient Bcl-2 expression in cells, significantly prolonged survival in response to a wide range of apoptotic stimuli such as growth factor withdrawal, chemotherapeutic drugs, metabolic toxins, viral infections and inappropriate oncogene expression (Minn *et al.*, 1998; Reed 1998). Moreover, expression of anti-sense Bcl-2 suppressed its normal function and induced or accelerated cell death (Reed *et al.*, 1990). Thus, Bcl-2 emerged as the first proto-oncogene, which contributed to neoplasia through its effects on cell survival, rather than on cell proliferation.

The subsequent cloning and molecular characterization of the anti-apoptotic *C. elegans* gene *CED-9* revealed that it encodes a 280 amino acid protein sharing overall 23% sequence identity with human Bcl-2 (Hengartner and Horvitz 1994a). The expression of Bcl-2 as a transgene in *C. elegans* not only suppressed the death of cells normally committed to programmed cell death during development, but partially rescued *C.elegans* mutants lacking CED-9 (Vaux *et al.*, 1992; Hengartner and Horvitz 1994a). These observations implicated Bcl-2 as a functional homologue of CED-9 and established that Bcl-2 governs an evolutionarily conserved step in the apoptotic pathway.

Since the discovery of Bcl-2, the family of related mammalian proteins has expanded significantly to include those with opposing function. The Bcl-2 family is comprised of two functional groups: those that inhibit cell death and others that promote cell death (Figure 6) (Adams and Cory 1998; Minn *et al.*, 1998; Reed 1998; Zamzami *et al.*, 1998 Gross *et al.*, 1999a). Several of the genes encoding human Bcl-2 homologues have been mapped and localize to various parts of the genome to include chromosome

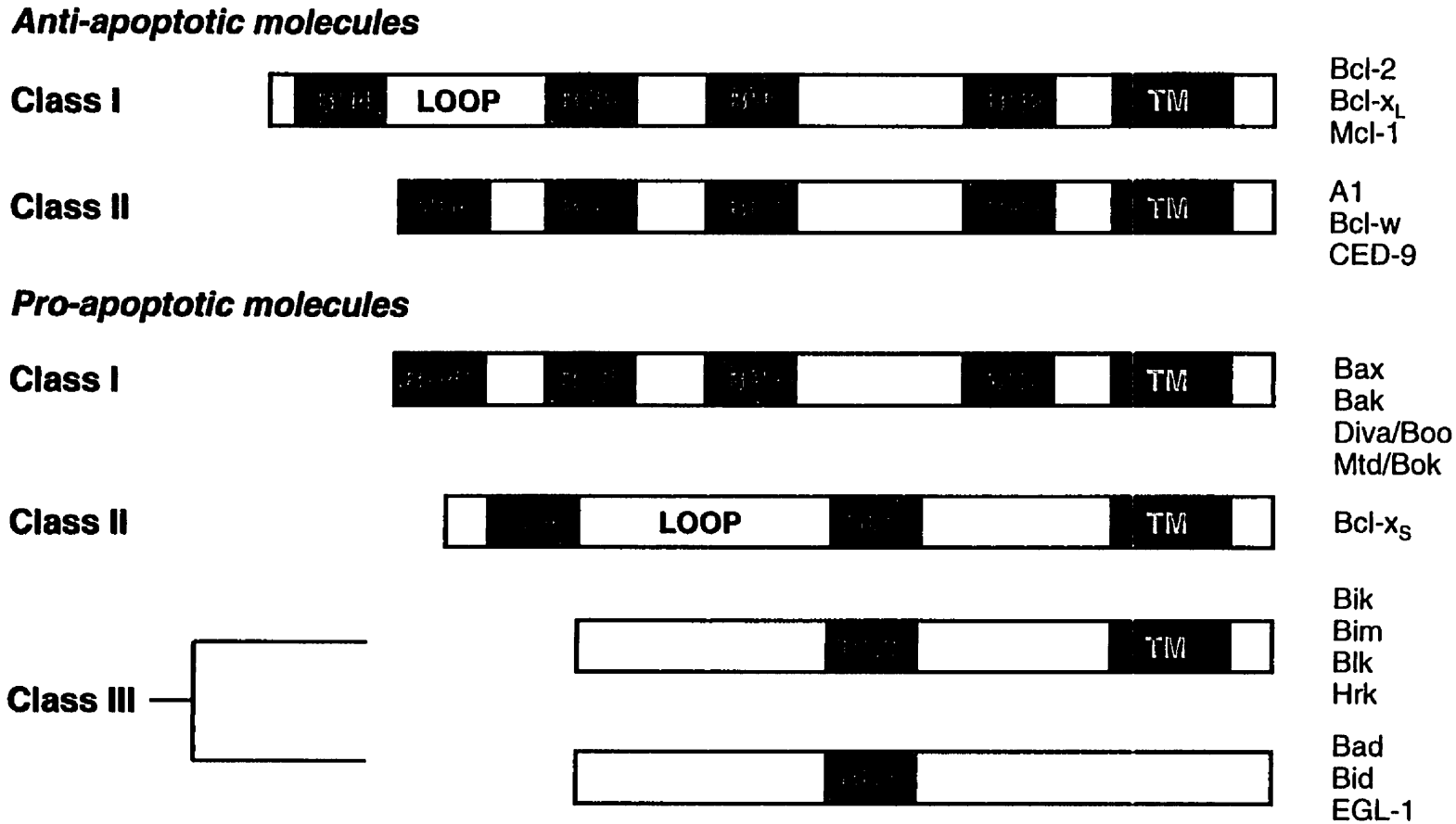

Figure 6. The classification of the Bcl-2 family members. The general organization of Bcl-2 homology domains (BH1-BH4) and transmembrane (TM) domain are indicated. The family is broadly divided into two functional groups: those that promote apoptosis and others that act as inhibitors. Within these two subgroups, further classification is possible based on the presence or absence of structural elements. The most well characterized mammalian Bcl-2 family members are shown. Among the *C. elegans* orthologues, CED-9 is classified as an anti-apoptotic member belonging to Class I and EGL-1 belonging to Class III of the pro-apoptotic subfamily. The viral orthologues are not included. The diagram is not drawn to scale. *(Adapted from Kelekar and Thompson 1998)*

6, 14, 18 and 19 (Online Mendelian Inheritance in Man available at http://www.ncbi.nlm.nih.gov/omim/). Orthologues have been found in avian species (Cazals-Hatem *et al.*, 1992; Gillet *et al.*, 1995), the frog (Cruz-Reyes and Tata 1995), the silkworm (Tambunan *et al.*, 1998) and recently, *Drosophila melanogaster* (Colussi *et al.*, 2000; Igaki *et al.*, 2000). It is important to note that a systematic nomenclature for assigning names to cell death-suppressing and promoting Bcl-2 homologues is yet to be established. In fact, many of the names originate from the laboratory where the protein was first characterized. The acronyms ascribed to Bcl-2 homologues reflect either function such as Bad, Bcl-x_L/Bcl-2-associated death promoter homologue, the ability to heterodimerize with Bcl-2 homologues such as Bid, BH3 interacting domain death agonist and/or shared homology with other Bcl-2 homologues such as Bak, Bcl-2-homologous antagonist/killer.

Several human herpesviruses encode orthologues of Bcl-2, these include BHRF-1 from Epstein-Barr virus (Henderson *et al.*, 1993), ORF16 in herpesvirus Saimiri (Nava *et al.*, 1997), KBcl-2 within the Kaposi sarcoma-associated virus and human herpes virus-8 (HHV8) (Cheng *et al.*, 1997b; Sarid *et al.*, 1997). The African swine fever virus gene 5HL also shares homology with Bcl-2 (Neilan *et al.*, 1993). The adenovirus E1B 19K shares limited homology with Bcl-2, however, it is the best characterized functional Bcl-2 orthologue encoded by a DNA-virus (Rao *et al.*, 1992). The role of viral cell death-suppressing orthologues is likely to maintain host viability by counteracting the propensity of host cells to undergo apoptosis by while an infection is being established (Minn *et al.*, 1998).

The Bcl-2 family has emerged as a multifunctional class of proteins capable of regulating apoptosis through several mechanisms (Figure 7). For example, Bcl-2

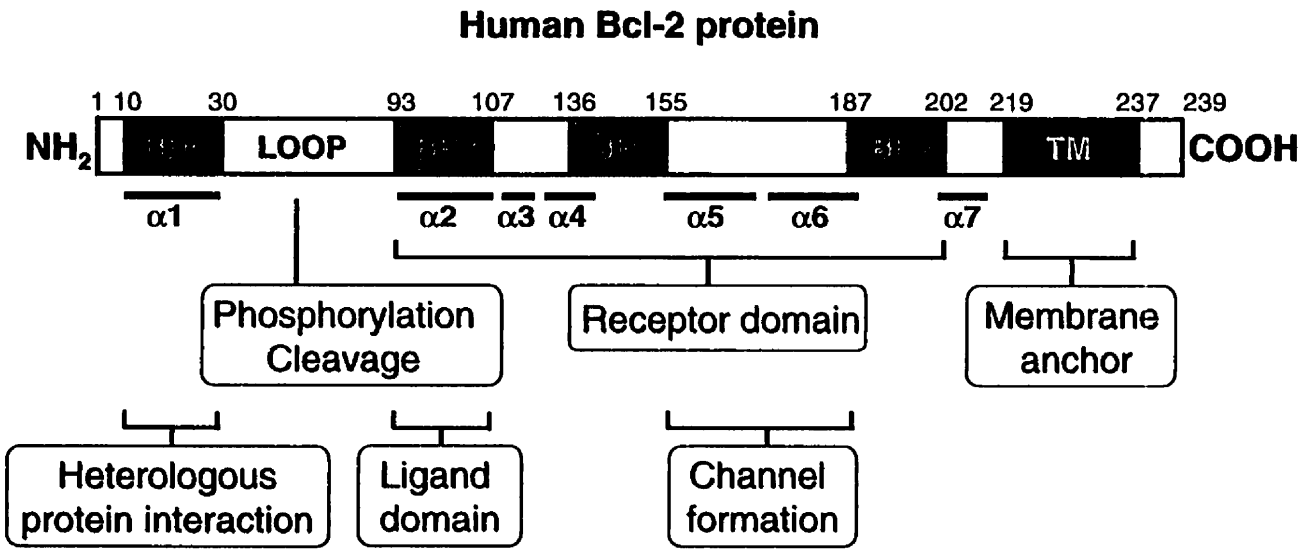

Figure 7. The predicted secondary structure and functional domains of human Bcl-2. The Bcl-2 homology (BH) domains and COOH-terminal transmembrane (TM) anchoring domain are depicted relative to the α-helical segments in Bcl-2. The BH4 domain (α1) mediates interactions with heterologous proteins such as Raf-1 and Bag-1. The long flexible loop (between α1 and α2) represents a negative regulatory domain subject to phosphorylation and caspase cleavage. The BH domains 1, 2, and 3 juxtapose to form a hydrophobic cleft that acts as a receptor into which the ligand, BH3 domain inserts to form the a receptor-ligand interaction critical to heterodimerizing Bcl-2 homologues. The fifth and sixth α-helices are predicted to participate in channel formation by inserting into intracellular membranes. *(Adapted from Reed 1997)*

homologues can act as adaptors or docking proteins. Bcl-2-related proteins have a propensity to form homo- and heterodimeric complexes forming a dynamic network that promotes apoptosis by bringing together essential elements or conversely, prevent cell death-inducing interactions. The many non-homologous Bcl-2 interacting proteins could potentially sequester Bcl-2 homologues to inhibit their function or promote cell death by activating an alternate cell death pathway. Furthermore, the homo- and heterodimeric complexes might act as chaperones or gatekeepers. The pro-apoptotic members would tend to promote movement in a death signalling direction, such as targeting proteins to the mitochondria and causing mitochondrial dysfunction, whereas anti-apoptotic members would suppress the permeation of a death signal through the cell. Many of the Bcl-2 family members localize to intracellular membranes, therefore influencing membrane stability. Pro-apoptotic members destabilize the membranes by promoting the release of intermembrane components such as cytochrome *c* from the mitochondria. In contrast, anti-apoptotic proteins function to stabilize intracellular membranes and can prevent the release of cytochrome *c* from the mitochondria. Lastly, select Bcl-2 family members could form anion-cation specific channels affecting both membrane stability and integrity of subcellular organelles, such as the mitochondria. These functions will be discussed in detail with particular emphasis on the contribution of structural elements mediating the functions of Bcl-2 homologues.

1.5.1. Subcellular localization of Bcl-2 homologues

In the absence of a death signal, proteins encoded by Bcl-2-related genes localize to separate subcellular compartments or reside in the cytosol. With the exception of a few homologues, the majority of Bcl-2 family members possess a COOH-terminal transmembrane (TM) domain that targets the protein to its correct intracellular membrane(s). However, it seems unlikely the subcellular localization of Bcl-2

homologues is determined merely by TM-lipid interactions. Rather, the preferential localization of Bcl-2-related proteins might be dictated by protein-protein interactions at various subcellular membranes. Although such proteins remain unidentified, these potential interactions would confer a level of regulation to this class of proteins prior to an apoptotic signal (Zamzami *et al.*, 1998).

By immunoelectron microscopy, Bcl-2 and Bcl-x_L were found in similar subcellular compartments: the outer mitochondrial membrane, endoplasmic reticulum (ER) and nuclear envelope (Krajewski *et al.*, 1993; Akao *et al.*, 1994; Lithgow *et al.*, 1994). In cells, staining for Bcl-2 appeared in a punctate pattern, suggesting Bcl-2 localized to the mitochondria, specifically the contact sites where the inner and outer mitochondrial membrane meet (de Jong *et al.*, 1994). *In vitro* targeting studies demonstrated that the Bcl-2 COOH-terminal TM domain spanning 19 amino acids was necessary and sufficient to target the protein to isolated mitochondria in a temperature-sensitive, dATP/ATP-dependent manner. The targeted Bcl-2 protein inserted into the outer mitochondrial membrane orienting its NH_2-terminus to the cytosol (Nguyen *et al.*, 1993). Upon deletion of the Bcl-2 TM domain, truncated mutants remain in the cytosol (Sedlak *et al.*, 1995) with either partially diminished (Hockenbery *et al.*, 1993; Hunter *et al.* 1996) or completely ablated cell death-suppressing activity (Alnemri *et al.*, 1992; Nguyen *et al.*, 1994; Tanaka *et al.*, 1993). Cytosolic Bcl-2 prevented the targeting of heterologous proteins such as kinase Raf-1 to the mitochondria (Wang *et al.*, 1996a). Similarly, truncated Bcl-x_L would not be able to sequester CED-4 and presumably Apaf-1 to the mitochondria (Chinnaiyan *et al.*, 1997; Wu *et al.*, 1997b). Both the protective effects and mitochondrial localization of Bcl-2 can be restored by fusing the cytosolic portion to an equivalent targeting sequence from the yeast outer mitochondrial membrane protein Mas70p (Zhu *et al.* 1996).

Bcl-2 is able to exert some of its protective effects on organelles other than the mitochondria. The forced localization and expression of Bcl-2 from the ER using the cytochrome b_5 targeting sequence suppresses cell death in a cell-type, signal-specific manner (Zhu *et al.* 1996). However, the partial activity observed with a Bcl-2 lacking its TM domain or by forced expression from the ER is difficult to interpret. First, Bcl-2 mutants without a targeting sequence retain their ability to heterodimerize with other mitochondrial-based proteins, including Bcl-2 homologues, thus able to act on the mitochondria and influence its function. Furthermore, Bcl-2 expressed from non-mitochondrial sites might prevent cell death by sequestering other death-promoting members away from the mitochondria (Kroemer 1997; Minn *et al.*, 1998; Zamzami *et al.*, 1998). Overall, these observations indicate that mitochondrial localization is optimal for Bcl-2/Bcl-x_L to act efficiently as an anti-apoptotic protein in the cell death pathway.

Among Bcl-2 homologues that promote cell death, the majority have a COOH-terminal TM domain that localize the proteins to various intracellular membranes (mitochondrial membrane, ER and nuclear envelope) in normal and dying cells (Adams and Cory 1998; Reed 1998; Gross *et al.*, 1999a). Others, such as Bax remain in the cytosol or loosely associated with the mitochondrial membrane and become integral membrane proteins in response to an apoptotic signal. The removal of the Bax TM domain eliminates both its cytotoxic activity and ability to localize to the mitochondria (Hsu and Youle 1997; Wolter *et al.*, 1997; Goping *et al.*, 1998). Fusion to a heterologous TM domain mitochondrial targeting sequence restores its function (Goping *et al.*, 1998). The protein Bim contains a TM domain, but is associated with the cytoskeleton in normal cells and redistributes to the mitochondria in response to an apoptotic signal.

Similarly, Bid (Wang *et al.*, 1996b) and Bad (Yang *et al.*, 1995) also translocate to the mitochondria in dying cells, but lack a membrane anchoring TM domain.

1.5.2. Structural organization of Bcl-2 homologues

Early models suggested that the balance between death antagonists and agonists as well as their propensity to form homo- and heterodimers determined whether a cell lived or died (Oltvai and Korsmeyer 1994; Chao and Korsmeyer 1998). Moreover, the expression of Bcl-2 family members in a cell type, differentiation-specific and activation stage manner helps to create a network of interacting proteins whose competitive dimerizations in part modulate the cells' response to its environment (Sedlak *et al.*, 1995). These interactions have been observed and detailed in yeast two-hybrid or *in vitro* binding assays as well as by co-immunoprecipitation of membrane-solubilized mammalian cells (Oltvai *et al.*, 1993; Sedlak *et al*; 1995; Zha *et al.*, 1996a) (Table 1). *In vivo*, interactions have been observed by protein cross-linking (Gross *et al.*, 1998) and by fluorescence resonance energy transfer (FRET) (Mahajan *et al.*, 1998). The homo- and heterodimeric interactions occur through sequence motifs that characterize the Bcl-2 family.

The death antagonists and agonists that comprise the Bcl-2 family share homology in up to four regions designated Bcl-2 homology (BH) domains (BH1, BH2, BH3 and BH4) (Yin *et al.*, 1994; Chittenden *et al.*, 1995; Gibson *et al.*, 1996; Zha *et al.*, 1996a). The BH1 and BH2 domains span 21 and 16 amino acids, respectively and are generally 30 to 40 amino acids apart. The BH4 domain is found at the extreme NH_2-terminus of select Bcl-2 homologues. The BH3 domain is present in all family members, but sequence differences distinguish between anti- and pro-apoptotic function. Apart from shared homology in the BH domains, the overall degree of homology between Bcl-2 homologues varies from as high as 45% between Bcl-2 and Bcl-x_L to as low as 19% between other family members.

Table 1. Summary of potential interactions between mammalian Bcl-2 family members as determined by *in vitro* or *in vivo* co-immunoprecipitation or yeast two-hybrid assays

Mammalian Bcl-2 homologues		Bcl-2	Bcl-x_L	Bcl-w	Mcl-1	A1	Bax	Bak	Mtd/Bok	Diva	Bcl-x_S	Bad	Bid	Bik	Bim	Hrk
Anti-apoptotic **Class I**	**Bcl-2**	+	±		+	+	+	±	-	-	+	±	+	+	+	+
	Bcl-x_L	±	±		±	-	+	+	-	-	+	+	+	+	+	+
	Bcl-w						+	+	-	-		+		+	+	
	Mcl-1	+	±		±	-	+		+		+	-	+		+	
	A1	+	-		-		+	+	+	-		-	+	+	+	
Pro-apoptotic **Class I**	**Bax**	+	+	+	+	+	+	+		-	-	-	+	-	-	-
	Bak	±	+	+		+	+	+		-	-				-	
	Mtd/Bok	-	-	-	+	+			-	-						-
	Diva	-	-	-		-	-	-	-			-				-
Class II	**Bcl-x_S**	+	+		+		-	-			-	-		+		-
Class III	**Bad**	±	+	+	-	-	-			-	-	-			-	
	Bid	+	+		+	+	+						-		-	
	Bik	+	+	+		+	-				+				-	
	Bim	+	+	+	+	+	-	-				-	-	-		
	Hrk	+	+				-		-	-	-					

+', interactions detected; '-' interactions not detected; interactions not determined to date

Several characteristics are apparent when defining and classifying Bcl-2 family members based on function and domain organization (Figure 6) (Kelekar and Thompson 1998). Generally, the anti-apoptotic family members contain all four BH domains and a COOH-terminal TM domain. The amino acids spanning between the BH4 and BH3 domain distinguishes between proteins belonging to class I (Bcl-2, Bcl-x_L) and class II (Bcl-w, CED-9). A high degree of homology in the NH_2-terminus BH4 domain only exists among anti-apoptotic members with the exception of pro-apoptotic Bcl-x_S (Minn *et al.*, 1996). The BH domains 1, 2 and 3 are important for heterodimerization. The BH4 domain is not involved in heterodimeric interactions with family members, rather it probably facilitates interaction with heterologous proteins. Lastly, the TM domain is critical for subcellular localization and cell death-suppressing function of class I and II proteins.

The Bcl-2 death agonists can be divided into three classes. Those belonging to class I (Bax, Bak, Mtd/Bok, Diva/Boo) bear the greatest resemblance to Bcl-2, containing BH1, BH2 and BH3 domains as well as a COOH-terminal TM domain. Those members sharing weak homology in the BH4 domain are also included in this group (Mtd/Bok, Diva/Boo). Class I pro-apoptotic proteins function by interacting with Bcl-2/Bcl-x_L and antagonizing their protective effects. Currently, Bcl-x_S is the only member belonging to class II. It is a splicing variant of Bcl-x_L that lacks the BH1 and BH2 domains, leaving the BH4 and BH3 domains linked by the loop region and an intact TM domain (Minn *et al.*, 1996). Bcl-x_S promotes apoptosis exclusively by antagonizing the function of Bcl-x_L (Chang *et al.*, 1999). Most intriguing is the emergence of a third class of pro-apoptotic proteins sharing no similarity to Bcl-2 beyond the BH3 domain. Many have a TM domain that determines their subcellular localization. Class III, the 'BH3-only' branch of the Bcl-2 family or class III includes Bad (Yang *et al.*, 1995), Bid (Wang *et al.*, 1996b), Bik (Boyd *et al.*, 1995; Farrow *et al.*, 1995), Bim (O'Conner *et al.*, 1998), Blk (Hedge *et al.*, 1998), Hrk

(Inohara *et al.*, 1997a) and the *C. elegans* orthologue EGL-1 (Conradt and Horvitz 1998). These proteins require their BH3 domain to facilitate heterodimerization and to promote apoptosis (Kelekar and Thompson 1998).

1.5.2.1. The tertiary structure

1.5.2.1.1. Bcl-x_L

The structural basis for heterodimeric interactions between Bcl-2-related proteins was elucidated using a combination of X-ray crystallography and NMR methods (Muchmore *et al.*, 1996; Sattler *et al.*, 1997). The structure of biologically active human Bcl-x_L (residues 1-205) lacking its TM domain revealed it to be predominantly an α-helical protein, consisting of two central hydrophobic helices (α5 and α6) surrounded by five amphipathic helices (α1, α2, α3, α4 and α7) (Figures 7 and 8). The seven α-helices are joined together by flexible loops. The central hydrophobic helices (α5 and α6) are approximately 30Å long and correspond to parts of BH1 and BH2 domains. The BH4 and BH3 domains correspond to the first and second amphipathic α-helices, respectively. Further structural analysis revealed these domains to be separated by a flexible, unstructured loop that corresponded to a region of low sequence homology and variable length among anti-apoptotic Bcl-2 homologues. The BH1, BH2 and BH3 domains are positioned in close spatial proximity to each other, forming an elongated cleft (Muchmore *et al.*, 1996). Bcl-x_L complexed to a 16 amino acid residue peptide derived from the BH3 domain of Bak showed that the hydrophobic pocket formed by domains BH1, BH2 and BH3 of Bcl-x_L was the site of interaction. The Bak BH3 peptide exists as a random coil in solution, but forms an amphipathic α-helix binding with high affinity to the Bcl-x_L hydrophobic cleft. The heterodimeric complex is stabilized through hydrophobic and electrostatic interactions involving highly conserved residues of the BH1, BH2 and BH3 domains of Bcl-x_L and the BH3 domain of Bak (Sattler *et al.*, 1997) (Figure 9). Although,

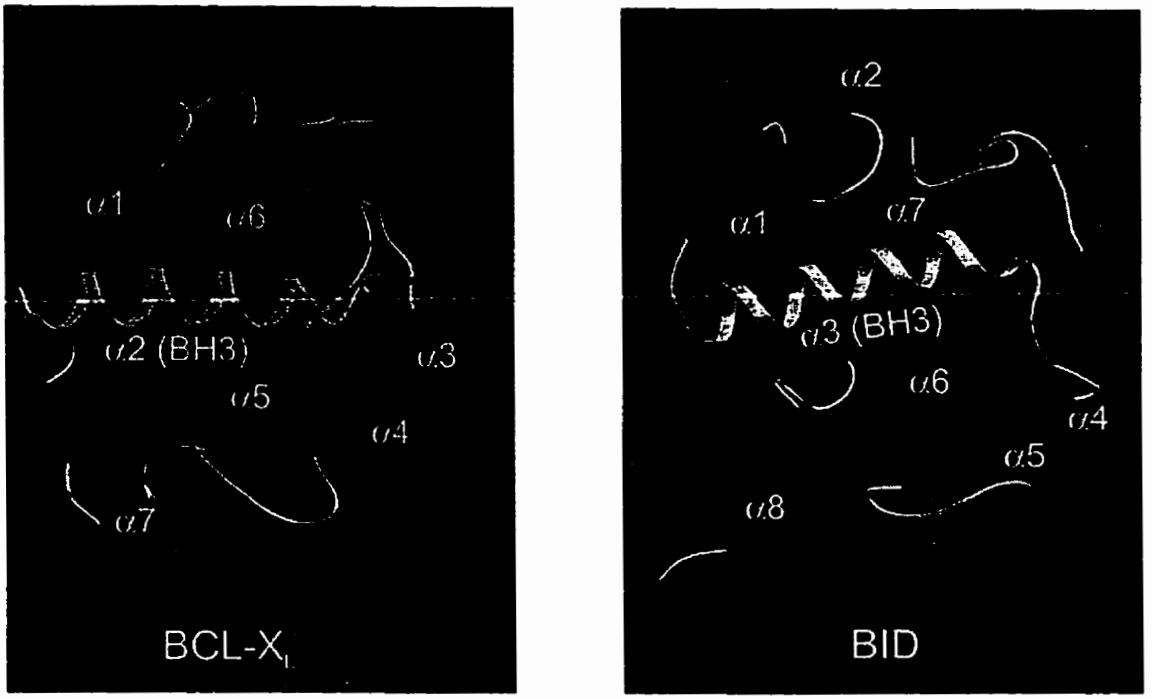

Figure 8. The three-dimensional structure of Bcl-x_L and Bid. The ribbon diagram represents the average minimized structure of Bcl-x_L and Bid using NMR. Both proteins contain an unstructured loop region that is not included, Bcl-x_L (residues 26 to 76) and Bid (residues 31 to 70). *(Adapted from Gross et al., 1999a)*

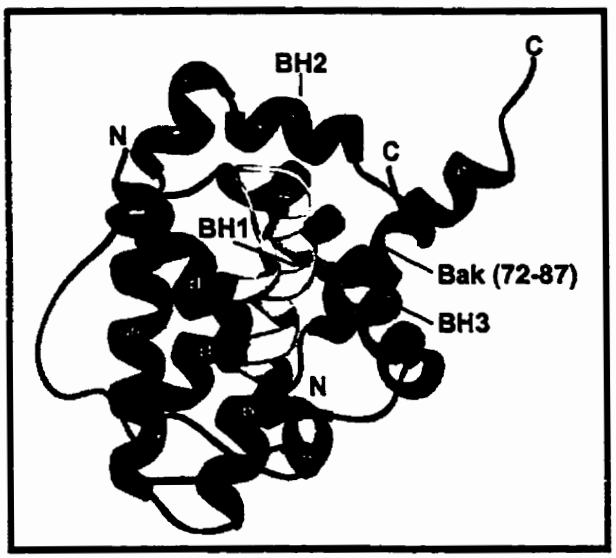

Figure 9. A model of Bak BH3 peptide complexed to the Bcl-x_L hydrophobic cleft. Ribbon structure depicting the average, minimized NMR structure of Bcl-x_L complexed with 16 amino acid peptide of the Bak BH3 domain. The BH domains of Bcl-x_L are indicated. The complex is stabilized through hydrophobic and electrostatic interactions. *(Adapted from Sattler et al., 1997)*

Bcl-x_L and many of its heterodimerizing partners have a membrane anchoring TM domain, to date, there is no structural information on how the TM domain influences the conformation of the BH domains.

1.5.2.1.2. Bid

Bid represents the only BH3-containing protein whose three-dimensional structure is known (Figure 8). It consists of eight α-helices arranged into three layers or compact folds. The two central hydrophobic helices (α6 and α7, equivalent to α5 and α6 of Bcl-x_L) are arranged in an anti-parallel fashion within the core of the protein. The other six amphipathic helices surround the central hydrophobic core. Within Bid, two regions spanning residues 1-12 and 43-77 remain structurally undefined. Overall, the structure of Bid exhibits striking similarity to Bcl-x_L, despite the fact the amino acid sequence of these two proteins is limited to the 16 residue BH3 domain (Figures 8 and 10). Two differences were noted between the structures of the two proteins: (1) Bid contains an extra helical domain (α2) in the NH_2-terminal region that appears to be a general characteristic for pro-apoptotic Bcl-2 homologues and (2) the flexible loop between the first and second helices of Bcl-x_L is almost twice as long as that in Bid (Chou *et al.*, 1999).

The BH3 domain of Bid occurs in the third α-helix, fixed in position through hydrophobic contacts with the first and last helix (α1 and α8). Biochemical studies have shown Bid exists in an inactive conformation through interaction between its NH_2-terminus and BH3 domain (Tan *et al.*, 1999). The strength of this interaction is evident from *in vitro* studies using recombinant Bid. Following caspase 8 cleavage at Asp^{59} in the large flexible loop between the second and third α–helices, the two fragments do not disassociate easily (Chou *et al.*, 1999). However, *in vivo*, truncated Bid exists without an associated NH_2-

terminal fragment. The cleavage of Bid results in the loss of the first and second helices (α1 and α2) and a portion of the unstructured loop. The removal of the NH_2-terminus leads to a change in surface hydrophobicity of Bid by exposing greater than 200Å of previously buried surface area that encompasses the BH3 domain and the two central hydrophobic helices. This is accompanied by a change in electrostatic charge from -13 in the full-length Bid to -4 in the truncated product. The changes in hydrophobic exposure and electrostatic charge are potentially the driving forces that contribute to the translocation and integration of Bid into the mitochondrial membrane which is one of the most strongly charged biological membranes (Darnell *et al.* 1990). Although, some uncertainty remains as to whether cleavage of Bid results in a direct conformational change (Chou *et al.*, 1999; McDonnell *et al.*, 1999), there is substantial biochemical evidence to support that Bid cleavage is essential for its activation (Li *et al.*, 1998; Luo *et al.*, 1998).

1.5.2.2. The BH1 and BH2 domains

The regions mediating dimerization among Bcl-2 family members were initially elucidated by mutational analysis. Further insight came from the structural determination of Bcl-x_L complexed to Bak BH3 peptide (Muchmore *et al.*, 1996; Sattler *et al.*, 1997) using combined X-ray crystallography and NMR methods. The BH1 and BH2 domains are present in all death antagonists of the Bcl-2 family and only one class of death agonists. The BH1 and BH2 domains are essential for the survival function Bcl-2/Bcl-x_L and for interaction with death agonists such as Bax and Bak (Yin *et al.*, 1994; Sedlak *et al.*, 1995). The structural data suggests the formation of the hydrophobic pocket and proper positioning of charged residues within the BH domains is necessary for anti-apoptotic function of Bcl-x_L and presumably Bcl-2. Consistent with this, mutations predicted to alter the accessibility or binding properties of Bcl-x_L such as Gly^{138}→Ala (Yin *et al.*, 1994) and Arg^{139}→Gln (Sattler *et al.*, 1997) have been found to inhibit protein function. For example,

mutation of the highly conserved BH1 domain residue Gly^{138} that lines the pocket, inhibits both the cell death suppressor activity of Bcl-x_L and abrogates its ability to interact with Bax (Yin *et al.*, 1994; Sattler *et al.*, 1997) or with Bak (Yang *et al.*, 1995). Structurally, glycine is important in this position because amino acids with bulkier side chains would potentially block access to the hydrophobic pocket (Muchmore *et al.*, 1996). The NMR studies of the Bak BH3 peptide complexed to Bcl-x_L revealed the charged side chain of Asp^{83} of the Bak BH3 peptide interacts with the oppositely charged Arg^{139} of the Bcl-x_L BH1 domain. In this position, the arginine residue or similar amino acid, lysine, is conserved among all the Bcl-2 pro-survival proteins. The substitution of a glutamine residue results in the loss of Bcl-x_L anti-apoptotic function and ability to interact with Bak (Sattler *et al.*, 1997). Other critical residues of Bcl-x_L involved in direct hydrophobic interactions include Tyr^{101} (BH3 domain), Leu^{130} (BH1 domain) and Tyr^{195} (BH2 domain). Mutations of these residues prevented heterodimerization with Bax, but the anti-apoptotic function of Bcl-x_L remained intact (Minn *et al.*, 1999). Some of the previously reported mutants of Bcl-x_L that failed to bind Bax were mutated at residues not predicted to mediate direct interaction with the Bax BH3 domain, therefore those mutations presumably affected the overall structure of the protein (Sedlak *et al*, 1995; Cheng *et al.*, 1996).

1.5.2.3. The BH3 domain

The BH3 domain was first characterized as a stretch of 16 amino acids in Bak critical for heterodimerization with anti-apoptotic Bcl-2-related proteins and necessary for induction of cell death (Chittenden *et al.*, 1995; Sattler *et al.*, 1997). Deletion and mutation studies indicate the BH3 domain mediates heterodimerization with pro-survival proteins and serves as the critical death domain. The importance of the BH3 domain is underscored by the identification of 'BH3-only' containing proteins in mammals and *C. elegans* which strongly suggests their mechanism of action is evolutionarily conserved.

Although all Bcl-2 family members contain a BH3 domain, its role in promoting apoptosis distinguishes its function from anti-apoptotic homologues. For example, BH3 domain swapping studies have shown that the substitution of Bax BH3 domain (23 amino acid segment) and surrounding region into Bcl-2 converted the protein to a death agonist (Hunter and Parslow 1996), implying that sequences within the BH3 domain of death antagonists and agonists contribute to their functional dissimilarities. This is particularly the case for Bcl-x_s, a splicing variant of Bcl-x_L (Minn *et al.*, 1996). Although the BH3 domain is identical between Bcl-x_s and Bcl-x_L, the removal of a stretch of 62 amino acids encompassing the BH1 and BH2 domains allows the molecule to suppress the survival function of Bcl-x_L through heterodimerization or to independently promote apoptosis under some experimental conditions (Boise *et al.* 1993; Sumantran *et al.*, 1995; Minn *et al.* 1996; Chang *et al.* 1999). These observations suggest that sequences flanking the BH3 domain must also contribute to determining its role in suppressing or promoting cell death.

1.5.2.3.1. Sequence differences determine binding affinity

The BH3 domain plays a central role in defining the binding specificity of pro-apoptotic Bcl-2 homologues to their anti-apoptotic partners. The subtle differences between the BH3 domain sequences of cell death agonists and antagonists might not only reflect differences in function, but also relate to differences in binding affinity among Bcl-2 homologues, creating a hierarchy of weakly and strongly interacting pairs (Kelekar and Thompson 1998). The strength of interacting proteins is apparent by co-immunoprecipitation assays from solubilized transfected mammalian cells in which immunoprecipitation of one protein co-precipitates varying amounts of different binding partners depending on the strength of interaction (Chang *et al.*, 1999; R. Ray and A.H. Greenberg, *unpublished observations*). In the yeast two-hybrid assay, differences in the

level of reporter gene activity due to protein-protein interaction reflect the strength of interaction and can be measured (Wang *et al.*, 1998a R. Ray and A.H. Greenberg, *unpublished observations*).

An alternative method to quantify the strength of interaction is to directly measure the binding affinity between two Bcl-2-related proteins. The binding affinities of a 16 amino acid (16-mer) BH3 peptide to Bcl-x_L exhibited stronger interaction with Bak and weaker interaction with Bax or Bik (Sattler *et al.*, 1997). The length of the peptide used was noted to influence binding affinity. For example, the 16-mer BH3 peptide of Bad exhibited lower binding affinity for Bcl-x_L, although a deletion mutant of the identical region in full-length Bad was sufficient to abrogate interaction. By increasing the peptide length to encompass the entire second helix (approximately 26 residues), the binding affinity increased significantly (Kelekar *et al.*, 1997). Therefore, the amino acids outside the 16-mer region presumably are involved in anchoring the Bad BH3 domain in the Bcl-x_L hydrophobic pocket. Similar observations were made using a Bcl-x BH3 domain 16-mer peptide that failed to interact with Bcl-x_L. By extending the peptide to encompassing the entire second α-helix yielded in higher binding affinity (Chang *et al.*, 1999). The minimal helical constructs may have a greater affinity for Bcl-x_L due to the lack of steric hindrance which might be otherwise imposed upon the BH3 domain by other domains present in full-length Bcl-x_S (Chang *et al.*, 1999). The sequences flanking the second α-helix also contribute to BH3 heterodimerization for some Bcl-2 homologues (Evangovan and Chinnadurai 1997; Wang *et al.*, 1998a). The role of these sequences either in specific binding interactions or simply for stabilization of the α-helices remains unclear.

1.5.2.3.2. Structure

The solution structure of Bcl-x_L complexed to a 16 amino acid BH3 Bak peptide revealed the BH3 domain assumed an amphipathic α-helical conformation binding with high affinity to the hydrophobic cleft created by BH domains 1, 2 and 3 of Bcl-x_L. Overall, mutations on either the hydrophobic and/or hydrophilic face of the α-helical BH3 peptide disrupted both heterodimeric interaction and cell death activity (Sattler *et al.*, 1997; Wang *et al.*, 1998a; Chou *et al.*, 1999; McDonnell *et al.*, 1999). The observations from the tertiary structure and point mutation analysis coupled with functional studies has defined an 8 amino acid core within the BH3 domain (Figure 10) (Kelekar and Thompson 1998). Specifically, a leucine residue at position 1 and aspartic acid residue at position 6 appear to be most critical for interaction of death agonists with their antagonist-binding partners as well as pro-apoptotic function. Interestingly, by inspection, these residues occur in the identical position of anti-apoptotic Bcl-2 and Bcl-x_L. However, neither of these molecules exhibits pro-apoptotic activity, unless the BH4 domain is removed by activated caspases. The amino acid residue at position 4 in the core of the BH3 domain may set pro-apoptotic Bcl-2 homologues apart from their anti-apoptotic counterparts. All the residues in this position have non-polar side chains. For example, Bcl-2, Bcl-x_L and Bcl-w harbour an alanine residue and Bak contains an isoleucine residue. However, the substitution of isoleucine with alanine at position 81 of the Bak BH3 peptide significantly disrupted its interaction with Bcl-x_L (Sattler *et al.*, 1997).

According to both structure and mutagenesis studies, the side chain of four highly conserved hydrophobic residues Val^{74}, Leu^{78}, Ile^{81} and Ile^{85} of the Bak BH3 helix point into the hydrophobic cleft of Bcl-x_L and stabilize the complex (Figures 9 and 10). The replacement of these residues with alanine residues disrupted heterodimerization.

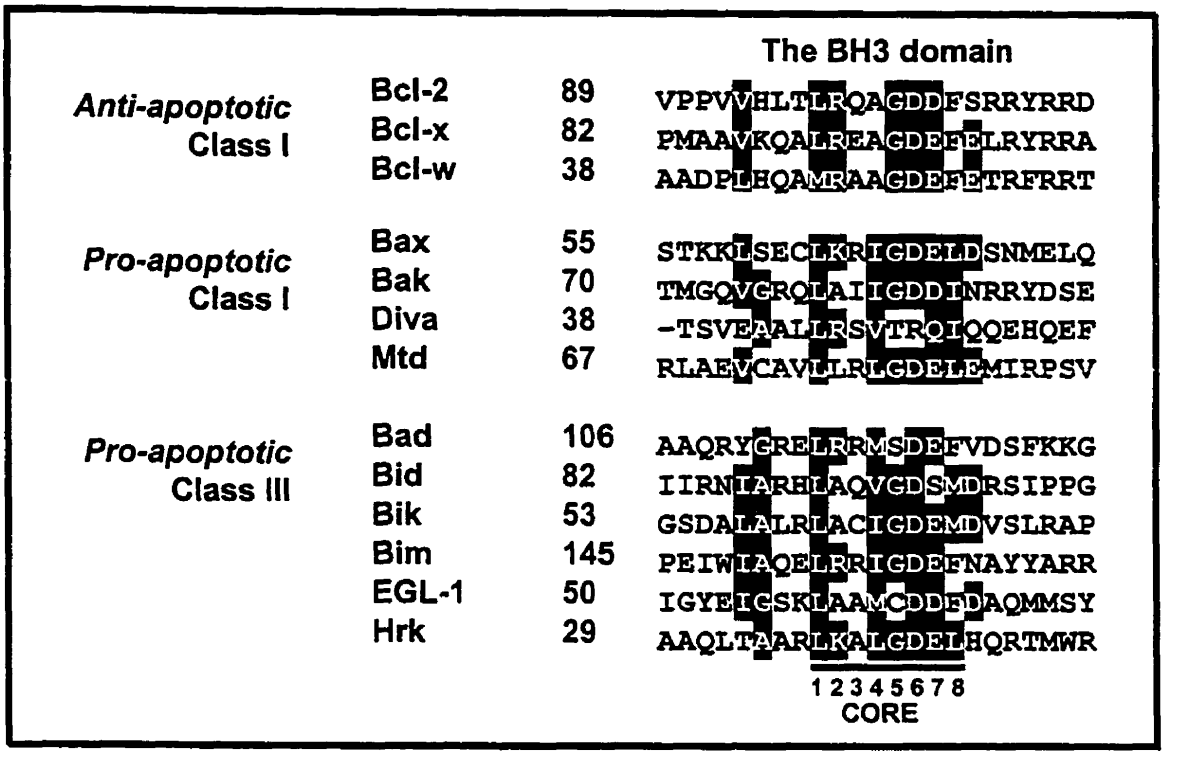

Figure 10. Sequence comparison of the BH3 domain from anti- and pro-apoptotic Bcl-2 homologues. Alignment of amino acid sequence in the Bcl-2 homology 3 (BH3) domain of several Bcl-2 homologues. The core residues, 1 to 8 are indicated. The numbers on the left denote the amino acid position in the context of the full-length protein. Identical residues are shaded in black and similar residues are shaded in grey. *(Adapted from Kelekar and Thompson 1998)*

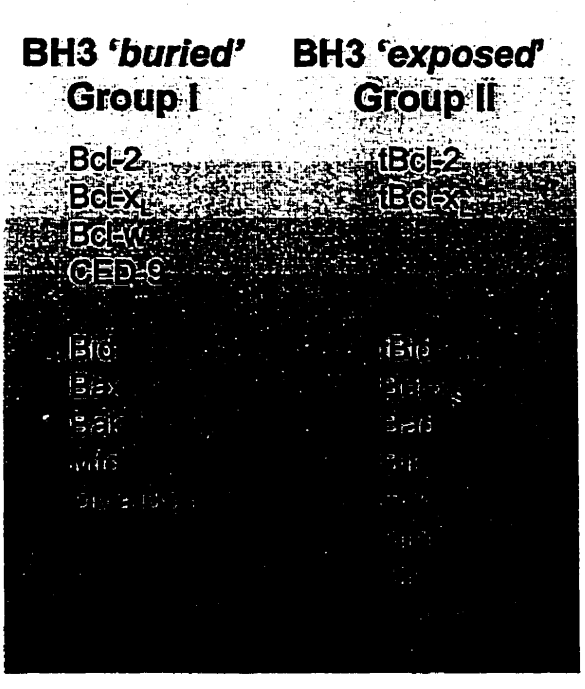

Figure 11. The classification of Bcl-2 homologues based on availability of the BH3 domain. The Bcl-2 family members can be grouped into two main structural categories. Group I consists of proteins with sequence pattern consistent with the conservation of Bid/Bcl-x_L topology and predicted to have their BH3 domain buried. These proteins rely on some mechanism to induce a conformational change for pro-apoptotic activity. Group II includes truncated Bcl-2-related proteins from group I and others having unrelated tertiary structure. These proteins are predicted to have their BH3 domain exposed and likely to be constituitively active as pro-apoptotic molecules. *(Adapted from Gross et al., 1999a McDonnell et al., 1999)*

By modelling full length Bak protein on the structural template of Bcl-x_L, residues Val^{74}, Leu^{78}, Ile^{81} and Ile^{85} would point towards the interior of the protein, therefore unavailable to interact with Bcl-x_L (Sattler *et al.*, 1997). This implies Bak heterodimerization requires a conformational change near the helical BH3 domain to expose its hydrophobic surface. A highly flexible loop that precedes the BH3 domain may facilitate the necessary conformational change (Sattler *et al.* 1997). Interestingly, the residues in Bid, Ile^{86}, Leu^{90}, Val^{93} and Met^{97} align with the conserved residues of the Bak BH3 domain, but are surface exposed forming an elongated patch on the third α-helix adjacent to the eighth α-helix (Figure 10). Further modelling studies revealed that the Bid BH3 domain readily inserts into the hydrophobic cleft of Bcl-x_L such that the binding surfaces complement each other without causing any significant stereo-chemical strain. Unlike, Bak and presumably Bax, the BH3 domain of Bid is exposed following proteolytic processing (Chou *et al.*, 1999; McDonnell *et al.*, 1999).

1.5.2.3.3. Accessibility: 'buried' vs. 'exposed'

Apart from classification of the Bcl-2 family based on domain organization, the three-dimensional structural characteristics of anti-apoptotic Bcl-x_L and pro-apoptotic Bid offer an alternative grouping based on two main conformational categories (Figure 11). The Bcl-2 antagonists (Bcl-2, Bcl-w, CED-9) and agonists (Bax and Bak) are predicted to have similar tertiary structure to Bcl-x_L based on shared amino acid sequence homology (BH1, BH2, BH3 domains) and similar secondary structural predictions (α-helical content). As described, the BH3 domain participates in intramolecular interactions with BH1 and BH2 domains to form the hydrophobic cleft. In the case of Bax and Bak, this conformation would bury the hydrophobic face of the BH3 α-helix, shielding its residues from interacting with Bcl-x_L. Therefore, when Bax or Bak heterodimerize, the intramolecular contacts

formed by the BH3 domain must be disrupted to allow rotation of the BH3 domain along its helical axis to form new interactions with the hydrophobic pocket of Bcl-x_L. Yet, Bid only shares similar tertiary structure with Bcl-x_L and requires proteolytic processing to expose its BH3 domain. Therefore, the Bcl-2 homologues belonging to group I that have their BH3 domain 'buried' would have a predicted structure consistent with Bcl-x_L/Bid topology. The anti-apoptotic proteins or the inactive pro-apoptotic proteins would require post-translational modification(s) to act as an "on/off" switch to convert these proteins into their active conformation by exposing their BH3 domain and potentially other hydrophobic surfaces. Group II molecules, BH3 domain 'exposed' proteins would encompass modified group I members such as tBid, tBcl-2, tBcl-x_L and Bcl-x_S. Other members in the second group would include the 'BH3-only' proteins sharing homology exclusively in the BH3 domain and unrelated tertiary structure. The proteins belonging to group II are predicted to have their BH3 domain surface exposed, thus, would be constitutively active (McDonnell *et al.*, 1999).

1.5.2.3.4. Bax homo- and heterodimeric interactions

Several structure-function studies have detailed the contribution of the Bax BH3 domain which mediates homo- and heterodimeric interactions necessary under certain conditions to promote cell death. Mutation analysis revealed the removal of four residues, Ile^{66}, Gly^{67}, Asp^{68} and Glu^{69} within the core of the Bax BH3 domain disrupted both homo- and heterodimeric interactions and its ability to promote apoptosis (Figure 10) (Zha and Reed 1997). The replacement of the hydrophobic face of the BH3 α-helix, specifically residues Leu^{63}, Gly^{67}, Leu^{70} and Met^{74} with alanine residues altered sufficient critical contacts along the binding surface to disrupt both homo-and heterodimeric interactions. Yet, this Bax mutant retained its ability to induce mitochondrial dysfunction and

subsequent cell death. Bax mutant/mutant homodimers were not detected. Thus, mutations along the hydrophobic face of the α-helical BH3 domain that maintain its amphipathic nature retain cell death activity (Wang *et al.*, 1998a). Single point mutations at residues Asp^{68} and Glu^{69} were noted to disrupt only Bcl-2 heterodimerization, but the mutants were still capable of homodimerizing and promoting cell death (Zha and Reed 1997).

The subcellular localization of many of these Bax mutants revealed that mutations on the hydrophobic face of its BH3 domain targeted the mutant to the mitochondria where it could potentially homodimerize similar to wild type Bax. Upon integration into mitochondrial membranes, wild type Bax is predicted to undergo a conformational change that may be mimicked by some Bax mutants that allow it to assume the homodimerized conformation within membranes. Mutants lacking the BH3 domain were observed to constituitively insert into mitochondria, but did not retain cell death activity (Nechushtan *et al.*, 1999). Overall, it appears the amphipathic nature of the second α-helix containing the BH3 domain is important for the death effector function of Bax and critical to initiating mitochondrial events that lead to cell death (Wang *et al.*, 1998a).

1.5.2.4. The BH4 domain

The BH4 domain at the NH_2 terminus represents the first α-helical region present in three of the most closely related pro-survival proteins Bcl-2, Bcl-x_L and Bcl-w. The structure from Bcl-x_L indicates that its BH4 domain encompasses an α-helical loop on the surface that forms extensive hydrophobic interactions with α2, α5 and α6 (Muchmore *et al.*, 1996). Bcl-2/Bcl-x_L lacking its BH4 domain no longer provides the cell with a survival advantage (Borner *et al.*, 1994; Borner *et al.* 1996; Huang *et al.*, 1997). In certain cell types, the removal of the BH4 domain converts Bcl-2/Bcl-x_L into proteins capable of

promoting cell death (Hunter *et al.*, 1996). The absence of the BH4 domain does not interfere with Bcl-2 heterodimerization with pro-apoptotic homologues Bax, Bad, Bid, Bik and Bim (Huang *et al.*, 1998). The BH4 domain is absent from virtually all cell death agonists, with the exception of Bcl-x_S which contains a BH4 domain identical to Bcl-x_L, yet it does not appear to contribute to its pro-apoptotic function (Minn *et al.*, 1996). Two other homologues, Diva/Boo (Inohara *et al.*, 1998a; Song *et al.*, 1999) and Mtd/Bok (Hsu *et al.*, 1997; Inohara *et al.*, 1998b) share very weak homology to the BH4 domain of anti-apoptotic Bcl-2 homologues.

1.5.2.4.1. Bcl-2 homodimeric interactions

Early studies to characterize Bcl-2 homo- and heterodimeric interactions found deletion of the NH_2-terminus of Bcl-2 in addition to BH1 and BH2 domains disrupted homodimerization between mutant proteins. However, these Bcl-2 deletion mutants were observed to interact with wild type Bcl-2, forming wild type/mutant heterodimers. This observation can be explained by the fact that Bcl-2 homodimerization involves a head to tail association in which the BH4 domain interacts with the distal portion of Bcl-2, presumably the hydrophobic cleft formed by BH1, BH2 and BH3 domain (Sato *et al.*, 1994; Hanada *et al.*, 1995; Sedlak *et al.* 1995). Bcl-x_L has not been reported to form homodimers by yeast-two hybrid (Sato *et al.*, 1994; Sedlak *et al.*, 1995), co-immunoprecipitation (Minn *et al.*, 1996) or structural analysis (Muchmore *et al.*, 1996). The role of cell death-suppressing proteins forming homodimers remains uncertain. It is conceivable that homodimers could contribute to channel-formation in intracellular membranes to regulate organelle function (Schendel *et al.*, 1998).

1.5.2.4.2. Interaction with heterologous proteins

The Bcl-2/Bcl-x_L BH4 domain mediates interaction with several unrelated proteins. These non-heterologous proteins interact with Bcl-2/Bcl-x_L, but have not been observed to interact with Bax or Bak that contain only BH domains 1, 2 and 3. This suggests that there is an important difference between death-suppressing and death promoting members of the Bcl-2 family. The anti-apoptotic members have likely evolved an additional domain to specifically interact with other non-related proteins that either participate in cell death or cell survival (Zamzami *et al.*, 1998). The functional significance of many of these interactions has not been fully elucidated. Some examples that specifically interact through the BH4 domain of Bcl-2/Bcl-x_L will be highlighted.

Bcl-2 has been found to indirectly regulate the phosphorylation of Bad. Bcl-2 promotes the targeting of Raf-1 kinase to the outer mitochondrial membrane where the kinase most likely phosphorylates mitochondrial-based Bad, thereby preventing its interaction with Bcl-2/Bcl-x_L. Forced mitochondrial expression of Raf-1 using the mitochondrial targeting sequence Mas70p demonstrated a marked increase in the anti-apoptotic effects of Bcl-2. In contrast, targeting Raf-1 to the plasma membrane resulted in a different set of proteins being phosphorylated and did not protect from cell death. Similarly using a dominant inhibitory form of Raf-1 abrogated the protective effects of Bcl-2 on cell survival. These observations demonstrate a role for the Bcl-2 BH4 domain in sequestering and targeting Raf-1 kinase to its proper site of action in the cell (Wang *et al.* 1996a).

Another molecule, Bag-1 acts as a liaison between apoptotic regulatory signals and Bcl-2. Bag-1 was originally identified to interact with Bcl-2 and promote cell survival (Takayama *et al.*, 1995). Recently, Bag-1 has been reported to interact with several other

proteins. For example, during growth factor withdrawal, it interacts with the cytoplasmic domains of the receptor for hepatocyte growth factor (HGP) and platelet derived growth factor (PDGF), therefore unable to bind mitochondrial-based Bcl-2 (Bardelli *et al.*, 1996). Bag-1 also modulates the activities of other proteins such as Raf-1 kinase. The interaction between Bag-1 with Raf-1 is a mechanism hypothesized to allow for the local action of the kinase within the vicinity of Bcl-2 and mitochondrial-based Bad (Wang *et al.*, 1994; Wang *et al.*, 1996a). Bag-1 interacts with another group of proteins, the Hsp70/Hsc70 chaperone system. These proteins are primarily involved in protein folding and refolding, assembly and disassembly of multiprotein complexes and protein translocation across biological membranes (Rasson *et al.* 1995; Hartl 1996). Both Bcl-2 and Bcl-x_L have structural and functional similarities to the pore-forming domains of bacterial toxins. Generally, these domains are able to switch back and forth from membrane integrated to unintegrated states through conformational changes in response to environmental triggers (Muchmore *et al.*, 1996; Minn *et al.*, 1997; Schendel *et al.*, 1997). The interaction between Bag-1 and the Hsc70/Hsp70 chaperone system may facilitate conformational changes of Bcl-2 homologues to enhance the probability of channel-formation through integration into mitochondrial and other intracellular membranes where many of these apoptotic regulatory proteins reside (Takayawa *et al.* 1997).

The BH4 domain of Bcl-2 also interacts with calcineurin, a calcium dependent protein phosphatase. The overexpression of calcineurin induces apoptosis in a Bcl-2 suppressible manner (Shibasaki *et al.*, 1995). Expression of Bcl-2 causes the redistribution of calcineurin from the cytosol to intracellular membranes and prevents its interaction with other cytosolic substrates. Apart from Bcl-2 control over calcium-induced apoptosis, these observations also suggest a role for phosphatases during calcium mediated cell death. The Bcl-2-calcineurin interaction may also have a role to play in Bcl-2 inhibition of cell

proliferation and the translocation of the transcription factor NF-AT to the nucleus to activate genes involved in cell proliferation of particular cell types (Pietenpol *et al.* 1994; Linette *et al.,* 1996). The Bcl-2-calcineurin interaction coupled with the role calcineurin plays in Bad phosphorylation, suggests that Bcl-2 contributes to regulating cell viability by indirectly modulating the phosphorylation states of Bcl-2 homologues.

1.5.3. Indirect interactions between Bcl-2/Bcl-x_L and caspases

Among all the potential proteins that Bcl-2/Bcl-x_L interacts with, those leading to caspase activation are potentially the most important. To date, three molecules have been identified to link interaction between Bcl-2/Bcl-x_L and caspases. These include p28Bap31 (Ng *et al.* 1997; Ng and Shore 1998), MRIT (Han *et al.*, 1997) and most notably the mammalian orthologue of CED-4, Apaf-1 (Zou *et al.*, 1997). p28Bap31 localizes to the ER and complexes with latent caspase 8 molecules, Bcl-2/Bcl-x_L and a CED-4-like adapter protein. The interaction suggests that Bcl-2 prevents the activation of precursor caspase 8 via its interaction with p28Bap31. As a result, the complex formed at the ER withdraws inactive caspase 8 from its physiological site of activation with adapter molecules that oligomerize following the ligation of cell surface death receptors at the plasma membrane (Ng *et al.* 1997; Ng and Shore 1998). Several groups have characterized another protein called MRIT (Han *et al.*, 1997), also known by several other names that likely plays a similar role to p28Bap31 by inhibiting the function of caspases or sequestering apoptotic regulatory proteins away from their site of action. Although the subcellular localization of MRIT remains to be determined, it interacts simultaneously and independently with Bcl-x_L and caspase 8 (Han *et al.*, 1997).

The evidence supporting a role for Bcl-x_L regulating Apaf-1 and subsequent caspase activation remains controversial. It appears that the NH_2-terminus of Bcl-x_L,

possibly involving the BH4 domain is important for interaction with CED-4 (Huang *et al.*, 1998) and presumably Apaf-1. Bax, which lacks a BH4 domain, and Bcl-x_S, which contains the identical BH4 domain as Bcl-x_L, do not interact with CED-4 (Chinnaiyan *et al.*, 1997). Although controversial, by analogy to the CED-9, CED-4 and inactive CED-3 trimolecular complex, often referred to as the 'apoptosome' (Hengartner 1998), Bcl-2/Bcl-x_L, Apaf-1 and inactive caspase 9 have been detected in a similar complex (Hu *et al.*, 1998a; Pan *et al.*, 1998). The current model proposes that inactive Apaf-1 is sequestered by anti-apoptotic Bcl-2 homologues, similar to CED-9-CED-4 interaction to form an inactive complex in healthy cells. Following an apoptotic stimulus, Apaf-1 is predicted to disassociate from Bcl-2/Bcl-x_L and in the presence of cytochrome *c* and dATP/ATP facilitate the activation of latent caspase 9, which in turn activates activation downstream caspases (Zou *et al.*, 1997). In fact, proteins such as Bax, Bak and Bik reportedly disrupt the interaction between Bcl-x_L and Apaf-1 (Chinnaiyan *et al.*, 1997). The viral counterpart of Bcl-2, E1B 19K has been detected in association with Apaf-1 (Han *et al.*, 1998). Diva, a pro-apoptotic Bcl-2 homologue containing a BH4 domain in addition to BH1, BH2 and BH3-like domain, also heterodimerizes with Apaf-1. The implication being that Diva and Bcl-x_L-like proteins complete for Apaf-1 binding, whereby Bcl-x_L homologues presumably inhibiting and Diva promoting cell death (Inohara *et al.*, 1998a). In another study, the identical protein termed Boo was found to interact with Apaf-1, but was not cytotoxic to cells. The co-expression of Bak or Bik was sufficient to disrupt the interaction between Boo and Apaf-1 (Song *et al.*, 1999).

A recent co-immunoprecipitation study systematically evaluated Apaf-1 interaction with a panel of anti-apoptotic Bcl-2 homologues reportedly to be potential binding partners. There was no detectable interaction between Apaf-1 and mammalian or viral Bcl-2 cell death inhibitors. Additionally, endogenous Apaf-1 failed to co-

immunoprecipitate with endogenous Bcl-2 and Bcl-x_L. Furthermore, Apaf-1 which was isolated as a cytosolic activator of caspases (Zou *et al.*, 1997) did not exhibit similar subcellular distribution as Bcl-2/Bcl-x_L by confocal microscopy and subcellular fractionation (Moriishi *et al.*, 1999). In *in vitro* assays, the addition of exogenous Bcl-x_L was insufficient to prevent Apaf-1 activation of latent caspase 9 (Deveraux *et al.*, 1998). Thus, the interaction between Bcl-x_L and Apaf-1 either is weak, therefore not detectable or does not occur as previously reported. Although the interaction between Bcl-x_L and Apaf-1 is disputed, this evolutionarily conserved mechanism appears to be a plausible means of regulating caspase activation in mammals. Based on the redundancy of Bcl-2 homologues and caspases, similarly the existence of additional CED-4 (Apaf-1)-like adaptor molecules cannot be excluded.

1.5.4. Post-translational modifications determine active and inactive conformations of Bcl-2 homologues

The Bcl-2 family is comprised of proteins with opposing functions: those that promote apoptosis and others that act as inhibitors. The active and inactive conformations are determined by post-translational modifications such as (de)phosphorylation and proteolysis. Once modified many of the proteins redistribute within the cell to initiate their cell death suppressing or promoting function. Others participate in homo- and heterodimeric interactions to regulate activity (Figure 12).

1.5.4.1. Homo- and heterodimeric interactions

The "life/death rheostat" set by Bcl-2 homologues is mediated in part by competitive dimerizations between selective pairs of antagonists and agonists. The question that remains is which structure forms the active molecule. For example, do homodimers of death antagonists such as Bcl-2/Bcl-2 or Bcl-2/Bcl-x_L actively inhibit cell death or conversely, do dimers of death agonists such as Bax/Bax promote apoptosis?

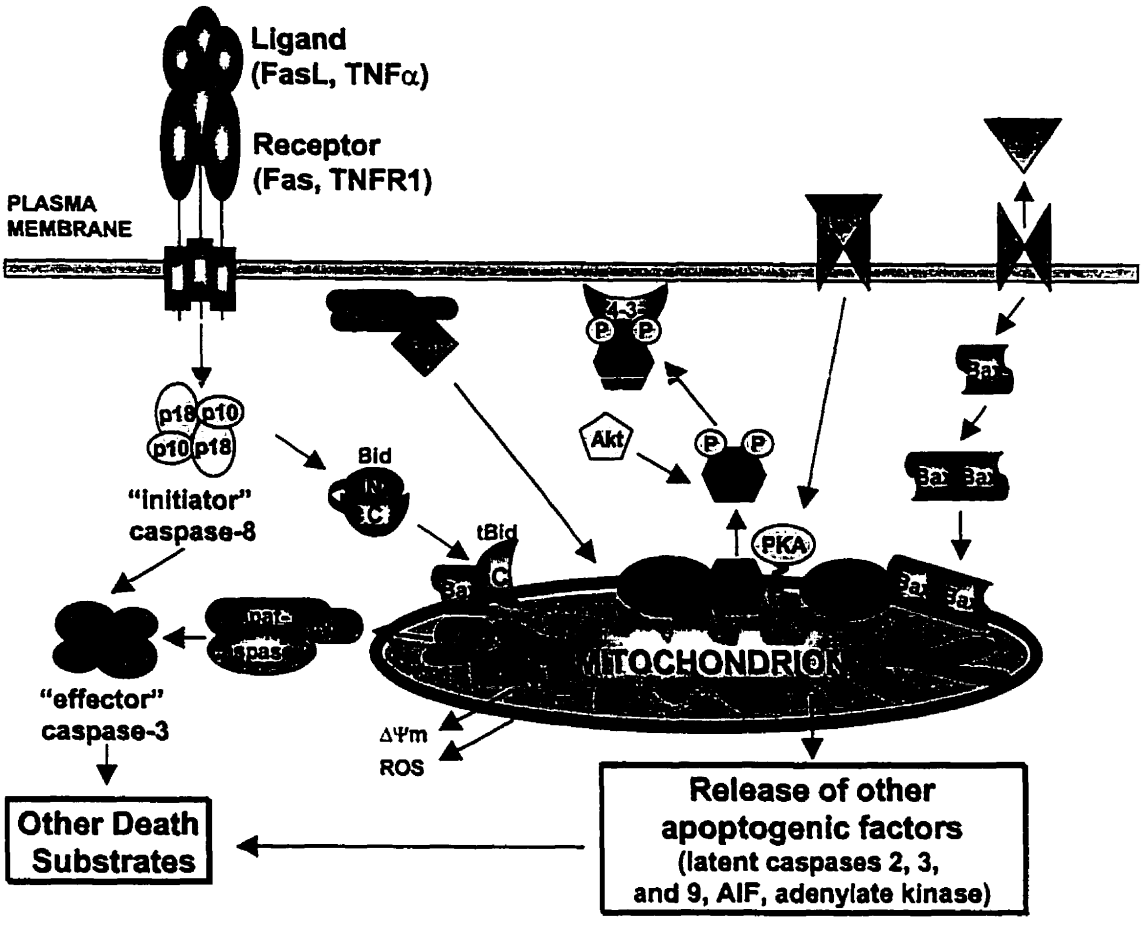

Figure 12. Model for regulation of pro-apoptotic Bcl-2 homologues via translocation from cytoplasmic to mitochondrial sites. The redistribution of Bcl-2 homologues, Bid, Bax, Bim and Bad to mitochondria in cells undergoing apoptosis resets the ratio of pro- and anti-apoptotic Bcl-2 homologues at the mitochondria. Ligation of death receptors TNFR1/FAS at the plasma membrane leads to activation of latent caspase 8 and subsequent cleavage of Bid. The COOH-terminal fragment of Bid translocates to the mitochondria to become an integral membrane protein to induce cytochrome *c* release. At the mitochondria, Bid has two potential binding partners: interaction with Bcl-2/Bcl-x_L counteracts the protective effects or interaction with Bax enhances its cytotoxicity. Bax resides in the cytosol or loosely associates with the mitochondrial membrane. Various apoptotic stimuli induce its translocation and integration into the mitochondrial membrane where it exists potentially as a homodimer and induces cytochrome *c* release. The cytotoxic effects of Bax proceed through Bcl-2/Bcl-x_L heterodimerization dependent and independent mechanisms similar to Bid. Bim is normally sequestered to the microtubule dynein motor complex. Following apoptotic stimuli, Bim redistributes to the mitochondria and antagonizes Bcl-2/Bcl-x_L function. In the presence of survival factors, Bad remains in the cytosol. It is phosphorylated by kinases Akt or PKA and sequestered by the phosphoserine binding protein 14-3-3. Calcineurin dephosphorylates Bad allowing it to translocate to the mitochondria to antagonize Bcl-2/Bcl-x_L and potentially to displace other interacting proteins. *(Adapted from Fadeel et al., 1999a and Gross et al., 1999a)*

Perhaps, the heterodimeric interactions between death antagonists and agonists such as Bcl-2/Bax or Bcl-2/Bcl-x_L/Bid are more critical to regulating the apoptotic response. To date, the stoichiometry of these interactions such as dimers, tetramers remain unknown. Therefore, it is possible that Bcl-2-related protein belong to a higher order of multiprotein complex involving Bcl-2 family members as well as other non-related apoptotic regulators (Zamzami *et al.*, 1998).

1.5.4.2. Phosphorylation

Protein phosphorylation is one of the most common cellular mechanisms to regulate proteins following translation. Although there is no conclusive evidence to show that kinases are required for execution of the cell death program, they have been observed to play an active role in regulating Bcl-2 function (Gjertson *et al.*, 1995; Anderson *et al.*, 1997). *In vivo*, select Bcl-2 family members have been found phosphorylated in response to stimuli such as growth factors or chemotherapeutic agents (Zamzami *et al.*, 1998; Fadeel *et al.*, 1999a; Gross *et al.*, 1999a).

The structural analysis of Bcl-x_L revealed a 60 amino acid loop domain between the BH4 and BH3 domains that lacks any defined structure. Although, the region is not conserved among anti-apoptotic Bcl-2 family members, structural modelling reveals that Bcl-2 shares a similar region. In the classification scheme for Bcl-2 homologues, the presence of the loop domain distinguishes between class I (Bcl-2/Bcl-x_L) and class II (Bcl-w) death antagonists (Figure 6). Although, the loop region is dispensable for anti-apoptotic function (Chang *et al.*, 1997), it does appear to serve as a negative regulatory site susceptible to phosphorylation, as described below and proteolytic processing (Section 1.5.4.3). There are several serine residues within the loop domain susceptible to phosphorylation by treatment with taxol and other chemotherapeutic agents that generally

target microtubules. The phosphorylation of Bcl-2/Bcl-x_L attenuates its anti-apoptotic activity (Haldar *et al.*, 1995; Chang *et al.*, 1997; Ito *et al.*, 1997; Poommipanit *et al.*, 1999) and removal of the loop domain leads to an increased resistance to drug-induced cell death and mitochondrial dysfunction in particular cell types (Fay *et al.*, 1998; Wang *et al.*, 1999b; Srivastava *et al.*, 1999). In contrast, other studies suggest that phosphorylation of Bcl-2 may be required for its anti-apoptotic function in growth factor dependent cell lines. In the presence of growth factors, a fraction of constitutively expressed Bcl-2 is phosphorylated on serine residues and localized primarily to the cytosol. The phosphorylated form of Bcl-2 was unable to interact with Bax (Poommipanit *et al.*, 1999). Thus, the phosphorylation of Bcl-2 within the flexible loop induces conformational changes that influence its function.

Another Bcl-2 homologue subjected to phosphorylation is the 'BH3-only' protein Bad (Figure 12). This TM domain less protein resides in the cytosol and is expressed in certain cell types (Kitada *et al.*, 1998). In the presence of growth factors, Bad is phosphorylated at two serine residues (Ser^{112} and Ser^{136}) which render the protein inactive. Specifically, Akt/PKB/RAC phosphorylates Bad at Ser^{136} (Datta *et al.*, 1997; del Peso *et al.*, 1997; Blume-Jensen *et al.*, 1998). Ser^{112} is specifically targeted by another cAMP-dependant protein kinase (PKA). PKA is anchored to the mitochondria through its association with an outer mitochondrial membrane protein, A-kinase anchoring protein (AKAP). In the presence of a survival factor, the tethered PKA is able to phosphorylate mitochondrial-based Bad resulting in its inactivation (Harada *et al.* 1999). Raf-1 kinase targeted to the mitochondria by Bcl-2 is also reported to phosphorylate BAD (Wang *et al.*, 1996a). The phosphorylated form of Bad is sequestered by 14-3-3 proteins that specifically recognize phosphoserine residues (Zha *et al.* 1996c). The complex remains in the cytosol as an inactive heterodimer, thereby allowing the cell to survive. Upon growth

factor withdrawal, Bad is dephosphorylated and exchanges binding partners from 14-3-3 to Bcl-2/Bcl-x_L. Following an increase in cytosolic calcium, the calcium activated protein phosphatase calcineurin has been reported to dephosphorylate Bad (Wang *et al.*, 1999a). Once dephosphorylated, Bad redistributes to the mitochondria through its interaction with Bcl-2/Bcl-x_L, thereby antagonizing Bcl-2/Bcl-x_L function (Minn *et al.* 1998; Gross *et al.*, 1999a). The BH3 domain of Bad is critical for interaction with Bcl-2/Bcl-x_L (Kelekar *et al.*, 1997; Ottilie *et al.*, 1997; Zha *et al.*, 1997). However, only the non-phosphorylated form of Bad is able to heterodimerize. These observations suggest that phosphorylation must alter the conformation of Bad and dephosphorylation would be predicted to render the hydrophobic face of the BH3 domain accessible. Thus, with the removal of phosphate groups, Bad becomes constituitively active and able to insert its BH3 domain into the hydrophobic pocket of Bcl-x_L to antagonize its function and displace other interacting proteins Bax or Bak to promote cell death (McDonnell *et al.* 1999; Gross *et al.*, 1999a).

1.5.4.3. Proteolytic processing

1.5.4.3.1. Caspase mediated cleavage of Bcl-2/Bcl-x_L

The cleavage of Bcl-2 in the variable loop region between the BH4 and BH3 domain results in loss of the BH4 domain and likely exposes the BH3 domain, converting the molecule from an anti-apoptotic to a pro-apoptotic protein (Cheng *et al.*, 1997a; Fujita *et al.*, 1998; Fujita and Tsuruo 1998). During viral infection, overexpressed 26 kDa Bcl-2 is cleaved to 23 kDa by activated caspase 3. The NH_2-terminal truncation of Bcl-2 is inhibited by caspase inhibitors or the introduction of mutations at the potential cleavage sites Asp^{31}, Asp^{34} and Asp^{36} (Grandgirand *et al.*, 1998). Caspase dependent cleavage of Bcl-2 and Bcl-x_L has been reported in response to numerous apoptotic triggers including growth factor withdrawal, death receptor ligation and DNA-damaging agents. The specific caspases that cleave and activate Bcl-2 in cells undergoing apoptosis remain to be

resolved. Various *in vivo* and *in vitro* studies have implicated initiator and/or effector caspases targeting Bcl-2/Bcl-x_L in a cell-type, signal specific manner (Cheng *et al.*, 1997a; Clem *et al.*, 1998; Grandgirand *et al.*, 1998; Kim *et al.*, 1998).

One of many roles of Bcl-2/Bcl-x_L is to preserve the function of the mitochondria by preventing the release of intermitochondrial proteins such as cytochrome *c* and AIF (Yang *et al.*, 1997; Kluck *et al.*, 1997; Susin *et al.*, 1999a). The cleavage of Bcl-2/Bcl-x_L early in apoptosis, not only disrupts the function of Bcl-2/Bcl-x_L, but likely accelerates the redistribution of intermitochondrial proteins, thus acting as an amplification step in the apoptotic pathway (Kirsch *et al.*, 1999). Furthermore, the sequestration of caspase precursors to similar subcellular compartments as Bcl-2/Bcl-x_L localization, such as the mitochondria and ER may expedite the preferential cleavage of Bcl-2/Bclx_L (Fadeel *et al.*, 1999b). The caspase-mediated cleavage of Bcl-2 appears to be an evolutionarily conserved event that has been observed in *C. elegans*, whereby CED-9 is proteolytically processed by CED-3. However, rather than amplifying the apoptotic signal, the cleavage of CED-9 produces a COOH-terminal fragment similar in sequence and function to Bcl-2, implying that the cleavage event further inhibits CED-3 function. A similar mechanism of action has not been noted among Bcl-2 homologues (Xue and Horvitz 1997).

1.5.4.3.2. Caspase mediated cleavage of Bid

The 'BH3-only' protein Bid has emerged as an intracellular messenger connecting cell death receptors and adaptor proteins at the plasma membrane to mitochondria (Li *et al.*, 1998; Luo *et al.*, 1998) (Figure 12). In healthy cells, Bid exists in the cytosol. Its NH_2- terminus contains a BH3-like motif that interacts with the BH3 domain, keeping the proteins in an inactive conformation that is likely a regulatory mechanism prior to cleavage (Tan *et al.*, 1999). Following Fas or TNFR1 receptor ligation, inactive full-

length Bid is cleaved at the NH_2-terminus by activated caspase 8 which exposes its BH3 domain. The truncated COOH-terminal fragment translocates to the mitochondria where its insertion triggers the release of cytochrome *c* (Li *et al.*, 1998; Luo *et al.*, 1998). In the presence of Bcl-2 or Bcl-x_L, the caspase 8 cleavage and translocation of Bid still occurs, however, Bcl-2/Bcl-x_L prevents the release of cytochrome *c* (Gross *et al.* 1999b).

The interaction of Bid with Bcl-2/Bcl-x_L requires an intact BH3 domain. Yet, its translocation to the mitochondria occurs independently of the BH3 domain (Li *et al.*, 1998; Luo *et al.*, 1998). The truncated from of Bid was found to bind to Bcl-x_L with higher affinity and induce the release of cytochrome *c* more efficiently than its full-length precursor (Li *et al.*, 1998). Recent studies proposed that Bid co-operates with Bax to cause mitochondrial dysfunction. The BH3 domain of Bid can interact with Bax through its hydrophobic pocket. The interaction likely induces a conformational change of Bax that enables its integration into the mitochondrial membrane (Desagher *et al.*, 1999; Eskes *et al.*, 2000). Mutations that disrupt the interaction between Bid and Bax have also been reported to compromise Bid-killing activity (Wang *et al.*, 1996b). In this context, Bid might function as a chaperone of Bax and facilitate its conformational change leading to Bax oligomerization, mitochondrial targeting and integration (Eskes *et al.*, 2000). Thus, Bid acts as a liaison between cell death receptors and mitochondrial dysfunction by complexing with survival factors to potentially attenuate their function or with pro-apoptotic proteins to enhance their function (Gross *et al.*, 1999b).

1.5.4.4. Redistribution of pro-apoptotic Bcl-2 homologues

Once a cell receives a death signal, several Bcl-2 family members transmit the signal to either avert or initiate a cell death response. The cytoplasmic compartments of cells contain the core components of the apoptotic machinery in a latent and/or

sequestered form (Takahashi and Earnshaw 1997). Apart from dimerization, the effect of many Bcl-2 family members also relies on intracellular movement during conditions of cellular stress. The activation of many of these proteins as described relies on post-translational modification such as the (de)phosphorylation of Bad, the cleavage of Bid or some other unidentified mechanism that triggers the redistribution of proteins such as Bim or Bax in response to a death signal (Figure 12).

1.5.4.4.1. Bim

Several 'BH3-only' proteins present in healthy cells are maintained by different mechanisms in a latent form until cued by cytotoxic signals. The 'BH3-only' protein Bim is expressed in many cell types, but usually sequestered to the microtubules dynein motor complex by interaction with dynein light chain LC8. Following an apoptotic signal, Bim coupled to LC8 disassociates from the dynein motor complex and translocates to the mitochondria where Bim interacts with Bcl-2 to antagonize its anti-apoptotic activity. The translocation of Bim does not involve caspase activation, possibly marking an early event in the apoptotic signalling pathway (Puthalakath *et al.*, 1999). The role of Bim as a component of the dynein motor complex remains to be determined. The release of Bim and possibly other unidentified Bcl-2 homologues from microtubules may be involved in apoptotic events responsible for microtubule disassembly (Fadeel *et al.*, 1999a).

1.5.4.4.2. Bax

Bax a pro-apoptotic Bcl-2 homologue undergoes considerable post-translational modification before exerting its cytotoxic effect. In certain cell types, Bax exists predominantly in the cytosol or loosely associates with the mitochondrial membrane. The induction of apoptosis induces a conformational change in Bax and targets the protein to mitochondria where it becomes an integral membrane protein (Hsu and Youle

1997; Wolter *et al.*, 1997; Gross *et al.*, 1998) (Figure 12). Although the immediate upstream events that lead to Bax conformational changes and targeting remain unknown, the NH_2-terminal region and TM domain are critical for Bax redistribution. The NH_2-terminus acts as a regulatory domain and the TM domain acts as a signal anchor sequence. Alterations to either region as well as interaction with 'BH3-only' protein Bid influences the overall conformation of Bax (Goping *et al.*, 1998; Eskes *et al.*, 2000).

Studies using specific antibodies to various Bax epitopes mapped regions of the NH_2-terminus not exposed in healthy cells, but detectable in dying cells. The removal of the COOH-terminal TM domain exposes the extreme NH_2-terminal region, suggesting the COOH-terminal tail of soluble, cytoplasmic Bax acts to shield the NH_2-terminal epitope through intramolecular interactions. Therefore, wild-type Bax undergoes conformational changes at the onset of apoptosis in which the COOH-terminus disengages from the NH_2-terminus (Nechushtan *et al.*, 1999). The first 20 NH_2-terminal amino acids appears to be required for regulated Bax targeting, as its deletion causes auto-insertion of the truncated protein into mitochondrial membranes (Goping *et al.*, 1998). The Bax COOH-terminal TM domain is essential for membrane integration. Under normal conditions, the activity of the TM domain is repressed. Its removal not only prevents Bax redistribution and mitochondrial integration, but also eliminates Bax cytotoxicity (Wolter *et al.*, 1997). The forced homodimerization of Bax also triggers its translocation to mitochondria resulting in mitochondrial damage leading to cell death. It is unclear whether Bax homodimerization is a consequence of translocation or insertion (Gross *et al.*, 1998).

1.5.5. Bcl-2 homologues share structural homology to bacterial pore-forming domains

Structural determination of Bcl-x_L and Bid revealed another possible mechanism of action. Although, these two proteins with opposing function share very limited sequence homology, they exhibit similar overall tertiary structure that resembles the pore forming domains found in various bacterial toxins such as diphtheria toxins and colicins (Muchmore *et al.*, 1996; Sattler *et al.*, 1997). The diphtheria toxin produced by *Corynebacterium diptheriae* is composed of two parts: the A fragment is involved in ADP-ribosylation and the B fragment is capable of forming pores (London 1992). The diphtheria toxin enters the cell by receptor medicated endocytosis. Inside the endosome, the acidic pH induces a conformational change allowing fragment B to form a channel in the endosome membrane through which fragment A passes into the cytosol and inhibits protein synthesis.

The other group of bacterial pore-forming toxins, the colicins are plasmid-encoded proteins secreted by bacteria to kill other bacteria. Following DNA damage, colicins are produced and bind to bacterial receptors on the outer membrane to translocate to the inner membrane. Colicins undergo a conformational change from water-soluble to membrane bound form. The ion channel forms initially by inserting the central two hydrophobic helices in response to the transmembrane potential of the inner membrane, followed by the insertion of additional two helices to create a four-helix bundle. This results in a highly conductive, voltage-gated ion channel that is capable of conducting non-specific, monovalent ions across the cytoplasmic membrane of the target cell. The formation of ion channels leads to depolarization of the cytoplasmic membrane, depletion of dATP/ATP thereby, ultimately killing the cell (Cramer *et al.*, 1995).

In order for the Bcl-2 homologues to form pores, they must contain helices that are long enough to span the membrane bilayer and must be largely devoid of charged residues. The average lipid bilayer has a hydrophobic cross-section of ~30Å (Montal and Mueller 1972). Therefore, the α-helix must be 20 amino acids in length to span a membrane bilayer and potentially participate in channel-formation (Schendel *et al.*, 1998). Bcl-x_L contains two α-helices (α5 and α6) COOH-terminus to the BH3 domain that meet this criterion (Figure 7). Consistent with the structural prediction of Bcl-x_L, molecular modelling of Bcl-2 and Bax reveal similar α-helical content and tertiary folds. The structural characterization of Bid also reveals two α-helices (α6 and α7) COOH-terminus to the BH3 domain capable of spanning a membrane. However, for Bcl-2 antagonists and agonists, the two α-helices are predicted to be insufficient to enclose an aqueous lumen. Therefore, Bcl-2, Bcl-x_L and even Bax may initially insert into the membrane via their TM domain to maintain an overall "globular" or receptor-like conformation. The propensity of Bcl-2-related proteins to form homo- and heterodimers suggests two or more molecules could come together, each contributing their hydrophobic helices to create a pore (Reed 1998; Schendel *et al.*, 1998; Zamzami *et al.*, 1998). The insertion of α5 and α6 could result in a conformational changes that liberates the BH4 and BH3 domains mediating interaction with other proteins and regulating pore-formation (Zamzami *et al.*, 1998).

As predicted from the tertiary structures, Bcl-x_L, Bcl-2, Bax and Bid were capable of incorporating into artificial membranes and forming channels under *in vitro* conditions previously shown suitable for channel-formation by diphtheria toxin and bacterial colicins (Antonsson *et al.*, 1997 Minn *et al.*, 1997; Schendel *et al.*, 1997; Schlesinger *et al.*, 1997; Schendel *et al.*, 1999). However, the ion selectivity, relative conductance and

dynamic characteristics of channels formed by Bcl-2 homologues appear to differ (Schendel *et al.*, 1998).

All four Bcl-2 family members were observed to form voltage and/or pH sensitive ion channels. Biologically active recombinant proteins, Bcl-x_L, Bcl-2 or Bax lacking its TM domain was used to assay for pore-formation. Similarly, Bid and the caspase 8 processed form tBid (Bid Δ1-55) were assayed for channel-forming activity. Low pH was found to promote ion channel-formation, similar to that observed with the bacterial pore forming domains. It is speculated that low pH conditions promote ion channel-formation by inducing a conformational change that exposes the two central α-helices allowing for membrane insertion. However, the pore-forming activity of Bid is unique, in that it occurred at low pH following proteolytic cleavage. Although, full-length Bid did exhibit some channel activity under low pH conditions, this observation likely stems from its NH_2-terminus becoming "unwrapped" from the protein similar to colicins in acidic conditions (Cramer *et al.* 1995; Schendel *et al.*, 1999).

The ion selectivity of channels formed by Bcl-2 homologues is also influenced by pH conditions. The channels formed by Bcl-2 and Bcl-x_L at neutral pH were more selective for cations than for anions whereas Bax was reported to be either mildly cation (Antonsson *et al.* 1997) or anion selective (Schlensinger *et al.*, 1997). Wild type Bid and the truncated form demonstrated no efflux of cations or anions at neutral pH (Schendel *et al.*, 1999). At lower pH, Bcl-2 and Bcl-x_L seemed to have lost their ability to discriminate between cations and anions, yet Bax apparently had enhanced selectivity (Antonsson *et al.* 1997; Minn *et al.*, 1997; Schendel *et al.*, 1997). In contrast, only cleaved Bid was observed to be cation selective (Schendel *et al.*, 1999). The apparent differences between pro- and anti-apoptotic members may be attributed to the

protination states of different amino acids lining the mouth and lumen of the pore. For example, α-helical wheel analysis of the putative membrane insertion domain of Bcl-x_L, shows a bias towards acidic residues on one face of the helix. At pH 4.0, these residues would be protinated and would not interfere with the passage of either cations or anions. At pH 7.2, the negatively charged residues would impede the passage of anions through the pore. Thus, the pH of the surrounding environment influences the ion selectivity of Bcl-2 homologues *in vitro*.

The ion channels formed by cell death antagonists, Bcl-2 and Bcl-x_L and agonists, Bax and Bid show differences in voltage conductance. The ion channels formed by Bcl-2 and Bcl-x_L behave in an ohmic fashion whereby plotting the current versus the applied voltage results in a linear relationship (Minn *et al.*, 1997; Schendel *et al.*, 1997). In contrast, Bax and Bid channel-formation do not display a linear relationship between current and voltage, rather channel-formation appears to be dependent on a membrane potential (Antonsson *et al.*, 1997; Schlesinger *et al.*, 1997; Schendel *et al.*, 1999). Similar to pores formed by colicins, Bid channel-formation is favoured by a positive potential (Schendel *et al.*, 1999) whereas Bax channel-formation is favoured by a negative potential (Antonsson *et al.*, 1997; Schlesinger *et al.*, 1997). The ion channels formed by Bcl-2, Bcl-x_L, Bax and Bid *in vitro* exhibit multiple conductance, either remaining predominantly open or closed or exhibiting flickering behaviour (Antonsson *et al.*, 1997 Minn *et al.*, 1997; Schendel *et al.*, 1997; Schlesinger *et al.*, 1997; Schendel *et al.*, 1999). This suggests that the presence of a proton gradient and membrane potential across the inner mitochondrial membrane could influence the process of protein insertion and/or channel-formation as well as its open or closed state (Schendel *et al.*, 1998). In this context, the mitochondrial contact sites where the inner and outer mitochondrial membranes meet contain a variety of channels. Many of the Bcl-2

proteins appear to congregate to these sites, thus Bcl-2 antagonists and agonists could potentially participate in the formation and/or regulation of mitochondrial conductance channels (Moran and Sorgato 1992; Kinnally *et al.*, 1996).

To explore whether the predicted fifth and sixth α-helices of Bcl-2 indeed contribute to pore-formation, a mutant lacking these two helices was constructed. The resulting Bcl-2 mutant was equivalent to the Bcl-x_L splicing variant, Bcl-x_S. The removal of the two central hydrophobic helices converted Bcl-2 into a pro-apoptotic molecule that initiated non-specific ion efflux from liposomes and failed to form discrete ion channels. These observations clearly demonstrated the central hydrophobic fifth and sixth α-helices to be involved in pore-formation by Bcl-2 and presumably Bcl-x_L and Bax (Schendel *et al.* 1997, 1998).

Further structure-function studies investigating the pore-forming properties of diphtheria toxin identified a stretch of charged amino acids connecting the two central hydrophobic helices. Mutations introducing a charge reversal within this region alter the channel-forming properties and prevents the full-length toxin from killing the target cell (Silverman *et al.* 1994). By inspection, Bcl-2, Bcl-x_L, Bax and Bak contain several charged residues within the region connecting α5 and α6. Among the anti-apoptotic members, the region contains a net negative charge, in contrast to a net positive charge among pro-apoptotic members Bax and Bak. The substitution of the 13 amino acid region between α5 and α6 from Bax into Bcl-x_L, resulted in a chimeric protein with an overall charge reversal in the substituted region. The mutant retained its anti-apoptotic activity and ability to interact with Bax. However, the Bcl-x_L chimeric protein exhibited altered ion channel-forming properties compared to wild type Bcl-x_L, whereby conductance was favoured by a positive potential. The mutation did not disrupt ion

selectivity of Bcl-x_L for cations. Overall, the substitution of Bax sequence into Bcl-x_L did not recapitulate the ion channel properties associated with Bax nor did it endow Bcl-x_L with Bax-like function (Minn *et al.*, 1999).

The contribution of the TM domain to the pore forming abilities of Bcl-2 homologues remains undetermined. The recombinant proteins used to date lack the hydrophobic COOH-terminal anchor, primarily because full-length proteins are insoluble and difficult to purify (Haldar *et al.*, 1994). Under experimental conditions, the absence of the TM domain likely relieves the protein of its normal means of membrane integration. Therefore, an acidic pH in *in vitro* studies is probably necessary to increase the likelihood the protein will find the membrane surface (Schendel *et al.*, 1998). For instance, at neutral pH, Bcl-2 and Bcl-x_L have a net negative charge. Under acidic conditions, the negatively charged residues would be protinated. The resulting charge neutralization would increase the hydrophobicity of the protein, making it more likely to insert into a membrane bilayer. The membrane lipid composition also appears to influence the ability of the proteins to become integrated. Recombinant Bcl-x_L and Bcl-2 lacking their TM domain only form channels when they are incorporated into membranes comprised of 30-40% acidic lipids (Minn *et al.*, 1997; Schendel *et al.*, 1997). Bid and truncated Bid normally exist without a TM domain are able to incorporate into vesicles of similar composition (Schendel *et al.*, 1999) whereas, Bax will incorporate into membranes containing neutral lipids (Antonsson *et al.*, 1997; Scheslinger *et al.*, 1997).

Evidence from biochemical studies indicate that Bcl-2-related antagonists and agonists form heterodimers to modulate their cell death activity which could conceivably be influenced by the ability of some members to form channels. *In vitro*, the Bcl-2 protein was found to prevent channel-formation by Bax. At neutral pH, pre-incubation of

Bax with tenfold excess of Bcl-2 prevented Bax channel-forming activity when added to liposomes. The antagonism of Bax channel activity was specific to Bcl-2, as incubation with control proteins had no effect on channel-formation (Antonsson *et al.*, 1997). At lower pH, Bcl-2 characteristically forms channels in liposomes, but exhibited an enhanced rate of ion efflux with the addition of Bcl-2 and Bax together (Schendel *et al.*, 1998). These findings suggest at neutral pH, Bax can form a pore and Bcl-2 will not form a pore, but prevent Bax from doing so. Bcl-2 may impede Bax from forming channels by heterodimerization which hinders the proteins (Bcl-2/Bax) from undergoing the necessary conformational change to promote transition from a soluble state to membrane bound state (Reed 1998; Zamzami *et al.*, 1998).

The ability to form pores appears to be a trait shared by several Bcl-2 family members. An apoptotic trigger is predicted to initiate the necessary conformational changes leading to pore-formation and organelle damage. Bcl-x_L and Bcl-2 are integrated membrane proteins localizing primarily to the mitochondria as well as the ER and nuclear envelope (Krajewski *et al.*, 1993; Akao *et al.*, 1994; Lithgow *et al.*, 1994). Bid and Bax become integral mitochondrial proteins following a death signal. It remains to be determined whether these pores actually form *in vivo* and if they do, their functional significance. *In vivo*, the pH essentially does not drop below pH 6.0, under these conditions, the results from *in vitro* studies suggest that anti- and pro-apoptotic members have different pore forming capabilities (Schendel *et al.*, 1999). Therefore, how channel-formation relates to the mutual antagonism displayed by anti-apoptotic proteins such as Bcl-2 and Bcl-x_L and pro-apoptotic proteins such as Bax and Bid remains to be determined. Although direct evidence is not available, several possibilities of how these proteins can regulate apoptosis by forming channels at the mitochondria can be envisioned.

1.5.6. Bcl-2 homologues at the mitochondria
1.5.6.1. Activity upstream and downstream of the mitochondria

There is increasing evidence from structure-function studies that many of the pro-apoptotic Bcl-2 homologues possess up to two independent mechanisms for promoting apoptosis. One mechanism relies on the BH3 domain functioning as the death ligand which heterodimerizes with Bcl-2/Bcl-x_L to antagonize its death-suppressing activity. The second mechanism is a heterodimerization-independent function, likely related to the ability of several Bcl-2 homologues to insert into membranes through hydrophobic regions other than their COOH-terminal TM domain to form ion channels (Minn *et al.*, 1998; Gross *et al.*, 1999a).

As an apoptotic signal permeates through the cell, molecules belonging to the 'BH3-only' branch of the Bcl-2 family lacking obvious hydrophobic core α-helices are predicted to serve as upstream death ligands (Gross *et al.*, 1999a). These proteins would function through Bcl-2/Bcl-x_L heterodimerization to inhibit their anti-apoptotic activity. Biochemical studies demonstrate that mutations in the BH3 domain of these pro-apoptotic proteins not only disrupts interaction with Bcl-2/Bcl-x_L, but also markedly impairs their death inducing ability (Kelekar and Thompson 1998). This hypothesis is consistent with the genetic pathway defined for *C. elegans* in which EGL-1 binds to CED-9, acting as an upstream negative regulator (Conradt and Horvitz 1998).

In contrast, pro-apoptotic molecules such as Bax and Bak share greater structural resemblance to Bcl-2/Bcl-x_L. These proteins have conserved BH1 and BH2 domains that correspond to α5 and α6 of the hydrophobic helical core that can become membrane integrated independent of anti-apoptotic molecules. In this context, pro-apoptotic Bax and Bak would work downstream of Bcl-2/Bcl-x_L, as well as function in parallel with their cell

death-suppressing partners, because of their propensity to heterodimerize via their BH3 domain. These observations are consistent with genetic evidence from gene deletion models of Bcl-2 and Bax that demonstrate either protein can regulate apoptosis in the absence of the other (Knudson and Korsemeyer 1997). Bid emerges as a unique 'BH3-only' protein. Similar to Bax/Bak it may function in parallel with Bcl-2/Bcl-x_L through heterodimerization to promote apoptosis. Alternatively, the shared tertiary structure between Bid and pore-forming bacterial toxins suggests that it can function independently of anti-apoptotic proteins by becoming an integral membrane protein and exert its effects downstream of Bcl-2/Bcl-x_L (Gross *et al.*, 1999a).

The hierarchy of binding affinities for BH3 peptides to Bcl-x_L provides support that many of these proteins act by competitively displacing other pro-apoptotic binding partners or heterologous interacting proteins from Bcl-2/Bcl-x_L. For example, the peptide encompassing the BH3 domain of Bad or Bcl-x competes for Bcl-x_L heterodimerization and is able to displace Bax, thereby releasing it to exert its cytotoxic effect (Yang *et al.*, 1995; Sattler *et al.*, 1997; Kelekar *et al.*, 1997; Chang *et al.*, 1999). Alternatively, the interaction between a 'BH3-only' protein and Bcl-x_L could displace heterologous interacting proteins such as Apaf-1-like molecules involved in caspase activation. Bcl-x_L has been found in association with Apaf-1 and some pro-apoptotic members have been shown to disassociate the complex (Hu *et al.*, 1998a; Pan *et al.*, 1998). This is consistent with the *C. elegans* model whereby EGL-1 binding to CED-9 displaces CED-4, releasing it to activate latent CED-3. Thus, the 'BH3-only' branch of the Bcl-2 family may act similarly by displacing CED-4/Apaf-1-like molecules as well as by inhibiting anti-apoptotic Bcl-2 homologues by heterodimerization (Gross *et al.*, 1999a).

1.5.6.2. Regulation of mitochondrial function

In the cell, the major site of Bcl-2 family activity is at the mitochondria. Here, death antagonists and agonists are implicated in several aspects of mitochondrial function, most importantly, regulating mitochondrial membrane permeability and the release of soluble intramembrane proteins via the outer mitochondrial membrane to the cytosol. The sequence of these alterations depends on the cell type and the apoptotic signal. Generally, the cytoprotective Bcl-2 homologues maintain the integrity of mitochondria through direct or indirect mechanisms by preserving mitochondrial membrane permeability and by preventing the redistribution of cytochrome *c* and presumably other apoptogenic factors. Conversely, the cytotoxic members of the Bcl-2 family trigger changes in membrane permeability and induce the release of cytochrome *c*, concomitantly with other proteins from the intermembrane space (Green and Kroemer *et al.*, 1998; Zamzami *et al.*, 1998).

The passage of cytochrome *c* through the outer mitochondrial membrane represents one of the most critical events responsible for mitochondrial-dependent caspase activation leading to an apoptotic cell death. Cytochrome *c* is a 12.3 kDa protein encoded by nuclear DNA that is synthesized in the cytosol and spontaneously inserts into the mitochondrial outer membrane via a non-receptor mediated process (Gonzales *et al.*, 1990; Stuart *et al.*, 1990). In the intramembrane space, cytochrome *c* is converted to a globular protein by the addition of a 'haem' group. Then, the functional cytochrome *c* shuttles electrons between complex III (ubiquinol:cytochrome *c* oxidoreductase) and complex IV (cytochrome oxidase) to maintain the inner membrane potential and cellular respiration. Cytochrome *c* cannot be released from the intermitochondrial space as long as the barrier function of the outer mitochondrial membrane remains intact. Once this has been disrupted, cytochrome *c* is released into

the cytosol and acts with additional co-factors, Apaf-1 and dATP/ATP to proteolytically process and activate latent caspase 9 (Li *et al.*, 1997b). In this context, the functional role of cytochrome *c* does not depend on its redox activity, rather only haem-binding halocytochrome *c* is competent to catalyze the activation of caspases (Kluck *et al.*, 1997; Hampton *et al.*, 1998). Studies of cytochrome *c* from difference species indicate that the release of this highly conserved molecule is a widespread and common response to a cell death signal. Furthermore, cytochrome *c* from various mammalian species (horse, bovine, rat, pigeon, tuna and *Drosophila melanogaster*) is able to reconstitute caspase activation *in vitro* (Liu *et al.*, 1996; Kluck *et al.*, 1997; Rodriguez *et al.*, 1999). Although the mechanism of cytochrome *c* release and its regulation by Bcl-2-related proteins remains unresolved, it has been proposed that its release is a consequence of either existing channels opening or through the formation of new channels. In addition, changes in channel regulation could conceivably lead to mitochondrial volume dysregulation whereby swelling of the matrix results in rupture of the outer membrane (Vander Heiden and Thompson 1999).

Mitochondria are well known to have a central role in energy metabolism. The active transport of protons creates an electrochemical gradient across the inner mitochondrial membrane that is essential for ATP production. The disruption of the transmembrane potential can be attributed to non-specific damage of the membrane or alternatively, to a more specific process involving pore forming proteins. The opening of proteaceous conductance channels called PT pores or megachannels alters membrane permeability. The PT pore is a dynamic multiprotein complex proposed to span both the inner and outer mitochondrial membranes at contact sites creating an inner membrane channel that allows the passage of molecules of approximately 1.5 kDa. Although the composition of the PT pore remains poorly defined, several known components include

proteins from the cytosol (hexokinase), intermembrane space (creatine kinase), the outer membrane (voltage dependent anion channel, VDAC), the inner membrane (adenine nucleotide translocator, ANT) and the matrix (cyclophilin D) (Zoratti *et al.*, 1995; Green and Kroemer 1998). The PT pore functions as a gated channel, participating in the regulation of matrix Ca^{2+}, pH and volume as well as membrane potential. The PT pore acts as a sensor to multiple physiological parameters and exhibits positive feedback since the disruption of mitochondrial function leads to the generation of many of the signals that contribute to PT in the first place, such as ROS production and increase in cytosolic calcium (Jacotot *et al.*, 1999).

Specifically, opening of the PT pore increases permeability of the inner mitochondrial membrane allowing for the movement of solutes less than 1.5 kDa. The equilibration of solutes across the membrane leads to a loss of inner mitochondrial membrane potential as well as uncoupling of the respiratory chain. A secondary consequence of PT pore opening includes ROS production and cessation of mitochondrial ATP production. In addition, entry of water into the mitochondrial matrix causes the matrix to expand. As the surface area of the inner membrane with its folded cristae is greater than the outer membrane, the increase in matrix volume eventually causes the outer membrane to rupture, thereby releasing proteins sequestered in the intermitochondrial space into the cytosol (Bernardi 1996; Petit *et al.*, 1996; Susin *et al.*, 1997). Apart from cytochrome *c*, other potential apoptotic regulatory proteins also escape from the intermitochondrial space. For example, in some cell types, a proportion of inactive caspase 2, 3 and 9 reside in the intermitochondrial space (Susin *et al.*, 1999a). Other proteins that are sequestered in the mitochondria include AIF that is responsible for caspase-independent nuclear changes associated with apoptosis (Susin *et al.*, 1999b). Adenylate kinase (Single *et al.*, 1998) as well as numerous other proteins

(Patterson *et al.*, 2000) are found to redistribute from the intermembrane space in response to a variety of death signals with no known involvement in apoptosis.

The potential role for the PT pore in apoptosis was demonstrated by treatment with agents such as calcium, GD3 ganglioside and atractyloside that induce PT pore opening (Zamzami *et al.*, 1996; Kristal *et al.*, 1999; Scorrano *et al.*, 1999) and likewise by other agents such as cyclosporin A or bongkrekic acid that prevent PT pore opening, thus, circumventing the release of cell death-inducing factors and subsequent cell death. Bcl-2 family members reportedly regulate the function of the PT pore in both isolated mitochondria and intact cells. The enforced dimerization or overexpression of Bax or Bak resulted in altered mitochondrial membrane potential, ROS production and in certain settings the release of cytochrome *c* (Xiang *et al.*, 1996; McCarthy *et al.*, 1997; Eskes *et al.*, 1998; Gross *et al.*, 1998; Pastorino *et al.*, 1998). Peptides derived from the BH3 region have also been shown to induce cytochrome *c* release from isolated mitochondria *in vitro* (Cosulich *et al.*, 1997). Expression of truncated Bid also had similar effects (Li *et al.*, 1998; Luo *et al.*, 1998). However, the expression of Bcl-2 or Bcl-x_L, confers protection to mitochondria and can in part neutralize the effect of its pro-apoptotic homologues (Li *et al.*, 1998; Luo *et al.*, 1998; Cosulich *et al.*, 1997). The mitochondrial effects of Bax could be countered by cyclosporin A or bongkrekic acid, suggesting that Bax may work at least in part through the PT pore (Shimizu *et al.*, 1998). Bax has been found to co-purify with ANT (Marzo *et al.*, 1998a) and VDAC (Narita *et al.*, 1998) and thought to induce channel opening to release cytochrome *c* and Bcl-x_L is proposed to maintain the channel in a closed conformation (Shimizu *et al.*, 1999; Marzo *et al.*, 1998a; Marzo *et al.*, 1998b).

Alternatively, Bcl-2 antagonists and agonists could form channels in the outer mitochondrial membrane altering its permeability. These large, non-specific channels could be responsible for the redistribution of cytochrome *c* as well as several other intermitochondrial proteins and small molecules that are released concomitantly following the induction of cell death (Vander Heiden and Thompson 1999). The multimerization of the Bcl-2 homologues appears in part to contribute to their function and has been shown to induce channel formation in *in vitro* systems (Antonsson *et al.*, 1997 Minn *et al.*, 1997; Schendel *et al.*, 1997; Schlesinger *et al.*, 1997; Schendel *et al.*, 1999). The channels would be predicted to form in response to an apoptotic signal. Proteins such as Bax and Bid could co-operatively form selective channels that allow the redistribution of cytochrome *c* in the absence of mitochondrial swelling (Luo *et al.*, 1998; Eskes *et al.*, 1998; Jurgensmeier *et al.*, 1998). Since the release of cytochrome *c* has been demonstrated to occur independently of changes in membrane potential, its release may reflect a more specialized mechanism attributable to the pore-forming capabilities of pro-apoptotic Bcl-2 homologues (Bossy-Wetzel *et al.*, 1998; Shimizu *et al.*, 2000).

Although the mechanism by which Bcl-2 homologues regulate existing channels or form new channels remains uncertain, the redistribution of cytochrome *c* and likely other proteins and/or molecules may in fact be bi-directional events. The release of intermitochondrial proteins by outer membrane rupture appears to be an uni-directional event. However, resealing of the outer mitochondrial membrane has been suggested to prevent complete loss of intermitochondrial proteins. Recent observations suggest that cytochrome *c* could cross the outer mitochondrial membrane aided by a cytosolic factor that co-operates with Bax and Bid to increase permeability of the outer mitochondrial membrane, (Kluck *et al.*, 1999). Thus, Bcl-2 homologues residing in the cytosol or

localized to organelles such as the mitochondria, ER and nuclear envelope function together either to maintain cellular homeostasis or initiate an apoptotic response.

1.5.7. Bcl-2 family knockout studies

The physiological role of several Bcl-2 family members has been explored by targeted gene disruption. To date, mice deficient in cell death antagonists include Bcl-2, Bcl-x_L and Bcl-w and cell death agonists include Bax, Bim and Bid. The deletion of either death inhibiting or death promoting Bcl-2 homologues suggests each likely plays different and possibly non-redundant roles during development and cellular homeostasis.

Newborn Bcl-2 deficient mice are viable, with the majority dying at a few weeks of age due to renal failure. At 5 to 6 days, the mice turn grey resulting from the premature death of melanocytes. Initially, the development of the hematopoietic system, including B and T lymphocyte selection and their population of lymphoid organs progresses normally. At later stages, the lymphoid organs become involuted due to massive cell death, indicating failure to maintain homeostasis of both B and T cell lineages (Veis *et al.*, 1995). While the absence of Bcl-2 allows viable mice to be born, the absence of Bcl-x_L results in a more severe phenotype with mice dying at embryonic day 13. These mice exhibit extensive cell death throughout the brain and spinal cord, specifically in regions of postmitotic, differentiating neurons where Bcl-x_L expression is normally high. In the hematopoietic system, massive cell death is apparent in the developing liver (Motoyama *et al.*, 1995). The deletion of a lesser known member of the Bcl-2 family, Bcl-w results in viable but sterile mice that exhibits progressive testicular degeneration leading to depletion of germ cells and subsequent failed spermatogenesis (Print *et al.*, 1998; Ross *et al.*, 1998).

Current interest has turned towards characterizing mice deficient in Bcl-2 homologues that promote cell death. In the absence of Bax, mice were viable indicating Bax does not play a role in development. However, Bax deficient males were infertile due to disordered seminiferous tubules with the accumulation of atypical pre-meiotic germ cells, but not mature haploid sperm. In contrast, Bax ovaries showed an accumulation of atrophic granulosa cells and primary follicles that presumably failed to undergo apoptosis (Knudson *et al*; 1995; Perez *et al.*, 1997). Hyperplasia of thymocytes and B-cells was apparent. Bax deficient sympathetic neurons survived growth factor withdrawal and motor neurons tolerated disconnection from their targets by axotomy. These neurons demonstrated reduced neurite outgrowth and had atrophic somas that could be reversed by the re-addition of trophic factors (Deckwerth *et al.*, 1996).

Two recent studies reported the outcome of deleting genes encoding 'BH3-only' proteins, Bim (Bouillet *et al.*, 1999) and Bid (Yin *et al.*, 1999). Bim appears to have an unidentified role in embryonic development. Although the penetrance of embryonic lethality appeared to be influenced by the genetic background of the mice, the loss of Bim deficient fetuses were noted before embryonic day 10. Bim is expressed in many hematopoietic cell types and its absence markedly affected homeostasis leading to the accumulation of lymphoid and myeloid cells as well as perturbation of thymic T cell development. With age, Bim deficiency led to an accumulation of plasma cells and systemic autoimmune disease. Eventually, the deficient mice died due to kidney disease (Bouillet *et al.*, 1999).

Interestingly, Bid deficient mice demonstrated no developmental abnormalities and all organs appeared normal according to weight and histology. Bid is best characterized for its role in the Fas/TNFR1 signalling pathway. Following injection with anti-Fas antibody,

most wild type mice died due to acute liver failure associated with massive hepatic apoptosis. In contrast, Bid deficient mice survived the anti-Fas antibody injection, with the majority showing no apparent liver injury. Immunohistochemical analysis confirmed the activation of caspase 8, but did not detect activation of downstream effector caspases 3 and 7 or the release of cytochrome *c*. Other Bid deficient mice survived with only moderate liver damage and activation of caspase 3, 7 and 8 without any apparent cytochrome *c* release. This loss-of-function model clearly implicates Bid as a critical substrate *in vivo* for signalling by death receptors which require the release of mitochondrial proteins to amplify the apoptotic signal in selected populations of dying cells (Yin *et al.*, 1999).

As discussed previously, Bcl-2 homologues function in part through dimerization. Extensive biochemical studies have not definitively established whether their function is interdependent or whether antagonists or agonists are dominant. To gain a better understanding whether cell death inhibitors or promoters function in the absence of the other, double knockout mice were bred. The targeted disruption of Bcl-x_L led to massive cell death of immature neurons in the developing mouse central nervous system. The deletion of Bax blocked cell death in specific populations of sympathetic and motor neurons. Mice deficient in both Bcl-x_L and Bax exhibited a reduction in levels of cell death because Bax deficiency countered the increased cell death in immature neurons caused by Bcl-x_L deficiency (Shindler *et al.*, 1997). These findings suggest that both Bax and Bcl-x_L contribute to regulating cell death in development of the central nervous system. Similarly, Bcl-2 and Bax appear to act along the same pathway in certain cell deaths. The thymic hypoplasia and cell death characteristic of Bcl-2 deficiency is largely absent in mice also deficient in Bax. Yet, either Bcl-2 or Bax can function efficiently in the absence of the other. Transient expression of Bcl-2 can function to block apoptosis in the absence of both

Bcl-2 and Bax. Similarly, Bax can promote apoptosis in the absence of Bcl-2. These findings imply the two proteins can exert their effects independently. Thus, Bcl-2/Bax heterodimerization is not exclusively required for either Bcl-2 repression or Bax promotion of cell death (Knudson and Korsmeyer 1997).

Since Bcl-2-related proteins represent a multigene family that is continually expanding, the contribution of unidentified homologues cannot be excluded in these models, especially in the double knockout mice. Overall, the absence of Bcl-2 homologues appears to affect the apoptotic pathway in specific cell types either in the course of development or in maintaining cellular homeostasis. It appears that the 'BH3-only' proteins are required to execute particular death responses in individual cell types, suggesting that certain death stimuli activate apoptosis through distinct 'BH3-only' proteins (Bouillet *et al.*, 1999).

1.5.8. Effect of mammalian Bcl-2 homologues expressed in yeast

Genetic studies in mice and recently those in yeast indicate that although competitive dimerizations between Bcl-2 family contribute to their function, some members inhibit or promote cell death independently of their antagonistic binding partner. The cell death activity of Bcl-2-related proteins has been studied in both budding yeast (*Sacchromyces cerevisiae*) and fission yeast (*Schizosaccharomyces pombe*). Yeast are a useful model to study cell death activity of Bcl-2-related proteins because the genomic sequence of yeast lacks known Bcl-2 homologues.

In yeast, the expression of either Bax or the closely related protein, Bak is lethal. The cell death induced by either protein can be suppressed by co-expression of Bcl-2 and Bcl-x_L (Sato *et al*, 1994; Hanada *et al.*, 1995; Greenhalf *et al.*, 1996; Zha *et al.*, 1996b; Ink *et al.*, 1997; Jurgensmeier *et al.*, 1997; Manon *et al.*, 1997). Bax expressed

in yeast is associated primarily with mitochondria and targeting of the proteins to these organelles appears to be important for its lethal phenotype. Bax expressed lacking its TM domain remains cytosolic and loses its toxicity in yeast cells. By fusing the truncated Bax to the yeast outer mitochondrial membrane targeting sequence, Mas70p restores its ability to localize to the mitochondria and cell death activity, suggesting that TM domain targeting of Bax to mitochondria is critical for its function. Yeast with mutations in the nuclear gene encoding mitochondrial F_0F_1-ATPase are resistant to the lethal effects of Bax (Matsuyama *et al.*, 1998).

Bax relies on two mechanisms to induce cell death in mammalian cells. One strategy relies on an intact BH3 domain that mediates heterodimerization with Bcl-2/Bcl-x_L. Specific, Bax BH3 domain point mutants found to be cytotoxic in yeast, had lost their ability to interact with Bcl-2. The co-expression of these BH3 mutants with Bcl-2 rescued the cells from the lethal effects of Bax (Zha and Reed 1997). Therefore, the cytotoxic activity of Bax is attributed to another mechanism, its intrinsic pore-forming abilities. Findings from Bax mutation analysis demonstrate that deletion of the α5 and α6 helices diminishes its ability to induce cell death in yeast (Matsuyama *et al.*, 1998). To date, the expression of the 'BH3-only' branch of the Bcl-2 family proteins do not appear to have any autonomous function in yeast (Zamzami *et al.*, 1998). Bcl-2 protein in yeast reportedly enhance survival in conditions of starvation and has a protective effect in superoxide dismutase deficient strains from cell death induced by oxidative stress (Kane *et al.*, 1993; Longo *et al.*, 1997).

Thus, it appears Bcl-2 homologues when expressed in yeast, each have a specific function in regulating cell viability. Apart from lacking endogenous Bcl-2 orthologues, yeast do not have caspases either. Therefore, yeast cells undergoing Bax-

induced cell death exhibit morphological changes that include massive cytosolic vacularization and mitochondrial disruption via the F_0F_1-ATPase proton pump (Ink *et al.*, 1997; Jurgensmeier *et al.*, 1997; Matsuyama *et al.*, 1998), similar to mammalian cells that express Bax in the presence of caspase inhibitors (Xiang *et al.*, 1996).

1.6. The BNip3 family

BNip3 (Bcl-2/E1B Nineteen kDa interacting proteins 3) was originally identified in a yeast two-hybrid screen using the adenoviral functional homologue of Bcl-2, E1B 19K as the bait protein (Boyd *et al.*, 1994). BNip3 (formerly Nip3) was the first characterized family member, sharing overall 56% homology with another mammalian homologue, Nip3-like protein x, Nix (BNip3L/BNip3α/B5) (Chen *et al.*, 1999; Imazu *et al.*, 1999; Ohi *et al.*, 1999; Yasuda *et al.*, 1999) (Table 2). Sequence comparison between the two proteins reveals BNip3 and Nix diverge in the NH_2-terminal region, but share up to 85% identity in the COOH-terminal region (Chen *et al.*, 1999). The mouse orthologues of BNip3 and Nix share 90% and 97% identity, respectively with their human counterparts. The *C. elegans* orthologue of BNip3, CeBNip3 shares 22% identity with both BNip3 and Nix (Yasuda *et al.*, 1998a; Cizeau *et al.*,2000).

BNip3 is detected as a major transcript of 1.7 kb in all mouse tissues a larger transcript of 2.5 kb was also detected in the brain, heart, kidney, liver and submaxillary gland. BNip3 mRNA is expressed in human breast carcinoma MCF-7 cells as a major transcript of 1.7 kb and two minor transcripts of 1.5 and 1.3 kb (Chen *et al.* 1997). In other human tissues examined, two transcripts were apparent, with the larger transcript being prominently expressed in brain and heart (Yasuda *et al.* 1998b). Two Nix transcripts, 1.6 kb, and 3.9 kb were ubiquitously expressed in human tissues studied, with notably lower levels in liver, skeletal muscle and the pancreas (Yasuda *et al.* 1999). The mRNA

transcript encoding BNip3 and Nix share approximately 50% nucleotide sequence homology. The presence of more than one transcript encoding BNip3 or Nix could be due to splicing variants, other potential homologues and/or the existence of pseudogenes. The BNip3 protein was found to be abundantly expressed in mouse skeletal muscle and has been detected in tongue and skin. BNip3 could not be detected in whole cell lysates prepared from several cell lines. However, following subcellular fractionation, BNip3 was detected in the mitochondrial fraction (Vande Velde *et al.*, 2000). The protein expression pattern of Nix remains to be determined. In *C. elegans*, the CeBNip3 mRNA is expressed as a major transcript of 1.4 kb and minor transcript of 1.6 kb. The 1.4 kb transcript is abundantly expressed at the embryonic stage and to a lesser extent at the larvae and young adult stages (Cizeau *et al.*, 2000). The pattern of CeBNip3 protein expression has not been determined.

Several structural elements are common to all BNip3 homologues (Figure 13). The NH_2-terminus contains PEST sequences. These regions are defined by frequent stretches of proline (P), glutamic acid (E), serine (S), threonine (T) and aspartic acid (D) flanked by charged amino acids such as histidine, arginine or lysine. The PEST sequences are associated with proteins that have a high turnover rate and whose degradation is controlled by the proteosome (Rogers *et al.* 1986). Another feature is a stretch of 16 amino acids that appear to be evolutionarily conserved from the *C. elegans* to mammalian orthologues of BNip3. Furthermore, BNip3, Nix and CeBNip3 contain two structural elements in common with Bcl-2 homologues: (1) a putative BH3 domain and (2) a COOH-terminal TM domain. Typically, Bcl-2-related agonists rely on their BH3 domain to facilitate heterodimerization with antagonists Bcl-2/Bcl-x_L and promote cell death. And the, TM domain ensures correct protein targeting to intracellular membranes critical for its function (Adams and Cory 1998; Reed 1998; Gross *et al.*, 1999a). Studies have shown

that the BNip3 family has emerged as a class of mitochondrial proteins that induce cell death when transiently expressed in mammalian cells (Chen *et al.*, 1997, 1999; Cizeau *et al.*, 2000). The contribution of the various structural elements to its function has been explored by mutational analysis (Section 4) and the findings summarized and discussed in detail (Sections 5 and 6).

Table 2. The BNip3 family

Protein	Species	GenBank Accession No.	References
[1]BNip3	human	NM_004052	Boyd *et al.*, 1994 Chen *et al.*, 1997 Yasuda *et al.*, 1998b
BNip3	mouse	NM_009760	D. Dubik and A.H. Greenberg[4]
[2]Nix	human	NM_004331	Matsushima *et al.*, 1998 Chen *et al.*, 1999 Yasuda *et al.*, 1999
Nix	mouse	NM_009761	Chen *et al.*, 1999
[3]CeBNip3	*C. elegans*	AF080583 AF133822	Yasuda *et al.*, 1998a Cizeau *et al.*, 2000

[1]BNip3, Bcl-2/E1B Nineteen kDa interacting protein 3
[2]Nix, BNip3-like protein x; also referred to as BNip3L, BNip3α or B5
[3]CeBNip3, C. elegans BNip3
[4]*unpublished observations*

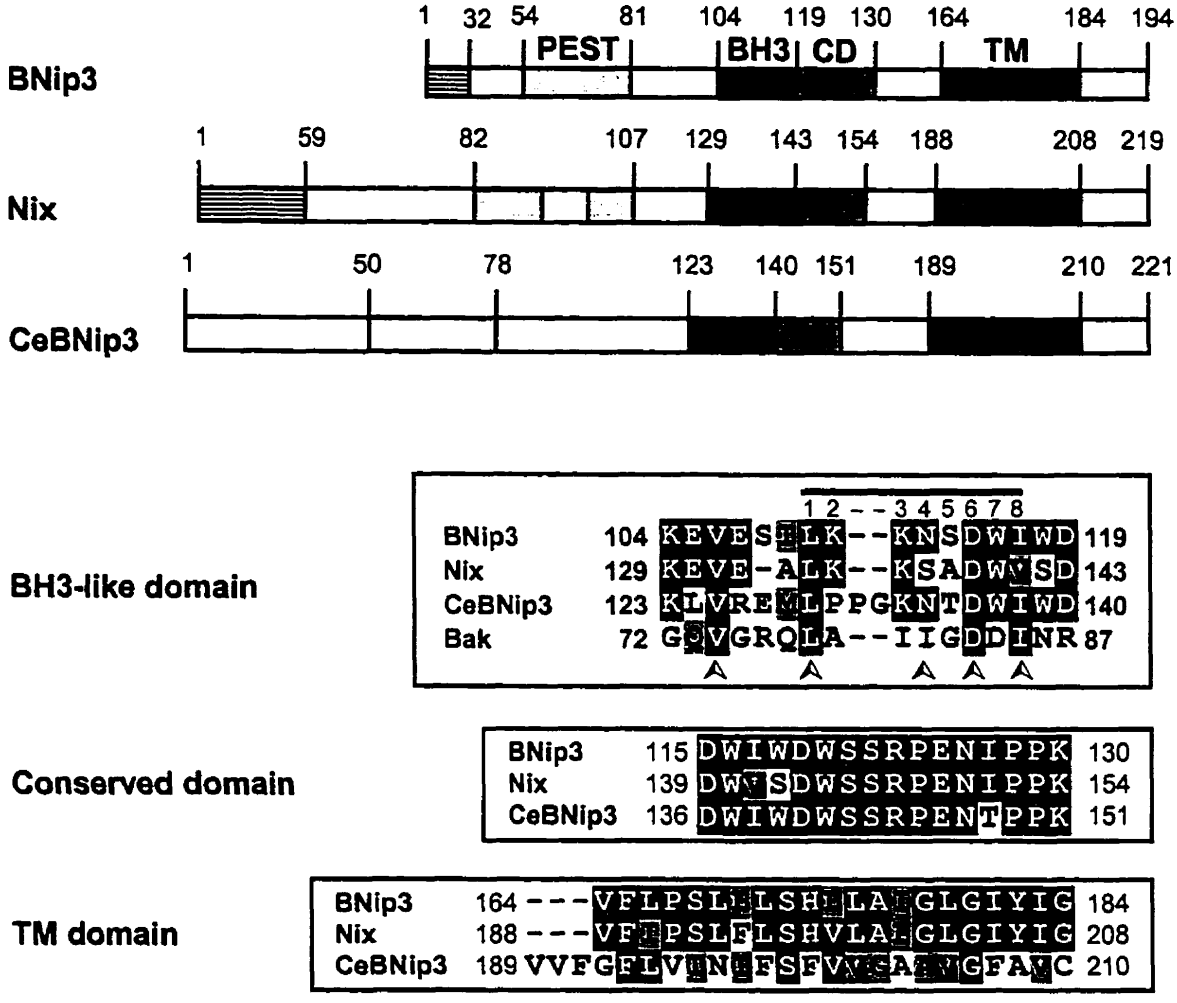

Figure 13. The structural arrangement of BNip3-related proteins. To date the BNip3 family of proteins consists of BNip3 and its homologue Nix (also called BNip3L, BNip3α or B5) as well as its orthologues found in mouse (mBNip3) and *C. elegans* (CeBNip3). BNip3 shares overall 56%, 90% and 21% identity with Nix, mBNip3 and CeBNip3 respectively. The NH_2-terminus of BNip3 and Nix are most divergent (hatched region). The proteins share structural similarity in the PEST region, Bcl-2 homology 3 (BH3) domain, conserved domain (CD) and COOH-terminal transmembrane (TM) domain. The aligned sequences of the BH3 domain, CD region and TM domain are shown for BNip3, Nix and CeBNip3. The identical and similar amino acids are shaded in black and grey, respectively. Among the BH3-only proteins, the BH3 domain facilitates heterodimerization and promotes apoptosis. Specifically, the residues valine (V), leucine (L), aspartic acid (D) and isoleucine (I) of BNip3 are conserved with the critical BH3 domain amino acids of Bak (marked ^) that determine Bak-Bcl-x_L heterodimerization. The bar across the top denotes the eight residue core of the BH3 domain. *(Adapted from Chen et al., 1997; 1999; Cizeau et al., 2000)*

2. Hypothesis

BNip3-related proteins based on structural similarities and mitochondrial localization, belong to a distinct subfamily of proteins related to the Bcl-2 family whose expression and homo- and heterodimeric interactions in part activate cell death in mammalian cells.

2.1. Objectives

1. To determine the region(s) of BNip3-related proteins critical for homodimerization and the role of homodimerization in BNip3-induced cell death.

2. To determine the region(s) of BNip3-related proteins critical for heterodimerization and the role of heterodimerization in BNip3-induced cell death.

3. To determine if the putative BNip3 BH3 domain, like typical 'BH3-only' proteins mediates Bcl-2/Bcl-x_L heterodimerization and promotes cell death.

4. To map BNip3 heterodimerization with other family members and heterologous protein, Bcl-x_S.

3. Materials and Methods

3.1. Reagents

Reagents, except as noted, were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A.). All solutions and buffers were filtered to remove any particulate matter using 0.22 μm pore sized filters (Nalge Nunc International, Rochester, NY, U.S.A.) and stored at room temperature, 4°C or -20°C as required.

3.2. Media

3.2.1. Bacterial media

Commercially available pre-mixed powders for Luria Bertani (LB) broth and LB agar were dissolved in the required volume of distilled deionized water (ddH_2O) and autoclaved at 121°C for 20 minutes. All media used for bacterial culture (liquid and solid) were supplemented with 1% ampicillin as described. The LB broth was stored at 4°C until required. Prior to use, the medium was pre-warmed to room temperature and supplemented with 1% ampicillin. The LB agar was cooled to 55°C, then supplemented with 1% ampicillin and poured into 100 mm plastic petri dishes (Fisher Scientific Canada, Nepean, ON, Canada). The medium was allowed to harden and dry at room temperature, then stored at 4°C until needed. The SOC medium used for bacterial transformations is commercially available from GIBCO BRL (Gaithersburg, MD, U.S.A.). For transformations requiring blue/white colony selection, 40 μl of 5-bromo-4-chloro-3-indolyl-ŧ-D-galactopyranoside (X-gal) dissolved in N,N-dimethylformamide (DMF) (40 mg/ml) was aseptically spread on to LB plates containing 1% ampicillin approximately 30 minutes prior to plating transformants.

3.2.2. Cell culture media

Dulbecco's Modified Eagle Media (DMEM, GIBCO BRL) and α-minimal essential medium (α-MEM, GIBCO BRL) were made according to manufacturers' instructions. The pH was adjusted to between 7.2 to 7.4 and then filter sterilized. All media was stored at 4°C until needed. Prior to use for cell culture, all media were supplemented with 10%v/v fetal bovine serum (FBS) (GIBCO BRL) and pre-warmed to 37°C.

3.2.3. Yeast media

3.2.3.1. Yeast extract-peptone-dextrose medium plus adenine

Yeast extract-peptone-dextrose medium plus adenine (YPAD) solid medium consists of 1% w/v yeast extract (Difco Laboratories, Detroit, MI, U.S.A.), 1% w/v peptone (Difco Laboratories), 1.2% w/v dextrose (Fisher Scientific), 0.1% w/v adenine hemisulphate and 1.5% w/v Bacto-agar (Difco Laboratories) dissolved in ddH_2O. After autoclaving at 121°C for 20 minutes, the medium was cooled and poured into 100mm plastic petri dishes (Fisher Scientific). Once hardened and dried, the plates were stored at 4°C until needed. For liquid YPAD, the agar was omitted. Following autoclaving, the medium was stored at room temperature and warmed to 30°C as needed.

3.2.3.2. Synthetic complete drop-out medium

To make synthetic complete (SC) media, the following were dissolved in 600 ml ddH_2O: 4 g yeast nitrogen base (without $(NH_4)_2SO_4$ (ammonium sulfate) or amino acids, Difco Laboratories), 3 g $(NH_4)_2SO_4$ (Fisher Scientific), 12 g dextrose (Fisher Scientific) and 0.5 g drop-out mix (Section 3.2.3.3). After adjusting the pH to 5.6 with 1M NaOH, 10 g of Bacto-agar (Difco Laboratories) was added to the solution and autoclaved. Once the medium was cooled, it was poured into plastic petri dishes (Fisher Scientific), allowed to harden and dry, then stored at 4°C.

3.2.3.3. Drop-out mix for synthetic medium

The synthetic complete (SC) medium contains all the amino acids in the appropriate amounts listed below omitting those marked (*) to produce synthetic complete minus (SC-) drop-out media: SC-Leu, SC-Trp, SC-Trp/Leu and SC-Trp/Leu/His. The components were combined together and stored at room temperature.

2 g Adenine hemisulfate
2 g Arginine HCl
2 g Histidine HCl*
2 g Isoleucine
4 g Leucine*
2 g Lysine HCl
2 g Methionine
3 g Phenylalanine
6 g Homoserine
3 g Tryptophan*
2 g Tyrosine
9 g Valine

3.2.3.4. 3-amino-1,2,4-triazole medium

To increase the selection pressure for histidine protophy, SC-Trp/Leu/His plates were supplemented with varying concentrations of 3-amino-1,2,4-triazole (3-AT) (Kishore and Shah 1988).

3.3. Antibodies

Hamster monoclonal antibody (mAb) raised against purified recombinant human Bcl-2 protein (Clone 6C8) and a mouse mAb raised against amino acids 61-76 of human Bcl-2 protein (Clone 4D7) were obtained from PharMingen (Mississauga, ON, Canada). Mouse mAb specific to amino acids 3-14 common to human and mouse Bcl-x_L (Clone 2H12) was also obtained from PharMingen. Rabbit (polyclonal antibody) pAb against Bcl-x_L was obtained from (Transduction Laboratories, Lexington, KY, U.S.A.). Mouse mAb against mitochondrial heat shock protein 60 (HSP60) was a gift from Dr. Radney Gupta (McMaster University, Hamilton, ON, Canada). Mouse mAb raised against the COOH-

terminus of human c-myc, amino acids 408-439 (Clone 9E10) was a gift from Dr. Jim Wright (University of Manitoba, Winnipeg, MB, Canada). Mouse mAb to the epitope tag T7 (MASMTGGQQMG) was obtained from Novagen (Madison, WI, U.S.A.). Two mouse mAbs were produced against human BNip3, amino acids 1-163: clone Ana40 recognizes amino acids 112-124 and clone Anb51 recognizes amino acids 98-109. The rabbit pAb generated against human BNip3, amino acids 1-163 recognizes amino acids 49-104. The specificity of anti-mouse and anti-rabbit BNip3 antibodies was determined by Western blot analysis of a series of BNip3 deletion mutants.

The primary antibodies were detected by goat anti-mouse or goat anti-rabbit antibody conjugated to horseradish peroxidase for Western blot analysis and goat anti-mouse or anti-rabbit antibody conjugated to fluorescein isothiocyanate (FITC) or cyanine fluorochrome, Cy3 (Bio-Can Ltd., Mississauga, ON, Canada) for immunofluorescence.

3.4. Bacterial strains

Plasmid DNA was transformed into commercially available competent *Escherichia coli* cells (Max efficiency DH5α competent cells, GIBCO BRL). All transformed bacterial stocks were maintained at -80°C in 20% glycerol. Transformants were recovered by streaking on to LB solid medium and incubating at 37°C overnight.

3.5. Yeast strains

Plasmid DNA was transformed into yeast strain KGY37 (Graham 1996). Yeast strains were stored at -80°C in 20% glycerol. Transformants were recovered by streaking on to solid YPAD or selective media and incubating at 30°C for 1-3 days.

3.6. Cell lines

Human embryonic kidney 293T epithelial cells were grown in DMEM (GIBCO BRL). Rat-1 and Rat-1/Bcl-2 (Chen *et al.*, 1997) fibroblasts were cultured in α-MEM (GIBCO BRL). 10T1/2 and 10T1/2 derived Bcl-X_L fibroblasts (Chen *et al.*, 1999) as well as MCF-7 breast carcinoma cells were cultured in α-MEM supplemented with 5% L-Glutamine (200 mM, GIBCO BRL), 5% MEM sodium pyruvate (100 mM, GIBCO BRL) and 5% HEPES buffer solution (1 M GIBCO BRL). Cells were cultured at 37°C in 5-7% CO_2 and split when confluent using 0.05% trypsin/0.53 mM EDTA (GIBCO BRL).

3.7. Construction of recombinant expression plasmids

3.7.1. Designing PCR primers

An important parameter for successful amplification by the polymerase chain reaction (PCR) is the correct design of oligonucleotide primers. With the aid of available computer programs such as Oligo 5.0 (National Biosciences Inc, Plymouth, MN), primer pairs were selected and analyzed for their suitability to PCR. The primer pair determines the location and size of the amplified product and the desired changes such as deletions, insertions or substitutions to be incorporated into the gene construct. Generally, the primers were designed to be 18 to 24 bases in length. Specific restriction endonuclease (RE) sequences that do not cut within the amplified region were added to the 5'-ends of the primers to modify the PCR products for cloning into expression vectors. Most REs require 2 to 3 extra, non-specific bases 5' to their RE sequence to cut efficiently. Therefore, a GC clamp was added to the 5'-end of all primers. The primers were scanned for complementary regions within and between primer pairs to prevent the formation of hairpin loops and primer-dimers. Primer pairs were also matched in terms of length and base composition (maximum 50% G+C content) to ensure that the T_M values were with in 2-3°C of each other to allow for efficient amplification of the region of interest.

3.7.2. Mutagenesis of cloned genes using PCR

Human BNip3 cDNA was used as template in the PCR with appropriately designed primers to generate BNip3 mutants suitable for cloning into yeast and mammalian expression vectors. Nix and CeBNip3 were also used as templates to generate mutants. Plasmids encoding other proteins were kindly provided by the following: pcDNA3-Bcl-2 (Dr. Lorrie Kirshenbaum, University of Manitoba, Winnipeg, MB, Canada), pcDNA3-myc-Bcl-x_L (Dr. Gordon C. Shore, McGill University, Montreal, PQ, Canada), yeast two hybrid vectors pGBT8-Bcl-2, pGBT8-Bcl-x_L, pGBT8-CED-9 and pGBT8-Bcl-X_S (Dr. Gabriel Núñez, University of Michigan, Ann Arbor, MI, U.S.A.) and additional yeast vectors, pACTII-MK and pACTII-PTP2 (Dr. R. Daniel Gietz, University of Manitoba, Winnipeg, MB, Canada). These are summarized in Table 3.

The full length BNip3 (residues 1-194) and truncated deletion mutants were generated by primers flanking the region of interest. Other BNip3 mutants containing deleted regions or chimeric BNip3 protein, BNip3-BclTM and Bcl-Cb5TM were generated by splice overlap extension method (Dieffenbach and Dveklsler, 1995). Briefly, 5'- and 3'-primers were designed to amplify BNip3 (residues 1-163) and TM domains of Bcl-2 (BclTM, residues 219-239) or cytochrome b_5 (Cb5TM, residues 100-134). Specifically, the 3'-primer of BNip3 and 5'-primer of BclTM or Cb5TM contained complementary regions. Following PCR to amplify BNip3 and BclTM or Cb5TM, aliquots of the two PCR reaction products were combined to generate the recombinant gene template (BNip3-BclTM or BNIP-Cb5TM) through the overlapping complementary regions. The overlap was extended through PCR and the recombinant gene was further amplified using 5'- and 3'- primers to the entire recombinant gene that incorporated RE sites suitable for cloning into pcDNA3 (Invitrogen Corp., San Diego, CA, U.S.A.). The point mutants, BNip3(L179→S) and

Table 3. Summary of genes cloned into yeast and mammalian expression vectors to study BNip3 homo- and heterodimeric interactactions and cell death

Insert name	Mutation (amino acid residues)	Expression vectors*
BNip3	none	pcDNA3, pGBT9, pACTII
BNip3ΔBH3	Δ104-119	pcDNA3, pACTII
BNip3ΔC	Δ185-194	pcDNA3, pGBT9, pACTII
BNip3ΔCD	Δ112-130	pcDNA3
BNip3ΔN	Δ1-49	pcDNA3, pACTII
BNip3ΔN;ΔTM	Δ1-49; Δ164-194	pcDNA3
BNip3ΔTM1	Δ164-184	pcDNA3
BNip3ΔTM2	Δ164-194	pcDNA3, pGBT9, pACTII
BNip3Δ179-185	Δ179-185	pcDNA3
BNip3Δ179-189	Δ179-189	pACTII
BNip3Δ179-194	Δ179-194	pACTII
BNip3(L179→ S)	L179→ S	pcDNA3
BNip3(G180→ E)	G180→ E	pcDNA3
BNip3-BclTM	BNIP3(1-163)+BclTM (219-239)	pcDNA3
BNip3-CybTM	BNIP3(1-163)+Cb5TM (100-134)	pcDNA3
Nix	none	pGBT9, pACTII
NixΔTM	Δ188-208	pGBT9, pACTII
CeBNip3	none	pGBT9, pACTII
CeBNip3ΔTM	Δ189-210	pGBT9, pACTII
[1]Bcl-2	none	pcDNA3
[2]Bcl-x_L	none	pcDNA3
[3]Bcl-2	none	pGBT8
[3]Bcl-x_L	none	pGBT8
[3]Bcl-x_S	none	pGBT8
[3]CED-9	none	pGBT8
[4]MK	none	pAS1
[4]PTP2	none	pAS1

*pcDNA3 (Invitrogen Corp., San Diego, CA, U.S.A.) is a mammalian expression vector; pAS1(Durfee *et al.*, 1993), pGBT8 and pGBT9 (Bartel *et al.*, 1993) are GAL4 binding domain yeast expression vectors and pACTII is a GAL4 activating domain yeast expression vector (Clontech Laboratories Inc. Palo Alto, CA, U.S.A.).

Vectors provided by Drs. Lorrie Kirshenbaum[1], Gordon C. Shore[2], Gabriel Nunez[3] and R. Daniel Gietz[4]

MK, myotonin kinase; PTP2, protein tyrosine phosphatase 2

BNip3(G180→E) were the result of a mismatched nucleotide being incorporated during the extension step in PCR.

To amplify the region of interest, PCR was set up as follows: 5 µl 10x Taq DNA polymerase reaction buffer (200 mM Tris-Hcl (pH 8.4), 500 mM KCl, GIBCO BRL), 5 µl 10x *Pfu* DNA polymerase reaction buffer (100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 nM Tris-Hcl (pH 8.75), 20 mM $MgSO_4$, 1% Triton X-100, 1000 µg/ml Stratagene, La Jolla, CA. U.S.A.), 100pmole appropriate forward primer, 100 pmole appropriate reverse primer, 3 µl $MgCl_2$ (50 mM, GIBCO BRL), 0.5 µl *Taq* DNA polymerase (GIBCO BRL), >1.75 units PFU (Stratagene) and 10 µl dNTP (2.5 mM of each dATP, dCTP, dGTP and dTTP), 500 ng template DNA and sterile ddH_2O to bring the final volume to 100 µl. The reaction was carried out in a thermocycler cycler (GeneAmp PCR System 2400, Perkin Elmer, Foster City, CA, U.S.A.) set for an initial denaturation at 94°C for 3 minutes, followed by 25 cycles at 94°C for 10 seconds, 55°C for 30 seconds and 72°C for four minutes and ending with a final extension of 10 minutes at 72°C. The annealing temperature and cycle number were adjusted to optimize the PCR conditions for a given primer pair. In addition, glycerol was added up to a maximum of 15% of the total volume to the PCR reaction mix to reduce non-specific amplification.

Aliquots of PCR products were electrophoresed on 1% w/v agarose gels (Section 3.7.4) to verify size, purity and yield. The PCR products were purified by agarose gel electrophoresis or commercially available kits (Section 3.7.8), then digested with suitable REs for cloning in-frame into yeast expression vectors, pGBT9 (Bartel *et al.*, 1993), pAS1 (Durfee *et al.*, 1993) and pACTII (Clontech Laboratories Inc. Palo Alto, CA, U.S.A.) or mammalian expression vector, pcDNA3 (Invitrogen Corp.) (Section 3.7.3). The recombinant plasmids were transformed in to bacterial cells (Section 3.7.7), then

recovered by alkali lysis (Section 3.7.8) followed by RE digestion analysis and sequencing to verify the authenticity of recombinant constructs (Sections 3.7.3 and 3.7.9).

3.7.3. Restriction endonuclease digestion of PCR products and plasmid DNA

RE digestion was used to linearize or excise inserts from plasmid DNA, identify specific recombinant plasmids or make the 5'- and 3'-ends of PCR products compatible for ligation. The digestions were carried out according to manufacturers' directions or as described in Sambrook *et al* 1989. For small scale plasmid preparations (Section 3.7.8), approximately 2-10 units of the appropriate RE and its compatible buffer was added to 10-15 µl of the plasmid preparation and ddH_2O to a final volume of 20 µl. The reactions were incubated at 37°C for 2 hours or overnight. The products of RE digestion were mixed with loading dye (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF, 50% v/v glycerol) to a final concentration of 10% v/v and electrophoresed on 1% w/v agarose gels (Section 3.7.4). Similarly, aliquots of PCR products were digested and electrophoresed in preparation for cloning into linearized expression vectors (Sections 3.7.4 and 3.7.6).

3.7.4. DNA fragment isolation and purification from agarose gels

PCR or RE digestion products were mixed with loading dye (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF, 50% v/v glycerol) to a final concentration of 10% v/v. The samples were electrophoresed through 1% w/v agarose gel (GIBCO BRL) made in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) with ethidium bromide (EtBr) added to a final concentration of 0.5 µg/ml. EtBr intercalates with the DNA helix and fluoresces when exposed to ultraviolet (UV) light allowing the DNA to be visualized on agarose gels. DNA marker (1µg 100-base pair ladder and/or 1 µg Kilobase DNA marker, Amersham Pharmacia Biotech, Buckinghamshire, England) mixed with loading dye was run alongside samples to determine DNA fragment size. The gels were run at 80 volts (V)

for 30 to 60 minutes to allow for adequate separation. The DNA banding pattern on the gels was visualized under UV light on a transilluminator and photographed for permanent record using Polaroid film (Polapan 667, Polaroid Corp. Cambridge, MA, U.S.A.).

Two approaches were used to purify PCR products and DNA fragments from EtBr-stained agarose gels. Following electrophoresis of PCR or RE digestions products, the DNA bands were visualized at 360 nm UV wavelength to minimize DNA damage and excised from the gels. Fragments of DNA ≤300 bp were run on 2% w/v low melting point agarose gels (GIBCO BRL) at 4°C in TAE buffer. The excised band was re-melted at 65°C and used directly in ligation reactions (Section 3.7.6). Fragments of DNA ≥300bp were run at room temperature on 1% w/v agarose gels in TAE buffer. The DNA was isolated and purified from excised bands using a silica matrix from the GENECLEAN II Kit (BIO 101, Inc., Vista, CA, U.S.A.) according to manufacturers' instructions and used directly in ligation reactions (Section 3.7.6).

3.7.5. Dephosphorylation of linearized plasmid DNA

In preparation for ligation reactions, purified linearized plasmid DNA was treated with calf intestinal alkaline phosphatase (CIP) to remove the terminal 5'phosphate group from the DNA and prevent self ligation (Sambrook *et al.*, 1989). Following RE digestion of up to 5 µg plasmid DNA, 1 unit CIP (Amersham Pharmacia Biotech) and the appropriate amount of 10x One-Phor-All Buffer (100 mM Tris-acetate, 100 mM magnesium acetate and 500 mM potassium acetate, Amersham Pharmacia Biotech) were added and incubated at 37°C for 30 minutes. An additional 1 unit CIP was added and incubated for another 30 minutes at 37°C. The digested vector was run on 1% w/v TAE agarose gel, excised and purified using the GENECLEAN II Kit (BIO 101 Inc.) according to manufacturers' instructions.

3.7.6. Ligation of linearized plasmid and insert DNA

Ligations were done essentially as described in Sambrook *et al.* (1989) with some modification. Following RE digest and purification of linearized vector and insert, aliqouts were run on 1% w/v agarose gels to determine approximate concentration (Section 3.7.4). The concentration of insert was maintained two fold greater than the concentration of vector to enhance ligation. The appropriate ratio of vector to insert was combined with 1 μl 10x ligation buffer (60 mM Tris-Hcl, pH 8.3, 60 mM $MgCl_2$, 50 mM NaCl, 1 mg/ml bovine serum albumin (BSA), 70 mM β-mercaptoethanol (β-ME), 1 mM ATP, 20 mM dithiothreitol (DTT), 10 mM spermidine) and 4-6 units T4 DNA ligase (Amersham Pharmacia Biotech) to a final volume of 10 μl. The reaction was incubated at 12°C for 4 hours or overnight.

3.7.7. Bacterial transformation of ligation products and plasmid DNA

Commercially available strains of competent *E. coli* (Max efficiency DH5α competent cells, GIBCO BRL) were used for bacterial transformations. A 20 μl aliquot of competent cells was rapidly thawed and mixed with 2 μl ligation reaction or 0.5-1 μg of plasmid DNA by gently pipetting up and down. The DNA/cell mixture was incubated on ice for 30 minutes then subject to heat shock for 45 seconds at 42°C and placed on ice for an additional 2 minutes. Following the addition of 500-1000 μl SOC medium (GIBCO BRL), the cells were cultured at 37°C in a shaking incubator set at 200 rpm for 1 hour. Aliquots of the transformed cells were plated on solid LB medium and incubated overnight at 37°C. Once colonies became apparent, plates were removed from 37°C and stored at 4°C until needed.

3.7.8. Plasmid DNA isolation and purification

Plasmid DNA was isolated from transformed *E. coli* following the alkali lysis method (Sambroock *et al.*, 1989) with some modification. All steps were carried out at

room temperature, except as indicated. Single colonies picked from LB solid medium were cultured in 5 ml of liquid LB broth overnight at 37°C in a shaking incubator. The overnight cultures were centrifuged for 8 minutes at 4°C at 2500g. After aspirating the medium, the pellet was resuspended in 100 µl cold glucose buffer (50 mM Tris-HCl, pH 8.0, 50 mM glucose, 0.5 M EDTA, pH 8.0) by vigorously vortex mixing and incubating on ice for 5 minutes. The cells were lysed by adding 200 µl of lysis buffer (4% (v/v) Triton X-100, 0.8 M NaOH) and incubated on ice for five minutes. To precipitate chromosomal DNA, high molecular weight RNA and protein, 150 µl of 3 M $NaOOCCH_3$ (sodium acetate) (pH 4.8) was added and incubated on ice for an additional five minutes. The precipitate was recovered by centrifugation at 12 000g in a microcentrifuge for 5 minutes. To extract the DNA, the recovered supernatant was added to 500 µl phenol:chloroform:isoamyl alcohol (25:24:1) and vigorously vortex mixed. The aqueous layer was separated by centrifugation at 12 000g for 5 minutes in a microcentrifuge. To precipitate the DNA, the aqueous layer was added to 450 µl isopropanol and mixed. The contents were left at room temperature for 30-60 minutes, then centrifuged for 15 minutes at 12 000g. After washing the pellet with 250 µl isopropanol, it was allowed to air dry for 10-20 minutes and dissolved in 30-50 µl sterile ddH_2O containing RNase A (1 µg/µl, Amersham, Pharmacia Biotech) to remove residual RNA. Diagnostic The authenticity of all plasmids was confirmed by RE mapping and DNA sequencing.

Plasmid DNA for transformations, transfections and coupled *in vitro* transcription/translation was produced by following a large-scale plasmid isolation and purification method. Initiating cultures of 5 ml LB broth inoculated with appropriate bacterial transformants were grown at 37°C for several hours with shaking, then added to 400 ml aliquots of LB broth and incubated overnight at 37°C with shaking. The plasmids were

isolated and purified using the QIAGEN Plasmid Kit (QIAGEN Inc., Missisauga, ON, Canada) according to manufacturers' instructions without modification. The quality and yield of the plasmid preparation was determined by RE digest as described (Section 3.7.3) and spectrophotometrically at 260nm. One optical density unit at 260nm (OD_{260}) corresponds to approximately 50 μg/ml double stranded DNA. The yield was calculated as follows: OD_{260} x 50 μg/ml x dilution factor. The purity of the DNA preparation was estimated by measuring the ratio of OD_{260} to OD_{280}. Preparations having a ratio of 1.8 do not contain protein contamination. Aliquots of purified plasmid preparations were stored at -20°C for later use.

3.7.9. Sequencing recombinant plasmid DNA

Recombinant plasmids were sequenced by automated fluorescent sequencing to determine the accuracy of the introduced genetic changes and to ascertain that there were no amplification errors. Following the cycle sequencing method, 125-400 ng plasmid DNA and 2.5-20 pmoles appropriate primer were added to 0.33 μl BigDye Terminator Cycle Sequencing Ready Reaction Mix (PE Applied Biosystems, Foster City, CA, U.S.A.) diluted in 1.67 μl sequence dilution buffer (PE Applied Biosystems) and sterile ddH_2O to a final volume of 5 μl. Alternatively, single bacterial colonies were sequenced directly by resuspending in 3 μl sequence dilution buffer (PE Applied Biosystems) and 20 pmoles of appropriate primer. Following an initial 10 minute denaturation step at 96°C, 0.5 μl BigDye Terminator Cycle Sequencing Ready Reaction Mix (PE Applied Biosystems) was added (Gladden *et al.*, 2000). The sequencing reactions were incubated in a thermal cycler (GeneAmp PCR System 2400, Perkin Elmer) set at: 96°C for 10 seconds, 40°C for 5 seconds and 60°C for 4 minutes for 25 cycles. The sequencing products were precipitated with 60% v/v ethanol for one hour to overnight. The precipitate was recovered by

centrifugation at top speed in a microcentrifuge for 15 minutes. The pellet was washed again with 70% v/v ethanol and then vacuum or air-dried. The precipitated, dried sequencing products were resuspended in 7 µl Template Suppression Reagent (PE Applied Biosystems) for sequencing on an ABI-310 Genetic Analyzer (PE Applied Biosystems).

3.8. Expression systems for cloned genes

3.8.1. Coupled *in vitro* transcription/translation

Expression plasmids encoding BNip3 and its mutants cloned into pcDNA3 were used as template for *in vitro* transcription/translation in the presence of translation grade ^{35}[S]-methionine by the TNT coupled Reticulocyte Lysate System (Promega, Madison, WI, U.S.A.) according to manufacturers' instructions. Briefly, 1 µg plasmid DNA, 4 µl L-^{35}[S]-methionine (1000 Ci/mmol, Amersham Pharmacia Biotech) and 35 units RNAguard RNase inhibitor (Amersham Pharmacia Biotech) were added to reagents provided in the kit, (25 µl TNT rabbit reticulocyte lysate, 2 µl TNT reaction buffer, 1 µl TNT T7 RNA polymerase and 1 µl 1mM amino acid mixture minus methione), then brought to a final volume of 50 µl with nuclease free ddH_2O. The reaction was incubated at 30°C for two hours and stored at -20°C for later use.

3.8.2. Transient transfection of mammalian cells

3.8.2.1. Calcium-phosphate precipitation method

In preparation for transient transfections, 293T cells were plated 24 hours in advance at a density of $2x10^6$ in 100mm culture dishes (Nunc, Roskilde, Denmark). Cells were co-transfected with indicated expression plasmids by the calcium phosphate precipitation method using a total of 15 ug plasmid DNA per transfection. The $CaCl_2$-DNA mix (250mM $CaCl_2$) was added in a drop-wise manner to 500 µl HeBs solution (280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4, pH 7.1). The calcium phosphate DNA co-

precipitate was allowed to form without further agitation for 20-30 minutes at room temperature, then overlaid onto 293T cells in 6 ml fresh DMEM medium. The cells were incubated for 4 hours with the transfection mix, then replaced with fresh medium and cultured for an additional 12-18 hours. The viability of transfected cells based on adherence to the culture dish was monitored by microscopy.

3.8.2.2. Cationic lipid method

In preparation for transient transfections in 6-well 35 mm plates (Nunc), cells were plated 24 hours in advance at a density of $1x10^5$ cells per well. Cells were transfected using the polycationic lipid LipofectAMINE reagent (GIBCO BRL). For each transfection, 0.75 μg DNA was diluted in 100 μl serum-free medium (OPTI-MEM I Reduced Serum Medium, GIBCO BRL). In another tube, 5 to 7 μl LipofectAMINE reagent was diluted in 100 μl serum-free medium. The diluted DNA was added drop-wise to the diluted LipofectAMINE reagent. The DNA-liposome complexes were allowed to form at room temperature for 20 to 30 minutes. An additional 0.8 ml serum-free media was added to each transfection mix and overlaid onto the cells washed once with OPTI-MEM I Reduced Serum Medium (GIBCO BRL). Following 4 to 5 hours incubation depending on the cell type, cells were placed in normal growth media. The transfected cells were cultured for an additional 12-48 hours. The viability of transfected cells based on adherence to the culture dish was monitored by microscopy.

3.9. Detection and analysis of proteins expressed from cloned genes
3.9.1. Immunofluorescence

To study protein expression and localization, mammalian cells were seeded onto glass coverslips (Fisher Scientific) and transfected using the calcium phosphate precipitation (Section 3.8.2.1) or LipofectAMINE method (Section 3.8.2.2) depending on the cell type. At the indicated time points, cells were fixed with 4% v/v formaldehyde in 0.1

M phosphate-buffered saline (PBS) for 20 minutes and washed twice with 0.1% v/v Nonidet-P40 (NP-40) in 0.1 M PBS at room temperature with agitation. After washing, slides were incubated for 2 hours in 0.1 M PBS supplemented with 0.1% v/v NP-40, 5% v/v FBS (GIBCO BRL) and the appropriately diluted antibody (Table 4). To detect the immune complexes, the slides were washed as before and incubated with FITC-conjugated goat anti-mouse or goat anti-rabbit antibody (dilution 1:1800) or Cy3-conjugated goat anti-rabbit antibody (dilution 1:2100) at room temperature shielded from light exposure. To determine protein subcellular localization, transfected cells were co-stained for expression of BNip3 or its mutants and HSP60, a mitochondrial matrix protein (Soltys and Gupta 1992). The slides were mounted in Fluoro-guard anti-fade reagent (Bio-Rad, Hercules, CA, U.S.A.) and viewed by fluorescence microscopy (Zeiss Axiophot).

Table 4. Summary of primary antibody dilutions used for Western blot and immunofluorescence analysis of expressed proteins

Primary antibody	Working dilution*	
	Western blotting	Immunofluorescence
mouse anti-BNip3 (Ana40)	1 in 10 000	1 in 2000
mouse anti-BNip3 (Ana50)	1 in 10 000	1 in 2000
rabbit anti-BNip3	1 in 8000	1 in 2000
mouse anti-Bcl-2 (Clone 4D7)	1 in 2000	1 in 2000
mouse anti-Bcl-x_L (Clone 2H12)	1 in 2500	1 in 2000
rabbit anti-Bcl-x_L	1 in 2500	1 in 2000
mouse anti-T7	1 in 10 000	1 in 1200-1500
rabbit anti-HSP60	ND	1 in 2000

*Antibodies for Western blotting were diluted in blocking buffer (0.1 M PBS, 3% w/v BSA, 0.2% v/v Tween-20) and for immunofluorescence in the appropriate buffer (0.1 M PBS, 0.1% v/v NP-40 and 5% v/v FBS)

3.9.2. Western blot analysis

Western blot analysis by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was used to identify specific proteins in cell lysates from transient transfections (Section 3.8.2) and co-immunoprecipitation assays using mAbs and pAbs (Section 3.10.2). Each sample (5-10 μl of mammalian cell lysates or 15 μl of co-immunoprecipitation reactions) was separated by electrophoresis at 140V through a 0.5 mm thick 12% SDS-polyacrylamide gel made according to standard protocol (Sambrook *et al.*, 1989) in Laemlli buffer (25 mM Tris, 250 mM glycine and 0.1% w/v SDS). Samples were run alongside a broad range pre-stained protein marker (New England BioLabs Inc. Beverly, MA, U.S.A.) to determine approximate molecular mass. The gels were run for 60-90 minutes to allow for adequate separation, then soaked in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol) along with the nitrocellulose transfer membrane (NitroPure, 0.22 micron, Micron Separations Inc. Westborough, MA, U.S.A.) and electrode paper (Munktell filter paper, grade 1F, Amersham Pharmacia Biotech). To transfer the proteins to the nitrocellulose membrane, the gel, the membrane and the electrode paper were layered in a semi-dry transfer apparatus (LKB 2117 Multiphor Apparatus) and set at 40 milliamperes (mA) per gel for 1-2 hours. The membranes were incubated in blocking buffer (3% w/v BSA in 0.1 M PBS supplemented with 0.2% v/v Tween 20) to block non-specific interactions for 1 hour at room temperature. The membrane was incubated overnight at 4°C with the appropriated antibody diluted in fresh blocking buffer. The working dilutions of primary antibodies are listed in Table 4. After removal of the primary antibody solution, the membranes were washed in 0.1 M PBS containing 0.2% v/v Tween 20 for 40 minutes with agitation, changing the buffer every 10 minutes. Next, membranes were incubated for an additional hour with appropriate secondary antibody conjugated to horseradish peroxidase diluted 1 in 10 000 in blocking buffer. The blots were washed as

before and developed using enhanced chemiluminescence as described by manufacturer (ECL, Amersham Pharmacia Biotech). Blots were exposed to X-ray film (Hyperfilm ECL, Amersham Pharmacia Biotech) for desired length of time and processed in a tabletop automated developer (AGFA, Etibicoke, ON, Canada).

3.9.3. Coomassie blue staining

To detect proteins by Coomassie blue which binds specifically to aromatic amino acids such as tyrosine and tryptophan (Congdon *et al.*, 1993), SDS-polyacrylamide gels were stained at room temperature for 1 hour with agitation in Coomassie blue staining solution (0.25 w/v Coomassie brilliant blue R250, 10% v/v glacial acetic acid, 90% v/v methanol). The gel was destained in 40% v/v methanol and 10% v/v glacial acetic acid for several hours until protein bands became evident. To make a permanent record, gels were sealed in plastic bags and placed in a desktop scanner to save as a computer graphic file.

3.9.4. Autoradiography of [^{35}S]-labeled proteins

To detect [^{35}S]-labeled *in vitro* transcription/translation protein products and labeled co-immunoprecipitation reactions, samples were run on 10 or 12% SDS-polyacrylamide gels in Laemlli buffer as described (Section 3.9.2). The gels were fixed with 25% v/v 2-propanol and 10% v/v glacial acetic acid for 20 minutes, followed by an additional 20 minutes in a commercial fluography enhancing solution (Amplify, Amersham Pharmacia Biotech). The gels were dried and exposed to film (BioMax film, Eastman Kodak Company, Rochester, NY, U.S.A.) for 2-24 hours and processed in a tabletop automated developer (AGFA).

3.10. Systems to identify and map protein-protein interaction of cloned genes

3.10.1. Yeast two-hybrid system

3.10.1.1. High efficiency yeast transformations

The yeast two-hybrid system was used to identify potential BNip3 interacting proteins and map the regions of interaction. The high efficiency lithium acetate (LiAc)/single stranded DNA polyethylene glycol (PEG) transformation protocol from the Gietz Lab Yeast Transformation Homepage (*available at* www.umanitoba.ca/faculties/medicine/units/biochem/gietz) and Gietz *et al.*, (1997) was used for all yeast transformations using the yeast strain KGY37 (Graham 1996). Briefly, initiating cultures of 10 ml YPAD were inoculated with freshly streaked KGY37 from solid medium. The yeast cells were grown overnight at 30°C with shaking at 200 rpm. The titre of the culture was determined and diluted into pre-warmed medium to a final density of $5x10^6$ cells/ml. The large cultures were incubated for 2-5 hours or until the cells had reached a titre of $2x10^7$ cells/ml which is equivalent to approximately 2 doublings. The cells were harvested by centrifugation at 3000g for 5 minutes, then washed once with 25 ml sterile ddH_2O and recovered by centrifugation. The cells were resuspended in 1 ml 100 mM LiAc and pelleted again, then resuspended in 500 µl 100 mM LiAc to bring the final concentration to $2x10^9$ cells/ml. Aliquots of 50 µl resuspended cells were pelleted and the LiAc removed. The transfection mix was layered on top of each pellet as follows: 240 µl PEG (50% w/v), 36 µl 1.0 M LiAc, 30 µl boiled single stranded carrier DNA (deoxyribonucleic acid sodium salt type III from salmon testes, dissolved in TE buffer (10 mM Tris-HCl pH 8.0, 1.0 mM EDTA) at 2 mg/ml), 100 ng to 5 µg plasmid DNA and sterile ddH_2O to a final volume of 380 µl. The cells were resuspended in the transformation mix by vigorously vortex mixing, then incubated at 30°C for 20-30 minutes without agitation. The cells were given heat shock treatment at 42°C for an additional 20-30 minutes with mixing by inverting every 5 minutes. Next, the cells were

pelleted by centrifugation at 8000g for 15 seconds, the transformation mix was removed and replaced with 500-1000 µl sterile ddH_2O. The cells were resuspended by gently mixing and aliquots spread on to appropriate media. To test for auto-activation, single transformations of BD or AD plasmids, respectively were plated on SC-Leu or SC-Trp medium. Co-transformations were plated on SC-Trp/Leu to select for both BD and AD plasmids, then plated on SC-Trp/Leu/His to detect protein-protein interaction by activation of the *HIS3* and *lacZ* reporter genes. The plates were incubated for 3-6 days at 30°C to allow for colony formation.

3.10.1.2. Detection of *lacZ* activation by β-galactosidase expression

3.10.1.2.1. Yeast colony filter lift assay

The activation of the *lacZ* reporter gene by autoactivation or interaction between two proteins was detected by filter lifting yeast colonies and staining for β-galactosidase expression (Breeden and Nasmyth, 1985). Circles of sterile filter paper (Whatman #1 75mm diameter, Whatman Inc., Tewksbury, U.S.A.) were overlaid on the surface of agar plates containing transformants, ensuring that all the colonies were in contact with the filter. The filters were carefully removed from the plates and immersed in a pool of liquid nitrogen with the colonies facing upward and allowed to freeze uniformly. The filters were thawed at room temperature, then frozen and thawed two additional times. Next, with the colonies facing upwards, the filters were placed on another circular filter saturated in 1.5 ml Z buffer (60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1mM $MgSO_4 \cdot 7H_2O$) containing 2.7 µl/ml β-mercaptoethanol (β-ME) and 25 µl X-gal solution (25 mg/ml in DMF) (Miller 1972) in plastic petri dishes (Fisher Scientific). The filters were incubated at 37°C for 30 minutes to overnight and monitored for blue colour development.

3.10.1.2.2. ONPG assay

The level of β-galactosidase activity was quantified by assaying the hydrolysis of ortho-nitrophenyl β-D-galactopyranoside (ONPG) (Miller 1972). Yeast transformants were inoculated into 2 ml of the appropriate SC- media lacking the specific nutrients to select for either the BD or AD plasmid or both. The cultures were incubated at 30• C overnight with agitation to reach a titre of approximately 1-2 $x10^7$ cells/ml. The absorbance at 600 nm of a 1 ml aliquot was determined and the remaining 1 ml was transferred to a microcentrifuge tube. The cells were pelleted by centrifugation at 8000g for 15 seconds and resuspended in 100 µl of Z buffer. The cells were lysed by freezing briefly in liquid nitrogen and allowed to thaw for 1 minute at 37°C. To each reaction tube, 700 µl Z buffer containing 2.7 µl/mL β-ME was added, followed immediately by the addition of 160 µl of ONPG (4mg/ml in Z buffer). The reactions were incubated at 30°C to allow for yellow colour development and quenched by the addition of 400 µl 1M Na_2CO_3. The time elapsed for yellow colour development was recorded in minutes. Following centrifugation at 14 000g for 1 minute to pellet the cellular debris, the absorbance at 420 nm was determined for the supernatant. The β-galactosidase activity was calculated as follows: 1000 x OD_{420}/reaction time x volume x OD_{600}, where 1 unit of β-galactosidase activity (Miller unit) is defined as the amount which hydrolyzes 1 µmole of ONPG to o-nitrophenol and D-galactose per minute (Miller, 1972). ONPG measurements were done in duplicate from two independent yeast cell cultures and the results averaged.

3.10.1.3. Detection of *HIS3* activation

To test activation of the *HIS3* reporter gene by protein-protein interaction, co-transformants from SC-Trp/Leu solid medium plates were streaked onto SC-Trp/Leu/His

plates containing varying concentrations of 3-AT and incubated for 3-4 days at 30°C. 3AT is a histidine analogue added to the selection medium to reduce background growth.

3.10.2. Co-immunoprecipitation assays

3.10.2.1. Co-immunoprecipitation of solubilized, transfected 293T cells

All steps for co-immunoprecipitation were carried out on ice or in the cold room. For each co-immunoprecipitation, $2x10^6$ co-transfected 293T cells were lysed 12 hours post-transfection in 500 µl 0.2% v/v NP-40 isotonic buffer with freshly added protease inhibitors (100 mM Tris-HCl, 2 mM EDTA, 100 mM NaCl, 0.2% v/v NP-40, 5 µg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin and 1 mM phenylmethylsulfonyl fluoride). Following sonication three times at 10 seconds, samples were centrifuged at top speed in a microcentrifuge for 10 minutes to remove nuclear and cellular debris. The lysates were pre-cleared with 15 µl protein A-Sepharose 4B (Zymed Laboratories Inc., San Francisco, CA, U.S.A.) for 30 minutes by rotation. The beads were recovered by centrifugation at 6000g for 2 minutes in a microcentrifuge. The pre-cleared lysates were combined with an equal volume of binding buffer (200 mM NaCl, 20% v/v glycerol and 0.2% v/v NP-40) and hamster monoclonal anti-Bcl-2 or mouse monoclonal anti-C-myc antibody for an additional 2 hours with rotation. To capture the immune complexes, 15 µl of protein A-Sepharose 4B beads (Zymed Laboratories Inc.) were added and the samples were rotated for an additional hour. The agarose beads were recovered by centrifugation at 6000g for 2 minutes and washed four times in excess combined lysis and binding buffer. The bound immune complexes were eluted from the beads by adding SDS sample buffer (50 mM Tris-HCl, pH 6.8, 10% v/v glycerol, 0.1% w/v bromophenol blue, 2% w/v SDS and 100 mM DTT), thorough vortex mixing and boiling for 3 minutes. Co-immunoprecipitation reactions

were separated by 10 or 12% Laemlli SDS-PAGE and analyzed by Western blotting as described (Section 3.9.2).

3.10.2.2. Co-immunoprecipitation of proteins expressed *in vitro*

Aliquots of labeled protein products of various BNip3 constructs were run on SDS-polyacrylamide gels then analyzed by autoradiography to determine efficiency of transcription/translation. Accordingly, adjustments were made to ensure that equivalent amounts of labeled BNip3 and its mutants were incubated with 3 μg purified Bcl-2 protein with an NH_2-terminus (His_6)-tag (gift from Dr. John C. Reed, Burnham Institute, La Jolla, CA, U.S.A.) and hamster monoclonal anti-Bcl-2 antibody in 250 μl 0.2% v/v NP-40 immunoprecipitation buffer (100 mM Tris-HCl, 2 mM EDTA, 100 mM NaCl, 0.2%v/v NP-P40, and 1.25 mg/ml BSA) for 2h with rocking at 4°C. Immune complexes were captured with 15 μl protein A-Sepharose 4B (Zymed Laboratories Inc.) by rocking at 4°C for an additional hour. The agarose beads were recovered by centrifugation at 6000g for 2 minutes and washed four time in excess NP-40 immunoprecipitation buffer and solubilized in SDS-sample buffer by thorough vortex mixing, then boiling for 3 minutes. Solubilized immune complexes were separated by 10 or 12% Laemlli SDS-PAGE and analyzed by autoradiography as described (Sections 3.9.2 and 3.9.4).

3.11. Cell death detection in transiently transfected mammalian cells

3.11.1. β-galactosidase assay

The cellular morphology of dying cells was determined by staining for β-galactosidase expression. Cells were co-transfected in six-well plates as described with 0.75 μg of indicated plasmid DNA and 0.2 μg β-galactosidase reporter (Section 3.8.2). Cells were fixed in 0.2% v/v glutaraldehyde in 0.1 M PBS for 10 minutes and washed three times with 0.1 M PBS, then stained in X-gal buffer (0.5 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 3 mM $K_3Fe(CN)_6$, 3mM $K_4Fe(CN)_6 \bullet 3H_2O$, 1 mM $MgCl_2$ in 0.1 M

PBS) at 37°C for 6-24 hours. The percentage of transfected dying cells was calculated by assessing the number of rounded, condensed, blue-staining cells in the total population of flat, blue-staining cells.

3.11.2. Nuclear staining assay

The apoptotic nuclear morphology of transfected cells was determined by immunofluorescence (Section 3.9.1) with an additional step staining for the nucleus to visualize chromatin condensation. MCF-7 breast carcinoma cells, as well as Rat-1 and Rat-1/Bcl-2, 10T1/2 and 10T1/2-Bcl-x_L fibroblasts were seeded onto glass slides (Fisher Scientific) and transfected with indicated plasmids using LipofectAMINE reagent (GIBCO BRL) as described (Section 3.8.2.2). After fixing at indicated time points, cells were stained for protein expression by immunofluorescence (Section 3.9.1). To assess the nuclear morphology of transfected cells, 1 μg/ml of Hoechst 33342 that binds to DNA was added to the final wash. Using a Zeiss Axiophot microscope, the transfected green or red fluorescent cells were enumerated by altered nuclear morphology based on Hoechst dye staining which emits blue fluorescence when excited by UV light at 350 nm. In total, 200-300 transfected cells were enumerated per sample.

4. Results

4.1. BNip3 homodimerization and cell death

4.1.1. BNip3-related proteins homodimerize through their transmembrane domain

Among the pro-apoptotic Bcl-2 family members, homodimeric interactions have been detailed for Bax (Oltvai *et al.*, 1993; Gross *et al.*, 1998; Wang *et al.*, 1998a). To date similar interactions have not been reported for the 'BH3-only' class of proteins (Table 1). An early observation suggested that BNip3 was capable of forming homodimers. *In vitro* transcribed/translated BNip3 was detected as a major band at 60 kDa and at 30 kDa under reducing and non-reducing conditions by Laemmli SDS-PAGE. Under similar conditions, the removal of the BNip3 TM domain resulted in a major band at 28 kDa and a minor band at 27 kDa (Chen *et al.*, 1997). The predicted molecular mass of BNip3 is 21.4 kDa, indicating the protein runs anomalously. Under, Tris-Tricine buffer conditions, dimeric BNip3 migrates at its correct relative molecular mass of 40 kDa by SDS-PAGE. Other BNip3-related proteins exhibit a similar phenomenon whereby the dimeric form is resistant to reduction and detected at its correct relative molecular mass using the Tris-Tricine buffered SDS-PAGE system. The reason for the differences in mobility between the two buffer systems is not clear (Chen *et al.*, 1997; 1999; Cizeau *et al.*, 2000). Peptide mapping of the 60 kDa BNip3 and 28 kDa BNip3ΔTM2 labeled products revealed by autoradiography identical trypsin digest fragments by two-dimensional electrophoresis and chromatography. Therefore, the BNip3 band of higher molecular mass represents the dimer and does not include additional interacting proteins (Chen *et al.*, 1997).

To determine if BNip3 is able to interact homologously in the yeast two-hybrid system, wild type BNip3 and COOH-terminal deletion mutants, BNip3ΔC and BNip3ΔTM2 were inserted into GAL4 BD and AD expression vectors, pGBT9 (Bartel *et*

al., 1993) and pACTII (Clontech Laboratories Inc.), respectively. Following co-transformation into yeast strain KGY37, interaction was determined by activation of two reporter genes: *HIS3* and *lacZ*. The activation of the *HIS3* reporter gene was selected for on medium lacking tryptophan, leucine and histidine in the presence of 1 mM 3AT. BNip3 was observed to interact with itself and BNip3ΔC (Figure 14A, *left panel*). There was no detectable interaction between BNip3 and BNip3ΔTM2 (Figure 14B, *left panel*). Similarly, BNip3ΔC was found to interact homologously, (Figure 14A, *left panel*), but no interaction was noted with BNip3ΔTM2 (not shown). The removal of the BNip3 TM domain disrupted homodimerization, as BNip3ΔTM2 did not interact with itself (Figure 14B, *left panel*). The observed interactions were specific, as no growth was detected on selection medium when BNip3 and its COOH-terminal deletion mutants were co-transformed with empty vector, pACTII (Figure 14A and 14B, *right panel*). The appearance of some colonies on selection medium for control transformations is likely due to growth supported by histidine derived from the innoculum. Similarly, the TM domain mediates homodimerization of other BNip3 family members, Nix (Table 5 and Figure 25) and CeBNip3 (Figure 14C). There was significantly reduced or no detectable interaction when yeast were co-transformed with BD and AD vectors expressing NixΔTM or CeBNip3ΔTM.

The homologous interaction of BNip3 was also assessed by the activation of the *lacZ* reporter gene. β-galactosidase expression was qualitatively determined by filter lifting yeast colonies, followed by X-gal staining and quantitatively by measuring the hydrolysis of ONPG to o-nitrophenol and D-galactose. Similar to the results obtained by nutrient selection, homologous interaction of BNip3 and BNip3ΔC yielded blue colonies (Table 6). By removing the TM domain, BNip3 no longer forms homodimers and the

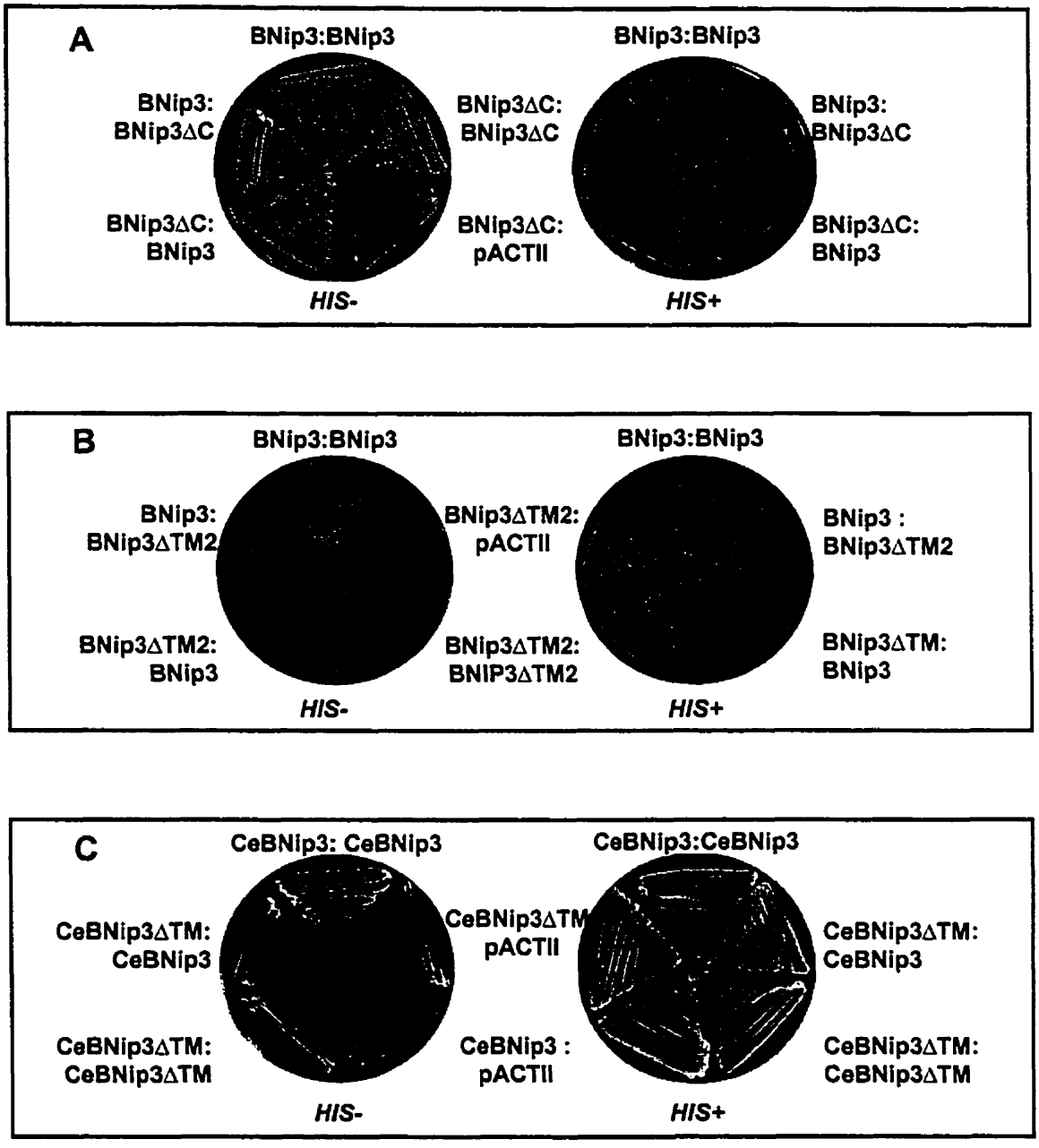

Figure 14. Homodimerization of BNip3-related proteins in the yeast two- hybrid system. Yeast strain KGY37 was co-transformed with GAL4 BD and AD vectors expressing indicated proteins. The BD constructs were co-transformed with pACTII an AD vector as a control. Protein-protein interaction was determined by growth on selection medium lacking tryptophan, leucine and histidine with 1 mM 3-amino-1,2,4-triazole (3AT) (*HIS-*). A, Both full length BNip3 and BNip3ΔC were observed to interact strongly. B and C, The removal of the TM domain of both BNip3 (BNip3ΔTM2) and its *C. elegans* orthologue, CeBNip3 (CeBNip3ΔTM) prevented homodimerization. There was significantly reduced or no detectable growth when BNip3, CeBNip3 or deletion mutants were co-transformed with empty vector indicating the specificity of observed interactions. All co-transformants grew on media lacking tryptophan and leucine (*HIS+*) (A, B and C, *right panel*). The co-transformations are labeled in the order, BD:AD expression vectors.

Table 5. Interaction of Nix in the yeast two-hybrid system

Transformation (BD:AD)	*HIS3* activation
Nix:Nix	+++
NixΔTM2:NixΔTM2	-
Nix:NixΔTM2	-
Nix:pACTII	-
pGBT9:NixΔTM2	-

Yeast strain KGY37 was co-transformed with indicated binding domain (BD) and activating domain (AD) constructs. The relative level of activation for the *HIS3* reporter gene was determined by growth (+) or no growth (-) on selection medium lacking tryptophan, leucine and histidine in the presence of 1 mM 3-amino-1,2,4-triazole (3AT).

Table 6. Interaction of BNip3ΔC in the yeast-two hybrid system

Transformation (BD:AD)	X-gal staining
BNip3:BNip3	B
BNip3:BNip3ΔC	B
BNip3ΔC:BNip3	B
BNip3ΔC:BNip3ΔC	B
BNip3ΔC:pACTII	W
BNip3:pACTII	W

*Yeast strain KGY37 was co-transformed with indicated GAL4 binding domain (BD) and activating domain (AD) constructs. The relative level of activation for the *lacZ* reporter gene was determined X-gal staining (B, blue, W, white).

Table 7. Interaction of BNip3ΔTM2 in the yeast two-hybrid system

Transformation (BD:AD)	X-gal staining
BNip3:BNip3	B
BNip3: BNip3ΔTM2	W
BNip3ΔTM2:BNip3	W
BNip3ΔTM2:BNip3ΔTM2	W
BNip3ΔTM2:pACTII	W
BNip3:pACTII	W

*Yeast strain KGY37 was co-transformed with indicated GAL4 binding domain (BD) and activating domain (AD) constructs. The relative level of activation for the *lacZ* reporter gene was determined X-gal staining (B, blue, W, white).

colonies remained white (Table 7). The level of β-galactosidase activity for BNip3 homodimerization was measured at 103 units. The interaction was specific as only 0.3 and 0.5 units, respectively of activity were measured when BNip3 was co-transformed with available heterologous proteins, myotonin kinase and protein tyrosine phosphatase 2, respectively (Table 8).

Table 8. β-galactosidase activitiy of BNip3 homodimerization in the yeast two-hybrid system

Transformation (BD:AD)	β-galactosidase activity	
	X-gal staining	ONPG assay
BNip3:BNip3	B	103.3
MK:BNip3	W	0.3
PTP2:BNip3	W	0.5
*BNip3 (BD)	W	1.0
*BNip3 (AD)	W	0.3

Yeast strain KGY37 was co-transformed with indicated GAL4 binding domain (BD) and activating domain (AD) constructs. The relative level of activation for the *lacZ* reporter gene was determined by X-gal staining (B, blue, W, white) and measuring hydrolysis of ortho-nitrophenyl β-D-galactopyranoside (ONPG) whereby, 1 unit of β-galactosidase activity (Miller unit) is defined as the amount which hydrolyzes 1 μmole of ONPG to o-nitrophenol and D-galactose per minute (Miller, 1972). *Denotes single tranformations to establish background levels of *lacZ* activation. MK, myotonin kinase; PTP2, protein tyrosine phosphatase 2

4.1.2. BNip3 transmembrane domain mutants that disrupt SDS-resistant homodimerization induce cell death

The TM domain of BNip3-related proteins has two identified roles: (1) it targets the protein to mitochondria (Chen *et al.*, 1997, 1999; Yasuda *et al.*, 1998a, 1998b, 1999; Cizeau *et al.*, 2000) and (2) it mediates homodimerization (Chen *et al.*, 1997). Furthermore, the cytotoxic activity of BNip3-related proteins requires the TM domain as its removal abrogates both the ability to homodimerize and induce cell death (Chen *et al.*, 1997, 1999; Cizeau *et al.*, 2000). The BNip3 homodimer is unusual, in that it is

resistant to SDS or 6M urea PAGE and reduction, suggesting that it may have a functional role (Chen *et al.*, 1997). To determine if TM domain-mediated homodimerization is necessary for BNip3-induced cell death, several TM domain deletion and point mutants were generated (Figure 15A). Following expression by coupled *in vitro* transcription/translation, the ^{35}[S]-labeled products were analyzed for dimerization under reducing and non-reducing conditions by Laemmli SDS-PAGE. As described, BNip3 migrates anomalously as a 60 kDa dimer and as a 30 kDa monomer (Figure 15B, *lanes 1 and 2*). The deletion mutants (BNip3ΔC, BNip3Δ179-185 and BNip3ΔTM2) and point mutants, BNip3(L179→S) and BNip3(G180→E) were expressed only as a monomer under both reducing and non-reducing conditions (Figure 15B, *lanes 3 to 12*).

The apoptotic activity of the TM domain mutants was determined by transient co-transfection with a β-galactosidase reporter in 293T human embryonic kidney cells. Following 25 hours post-transfection, cells were stained for β-galactosidase expression and the dying cells were enumerated in the transfected population. The expression of BNip3 resulted in approximately 35% cell death. The killing activity of the TM domain deletion mutants ranged from 20% for BNip3(L179→S) to 29% for BNip3(G180→E), in contrast to 8% cell death in BNip3ΔTM2 or control vector transfected cells (Figure 16A). The differences in killing activity of the various mutants were not due to variable levels of protein expression as verified by Western blotting. All of the mutants were expressed at levels comparable to wild type BNip3 (Figure 16B). Similarly, Rat-1 fibroblasts and MCF-7 breast carcinoma cells transiently transfected with the same series of BNip3 TM domain mutants underwent cell death in a similar manner (G. Chen and A.H. Greenberg, *unpublished observations*). Although some of the *in vitro* transcribed/translated BNip3

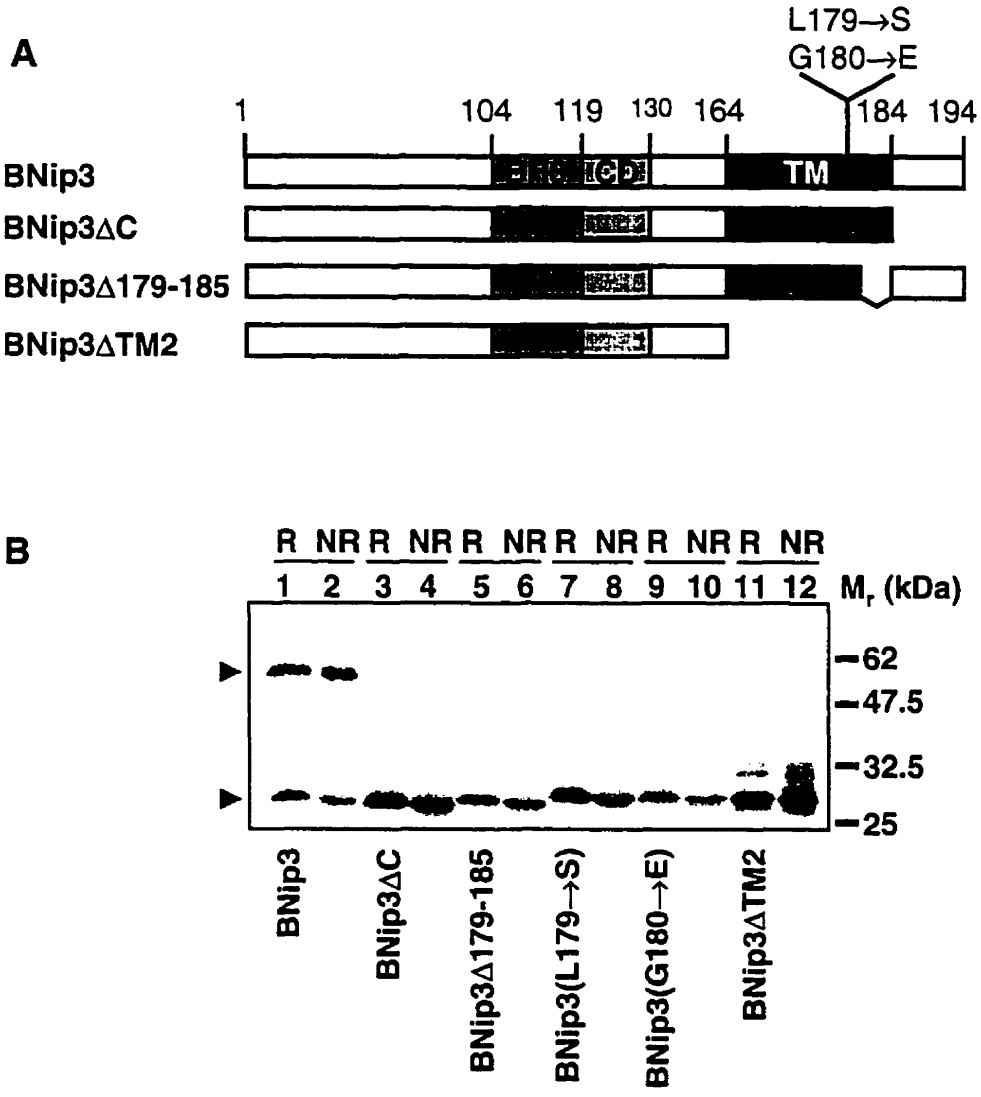

Figure 15. SDS-resistant homodimerization of *in vitro* transcribed/translated BNip3 and its mutants. A, Schematic representation of BNip3 and its COOH-terminal mutants cloned into mammalian expression vector, pcDNA3 to study BNip3 homodimerization and cell death-inducing activity. Both BNip3 point mutants are altered from a non-polar to a polar amino acid residue. B, The COOH-terminal BNip3 mutants were expressed by coupled *in vitro* transcription/translation. The ^{35}[S]-labeled protein products were electrophoresed on SDS-polyacrylamide gels under reducing (R) and non-reducing (NR) conditions. BNip3 migrates as a 60 kDa dimer and as a 30 kDa monomer (arrows). The remaining mutants were expressed only as a monomer under these conditions. The molecular mass standard (M_r) is indicated on the right.

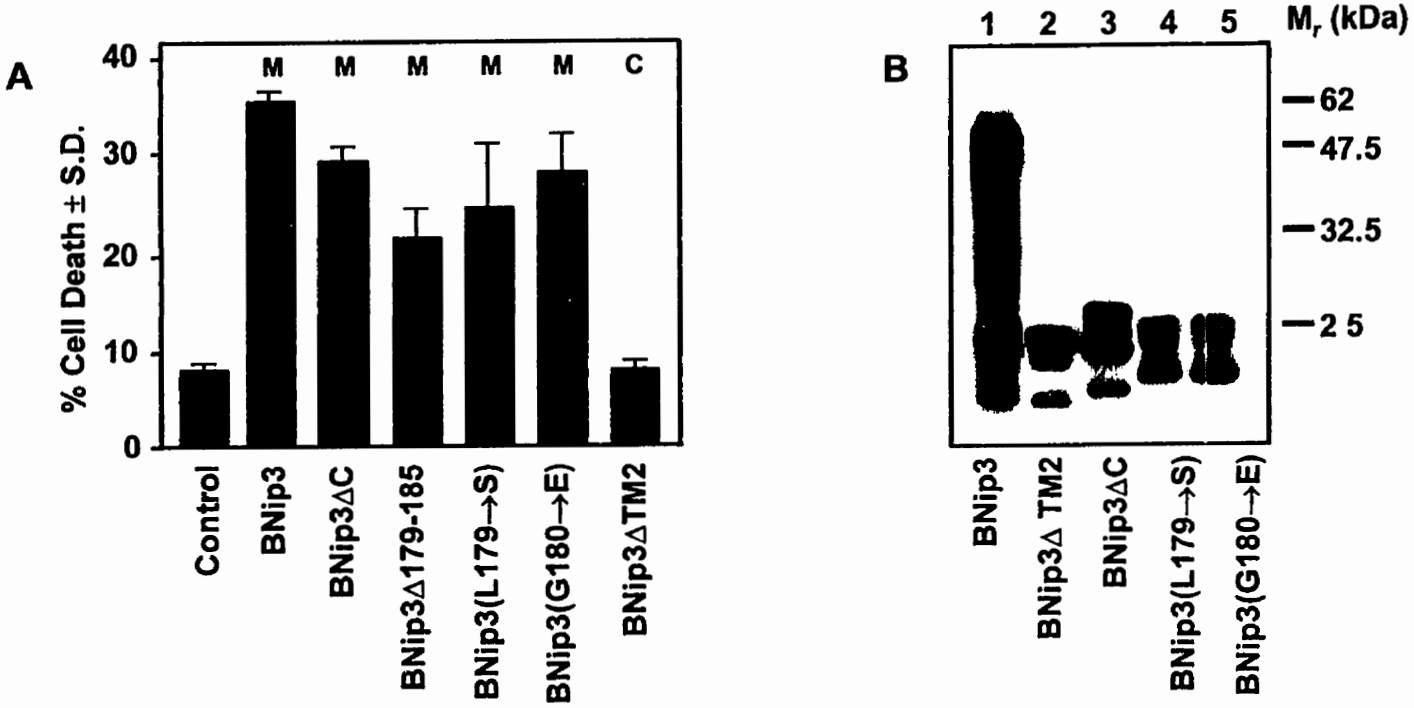

Figure 16. BNip3 TM domain mutants not resistant to SDS and reduction induce cell death. 293T cells seeded at $1x10^5$ per 35 mm plate were co-transfected with 0.2 μg pcDNA3-β-galactosidase reporter and or empty vector (control) using the LipofectAMINE reagent. Following 25 hours post-transfection, cells were fixed in 0.2% glutaraldehyde and stained for β-galactosidase expression. The percentage of dying cells was calculated by assessing the number of rounded, condensed blue cells in the total population of flat, blue cells. The results shown represent the mean ± standard deviation (S.D.) from replicate experiments. Apart from BNip3ΔTM2 which lost its pro-apoptotic activity, the other BNip3 COOH-terminal mutants induced varying levels of cell death. Using the t-test, all comparisons between BNip3 or its deletion mutants and control were significant at p-values less than 0.05. As expected there was no significant difference between cells transfected with empty vector (control) and BNip3ΔTM2. The subcellular localization of these mutants was determined by immunofluorescence (not shown). All were found to localize predominantly to the mitochondria (M), whereas BNip3 lacking its TM domain remained in the cytosol (C). B, These BNip3 TM domain mutants were expressed in 293T cells at levels comparable to BNip3, as determined visually by Western blot analysis.

mutants did not retain their ability to homodimerize following SDS-PAGE, they were all capable of inducing cell death in transient transfection assays.

From previous studies the mitochondrial localization of BNip3 appears to be important for its cell death function (Chen *et al.*, 1997). To determine if the TM mutants that induce cell death localize to the mitochondria, 293T cells seeded on glass slides were transiently transfected and fixed for immunofluorescence staining. Initially, the cells were stained with mouse monoclonal anti-BNip3 antibody, followed by Cy3-conjugated secondary antibody to detect BNIP3 and its mutants. Then, the cells were co-stained for HSP60, a mitochondrial matrix protein (Soltys and Gupta 1992) and FITC-conjugated secondary antibody. The staining pattern of BNip3, BNip3ΔC and TM mutants, BNip3Δ179-185, BNip3(L179→S) and BNip3(G180→E) resembled the punctuate mitochondrial distribution of HSP60. In contrast, BNip3ΔTM2 showed cytosolic distribution, distinct from the localization of HSP60. Computer-assisted overlay of the red and green fluorescent images of BNIP3 or TM mutants with HSP60 showed a uniform yellow staining pattern indicative of virtually complete coincidence of the two stains. The staining pattern of BNip3ΔTM2 did not coincide with the green fluorescence of HSP60, thus was not targeted to the mitochondria (not shown).

4.2. BNip3 heterodimerization and cell death

4.2.1. BNip3-related proteins heterodimerize with Bcl-2, Bcl-x_L and CED-9

BNip3 was originally identified in a yeast two-hybrid screen using the adenoviral orthologue of Bcl-2, E1B 19K as bait (Boyd *et al.*, 1994). Subsequently, it was shown that BNip3 interacts with Bcl-2 (Boyd *et al.*, 1994) and Bcl-x_L (Yasuda *et al.*, 1998b). Using the yeast two-hybrid system, we have been able to confirm these interactions and have shown interaction of BNip3 with CED-9. Yeast transformants co-expressing BNip3 with Bcl-2, Bcl-x_L or CED-9 grew in the absence of tryptophan, leucine and histidine,

indicating interaction between BNip3 and these cell death-repressors (Figure 17A, *left panel*). Co-transformation of Bcl-2, Bcl-x_L or CED-9 with an empty vector exhibited no detectable growth on selection medium (Figure 17A, *right panel*). Similarly, yeast co-transformants expressing CeBNip3 were found to interact with Bcl-x_L and CED-9, with no detectable interaction when co-transformed with empty vector and plated on to selection medium (Figure 17B). The appearance of some colonies on selection medium for control transformations is likely due to growth supported by histidine derived from the innoculum.

The activation of the *lacZ* reporter gene was detected by assaying for β-galactosidase expression. Filter lifts of yeast co-transformants expressing BNip3 and Bcl-2, Bcl-x_L or CED-9 turned blue with X-gal staining and the level of β-galactosidase activity measured using the ONPG assay was 10.9, 15.9 and 13.0 units respectively. There was no detectable interaction between BNip3 and two heterologous proteins, indicating BNip3 heterodimerization with the anti-apoptotic Bcl-2 proteins is specific (Table 9).

To confirm the interactions *in vivo*, 293T cells were transiently co-transfected with plasmids expressing BNip3 and Bcl-2 or Bcl-x_L (Figures 19 and 20). Following immunoprecipitation for Bcl-2 or Bcl-x_L, BNip3 co-immunoprecipitated with both Bcl-2 and Bcl-x_L, respectively as a dimer and as a monomer (Figure 19, *lane 1* and Figure 20, *lane 1*). Similarly, immunoprecipitation of lysates prepared from transiently co-transfected cells expressing BNip3 and CED-9 revealed that BNip3 also interacts with CED-9 (Cizeau *et al.*, 2000). The 60 kDa dimeric form of BNip3 migrates with the heavy chain of anti-Bcl-2 or anti-Bcl-x_L antibody used for co-immunoprecipitation. Therefore, the dimeric form of BNip3 was difficult to visualize using anti-BNip3 antibody on

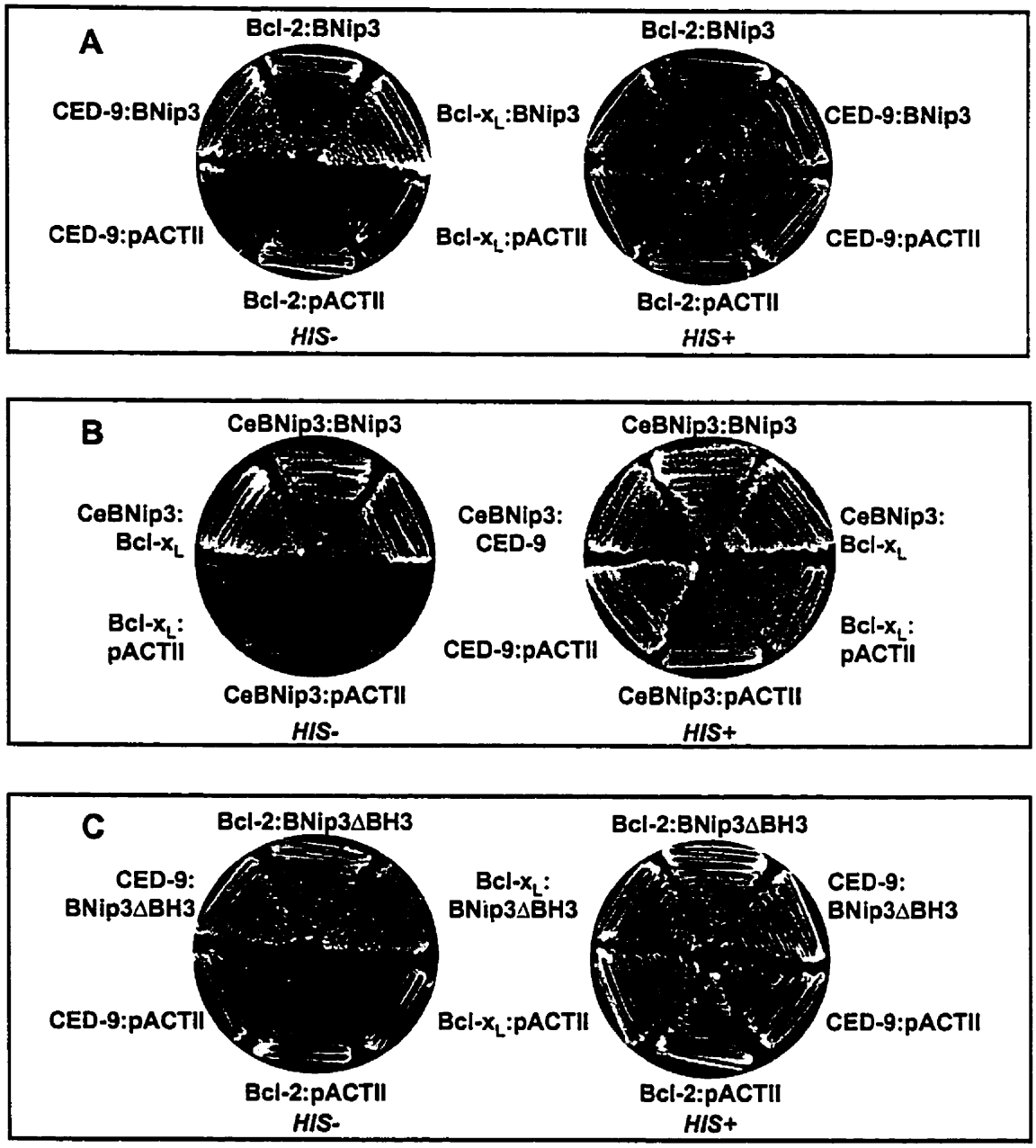

Figure 17. BNip3-related proteins heterodimerize with Bcl-2, Bcl-x_L and CED-9 in the yeast two-hybrid system. Yeast strain KGY37 was co-transformed with GAL4 BD vector expressing Bcl-2, Bcl-x_L or CED-9 with BNip3 (A), CeBNip3 (B) or BNip3ΔBH3 (C) fused to the GAL4 AD. The BD constructs were also co-transformed with empty vector, pACTII as a control. A, BNip3 interacts with Bcl-2, Bcl-x_L and CED-9 as determined by growth on selection medium lacking tryptophan, leucine and histidine with 1 mM 3AT(*HIS*-). B, Similarly, CeBNip3 interacts with Bcl-x_L and CED-9 on selection medium (*HIS*-). C, The removal of BNip3 BH3-like domain did not disrupt heterodimerization. There was significantly reduced or no detectable growth when Bcl-2, Bcl-x_L or CED-9 were co-transformed with empty vector indicating the specificity of observed interactions. All co-transformants grew on media lacking tryptophan and leucine (*HIS*+) (A, B and C, *right panel*). The co-transformations are labeled in the order, BD:AD vectors.

Western blots. There was no specific signal detected on Western blots if BNip3 was expressed alone, indicating that the co-immunoprecipitation of BNip3 in co-transfected cells was due to specific interaction with Bcl-2 or Bcl-x_L. Thus, BNip3 and CeBNip3 interact with Bcl-2, Bcl-x_L and CED-9 in both yeast and mammalian expression systems.

Table 9. β-galctosidase activity of BNip3 interaction with Bcl-2-related proteins in the yeast two-hybrid system

Transformation (BD:AD)	β-galactosidase activity	
	X-gal staining	ONPG assay
BNip3:Bcl-2	B	10.9
BNip3:Bcl-x_L	B	15.9
BNip3: CED-9	B	13.0
BNip3:MK	W	1.3
BNip3:PTP2	W	1.4
*BNip3 (BD)	W	1.4
*Bcl-2 (AD)	W	0.5
*Bcl-x_L (AD)	W	0.7
*CED-9 (AD)	W	0.3

Yeast strain KGY37 was co-transformed with indicated GAL4 binding domain (BD) and activating domain (AD) constructs. The relative level of activation for the *lacZ* reporter gene was determined by X-gal staining (B, blue, W, white) and measuring hydrolysis of ortho-nitrophenyl β-D-galactopyranoside (ONPG) whereby, 1 unit of β–galactosidase activity (Miller unit) is defined as the amount which hydrolyzes 1 μmole of ONPG to o-nitrophenol and D-galactose per minute (Miller, 1972). *Denotes single tranformations to establish background levels of *lacZ* activation. MK, myotonin kinase; PTP2, protein tyrosine phosphatase 2

4.2.2. BNip3 lacking a BH3 domain heterodimerizes with Bcl-2, Bcl-x_L and CED-9

To date, the majority of pro-apoptotic Bcl-2-related proteins interact with their death-repressing partners through their BH3 domain. Specifically, these proteins contain an eight amino acid residue core within the BH3 domain consisting of conserved leucine and aspartic acid residues at positions 1 and 6, respectively, that are critical for

heterodimerization (Figure 10) (Kelekar and Thompson 1998). Sequence analysis of BNip3 identified a BH3-like motif in which the core residues, Leu^{110} and Asp^{115} and flanking residues Val^{108}, and Ile^{117} are conserved with critical amino acids of the Bak BH3 domain that determines Bak-Bcl-x_L heterodimerization (Figure 13) (Sattler *et al.*, 1997). These residues are conserved in Nix as well as CeBNip3, with the exception that CeBNip3 contains two additional amino acids inserted between the leucine and aspartic acid residues (Figure 13).

To evaluate the role of the BH3-like domain in BNip3 heterodimerization with cell death-repressors, a 16 amino acid deletion mutant was constructed to encompass the flanking and most conserved residues of the BNip3 BH3-like domain. For the yeast two-hybrid assay, BNip3ΔBH3 was co-transformed with Bcl-2, Bcl-x_L or CED-9. The co-transformants grew in the absence of tryptophan, leucine and histidine, indicating the deletion of the BH3-like domain did not interfere with its ability to interact with Bcl-2, Bcl-x_L or CED-9 (Figure 17C, *left panel*). These interactions were specific, as there was no detectable growth on selection medium when Bcl-2, Bcl-x_L or CED-9 was co-transformed with an empty vector, pACTII (Figure 17C, *right panel*). The appearance of some colonies on selection medium for control transformations is likely due to growth supported by histidine derived from the innoculum. BNip3 lacking its BH3-like domain was also shown to interact with Bcl-2 as efficiently as wild type BNip3 in an *in vitro* binding assay, followed by Bcl-2 immunoprecipitation described in Section 4.2.3 (Figure 21, *lanes 1 and 3*).

To verify that BNip3ΔBH3 exhibits similar interactions in mammalian cells, 293T cells were transiently co-transfected with plasmids expressing BNip3ΔBH3 and Bcl-2 or Bcl-x_L. The co-immunoprecipitation reactions were prepared and analyzed by Western

blotting as described (Section 4.2.3). Similar to wild type BNip3, BNip3ΔBH3 co-precipitated as a dimer with Bcl-2 (Figure 19, *lanes 1 and 5*), Bcl-x_L (Figure 20, *lanes 1 and 5*) and CED-9 (Cizeau *et al.*, 2000). These findings confirm the interactions observed in the yeast two-hybrid and *in vitro* co-immunoprecipitation assays. The removal of CeBNip3 BH3-like domain yielded similar results, whereby CeBNip3ΔBH3 co-precipitated with CED-9 and Bcl-x_L in lysates prepared from transiently co-transfected 293T cells (Cizeau *et al.*, 2000).

4.2.3. BNip3 requires its NH_2-terminus to interact with Bcl-2 and Bcl-x_L

The exclusion of a BH3-like domain in BNip3 heterodimerization suggested that other regions must promote interaction with Bcl-2-related cell death antagonists. To map these regions, a series of deletion mutants were generated to encompass the NH_2-terminus (residues 1-49) and COOH-terminus (residues 164-194), as well as the region of shared identity between BNip3, Nix and CeBNip3 (CD, conserved domain; BNip3 residues 112-130) (Figure 18). Using the calcium phosphate precipitation method, 293T cells were transiently co-transfected with either wild type BNip3 or its deletion mutants and Bcl-2 or Bcl-x_L (Figure 19 and 20). Following immunoprecipitation of Bcl-2 or Bcl-x_L respectively, BNip3ΔCD was readily detected by Western blotting (Figure 19, *lane 6* and Figure 20, *lane 6*). The mutants BNip3ΔTM1, BNip3ΔTM2 and BNip3ΔN co-immunoprecipitated with Bcl-x_L (Figure 20, *lanes 3, 4 and 7*), but not with Bcl-2 (Figure 19, *lanes 3, 4 and 7*). Thus, the removal of the NH_2-terminus of BNip3 prevented Bcl-2, but not Bcl-x_L heterodimerization. Subsequently, the removal of both the NH_2-terminus and the TM domain (BNip3ΔN;ΔTM) was necessary to prevent BNip3 heterodimerization with Bcl-x_L (Figure 20, *lane 8*).

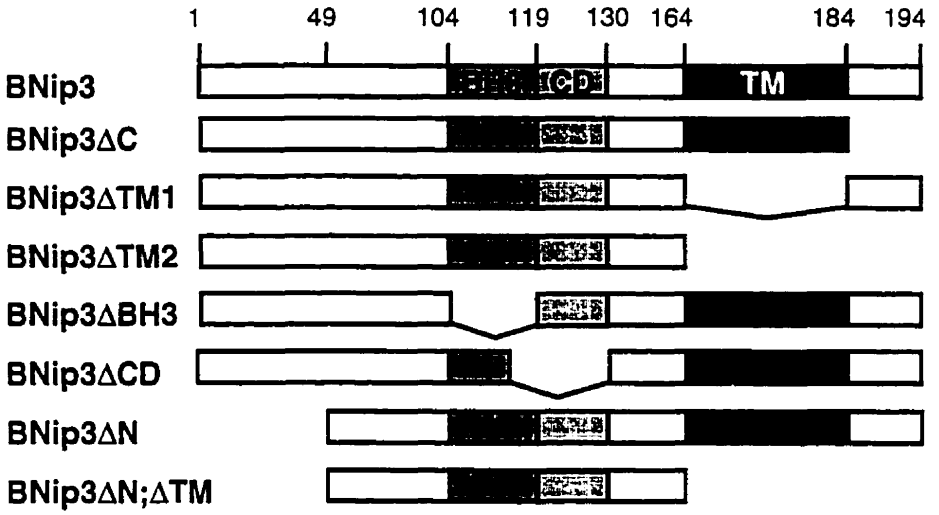

Figure 18. Schematic representation of BNip3 mutants generated to map Bcl-2/Bcl-x_L heterodimerization. BNip3 and its mutants were cloned into yeast and mammalian expression vectors to map Bcl-2/Bcl-x_L heterodimerization. The series of BNip3 deletion mutants encompass the NH_2-terminus (amino acids 1-49) and the extreme COOH-terminus (amino acids 184-194), as well as the BH3-like domain (BH3, amino acids 104-119), the conserved domain (CD, amino acids 112-130) and the TM domain (amino acids 164-184).

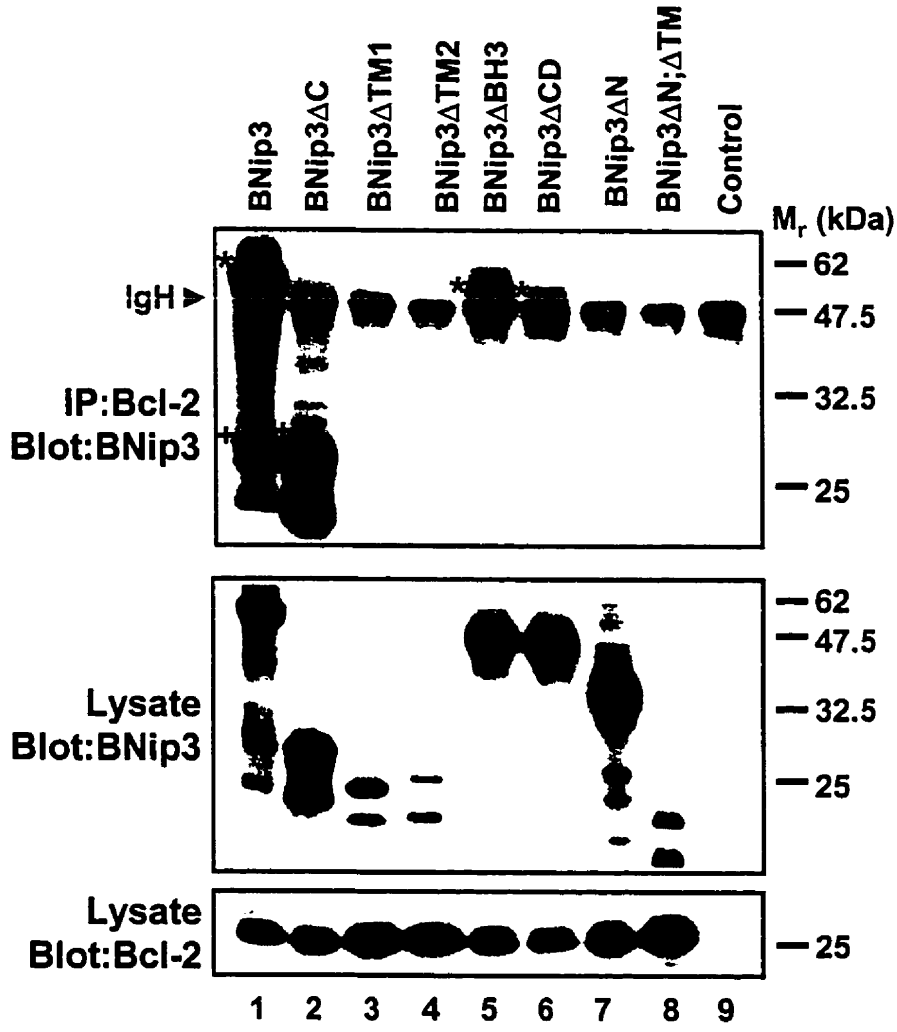

Figure 19. Mapping BNip3-Bcl-2 heterodimerization by *in vivo* co-immunoprecipitation assay. Using the calcium phosphate precipitation method, 293T human embryonic kidney cells ($2x10^6$ per 100 mm plate) were transiently co-transfected with pcDNA3-Bcl-2 and pcDNA3 encoding BNip3 or indicated deletion mutants. The total amount of DNA used was always 15 μg. Lysates were immunoprecipitated (IP) with hamster anti-Bcl-2 antibody from co-transfected cells as well as empty vector transfected (control) cells. Immunoprecipitates were immunoblotted with rabbit anti-BNip3 antibody. The dimeric (*) and monomeric (+) forms of BNip3 and its deletion mutants are indicated. Transiently expressed in 293T cells, BNip3 migrates as a 60 kDa dimer and as a 30 kDa monomer determined from the molecular mass standard (M_r). The immunoglobulin heavy chain (IgH) is present in all lanes. Total cell lysates were analyzed by immunoblotting with rabbit anti-BNip3 (*middle panel*) or mouse anti-Bcl-2 (*lower panel*) antibody. BNip3, as well as mutants lacking the BH3 domain or conserved domain co-precipitate with Bcl-2. The deletion of either the NH_2-terminal region or the TM domain prevents BNip3-Bcl-2 heterodimerization.

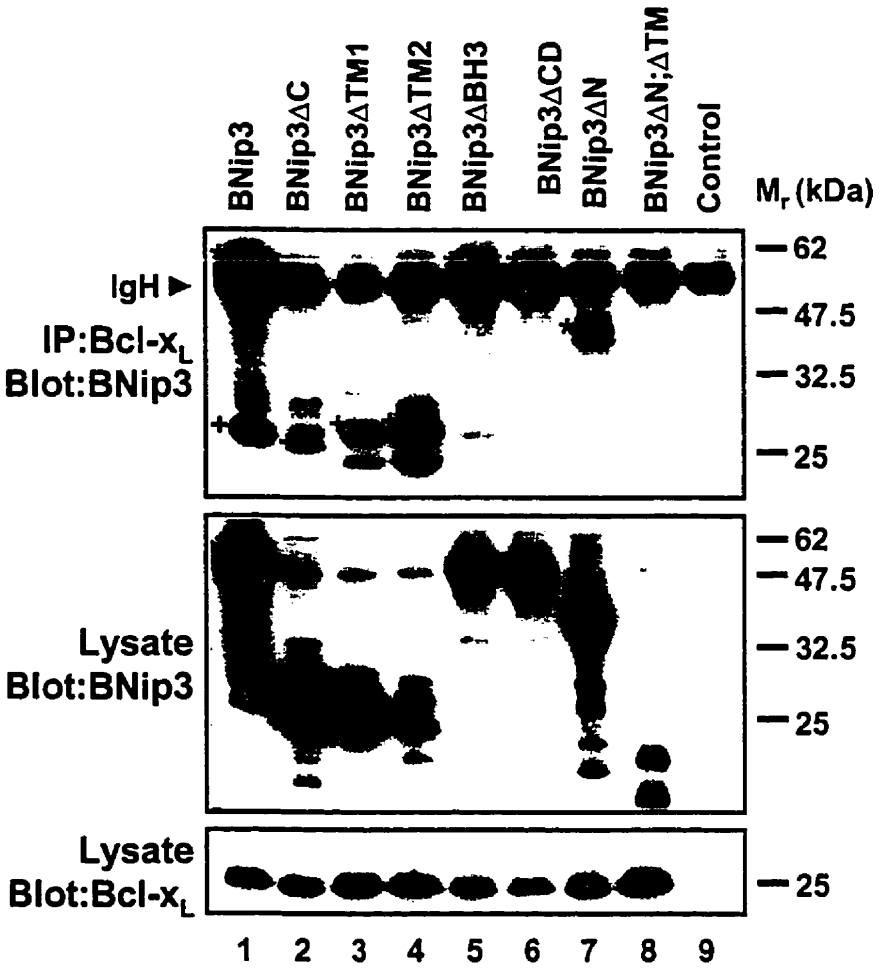

Figure 20. Mapping BNip3-Bcl-x_L heterodimerization by *in vivo* co-immunoprecipitation assay. The *in vivo* co-immunoprecipitation assay as described was repeated whereby pcDNA3-myc-Bcl-x_L was co-expressed with BNip3 or indicated deletion mutants in 293T cells. Following immunoprecipitation (IP) with mouse anti-C-myc antibody, full length and truncated BNip3 proteins were detected by immunoblotting with rabbit anti-BNip3 antibody. The dimeric (*) and monomeric (+) forms of BNip3 and its deletion mutants are indicated. Transiently expressed BNip3 migrates as a 60 kDa dimer and as a 30 kDa monomer determined from the molecular mass standard (M_r). The immunoglobulin heavy chain (IgH) is present in all lanes. Total cell lysates were analyzed by immunoblotting with rabbit anti-BNip3 (*middle panel*) or rabbit anti-Bcl-x_L (*lower panel*) antibody. Bcl-x_L interacts with all the BNip3 mutants that contain an intact NH_2-terminal region or TM domain.

The direct role for the NH_2-terminus in BNip3 heterodimerization is further demonstrated by interaction of *in vitro* transcribed/translated ^{35}[S]-labeled BNip3 or its mutants with purified His-tagged Bcl-2 protein. Equivalent amounts of labeled BNip3 protein products were incubated with Bcl-2 protein in the appropriate buffer. Following immunoprecipitation for Bcl-2, the immune complexes were separated by Laemmli SDS-PAGE and analyzed by autoradiography. Both BNip3ΔBH3 and BNip3ΔCD co-immunoprecipitated with Bcl-2 protein (Figure 21A, *lanes 3 and 4*), thus, excluding the central region of BNip3 encompassing the BH3-like domain and conserved domain in heterodimerization. BNIPΔTM2 also co-immunoprecipitated with Bcl-2 (Figure 21A, *lane 2*). In contrast, removal of NH_2-terminal sequences (BNip3ΔN) or both the NH_2-terminus and TM domain (BNip3ΔN;ΔTM) disrupted interaction with Bcl-2 (Figure 21A, *lanes 5 and 6*). The observed interactions were specific, as BNip3 and its mutants were not detected when co- immunoprecipitated with anti-Bcl-2 antibody and a non-specific protein, GAPDH (glyceraldehyde 3-phosphate dehydrogenease, not shown). The presence of Bcl-2 protein in each co-immunoprecipitation reaction was confirmed by Coomassie blue staining of SDS-polyacrylamide gels (Figure 21A, *lower panel*). Detergents used to solubilize proteins have been reported to facilitate dimerization between Bcl-2-related proteins (Hsu and Youle 1997 and 1998). The *in vitro* co-immunoprecipitation experiment was repeated substituting NP-40 with another non-ionic detergent, Triton X-100 in the immunoprecipitation buffer. Under these conditions, this 'detergent effect' was not observed to facilitate interaction of BNip3 or its mutants with Bcl-2 (Figure 21B, *lanes 1 to 7*). The results are identical using NP-40 or Triton X-100 in the immunoprecipitation buffer.

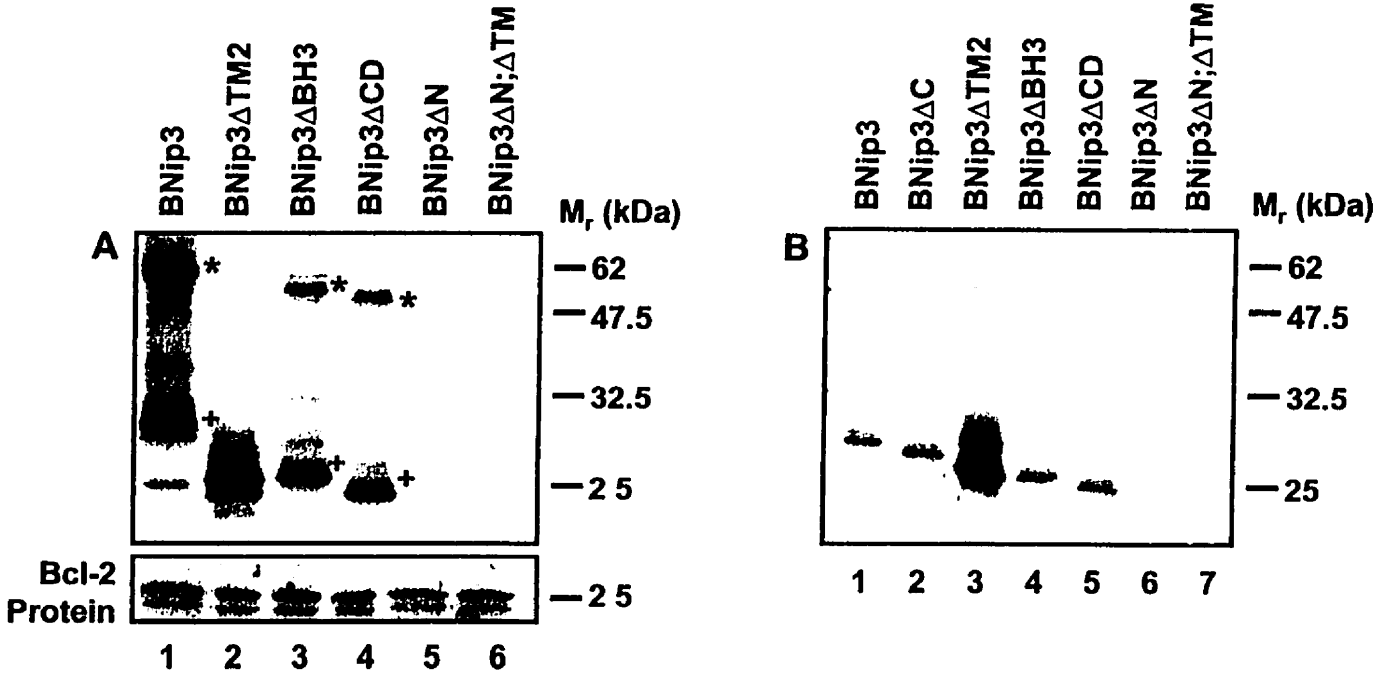

Figure 21. Mapping BNip3-Bcl-2 heterodimerization by *in vitro* co-immunoprecipitation assay. BNip3 and indicated deletion mutants were *in vitro* transcribed/translated in the presence of ^{35}[S]-methionine. The equivalent amounts of labeled products were incubated with purified Bcl-2 protein in 0.2% NP-40 immunoprecipitation buffer, followed by immunoprecipitation with hamster anti-Bcl-2 antibody, The immune complexes were electrophoresed though SDS-polyacrylamide gels and detected by autoradiography. The dimeric (*) and monomeric (+) forms of BNip3 and its mutants are indicated. BNip3 migrates as a 60 kDa dimer and as a 30 kDa monomer as determined from the molecular mass standard (M_r). The presence of Bcl-2 protein in each co- immunoprecipitation reaction was verified by Coomassie blue staining (*lower panel*). B, The experiment was repeated, whereby 0.2% NP-40 was replaced in the immunoprecipitation buffer with 1% Triton X-100 as the non-ionic detergent. There was no difference noted among the BNip3 mutants that co-immunoprecipitate with Bcl-2 using either detergent. The presence of Bcl-2 protein in each co-immunoprecipitation reaction was verified by Coomassie blue staining (not shown).

4.2.4. BNip3 NH2-terminal interaction with Bcl-2/Bcl-xL is not detectable in the yeast two-hybrid system

Initial studies to map the region(s) of BNip3 critical for Bcl-2/Bcl-x_L heterodimerization were done in the yeast two-hybrid system. Bcl-2 or Bcl-x_L was co-transformed into yeast strain KGY37 with a series of BNip3 deletion mutants encompassing the NH_2-terminus, the BH3-like domain, conserved domain (CD) and the COOH-terminus (Figure 18). The heterodimeric interactions observed with BNip3 or its mutants were identical for both Bcl-2 and Bcl-x_L. Consistent with the *in vivo* and *in vitro* co-immunoprecipitation studies described earlier, full length BNip3 or BNip3 lacking its conserved domain or its BH3-like domain formed heterodimers with Bcl-2/Bcl-x_L (Table 10). Deletion of the first 49 amino acids of BNip3 (BNip3ΔN) did not disrupt its interaction with Bcl-2/Bcl-x_L. Rather, the removal of the BNip3 TM domain (BNip3ΔTM2) disrupted Bcl-2/Bcl-x_L heterodimerization in the yeast two-hybrid assay (Table 10). Similarly, the removal of the CeBNip3 TM domain prevented interaction with Bcl-x_L and CED-9 (Table 11). For, BNip3 the latter two findings are in contrast to the observations made by *in vivo* co-immunoprecipitation whereby, BNip3ΔN and BNip3ΔTM2 interact with Bcl-x_L but not Bcl-2. In the *in vitro* co-immunoprecipitation assay, BNip3ΔTM2 was detected to interact with Bcl-2. Therefore, in yeast the NH_2-terminal BD and AD fusion proteins of BNip3 lacking residues 1-49 or its TM domain must be subject to conformational changes that mask interaction with Bcl-2/Bcl-x_L. For, CeBNip3 lacking its TM domain, the yeast two-hybrid results are consistent with *in vivo* co-immunoprecipitation studies (Cizeau *et al.*, 2000). Although, the BNip3 NH_2-terminal interaction was not detected by the yeast two-hybrid assay, these findings confirm a role for its TM domain in heterodimerization with Bcl-2, Bcl-x_L and CED-9. The lack of detectable growth on selection medium was not due to insufficient levels of protein expression from BD and/or AD vectors in co-transformations, as all constructs were

Table 10. Mapping yeast two-hybrid interaction of BNip3 with Bcl-2, Bcl-x_L and CED-9

	Constructs		
AD BNip3 mutants	BD Bcl-2	BD Bcl-x_L	BD CED-9
	HIS3 activation		
BNip3	+++	+++	+++
BNip3ΔC	+++	+++	ND
BNip3ΔN	+++	+++	+++
BNip3ΔTM2	-	-	-
BNip3ΔBH3	+++	+++	+++
BNip3ΔCD	+++	+++	ND
pACTII	-	-	-

Yeast strain KGY37 was co-transformed with indicated GAL4 binding domain (BD) and activating domain (AD) constructs. The relative level of activation for the *HIS3* reporter gene was determined by growth (+) or no growth (-) on selection medium lacking tryptophan, leucine and histidine in the presence of 1 mM 3 amino-1,2,4-triazole (3AT). ND, not determined

Table 11. Interaction of CeBNip3 with CED-9 and Bcl-x_L in the yeast-two hybrid system

Transformation (BD:AD)	*HIS3* activation
CED-9:CeBNip3	+++
CED-9:CeBNip3ΔTM	-
Bcl-x_L:CeBNip3	+++
Bcl-x_L:CeBNip3ΔTM	-
CED-9:pACTII	-
Bcl-x_L:pACTII	-
pGBT9:CeBNip3	-
pGBT9:CeBNip3ΔTM	-

Yeast strain KGY37 was co-transformed with indicated GAL4 binding domain (BD) and activating domain (AD) constructs. The relative level of activation for the *HIS3* reporter gene was determined by growth (+) or no growth (-) on selection medium lacking tryptophan, leucine and histidine in the presence of 1 mM 3-amino-1,2,4-triazole (3AT). ND, not determined

verified to interact with known positives such as full length BNip3 or granzyme B (not shown).

4.2.5. BNip3 lacks a cell death inducing BH3 domain

BNip3-related proteins transiently expressed in several cell lines induce cell death (Chen *et al.*, 1997, 1999; Yasuda *et al.*, 1998a, 1998b, 1999; Cizeau *et al.*, 2000). The BH3 domain confers pro-apoptotic activity to several Bcl-2 homologues such as Bik (Boyd *et al.*, 1995; Farrow *et al.*, 1995), Blk (Hedge *et al.*, 1998), Hrk (Inohara *et al.*, 1997a), BimL (O'Conner *et al.*, 1998), Bad (Yang *et al.*, 1995), Bid (Wang *et al.*, 1996b) and EGL-1 (Conradt and Horvitz 1998). The BH3 domain of Bax and Bak is sufficient to induce apoptosis in cell free systems (Cosulich *et al.*, 1997). To determine if the BH3-like domain of BNip3 plays a similar role, the cell death activity of wild type BNip3 was compared to BNip3ΔBH3. Two cell lines, Rat-1 fibroblasts and MCF-7 breast carcinoma cells were transiently transfected with T7-epitope tagged BNip3 or BNip3ΔBH3. At the indicated time-points, cells were stained with anti-T7 antibody, followed by FITC-conjugated secondary antibody to identify transfected cells as well as Hoechst dye to assess dying cells by nuclear chromatin condensation. Rat-1 fibroblasts became apoptotic by 12 hours following BNip3 or BNip3ΔBH3 transfection and reached a peak of 55% and 50%, respectively at 36 hours, although BNip3ΔBH3 cell death was reduced somewhat at earlier time-points (Figure 22A). Dying MCF-7 cells expressing BNip3 or BNip3ΔBH3 were detected by 12 hours reaching a maximum by 36 hours with BNip3ΔBH3 exhibiting no difference except at the 36 hour time-point (Figure 22B). The experiment was repeated in 10T1/2 fibroblasts. The cell death activity of BNip3 and BNip3ΔBH3 reached 60% and 69%, respectively, 24 hours post-transfection using the β-galactosidase assay to detect dying cells (Figure 22D).

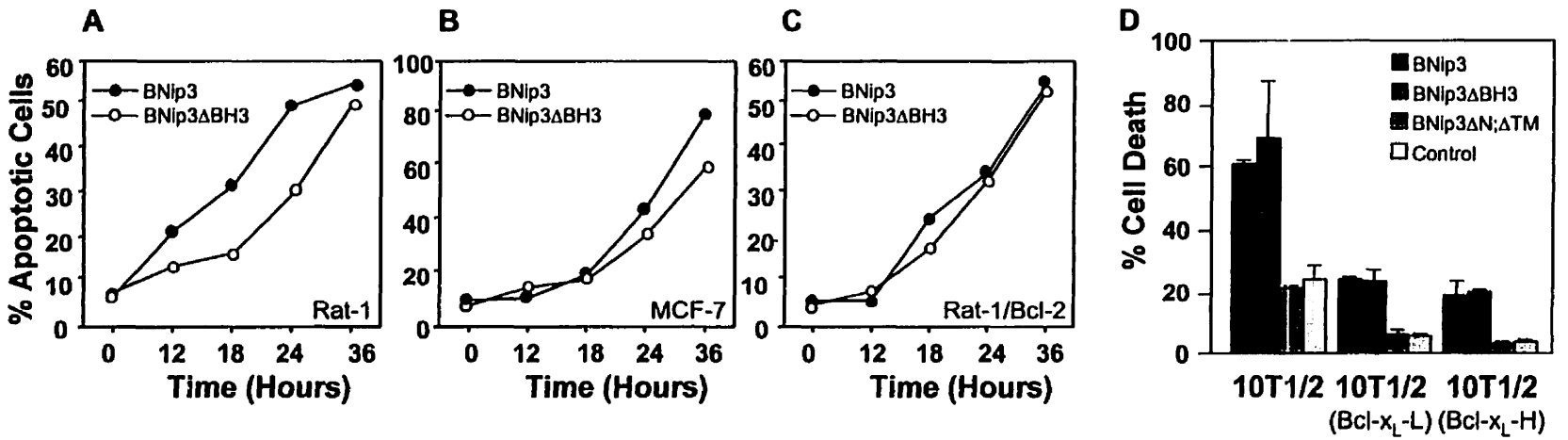

Figure 22. Overexpression of BNip3 lacking its BH3-like domain induces cell death. Rat-1 fibroblasts (A), MCF-7 breast carcinoma cells (B) or Rat-1 fibroblasts constitutively expressing Bcl-2 (C) were transiently transfected with T7-BNip3 (•) or T7-BNip3ΔBH3 (o) using the LipofectAMINE reagent. At the indicated time-points, cells were fixed with 4% formaldehyde and stained with mouse monoclonal anti-T7 antibody followed by FITC-conjugated secondary antibody to detect transfected cells by immunofluorescence. BNip3 and BNip3ΔBH3 dying cells were enumerated by altered nuclear morphology based on Hoechst dye staining. In total, 200-300 transfected cells were evaluated per sample. D, 10T1/2 fibroblasts and two 10T1/2 derived lines constitutively expressing low (10T1/2-Bcl-x_L-L) and high levels (10T1/2-Bcl-x_L-H) of Bcl-x_L were co-transfected with 0.2 μg pcDNA3-β-galactosidase reporter and 0.75 μg pcDNA3 expressing BNip3, BNip3ΔBH3, BNip3ΔN;ΔTM or empty vector (control) using the LipofectAMINE reagent. Following 36 hours post-transfection, cells were fixed in 0.2% glutaraldehyde and stained for β-galactosidase expression. The percentage of dying cells was enumerated by assessing the number of rounded, condensed blue cells in the total population of flat, blue cells. The results shown represent the mean±standard deviation (S.D.) from replicate experiments. Using the t-test, all comparisons between BNip3 or its deletion mutants and control were significant at p-values of less than 0.05. As expected, there was no significant difference between cells transfected with empty vector (control) and BNip3ΔN;ΔTM.

The pro-apoptotic activity of BH3-only proteins, Bik (Boyd *et al.*, 1995; Farrow *et al.*, 1995), Blk (Hedge *et al.*, 1998), Hrk (Inohara *et al.*, 1997a) and BimL (O'Conner *et al.*, 1998) is blocked by simultaneous expression with Bcl-2/Bcl-x_L. In BNip3-, Nix- or CeBNip3-induced cell death, Bcl-2/Bcl-x_L overexpression initially delays the onset of cell death, but the resistance is overcome in time (Chen *et al.*, 1997, 1999; Cizeau *et al.*, 2000). To determine the effect of Bcl-2 overexpression on BNip3ΔBH3 induced cell death compared to wild type BNip3, Rat-1/Bcl-2 fibroblasts were transiently transfected with T7-BNip3 or T7-BNip3ΔBH3. Although the appearance of BNip3 and BNip3ΔBH3 dying cells was delayed in Bcl-2 transfected (Figure 22C), compared to parental Rat-1 fibroblasts (Figure 22A), there was no observable difference between BNip3 and BNip3ΔBH3 cell death activity. Using the β-galactosidase assay to detect dying cells, both BNip3 and BNip3ΔBH3 induced comparable levels of cell death in 10T1/2 fibroblasts constitutively expressing low and high levels of Bcl-x_L (Figure 22D). Similar findings were also made in transient cell death assays expressing CeBNip3ΔBH3 (Cizeau *et al.*, 2000).

4.2.6. BNip3 targeted to mitochondrial and nonmitochondrial sites interacts with Bcl-2 and Bcl-x_L

The COOH-terminal TM domain of the Bcl-2 class of proteins targets both anti- and pro-apoptotic members to various intracellular membranes. Apart from targeting BNip3-related proteins to the mitochondria, the TM domain and NH_2-terminal region were identified to mediate BNip3 heterodimerization and the TM domain to exclusively mediate CeBNip3 heterodimerization (Cizeau *et al.*, 2000). The *in vitro* and *in vivo* co-immunoprecipitation studies were repeated using BNip3 substituted with heterologous TM domain sequences from Bcl-2 and cytochrome b_5 to determine the following: (1) if BNip3 targeted to various subcellular sites is able to heterodimerize with Bcl-2 and Bcl-

x_L and (2) if BNip3 heterodimerization is independent of its TM domain. The BNip3 chimera, BNip3-Bcl-2 was constructed by fusing the cytoplasmic region of BNip3 (residues 1-163) to 21 amino acids of the Bcl-2 TM domain, which is sufficient to target heterologous proteins to the outer mitochondrial membrane orienting the protein towards the cytosol (Figure 23) (Nguyen *et al.*,1993; Janiak *et al.*, 1994). Similarly, the COOH-terminally truncated BNip3 was fused to a 35 amino acid residue segment of rat hepatic cytochrome b_5 which has been previously shown to target heterologous proteins to the cytoplasmic face of the ER in transfected cells (Figure 23) (Mitoma and Ito 1992; Zhu *et al.*, 1996). Equivalent amounts of *in vitro* transcription/translation products of BNip3 and its chimeric proteins were incubated with purified Bcl-2 protein. Following Bcl-2 immunoprecipitation, both BNip3-BclTM and BNip3-Cb5TM were detected in the immune complexes (Figure 24A, *lanes 3 and 4*). The observed interactions were specific as no co-immunoprecipitation was apparent when labeled products were incubated with a non-specific protein (GAPDH) and immunoprecipitated with anti-Bcl-2 antibody (not shown). The presence of Bcl-2 in each co-immunoprecipitation reaction was confirmed by Western Blotting (Figure 24A, *lower panel*). Similar interactions were observed in 293T cells co-transfected with BNip3-BclTM or BNip3-Cb5TM and Bcl-x_L. Co-immunoprecipitation reactions were prepared and analyzed by Western blotting as described (Section 4.2.3). BNip3 fused to either the Bcl-2 or cytochrome b_5 TM domain sequence co-immunoprecipitated with Bcl-x_L (Figure 24B, *lanes 3 and 4*).

4.3. Other BNip3 heterodimerizing partners

4.3.1. BNip3-related proteins heterodimerize through their transmembrane domains

The Bcl-2 family of proteins characteristically form heterodimers between pro- and anti-apoptotic members through their BH3 domains (Kelekar and Thompson 1998).

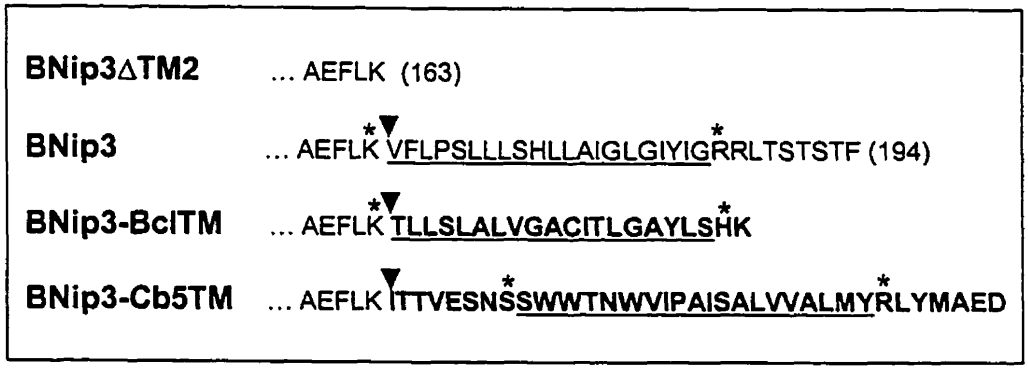

Figure 23. Schematic representation of BNip3 substituted with heterologous TM domain sequences. The TM domain of BNip3 was substituted with heterologous TM domain sequences from Bcl-2 and cytochrome *b5*. The COOH-terminal amino acid sequences of BNip3 and chimeric proteins, BNip3-BclTM and BNip3-Cb5TM are shown (bold) with the hydrophobic TM domain (underlined). The arrowhead indicates the fusion junction between BNip3 and heterologous TM domain sequences. The (*) indicates the charged amino acids flanking the TM domain. The Bcl-2 TM domain targets heterologous proteins primarily to the outer mitochondrial membrane, ER and nuclear envelope. The cytochrome *b5* TM domain targets heterologous proteins to the ER. Both targeting sequences orient the heterologous protein towards the cytosol.

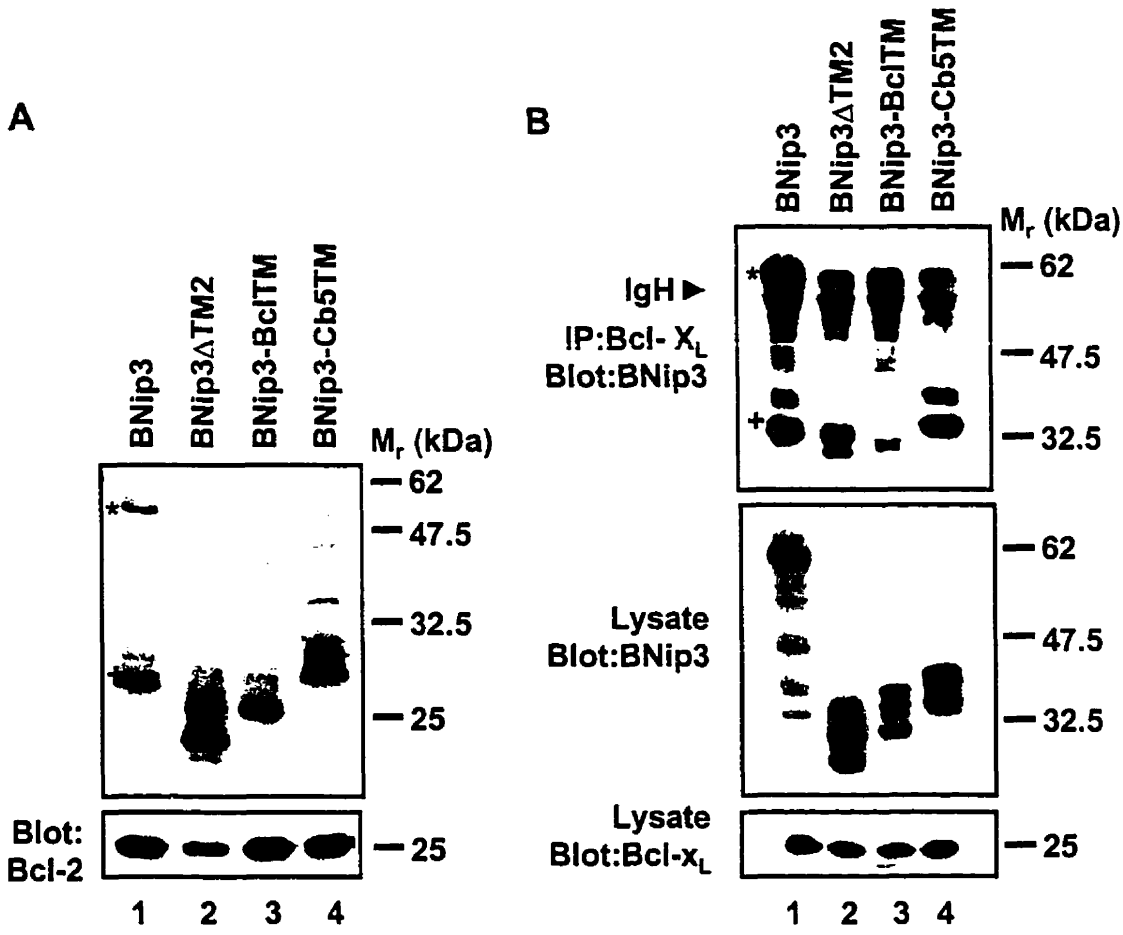

Figure 24. BNip3 substituted with heterologous TM domains interacts with Bcl-2 and Bcl-x_L. A, BNip3 and chimeric proteins, BNip3-BclTM and BNip3-Cb5TM were *in vitro* transcribed/translated in the presence of ^{35}[S]-methionine and immunoprecipitated as described. Equivalent amounts of labeled products were incubated with purified Bcl-2 protein in 0.2% NP-40 immunoprecipitation buffer, followed by immunoprecipitation with hamster anti-Bcl-2 antibody, The immune complexes were electrophoresed through SDS-polyacrylamide gels and detected by autoradiography. The presence of Bcl-2 protein in each immunoprecipitation reaction was verified by Western blotting (*lower panel*). B, 293T cells ($2x10^6$ per 100 mm plate) were transiently co-transfected by the calcium phosphate precipitation method with Bcl-x_L and BNip3, BNip3-BclTM or BNip3-Cb5TM. The total amount of DNA used was always 15 μg. Lysates were immunoprecipitated with mouse anti-C-myc antibody from co-transfected cells as well as empty vector transfected cells (control, not shown). Immunoprecipitates were immunoblotted with rabbit anti-BNip3 antibody. The immunoglobulin heavy chain (IgH) is present in all lanes. Total cell lysates were analyzed by immunoblotting with rabbit anti-BNip3 (*middle panel*) or mouse anti-Bcl-x_L (*lower panel*) antibody. The dimeric (*) and monomeric (+) forms of BNip3 are indicated. BNip3 migrates as a 60 kDa dimer and as a 30 kDa monomer determined from the molecular mass standard (M_r).

Heterodimerization among the pro-apoptotic members has been observed between some family members such as Bax and the 'BH3-only' protein Bid (Wang *et al.*, 1996b). To determine if BNip3-related proteins are able to heterodimerize, BNip3 was co-transformed with Nix or CeBNip3 into yeast strain KGY37. BNip3 was observed to interact with Nix based on growth on medium lacking tryptophan, leucine and histidine in the presence of 1mM 3AT (Figure 25). Similarly, CeBNip3 was found to interact with BNip3 as determined by growth of yeast co-transformants on selection medium (Figure 17B). The TM domain of BNip3-related proteins mediates homo- and heterodimeric interactions. To determine if the TM domain also facilitates interaction between BNip3 family members, the yeast two-hybrid assay was repeated. BNip3 was co-transformed with either NixΔTM, CeBNip3ΔTM or empty vector as a control. The removal of the TM domain of Nix or CeBNip3 was found to disrupt interaction with BNip3 as determined by no growth on selection medium. The interactions are specific as there was no detectable growth on selection medium when BD vectors were co-transformed with empty vector, pACTII (Table 12).

4.3.2. BNip3 heterodimerizes with Bcl-x_S, a splicing variant of Bcl-x_L

Structural studies of Bak-BH3 peptide complexed to Bcl-x_L reveal the BH3-domain forms an α-helix that inserts into the hydrophobic pocket created by the BH1, BH2 and BH3 domains of Bcl-x_L (Muchmore *et al.*, 1996; Sattler *et al.*, 1997). Systematic mutational analysis has shown that mutations in the BH3 domain of the pro-apoptotic binding partner or in the BH1 or BH2 domains of Bcl-2/Bcl-x_L disrupt heterodimerization (Yin *et al.*, 1994; Sedlak *et al.*, 1995; Yang *et al.*, 1995; Cheng *et al.*, 1996; Sattler *et al.*, 1997), suggesting Bcl-2-related proteins require intact BH domains for interaction. To provide further evidence that the BH domains of Bcl-x_L are not

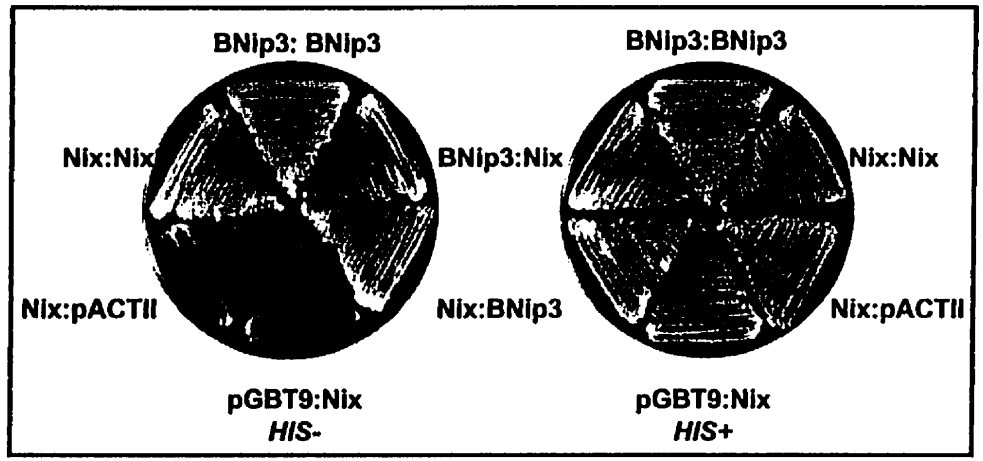

Figure 25. BNip3 heterodimerizes with its homologue, Nix. Yeast strain KGY37 was co-transformed with indicated GAL4 BD and AD expression vectors. BNip3 interacts with Nix as determined by growth on selection medium lacking tryptophan, leucine and histidine with 1 mM 3AT (*HIS-*). There was significantly reduced or no detectable growth when BNip3 or Nix were co-transformed with empty vector, pACTII indicating the specificity of observed interactions. All co-transformants grew on media lacking tryptophan and leucine (*HIS+*) (*right panel*). The co-transformations are labeled in the order, BD:AD vectors. All co-transformants grew on media lacking tryptophan and leucine (*HIS+*).

Table 12. Interaction of BNip3-related proteins in the yeast two-hybrid system

Transformation (BD:AD)	*HIS3* activation
BNip3:BNip3	+++
Nix:BNip3	+++
CeBNip3:BNip3	+++
BNip3ΔTM2:BNip3	-
NixΔTM:BNip3	-
CeBNip3ΔTM:BNip3	-
Nix:pACTII	-
ceBNip3:pACTII	-
BNip3ΔTM2:pACTII	-
NixΔTM:pACTII	-
CeBNip3ΔTM:pACTII	-
pGBT9:BNip3	-

Yeast strain KGY37 was co-transformed with indicated GAL4 binding domain (BD) and activating domain (AD) constructs. The relative level of activation for the *HIS3* reporter gene was determined by growth (+) or no growth (-) on selection medium lacking tryptophan, leucine and histidine in the presence of 1 mM 3-amino-1,2,4-triazole (3AT).

involved in BNip3 heterodimerization, BNip3 was co-transformed with Bcl-x_S into yeast strain KGY37. Bcl-x_S is a splicing variant of Bcl-x_L in which a stretch of 62 amino acids including the BH1 and BH2 domains is not present (Minn *et al.*, 1996). BNip3 was observed to interact strongly with Bcl-x_S as determined by growth on selection medium. There was no detectable growth on selection medium when Bcl-x_S was co-transformed with empty vector, pACTII (Table 13).

In the yeast two-hybrid system, Bcl-2/Bcl-x_L heterodimerization with BNip3 was mapped to its TM domain. To identify specific amino acids involved in heterodimerization, additional BNip3 TM domain mutants were co-transformed with Bcl-x_S (Figure 26). Bcl-x_S was used as the interacting partner, as opposed to Bcl-x_L, because results from these co-transformations were more consistent. The interactions summarized in Table 13 indicate amino acids 180-185 of BNip3 to be involved in TM domain mediated heterodimerization. Some of these residues are conserved among BNip3 family members (Figure 13). These observations were not confirmed by co-immunoprecipitation studies, nor, was a BNip3 mutant lacking both the NH_2-terminus and residues 180-185 constructed to test for interaction with Bcl-x by either yeast two-hybrid or co-immunoprecipitation assays.

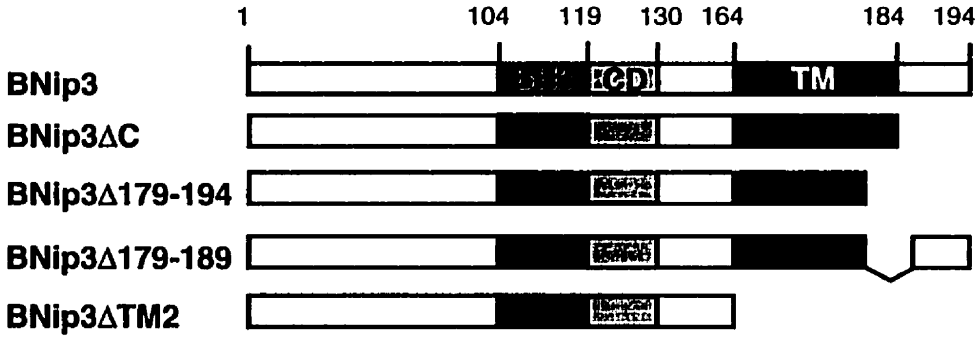

Figure 26. Schematic representation of BNip3 COOH-terminal mutants. Full length BNip3 and its mutants were cloned into yeast AD vector, pACTII to map interaction with Bcl-x_S, a splicing variant of Bcl-x_L cloned into BD vector, pGBT8.

Table 13. Mapping yeast-two hybrid interaction of BNip3 with Bcl-x_S, a splicing variant of Bcl-x_L

Constructs	
AD BNip3 mutants	**BD Bcl-x_S**
	***HIS3* activation**
BNip3	+++
BNip3ΔC	+++
BNip3ΔTM2	-
BNip3Δ179-194	-
BNip3Δ179-189	-
pACTII	-

Yeast strain KGY37 was co-transformed with indicated GAL4 binding domain (BD) and activating domain (AD) constructs. The relative level of activation for the *HIS3* reporter gene was determined by growth (+) or no growth (-) on selection medium lacking tryptophan, leucine and histidine in the presence of 1 mM 3-amino-1,2,4-triazole (3AT).

5. Summary

The work described herein delineates the regions within BNip3 that mediate homo- and heterodimeric interactions and their contribution to cell death function. Nix and CeBNip3 have been similarly characterized. The COOH-terminal TM domain of BNip3 homologues targets the protein to mitochondria and is necessary for cytotoxicity (Chen *et al.*, 1997; 1999; Cizeau *et al.*, 2000). The TM domain also mediates homodimerization of BNip3-related proteins. However, the strong homologous interaction observed for BNip3 is not required for its cytotoxic effect when transiently expressed. Furthermore, the substitution of the BNip3 TM domain with heterologous TM domain sequences disrupted homodimeric interaction, but did not significantly affect its cell death activity from mitochondrial and nonmitochondrial sites (Ray *et al.*, 2000).

BNip3 shares homology with the Bcl-2 family in the BH3 domain, a region that appears to be necessary for 'BH3-only' proteins to interact with anti-apoptotic Bcl-2 homologues and to regulate cell death. The removal of the BNip3 BH3-like domain did not disrupt its ability to heterodimerize with Bcl-2, Bcl-x_L or CED-9 nor diminish its ability to induce cell death even in the presence of Bcl-2 or Bcl-x_L. Similar observations were made with CeBNip3 which bound to CED-9 and induced cell death in the absence of its BH3-like domain (Cizeau *et al.*, 2000). Furthermore, the BH1 and BH2 domains of Bcl-x_L are dispensable for BNip3 heterodimerization, as BNip3 was found to interact with Bcl-x_S, a splicing variant of Bcl-x_L lacking these regions. In fact, the BNip3 NH_2-terminus and TM domain were identified to be critical for Bcl-2 heterodimerization, and either region sufficient for Bcl-x_L interaction. Similar mapping studies of CeBNip3 demonstrated its association with CED-9 and Bcl-x_L occurs exclusively through its TM domain. Thus, BNip3 and presumably its homologues function without a typical BH3 domain to regulate cell death by selective Bcl-2/Bcl-x_L interactions.

6. DISCUSSION

6.1. The BNip3 TM domain: mitochondrial targeting, homodimerization and cell death

Among Bcl-2 family members, the COOH-terminal TM domain is critical for targeting proteins to their correct subcellular site(s) and facilitating membrane association and/or integration essential to their function. The deletion of the Bcl-2 TM domain not only altered its distribution, but also dramatically reduced its anti-apoptotic activity (Hockenbery *et al.*, 1990; Alnemri *et al.*, 1992). Similarly, removal of the Bax TM domain prevented its targeting and integration into the mitochondrial membrane in response to an apoptotic signal (Wolter *et al.*, 1997). The function of both proteins could be restored by substituting heterologous mitochondrial targeting TM domain sequences (Nguyen *et al.*, 1994; Zhu *et al.*, 1996; Goping *et al.*, 1998; Lee *et al.*, 1999). Similar to Bcl-2 homologues, BNip3-related proteins have a COOH-terminal TM domain that targets the proteins to their correct subcellular sites and acts as a membrane anchor. Inspection of the amino acid sequence of BNip3, Nix and CeBNIP3 does not reveal any additional targeting sequences apart from the COOH-terminal TM domain. Similar to Bcl-2, the COOH TM domain is flanked on either side with positively charged amino acids (Nguyen *et al.* 1993; R. Ray and A.H. Greenberg, *unpublished observations*) (Figures 13 and 23). The expression of BNip3-related proteins appears in a punctate staining pattern, co-localizing with the mitochondrial matrix protein, HSP60 (Chen *et al.*, 1997, 1999; Cizeau *et al.*, 2000), the mitochondrial inner membrane specific dye, Mito-Tracker Red (Yasuda *et al.*, 1998b and 1999; Ohi *et al.*, 1999) or the inner mitochondrial membrane proton pump protein, F_0F_1-ATPase (Imazu *et al.*, 1999). The removal of the TM domain shifts the protein distribution from mitochondria to the cytoplasm (Chen *et al.*, 1997, 1999; Imazu *et al.*, 1999; Ohi *et al.*, 1999; Cizeau *et al.*, 2000). A small proportion of BNip3

lacking its TM domain co-localized with HSP60, perhaps through interaction with other mitochondrial-based proteins (G. Chen and A.H. Greenberg, *unpublished observations*).

Several Bcl-2 homologues form homodimers to regulate their capacity to suppress or promote cell death (Oltvai and Korsmeyer 1994; Chao and Korsmeyer 1998). The observations from the yeast two-hybrid and *in vitro* binding assays demonstrated that Bcl-2 forms homodimers through a head-to-tail association in which the BH4 domain of one monomer interacts with BH domains 1 and 2 of another monomer (Reed *et al.*, 1996). Mutations in any one of these three domains prevented the Bcl-2 mutant protein from forming homodimers, yet, endogenous Bcl-2 protein could still form mutant/wild type heterodimers (Hanada *et al.*, 1995). These mutants were also found to have lost their death-suppressing properties in mammalian cells (Borner *et al.*, 1994). In contrast, homodimerization of Bcl-x_L has not been observed in yeast and mammalian expression systems (Sato *et al.*, 1994; Sedlak *et al.*, 1995; Minn *et al.*, 1996; Muchmore *et al.*, 1996). A recent study suggests that structural constraints likely prevent Bcl-2 from forming homodimers, therefore, probably acting as a monomer form to protect cells from apoptosis (Conus *et al.*, 2000). Among the pro-apoptotic members, homodimeric interactions have been most systematically defined for Bax, which interacts homologously through its BH3 domain (Zha *et al.*, 1996a and 1996b; Wang *et al.*, 1998a). Specific BH3 domain mutants of Bax were identified which no longer formed homodimers, but retained their ability to induce cell death, implying that its pro-apoptotic function did not depend on homodimerization (Wang *et al.*, 1998a). In contrast, other studies reported that the oligomerization of Bax alone or through its interaction with Bid promoted mitochondrial integration and subsequent cell death. Therefore, Bax dimerization is suspected to be another mechanism through which the protein exerts its

cytotoxic effects (Gross *et al.*, 1998; Eskes *et al.*, 2000). To date, homodimeric interactions have not been observed among the 'BH3-only' class of Bcl-2 homologues.

The homodimerization of BNip3, as well as its homologues, Nix and CeBNip3 occurs exclusively through their TM domain, as determined by yeast two-hybrid analysis and resistance to reduction by SDS-PAGE. The TM domain is also critical for targeting BNip3-related proteins to the mitochondria and their cell death activity (Chen *et al.*, 1997, 1999; Cizeau *et al.*, 2000). The removal of the TM domain shifts the distribution of BNip3, Nix and CeBNip3 to the cytosol diminishing both their cytotoxicity and homologous interaction (Chen *et al.*, 1997, 1999; Cizeau *et al.*, 2000). The mutant BNip3ΔC which lacks the last ten amino acids of the protein was observed to interact homologously in the yeast two-hybrid assay. In contrast, specific BNip3 TM domain mutants characterized to date, including BNip3ΔC, were no longer able to form homodimers as determined by SDS-PAGE, yet localized to the mitochondria and induced cell death. Furthermore, the chimeric protein BNip3 fused to Bcl-2 TM domain did not form SDS-resistant homodimers, but localized primarily to the mitochondria and induced cell death as efficiently as wild type BNip3 (Ray *et al.*, 2000). Therefore, homologous interaction of BNip3-related proteins likely does not contribute to cell death function in mammalian cells.

The TM domains of BNip3 and Nix share 80% identity (Figure 13). Apart from localization to the mitochondria, Nix has also been detected in the ER and nuclear envelope (Ohi *et al.*, 1999). Based on the high degree of homology between BNip3 and Nix TM domain sequences, BNip3 may also be associated with these intracellular membranes in much smaller quantities. BNip3 has been reported to localize to the nuclear envelope when co-expressed with E1B 19K (Boyd *et al.*, 1994). The substitution

of heterologous TM domain sequences into BNip3 altered its protein distribution, yet it did not lead to loss of function. BNip3 expressed from mitochondrial and nonmitochondrial sites using the Bcl-2 TM domain induced cell death as efficiently as wild type BNip3, whereas cytochrome b_5 TM domain targeted BNip3 to the nonmitochondrial sites induced cell death to a slightly lesser degree (Ray *et al.*, 2000). These findings indicate that both BNip3 chimeric proteins are functional and the substitution of its TM domain does not result in the ablation of essential sequences. Therefore, the first 163 amino acids of BNip3 must be sufficient for its cytotoxic activity, provided it is fused to a TM domain that allows for membrane association and/or integration. The BNip3 TM domain sequence is necessary for its correct subcellular localization. It has also been found to be sufficient in targeting heterologous proteins to mitochondria (Yasuda *et al.*, 1998b). Interestingly, endogenous BNip3 is expressed highly in muscle tissue, remaining loosely associated with mitochondrial membranes as an unintegrated protein susceptible to alkali extraction. When overexpressed, BNip3 integrates into the mitochondrial membrane through its COOH-terminal TM domain and becomes resistant to alkali elution. The NH_2-terminus orients to the cytosol as confirmed by anti-BNip3 antibodies detecting the epitope spanning residues 112-124 (Vande Velde *et al.*, 2000). Therefore, the primary role of the BNip3 TM domain in addition to protein targeting appears to be association and integration into membranes in response to as yet, unidentified cell death signals.

6.2. The BNip3 BH3-like domain: heterodimerization and cell death

The susceptibility of cells to apoptotic signals is in part regulated by relative levels and competing interactions between death-suppressing and death-promoting Bcl-2 homologues (Oltvai and Korsmeyer 1994; Chao and Korsmeyer 1998). The BH3 domain of pro-apoptotic proteins typically plays a dual role by promoting

heterodimerization with cell death-repressors and inducing cell death. Among the majority of Bcl-2 pro-apoptotic proteins characterized to date, these two functions appear to be inseparable (Wang *et al.*, 1996b; Yang *et al.*, 1995; Inohara *et al.*, 1997a; Conradt and Horvitz 1998; Hedge *et al.*, 1998 O'Conner *et al.*, 1998). Apart from the COOH-terminal TM domain, BNip3-related proteins share limited sequence homology in the BH3 domain (Figure 13). The results described clearly indicate that the removal of the BH3-like domain in BNip3 does not disrupt Bcl-2/Bcl-x_L heterodimerization by both yeast two-hybrid or co-immunoprecipitation studies. Moreover, BNip3 lacking its BH3-like domain induced cell death almost as efficiently as wild type BNip3 in several different cell lines commonly used in cell death assays. Similarly, CeBNip3 lacking its BH3-like domain retained its ability to heterodimerize with CED-9 and Bcl-x_L and to induce cell death in several cell lines (Cizeau *et al.*, 2000). The heterodimeric interactions between BNip3 and Bcl-2/Bcl-x_L would suggest that Bcl-2/Bcl-x_L could suppress the death inducing activity of BNip3 and presumably Nix and CeBNip3. The overexpression of Bcl-2 or Bcl-x_L initially delays the onset of BNip3-, Nix- and CeBNip3-induced cell death, but does not completely block it except at very high expression levels (Chen *et al.*, 1997 and 1999; Yasuda *et al.*, 1999; Cizeau *et al.*, 2000). Taken together, these observations suggest that BNip3-related proteins do not heterodimerize or induce cell death similar to previously characterized 'BH3-only' Bcl-2 homologues.

Previous reports demonstrated the removal of the putative BH3 domain of BNip3 completely disrupted its ability to interact with Bcl-x_L and E1B 19K and partially reduced its cell death activity (Yasuda *et al.*, 1998b). Similarly, the removal of the Nix BH3 domain showed reduced efficiency in Bcl-2 heterodimerization and cell death function (Imazu *et al.*, 1999). Further studies demonstrated that the substitution of the BNip3 BH3-like domain into Bax recapitulated heterodimerization with Bcl-x_L and cell death activity

(Yasuda *et al.*, 1998b). As described, Bax contains BH domains 1 and 2 in addition to the BH3 domain which imposes conformational constrains on the protein allowing it to exist in two possible conformations, either with its BH3 domain 'buried' or 'exposed' in healthy or dying cells, respectively (Gross *et al.*, 1999; McDonnell *et al.*, 1999). In response to a cell death signal, Bax is subject to conformational changes at its NH_2-terminus (Goping *et al.*, 1998; Wood *et al.*, 2000) or through its interaction with Bid (Eskes *et al.*, 2000). Either or both event results in Bax being targeted to the mitochondria to become an integral membrane protein (Goping *et al.*, 1998; Eskes *et al.*, 2000; Wood *et al.*, 2000). Therefore, the BNip3 BH3-like domain substituted into Bax would be subject to conformational changes that normally do not affect its function. Moreover, systematic mutational analysis of the Bax BH3 domain demonstrated that mutations at some of the conserved sites did not disrupt heterodimerization with Bcl-x_L (Wang *et al.* 1998a). Thus, it is conceivable that the substitution of its BH3 domain from BNip3 would be tolerated, thereby, not significantly altering Bax function.

By inspection, several critical residues for Bak-Bcl-x_L heterodimerization are conserved among the BNip3 class of proteins (Figures 10 and 13). The core residues of the BH3 domain, leucine at position 1 and aspartic acid at position 6 define the putative BH3 domain in BNip3 and Nix. These residues are also conserved in CeBNip3 with the exception of two additional proline residues inserted between residues at position 1 and position 6. The residues flanking the core BH3 domain (valine and isoleucine) are also conserved in BNip3, Nix and CeBNip3. Additional residues, glycine and glutamic acid at positions 5 and 7, respectively, found in the 'BH3-only' proteins having a COOH-terminal TM domain are not conserved in BNip3. Moreover, secondary structural analysis of the BNip3 BH3-like domain reveals that it is not compatible with an amphipathic α-helix characteristic of typical BH3 domains (R. Ray and A.H. Greenberg, *unpublished*

observations). In order for the BNip3 BH3-like domain to resemble a typical BH3 domain, it is preferable to have a hydrophobic or serine residue, instead of an asparagine at position 4 (Gabriel Núñez, University of Michigan, Ann Arbor MI, *personal communication*). In CeBNip3, the insertion of two additional proline residues makes it unlikely that this region forms an α-helix. The substitution of one of the proline residues with a leucine would be more compatible to forming an α-helix (Cizeau *et al.*, 2000; Gabriel Núñez, *personal communication*).

Studies of class II pro-apoptotic Bcl-2 homologues, Mtd/Bok (Hsu *et al.*, 1997; Inohara *et al.*, 1998b) and Diva (Inohara *et al.*, 1998a) reveal that the BH3 domain of these proteins does not play its characteristic dual role in mediating heterodimerization and promoting apoptosis. Mtd/Bok interacts through its BH3 domain selectively with anti-apoptotic proteins, Mcl-1, Bfl-1 and BHRF-1 (Table 1). Yet, its BH3 domain is not required for pro-apoptotic activity as shown by deletion analysis and characterization of a splicing variant of Bok lacking this domain (Hsu and Youle 1997 and 1998; Inohara *et al.*, 1998b). Diva shows limited homology within the BH3 domain. It induces apoptosis and heterodimerizes with the viral orthologue of Bcl-2, KBcl-2 encoded by the Kaposi's sarcoma-associated herpesvirus in the absence of its BH3 (Inohara *et al.*, 1998a). Another group, characterized the identical protein, referred to as Boo and reported to act as a cell death suppressor and interacting preferentially with Bcl-x_L, Bak and Bik (Song *et al.*, 1999) (Table 1). The discrepancy in function of Diva/Boo is likely cell type dependent and signal specific. Systematic mutational analysis of another Bcl-2 homologue, Bik suggests that its BH3 domain is insufficient for heterodimerization and other flanking regions are likely involved (Elangovan and Chinnadurai 1997).

For BNip3, two other findings support the observation that heterodimerization does not occur through its BH3-like domain and the hydrophobic pocket created by BH domains 1, 2 and 3 of Bcl-2/Bcl-x_L. In the yeast two-hybrid assay, BNip3 exhibited strong interaction with the Bcl-x_s, a splicing variant, Bcl-x_L. In Bcl-x_S, a stretch of 62 amino acids including the BH1 and BH2 domains is not present (Minn *et al.*, 1996), therefore, excluding their involvement in forming BNip3-Bcl-x_L heterodimers. The heterodimerization of Bcl-x_S has been evaluated for only selected pro-apoptotic Bcl-2 homologues (Table 1). Although Bax and Bak are well known binding partners of Bcl-x_L, both are unable to interact with Bcl-x_S (Reed 1997), implying that the interaction depends upon the BH domains. Among the 'BH3-only' proteins co-expressed with Bcl-x_S, Bik heterodimerizes (Boyd *et al.*, 1995), whereas Hrk (Inohara *et al.*, 1997a) and Bad (Yang *et al.*, 1995) demonstrate no interaction. The lack of interaction between Bcl-x_S and 'BH3-only' proteins suggests that these proteins also rely on the BH domains of their heterodimerizing partner. Earlier studies by Boyd *et al.* (1994) mapped two regions within Bcl-2 based on homology to E1B 19K required for heterodimerization with BNip3. The first segment is found within the loop region of Bcl-2 spans amino acids 42 to 48 in which Ala^{43} and Pro^{44} are conserved with Bcl-x_L/Bcl-x_S. The loop region of Bcl-2/Bcl-x_L is not present in CED-9. The second region of Bcl-2 (residues 106 to 115) immediately follows the BH3 domain and precedes the third α-helical region in which Arg^{106}, Tyr^{107}, Arg^{108}, Arg^{109} and Phe^{111} are conserved with Bcl-x_L /Bcl-x_S and Glu^{113} and Met^{114} are similar to Asp^{107} and Leu^{108} of Bcl-x_L/Bcl-x_S, respectively. In the second interaction site, Lys^{127} and Phe^{132} of CED-9 are conserved with Arg^{106} and Phe^{111} of Bcl-2. Taken together, the BH domains are not involved in heterodimeric interactions between BNip3 and Bcl-2/Bcl-x_L.

6.3. A role for the BNip3 NH_2-terminus and TM domain in Bcl-2/Bcl-x_L heterodimerization

Further deletion mapping studies of BNip3 elucidated two regions of interaction with Bcl-2 and Bcl-x_L, specifically, the NH_2-terminus (residues 1-49) and the TM domain. In the *in vitro* co-immunoprecipitation assay, removal of the BNip3 TM domain or substitution with heterologous TM domain sequences did not interfere with Bcl-2 heterodimerization. Yet, when BNip3 lacking its TM domain was co-expressed with Bcl-2 in 293T embryonic kidney cells, an interaction between these two proteins was not detected. Similarly, BNip3 substituted with a heterologous TM domain did not interact with Bcl-2 when transiently co-expressed in 293T cells and immunoprecipitated (R. Ray and A.H. Greenberg, *unpublished observations*). Therefore, the interaction through the BNip3 TM domain with Bcl-2 may facilitate the NH_2-terminal interaction when the proteins are associated with membranes, but occurs independently of the TM domain *in vitro*. In contrast, either the NH_2-terminus or TM domain of BNip3 interacts with Bcl-x_L. The observation that both BNip3 fused to heterologous TM domain sequences from Bcl-2 or cytochrome b_5 interacts with Bcl-2 as well as Bcl-x_L implies that the substitution of its TM domain does not lead to protein misfolding. Therefore, BNip3 expressed from various subcellular sites must be accessible to a common set of cellular components that can interact with and/or modify the protein. CeBNip3 was observed to interact with CED-4 (Cizeau *et. al.*, 2000) and reportedly binds simultaneously to CED-9 and the CED-3 precursor to form a ternary complex (Yasuda *et al.*, 1998a). Bap31, an ER pro-apoptotic protein interacts with Bcl-2 through its NH_2-terminal TM domain and COOH-terminal sequences (Ng *et al.*, 1997; Ng and Shore 1998). It also interacts with a CED-4-like protein and latent caspase 8 (Ng *et al.*, 1997). Although, the functional significance of these complexes has not been fully elucidated, it could serve as a

possible mechanism to sequester and alter the distribution of apoptotic regulatory proteins until an appropriate signal triggers their lethal effects.

6.4. Limitations of protein-protein interaction assays

It is important to note that to date, all the biochemical studies and structural determination of Bcl-2 family members involve proteins lacking their TM domain. The targeting function of the COOH-terminal TM domain is well characterized for many Bcl-2 homologues, yet its contribution to the overall structure of the protein and to either intra- or intermolecular interactions remains limited. For example, indirect evidence from Bax using specific antibodies to detect NH_2-terminal epitopes reveals that its TM domain masks the NH_2-terminus acting as a regulatory mechanism in healthy cells. The dimerization of Bcl-2 family members is typically assayed in solution. For technical reasons, the GST (glutathione-*S*-transferase) fusion proteins used for *in vitro* binding assays generally lack their TM domain. In the preparation of GST fusion proteins, the TM domain is removed to facilitate isolation and purification. When, expressed in bacterial cells, proteins containing long stretches of hydrophobic amino acids tend to accumulate in inclusion bodies and cannot be isolated (Haldar *et al.*, 1994). Even proteins transiently expressed in mammalian cells for co-immunoprecipitation studies lack their TM domain. In the yeast two-hybrid assay, the TM domain is generally removed to prevent targeting to intracellular membranes and facilitate targeting to the nucleus to activate transcription of reporter genes by interaction of the bait protein and its potential binding partner. The studies of Bcl-2 family interactions *in vitro* and *in vivo* lacking the TM domain may in fact preclude interactions that are mediated by the membrane anchoring sequence. In fact, TM domain integration and/or membrane association may influence the conformation of the remainder of the protein, thereby promoting or preventing potential interactions. Other proteins may interact within the

vicinity of the TM domain, presumably blocking membrane association and/or integration. In the case of Bax, it retains its ability to interact with Bcl-2/Bcl-x_L in membranes, despite deletions or mutations in the BH3 domain (Reed 1998). This indicates that for some members of the Bcl-2 family, other regions of the protein likely contribute to heterodimerization apart from the BH3 domain. Although the BNip3-related proteins bear some resemblance in this region and other studies support a role for its BH3-like domain in heterodimerization and cell death (Yasuda *et al.*, 1998a, 1998b and 1999). The results described herein clearly exclude the putative BH3 domain and demonstrate a role for the BNip3 as well as CeBNip3 TM domain in mediating interaction with Bcl-2/Bcl-x_L.

In this study, the results from BNip3 deletion mapping for homo- and heterodimeric interactions by yeast two-hybrid and co-immunoprecipitation assays utilized full length proteins with intact TM domains. With some exceptions, the results using the two approaches to assay for protein–protein interaction were identical. For instance, interactions of BNip3 or CeBNip3 mediated by the TM domain and not the BH3-like domain with Bcl-2, Bcl-x_L or CED-9 were detected in both yeast and mammalian expression systems. Similarly exclusion of the conserved domain in heterodimerization was demonstrated by both methods. Some exceptions were noted in detecting BNip3 NH_2-terminal interactions. For example, BNip3 lacking its TM domain co-immunoprecipitated with Bcl-2 in the *in vitro* assay, but was not detectable by co-immunoprecipitation of solubilized, transfected mammalian cells. Even the interaction of BNip3 fused to heterologous TM domain sequences with Bcl-2 was consistently observed by *in vitro*, but not by *in vivo* co-immunoprecipitation assays. *In vitro*, the BNip3(Δ1-49) mutant with an intact TM domain did not heterodimerize with Bcl-2, confirming the interaction requires the extreme NH_2-terminus. These differences in

interaction suggest the conformation of either or both proteins must differ when assayed in solution in either the membrane bound or unbound states. In mammalian cells, BNip3 lacking its TM domain is unable to interact with Bcl-2 likely due to conformational changes and/or sequestration by another unidentified cellular protein. These limitations were not encountered when BNip3 lacking its TM domain was assayed *in vitro* using purified proteins. In the yeast two-hybrid assay, the NH_2-terminal interaction of BNip3 with Bcl-2 or even Bcl-x_L was not detected. The BNip3 constructs were all NH_2-terminally fused either to the binding or activating domain of yeast expression vectors, possibly rendering the Bnip3 NH_2-terminus inaccessible for Bcl-2/Bcl-x_L interaction.

6.5. NH_2-terminal degradation of BNip3-related proteins by the proteosome

For many Bcl-2-related proteins, the NH_2-terminus is an important regulatory region to keep the molecule in a closed, inactive conformation until activated by a death stimuli. During apoptosis, both cell death antagonists and agonists are modified by proteolytic processing to either attenuate or enhance their function. For example, the cleavage of Bcl-2/Bcl-x_L probably serves as an amplification mechanism by diminishing its anti-apoptotic effects and disrupting its ability to block cytochrome *c* and AIF release from mitochondria. The NH_2-terminal changes of Bid and Bax appears to remove an inhibitory function allowing mitochondrial membrane integration and promoting mitochondrial dysfunction (Goping *et al.*, 1998; Li *et al.*, 1998; Luo *et al.*, 1998; Wood *et al.*, 2000). Similarly, the removal of the NH_2-terminus of Bim which keeps it bound to the cytoskeleton and away from the mitochondria converts it into a more potent killer (Puthalakath *et al.*, 1999).

The NH_2-terminal region of BNip3-related proteins contains PEST sequences. Characteristically, these regions are defined by frequent stretches of proline (P), glutamic

acid (E), serine (S), threonine (T) and aspartic acid (D) flanked by charged amino acids such as histidine, arginine or lysine. The PEST sequences are associated with proteins that have a high turnover rate and whose degradation is controlled by the proteosome (Rogers *et al.* 1986). Transient expression of BNip3, Nix or CeBNip3 reach peak levels between 12 to 24 hours, followed by progressive degradation over time that does not correlate with increased cell death. The TM domain mutants also follow a similar pattern of expression and degradation. (Chen *et al.*, 1997; 1999; Cizeau *et al.*, 2000). It appears the degradation step is dependent on an active proteosome. Proteolysis of BNip3 begins at its NH_2-terminus, yielding a final product of 11 kDa predicted to be a homodimer by SDS-PAGE. In the presence of lactacystin, a *Streptomyces* product that binds and inhibits proteosome threonine protease activity (Fenteany *et al.*, 1998), transiently expressed BNip3-related proteins accumulate over time. Further experiments in an ubiquination defective cell line resulting from a mutation in the E1 enzyme necessary to ubiquinate proteins marked for degradation, revealed that BNip3-related proteins are not only degraded through a ubiquination-dependent step, but also require an active proteosome (Chen *et al.*, 1999; Cizeau *et al.*, 2000). The involvement of the NH_2-terminus in BNip3 heterodimerization suggests that its removal would interfere with its ability to bind Bcl-2 and to a much lesser extent Bcl-x_L. The 11 kDa fragment of BNip3 includes the TM domain, thus retaining its ability to heterodimerize with Bcl-x_L and induce cell death unlike BNip3 lacking its TM domain. Although the significance of this rapid degradation remains to be elucidated in relation to cell death, the post-translational control of BNip3 expression through rapid protein degradation may constitute a mechanism for regulating intracellular levels of a potentially lethal protein. Furthermore, not only does the degradation of the NH_2-terminus disrupt interaction with Bcl-2/Bcl-x_L, it may induce

conformational changes and/or disrupt interaction with other regulatory proteins to accelerate the cell death process.

6.6. BNip3 induces cell death from mitochondrial and nonmitochondrial sites

In healthy cells, many of the pro-apoptotic Bcl-2 homologues either exist in an inactive conformation through intramolecular interactions or sequestered by other proteins. The redistribution of these proteins from the cytosol to the mitochondria is a useful strategy to modulate their distribution and interaction with cell death suppressors at the mitochondria. Following an apoptotic signal, post-translational modifications such as dephosphorylation, dimerization or proteolytic cleavage activate the protein whereby the protein becomes an integral membrane protein and antagonize Bcl-2/Bcl-x_L to initiate apoptotic events. In healthy cells, Bax resides in the cytosol in an inactive conformation. Following a death signal, Bax becomes membrane integrated and cannot be eluted by alkali extraction similar to BNip3. The integration event for Bax is suspected to be initiated as a result of conformational change of an NH_2-terminal inhibitory region (Goping *et al.*, 1998; Wood *et al.*, 2000) or through its interaction with pro-apoptotic Bid (Eskes *et al.*, 2000). Endogenous BNip3 remains in an inactive, unintegrated state, yet its overexpression in mammalian cells leads to its integration and subsequent cell death. Therefore, apart from heterodimeric interactions, it remains to be determined whether post-translation modifications alter the state of endogenous, unintegrated BNip3 and allow for TM domain mediated integration. Alternatively, an unidentified interacting protein could maintain BNip3 loosely associated with the mitochondria until a signal removes the inhibitory protein.

In some apoptotic signaling pathways, mitochondria play a central role whereby the release of intermitochondrial proteins such as cytochrome *c* (Liu *et al.*, 1996) and

AIF (Susin *et al.*, 1999b) as well as the disruption of mitochondrial membrane permeability (Kroemer *et al.*, 1997) lead to caspase activation and disassembly of the cell. Alternate pathways, proceed in the absence of released intermitochondrial proteins and caspase activation (Kitanaka and Kuchino 1999). Apart from the mitochondria, apoptotic regulatory proteins also reside on the ER. The role of the ER in apoptosis is less well defined. The ER stores intracellular calcium (Carafoli 1987) and its release reportedly triggers apoptosis (Shibasaki and McKeon 1995). Furthermore, a recent study demonstrated that there are numerous close contacts between mitochondria and ER, and opening of gated channels on the ER exposes the mitochondria to higher levels of calcium (Rizzuto *et al.*, 1998). Bcl-2 can act at the mitochondria by blocking the release of cytochrome *c* and suppressing changes in mitochondrial membrane permeability (Zamzami *et al.*, 1998; Green and Reed 1998). At the ER, Bcl-2 can regulate the release of calcium from intracellular stores (Kuo *et al.*, 1998; He *et al.*, 1997). BNip3 expressed from mitochondrial and nonmitochondrial sites using heterologous targeting sequences induced cell death, suggesting its cytotoxic effect can be localized to different spatial areas within the cell (Ray *et al.*, 2000). When a cell receives a death signal, proteins such as Bap31 at the ER and BNip3 primarily at the mitochondria may exert a co-operative effect from different membrane sites by forming complexes with surrounding proteins including Bcl-2 family members and those in the cytosol. Alternatively, these proteins may respond differently to various signals, but still play an important role in sequestering Bcl-2-related proteins, CED-4/Apaf-1-like molecules, caspases and other regulators of cell death to mitochondrial and non-mitochondrial sites.

6.7. Defining a new class of proteins: the BNip3 family
6.7.1. The conservation of cell death genes

Evolutionary comparison of genes among organisms helps to identify sequences that play an important role in protein structure or gene regulation and highlight those that have been retained unaltered over long periods of time. These comparisons reveal that the number of distinct genes required to template an organism does not differ significantly from organism to organism (Lander and Weinberg 2000). The human genome consists of 3 billion bases of DNA, of which 95% does not code for proteins or regulatory information. The remaining 5% is predicted to encode approximately 100 000 genes. In comparison, the *C.elegans* genome is considerably smaller, comprised of 97 million bases of DNA containing approximately 19 000 protein-coding genes, with more than 40% of the predicted protein products in *C. elegans* having significant matches in other organisms (The *C. elegans* Consortium, 1998). Therefore, despite, the differences in genome size and complexity, the identification of Bcl-2 and Bid orthologues in *C. elegans*, undoubtedly sets the precedence that these are functionally important genes, as proven by extensive biochemical and physiological investigations of these proteins in cell death (Veis *et al.*, 1995; Kelekar and Thompson 1998; Gross *et al.*, 1999; Yin *et al.*, 1999). The fact that mammalian BNip3 has a counterpart in *C. elegans* suggests that these proteins must also contribute significantly to the developing and mature organism.

Often the genes encoding early proteins have multiplied and diversified over the course of evolution. This gives rise to large families of related genes and proteins with diverse functions, such as the antagonists and agonists of the Bcl-2 family. The recognition of gene families produces enormous synergy in the characterization of new genes, as the function of one member can often be deduced from that of its known relatives (Lander and Weinberg 2000). All the BNip3 homologues studied to date have

several structural elements in common, such as the PEST sequences, a BH3-like domain and TM domain. Furthermore, BNip3, Nix and CeBNip3 all contain a stretch of 8 identical amino acids (Figure 13). Although the region is evolutionarily conserved from *C. elegans* to mammals, it has no apparent role in heterodimerizing with known BNip3 binding partners or in promoting cell death as determined by mutational analysis (Chen *et al.*, 1997; 1999; Cizeau *et al.*, 2000; Ray *et al.*, 2000). The region likely contributes to some unidentified function, acting as a regulatory domain, site for post-translational modification and/or site for protein interaction. Preliminary mapping studies localize BNip3 to chromosome 10 and a possible pseudogene to chromosome 14 (D. Dubik and A.H. Greenberg, *unpublished observations*). Nix maps proximal to the short arm of chromosome 8, specifically band p21 (8p21) (Matsushima *et al.*, 1998). Another BNip3 homologue appears to map to chromosome 21 (J. Cizeau and A.H. Greenberg, *unpublished observations*). Further, examination of the expressed sequenced tagged (EST) database reveals conserved sequences to both BNip3 and Nix suggesting that the family may be even larger and not limited to the two mammalian genes currently characterized.

6.7.2. Preferential binding partners

The complexity of the mammalian organism is reflected in the vast number of Bcl-2 homologues being identified and characterized in comparison to the *C. elegans* orthologues, CED-9 and EGL-1. The existence of multiple Bcl-2 family members in mammalians is likely due to the need to regulate cell death in a tissue-specific manner thereby allowing cells to rapidly alter their apoptotic threshold in response to changing environmental signals. Consistent with this, both Bcl-2 antagonists and agonists exhibit difference in tissue distribution, developmental expression and inducibility in response to cell death cues. In part, the function of these proteins is regulated by heterodimerization

between Bcl-2 homologues of opposing function (Oltvai and Korsmeyer 1994; Chao and Korsmeyer 1998). Yet, biochemical studies demonstrate that all antagonists do not simply bind all agonists (Table 1). Rather, it appears the death promoters may preferentially target subsets of death-repressors. For example, the Bcl-2 homologue, Mtd/Bok only binds to death-repressors Mcl-1 and BHRF1 but not to Bcl-2, Bcl-x_L or Bcl-w (Table 1) (Inohara *et al.*, 1998b). Similarly, Diva only binds to the viral Bcl-2 homologue encoded by Kaposi's sarcoma-associated herpesevirus (Table 1) (Inohara *et al.*, 1998a). Both of these proteins are expressed primarily in the granulosa cells of the ovary and in the testis. Bax is also expressed in germ cells, but exhibits a different pattern of expression and interacts with a broader range of cell death suppressors (Table 1) (Knudson *et al.*, 1995). Thus, particular cell types depend upon different pro-apoptotic Bcl-2 homologues to antagonize anti-apoptotic Bcl-2 family members during oogenesis and spermatogenesis, suggesting that each pro-apoptotic member likely has a preferred anti-apoptotic binding partner expressed in a tissue-specific manner. It is conceivable that similar scenarios and preferential interactions of Bcl-2 homologues occur in other tissues. BNip3 heterodimerizes with Bcl-2, Bcl-x_L and CED-9 as well as E1B 19K (Boyd *et al.*, 1994). Nix is also reported to demonstrate similar interactions (Imazu *et al.*, 1999; Ohi *et al.*, 1999; Yasuda *et al.*, 1999). In certain human tissues, BNip3 and Nix are differentially expressed (Chen *et al.*, 1997; Yasuda *et al.*, 1999), thus contributing to cell death with some degree of specificity and selectivity. Therefore, BNip3-related proteins likely play different roles in development and cellular homeostasis either independently or through heterodimeric interactions with other BNip3 homologues and/or the Bcl-2 class of proteins.

6.7.3. Splicing variants: genes encoding their own antagonists and agonists

Interestingly, several members of the Bcl-2 family encode their own antagonists. For example, the Bcl-x gene encodes anti-apoptotic Bcl-x_L and a splicing variant that yields its agonist, the 'BH3-only' protein, Bcl-x_S (Boise *et al.*, 1993). Similarly, translation of the Mcl-1 gene results in a protein product that promotes survival whereas exon skipping leads to a 'BH3-only' cell death-inducing protein product (Bingle *et al.*, 2000). Unspliced transcripts of Bcl-2 and Bcl-x_L lacking their COOH-terminal TM domain have been detected, although their function remains uncertain (Tsujimoto and Croce 1986; Gonzalez-Garcia *et al.*, 1994). Among pro-apoptotic Bcl-2 proteins, Bim has three isoforms, Bim_{EL}, Bim_L and Bim_S. These products of alternative splicing are progressively shorter in length from the NH_2-terminus. Bim_S is the most potent death promoter, whereas the other two might serve to negatively regulate the ability of the proteins to promote death (O'Conner *et al.*, 1998). Although the splicing variants of these proteins are probably differentially expressed in various tissues types, the fact that a cell can potentially express both a death antagonist and its agonist from the same gene suggests another level of regulation for the Bcl-2 class of proteins.

The knowledge of the intron/exon boundaries and regulatory elements that flank BNip3-related genes will help to elucidate if sequences for BNip3 or Nix also harbour splicing variants. Determination of the genomic organization for BNip3 and Nix is currently underway (D. Dubik and A.H. Greenberg, *unpublished observations*). The BNip3 homologues characterized to date appear to induce cell death. It is conceivable that the BNip3 class of proteins, by analogy to the Bcl-2 family may be comprised of binding partners that prevent cell death in addition to those that promote cell death. These proteins may be encoded as BNip3 splicing variants similar to the gene encoding Bcl-x or Bim. Although such findings would be of interest, it can not be excluded that

proteins suppressing BNip3 function may map to other regions of the genome. One report suggested that Nix found on chromosome 8 functions to inhibit BNip3 induced cell death (Ohi *et al.*, 1999), however, this observation contradicts other reports (Chen *et al.*, 1999; Yasuda *et al.*, 1999).

6.7.4. The classification of BNip3: an apoptotic versus a necrotic protein

Initially, BNip3 was thought to induce an apoptotic cell death response when overexpressed in mammalian cells (Chen *et al.*, 1997). This assertion was based on the observation of condensed nuclei in BNip3 transfected cells, one of the hallmarks of apoptotic cells. The structural elements of BNip3, such as a BH3-like domain and COOH-terminal TM domain, further suggested that BNip3 functions similar to the pro-apoptotic 'BH3-only' class of Bcl-2 homologues. Yet, the studies described herein, clearly demonstrate that the BNip3 BH3-like domain is not responsible for either heterodimeric or cell death function, two properties attributed to the BH3 domain of the 'BH3-only' proteins. Despite the initial similarities, these findings suggest that the BH3-like domain of BNip3 and presumably Nix and CeBNip3 may have lost its original function, evolving alternate mechanism(s) to exert their cytotoxic effects. A closer examination of BNip3-induced cell death to include other indicators of an apoptotic death response such as caspase activation, intact plasma membrane integrity and redistribution of intramitochondrial proteins suggested the cytotoxic effects of BNip3 proceeded through an alternative mechanism. The absence of both caspase activation and release of intermitochondrial proteins such as cytochrome *c* and AIF, coupled with observable early loss of plasma membrane integrity, changes in mitochondrial membrane permeability and ROS production resembled a necrotic rather than an apoptotic cell death (Vande Velde *et al.*, 2000).

The mode of BNip3-Bcl-2/Bcl-x_L heterodimerization and cytotoxicity sets the precedence for defining a new class of cell death-inducing proteins. This class would also include two other Bcl-2 homologues, Bax (Xiang *et al.*, 1996) and Mtd/Bok (Inohara *et al.*, 1998b). Typically, the BH3 domain of Bax heterodimerizes with Bcl-2/Bcl-x_L and promotes apoptosis. Yet, selected Bax BH3 domain mutations not only disrupt Bcl-2/Bcl-x_L interaction, but retain cell death activity. Moreover, in the presence of peptide caspase inhibitors, Bax-induced cell death resembled a necrotic rather than an apoptotic cell death. Similarly, characterization of Mtd/Bok revealed its BH3 domain is dispensable for both heterodimerization and cell death function. Furthermore, the cytotoxicity of Mtd/Bok was not inhibited by peptide caspase inhibitors and CrmA (Inohara *et al.*, 1998b). Other similarities among BNip3 homologues (Chen *et al.*, 1997; 1999; Cizeau *et al.*, 2000 Ray *et al.*, 2000), Mtd/Bok (Inohara *et al.*, 1998b) and Bax (Gross *et al.*, 1998) include their ability to overcome Bcl-2/Bcl-x_L suppression as well as induce DNA fragmentation in dying cells (Inohara *et al.*, 1998b; Vande Velde *et al.*, 2000). Taken together, it appears the BNip3 class of proteins favours a necrotic cell death. And proteins such as Bax and perhaps to a lesser extent Mtd/Bok have retained two mechanisms, initiating either an apoptotic or necrotic demise in response to environmental cues.

6.8. Future studies

Often the function of a gene is not obvious even after comprehensive study of its structure and expression patterns. In such cases, it may be advantageous to identify other interacting proteins, especially if those turn out to be ones that have been previously studied and whose functions are known. In part, this has been explored for BNip3 homologues based on their structural resemblance to the 'BH3-only' class of Bcl-2-related proteins. The yeast two-hybrid system and co-immunoprecipitation studies were used to

exclude the BNip3 class of proteins as homologues of Bcl-2 agonists and to delineate regions of BNip3 critical for homo- and heterodimerization. The activity of BNip3 does not occur in isolation and must be influenced by other interacting proteins apart from Bcl-2/Bcl-x_L to form an interconnected dynamic network within the cell. To gain further insight, the yeast two-hybrid system is a useful strategy employed to identify additional BNip3 binding partners and provide hints to potential mechanism(s) of action through which BNip3 exerts its cytotoxic effects. The mitochondrial localization of BNip3 and subsequent mitochondrial damage initiated by BNip3 overexpression suggests that mitochondrial associated and/or integrated proteins would be of particular interest. Briefly, in the yeast two-hybrid system, the protein of interest or bait, such as BNip3 is co-expressed with proteins from a tissue-specific cDNA library. Those proteins that physically interact with BNip3 are detected by their ability to activate transcription of reporter genes. Once a positive interacting partner is identified by selection on appropriate media, the cDNA containing plasmid is isolated from yeast transformants and sequenced to determine its identity. The resulting sequence data is compared against sequences available from public databases. A match with a previously cloned and characterized gene provides a connection between BNip3 and existing pathways or suggests other possible roles of BNip3 in regulating cell function. The interaction identified in the yeast two-hybrid system must be confirmed by co-immunoprecipitation studies in mammalian cells. Then, potential BNip3-interacting proteins can be overexpressed or suppressed by antisense expression to determine its effect on BNip3 cell death function.

Present molecular and biochemical techniques helped to systematically define the structural elements of BNip3 and their contribution to cell death function using yeast and mammalian expression systems. However, structure-function data do not provide

insight into how expression of a particular protein contributes to the whole organism and its role in various differentiated cell types that make up the organism. Two strategies to study the role of a particular protein in the developing and the mature organism include disruption of gene expression either in the germline by targeted homologous recombination or through the use of antisense technology. Presently, characterization of the BNip3 gene structure is under way (D. Dubik and A.H. Greenberg, *unpublished observations*). Once completed, appropriate targeting vectors can be constructed to produce and breed mice that do not express BNip3 to evaluate the development and cellular homeostasis of BNip3 deficient mice. In *C. elegans*, CeBNip3 mRNA is abundantly expressed at the embryonic stage, when 113 of 131 somatic cell deaths occur. The transcripts of two other cell death genes, ced-4 and ced-3 are also expressed at this stage (Yuan *et al.*, 1992 and 1993). To determine the effects of the absence of CeBNip3 on nematode development, CeBNip3 deficient nematode can be created using anti-sense technology. Similar studies can be carried out in nematode strains expressing low levels or deficient in CED-3 or CED-4 to determine if CeBNip3 acts along the same cell death execution pathway. Taken together, the identification of new BNip3-interacting proteins and an understanding of its physiological role will provide further insight into mechanism(s) utilized by BNip3-related proteins in maintaining normal cell function and initiating its demise.

6.9. Conclusion

In conclusion, the BNip3 class of proteins was predicted to belong to the 'BH3-only' branch of the Bcl-2 family. The present study demonstrates that BNip3 differs from many of the previously characterized 'BH3-only' Bcl-2 homologues, by its ability to heterodimerize with Bcl-2, Bcl-x_L and CED-9 and induce cell death through a mechanism that does not rely on its BH3-like domain. Rather, the cytotoxicity of BNip3

depends on its membrane association and integration at either mitochondrial or nonmitochondrial sites and selective interaction with Bcl-2/Bcl-x_L through an NH_2-terminal domain (Ray *et al.,* 2000). Furthermore, recent findings indicate the cell death pathway initiated by BNip3, unlike BH3-only proteins, is independent of caspase activation and release of AIF and cytochrome c. BNip3 expression triggers mitochondrial dysfunction through opening of the PT pores and damages the plasma membrane leading to necrotic cell death (Vande Velde *et al.,* 2000). The heterodimerizing and cytotoxic properties of BNip3 and presumably Nix and CeBNip3 do not resemble the Bcl-2 proteins characterized to date, with the exception of Bax and Mtd/Bok. Thus, BNip3-related proteins represent a new class of mitochondrial proteins involved in a necrotic cell death pathway activated in a cell-type, signal specific manner.

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