BNIP3 Heterodimerizes with Bcl-2/Bcl-XL and Induces Cell Death Independent of a Bcl-2 Homology 3 (BH3) Domain at Both Mitochondrial and Nonmitochondrial Sites*

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Reena Ray¶¶¶, Gao Chen†, Christine Vande Velde‡‡, Jeannick Cizeau‡, Jae Hoon Park‡‡‡, John C. Reed¶¶, R. Daniel Gietz§§, and Arnold H. Greenberg¶¶¶ §§

From the ¶¶¶Manitoba Institute of Cell Biology, University of Manitoba, Winnipeg, Manitoba R3E 0V9, Canada, the §Institute for Biological Sciences, Ottawa K1A 0R6, Canada, the ‡‡Department of Pathology, Kyung Hee University College of Medicine, Seoul 130-701, Korea, the ‡Burnham Institute, La Jolla, California 92037-1062, and the §§Department of Biochemistry and Medical Genetics, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba R3E 0W3, Canada

BNIP3 (formerly NIP3) is a pro-apoptotic, mitochondrial protein classified in the Bcl-2 family based on limited sequence homology to the Bcl-2 homology 3 (BH3) domain and COOH-terminal transmembrane (TM) domain. BNIP3 expressed in yeast and mammalian cells interacts with survival promoting proteins Bcl-2, Bcl-XL, and CED-9. Typically, the BH3 domain of pro-apoptotic Bcl-2 homologues mediates Bcl-2/Bcl-XL heterodimerization and confers pro-apoptotic activity. Deletion mapping of BNIP3 excluded its BH3-like domain and identified the NH2 terminus (residues 1–49) and TM domain as critical for Bcl-2 heterodimerization, and either region was sufficient for Bcl-XL interaction. Additionally, the removal of the BH3-like domain in BNIP3 did not diminish its killing activity. The TM domain of BNIP3 is critical for homodimerization, pro-apoptotic function, and mitochondrial targeting. Several TM domain mutants were found to disrupt SDS-resistant BNIP3 homodimerization but did not interfere with its killing activity or mitochondrial localization. Substitution of the BNIP3 TM domain with that of cytochrome b6 directed protein expression to nonmitochondrial sites and still promoted apoptosis and heterodimerization with Bcl-2 and Bcl-XL. We propose that BNIP3 represents a subfamily of Bcl-2-related proteins that function without a typical BH3 domain to regulate apoptosis from both mitochondrial and nonmitochondrial sites by selective Bcl-2/Bcl-XL interactions.

Apoptosis is a genetically encoded program of cell death critical for development and tissue homeostasis as well as a defense against pathogens (1). The core components of the cell death pathway were originally identified in the nematode Caenorhabditis elegans (2). Specifically, three C. elegans gene products are essential: CED-3 and CED-4, which promote apoptosis (3), and CED-9, which inhibits apoptosis (4). In vertebrates, entire gene families have evolved to resemble the C. elegans death genes. The mammalian counterpart of CED-3 is the family of caspases, which are cysteine proteases that cleave after aspartic acid residues (5). Apaf-1 and the recently identified CARD4/Nod1 (6, 7) found in mammalian cells share homology with CED-4 (8). The Bcl-2 family of genes originally identified as repressors of cell death share structural and functional similarity to CED-9 (9–12).

The Bcl-2 family of proteins acts at a central decision point in the apoptotic pathway. The family is divided into two functional groups: 1) anti-apoptotic members (Bcl-2, Bcl-XL, Bcl-1, A1, Bcl-W, and CED-9), which suppress cell death triggered by a diverse array of stimuli, and 2) pro-apoptotic members include Bax, Bak, Bcl-XL, Diva, and Mtd/Bok as well as the BH3-only subfamily described below, which antagonize the activity of pro-survival proteins and promote apoptosis in transfected cells (10–12). The intracellular distribution of these proteins is primarily to the outer mitochondrial membrane, endoplasmic reticulum (ER)1 and nuclear envelope anchored by a COOH-terminal transmembrane (TM) domain (10–12). Other family members remain in the cytosol or loosely associated with the mitochondria until the delivery of a death signal and then translocate (13–17) and integrate into the mitochondrial outer membrane (13–16).

Early models suggested that the balance between pro-apoptotic and anti-apoptotic Bcl-2-related proteins and their propensity to form homo- and heterodimers determined whether a cell lived or died (18). Mice deficient in Bcl-2 exhibit developmental defects characteristic of increased cell death, with the majority dying at a few weeks of age (19). The absence of Bcl-XL as well as its C. elegans orthologue, CED-9, leads to embryonic lethality (4, 20). Sequence alignment of the Bcl-2-related proteins highlights four regions of homology designated Bcl-2 homology domains 1, 2, 3, and 4 (BH1, BH2, BH3, and BH4) (21–24). The tertiary structure of the Bcl-XL monomer reveals the BH1, BH2, and BH3 domains in close proximity to create a hydrophobic pocket (25). Further analysis of Bcl-XL complexed to the Bak BH3 peptide demonstrates electrostatic and hydrophobic interactions between the hydrophobic pocket of Bcl-XL and the amphipathic a-helix of the Bak-BH3 peptide (26). The interactions between pro- and anti-apoptotic members have been extensively detailed in yeast two-hybrid or in vitro binding assays as well as by co-immunoprecipitation (IP) of mem-

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1 The abbreviations used are: ER, endoplasmic reticulum; BH, Bcl-2 homology; CD, conserved domain; FITC, fluorescein isothiocyanate; HSP60, heat shock protein 60; IP, immunoprecipitation; IPed, immunoprecipitated plasmid, endoplasmic reticulum; TM, transmembrane; X-gal, 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside; GST, glutathione S-transferase.
BNIP3 Induces a BH3- and Mitochondria-independent Cell Death

Earlier mutagenesis studies of Bcl-2 and Bcl-XL determined that BH1 and BH2 domains are critical for heterodimerizing with pro-apoptotic molecules Bak and Bak and sustaining their ability to suppress cell death (21, 29). Therefore, Bcl-2 and Bcl-XL mutants that fail to bind Bak or Bak also fail to protect against apoptosis (21, 28). However, mutants of Bcl-XL have been identified that no longer bind to Bak or Bak yet retain their anti-apoptotic activity (29, 30). Similar examples are evident among the pro-apoptotic Bcl-2-related proteins. For example, BH3 mutants of Bak have been identified that are unable to heterodimerize with Bcl-2/Bcl-XL but induce cell death (31–33). The observation of mutants that have lost their ability to heterodimerize yet still retain their cell death function indicates that the two roles of these proteins are separable. Studies in Bcl-2- and Bax-deficient mice also show that these proteins can function independently of one another (34).

The importance of the BH3 domain in mediating heterodimerization with pro-survival proteins and facilitating apoptosis is underscored by the identification of BH3-only containing proteins: Bik, Bik, Hrk, BimL, Bad, Bid, and the C. elegans, EGL-1 (10–12). BNIP3 (formerly called NIP3) is currently classified into this group based on limited homology with the BH3 domain and presence of a COOH-terminal TM domain (10–12, 35). Earlier studies of BNIP3 suggested that the removal of the proposed BH3 domain reduced its ability to interact with Bcl-XL or E1B 19K and resulted in a partial loss of its cell death-inducing activity (36). The pro-apoptotic activity of BNIP3 is also dependent on its TM domain, because its removal completely ablated apoptotic activity (35). Homologues of BNIP3 sharing both structural and functional similarity have been identified in mammals: Nix (also called BNIP3L/BNIP3a/B5) (37–40) and, in C. elegans, ceBNIP3 (41).

In this report, we have characterized the regions within BNIP3 that are required for Bcl-2 family interaction and cell death. We have demonstrated that BNIP3 lacking its BH3-like domain is still able to heterodimerize with Bcl-2, Bcl-XL, or CED-9 and efficiently induce cell death and that this interaction occurs at the NH2 and COOH termini of the BNIP3 protein. Furthermore, although the BNIP3 TM domain targets it to the mitochondrion, the induction of apoptosis is nearly as efficient when BNIP3 is directed to nonmitochondrial sites following TM domain swapping. These data demonstrate that BNIP3 is part of a functionally unique subset of the Bcl-2 family, which also includes Mtd/Bok (42, 43) and Diva (44), that does not require a BH3-like domain to promote cell death at mitochondrial and nonmitochondrial sites.

EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids— Recombinant expression plasmids were constructed as described (35). Briefly, human BNIP3 cDNA was used as template with appropriate primers to incorporate suitable restriction sites at the 5′- and 3′-ends by polymerase chain reaction (PCR) to amplify full-length BNIP3 (1–194) and deletion mutants: BNIP3ΔN (Δ1–49), BNIP3ΔTM2 (Δ164–194), BNIP3ΔC (Δ184–194), and BNIP3ΔNΔTM (Δ1–49, Δ164–194). Following restriction digestion, the inserts were ligated in-frame into modified pcDNA3 (In-vitrogen Corp., San Diego, CA) expression vectors with a 5′- and 3′-end primer of BNIP3 and 5′-and 3′-primer of the entire TM2 cleaved from pCB2 (45). The relative binding affinities and activity of interacting proteins was measured by filter binding colonies followed by X-gal staining or by assaying the hydrolysis of ortho-nitrophenyl β-D-galactopyranoside according to the manufacturer’s instructions.

Transient Transfections, Co-immunoprecipitation, and Western Blot Analysis— Cultures (100 mm) were seeded with 2 × 10^7 293T cells the day prior to transfection. Cells were cotransfected with wild-type or mutant BNIP3 expression plasmids (100 ng) and the wild-type or mutant Bax expression plasmids (100 ng) in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.). Cells were co-transfected with indicated expression plasmids by the calcium phosphate precipitation method. The total amount of DNA was maintained at 15 μg. After 12 h post-transfection, cells were lysed in 1 volume of 0.2% Nonidet P-40 isotonic buffer with freshly added protease inhibitors (100 mM Tris-HCl, 2 mM EDTA, 100 mM NaCl, 0.2% Nonidet-P40, 5 μg/ml apotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml phenylmethylsulfonyl fluoride). Following sonication three times at 10 s, samples were centrifuged at 14,000 rpm for 10 min to remove cellular debris. Lysates were precleared with protein A-Sepharose 4B (Zymed Laboratories Inc., San Francisco, CA) for 30 min and then incubated for 2 h with an equal volume of binding buffer (200 mM NaCl, 2% glycerol, and 0.2% Nonidet-P40) and hamster monoclonal anti-Bcl-2 (Clone 6C8, Pharmingen, Mississauga, ON, Canada) or mouse monoclonal anti-c-Myc (Clone 9E10, gift from Dr. Jim Wright, University of Manitoba, Winnipeg, MB, Canada). Immune complexes were captured with protein A-Sepharose 4B beads for an additional hour, centrifuged, and washed four times in equal volumes of lysis and binding buffer, and then solubilized in SDS loading buffer. Protein samples were analyzed on Laemmli 10–12% SDS-PAGE and immunoblotted with rabbit polyclonal anti-BNIP3, mouse monoclonal anti-Bcl-2 (Clone 4D7, Pharmingen), or rabbit polyclonal anti-Bcl-XL (Transduction Laboratories, Lexington, KY) antibody. The immune complexes were detected by horseradish-conjugated secondary antibody and developed using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).
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In Vitro Transcription/Translation and Co-immunoprecipitation—Expression plasmids encoding BNIP3 and its mutants cloned into pcDNA3 were used as templates for in vitro transcription/translation in the presence of [35S]methionine (Amersham Pharmacia Biotech) by the Tri-T-coupled Reticulocyte Lysate System (Promega, Madison WI) according to the manufacturer’s instructions. For co-IP, equivalent amounts of in vitro protein products were incubated with 3 μg of purified Bcl-2 protein and hamster monoclonal anti-Bcl-2 antibody in 250 μl of 0.2% Nonidet-P40 IP buffer (100 mM Tris-HCl, 2 mM EDTA, 100 mM NaCl, and 0.2% Nonidet-P40) for 2 h at 4° C. Immune complexes were captured with protein A-Sepharose 4B for an additional 90 min, washed four times in excess IP buffer, and resuspended in SDS sample buffer. In vitro protein products or solubilized immune complexes were separated by Laemmli 12% SDS-PAGE. Gels were fixed with 25% 2-propanol and 10% acetic acid for 20 min, followed by an additional 20 min in Amplify solution (Amersham Pharmacia Biotech) and then analyzed by autoradiography. To detect Bcl-2 protein in each co-IP reaction, gels were stained with Coomassie Blue.

Transient Transfections and Apoptosis Assay—Rat-1 and Rat-1/Bcl-2 fibroblasts or MCF-7 breast carcinoma cells were transfected as described (35) with pcDNA3 constructs T7-BNIP3 and T7-BNIP3ΔBH3. Cells were fixed with 4% formaldehyde and then stained with mouse monoclonal anti-IT (Novagen, Madison, WI) followed by FITC-conjugated goat anti-mouse (Sigma-Aldrich Corp.). Transfected apoptotic cells were detected by altered nuclear morphology followed by Hoechst dye staining. In total, 200–300 transfected cells/sample were evaluated using a Zeiss Axiohot microscope. For the β-galactosidase cell death assay, 1 × 10^5 293T cells seeded in 6-well 35-mm plates were co-transfected with 0.75 μg of indicated expression plasmid and 0.2 μg of pcDNA3-β-galactosidase in duplicate using the LipofectAMINE reagent (Life Technologies, Inc.). Cells were then fixed in 0.2% glutaraldehyde and washed three times with 0.1 M phosphate-buffered saline and stained in X-gal buffer as described to detect β-galactosidase expression (49). The percentage of dying cells was calculated by assessing the number of rounded, condensed, blue cells in the total population of flat, blue cells.

Subcellular Localization of BNIP3 Chimeric Proteins—MCF-7 breast carcinoma cells were transfected with indicated expression plasmids using LipofectAMINE reagent for 5 h, and then fixed in 4% formaldehyde for 24 h post-transfection. Cells were co-stained with mouse monoclonal anti-BNIP3 antibody to detect expression of BNIP3, BNIP3-BclTM, and BNIP3-Ch5TM and rabbit polyclonal anti-heat shock protein 60 (HSP60) antibody (gift from Dr. Radney Gupta, McMaster University, Hamilton, ON, Canada) and visualized by goat anti-mouse Cy3-conjugated antibody (Bio-Rad Inc., Mississauga, ON, Canada) and goat anti-rabbit FITC-conjugated antibody using a confocal fluorescence microscope equipped with an argon laser and dual detectors (Molecular Dynamics, Sunnyvale, CA). The images were acquired with Image Space software using a 60× objective lens and transferred to graphics program for printing.

RESULTS

Heterodimerization of BNIP3 with Bcl-2, Bcl-XL, and CED-9—BNIP3 was originally identified in a yeast two-hybrid screen using E1B 19K as bait (50). Subsequently, it was shown that BNIP3 interacts with Bcl-2 (50) and Bcl-XL (36). Using the yeast two-hybrid system we have been able to confirm these interactions and have shown interaction of BNIP3 with CED-9 (Table I). Yeast transformants co-expressing BNIP3 with Bcl-2, Bcl-XL, or CED-9 activated both the lacZ and HIS3 reporter genes, respectively, as determined by relative β-galactosidase expression and growth in the absence of histidine. There was no detectable interaction between BNIP3 and two heterologous proteins, indicating that BNIP3 heterodimerization with these cell death repressors is specific (Table I).

To confirm the interactions in vitro, 293T cells were transiently co-transfected with plasmids expressing BNIP3 and Bcl-2 or Bcl-XL (Fig. 1). Following IP for Bcl-2 or Bcl-XL respectively, BNIP3 co-IPed with both Bcl-2 and Bcl-XL as a dimer and as a monomer (Fig. 1, B, lane 1 and C, lane 1). Similarly, IP of lysates prepared from transiently co-transfected cells expressing BNIP3 and CED-9 revealed that BNIP3 also interacts with CED-9.2 There was no specific signal detected on Western blots if BNIP3 was expressed alone, indicating that the IP of BNIP3 in co-transfected cells was due to interaction with Bcl-2 or Bcl-XL. Detergents used to solubilize mammalian cell membranes have been reported to facilitate dimerization between Bcl-2-related proteins (51, 52). This “detergent effect” was not observed to facilitate BNIP3 interaction with Bcl-2.2 Thus, BNIP3 interacts with Bcl-2, Bcl-XL, and CED-9 in both yeast and mammalian systems.

BNIP3 Lacks a BH3 Domain That Facilitates Heterodimerization with Bcl-2, Bcl-XL, and CED-9—To date, the majority of pro-apoptotic Bcl-2-related proteins interact with their death-repressing partners through the BH3 domain, which contains an eight-amino acid residue core with conserved Leu and Asp residues at positions 1 and 6, respectively, that are critical for heterodimerization (53). Sequence analysis of BNIP3 identified a BH3-like motif in which the core residues, Leu^110 and Asp^115, and flanking residues, Val^106 and Ile^117, are conserved with critical amino acids of the Bak BH3 domain that determine Bak-Bcl-XL heterodimerization (26). To evaluate the role of the BH3-like domain in BNIP3 heterodimerization with cell death repressors, we constructed a 16-amino acid deletion mutant to encompass the flanking and most conserved residues of the BH3 domain and Bcl-2, Bcl-XL, and CED-9 in both yeast and mammalian systems.

| Transformation (BD:AD) | β-Galactosidase activity | HIS3 activation |
|------------------------|--------------------------|----------------|
| BNIP3:Bcl-2            | 10.9                     | ++            |
| BNIP3:Bcl-XL           | 15.9                     | ++            |
| BNIP3:CED-9            | 13.0                     | ++            |
| BNIP3:MK               | 1.3                      | -             |
| BNIP3:PTP2             | 1.5                      | -             |
| *BNIP3:BD              | 1.4                      | -             |
| *Bcl-2 (AD)            | 0.5                      | -             |
| *Bcl-XL (AD)           | 0.7                      | -             |
| *CED-9 (AD)            | 0.3                      | -             |

2R. Ray and A. H. Greenberg, unpublished observations.

To verify that BNIP3ΔBH3 exhibits similar interactions in mammalian cells, 293T cells were transiently co-transfected with plasmids expressing BNIP3ΔBH3 and Bcl-2 or Bcl-XL. The co-IP reactions were prepared and analyzed by Western blotting as described. Similar to wild type BNIP3, BNIP3ΔBH3 co-IPed with Bcl-2 (Fig. 1B, lanes 1 and 5), Bcl-XL (Fig. 1C, lanes 1 and 5), and CED-9 as a dimer. This confirmed the interactions observed in the yeast two-hybrid and in vitro co-IP assays.

BNIP3 Requires the NH2 Terminus to Interact with Bcl-2 and
Bcl-X\(_L\)—The exclusion of a BH3-like domain mediating BNIP3 heterodimerization suggested that other regions must promote interaction with Bcl-2-related cell death agonists. To map these regions, we generated a series of deletion mutants encompassing the NH\(_2\) terminus (residues 1–49) and COOH terminus (residues 164–194), as well as the region of shared identity between BNIP3, Nix, and ceBNIP3 (CD, conserved domain; BNIP3 residues 112–130) (Fig. 1A). 293T cells were transiently co-transfected with either wild type BNIP3 or its deletion mutants and Bcl-2 or Bcl-X\(_L\) (Fig. 1, B and C). Following IP of Bcl-2 or Bcl-X\(_L\) respectively, BNIP3 was readily detected by Western blotting (Fig. 1, B, lane 6, and C, lane 6). The mutants BNIP3\(_{\Delta T M1}\), BNIP3\(_{\Delta T M2}\), and BNIP3\(_{\Delta N}\) co-IPed with Bcl-X\(_L\) (Fig. 1C, lanes 3, 4, and 7) but not with Bcl-2 (Fig. 1B, lanes 3, 4, and 7). Thus, the removal of the NH\(_2\) terminus of BNIP3 prevented Bcl-2 but not Bcl-X\(_L\) heterodimerization. We subsequently determined that removal of both the NH\(_2\) terminus and the TM domain (BNIP3\(_{\Delta N;\Delta T M}\)) were necessary to prevent BNIP3 heterodimerization with Bcl-X\(_L\) (Fig. 1C, lane 8).

The direct role for the NH\(_2\) terminus in BNIP3 heterodimerization is further demonstrated by interaction of in vitro transcribed/translated \(^{35}\)S-labeled BNIP3 or its mutants with purified Bcl-2 protein. Following Bcl-2 IP, the immune complexes were separated by SDS-PAGE. Co-IP of BNIP3 or its mutants was detected by autoradiography. The dimeric (*) and monomeric (+) forms of BNIP3 and its deletion mutants are indicated. BNIP3 migrates as a 60-kDa dimer and a 30-kDa monomer determined from the molecular mass standard (\(M_r\)).

Table II

| Transformation (BD:AD) | HIS3 activation |
|------------------------|----------------|
| CED-9:BNIP3\(_{\Delta BH3}\) | ++ + |
| Bcl-2:BNIP3\(_{\Delta BH3}\) | ++ + |
| Bcl-X\(_L\):BNIP3\(_{\Delta BH3}\) | + + + |
| CED-9:pACTII | - |
| Bcl-2:pACTII | - |
| Bcl-X\(_L\):pACTII | - |
| pGBT9:BNIP3\(_{\Delta BH3}\) | - |

Yeast two-hybrid interaction of BNIP3 lacking its BH3-like domain with Bcl-2-related proteins

Yeast strain KGY37 was co-transformed with indicated GAL4 binding domain (BD) and activating domain (AD) constructs. The relative level of activation for the HIS3 reporter gene was determined by growth (+) or no growth (−) on selection medium lacking tryptophan, leucine, and histidine in the presence of 1 mM 3-amino-1,2,4-triazole.
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Fig. 2. BNIP3 lacking its BH3-like domain induces cell death. Rat-1 fibroblasts (A), MCF-7 breast carcinoma cells (B), and Rat-1 fibroblasts constitutively expressing Bcl-2 (C) were transiently transfected with T7-BNIP3 (∙) or T7-BNIP3ΔBH3 (○). At the indicated time points, cells were fixed and stained with mouse monoclonal anti-T7 antibody followed by FITC-conjugated secondary antibody to detect transfected cells. Apoptotic cells were evaluated by nuclear morphology following Hoechst dye staining.

Complexes were separated by SDS-PAGE and analyzed by autoradiography. Both BNIP3ΔBH3 and BNIP3ΔCD co-IPed with Bcl-2 protein (Fig. 1D, lanes 3 and 4), thus excluding the central region of BNIP3 encompassing the BH3-like domain and conserved domain in heterodimerization. BNIP3ΔTM2 also co-IPed with Bcl-2 (Fig. 1D, lane 2). In contrast, removal of NH2-terminal sequences (BNIP3ΔN) or both the NH2 terminus and TM domain (BNIP3ΔN;ΔTM) disrupted interaction with Bcl-2 (Fig. 1D, lanes 5 and 6). The observed interactions were specific, because BNIP3 or its mutants were not detected when co-IPed with anti-Bcl-2 antibody and a nonspecific protein (not shown). Equal loading of Bcl-2 for each co-IP reaction was confirmed by Coomassie Blue staining of SDS-polyacrylamide gels (not shown).

BNIP3 Lacks a BH3 Domain That Induces Cell Death—
BNIP3 transiently expressed in several cell lines induces cell death (35, 36). The pro-apoptotic activity of several Bcl-2 homologues such as Bik, Bik, Hrk, BimL, Bad, Bid, and EGL-1 (10–12) is conferred by the BH3 domain. To determine whether the BH3-like domain of BNIP3 plays a similar role, the cell death activity of wild type BNIP3 was compared with BNIP3ΔBH3. Two cell lines, Rat-1 fibroblasts and MCF-7 breast carcinoma cells were transiently transfected with T7-epitope tagged BNIP3 or BNIP3ΔBH3. At the indicated time points, cells were stained with anti-T7 antibody, followed by FITC-conjugated secondary antibody to identify transfected cells and Hoechst dye to assess apoptotic cells by nuclear chromatin condensation. Rat-1 fibroblasts became apoptotic by 12 h following BNIP3 or BNIP3ΔBH3 transfection and reached a peak of 55 and 50%, respectively at 36 h, although BNIP3ΔBH3 apoptosis was reduced somewhat at earlier time points (Fig. 2A). Apoptotic MCF-7 cells expressing BNIP3 or BNIP3ΔBH3 was detected by 12 h, reaching a maximum by 36 h with BNIP3ΔBH3 exhibiting no difference except at the 36-h time point (Fig. 2B). The experiment was repeated in 10T1/2 fibroblasts, where no difference was observed between BNIP3 and BNIP3ΔBH3 using a β-galactosidase cell death assay.

The pro-apoptotic activity of BH3-only proteins, Bik, Bik, Hrk, and BimL, is blocked by simultaneous expression with Bcl-2/Bcl-XL (54–58). In BNIP3-induced cell death, Bcl-2 overexpression initially delays the onset of apoptosis, but the resistance is overcome in time (35). To determine the effect of Bcl-2 overexpression on BNIP3ΔBH3-induced cell death compared with wild type BNIP3, Rat-1/Bcl-2 fibroblasts were transiently transfected with T7-BNIP3 or T7-BNIP3ΔBH3. Although the appearance of BNIP3 and BNIP3ΔBH3 apoptotic cells were delayed in Rat-1 fibroblasts expressing Bcl-2 (Fig. 2C), compared with the parental cell line (Fig. 2A), there was no observable difference between BNIP3 and BNIP3ΔBH3 apoptotic activity (Fig. 2C). Using the β-galactosidase assay, BNIP3 and BNIP3ΔBH3 induced similar levels of cell death in 10T1/2 fibroblasts overexpressing Bcl-XL (38).

BNIP3 TM Domain Mutants That Disrupt SDS-resistant Homodimerization Induce Cell Death—The TM domain of BNIP3 has two identified roles: 1) it targets BNIP3 to the mitochondria (35, 36) and 2) it mediates homodimerization (35). Furthermore, the pro-apoptotic activity of BNIP3 requires the TM domain because its removal abrogates both cell death activity and the ability to homodimerize (35). The BNIP3 homodimer is unusual in that it is resistant to reduction, suggesting that it may have a functional role in cell death (35). To determine whether TM domain-mediated homodimerization is necessary for BNIP3-induced apoptosis, we generated several TM domain deletion and point mutants (Fig. 3A). Following expression by coupled in vitro transcription/translation, BNIP3 and its mutants were analyzed for dimerization under reducing and nonreducing conditions. The predicted molecular mass of BNIP3 is 21.4 kDa; however, wild type BNIP3 migrates anomalously as a 60-kDa dimer and as a 30-kDa monomer in Laemmli buffer (Fig. 3B, lanes 1 and 2). The deletion mutants BNIP3ΔC, BNIP3Δ179–185 and BNIP3TM2 and point mutants, BNIP3(L179→S) and BNIP3(G180→E) were expressed only as a monomer under both reducing and nonreducing conditions (Fig. 3B, lanes 3–12).

The apoptotic activity of the BNIP3 TM domain mutants was determined by transient co-transfection with a β-galactosidase reporter in 293T cells. Following 25 h post-transfection, cells were stained for β-galactosidase expression, and dying cells were enumerated in the transfected population. The expression of BNIP3 resulted in approximately 35% cell death. The killing activity of the TM domain mutants ranged from 20% for BNIP3(L179→S) to 29% for BNIP3(G180→E), in contrast to 8% cell death in BNIP3ΔTM2 or control vector transfected cells (Fig. 4). The differences in killing activity of the various mutants were not due to variable levels of protein expression as verified by Western blotting. All of the mutants expressed at levels comparable with that of wild type BNIP3 (not shown). Similarly, Rat-1 fibroblasts transiently transfected with the same series of BNIP3 TM domain mutants underwent cell death in a similar manner. Although some of the in vitro transcribed/translated BNIP3 mutants did not retain their ability to homodimerize following SDS-PAGE, they were all capable of inducing cell death in transient assays.

Mitochondrial and Nonmitochondrial Targeting Forms of BNIP3 Induce Cell Death—The COOH-terminal TM domain of Bcl-2 family proteins targets both anti- and pro-apoptotic members to various intracellular membranes (10–12). Bcl-2 localizes primarily to the outer mitochondrial membrane as well as the ER and nuclear envelope (59–61). Studies have shown that restricted expression of Bcl-2 to the mitochondria or ER enhances or attenuates its protective effects depending upon cell type and apoptotic signal (62, 63). To address the physiological relevance of BNIP3 membrane targeting, its TM domain was
substituted with heterologous TM domain sequences from Bcl-2 and cytochrome b₅ (Fig. 5A). The cytoplasmic region of BNIP3 (residues 1–163) was fused to 21 amino acid residues of the Bcl-2 TM domain, which is sufficient to target heterologous proteins to the outer mitochondrial membrane orienting the protein toward the cytosol (64, 65). Similarly, the COOH-terminally truncated BNIP3 was fused to a 35-amino acid residue segment of rat hepatic cytochrome b₅, which has been previously shown to target heterologous proteins to the cytoplasmic face of the ER in transfected cells (62, 66).

To determine the subcellular localization of BNIP3 chimeric proteins, MCF-7 cells were transfected with wild type BNIP3, BNIP3-BclTM, and BNIP3-Cb5TM. The BNIP3 chimeric proteins were detected by staining with mouse monoclonal anti-BNIP3 antibody, followed by Cy3-conjugated secondary antibody. The cells were co-stained for HSP60, a mitochondrial matrix protein (67), and a FITC-conjugated secondary antibody. The staining pattern of BNIP3 and BNIP3-BclTM (Fig. 5, B and D) resembled the punctuate mitochondrial distribution of HSP60 (Fig. 5, C and E). In contrast, BNIP3-Cb5TM showed a globular staining pattern (Fig. 5F) distinct from the localization of HSP60 (Fig. 5G). Using confocal laser microscopy (not shown), overlay of the red and green fluorescent images of BNIP3 and HSP60, respectively, showed a uniform yellow staining pattern indicative of virtually complete coincidence of the two stains. The staining pattern of BNIP3-BclTM did not completely coincide with HSP60, as indicated by partial yellow fluorescence in the overlay image of BNIP3-BclTM and HSP60. This suggests that the Bcl-2 TM domain is targeting BNIP3 to the mitochondria and other areas consistent with reports of Bcl-2 localizing to the mitochondria, ER, and nuclear envelope (59–61). In contrast, the red fluorescence of BNIP3-Cb5TM did not co-localize with the green fluorescence of HSP60 (Fig. 5D) thus was not targeted to the mitochondria.

**FIG. 3.** *In vitro* expression and homodimerization of BNIP3. A, schematic representation of BNIP3 TM domain deletion and point mutants. B, BNIP3 and its mutants were expressed by coupled *in vitro* transcription/translation. The 35S-labeled products were separated on SDS-PAGE under reducing (R) and nonreducing (NR) conditions and detected by autoradiography. BNIP3 migrates as a dimer and as a monomer (arrows). The remaining mutants were expressed only as a monomer. The molecular mass standard (Mₖ) is indicated on the right.

**FIG. 4.** BNIP3 TM domain mutants induce cell death. 293T cells (1 × 10⁵/35-mm plate) were co-transfected with 0.75 μg of pcDNA3 (Control) or pcDNA3 expressing the indicated BNIP3 mutant plus 0.2 μg of pcDNA3-β-galactosidase. 25 h post-transfection, cells were stained for β-galactosidase expression, and the percentage of dying cells was quantified in the transfected population. The results shown represent the means ± S.D. from replicate experiments.

**FIG. 5.** Heterologous TM domains target BNIP3 to mitochondrial and nonmitochondrial sites. A, the COOH-terminal amino acid sequences of BNIP3 and chimeric proteins are shown. The sequences of Bcl-2 and cytochrome b₅ (bold) are shown with the hydrophobic TM domain (underlined). The arrowhead indicates the fusion junction between BNIP3 and the heterologous TM domain. MCF-7 cells were transfected with BNIP3, BNIP3-BclTM, or BNIP3-Cb5TM. Cells were co-stained with anti-BNIP3 and anti-HSP60 antibodies and then visualized by Cy3 and FITC-conjugated antibodies. The staining pattern for BNIP3 (B) and BNIP3-BclTM (D) resembles the punctate mitochondrial staining pattern characteristic of HSP60 in corresponding cells (C and E). BNIP3-Cb5TM shows a globular staining pattern (F) distinct from the distribution of HSP60 (G).
FIG. 6. BNIP3 induces cell death from mitochondrial and nonmitochondrial sites. 293T or MCF-7 cells were co-transfected with BNIP3, BNIP3-BclTM, or BNIP3-Cb5TM and β-galactosidase reporter as described. The results of cell death are representative of the means ± S.D. of replicate experiments.

To date, the pro-apoptotic activity of BNIP3 and its homologues has been shown to require TM domain-mediated mitochondrial targeting (35, 38). To determine whether BNIP3 targeted by Bcl-2 or cytochrome b$_5$ TM domain sequences is able to heterodimerize with Bcl-2, BNIP3-BclTM, or BNIP3-Cb5TM and the β-galactosidase reporter. Following 24 h post-transfection, cells were stained for β-galactosidase expression, and dying cells were enumerated in the transfected population. The expression of BNIP3 resulted in approximately 47% cell death in comparison with 50 and 27% induced by BNIP-BclTM and BNIP3-Cb5TM, respectively. In contrast, the removal of the BNIP3 TM domain diminished its cell death activity to 8% (Fig. 6). Similar transfections in MCF-7 cells confirmed these findings, showing little difference between cell death induced by each of the two mutants and wild type BNIP3 (Fig. 6). The level of protein expression was determined by Western blotting and found to be equivalent for all constructs (not shown).

Mitochondrial and Nonmitochondrial Localized Forms of BNIP3 Interact with Bcl-2 and Bcl-X$_L$—BNIP3 heterodimerizes with Bcl-2 and Bcl-X$_L$ both by in vitro and in vivo co-IP as well as in the yeast two-hybrid system. Our findings consistently exclude the region proposed to be a BH3 domain (36) and support the role for the NH$_2$ terminus mediating BNIP3 heterodimerization. We repeated the in vitro and in vivo co-IP studies using BNIP3 substituted with heterologous TM domains to determine the following: 1) whether BNIP3 targeted to various subcellular sites is able to heterodimerize with Bcl-2 and Bcl-X$_L$ and 2) whether BNIP3 heterodimerization is independent of its TM domain. In vitro transcription/translation products of BNIP3 and its chimeric proteins were incubated with purified Bcl-2. Following Bcl-2 IP, both BNIP3-BclTM and BNIP3-Cb5TM were detected in the immune complexes (Fig. 7A, lanes 3 and 4). The observed interactions were specific because no co-IP was apparent when labeled products were incubated with a nonspecific protein and IPed with anti-Bcl-2 antibody (not shown). Equal amounts of Bcl-2 protein in each co-IP reaction was confirmed by Coomassie Blue staining (not shown). Similar interactions were observed in 293T cells co-transfected with BNIP3-BclTM or BNIP3-Cb5TM and Bcl-X$_L$. Co-IP reactions were prepared and analyzed by Western blotting as described. BNIP3 fused to either the Bcl-2 or cytochrome b$_5$ TM domain sequence co-IPed with Bcl-X$_L$ (Fig. 7B, lanes 3 and 4).

FIG. 7. BNIP3 substituted with heterologous TM domains interacts with Bcl-2 and Bcl-X$_L$. A. Equivalent amounts of $^35$S-labeled BNIP3, BNIP3-BclTM, or BNIP3-Cb5TM was incubated with purified Bcl-2 protein. Following Bcl-2 IP, the immune complexes were separated by SDS-PAGE. Co-IP of BNIP3 or its mutants was detected by autoradiography. B, in vivo co-IP was repeated whereby Bcl-X$_L$ was co-expressed with BNIP3, BNIP3-BclTM, or BNIP3-Cb5TM in 293T cells. Following IP with mouse anti-c-Myc antibody, the BNIP3 chimeric proteins were detected by immunoblotting with rabbit anti-BNIP3 antibody. Total cell lysates were analyzed by immunoblotting with rabbit anti-BNIP3 (middle panel) or rabbit anti-Bcl-X$_L$ (bottom panel) antibody. The dimeric (*) and monomeric (+) forms of BNIP3 and molecular mass standard ($M_r$) are indicated.

DISCUSSION

In the present study, we have delineated regions within BNIP3 that are responsible for homo- and heterodimeric interactions and for inducing cell death. The BNIP3 TM domain targets the protein to the mitochondria and is necessary for its apoptotic function as well as homodimerization (35). However, we show here that the strong homologous interaction based on resistance to SDS-reduction is not required for promoting cell death. Even the substitution of the BNIP3 TM domain with heterologous TM domain sequences, which targets the protein to mitochondrial and nonmitochondrial sites, did not significantly affect its pro-apoptotic activity. This indicates that BNIP3 can initiate apoptosis from nonmitochondrial sites and that the NH$_2$ terminus of the protein is critical to its apoptotic function. BNIP3 shares limited homology with the Bcl-2 family in the BH3 domain, a region that appears to be necessary for BH3-only proteins to interact with anti-apoptotic Bcl-2 homologues and to regulate apoptosis. Removal of the BH3-like domain in BNIP3 does not disrupt its ability to heterodimerize or to induce cell death. Thus, regions in both the NH$_2$ terminus and TM domain of BNIP3 are required or are sufficient for interaction with Bcl-2 and Bcl-X$_L$.

Bcl-2 and its homologues form homodimers that regulate their pro- or anti-apoptotic function (18). Yeast two-hybrid and in vitro binding assays demonstrate that Bcl-2 forms homodimers through a head-to-tail association in which the BH4 domain of one monomer interacts with domains BH1 and BH2 of another monomer (68). Mutations involving any of these three domains prevent the Bcl-2 mutant protein from forming homodimers, although they can still bind to endogenous Bcl-2 protein forming mutant/wild type heterodimers (69). These mutants are also deficient in anti-apoptotic function in mammalian cells (70). In contrast, homodimerization of Bcl-X$_L$ has not been observed by the yeast two-hybrid assay (28, 71), co-IP (72), or structural analysis (25). Among the pro-apoptotic members, homodimeric interactions have been most systematically
defined for Bax, which interacts homologously through its BH3 domain (24, 33). Specific BH3 mutants of Bax do not homodimerize but retain their ability to induce cell death, confirming that its pro-apoptotic function does not depend on homodimerization (33). To date, homodimeric interactions have not been noted among the BH3-only proteins. Homodimerization of BNIP3, as well as its homologues, Nix and ceBNIP3, occurs exclusively through the TM domain, which is also critical for targeting BNIP3-related proteins to the mitochondria and their pro-apoptotic activity (35, 38). The removal of the TM domain shifts the distribution of BNIP3, Nix, and ceBNIP3 to the cytosol and ablates their cell death activity as well as their ability to homodimerize (35, 38). The mutant BNIP3ΔC lacking the last 10 amino acids of BNIP3 was observed to interact homologously in the yeast two-hybrid assay. Yet, specific BNIP3 TM domain mutants described in this study, including BNIP3ΔC, were no longer able to form homodimers as determined by SDS-PAGE but localized to the mitochondria and induced cell death. Furthermore, the chimeric protein BNIP3-BclTM, which does not form SDS-resistant homodimers, localizes primarily to the mitochondrial membrane and induces cell death as efficiently as wild type BNIP3. The TM domain of Bcl-2 is not predicted to promote dimerization. Therefore, homodimerization of BNIP3 is not required for cell death induction in mammalian cells.

Among Bcl-2 family members, the COOH-terminal TM domain targets proteins to their correct subcellular site(s) and facilitates membrane association and/or integration critical to their function (10–17). Deletion of the Bcl-2 TM domain not only alters its distribution but also dramatically reduces its anti-apoptotic activity (73, 74). Similarly, removal of the Bax TM domain prevents it from being targeted and integrated into the mitochondrial membrane in response to an apoptotic signal (13). The function of both proteins can be restored by substituting heterologous TM domain sequences (14, 62, 63, 75). Similar to Bcl-2-related proteins, BNIP3 has a COOH-terminal TM domain that targets the protein primarily to mitochondria. The TM domain of BNIP3 and Nix share 80% identity. Apart from localization to the mitochondria, Nix has also been detected in the ER and nuclear envelope (40). Based on the high degree of homology between BNIP3 and Nix TM domain sequences, BNIP3 may also be associated with these other intracellular membranes in much smaller quantities. BNIP3 has been reported to localize to the nuclear envelope when co-expressed with E1B 19K (50). The substitution of heterologous TM domain sequences into BNIP3 altered its protein distribution, yet it did not lead to loss of function. BNIP3 expressed from mitochondrial and nonmitochondrial sites using the Bcl-2 TM domain induced apoptosis as efficiently as wild type BNIP3, whereas cytochrome b6 TM domain targeted BNIP3 induced apoptosis to a slightly lower level. This demonstrates that both chimeric proteins are functional, and the substitution of the BNIP3 TM domain did not result in the ablation of essential sequences. Thus, the first 163 amino acids of BNIP3 are sufficient for pro-apoptotic function, provided it is fused to a heterologous TM domain. The TM domain of BNIP3 is sufficient to target heterologous proteins to the mitochondria (36). At the mitochondria, we have recently found that BNIP3 is fully integrated into the membrane in cells undergoing apoptosis following protein overexpression based on resistance to alkali elution. Therefore, the primary role of the BNIP3 TM domain appears to be protein targeting and membrane association.

The susceptibility of a cell to apoptotic signals is in part regulated by relative levels and competing interaction between death suppressing and death promoting Bcl-2 family members (11). The BH3 domain of pro-apoptotic proteins plays a dual role by promoting heterodimerization with cell death suppressors and inducing cell death (53). Among the majority of Bcl-2 pro-apoptotic homologues characterized to date, these two functions appear to be inseparable. The heterodimeric interactions between BNIP3 and Bcl-2/Bcl-XL would suggest that Bcl-2/Bcl-XL could suppress the death inducing activity of BNIP3. Our studies have shown that the overexpression of Bcl-2 or Bcl-XΔC delays the onset of BNIP3-, Nix-, or ceBNIP3-induced cell death but does not completely block it except at very high levels (35, 38, 39). This suggests that BNIP3 and its homologues may not heterodimerize or induce apoptosis similar to other BH3-containing proteins. Previous reports demonstrated that substitution of the BNIP3 BH3-like domain into Bax facilitates heterodimerization with Bcl-XL and promotes apoptosis (36). However, Bax contains both BH1 and BH2 domains, which permits its BH3 domain to exist in a buried or exposed conformation (53). Therefore, the BNIP3 BH3-like domain in the context of Bax would be subject to conformational changes that do not normally affect its function as a part of BNIP3. Our findings indicate BNIP3 lacks a BH3 domain that would mediate heterodimerization with Bcl-2, Bcl-XΔC, or CED-9 and promote cell death, even in the presence of constitutively expressed Bcl-2/Bcl-XL. Although residues Leu110 and Asp115 are conserved in the core of the BH3-like domain in BNIP3, additional residues G and E at positions 5 and 7, respectively, found in the BH3-only proteins with a COOH-terminal TM domain are not conserved in BNIP3. Moreover, secondary structural analysis of the BNIP3 BH3-like domain shows that it is not compatible with an amphipathic a-helix. Two new Bcl-2 homologues have been identified, Mtd/Bok (42, 43) and Diva (44), in which the BH3 domain does not play its characteristic dual role in heterodimerization and apoptosis. Mtd/Bok interacts through the BH3 domain with selective anti-apoptotic proteins, Mcl-1, Bcl-1, and BHRF-1, yet the BH3 domain is not required for its apoptotic activity as shown by deletion analysis and characterization of a splicing variant of Bok lacking the BH3 domain (42, 43, 76). Diva shows limited homology within the BH3 domain. It induces apoptosis and heterodimerizes with vBcl-2, a homologue of Bcl-2 encoded by the Kaposi’s sarcoma-associated herpesvirus in a BH3-independent manner (44). Additionally, mutational analysis of Bik suggests that its BH3 domain is insufficient for heterodimerization and that other flanking regions are involved (77).

Two other findings support our observations that heterodimerization does not occur through the BH3-like domain of BNIP3 and the hydrophobic pocket created by domains BH1, BH2, and BH3 of Bcl-2/Bcl-XΔC. In the yeast two-hybrid assay, we observed that BNIP3 interacts strongly with Bcl-XΔC. Bcl-XΔC is a splicing variant of Bcl-XΔC in which a stretch of 62 amino acids including the BH1 and BH2 domains is not present (72), therefore excluding their involvement in forming BNIP3-Bcl-XΔC heterodimers. Heterodimerization of Bcl-XΔC has been evaluated for only selected pro-apoptotic Bcl-2 homologues. Both Bak and Bax are unable to interact with Bcl-XΔC (78). Among the BH3-only proteins co-expressed with Bcl-XΔC, Bik heterodimerizes (54), whereas Hrk and Bad demonstrate no interaction (57, 59). Earlier studies by Boyd et al. (50) mapped two regions within Bcl-2 based on homology to E1B 19K required for heterodimerization with BNIP3. The first segment found within the loop region of Bcl-2 spans amino acid residues 42–48 in which Ala42 and Pro44 are conserved with Bcl-XΔC/Bcl-XΔC. The loop region of Bcl-2/Bcl-XΔC is not present in CED-9. The second region of Bcl-2 (residues 106–115) immediately follows

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the BH3 domain and precedes the third α-helical region in which Tyr<sup>107</sup>, Arg<sup>108</sup>, Arg<sup>109</sup>, and Phe<sup>111</sup> are conserved with Bcl<sub>X<sub>L</sub></b>-Bcl<sub>X<sub>L</sub></b> and Glu<sup>113</sup> is similar to Asp<sup>107</sup> of Bcl<sub>X<sub>L</sub></b>-Bcl<sub>X<sub>L</sub></b>. In the second interaction site, only Phe<sup>132</sup> of CED-9 is conserved with Phe<sup>111</sup> of Bcl-2. Taken together, the BH domains are not involved in heterodimeric interactions between BNIP3 and Bcl-2-Bcl-X<sub>L</sub>.

Further mapping studies of BNIP3 elucidated two regions of interaction with Bcl-2 and Bcl-X<sub>L</sub>, the NH<sub>2</sub>-terminal (residues 1–49), and the TM domain. In the <i>in vitro</i> co-IP assay, removal of the BNIP3 TM domain or substitution with heterologous TM domain sequences did not interfere with Bcl-2 heterodimerization. Yet when BNIP3 lacking its TM domain was co-expressed with Bcl-2 in 293T cells, an interaction between these two proteins was not detected. Similarly, BNIP3 substituted with a heterologous TM domain did not interact with Bcl-2 when co-expressed in 293T cells and co-IPed. Therefore, the interaction through the BNIP3 TM domain with Bcl-2 may facilitate the NH<sub>2</sub>-terminal interaction when the proteins are associated with membranes but occur independent of the TM domain <i>in vitro</i>. In contrast, either the NH<sub>2</sub>-terminal or TM domain of BNIP3 interacts with Bcl-X<sub>L</sub>. The observation that both BNIP3 chimeric proteins interact with Bcl-2 and Bcl-X<sub>L</sub> suggests that substitution of its TM domain does not lead to protein misfolding. Therefore, BNIP3 expressed from various subcellular sites is accessible to a common set of cellular components that can interact with and/or modify BNIP3. The C. elegans orthologue of BNIP3, cepBNIP3, interacts with CED-4<sup>2</sup> and has been reported to bind simultaneously to CED-9 and pro-CED-3 forming a ternary complex (41). Bap31, an ER pro-apoptotic protein interacts with Bcl-2 through an NH<sub>2</sub>-terminal TM domain and COOH-terminal sequences (80, 81). It also interacts with a CED-4-like protein and pro-caspase 8 (80). Although the functional significance of these complexes has not been determined, it does suggest a possible mechanism to regulate apoptosis.

Many proteins involved in regulating apoptosis reside on the mitochondria and ER. In some apoptotic signaling pathways, mitochondria play a central role whereby the release of cytochrome c (82), and the disruption of mitochondrial membrane potential (83) leads to caspase activation. Alternative cell death pathways bypass the mitochondria and caspase activation (84). The role of the ER in apoptosis is less well defined. The ER plays a role in regulating apoptosis by blocking the release of cytochrome c and suppressing changes in mitochondrial membrane permeability (87, 88). At the ER, Bcl-2 regulates the release of calcium from intracellular stores (89, 90). The observation that BNIP3 induces apoptosis from mitochondrial and nonmitochondrial sites suggests that the pro-apoptotic function of BNIP3 can be localized to different spatial areas within a cell. When a cell receives a death signal, proteins such as Bap31 and BNIP3 may exert a co-operative effect from the different membrane sites by forming complexes with surrounding proteins including Bcl-2 family members and those in the cytosol. Alternatively, these proteins may respond differently to various apoptotic stimuli but still play an important role in sequestering Bcl-2-related proteins, CED-4/Apaf-1-like molecules, caspases, and other regulators of apoptosis.

In conclusion, BNIP3 does not function like previously characterized BH3-only Bcl-2 homologues. The region proposed as a typical BH3 domain in BNIP3 neither facilitates heterodimerization with Bcl-2, Bcl-X<sub>L</sub>, or CED-9 nor promotes cell death. Instead, the pro-apoptotic function of BNIP3 depends on its membrane association at either mitochondrial or nonmitochondrial sites and selective interaction with Bcl-2/Bcl-X<sub>L</sub> through an NH<sub>2</sub>-terminal (amino acid 1–49) region to regulate the induction of apoptosis.

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