Interaction Between the Mitochondrial Protein BNIP3 and Caspase-2 Enhances Cell Death and DNA Fragmentation

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LIST OF ABBREVIATIONS

A	alanine
Ac	acetyl
AIF	apoptosis-inducing factor
AMC	amino-methyl coumarin
Apaf-1	apoptotic protease activating factor-1
ARC	apoptosis repressor with CARD
ATA	aurinetricarboxylic acid
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma/leukemia-2
BH1-4	Bcl-2 homology domains 1-4
Bid	BH3-interacting domain death agonist
BNIP3	Bcl-2/nineteen kD-interacting protein-1
BSA	bovine serum albumin
C	cysteine
C. elegans	*Caenorhabditis elegans*
CARD	caspase recruitment domain
CARD4	caspase recruitment domain-containing protein-4
CARDIAK	CARD-containing ICE-associated kinase
ceBNIP3	*C. elegans* BNIP3
ced/CED	cell death abnormal
co-IP	co-immunoprecipitation
COOH	carboxy
CRADD	caspase and RIP adaptor protein with a death domain
D	aspartate
dATP/ATP	deoxyadenosine triphosphate
DD	death domain
DED	death effector domain
DFF	DNA fragmentation factor
DISC	death inducing signaling complex
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
egl-1/EGL-1	egg-laying abnormal-1
FADD	Fas-associated protein with a death domain
fmk	fluoro-methyl ketone
G	glycine
ICAD/CAD	inhibitor of caspase-activated deoxyribonuclease
ICE	interleukin-1β-converting enzyme
Ich-1	ICE and CED-3 homologue-1
IL-1β	interleukin-1β
MEFs	mouse embryonic fibroblasts
Nedd2	neural precursor cell-expressed developmentally down-regulated gene 2

NH_2	amino
NIX	BNIP3-like protein X
PBS	phosphate-buffered saline
PSB	protein sample buffer
PT	permeability transition
Q	glutamine
R	arginine
RAIDD	RIP-associated Ich-1-homologous protein with a death domain
RIP	receptor-interacting protein
ROS	reactive oxygen species
S	serine
SDS	sodium dodecyl sulfate
tBid	truncated Bid
TM	transmembrane
TNF(R)	tumor necrosis factor (receptor)
TRADD	TNFR-associated protein with a death domain
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
V	valine
W	tryptophan
X	any amino acid
z	benzyloxycarbonyl
$\Delta\psi_m$	mitochondrial membrane potential

ABSTRACT

BNIP3 is a cell death-inducing mitochondrial protein that is part of a Bcl-2 subfamily with NIX and ceBNIP3. BNIP3-induced cell death morphologically resembles necrosis that is characterized by rapid plasma membrane damage and mitochondrial dysfunction in the early stages, followed by DNA fragmentation and chromatin condensation characteristic of apoptotic cell death in the later stages. DNA fragmentation during most types of apoptosis is predominantly due to caspase-3 activation. However, BNIP3-induced cell death is independent of caspase-3 activity, thus the mechanism of BNIP3-induced DNA fragmentation remains unknown. Co-immunoprecipitation analysis revealed an interaction between BNIP3, NIX, and caspase-2. The interaction is unique in that it is not mediated through the caspase-2 prodomain. Caspases-1, -8, and –9, which have homologous prodomains to caspase-2, do not bind BNIP3 or NIX, indicating specificity for caspase-2. Further structural analysis indicated that the transmembrane domain and NH_2-terminus of BNIP3, which mediate binding to Bcl-2 and Bcl-X_L, are not required for binding caspase-2. An *in vitro* caspase assay detected activation of endogenous caspase-2 in BNIP3-transfected cells. This activation corresponded with enhanced cell death and DNA fragmentation in cells co-expressing BNIP3 and caspase-2. Furthermore, both a broad-spectrum caspase inhibitor and a caspase-2-specific inhibitor blocked BNIP3-induced DNA fragmentation *in vivo*, and cleavage of a caspase-2-specific substrate *in vitro*, indicating a role for caspase-2 in BNIP3-induced cell death. Taken together, our results suggest that BNIP3 induces DNA fragmentation and subsequent cell death through a novel mechanism involving the specific recruitment and activation of caspase-2.

INTRODUCTION

Apoptosis, or programmed cell death, is an evolutionarily conserved, genetically regulated form of cell suicide essential for development and maintaining homeostasis in multicellular organisms (99). Apoptosis is important for the elimination of cells that are in excess, misplaced, nonfunctional, or self-reactive, as well as for cells that have sustained genetic damage or have been infected by virus (42). Hence, the dsyregulation of apoptosis contributes to the pathogenesis of a variety of human diseases (91). The failure of cells to induce apoptosis has been associated with cancer, autoimmune diseases, and viral infections. In contrast, excessive apoptosis has been associated with neurodegenerative disorders, AIDS, aplastic anemia, Alzheimer's disease, and osteoporosis. Apoptosis was originally classified by Kerr et. al. (44) as an active and intrinsic cell death pathway distinct from necrosis. Apoptosis is triggered by a variety of extrinsic and intrinsic signals which include growth factor withdrawal, TNFα, Fas ligand, loss of matrix attachment, heat shock, viral infection, chemotherapeutic drugs, radiation, hypoxia, DNA damage, oncogenes, and tumor suppressors (75,100). Necrosis on the other hand is generally considered to be an acute and passive form of cell death triggered by environmental insults that cause physical cellular injury (45). Necrotic cell death is associated with an early loss of plasma membrane integrity, allowing the leakage of cytoplasmic contents, rapid cell swelling, and lysis, resulting in the induction of an inflammatory response (45). In contrast, apoptotic cell death is characterized by distinct morphological features including an intact plasma membrane, chromatin condensation, DNA fragmentation, cytoskeleton disruption, cell shrinkage, and plasma membrane

blebbing resulting in the formation of apoptotic bodies that are rapidly phagocytosed by macrophages, thus avoiding an inflammatory response (106).

Apoptosis in the nematode *Caenorhabditis elegans*

The first evidence for the existence of a genetically controlled cell death pathway came from studying the development of the nematode *Caenorhabditis elegans*. During *C. elegans* development, 1090 somatic nuclei are generated from a fertilized egg by an essentially invariant pattern of cell divisions (34). Precisely 131 cells rapidly undergo programmed cell death to generate the 959 somatic nuclei of the adult hermaphrodite (35). Genetic analysis of programmed cell death during development in *C. elegans* has identified several core components of the *C. elegans* death machinery that establish a paradigm for cell death in all multicellular organisms. Three genes define the killing or execution step of programmed cell death; *ced-3* (cell death abnormal), *ced-4*, and *ced-9* (35). CED-4 and CED-3 are required for initiation and execution of the cell death pathway, respectively (24), while CED-9 functions to protect cells from cell death (32).

Several mutations have been identified that alter the cell death process in all 131 cells, indicating that all programmed cell deaths that occur during *C. elegans* development involve the same mechanism. Mutations that result in a reduction or loss of function in either *ced-3* or *ced-4* cause cells that normally die to survive (24). However, neither mutation results in embryonic lethality, nor do the adult nematodes have any obvious deleterious effects on behavior, although they may have up to 20% more neural cells than wild-type nematodes (24). In contrast, a *ced-9* gain-of-function mutation prevents normal cell death, whereas a *ced-9* loss-of-function mutation causes cells that normally live to undergo programmed cell death and results in embryonic lethality (32).

Furthermore, mutations in either *ced-3* or *ced-4* completely suppress all defects observed in *ced-9* loss-of-function mutants (32). Thus, in order for *ced-9* activity to have an effect, *ced-3* and *ced-4* must also be functional, hence *ced-9* functions upstream as a negative regulator by antagonizing the activities of *ced-3* and *ced-4* (32,35). Agonist and antagonist effects of these three proteins are mediated through protein-protein interactions. CED-4 binds to and activates CED-3, while CED-9 can bind CED-4 forming an inactive ternary complex that inhibits CED-3 activation (13,105). Recently, a new *C. elegans* protein has been identified called EGL-1 (egg-laying abnormal-1) (19). Like the loss-of-function mutations in *ced-3* and *ced-4*, loss-of-function mutations in *egl-1* cause all 131 cells that normally die to instead survive (19). EGL-1 induces cell death by binding CED-9, and consequently disrupting the association between CED-9 and CED-4, allowing CED-4 to activate of CED-3 (21). A model for the activation of programmed cell death in *C. elegans* evolved from the genetic and molecular data described above. Upon activation by upstream signals, EGL-1 physically interacts with CED-9, thereby releasing CED-4 from the CED-9/CED-4 complex. Free CED-4 can then promote activation of CED-3, which induces cell death by activating a discrete set of proteins that function to kill cells, and by inactivating other proteins that function to protect cells from death (34).

The molecular cell death pathway delineated in *C. elegans* is highly conserved in mammalian cells. The mammalian counterparts of the *C. elegans* death genes have evolved into large gene families. The mammalian interleukin-1β-converting enzyme (ICE) was the first CED-3 homologue to be identified (116), which subsequently grew into a family of cysteinyl aspartate-specific proteases, or caspases (4). Apaf-1 (apoptotic

protease activating factor-1) and the recently identified CARD4 (caspase recruitment domain-containing protein-4) constitute a new mammalian family homologous to CED-4 (7,119). The mammalian homologue of CED-9 is Bcl-2 (33), which serves as the prototype for a family of anti- and pro-apoptotic members that also includes EGL-1 homologues (19).

The Caspases

ICE is a cysteine protease that cleaves the inactive IL-1β precursor at aspartate residue 116 to release the mature IL-1β peptide, a cytokine involved in a wide range of biological responses including inflammation and hematopoiesis (92). ICE and CED-3 share 29% identity overall, and 43% within a 115 amino acid region that includes a completely conserved pentapeptide QACRQ, which contains the active site cysteine residue essential for ICE proteolytic activity (92,116). Mutations altering the pentapeptide sequence abolished CED-3 function, indicating for the first time that CED-3, like ICE, functions as a cysteine protease in executing cell death in *C. elegans* (108,116). This discovery lead to the identification of a family of cysteinyl aspartate-specific proteases now known as caspases (4), which function in inflammation and apoptosis (69,93). The caspase family thus far consists of 14 members that are divided into two subfamilies based on their homology to either ICE or CED-3 (2,69,93). The ICE subfamily, consisting of caspases-1, -4, -5, -11, -12, and –13, functions primarily in inflammation, whereas the CED-3 subfamily, consisting of the remaining caspases, functions in apoptosis (69,93). The CED-3 subfamily is further subdivided into initiator, or upstream caspases (caspases-2, -8, -9, and –10) and effector caspases (caspases-3, -6, -7, -14) that typically function further downstream in the pathway to execute cell death

(93). Downstream caspases are responsible for the cleavage of a discrete fraction of cytoplasmic and nuclear substrates, which ultimately lead to the systematic disassembly of the cell and characteristic apoptotic morphology (23). The net effect of caspase substrate cleavage is to: (i) arrest cell cycle progression, (ii) disable homeostatic and repair mechanisms, (iii) detach the cell from surrounding tissue, (iv) disassemble cytoskeletal and nuclear structural components, and (v) mark the cell for phagocytosis (70).

Caspase Structure

Caspases are expressed as inactive precursors consisting of three domains: an NH_2-terminal prodomain, a large subunit (~20kD), and a small subunit (~10kD) (93). The initiator caspases are structurally distinct from the effector caspases in that they have a significantly longer prodomain that is important for activation, as discussed below. Conversion of the caspase precursor to the mature protease requires two cleavage events at specific aspartate residues found within caspase consensus sequences at the domain junctions. Processing occurs in an ordered fashion, with cleavage between the small and large subunits preceding removal of the prodomain (23). The free large and small subunits associate to form a heterodimer with one catalytic domain in which both subunits contribute residues necessary for substrate specificity, binding, and catalysis (69,93). The crystal structures of mature caspase-1 and caspase-3 reveal a tetrameric conformation, consisting of two heterodimers and two independent catalytic sites (79,101). The catalytic site recognizes a very short tetrapeptide sequence in target substrates. All caspases have an absolute requirement for aspartate in P_1, are flexible with P_2, prefer glutamate in P_3, and are further subdivided into three groups based on their

amino acid preference in P_4: (i) a hydrophobic residue (caspases-1, -4, -5, and –13), (ii) aspartate (caspases-2, -3, -7, and CED-3), (iii) an aliphatic residue (caspases-6, -8, -9, and –10) (94). Caspase-2 is unique in that it also appears to have a requirement for valine in P_5 (89). Given that caspases are among the most specific of proteases, with an absolute requirement for cleavage after an aspartate residue, it serves to reason that these enzymes are activated either autocatalytically or in a cascade by other caspases.

Caspase Activation

There are three distinct pathways for caspase activation: (1) trans-activation, (2) autoactivation, and (3) recruitment-activation (69). Trans-activation is the primary mechanism for activation of effector procaspases by other upstream caspases, resulting in a proteolytic caspase cascade (69). Typically, upstream initiator caspases (e.g. caspase-8 or-9) cleave and activate downstream effector caspases (e.g. caspases-3 or –7), which subsequently activate other downstream caspases and may in turn initiate a feedback amplification loop (84).

Autocatalytic activation has been observed by overexpression for most procaspases, although there is no definitive evidence that it occurs in the absence of adaptor molecules under physiological conditions (69). For example, upon overexpression, caspase-2 homodimerization mediated by the prodomain is sufficient for autocatalytic processing (9). In addition, procaspase-3 can be converted to an autoactivating caspase by fusion to the caspase-2 prodomain (18). Under physiological conditions, adaptor molecules are likely required to promote autocatalytic activation of initiator procaspases by recruitment and oligomerization.

Recruitment through adaptor molecules brings procaspases into close proximity enabling oligomerization and inducing auto-catalytic processing and activation, a process described as the induced-proximity model (81), also known as scaffold-mediated activation (23). The interaction of adaptor molecules and procaspases is mediated through homophilic interactions between specific domains collectively known as caspase recruitment domains (CARDs), which reside in the caspase prodomain and the corresponding adaptor molecule (50). Oligomerization mediated by CARDs has been observed for the activation of CED-3, caspase-1, caspase-2, caspase-8, and caspase-9, with their corresponding adaptor proteins, CED-4 (110), CARDIAK (90), RAIDD (22), FADD (67), and Apaf-1 (85), respectively. The exact mechanism of activation is unknown, but likely involves intrinsic proteolytic activity that resides in the caspase zymogen (81). For example, the zymogenicity, the ratio of the activity of a processed protease to the activity of the zymogen on any given substrate, of procaspase-3 is greater than 10,000, thus the zymogen is completely inactive compared to the activated caspase-3. In contrast, the initiator procaspases-8 and –9 have zymogenicity ratios of 100 and 10, respectively, thus the zymogens have much more intrinsic activity than compared to procaspase-3 that can be utilized for autocatalytic processing (81). There are two main, non-exclusive, pathways described for the activation of initiator caspases by the induced-proximity model: (i) the death receptor pathway, and (ii) cytochrome *c*/Apaf-1 pathway.

(i) The Death Receptor Pathway

Death receptors belong to the tumor necrosis factor receptor (TNFR) family, and are cell surface receptors consisting of cysteine-rich extracellular domains and homologous cytoplasmic sequences called death domains (DDs) that transmit an

apoptotic signal upon binding of a death ligand (5). Death receptors activate apical caspases, primarily caspase-8 and to a lesser extent caspase-10, within seconds of ligand binding, inducing a swift apoptotic demise of the cell (5). The best characterized death receptors include TNFR1 (also called p55 or CD120a), and Fas (also called Apo1 or CD95), and their corresponding death ligands, TNF, and Fas ligand (FasL or CD95L), respectively (5). Activation of TNFR1 by TNF is complex in that ligand binding typically activates cell survival pathways mediated by NF-κB, but can also induce apoptosis in certain cell types, although only when protein synthesis is impaired (5). In contrast, activation of Fas by either FasL, or receptor agonist antibodies, rapidly transduces an apoptotic signal through the formation of the DISC (death inducing signaling complex), which comprises Fas, the adaptor molecule FADD (Fas-associated protein with death domain), and procaspase-8 (67). FasL binding induces clustering of the Fas receptors, allowing for recruitment of FADD via homologous DD sequences (14). In turn, FADD recruits procaspase-8 via homologous death effector domains (DEDs), a specific type of CARD found in both FADD and the prodomain of caspase-8 (67). FADD mediated oligomerization promotes autocatalytic processing and activation of caspase-8, which subsequently activates downstream effector caspases committing the cell to apoptosis (67).

FADD is also recruited to TNFR1 through homologous interaction with the DD in the adaptor protein TRADD (TNFR-associated protein with death domain), and thus transduces apoptotic signals from ligand-bound TNFR1 (15,36). Similarly, TNFR1 can recruit RAIDD (RIP-associated Ich-1-homologous protein with a death domain), also known as CRADD (caspase and RIP adaptor with death domain), through TRADD and

another adaptor protein called RIP (receptor-interacting protein) (3,22). Although there is no direct evidence, RAIDD can recruit caspase-2 and may induce autocatalytic activation in a similar fashion as FADD and caspase-8 (3,22). However, a dominant negative FADD mutant effectively blocks TNF- and FasL-induced apoptosis, indicating an obligatory role for FADD in both cytokine-mediated pathways of caspase activation (36).

The importance of caspase-8 during development is evident in mouse embryos with targeted disruption of caspase-8 that die at about day E11 due to impaired heart development and abdominal hemorrhage (98). Embryonic fibroblasts derived from caspase-8 $^{-/-}$ mice are resistant to apoptosis induced by TNFR1 and Fas, but remain sensitive to other apoptotic triggers such as treatment with dezamethosone, etoposide, staurosporine, or serum-starvation (98). Thus, while caspase-8 is the primary initiator caspase activated by the death receptors, there exists an alternative pathway for caspase activation in response to other apoptotic stimuli.

(ii) The Cytochrome *c*/Apaf-1 Pathway

Another example of induced-proximity activation of initiator caspases is found in the cytochrome *c*/Apaf-1 pathway. Earlier studies utilizing cell-free extracts resulted in the identification of cytochrome *c* as an essential cofactor required for triggering activation of caspase-3 upon addition of dATP (56). Newly translated apocytochrome *c* exists in the cytosol and has no apoptosis promoting activity. However, upon its translocation into the mitochondrial intermembrane space, a heme group is attached forming holocytochrome *c*. Treatment of isolated intact mitochondria with staurosporine, a broad-spectrum protein kinase inhibitor and potent apoptosis-inducing agent, induces the release of holocytochrome *c* into the cytosol (56). Subsequently, caspase-9, and a new

protein called Apaf-1, were identified as the other two factors necessary for caspase-3 activation *in vitro* (7). Apaf-1 consists of three distinctive domains: (i) an NH_2-terminal CARD that exclusively binds to the prodomain of procaspase-9, (ii) a 320 amino acid sequence with extensive homology to CED-4 that includes a putative ATPase domain with Walker's A and B motifs required for nucleotide binding, and (iii) 12 WD-40 repeats at the COOH-terminus that are known to mediate protein-protein interactions (7).

Multiple extrinsic and intrinsic apoptotic stimuli that do not activate death receptors, instead act directly on the mitochondria, inducing the release of cytochrome *c*. Once released into the cytosol, cytochrome *c* along with dATP bind to and induce a conformational change in Apaf-1, which allows it to recruit and oligomerize procaspase-9 molecules resulting in autocatalytic activation (55,85). Active caspase-9 trans-activates caspase-3 thereby initiating a proteolytic caspase cascade. dATP/ATP hydrolysis is essential for cytochrome *c* binding to Apaf-1, Apaf-1 self-association, interaction of Apaf-1 and procaspase-9, and ultimately for caspase-9 activation (37). The WD-40 repeats function to negatively regulate Apaf-1 function by interacting with the ATPase-like domain thereby preventing dATP/ATP hydrolysis and maintaining Apaf-1 in an inactive conformation (39). A truncated Apaf-1 lacking the WD-40 repeats is constitutively active independently of cytochrome *c* and dATP (39). A three-step reaction culminates in the formation of a multimeric complex called the apoptosome that activates caspase-9 (120). Initially, dATP/ATP binds to Apaf-1 and is hydrolyzed to dADP/ADP, which fuels the binding of cytochrome *c* and the multimerization of Apaf-1/cytochrome *c* complexes to form the apoptosome (120). Procaspase-9 is recruited to the apoptosome in a 1:1 molar ratio to Apaf-1 and becomes activated through autocatalytic processing (120).

The active caspase-9 is released from the complex, allowing it to cleave caspase-3 and new procaspase-9 to enter the complex (120). However, an alternative mechanism has been suggested in which Apaf-1 and active caspase-9 remain in a complex and function as a holoenzyme where caspase-9 is the catalytic subunit and Apaf-1 is the allosteric regulator (78). This is supported by the observation that caspase-9 activity is at least 1000-fold greater when complexed with Apaf-1 than compared to the free caspase (78,86).

The importance of Apaf-1 as an apoptosis activator is evident in Apaf-1-deficient mice, which develop severe craniofacial malformations, brain hyperplasia, and abnormal eye development, resulting in embryonic lethality at day 16.5 (10,115). Similarly, targeted disruption of either caspase-9 or caspase-3 results in perinatal lethality due to severe brain abnormalities (48,49). Although a loss-of-function mutation in CED-3 is not lethal, the adult nematode does exhibit superfluous neuronal cells (24), indicating an evolutionarily conserved function for these caspases. The similarities in the phenotypes of Apaf-1 $^{-/-}$, caspase-9 $^{-/-}$, and caspase-3 $^{-/-}$ mice supports a model in which the pro-apoptotic activity of these proteins is dependent on one another. Cells from Apaf-1- or caspase-9-deficient mice have impaired activation of caspase-3, and exhibit resistance to a variety of apoptotic stimuli including chemotherapeutic drugs, dexamethosone, and γ-irradiation even though cytochrome *c* is still released (10,30,48,115). However, Apaf-1- and caspase-9-deficient cells remain susceptible to Fas-mediated killing, indicating that the two pathways can function independently (10,30,48,115).

Caspase-2

Caspase-2 (Ich-1/Nedd2) is an obscure member of the caspase family given that its role in apoptosis has remained somewhat elusive and ambiguous. The mouse caspase-2 homologue, Nedd2 (neural precursor cell-expressed developmentally down-regulated gene 2), was first to be discovered as an mRNA expressed during early embryonic brain development and down-regulated in the adult brain (51,52). Using the *Nedd2* cDNA as a probe, the human caspase-2 homologue was identified as Ich-1 (ICE and CED-3 homologue-1), based on 27 % and 28% identity with caspase-1 (ICE) and CED-3, respectively (104). Given that Ich-1 was the second mammalian homologue of this family to be discovered it was re-classified as caspase-2 (4).

Caspase-2 mRNA is alternatively spliced into long (Ich-1_L) and short (Ich-1_S) isoforms in a tissue-specific manner (104). Expression of both caspase-2 isoforms can be detected in heart, kidney, and embryonic and adult brain, with expression of the short isoform being highest in the embryonic brain, and only the long isoform expressed in the adult thymus (104). In Rat-1 cells, overexpression of caspase-2 (Ich-1_L) induces apoptosis that is inhibited by Bcl-2, while overexpression of the short isoform (Ich-1_S) prevents apoptosis induced by serum deprivation (104). Mice deficient in caspase-2 are endowed with excess oocytes in the ovaries, which are resistant to doxorubicin-induced apoptosis (6). B lymphoblasts from caspase-2-deficient mice are resistant to apoptosis mediated by granzyme B and perforin, but are equally sensitive to apoptosis induced by anti-Fas, doxorubicin, etoposide, γ-irradiation, and staurosporine as compared to wild type cells (6,54). Although activation of caspase-2 has been observed as an early response to all of these factors (31), the sensitivity of the knockouts suggests that these

agents can induce cell death through alternate pathways that do not require caspase-2 activation. In contrast, facial motor neurons in caspase-2 knockout mice exhibit accelerated apoptosis during development (6). In addition, sympathetic neurons from caspase-2-deficient mice are more sensitive to nerve growth factor (NGF) withdrawal than wild type neurons (6). Although the molecular basis for these developmental abnormalities is entirely unknown, it may in part be due to the tissue specificity of alternative splicing of caspase-2 that yields the long pro-apoptotic isoform or short anti-apoptotic isoform (104).

The subcellular location of caspase-2 is also varied. Endogenous procaspase-2 has been identified within the intermembrane space of isolated mouse liver mitochondria along with procaspase-9 and procaspase-3 (87,61). In response to multiple apoptotic stimuli, procaspase-2 and –9 redistribute from the mitochondria to the cytosol and are processed into the active protease in a variety of cell lines (87). However, when overexpressed, caspase-2 displays a predominantly prodomain-dependent nuclear location with minimal cytoplasmic distribution (17). Recently, Mancini et. al. confirmed a nuclear localization for endogenous caspase-2, as well as a significant fraction present in the Golgi complex of HeLa cells (60).

Structurally, caspase-2 is classified as an initiator caspase based on its long prodomain that is highly homologous to the caspase-9 prodomain (93). However, caspase-2 has also been classified as an effector caspase based on the observation that cleavage and activation of caspase-2 is a caspase-3-dependent event both *in vitro* and *in vivo* (54,84). Caspase-2 represents a unique scenario in that its activation *in vivo* has been observed by three nonexclusive mechanisms: (i) autocatalytic processing by RAIDD-

mediated oligomerization (22), (ii) autocatalytic processing by prodomain-dependent homodimerization (9), and (iii) trans-activation by caspase-3-dependent cleavage (54,84). Processing of caspase-2 *in vitro* has also been attributed to caspase-8 and to a lesser extent caspase-7, while the active caspase-2 is only capable of processing its own precursor and no other known procaspases (96). Hence, caspase-2, whether activated upstream or downstream, may not function to initiate a wide proteolytic caspase cascade. However, it is plausible that caspase-2 can function both upstream as an initiator caspase, as well as functioning downstream as a caspase-3 substrate perhaps as part of a feedback amplification loop, or in response to other stimuli that do not directly activate caspase-2. Recently, the first caspase-2 substrate *in vivo* was identified as golgin-160, an autoantigen of the Golgi complex (60). Caspase-2 cleaves golgin-160 at a unique site not utilized by caspase-3 or caspase-7, which cleave at other shared sites (60). The presence of a caspase substrate in the Golgi complex suggests the potential for a novel apoptotic pathway, generated and transduced from the Golgi complex (60).

Caspase-2 is known to physically interact with only two other proteins, RAIDD also known as CRADD (3,22), and ARC (apoptosis repressor with CARD) (47). Both interactions are mediated through homophilic binding to the caspase-2 prodomain. RAIDD has a dual-domain structure consisting of an NH_2-terminal CARD that mediates binding exclusively to the prodomain of caspase-2 and CED-3, and a COOH-terminal DD that interacts specifically with the DD in RIP (3,22). In the presence of RIP, RAIDD is able to complex with the TNFR-1 signaling complex and simultaneously recruit caspase-2 in a similar fashion to the FADD mediated recruitment of caspase-8 (22). Overexpression of RAIDD induces cell death that is blocked by broad-spectrum caspase

inhibitors, CrmA and Ac-zVAD.fmk, as well as by a catalytically inactive caspase-2, presumably by acting as a dominant negative inhibitor of endogenous caspase-2 (22). Although there is no direct evidence, it does suggest that RAIDD can function as an adaptor molecule by recruiting caspase-2 to the TNFR-1 signaling complex via RIP, and thereby establishing an alternative, albeit redundant, link to the activation of downstream effector caspases. However, putative dominant-negative versions of RAIDD do not block TNFR-1-induced cell death, further supporting the observation that the FADD-caspase-8 pathway predominates (22). In addition, since caspase-2 does not activate any other known caspases to date, RAIDD-induced apoptotic cell death may be executed exclusively by caspase-2, or may involve other unidentified caspases (3). ARC also contains an NH_2-terminal CARD with significant homology to the CARDs found within Apaf-1 and RAIDD (47). ARC interacts with caspase-2, caspase-8, and CED-3 via their prodomains, but not with caspase-1, -3, or -9 (47). Overexpression of ARC effectively inhibits apoptosis induced by caspase-8, CED-3, FADD, and TRADD, but not caspase-9 (47). The effect of ARC on caspase-2 and RAIDD-induced apoptosis has not been established, although the evidence suggests that ARC may function similarly with caspase-2 and RAIDD as it does with caspase-8 and FADD. The mechanism by which ARC inhibits apoptosis remains unclear, but may be due to the disruption of caspase interaction with their respective adaptor proteins, thereby preventing proximity-induced autocatalytic activation (47).

The Bcl-2 Family

The Bcl-2 family of proteins plays an important role in regulating apoptotic pathways, both to inhibit and promote cell death. The *bcl-2* (B-cell lymphoma/leukemia-

2) gene was first discovered at the breakpoint of a t(14:18) chromosomal translocation in B-cell malignancies as a novel proto-oncogene that promoted cell survival by blocking apoptosis rather than promoting cell proliferation (95). Early studies revealed that the overexpression of Bcl-2 significantly prolonged the survival of a variety of cells subjected to a variety of conditions that usually lead to cell death, for example, (i) lymphokine deprivation from factor-dependent hematopoietic cells, (ii) glucocorticoid treatment of thymocytes, (iii) γ-irradiation of thymocytes, and (iv) withdrawal of nerve growth factor from fetal sympathetic neurons (77). The wide range of circumstances in which Bcl-2 functions suggests that it controls a distal step at which different signaling pathways converge into a common pathway for executing cell death (75). Subsequently, the Bcl-2 protein was found to be structurally and functionally homologous to CED-9. Overexpression of Bcl-2 blocks normal programmed cell death in *C. elegans*, and partially rescues CED-9 loss-of-function mutants, indicating an evolutionary conserved mechanism that allows Bcl-2 to substitute for Ced-9 (33).

Anti- and Pro-apoptotic Bcl-2 Subfamilies

Additional Bcl-2 homologues have been identified based on their ability to heterodimerize with Bcl-2 and on their sequence similarity with one or more of the four Bcl-2 homology (BH1 to BH4) domains, resulting in the formation of a large family including both anti-apoptotic proteins (eg. Bcl-2, Bcl-X_L, Mcl-1, Bcl-W, and CED-9), and pro-apoptotic proteins (eg. Bax, Bak, Bim, Bid, Bad, Bok, BNIP3, NIX, and EGL-1) (1,77). All of the anti-apoptotic subfamily members contain at least the BH1 and BH2 domains, while those most similar to Bcl-2 have all four domains (1,77). In contrast, most of the pro-apoptotic members lack the BH4 domain, and are further subdivided into the

BH3-only subfamily (eg. Bad, Bid, Bim, BNIP3, NIX, EGL-1), and Bax-like subfamily which contain BH1, BH2, and BH3 (Bax, Bak, and Bok) (1,77). Most Bcl-2 family members also share a conserved transmembrane (TM) domain that localizes these proteins to the outer mitochondrial membrane, endoplasmic reticulum, and nuclear envelope (1,77).

Mechanisms of Action of Anti- and Pro-apoptotic Bcl-2 Proteins

(i) Homodimerization and Heterodimerization

The BH1, BH2, and BH3 domains are involved in mediating homo- and heterodimerizations between anti- and pro-apoptotic proteins. The pro-apoptotic activity of Bax (Bcl-2 associated X protein) is dependent on homodimerization mediated by its BH3 domains (102,114). In viable cells, inactive Bax is predominantly monomeric and loosely associated with the mitochondria (27). Following a death stimulus, Bax forms homodimers that integrate into the mitochondrial outer membrane resulting in the release of cytochrome *c* and subsequent caspase activation (27). Bax pro-apoptotic activity can be neutralized by heterodimerization with Bcl-2 or Bcl-X_L (72). The heterodimerization of Bcl-2/Bcl-X_L with Bax requires the BH1 and BH2 domains of Bcl-2/Bcl-X_L, and the BH3 domain of Bax (114,117). The three dimensional structure of Bcl-X_L revealed that the BH1, BH2, and BH3 domains are in close spatial proximity and form a hydrophobic pocket to which the amphipathic α-helix of the Bax BH3 domain can bind (66). The heterodimerization between Bcl-2 and Bax, and the observation that Bax overexpression can overcome Bcl-2 repression, suggested a model in which the ratio of anti-apoptotic to pro-apoptotic proteins determined cell survival or death (72). If sufficient Bcl-2/Bcl-X_L is present in the cell to bind and effectively neutralize all of the Bax, the cell will be

protected against cell death. In turn, insufficient amounts of Bcl-2/Bcl-X_L will not be able to sequester Bax in an inactive heterodimer, hence Bax can form active homodimers that induce cell death. This model was further supported by the observation that deletion of the BH1 and BH2 domains in Bcl-2 abolished both anti-apoptotic activity and heterodimerization with Bax (114). However, deletion of the BH4 domain abrogates Bcl-2 activity, but does not interfere with Bax binding (40). Similarly, BH3 mutants of Bax have been identified that retain their pro-apoptotic activity, but no longer bind Bcl-2 (102). Thus, alternative mechanisms exist for the function of Bcl-2 and Bax that may involve heterodimerization, but are more complex than simple neutralization.

(ii) Displacement

In *C. elegans* CED-9 binds to CED-4 thereby displacing it from the CED-4/CED-3 complex and inhibiting CED-3 activation (13). A similar mechanism was proposed for the action of Bcl-2/Bcl-X_L on the Apaf-1/caspase-9 interaction. Co-immunoprecipitation analysis revealed that Bcl-X_L binds Apaf-1, thereby blocking the association of Apaf-1 with caspase-9 (38). In addition, recombinant Bcl-X_L blocked Apaf-1-mediated processing of caspase-9 in a cell-free system (38). However, recent studies have provided conflicting evidence to suggest that Bcl-2/Bcl-X_L do not function in this fashion. Moriishi et. al. (65) showed that under physiological conditions none of the anti-apoptotic Bcl-2 family members interacted with Apaf-1, and suggested that Bcl-2/Bcl-X_L acted indirectly on Apaf-1 to inhibit its activity. In contrast, Newmeyer et. al. (68) showed that although Bcl-X_L did associate with Apaf-1, it could not prevent caspase activation upon the addition of cytochrome *c* to a cell free system. Although the displacement model is valid

for the function of CED-9 in the *C. elegans'* cell death pathway, the mammalian homologues appear to have a more complex function.

(iii) Cleavage and Translocation

Bid (BH3 interacting domain death agonist) is a BH3-only member of the Bcl-2 family that is known to interact with both Bcl-2 and Bax through its BH3 domain (103). In viable cells, Bid exists in an inactive conformation in the cytosol. Upon activation of cell surface death receptors, Bid is cleaved by caspase-8, resulting in the release of a COOH-terminal fragment, called tBid (truncated Bid), that translocates to the mitochondria and integrates into the outer membrane triggering the release of cytochrome *c* (53). Interestingly, in the presence of Bcl-2 and Bcl-X_L, death ligand-induced cleavage, translocation, and integration of tBid into the mitochondrial outer membrane and cell death still occur, but the release of cytochrome *c* is blocked (29). Thus, while Bid is required for death receptor-induced cytochrome *c* release, it is not essential for cell death since caspase-8 can directly cleave downstream effector caspases.

(iv) Formation of Ion Channels or Pores

A well-established role for Bcl-2/Bcl-X_L-mediated inhibition of apoptosis is in blocking the release of cytochrome *c* from the mitochondrial intermembrane space, thus preventing formation of the apoptosome and caspase activation. Overexpression of Bcl-2/Bcl-X_L prevents cytochrome *c* release from the mitochondria in response to multiple death-inducing stimuli (46,109), while Bax overexpression has been shown to promote cytochrome *c* release (43). The mechanism by which Bcl-2/Bcl-X_L and Bax regulate cytochrome *c* release has been based on the three-dimensional structure of Bcl-X_L. The structure of Bcl-X_L exhibits strong similarity to the pore-forming domains of certain

bacterial toxins that dimerize and form transmembrane channels, suggesting that Bcl-2 family proteins may be capable of forming pores in the cytoplasmic membranes to which they localize (66). Bcl-2, Bcl-X_L, Bax, and tBid all form ion channels in synthetic membranes *in vitro* (76,82,83). Furthermore, Bcl-2 can block the formation of Bax channels in liposomes (76). Thus, it is possible that Bax creates pores in the mitochondrial outer membrane that allow for release of cytochrome *c* (43). In turn, Bcl-2 heterodimerization with Bax may prevent Bax channel formation, or alternatively may form a heteromeric channel that is not a conduit for cytochrome *c* (76). Although there is no evidence of channel formation *in vivo*, the *in vitro* studies suggest that the Bcl-2 proteins may participate in mitochondrial permeability transition, an event that involves opening of a large pore at the junction of the inner and outer membranes (20). Opening of the permeability transition (PT) pore occurs almost universally during apoptosis resulting in, (i) the dissipation of the mitochondrial membrane potential ($\Delta\psi_m$), (ii) generation of reactive oxygen species (ROS), (iii) swelling and rupture of the outer membrane, and (iv) release of mitochondrial intermembrane space proteins that may include cytochrome *c*, AIF (apoptosis inducing factor), and procaspases (20). Bcl-2 inhibits PT pore opening, whereas Bax induces it, possibly by directly interacting with pore components (88,107).

The BNIP3 Subfamily

BNIP3 (Bcl-2/nineteen kD-interacting protein-1; formerly NIP3) (12) forms a Bcl-2 subfamily of death-inducing mitochondrial proteins that includes the mammalian homologue NIX (BNIP3-like protein X; also called BNIP3α/BNIP3L/B5) (11,62,71,112) and the *C. elegans* homologue ceBNIP3 (111) (16). BNIP3 was isolated from a yeast two-hybrid screen using the adenovirus E1B 19kD protein as bait, and was subsequently

found to also interact with Bcl-2 (8). NIX and ceBNIP3 share 56% and 21% identity with BNIP3, respectively (11,111). All three family members share similar structure and function including, (i) a NH_2-terminal PEST sequence that labels these proteins for degradation by the proteosome (11,16), (ii) a region with limited sequence homology to the BH3 domain of Bcl-2, hence their classification as BH3-only members of the Bcl-2 family (16,28,41,74,113), (iii) a highly conserved domain of 19 amino acids with unknown function (16,16,74), and (iv) a COOH-terminal TM domain that is essential for mitochondrial localization, homodimerization, and pro-apoptotic activity (11,12,16,113). It is only at the NH_2-terminal where the sequences exhibit distinct differences (11,111). In particular, NIX has a significantly longer NH_2-terminus that contains five sequential asparagine residues and is rich in proline (11).

Heterodimerizations with Bcl-2, Bcl-X_L, and CED-9 are also conserved within the BNIP3 subfamily (8,16,74,112). Typically the BH3 domain of pro-apoptotic Bcl-2 proteins mediates heterodimerization with Bcl-2/Bcl-X_L, however BNIP3 and ceBNIP3 bind Bcl-2/Bcl-X_L independently of their BH3 domains (41,74,111). Instead, the NH_2-terminus (residues 1-49) and TM domain of BNIP3 are required for binding Bcl-2, and either region is sufficient for heterodimerization with Bcl-X_L (74). ceBNIP3 has also been shown to heterodimerize with CED-3 and CED-4 (16,111), although the homologous interactions for BNIP3 and NIX have not yet been reported.

Overexpression of BNIP3, NIX and ceBNIP3 induces cell death in a variety of mammalian cell lines (11,12,111). Although Bcl-2 and Bcl-X_L can heterodimerize with these BNIP3 family members, overexpression of Bcl-2 and Bcl-X_L only delays the onset of cell death, and can completely surppress it only when expressed at very high levels

(11,12,16,112). Like Bax, endogenous BNIP3 is loosely associated with the mitochondria, but is integrated into the outer membrane when overexpressed (97). This would suggest that active BNIP3 is integrated into the outer mitochondrial membrane in response to an apoptotic stimulus, although the mechanism remains unknown. Deletion of the TM domain abolishes the apoptotic activity of all three BNIP3 family proteins (11,12,16). Although the TM domain is required for mitochondrial localization, chimeric BNIP3 proteins that are targeted to nonmitochondrial sites by TM domain swapping are nearly as efficient at inducing cell death as the wild type (74). In addition, unlike other BH3-only proteins, BNIP3 and ceBNIP3 do not require the BH3 domain to initiate cell death (16,74). However, substitution of the BNIP3 BH3 domain for the corresponding sequences in Bax functionally restores Bax pro-apoptotic activity and heterodimerization with Bcl-X_L (113). Thus, the BH3 domain of BNIP3 appears to be functionally equivalent to the Bax BH3 domain, but only in the context of the Bax peptide, which may be due to conformational changes that differ from BNIP3. This suggests that BNIP3, ceBNIP3, and presumably NIX, induce cell death in a unique fashion unseen in other BH3-only proteins

Recently, Vande Velde et. al. (97) described a necrotic-like cell death in BNIP3-expressing cells as opposed to apoptotic cell death. Overexpression of BNIP3 induced early plasma membrane permeability and mitochondrial damage initiated by rapid opening of the mitochondrial PT pore, $\Delta\psi m$ suppression, and increased ROS production (97). The PT pore inhibitors cyclosporin A and bongkrekic acid efficiently blocked BNIP3-induced cell death, indicating that mitochondrial dysfunction is the primary cause of death (97). In addition, electron microscopy of BNIP3-transfected cells revealed extensive cytoplasmic vacuolation, autophagosomes, and mitochondrial deformation in

which the internal cisternae were destroyed, but minimal nuclear damage characteristic of necrosis (97). Furthermore, BNIP3-induced cell death was independent of cytochrome *c* release, Apaf-1, caspase-9, and caspase-3 (97). Only during the later stages of BNIP3-induced cell death were features characteristic of apoptosis observed, specifically chromatin condensation and DNA fragmentation (97).

Hypothesis and Approach

BNIP3 overexpression induces DNA fragmentation through an unknown mechanism (97). Typically, DNA fragmentation is attributed to caspase-3 activation, however BNIP3-expressing cells do not exhibit any caspase-3 activity (97). Thus, BNIP3 utilizes an alternative pathway for initiating DNA fragmentation. Given that caspases are the primary executioners of apoptotic cell death, we postulate that an unidentified caspase activated by BNIP3 may be responsible for mediating DNA fragmentation.

Protein-protein interaction is the predominant mechanism utilized by apoptotic regulatory proteins to exert their pro- and anti-apoptotic effects. The *C. elegans* homologue ceBNIP3 heterodimerizes with CED-3 thereby enhancing its activation (16,111). Hence, co-immunoprecipitation analysis will be employed to screen members of the caspase family for interaction with BNIP3 and its homologue NIX. An *in vitro* caspase assay will be utilized to detect activation of endogenous caspases in BNIP3-transfected cells. The effect of caspase expression on BNIP3-induced cell death will be measured using the β-galactosidase cell death assay. To investigate the role of caspase activity in BNIP3-induced DNA fragmentation, the TUNEL assay will be used to compare the extent of DNA fragmentation in cells expressing BNIP3 alone with cells co-expressing BNIP3 and either wild type caspase or a catalytically inactive caspase.

Finally, the effect of a caspase-specific inhibitor on DNA fragmentation in BNIP3-transfected cells will further substantiate a requirement for endogenous caspase activity in BNIP3-induced DNA fragmentation.

MATERIALS AND METHODS

Cell Culture and Antibodies

Human embryonic kidney cells (293T) were cultured in Dulbecco's Modified Eagle Medium (GIBCO-BRL, Burlington, ON), supplemented with 10% fetal calf serum (CanSera, Rexdale, ON). Murine monoclonal anti-T7 and rabbit polyclonal anti-HA antibodies used for immunoprecipitation and Western blot were purchased from Novagen (Madison, WI) and Santa Cruz Biotechnology Inc. (Santa Cruz, CA), respectively.

Expression Plasmids

Construction of recombinant expression plasmids HA-tagged pcDNA3-BNIP3, pcDNA3-BNIP3Δ1-49, pcDNA3-BNIP3Δ164-194, pcDNA3-BNIP3Δ1-49/Δ164-194, and pcDNA3-NIX has been previously described (11,12). pcDNA3 constructs containing T7-tagged catalytically inactive full length caspase-2 and prodomain deletion mutant (Δpro), as well as pFLAG-CMV-5a-tBid were provided by Dr. Junying Yuan (Harvard Medical School, Boston, Mass.). Myc-tagged pcDNA3-caspase-2 wild type, FLAG-tagged pcDNA3-caspase-1, and AUI-tagged pcDNA3-caspase-8 were donated by Dr. Gabriel Nunez (University of Michigan Medical School, Ann Arbor). pcDNA3-caspase-9-His_6 and pcDNA3-Apaf-1 were provided by Dr. Emad Alnemri (Thomas Jefferson University, Philadelphia) and Dr. Xiaodong Wang (Howard Hughes Medical Institute, Dallas), respectively.

Transient Transfections, Co-immunoprecipitation, and Western Blot Analysis

Transient transfections, co-immunoprecipitation, and Western blot analysis were performed essentially as previously described (74). Human embryonic kidney cells were seeded at 2×10^6 in 100 mm culture dishes 24 hours prior to transfection. Cells were co-

transfected with 5 μg of each of the indicated expression plasmids by the calcium phosphate precipitation method. The total amount of DNA was maintained at 10 μg using empty pcDNA3 vector.

Cells were collected 12 hours post-transfection, and washed two times in phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) followed by centrifugation at 330 g for 5 minutes at 4°C. The pellets were resuspended in 250 μl of 0.2% Nonidet P-40 isotonic lysis buffer (100 mM Tris-HCl pH 7.5, 2 mM EDTA, 100 mM NaCl, 0.2% NP-40, 5 μg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride), transferred to a clean microcentrifuge tube, and incubated on ice for 20 minutes. The cell lysates, maintained on ice, were sonicated three times for 10 seconds, followed by centrifugation at 16,000 g for 10 minutes at 4°C. The supernatant was transferred to a clean microcentrifuge tube and precleared with 15 μl of protein A sepharose beads (Zymed Laboratories Inc., San Francisco, CA) for 30 minutes at 4°C with rotation. The precleared lysates were centrifuged at 6,000 g for 2 minutes at 4°C to pellet the protein A sepharose beads. To confirm protein expression, a 20 μl aliquot of lysate was removed, mixed with 4 μl of 5X protein sample buffer (PSB) (2.5 M Tris, pH 6.8, 500 mM DTT, 10% SDS), boiled for 5 minutes, and stored at –20°C until ready for electrophoresis. The remaining cell lysate was mixed with an equal volume, approximately 200 μl of binding buffer (200 mM NaCl, 20% Glycerol, and 0.2% NP-40), and 2 μg of either mouse monoclonal anti-T7 or rabbit polyclonal anti-HA antibody, and incubated for 2-3 hours at 4°C with rotation. Immune complexes were collected by the addition of 15 μl of protein A sepharose beads followed by an additional one hour incubation at 4°C with rotation. The

beads were collected by centrifugation at 6,000 g for 2 minutes at 4°C, and washed three times with 1 ml of a 1:1 mixture of lysis buffer and binding buffer. Finally, the beads were mixed with 40 µl of PSB, boiled for 5 minutes, and stored at –20°C until ready for electrophoresis.

A 10 µl aliquot of immunoprecipitate and 2 µl aliquot of lysate were separated by electrophoresis on an 8-10% Laemlli SDS-polyacrylamide gel, and transferred to nitrocellulose membranes. Following transfer, the membranes were incubated for one hour with shaking at room temperature with a blocking solution containing 3% bovine serum albumin (BSA) and 0.2% TWEEN-20 in PBS. The membranes were probed with either mouse monoclonal anti-T7 or rabbit polyclonal anti-HA antibody diluted to 1 in 10,000 in blocking buffer and incubated overnight at 4°C with gently rocking. Following three 10 minute washes in PBS with 0.2% TWEEN-20, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody diluted to 1 in 5,000 with blocking solution for 1 hour at room temperature. After washing the membranes three times in PBS with 0.2% TWEEN-20 for 10 minutes each, the immune complexes were detected using the enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia Biotech, Amersham, UK) according to manufacturer's instructions.

***In vitro* Ac-VDVAD.AMC Fluorometric Assay**

Activation of endogenous caspase-2 was assayed *in vitro* using the fluorometric caspase-2-specific substrate Ac-VDVAD.AMC (amino-methyl coumarin) (Peptide Institute Inc., Osaka, Japan) (89). Cleavage of Ac-VDVAD.AMC by caspase-2 releases the fluorescent AMC product. Using an optimized calcium phosphate precipitation

method (73), 293T cells seeded at 10^6 in 100mm culture dishes were transiently transfected with 7.5 μg BNIP3 or mock transfected with reagents alone. Cells were collected 18 hours post-transfection, washed twice in PBS and incubated with 200 μl of a 1% Nonidet P-40 lysis buffer (10 mM Hepes pH7.5, 2 mM EDTA, 1 mM DTT, and 1% NP-40, 5 μg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride) (61) for 15 minutes at 4°C with rotation. Lysates were collected by centrifugation at 14,000 g for 8 minutes and the concentration was adjusted to 5 μg/μl. To confirm the specificity of proteolytic activity, 1 mg of lysate was pre-incubated in the presence or absence of 1 μM Ac-zVAD.fmk (benzyloxycarbonyl-VAD-fluoro-methyl ketone), Ac-VDVAD.fmk, or Ac-FA.fmk (Enzyme System Products, Livermore, CA) for 30 minutes at 37°C in a black 96-well plate. 100 μM of Ac-VDVAD.AMC was added to the lysate in a total volume of 300 μl of assay buffer (100 mM Hepes pH 7.5, 10% sucrose 0.1% CHAPS, 0.1 mM EDTA, and 1 mM DTT) (61), and allowed to incubate for up to 8 hours at 37°C. Substrate cleavage was monitored using a Titertek® Fluoroskan II (Flow Laboratories Inc., Lugano, Switzerland) multiwell plate reader at an excitation wavelength of 355 nm and an emission wavelength of 460 nm at the indicated time points.

β-galactosidase Cell Death Assay

Using 35mm 6-well plates, 293T cells were seeded at 10^5 24 hours prior to transfection. Cells were co-transfected with 0.1 μg of each of the indicated expression plasmids plus 0.04 μg of a *lacZ* reporter plasmid using the LipofectAMINE reagent (GIBCO-BRL, Burlington, ON) according to manufacturer's instructions. The total DNA concentration per well was adjusted to 0.5 μg with empty pcDNA3 vector. Where

indicated the medium was replaced with fresh medium containing 100 μM Ac-zVAD.fmk (Enzyme System Products, Livermore, CA) 4 hours post-transfection. After 24 hours, cells were fixed with 0.2% glutaraldehyde in PBS for 10 minutes, rinsed three times in PBS, and stained with X-Gal buffer (0.5 mg/ml 5-bromo-4-chloro-3-indoxyl β-galactoside, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$-$3H_2O$, 1 mM $MgCl_2$, 10 mM KCl, 0.1% Triton X-100 in 0.1 M sodium phosphate buffer, pH 7.5) (64) for at least 4 hours at 37°C to detect β-galactosidase expression. The percent of cell death was calculated based on the number of rounded, condensed blue cells out of at least 300 total blue cells.

DNA Fragmentation Assay

293T cells were seeded at 10^6 in 100 mm culture dishes 24 hours prior to transfection with 7.5 μg of the indicated expression plasmid using an optimized calcium phosphate precipitation method (73). Where indicated, transfected cells were incubated in the presence of 50 μM Ac-VDVAD.fmk, Ac-zVAD.fmk, or Ac-FA.fmk (Enzyme System Products, Livermore, CA). After a 24 hour incubation, cells were collected on ice, washed in PBS, and resuspended in PBS at a concentration of 5 x 10^5 cells/ml. Aliquots of 100 μl were allowed to adhere onto poly-lysine coated slides within inscribed circles for 30 minutes at room temperature. The suspension was aspirated off without disrupting the cells, replaced with 100 μl of 3.7% formaldehyde solution in PBS, and allowed to fix overnight at 4°C in a humid box. The fixed cells were rinsed two times with PBS, and permeabilized with 0.1% Triton X-100/0.1% sodium citrate in PBS for 2 minutes on ice. After rinsing the cells twice with 1X PBS, the extent of DNA fragmentation was determined using the *In Situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Mannheim, Germany), also known as the TUNEL (terminal deoxynucleotidyl

transferase-mediated dUTP nick end labeling) assay, according to manufacturer's instructions. Following incubation for 1 hour at 37°C with the TUNEL reagent, the cells were washed twice for 5 minutes in PBS, and subsequently stained with Hoechst dye for visualization of the total cell population. After rinsing two times in PBS, FluoroGuard antifade reagent (Bio-Rad Laboratories, Hercules, CA) and coverslips were placed over the cells, and the slides were stored at 4°C away from light until ready for analysis. The percentage of TUNEL positive cells was scored out of 200-300 cells in the total population.

RESULTS

Heterodimerization of BNIP3 and NIX with Caspase-2

The observation that ceBNIP3 physically interacts with CED-3 (111) prompted the examination of the mammalian homologues BNIP3 and NIX for potential heterodimerizations with members of the caspase family. Using co-immunoprecipitation (co-IP) analysis, catalytically inactive long prodomain caspases-1, -2, -8, and -9 were screened for interaction with BNIP3 and NIX. Figure 1 illustrates the structure of the catalytically inactive caspase-2 construct utilized for co-IP analysis. Caspases-1, -8, and -9 have a similar structure (not shown). The catalytically inactive caspases have the active site cysteine (C) residue replaced with an inactive serine (S) residue (Fig. 1) to reduce the extent of cell death in transfected cells. The top left panel of Figure 2 *A* is a Western blot for T7-tagged caspase-2 on anti-HA immunoprecipitates from 293T cells transiently expressing caspase-2 alone, or co-expressing caspase-2 and either HA tagged BNIP3 or NIX. Following an anti-T7 IP for caspase-2 in lysates from 293T cells co-transfected with BNIP3 and caspase-2, the BNIP3 dimer is detected by Western blot (Fig. 2 *A*, bottom left panel). However, as shown in the top left panel of Figure 2 *A*, there is no caspase-2 detected in anti-HA immunoprecipitates from co-transfected cells. The opposite scenario is observed in NIX and caspase-2 co-expressing cells. Both the p48 full-length caspase-2, and the p34 intermediate cleavage product consisting of the prodomain and large subunit (54), are detected by Western blot following an IP for NIX (Fig.2 *A*, top left panel). Conversely, an IP for caspase-2 does not successfully co-precipitate NIX (Fig.2 *A*, bottom left panel). In contrast, IPs for either BNIP3 or NIX failed to co-precipitate caspases-1, 8, and 9 as indicated in Figure 2 *B-D* (left panels,

second and third lanes). To confirm that caspase-2 does not non-specifically interact with the anti-HA antiserum or to the protein A sepharose beads used for the IP, caspase-2 was blotted for in anti-HA immunoprecipitates from 293T cells expressing caspase-2 alone. As shown in the first lane of Figure 2 *A*, top left panel, there is no caspase-2 detected. Similarly, niether BNIP3 nor NIX bind non-specifically to the anti-T7 antibody or to the beads used for the IPs (data not shown). Like caspase-2, none of caspases-1, -8, or –9 interacted non-specifically with the anti-HA antibody or the beads used for the IP (Fig. 2 *B-D*, first lane left panels). Western blot of the lysates confirms efficient expression of caspases-2, -1, -8, and –9 in all transfections (Fig. 2 *A-D*, right panels). BNIP3 and NIX are also expressed at significant levels in all co-transfections similar to that depicted in the bottom right panel of Figure 2 *A*.

Heterodimerization is Independent of the Caspase-2 Prodomain

All caspase heterodimerizations identified to date are mediated through the prodomain. For example, the heterodimerization of caspase-9 with Apaf-1 (55), caspase-8 with FADD (67), caspase-2 with RAIDD (22), and ceBNIP3 with CED-3 (111) are all dependent on the caspase prodomain. To determine the necessity of the caspase-2 prodomain for heterodimerization with BNIP3 and NIX, 293T cells were co-transfected with either HA-tagged BNIP3 or NIX and a catalytically inactive T7-tagged caspase-2 p32 mutant with a deletion of the NH_2-terminal prodomain 152 amino acid residues (Δpro) (Fig.1). In co-transfected 293T cells, caspase-2Δpro is detected by Western blot following an IP for both BNIP3 and NIX (Fig. 3 *A*, top panel). Similarly, both the dimer and monomer of BNIP3 and NIX co-precipitated with a caspase-2Δpro IP (Fig. 3 *A*, bottom panel). As with the full-length caspase-2, caspase-2Δpro does not bind non-

specifically with either the anti-HA antiserum or the protein A sepharose beads used for the IP (Fig. 3 *A*, first lane, top panel). Western blot of the lysates confirms efficient expression of BNIP3, NIX, and caspase-2Δpro (Fig.3 *B*, top and bottom panels).

Heterodimerization is Independent of the NH_2-terminus and Transmembrane Domain of BNIP3

The NH_2-terminus and TM domain of BNIP3 are required for heterodimerization with Bcl-2 and Bcl-X_L (74). To determine if these same regions are involved in binding caspase-2, three deletion mutants of BNIP3 were examined for their binding potential with caspase-2: (i) BNIP3Δ1-49 mutant with a deletion of the NH_2-terminal 49 amino acid residues, (ii) BNIP3Δ164-194 mutant with the C-terminus truncated at amino acid residue 163 and thus lacking the transmembrane (TM) domain, and (iii) BNIP3Δ1-49/Δ164-194 double mutant lacking both the amino and carboxy terminals (Fig. 4 *A*). Each BNIP3 deletion mutant was co-transfected into 293T cells with full-length catalytically inactive caspase-2. Following an IP for each of the three BNIP3 deletion mutants, both the full-length p48 caspase-2 and the p34 intermediate cleavage product were detected (Fig. 4 *B*, top left panel). Thus, the NH_2-terminus and TM domain of BNIP3 are not involved in binding caspase-2. All three BNIP3 deletion mutants and caspase-2 are efficiently expressed as shown by Western blot of the cell lysates (Fig.4 *B*, top and bottom right panels).

Activation of Endogenous Caspase-2 in BNIP3-expressing Cells

The physiological effect of caspase binding is typically activation or inhibition of caspase activity. In the *C. elegans*' homologues ceBNIP3 and CED-3, co-expression results in accelerated processing and activation of CED-3 (111). Given the pro-apoptotic

nature of BNIP3, the binding of BNIP3 to caspase-2 may similarly function to promote activation of caspase-2. An *in vitro* fluorometric assay was employed using the peptide Ac-VDVAD.AMC, a caspase-2-specific substrate (89), to detect activation of endogenous caspase-2 in 293T cells transiently expressing BNIP3. Cleavage of Ac-VDVAD.AMC by caspase-2, or caspase-2-like proteases, releases the fluorescent AMC product, which can be detected and quantified in relative fluorescent units using a multiwell plate reader at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Lysates from BNIP3-transfected cells exhibited a significant increase in Ac-VDVAD.AMC cleavage activity over lysates from mock-transfected control cells ($P<0.02$) (Fig. 5). To determine the specificity of the substrate cleavage, lysates were treated with 1 μM of Ac-zVAD.fmk, Ac-VDVAD.fmk, or Ac-FA.fmk for 30 minutes prior to incubation with the substrate. Ac-zVAD.fmk is a broad-spectrum caspase inhibitor and thus should inhibit all caspase activity. Conversely, Ac-VDVAD.fmk is a caspase-2-specific inhibitor (89) with identical sequence to the substrate, and thus will specifically block cleavage by caspase-2 or caspase-2-like proteases. The proteolytic activity observed in BNIP3 lysates is reduced to background levels by pre-treatment with either Ac-VDVAD.fmk or Ac-zVAD.fmk (Fig. 5), indicating that endogenous caspase-2, or caspase-2-like proteases, are responsible for the increased activity. Ac-FA.fmk is a non-specific peptide that serves as a negative control, and thus should have minimal to no effect on caspase activity. As expected, Ac-FA.fmk has only a minimal effect on Ac-VDVAD.AMC cleavage by BNIP3 lysates (Fig. 5). Background levels of Ac-VDVAD.AMC cleavage by mock transfected cell lysates are unaffected by any of the inhibitors (Fig. 5).

Enhanced Cell Death in Cells Co-expressing BNIP3 and Caspase-2

Given the observation that ceBNIP3 and CED-3 physically interact and cooperate to induce enhanced apoptosis (111), the interaction of caspase-2 with BNIP3 and NIX may also serve to enhance cell death. Using the β-galactosidase cell death assay, the extent of cell death was compared in 293T cells transiently expressing either BNIP3, NIX, or wild type caspase-2 alone with those cells co-expressing BNIP3 and caspase-2, or NIX and caspase-2. After 24 hours, BNIP3 and NIX expressed alone induce about 30% cell death, while caspase-2 induces about 25% cell death (Fig. 6). Co-expression of BNIP3 with caspase-2 results in a significant increase in cell death to about 50% ($P<0.006$) (Fig. 6). However, in cells co-expressing NIX and caspase-2, there is no significant increase in cell death as compared to NIX alone ($P>0.1$) (Fig. 6). A background of about 10% cell death is observed for cells transfected with empty pcDNA3 (Fig. 6).

To determine the requirement for caspase-2 activity, the effectiveness of 100 μM Ac-zVAD.fmk on BNIP3 and caspase-2-induced cell death was examined using the β-galactosidase cell death assay (Fig. 7). Cells transiently transfected with BNIP3 and caspase-2 were incubated with 100 μM Ac-zVAD.fmk for 24 hours, then stained and analyzed for the percentage of cell death. Expression of either caspase-2 or BNIP3 induces cell death in 20-30% of untreated cells respectively, whereas co-expression of both proteins induces 40-45% cell death (Fig. 7). Surprisingly, treatment with 100 μM Ac-zVAD.fmk had no effect on either caspase-2- or BNIP3-induced cell death (Fig. 7). Thus it follows that cells co-expressing BNIP3 and caspase-2 are also unaffected by 100 μM Ac-zVAD.fmk (Fig. 7). As a positive control, 293T cells were co-transfected with

Apaf-1 and caspase-9, which are known to induce cell death by an Ac-zVAD.fmk inhibitable pathway. In the absence of inhibitor, Apaf-1 and caspase-9 induce almost 50% cell death, but only about 20% in the presence of 100 μM Ac-zVAD.fmk, hence confirming the effectiveness of the inhibitor (Fig.7). Transfection of empty pcDNA3 induces minimal cell death and is unaffected by the inhibitor (Fig.7). Thus, the β-galactosidase cell death assay and Ac-zVAD.fmk are not suitable for evaluating the requirement for caspase-2 activity in BNIP3-induced cell death.

Enhanced DNA Fragmentation in Cells Co-expressing BNIP3 and Caspase-2

BNIP3-induced cell death is characterized by late DNA fragmentation through an unknown mechanism (97). The interaction with and activation of caspase-2 may play a role in BNIP3-induced DNA fragmentation. Using the TUNEL assay, the extent of DNA fragmentation in 293T cells transiently transfected with either BNIP3 or caspase-2 is compared to co-transfected cells. BNIP3 induces DNA fragmentation in about 25% of the total cell population 24 hours post-transfection (Fig. 8). Wild type caspase-2 induces DNA fragmentation in about 15% of cells, whereas the catalytically inactive caspase-2 exhibits only a minor increase over control cells transfected with empty pcDNA3 (Fig. 8). Co-expression of BNIP3 with wild type caspase-2 results in a significant enhancement in DNA fragmentation, up to 35% of the total cell population ($P<0.001$) (Fig. 8), similar to the pattern observed in the β-galactosidase cell death assay. However, when BNIP3 is co-expressed with catalytically inactive caspase-2, there is no increase in the amount of DNA fragmentation (Fig. 8), indicating that caspase-2 activity is required for the enhanced DNA fragmentation observed. Minimal DNA fragmentation is observed in control cells transfected with empty pcDNA3 (Fig. 8)

BNIP3-induced DNA Fragmentation Requires Endogenous Caspase-2 Activity

In order to substantiate a role for endogenous caspase-2 activity in BNIP3-induced cell death, the effect of Ac-VDVAD.fmk on BNIP3-induced DNA fragmentation was determined using the TUNEL assay. Cells were transfected with either BNIP3, caspase-2, or tBid (truncated Bid), and incubated for 24 hours with 50 μM of Ac-VDVAD.fmk, Ac-zVAD.fmk, or Ac-FA.fmk. As previously observed in Figure 8, BNIP3 induces DNA fragmentation in about 30% of the total cell population (Fig.9). Incubation of BNIP3-transfected cells with either Ac-VDVAD.fmk or Ac-zVAD.fmk significantly reduces the percentage of cells exhibiting DNA fragmentation by half ($P<0.02$) (Fig. 9). In contrast, BNIP3-induced DNA fragmentation is relatively unaffected by treatment with Ac-FA.fmk ($P>0.3$) (Fig. 9). Similarly, both Ac-VDVAD.fmk and Ac-zVAD.fmk, but not Ac-FA.fmk, reduce caspase-2-induced DNA fragmentation by about 50%, confirming the effectiveness of Ac-VDVAD.fmk against caspase-2 activity (Fig. 9). tBid induces DNA fragmentation through the classical Apaf-1/caspase-9/caspase-3 pathway, thus it serves as both a positive control for Ac-zVAD.fmk inhibition, and a negative control for Ac-VDVAD.fmk inhibition. tBid induces DNA fragmentation in up to 75% of cells, which is almost completely abolished by Ac-zVAD.fmk (Fig. 9). In contrast, incubation of tBid-transfected cells with Ac-VDVAD.fmk or Ac-FA.fmk has little effect on DNA fragmentation, exhibiting less than a 10% reduction (Fig. 9). Mock transfected control cells exhibit no TUNEL positive signals, and are unaffected by the caspase inhibitors (data not shown).

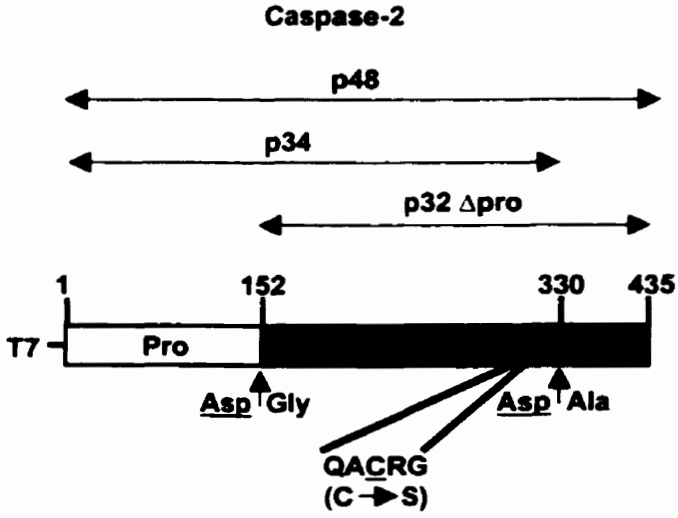


Figure 1: Structure of the catalytically inactive caspase-2 construct used for co-immunoprecipitation analysis.

Full-length p48 procaspase-2, intermediate cleavage product p34 consisting of the prodomain and large subunit, and prodomain deletion mutant p32 (Δpro) are indicated by arrows. The active cysteine (C) residue that is replaced with an inactive serine (S) residue is underlined. Cleavage sites are indicated by arrows at aspartate (Asp) residues. Caspases-1, -8, and –9 have similar structure (not shown).

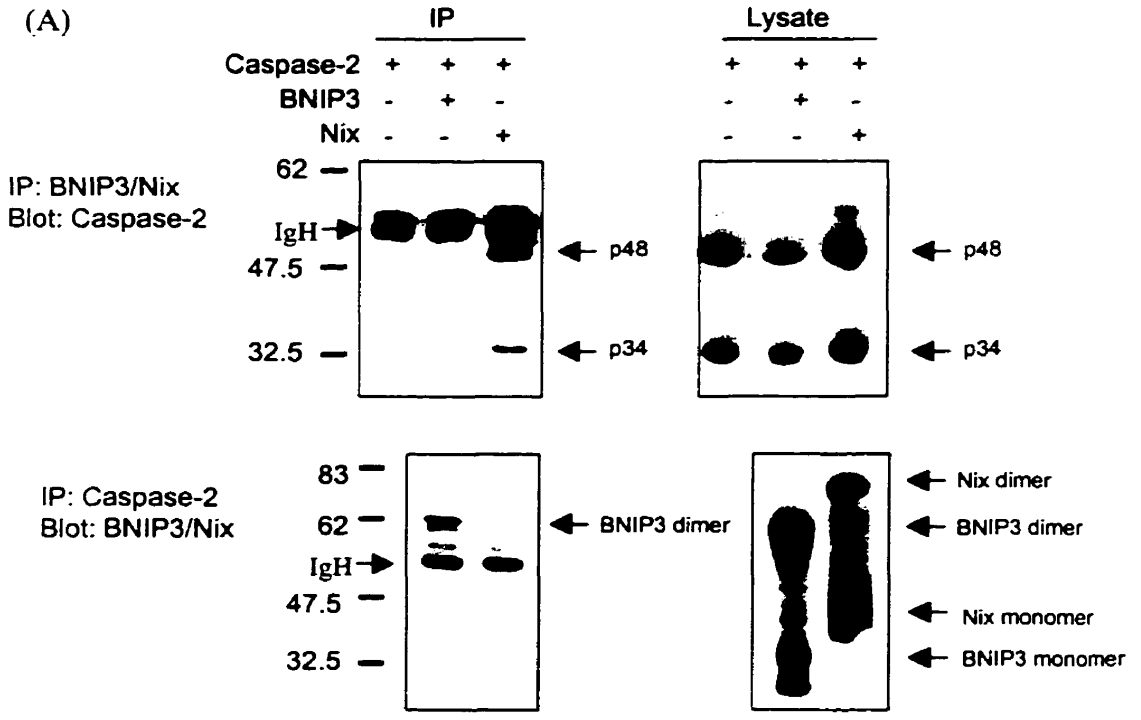
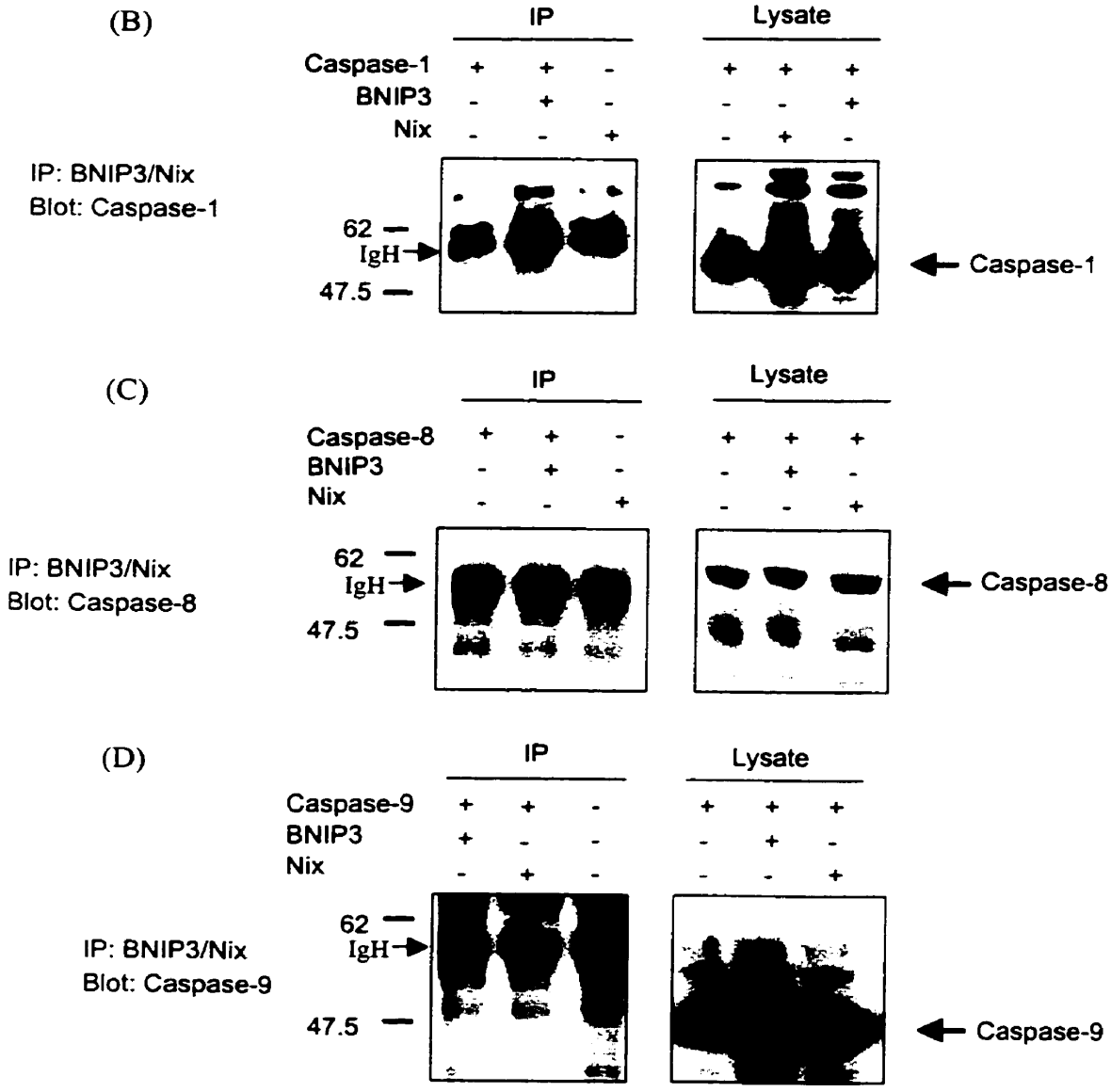


Figure 2: Co-immunoprecipitation analysis of BNIP3 and NIX with caspases-1, -2, -8, and -9.

293T cells were co-transfected by the calcium phosphate method with HA-tagged BNIP3, or NIX, and (*A*) catalytically inactive caspase-2, (*B*, p.46) caspase-1, (*C*, p.46) caspase-8, or (*D*, p.46) caspase-9. Lysates were collected 12 hours post-transfection, immunoprecipitated with rabbit polyclonal anti-HA antibody, and immunoblotted with the appropriate caspase antibody, as described under Material and Methods. In (*A*), immunoprecipitation of T7-tagged caspase-2 with mouse monoclonal anti-T7 antibody and immunoblot with rabbit polyclonal anti-HA antibody for BNIP3 or NIX was also performed. Total cell lysates were analyzed for expression of BNIP3 and NIX (*A*, bottom right panel), and for expression of the indicated caspase (*A-D*). BNIP3 migrates as a 60kDa dimer and 30kDa monomer. NIX migrates as an 80kDa dimer and 40kDa monomer. Approximate sizes of the caspases as indicated by an arrow are as follows, 48kDa, 48kDa, 55kDa, and 45kDa for caspase-2, -1, -8, and –9, respectively. The immunoglobulin heavy chain (IgH) is present in all lanes at approximately 55-60kDa as indicated by the arrow.


(B)
IP
Lysate
Caspase-1 + + - + + +
BNIP3 - + - - - +
Nix - - + - + -
IP: BNIP3/Nix
Blot: Caspase-1
62
IgH
47.5
Caspase-1
(C)
IP
Lysate
Caspase-8 + + - + + +
BNIP3 - + - - + -
Nix - - + - - +
IP: BNIP3/Nix
Blot: Caspase-8
62
IgH
47.5
Caspase-8
(D)
IP
Lysate
Caspase-9 + + - + + +
BNIP3 + - - - + -
Nix - + - - - +
IP: BNIP3/Nix
Blot: Caspase-9
62
IgH
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Caspase-9

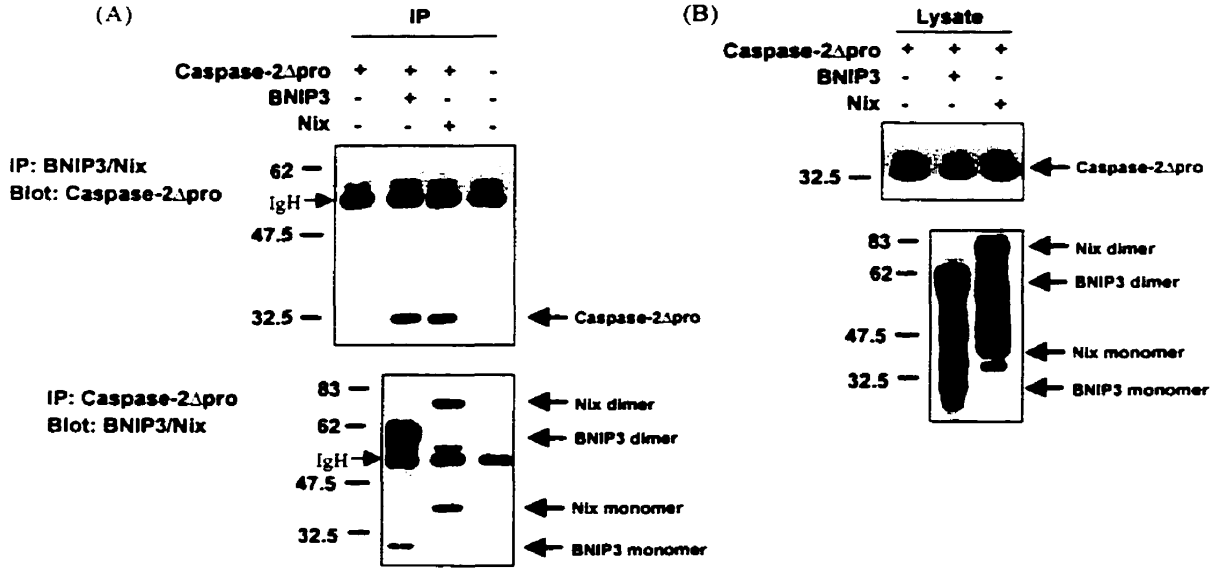

Figure 3: Heterodimerization of BNIP3 and NIX with caspase-2 is independent of the prodomain.

293T cells were co-transfected with HA-tagged BNIP3 or NIX and a T7-tagged catalytically inactive caspase-2 mutant lacking the 152 amino acid NH_2-terminal prodomain (Δpro, Fig. 1). Lysates were collected 12 hours post-transfection. immunoprecipitated with rabbit polyclonal anti-HA antibody, and immunoblotted with mouse monoclonal anti-T7 antibody (*A*, top panel), and vice versa (*A*, bottom panel). Lysates confirm efficient expression of all proteins (*B*).

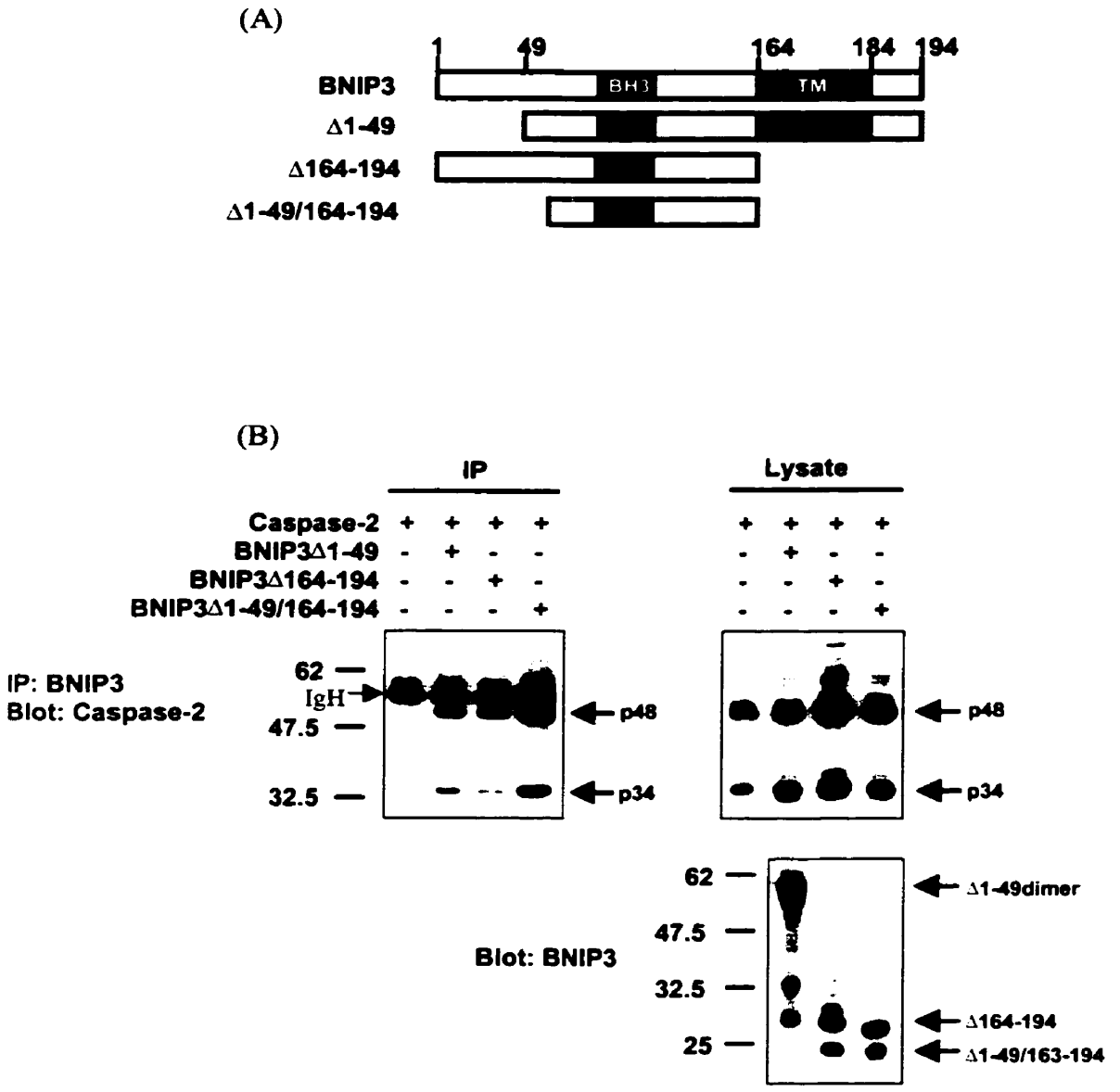

Figure 4: Heterodimerization of caspase-2 with BNIP3 is independent of the NH_2-terminus and transmembrane domain.

(*A*) Schematic representation of BNIP3 deletion mutants. (*B*) 293T cells were co-transfected with HA-tagged BNIP3 deletion mutants and T7-tagged catalytically inactive full length caspase-2 (Fig.1 *A*). 12 hours post-transfection, lysates were immunoprecipitated with rabbit polyclonal anti-HA antibody, and immunoblotted with mouse monoclonal anti-T7 antibody (top left panel). Total cell lysates were immunoblotted for expression of caspase-2 (top right panel) and BNIP3 deletion mutants (bottom panel) as indicated by arrows.

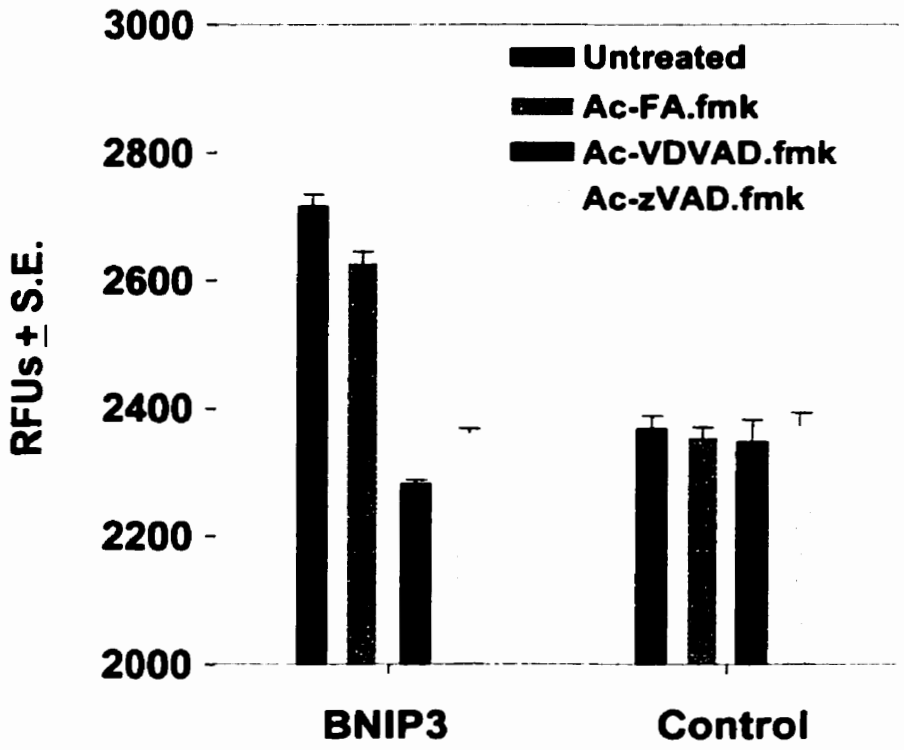

Figure 5: BNIP3 activates endogenous caspase-2.
293T cells were transiently transfected with BNIP3 or mock transfected by the calcium phosphate method. Cell lysates were collected 18 hours post-transfection and incubated with 1 μM of Ac-FA.fmk, Ac-zVAD.fmk, or Ac-VDVAD.fmk at 37°C for 30 minutes. 100 μM Ac-VDVAD.AMC was added to the lysates and allowed to incubated up to 8 hours at 37°C. Results are expressed as relative fluorescent units (RFUs) ± S.E. from three independent experiments. The increase in Ac-VDVAD.AMC cleavage by BNIP3 lysates is significantly higher as compared to control lysates ($P < 0.02$).

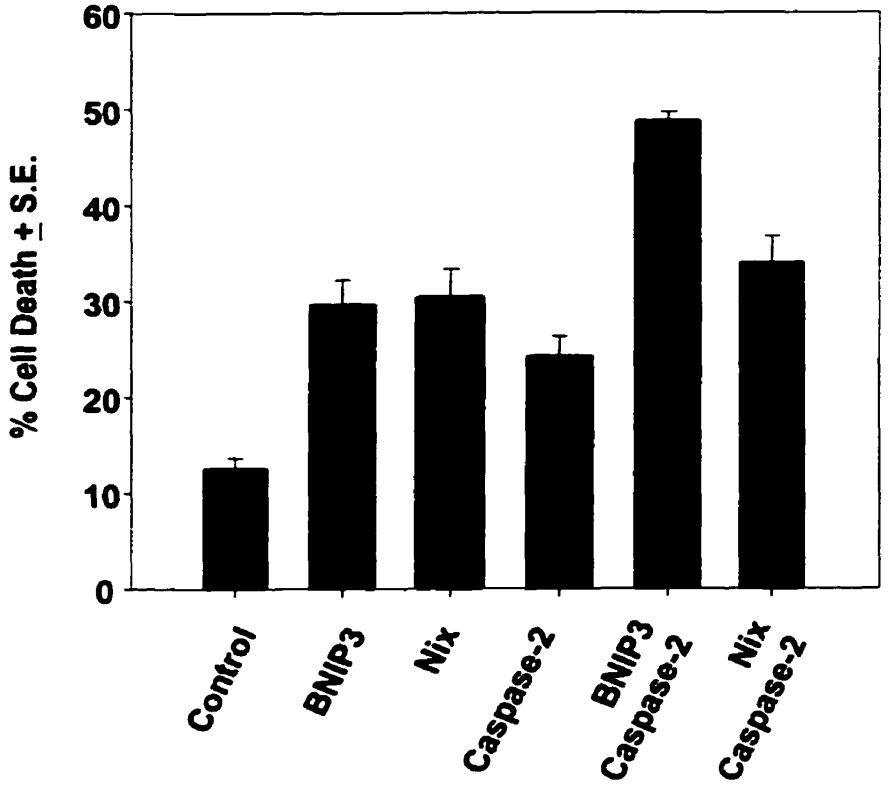

Figure 6: Co-expression of BNIP3 and caspase-2 results in enhanced cell death in 293T cells.

293T cells were transiently co-transfected with reporter plasmid, pcDNA3-βgal, and BNIP3, NIX, or caspase-2 alone or in combination as indicated. 24 hours post-transfection cells were stained with X-gal and percent cell death determined. The results shown represent the mean ± S.E. from three independent experiments. Co-transfection of BNIP3 and caspase-2 results in a significant increase in cell death as compared to cells transfected with BNIP3 alone ($P < 0.006$), whereas the increase observed in cells co-transfected with NIX and caspase-2 is insignificant as compared to cells transfected with NIX alone ($P > 0.1$).

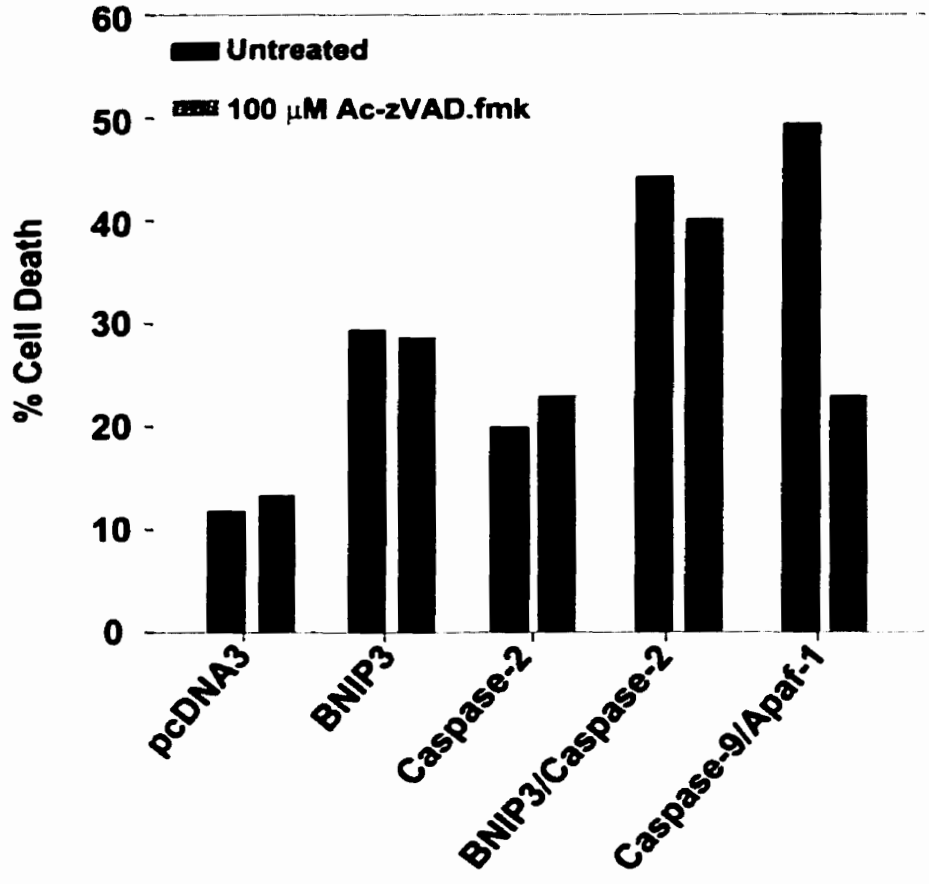

Figure 7: BNIP3 and caspase-2-induced cell death is insensitive to Ac-zVAD.fmk. 293T cells transiently transfected as described in Figure 6, were incubated with 100 μM Ac-zVAD.fmk for 24 hours and then stained for β-galactosidase expression. Cells transfected with caspase-9 and Apaf-1 served as positive controls. Results representative of three independent experiments.

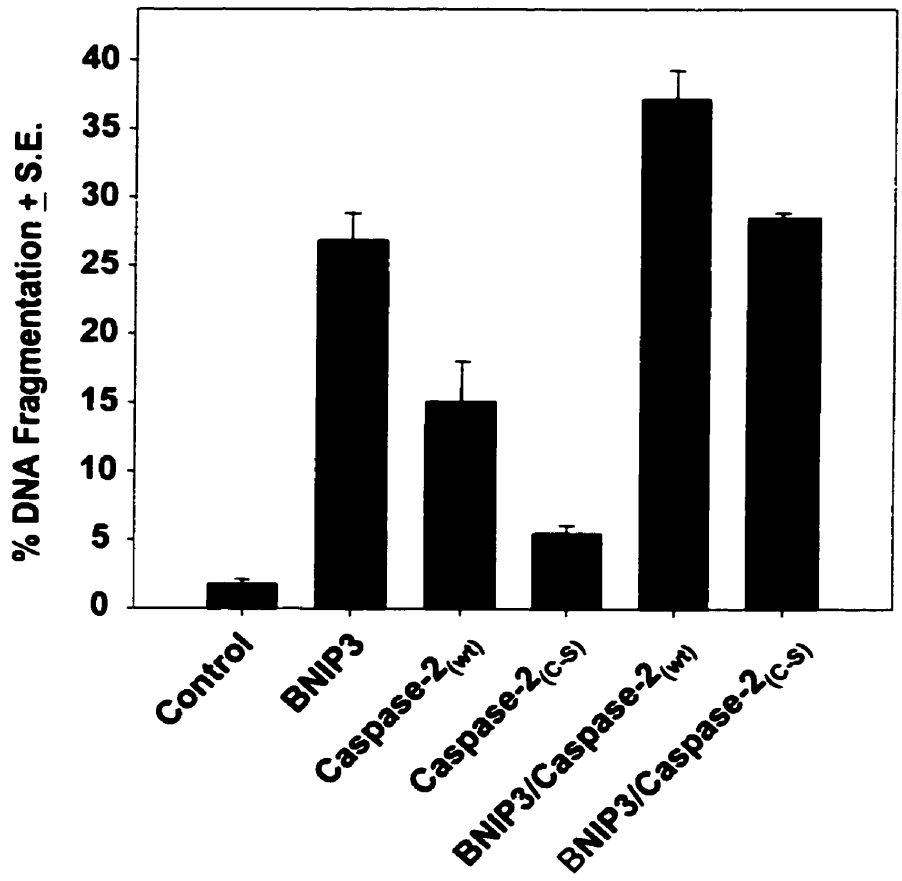

Figure 8: Co-expression of BNIP3 and caspase-2 results in enhanced DNA fragmentation in 293T cells.

293T cells were transiently transfected with BNIP3, wild type caspase-2, or catalytically inactive caspase-2 alone or in combination. 24-hours post-transfection, cells were stained with TUNEL as described in Material and Methods, and the percentage of DNA fragmentation $\pm$ S.E. was determined for three independent experiments. The increase in DNA fragmentation observed in BNIP3 and caspase-2 co-transfected cells is significantly greater than that observed in cells transfected with BNIP3 alone ($P < 0.001$).

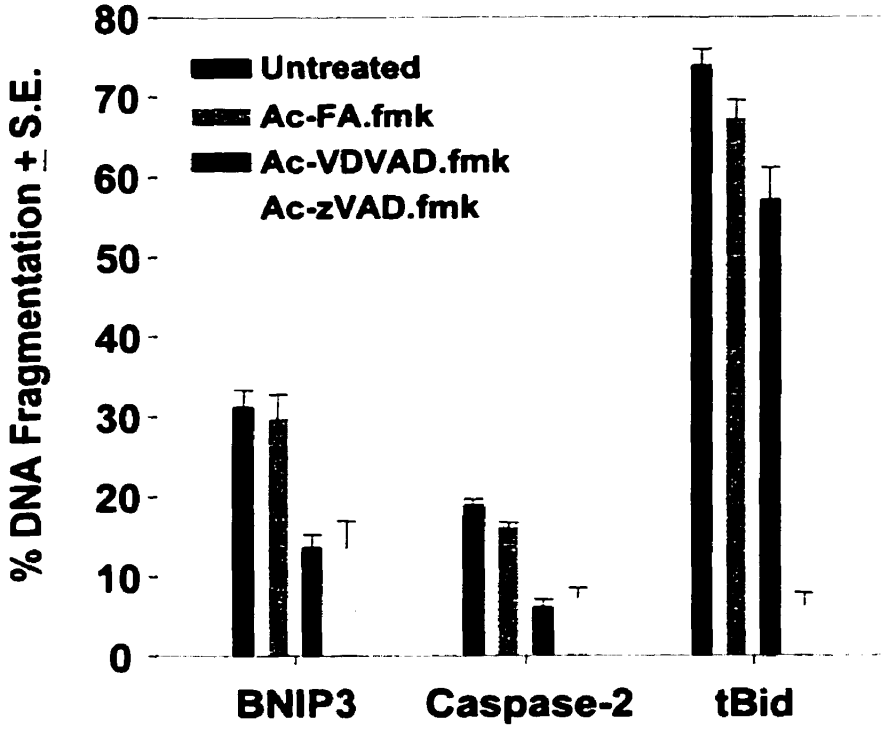

Figure 9: BNIP3-induced DNA fragmentation is blocked by a caspase-2-specific inhibitor.

293T cells transiently expressing BNIP3, caspase-2, or tBid, were incubated with 50 μM of Ac-FA.fmk, Ac-VDVAD.fmk, or Ac-zVAD.fmk for 24 hours, and then assayed for percentage DNA fragmentation as in Figure 7. The percentage of DNA fragmentation ± S.E. was determined from three independent experiments. Incubation of BNIP3-transfected cells with either Ac-VDVAD.fmk or Ac-zVAD.fmk results in a significant reduction in DNA fragmentation as compared to untreated cells transfected with BNIP3 ($P < 0.02$), whereas the reduction observed in cells incubated with Ac-FA.fmk is not significant ($P > 0.3$).

DISCUSSION

Identification of heterodimerizations among apoptotic regulatory proteins has served as an important means for determining the mechanism of action of such proteins. Many of these interactions are structurally and functionally conserved between mammalian and *C. elegans'* proteins. For example, the interaction between Apaf-1 and caspase-9 resulting in caspase-9 activation (55) is homologous to the CED-4/CED-3 interaction in *C. elegans* (13). Similarly, BNIP3 and NIX heterodimerize with Bcl-2 (8,41,74,112), which is homologous to the interaction observed between ceBNIP3 and CED-9 (16). Given that ceBNIP3 also heterodimerizes with CED-3 (111), it was not surprising to find that BNIP3 and NIX interact with a mammalian CED-3 homologue, caspase-2.

Caspase-2 is known to physically interact with only two other proteins, RAIDD and ARC (3,22,47). Both interactions are mediated through homophilic binding to the caspase-2 prodomain. As well, the interaction between CED-3 and ceBNIP3 is mediated by the prodomain (111), as are all caspase interactions in general, hence the necessity of the caspase-2 prodomain for binding BNIP3 and NIX was determined. Interestingly, the interaction of caspase-2 with BNIP3 and NIX is independent of the prodomain. In addition, the other long prodomain caspases examined, caspase-1, -8, and –9 do not bind to either BNIP3 or NIX. Thus, this interaction is unique to caspase-2 and is not characteristic of the typical prodomain-mediated binding observed for all other caspase interactions identified to date. However, co-IP analysis does not distinguish between direct and indirect interactions, hence another protein may be required for bridging the interaction between caspase-2, BNIP3, and NIX.

To further characterize the interaction structurally, three deletion mutants of BNIP3 were examined for binding potential with caspase-2: (i) BNIP3Δ1-49, (ii) BNIP3Δ164–194 (TM), and (iii) BNIP3Δ1-49/Δ164-194 (TM). As observed in the ceBNIP3 and CED-3 interaction (111), the TM domain of BNIP3 is not required for binding caspase-2. In addition, the NH_2-terminal 49 amino acid residues of BNIP3 are also not required for binding caspase-2. Thus, the regions of BNIP3 required for binding Bcl-2 and Bcl-X_L, specifically the NH_2-terminus and TM domain (74), are not involved in caspase-2 binding. This implies that the region within amino acid residues 50-163, containing the BH3-like domain and conserved domain, may also contain a putative caspase-2 binding domain, although further co-IP analysis of deletion mutants from this region are required to determine the precise caspase-2 binding domain.

The physiological effect of caspase binding is typically activation or inhibition of caspase activity. In the *C. elegans*' homologues ceBNIP3 and CED-3, co-expression results in accelerated processing and activation of CED-3 (111). Given the pro-apoptotic nature of BNIP3, the binding of BNIP3 and caspase-2 may result in activation of caspase-2. Until only recently there were no known substrates for caspase-2 *in vivo*, hence an *in vitro* fluorometric assay was employed using a caspase-2-specific peptide substrate in order to detect activation of endogenous caspase-2 in BNIP3-expressing cells. All caspases have a strict requirement for a minimum 4 amino acid residue substrate recognition sequence with aspartate in the P_1 position, but only caspase-2 exhibits optimal specificity for a 5 amino acid residue target sequence with valine in P_5 (89). Using a series of defined peptide sequence variants, Talanian et. al. (89) determined the substrate specificity of several purified caspases by measuring relative V_{max}/K_m values,

and found VDVAD to be the optimal substrate for caspase-2. Cleavage of Ac-VDVAD.AMC by caspase-2 releases the fluorescent AMC product, which can be quantified using a fluorometer. The ability of BNIP3-expressing cell lysates to specifically cleave the caspase-2-specific substrate Ac-VDVAD.AMC *in vitro*, indicates activation of endogenous caspase-2, or caspase-2-like proteases, in BNIP3-transfected cells. Furthermore, the endogenous VDVADase activity detected in BNIP3-expressing cell lysates is efficiently blocked by Ac-zVAD.fmk and Ac-VDVAD.fmk. Fluoro-methyl ketones (fmk) are irreversible caspase inhibitors, whose specificity depends on the conjugated peptide (26). The Ac-zVAD.fmk peptide functions as a broad-spectrum caspase inhibitor due to the lack of a P_4 amino acid residue, thereby allowing it to bind irreversibly to a wide range of caspase catalytic sites and thus block the interaction of potential substrates (26). In contrast, Ac-VDVAD.fmk is of identical sequence to the substrate used in the assay, hence it will specifically block caspase-2-mediated cleavage. The ability of Ac-VDVAD.fmk to completely block cleavage of Ac-VDVAD.AMC down to levels observed in control cell lysates, indicates the specificity of caspase activity in BNIP3-transfected cells. Ac-zVAD.fmk also inhibits VDVADase activity in BNIP3-transfected cell lysates. However, caspase-2 has been observed to be significantly less sensitive to Ac-zVAD.fmk as compared to caspases-3, -7, -8, and –9 (26). Thus, Ac-zVAD.fmk may be inhibiting the activity of other unidentified caspases in addition to, or instead of, caspase-2. Based on the current models for caspase activation and the observed heterodimerization of BNIP3 and caspase-2, three potential mechanisms for BNIP3-induced caspase-2 activation are implied: (i) BNIP3-induced oligomerization and auto-catalytic activation of caspase-2, (ii) BNIP3-induced recruitment of another

molecule which promotes activation of caspase-2, and (iii) BNIP3-enhanced activity of the caspase-2 zymogen.

Concordant with the induced-proximity model (81), BNIP3 may function as an adaptor protein and induce oligomerization of procaspase-2 thereby bringing two procaspase-2 molecules into close proximity and promoting auto-catalytic processing and activation. The TM domain mediates BNIP3 homodimerization (12), however the TM domain is not required for binding caspase-2, thus it may serve to mediate oligomerization of BNIP3/caspase-2 heterodimers. Although the zymogenicity ratio for caspase-2 has not been reported, prodomain-mediated homodimerization during overexpression is sufficient to induce auto-catalytic activation of procaspase-2 (9), indicating that the zymogen does have sufficient intrinsic activity for self-processing.

Another possible mechanism is that BNIP3 may function to recruit, or favorably present procaspase-2 to another molecule that could promote processing and activation. Since the prodomain of caspase-2 is not required for binding BNIP3, it is available to participate in CARD-CARD interactions with other adaptor proteins such as RAIDD or possibly Apaf-1, resulting in the formation of a tertiary complex that may be able to induce oligomerization and hence promote auto-catalytic activation of caspase-2. Alternatively, another caspase may be responsible for processing caspase-2 presented by BNIP3, although likely not caspase-3 since its activity is not detected in BNIP3-expressing cells (97).

Recently, caspase activation has been observed without proteolytic processing. Stennicke et. al. (86) demonstrated that procaspase-9 mutants in which one or both of the interdomain processing sites are disabled, still activated downstream caspases in a cell-

free system, but only in the presence of cytosolic factors Apaf-1, cytochrome c, and dATP. Processing of procaspase-9 resulted in only a 10-fold increase in activity, hence a zymogenicity ratio of 10, but was further activated 2000-fold in the presence of cytosolic factors, presumably Apaf-1, cytochrome *c*, and dATP (86). This supports the model in which Apaf-1 and active caspase-9 remain in a complex and function as a holoenzyme where caspase-9 is the catalytic subunit and Apaf-1 is the allosteric regulator (78). Moreover, it raises the question of how to distinguish between processing, which may be advantageous, and activation, which is not simply due to proteolytic processing as previously suggested (86). A similar mechanism may apply to the activation of caspase-2 in BNIP3-expressing cells. Overexpression of caspase-2 results in processing, hence it was not possible to measure the effect of co-expression with BNIP3 on caspase-2 processing. In addition, we were not able to detect processing of endogenous caspase-2 in 293T cells expressing BNIP3 (data not shown). However, the *in vitro* fluorometric assay clearly demonstrated specific activation of endogenous caspase-2 in BNIP3-expressing cells. Thus, the BNIP3/caspase-2 heterodimer may function as a holoenzyme similar to Apaf-1 and caspase-9, in which BNIP3 enhances the activity of the caspase-2 zymogen.

Regardless of the mechanism, activation of caspase-2 by BNIP3 may contribute to an increase in cell death in cells co-expressing both proteins. Since the ceBNIP3/CED-3 interaction results in enhanced CED-3 activation and cell death (111), the interaction of BNIP3 and NIX with caspase-2 may have a similar function. Using the β-galactosidase cell death assay, an increase in BNIP3-induced cell death upon co-expression with caspase-2 was observed. However, no increase in NIX-induced cell death was observed when co-expressed with caspase-2. The enhanced cell death observed in BNIP3/caspase-

2 co-expressing cells appears to be an additive effect, which implies two different potential mechanisms. One possible interpretation is that BNIP3 and caspase-2 are acting independently in distinct cell death pathways. In this scenario, co-expression would promote both BNIP3- and caspase-2-induced cell death pathways independently, resulting in an increase cell death. Alternatively, BNIP3 and caspase-2 may be functioning within the same cell death pathway, a possibility that is further supported by the observed physical interaction. Overexpression of both BNIP3 and caspase-2 would allow such a pathway to function at a greater flux, limited only by other endogenous proteins that may be involved, resulting in the observed enhancement in cell death. In turn, inhibition at one step in this pathway would coincide with a reduction in cell death.

Lack of enhanced cell death in NIX/caspase-2 co-expressing cells may be due to structural differences between BNIP3 and NIX that impair caspase-2 activation. The larger NH_2-terminus in NIX may impair activation of caspase-2, perhaps by blocking the prodomain of caspase-2 and impairing recruitment of a necessary co-activator, or by adopting a conformation that does not promote caspase-2 zymogen activity. However, the possibility that NIX and caspase-2 are functioning within the same cell death pathway is not entirely eliminated. A pathway involving both proteins may be operating at optimal levels with NIX overexpression alone, thus no increase is observed upon co-expression of caspase-2. NIX may be able to functionally substitute for BNIP3 in a caspase-2-dependent cell death pathway, however investigation of those questions is beyond the scope of this thesis.

To determine the requirement for caspase activity in BNIP3-induced cell death, transfected cells were incubated with Ac-zVAD.fmk prior to performing the β-

galactosidase cell death assay. The broad-spectrum caspase inhibitor had no effect on either BNIP3 or caspase-2 killing activity, and thus no effect on the cooperate action of BNIP3 and caspase-2 in co-transfected 293T cells. In contrast, cells co-expressing caspase-9 and Apaf-1 were rescued by the inhibitor, confirming previous observations of its effectiveness at blocking cell death initiated from these proteins. The primary mechanism of BNIP3-induced cell death involves early plasma membrane permeability and mitochondrial damage, events that are caspase-independent and hence insensitive to Ac-zVAD.fmk (97). DNA fragmentation and chromatin condensation, typically caspase-dependent events, are only during the later stages of BNIP3-induced cell death, likely occurring after the cell is committed to die (11,12,97). In addition, caspase-2 is relatively insensitive to Ac-zVAD.fmk (26). Thus, the β-galactosidase cell death assay, which does not distinguish between caspase-dependent and independent cell death, is inadequate for measuring the effect of Ac-zVAD.fmk on BNIP3-induced caspase-dependent events, and subsequently inadequate for measuring the effect of co-expression with caspase-2. Hence, an assay to specifically examine the role of caspase-2 in a BNIP3-induced DNA fragmentation pathway was performed.

DNA fragmentation and chromatin condensation are hallmarks of caspase-dependent apoptosis. We have previously shown that BNIP3 induces DNA fragmentation and chromatin condensation that is sensitive to Ac-zVAD.fmk in a variety of cell lines (11,12,97). However, the mechanism of BNIP3-iduced DNA fragmentation remains unknown. During apoptosis, DNA fragmentation is almost exclusively attributed to the activation of the DNA fragmentation factor (DFF), a heterodimer consisting of a 40kDa caspase-3 activated nuclease (DFF40/CAD; caspase-activated deoxyribonuclease), and a

45kDa inhibitor (DFF45/ICAD; inhibitor of CAD) (25,57,58,80). Caspase-3 specifically cleaves DFF45/ICAD, releasing the active nuclease DFF40/CAD (25,57,58,80). Expression of a caspase-3-resistant DFF45/ICAD mutant inhibits DNA fragmentation induced by a wide variety of stimuli, confirming that DFF40/CAD is the primary nuclease responsible for DNA fragmentation in apoptotic cells (63,80). In addition, DFF45/ICAD appears to be cleaved exclusively by caspase-3 (80,118). However, caspase-3 is not active in BNIP3-expressing cells (97), suggesting that BNIP3 initiates an alternative pathway for DNA fragmentation. BNIP3-induced DNA fragmentation may be due to the activation of a nuclease other than DFF40/CAD. Susin et. al. (87) have described a unique DNase sequestered in the mitochondrial intermembrane space that is released into the cytosol upon PT pore opening, which is distinct from DFF40/CAD based on its insensitivity to ATA (aurinetricarboxylic acid), indicating the existence of caspase-3-independent DNases capable of inducing DNA damage during apoptosis. Alternatively, BNIP3 may activate another unidentified caspase capable of cleaving DFF45/ICAD and activating DFF40/CAD. This lead to the hypothesis that caspase-2 may play a role in BNIP3-induced DNA fragmentation.

Using the TUNEL assay, an increase in DNA fragmentation was observed upon co-expression of BNIP3 and caspase-2. In contrast, co-expression of BNIP3 with a catalytically inactive caspase-2 did not effect the percentage of DNA fragmentation, indicating a requirement for caspase-2 activity. Furthermore, Ac-zVAD.fmk and Ac-VDVAD.fmk each reduced the percentage of DNA fragmentation by half in BNIP3-transfected cells. Thus, endogenous caspase-2 is partially responsible for BNIP3-induced DNA fragmentation, although other unidentified caspases may also be involved that are

inhibited by Ac-zVAD.fmk. A similar effect was observed in caspase-2-expressing cells, supporting a role for caspase-2 in apoptotic DNA fragmentation, both directly and indirectly by activating other Ac-zVAD.fmk-sensitive caspases. tBid, the COOH-terminal product of the caspase-8-mediated cleavage of Bid, translocates to the mitochondria where it triggers cytochrome *c* release and apoptosis through the classical Apaf-1/caspase-9/caspase-3 pathway (53,59). Thus, it serves as both a positive control for Ac-zVAD.fmk inhibition, and a negative control for Ac-VDVAD.fmk inhibition. tBid-induced DNA fragmentation was almost completely abolished by Ac-zVAD.fmk, but relatively unaffected by Ac-VDVAD.fmk, indicating the specificity of Ac-VDVAD.fmk for caspase-2-dependent pathways. Caspase-2 has reduced affinity for DXXD-substrates *in vitro* (89,94), but potentially could cleave DFF45/ICAD *in vivo* at caspase-3 target sequences, or at other unique sites under certain conditions. Alternatively, caspase-2 may activate an entirely different nuclease, given that the DNA fragmentation observed in BNIP3-expressing cells is morphologically different than the classical caspase-3-induced DNA fragmentation, displaying fewer and more dispersed foci (97).

Although PT pore opening is the primary mechanism of BNIP3-induced cell death, under conditions in which PT pore opening is blocked, BNIP3 may still induce cell death solely through nuclear damage. However, determining the effect of BNIP3 expression on cells in which PT pore opening is blocked is hindered by the toxicity of PT pore inhibitors (97). In addition, the release of procaspase-2 from the mitochondrial intermembrane space is dependent on PT pore opening (87), thus BNIP3-induced PT pore opening may be linked to the subsequent DNA fragmentation observed at later stages. Once released from the mitochondrial intermembrane space, the activation of

caspase-2 occurs independently of cytochrome *c*, although the mechanism of activation remains unresolved (87). It is not known if caspase-2 is activated during or after release from the intermembrane space, or if it may involve mitochondrial surface proteins or extramitochondrial factors. The presence of BNIP3 in the mitochondrial outer membrane provides an opportunity for physical interaction with procaspase-2 either directly or indirectly, and makes it an ideal candidate for promoting caspase-2 activation independently of cytochrome *c*.

In conclusion, the evidence presented here demonstrates that BNIP3 physically interacts with caspase-2, that overexpression specifically activates endogenous caspase-2, and that co-expression with caspase-2 results in the cooperative enhancement of cell death and DNA fragmentation. In addition, BNIP3-induced DNA fragmentation is efficiently blocked by a caspase-2-specific inhibitor, indicating a specific requirement for endogenous caspase-2. Taken together these findings suggest that BNIP3 initiates cell death and DNA fragmentation by the specific binding and activation of caspase-2. A model for BNIP3-induced cell death could be as follows; an apoptotic stimulus induces integration of BNIP3 into the mitochondrial outer membrane through its TM domain, BNIP3 then initiates PT pore opening, which results in mitochondrial dysfunction as well as the release of intermembrane space proteins including caspase-2. Free caspase-2 may bind to BNIP3, allowing for autocatalytic activation of caspase-2, which could subsequently activate a DNase either directly or indirectly through an effector caspase, ultimately contributing to the demise of the cell.

FUTURE STUDIES

In order to substantiate a role for caspase-2 in a BNIP3-induced DNA fragmentation pathway and to identify other potential caspases involved, the effect of Ac-zVAD.fmk can be further examined. As discussed earlier, caspase-2 activity is minimally effected by Ac-zVAD.fmk (26). Thus, the 50% reduction in DNA fragmentation observed in BNIP3-transfected cells treated with Ac-zVAD.fmk may be due to inhibition of endogenous caspase-2, or may be due to the inhibition of other caspases or proteases more sensitive to Ac-zVAD.fmk. Furthermore, since Ac-VDVAD.fmk also only reduced BNIP3-induced DNA fragmentation by about 50%, it suggests that other caspases may be involved. Treating BNIP3-transfected cells with both Ac-zVAD.fmk and Ac-VDVAD.fmk simultaneously can give some indication as to whether these inhibitors have shared or distinct targets. If a similar 50% reduction in DNA fragmentation were observed with both inhibitors, it would suggest that Ac-zVAD.fmk and Ac-VDVAD.fmk are targeting the same caspases, primarily caspase-2 or caspase-2-like proteases. On the other hand, if there were an additive effect resulting in a complete reduction in DNA fragmentation, it would suggest that Ac-zVAD.fmk and Ac-VDVAD.fmk have distinct cellular targets and that more than one caspase is activated by BNIP3 overexpression. Similarly, the presence or absence of DNA fragmentation in caspase-$2^{-/-}$ mouse embryonic fibroblasts (MEFs) transfected with BNIP3 will further distinguish between caspase-2-dependent and independent pathways. If there is any residual DNA fragmentation observed in caspase$2^{-/-}$ MEFs transfected with BNIP3, it may be blocked by Ac-zVAD.fmk or other specific caspase inhibitors, further supporting a role for other caspases and possibly allowing for further identification of specific caspases. In addition,

the requirement for caspase-2 in BNIP3-induced cell death could also be examined in the knockouts. Also of interest would be to assay for cleavage of golgin 160, the Golgi complex autoantigen that is specifically cleaved by caspase-2 *in vivo* (60), in BNIP3-transfected cells.

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