Adenovirus E1B-19K/BCL-2 Interacting Protein BNIP3 Contains a BH3 Domain and a Mitochondrial Targeting Sequence*

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Motoaki Yasuda‡, Paul Theodorakis‡, T. Subramanian‡, and G. Chinnadurai§

From the Institute for Molecular Virology, St. Louis University Medical Center, St. Louis, Missouri 63110

Adenovirus E1B-19K and BCL-2 anti-apoptosis proteins interact with certain BCL-2 family pro-apoptotic proteins. A conserved domain, BH3, present in these proteins is essential for their pro-apoptotic activity and for heterodimerization with anti-apoptosis proteins. Cellular protein BNIP3 (previously NIP3) interacts with E1B-19K, BCL-2, BCL-xL, and EBV-BHRF1. BNIP3 contains a motif similar to the BH3 domain. Substitution of the BH3 domain of BNIP3 for the corresponding sequences of BAX functionally restores the pro-apoptotic and protein heterodimerization activities of BAX. BNIP3 exhibits a delayed cell death activity that is partially relieved by deletion of the BH3 domain. BNIP3 suppresses the anti-apoptosis activity of BCL-xL in a BH3-dependent manner. BNIP3 contains a C-terminal trans-membrane (TM) domain similar to other BCL-2 family proteins and BNIP1 (previously NIP1). The TM domains of BNIP3 and BNIP1 can functionally substitute for the TM domain of a BCL-2 family member EBV-BHRF1. The BNIP3 TM domain exclusively targets the heterologous green fluorescent protein (GFP) to mitochondria. These results suggest that BNIP3 is a member of the BH3-containing BCL-2 family of pro-apoptotic proteins and functions in mitochondria.

Apoptosis is an essential physiological process of selective elimination of cells in multicellular organisms. This process is invoked during normal organ development and tissue homeostasis and also during certain pathological conditions that result in degenerative diseases. Apoptosis plays an important role in regulating the multiplication of and pathogenesis by a number of viruses (for review, see Ref. 1). In virus-infected cells, apoptotic paradigms are initiated as a cellular defensive mechanism to eliminate infected cells. However, a number of viruses encode proteins that suppress apoptosis resulting in efficient viral replication and pathogenesis. Human adenoviruses have evolved multiple strategies employing proteins coded by three different gene blocks, E1B, E3, and E4, to overcome the effect of apoptosis of infected permissive cells (for review, see Ref. 2). One of the proteins coded by the E1B region, the 19K protein, efficiently suppresses apoptosis manifested during viral infection and in cells exposed to several different heterologous stimuli. E1B-19K is a functional homolog of the cellular anti-apoptosis protein BCL-2. The mechanism by which E1B-19K and the related BCL-2 family anti-apoptosis proteins promote cell survival remains to be elucidated. In order to gain insight into the possible mechanism(s) by which these proteins exert their activity, several investigators have searched for cellular proteins that complex with E1B-19K and BCL-2. Such searches have thus far identified seven cellular proteins that complex with E1B-19K (3–9). Among these seven proteins, six can also interact with BCL-2/BCL-xL. Three of these proteins, BAK, BAX, and BIK, are potent pro-apoptotic proteins and are members of the BCL-2 family proteins. Coexpression of the anti-apoptosis proteins with the pro-apoptotic proteins results in suppression of cell death to varying extents. Thus, one of the mechanisms by which E1B-19K, BCL-2, and related proteins promote cell survival may involve antagonizing the activities of pro-apoptotic proteins through direct protein heterodimerization.

Among the three BCL-2 family pro-apoptotic proteins that complex with E1B-19K and BCL-2, BAK and BAX are more closely related to BCL-2 and share more extensive homology with other BCL-2 family proteins. In contrast, BIK shares a single domain (designated BH3 domain) with other BCL-2 family proteins (in addition to a C-terminal trans-membrane domain characteristic of most BCL-2 family proteins; Ref. 4). Since the discovery of BIK, two other pro-apoptotic proteins, BID (10) and HRK (11) that share only the BH3 domain with BCL-2 family proteins have been identified. In addition, a previously identified pro-apoptotic protein BAD (12) has recently been recognized as a BH3 domain-containing protein; Ref. 13–15).

The BH3 domain is conserved both in the pro-apoptotic and anti-apoptotic BCL-2 family proteins. The BH3 domain of the pro-apoptotic proteins serves a dual function. It is essential for their cell death activity and for mediating heterodimerization with anti-apoptosis proteins (4, 10, 11, 16). Since BH3 alone-containing pro-apoptotic proteins (BIK, BID, HRK, and BAD) share only the BH3 domain in common, it is believed that the BH3 domain is the death effector domain and is postulated to elicit its cell death activity by inactivating the anti-apoptotic proteins through heterodimerization. However, detailed mutational analysis of one pro-apoptotic protein, BIK, suggests that heterodimerization with survival proteins alone is insufficient for its cell death activity (17). In the present communication, we report that the cellular protein BNIP3 (previously NIP3, according to Human Gene Nomenclature Committee) identified and cloned in our laboratory (3) also contains a functional BH3 domain. The BH3 domain of BNIP3 mediates protein heterodimerization with anti-apoptosis proteins, and when substituted in a different pro-apoptotic protein, BAX, can elicit both cell death and heterodimerization activities. The BH3 domain of BNIP3 is required for its ability to antagonize the activity of BCL-xL and is partially required for its pro-apoptotic activity.
Most of the BCL-2 family proteins contain a characteristic C-terminal trans-membrane (TM) domain that appears to target these proteins to subcellular regions such as mitochondria and nuclear envelope/endoplasmic reticulum regions. Deletion of the TM domain results in varying effects on the activity of various anti-apoptotic proteins. In certain members like EBV-BHRF1, it is essential (18) while in other members, like BCL-2 and BCL-xL, it is not always required. Deletion of the TM domain of BCL-2 results in partial reduction of the anti-apoptotic activity under certain conditions (19), whereas under other conditions, no significant reduction results (20). In this communication, we report that both BNIPI and BNIPIII also contain functional TM domains suggesting that these proteins function at subcellular locations where other BCL-2 family proteins function. Further, the domain of BNIPIII can specifically target a heterologous non-BCL-2 family protein to mitochondria.

**EXPERIMENTAL PROCEDURES**

**Plasmids—** Plasmids pMA42-19k, pACT-BNIPIII, and pcDNA3-BNIPIII have been described (3). Plasmid pBNIPI (puro) was constructed by cloning BNIPI, the corresponding TM domain (21). Plasmid pGSTD-BNIPI (wt) was constructed by cloning PCR-amplified BCL-xL DNA in frame with the GAL4 DNA binding (DB) domain. Plasmids pcDNA3-BAX and pcDNA3-BAXΔGD (referred to here as AΔB3H3) were gifts from Tom Chittenden (16). Plasmid pGSTD-BCL-xL, was a gift from Robert Lutz. Deletion mutants of BNIPIII as well as BAX-BH3 domain substitution mutants were constructed by PCR. Plasmids expressing EBV-BHRF1 and BHRF1-TM (previously mutant Y164–91) have been described (18). To construct various TM domain substitution mutants, the DNA sequences encoding the various TM domains were amplified by PCR. The PCR primers were engineered to contain a BamHI site at the 5′ end and an XhoI site at the 3′ end. The PCR-generated DNA fragments were used to replace the corresponding sequences in pcDNA3-HABHRF1. pcDNA3-GFP was constructed by cloning the GFP gene from Alpha Fluoroprobe Cycle (22) in pcDNA3. The GFP-TM chimeric genes were constructed by substituting the GFP gene (engineered to contain a RspHI site at the 3′ end) for the BHRF1 open reading frame in plasmids expressing the BHRF1/BNIPI-TM or BHRF1/BNIPIII-TM fusion proteins.

Two-hybrid Analysis—Yeast indicator strain GGY1::171 was transformed with a bait plasmid expressing a fusion protein consisting of E1B-19K or BCL-xL, tagged with the GAL4 DB domain (pMA42-19k, Ref. 3, or pMA42-19k-BCH-xL,) and prey plasmids (pACT-BNIPII (wt) or the BH3 deletion mutant)) expressing BNIPII protein tagged with the GAL4 activation domain (AD) using established procedures (23, 24). The relative levels of protein interaction were determined by estimating the levels of expression of the reporter gene Escherichia coli lacZ. A comparable number of yeast transformants were pooled and grown in liquid selective media (lacking histidine and leucine), equal number of cells were lysed, and the levels of β-galactosidase were determined by β-galactosidase assay (25), and these results were expressed as units defined by Miller (26).

**Protein Binding—** To examine the in vitro protein binding, 35S-labeled proteins were prepared by in vitro transcription and translation of various BAX mutants cloned in pcDNA3 vector using a commercially available kit (Promega). The labeled proteins were incubated with GST-BCL-xL fusion protein immobilized on glutathione-agarose beads (Sigma) in a buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.2% Nonidet P-40, washed five times in the same buffer, re-suspended in sample buffer, and analyzed by SDS-PAGE.

**Immunofluorescence and GFP Fluorescence—** Indirect immunofluorescence studies were carried out in COS-7 cells (plated on coverslips in 35-mm dishes) transfected with 5 μg of pcDNA3-HA BHRF1 wt or various mutants expressing TM domain fusion proteins. For mitochondrial staining, 25 nM of MitoTracker Red (Molecular Probes, Inc.) was added to the media for 30 min at 24 h post-transfection. Cells were immediately fixed with 3.7% formaldehyde, permeabilized with ice-cold methanol, and then were stained with monoclonal anti-HA (12CA5) antibody, followed by fluorescein isothiocyanate-conjugated secondary goat anti-mouse IgG (Pierce). Cells were photographed using a 485-nm filter to visualize BHRF1 and a 546-nm filter to visualize mitochondria. For GFP fluorescence, COS-7 cells were transfected as above, with 5 μg of pcDNA3-GFP wt or pcDNA3 containing various GFP-TM domain chimeric genes. Twenty-four h after transfection, cells were stained with MitoTracker Green and photographed using a 495-nm (for GFP fluorescence) or 546-nm filter (to visualize mitochondria).

**Cell Survival Assays—** Cell survival assays were carried out using a baby rat kidney (BRK) cell line (BRK/p53val135-E1A) transformed by cointegration of p55val135 (27) and adenovirus E1A (28). To assay the effect of BHRF1, pRcCMV-based plasmids expressing wt or mutant proteins were transfected in BRK/p53val135-E1A cells, and G418-resistant colonies (~100) from each transfection were pooled (at 37.5 °C). These cells were assayed for survival at 32.5 °C at 24, 48, and 72 h by counting the Trypan-blue excluding cells as described earlier (18). To determine the effect of BNIPII on the anti-apoptotic activity of BCL-xL, the BRK/p53val125-E1A cell line was transfected with pcDNA3-BCL-xL, (neo) and pBabe-BNIPII (puro), and the transfected cells were maintained at 32.5 °C for 5 days. The surviving cells were then allowed to form visible colonies by growing at 37.5 °C in the presence of 100 μg of G418 and 1 μg of puromycin, stained with Giemsa, and counted.

**Transient Cell Death Assay—** MCF-7 cells were transfected with pcDNA3-based plasmids expressing BAX or BAX mutants or BNIPII (2 μg) along with the reporter plasmid pCMV-Flg (0.5 μg). After 12 or 48 h of transfection, the cells were fixed, stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and microscopically examined. 100 to 200 blue color cells were microscopically scored as flat (live) and round (apoptotic) cells.

**RESULTS**

BH3 Domain in BNIPII—Since the BH3 domain of several BCL-2 family proteins mediates heterodimerization with BCL-2 and related proteins, we searched BNIPII–3 for BH3-like sequence motifs. We noted a BH3-like motif in BNIPIII between residues 110 and 118 (Fig. 1A). This motif of BNIPIII resembles the known BH3 domains of various BCL-2 family proteins, particularly the recently identified BH3 domain of BAD (Fig. 1D). In order to determine if the putative BH3 domain in BNIPIII mediates heterodimerization with anti-apoptotic BCL-2 family proteins, we constructed a 6-amino acid (residues 110–115) deletion mutant replacing the BH3 domain of BNIPIII (Fig. 1A). We then examined the ability of this mutant to heterodimerize with E1B-19K and BCL-xL by two-hybrid analysis in yeast. The relative levels of interaction of BNIPIII or BNIPIIIΔBH3 were determined by quantitating the levels of expression of the E. coli lacZ reporter gene. Ten to twenty independent transformed yeast colonies were pooled,

![Fig. 1. Domain map of BNIPI and BH3 domains of BCL-2 family proteins.](http://www.jbc.org)
and the relative levels of β-galactosidase expression were colorimetrically determined (Fig. 2). Cells expressing either E1B-19K or BCL-xL (as GAL4 DB domain fusion protein) and BNIP3 wt (tagged with the GAL4 AD) induced significant levels of β-galactosidase compared with the control cells expressing the respective DB fusion protein and AD vector (pACT). In contrast, cells expressing the DB fusion proteins and the respective AD-tagged BH3 deletion mutant of BNIP3 did not induce significant levels of β-galactosidase. These results suggest that the BH3-like motif deleted in BNIP3 DBH3 mutant is essential for heterodimerization with E1B-19K and BCL-xL.

Functional Substitution of BH3 Domain of BNIP3 in BAX—To further substantiate the presence of a BH3 domain in BNIP3, we carried out a domain substitution study with the pro-apoptotic protein BAX. We constructed two substitution mutants of BAX in which a 9- (BAX/BNIP3BH3–9) or a 16-amino acid region (BAX/BNIP3BH3–16) corresponding to the BH3 domain of BNIP3 was substituted for the corresponding sequences of BAX (Fig. 3A). The 16-amino acid region containing the BH3 region was chosen since this region of BAX and BAK corresponds to a BH3-containing α-helical region of BCL-xL and since peptides of BAK and BAX containing the 16-amino acid region efficiently bind to BCL-xL (29). The effect of these substitution mutations on the apoptotic activity of BAX was determined by transient transfection (16, 30) in MCF-7 cells. Plasmids expressing various BAX mutants or BNIP3 were cotransfected with a plasmid expressing the E. coli lacZ reporter gene. Transfected cells were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and the percentage of apoptotic cells was quantitated by microscopically counting the number of round (apoptotic) blue cells from the total number of blue cells (Fig. 3B). Transfection of BAX wt resulted in 50–60% of apoptotic cells while transfection of a BH3 deletion mutant (BAXNBH3) of BAX (16) severely impaired apoptosis. Substitution of the 9- or 16-amino acid region of BNIP3 also fully restored the pro-apoptotic activity of BAX. We also determined the ability of the various BAX mutants to heterodimerize with BCL-xL. Various mutant proteins were prepared by in vitro...
transcription and translation and incubated with GST-BCL-xL fusion protein. The protein complexes were separated by chromatography on the glutathione-agarose affinity matrix and analyzed by SDS-PAGE (Fig. 3C). As expected, BAX bound to BCL-xL, while BAXΔBH3 mutant did not. Both chimeric BAX proteins containing the BH3 domain of BNIP3 also bound to BCL-xL. These results suggest that the BH3 domain of BNIP3 is functionally equivalent to the BH3 domain of BAX based on two functional criteria (i.e., induction of apoptosis and heterodimerization with anti-apoptosis proteins).

Pro-apoptotic Activity of BNIP3—In cells transfected with BNIP3, only a marginal amount of apoptosis, compared with cells transfected with the empty vector, was observed at 12 h after transfection (under conditions where BAX induces efficient apoptosis) (Fig. 3B). To determine if BNIP3 can induce apoptosis after extended periods of time, we examined the transfected cells 48 h after transfection. Under these assay conditions, BNIP3 induced significant cell death (Fig. 4A). Cells transfected with a BH3 deletion mutant of BNIP3 exhibited reduced levels of apoptosis, albeit detectable residual cell death activity. These results suggest that the BH3 domain may only be partially required for the cell death activity manifested by BNIP3.

We then determined if BNIP3 could antagonize the activity of an anti-apoptosis protein. For this purpose, BRK/p53val135-E1A cells were cotransfected with plasmids expressing BCL-xL (and the neo selection marker) or BNIP3 (and the puro selection marker) and maintained at 37.5 °C for 24 h and shifted to 32.5 °C for 5 days. The surviving cells were then allowed to proliferate (in the presence of G418 and puromycin) and form visible colonies at 37.5 °C and then were quantitated. In the absence of any anti-apoptosis protein, the BRK/p53val135-E1A cells undergo total apoptosis and do not result in detectable colony formation, suggesting that it is a sensitive assay for the anti-apoptosis proteins (Fig. 4B). Transfection of BCL-xL resulted in significant colony formation while cotransfection with BNIP3 reduced the colony formation by about one-half. This inhibitory effect of BNIP3 is relieved by the ΔBH3 mutation (Fig. 4B). These results suggest that coexpression of BNIP3 may directly or indirectly suppress the activity of BCL-2 family anti-apoptosis proteins through the BH3 domain.

Subcellular Targeting by TM Domain—Previous indirect immunofluorescence analysis revealed that BNIP3 localizes primarily in punctate cytoplasmic structures characteristic of mitochondria while two other M, ~19,000-interacting proteins (BNIP1 and BNIP2) were found to localize in the nuclear envelope/endoplasmic reticulum regions (3). Computer-assisted analysis of the BNIP3 sequence suggests that the C-terminal region could potentially form a trans-membrane (TM) domain similar to other BCL-2 family proteins. In order to determine if the TM domain of BNIP3 could determine the specific subcellular localization pattern previously observed with BNIP3, we carried out a domain substitution study with EBV-BHRF1. For this study, we chose BHRF1 since the TM domain is essential for the anti-apoptosis activity of BHRF1 (18) and its subcellular localization pattern (punctate) appears to resemble that of BNIP3 (31, 32). In the present study, the TM domains of BCL-2 and BNIP1 were also used for comparison. The TM domains of BNIP3, BCL-2, or BNIP1 were fused to BHRF1, taking advantage of a unique BamH1 site at the C terminus of BHRF1 just upstream of the TM domain (Fig. 5A). We then determined if the various TM domains could functionally substitute for the TM domain of BHRF1 in a functional anti-apoptosis assay and then determined the subcellular localization by indirect immunofluorescence. The effect of various BHRF1 mutants on apoptosis induced by a ts allele of p53 tumor suppressor protein (p53val135) was determined (18). A BRK cell line transformed with E1A and the ts mutation of p53 (BRK/p53val135-E1A) was transformed with various BHRF1 mutants, and pooled cell lines were established from a comparable number of G418-resistant colonies. The effect of BHRF1 on apoptosis was de-

![Fig. 4. Pro-apoptotic activity of BNIP3. A, induction of apoptosis by BNIP3. The apoptosis assay was carried out at 48 h after transfection as in Fig. 3. B, suppression of BCL-xL activity by BNIP3. BRK/p53val135-E1A cells were transfected with pBabe-puro empty vector (indicated as Vector) or vectors (pBabe-puro) expressing either BNIP3 or BNIP3ΔBH3 along with pDNA-3 vector or pcDNA3-BCL-xL. Colonies of cells resistant to apoptosis induced by the combinatorial effects of E1A and p53 were selected (by neo and puro selection), stained with Giemsa, and counted (mean ± S.D.).](http://www.jbc.org/)

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BH3 and Trans-membrane Domains of BNIP3

Fig. 5. Trans-membrane domain substitutions mutants of BHRF1 and their anti-apoptosis activities. A, amino acid sequences of the various C-terminal trans-membrane domains substituted in BHRF1. The hydrophobic regions are underlined. B, protein expression by BHRF1-TM domain substitution mutants. Protein extracts prepared from BRK/pS3val135-E1A cells stably expressing various mutants were examined by Western blot analysis using the HA monoclonal antibody. The BHRF1-TM protein is not visible in the blot shown due to the presence of a background band. Significant expression of this mutant protein was observed when analyzed by immunoprecipitation analysis (not shown). C, anti-apoptosis activity of BHRF1 and BHRF1-TM substitution mutants. BRK/pS3val135-E1A cells stably transfected with various plasmids were assayed for cell survival at various times after shifting to 32.5 °C. The percentages of surviving cells in three independent experiments (mean ± S.D.) are presented.

BHRF1 expression plasmids and analyzed by indirect immunoprecipitation (not shown). Antibodies recognizing the endogenous BHRF1 or the chimeric protein were used. The BHRF1 chimeric protein containing the TM domain of BCL-2 targets a heterologous protein, dihydrofolate reductase (DHFR), to mitochondria (33). Cells expressing the GFP/BCL2-TM or GFP/BNIP1-TM fusion proteins, the fluorescence was primarily associated with the nucleus (Fig. 6, A-E). In cells expressing the GFP/BCL2-TM or GFP/BNIP1-TM fusion proteins, the fluorescence was primarily associated with the nucleus (Fig. 6, A-E). In contrast, in cells expressing the GFP/BNIP1-TM chimeric protein, the fluorescence was primarily associated with the mitochondria (Fig. 6, F-J). The subcellular localization of the various BHRF1 mutant proteins (tagged with HA) was determined in comparison to wt BHRF1. COS-7 cells were transiently transfected with various BHRF1 expression plasmids and analyzed by indirect immunofluorescence using HA monoclonal antibody (Fig. 6, A-E). Since BHRF1 (31) and BNIP3 (3) appear to be localized significantly to the mitochondria, cells were double stained with MitoTracker Red, a mitochondrial specific dye (Fig. 6, B). In cells transfected with the BHRF1 mutant lacking the TM domain (BHRF1ΔTM), the HA-specific fluorescence was observed throughout the cell including the nucleus (Fig. 6A), whereas in cells expressing wt BHRF1, primarily cytoplasmic fluorescence (Fig. 6B) that coincides with MitoTracker fluorescence (Fig. 6G) was observed. A pattern similar to wt BHRF1 was also observed in cells expressing the BHRF1/BNIP3-TM chimeric protein (Fig. 6, E and J). In cells expressing the chimeric proteins BHRF1/BCL2-TM and BHRF1/BNIP1-TM, both punctate cytoplasmic fluorescence and nuclear envelope fluorescence (HA-specific) was observed (Fig. 6, C and D). The cytoplasmic HA-specific punctate fluorescence appears to coincide with the MitoTracker-specific fluorescence (Fig. 6F). In these results suggest that the TM domain of BNIP3 appears to target BHRF1 primarily to the mitochondria, whereas the TM domains of BCL-2 and BNIP1 can target BHRF1 to nuclear envelope/endoplasmic reticulum and mitochondrial regions.

In addition, we also determined if the TM domain of BNIP3 by itself could target a heterologous (non-BCL2 family) protein to mitochondria. For this purpose, we fused the TM domain of BNIP3 to the C terminus of GFP and analyzed the GFP fluorescence in transiently transfected COS-7 cells. For comparison, we also used the GFP fusion proteins containing the TM domains of BCL-2 or BNIP1. It has previously been shown that the TM domain of BCL-2 targets a heterologous protein, dihydrofolate reductase (DHFR), to mitochondria (33). Cells expressing GFP showed fluorescence throughout the cell, which was concentrated in the nucleus (Fig. 7A). In contrast, in cells expressing the GFP/BCL2-TM or GFP/BNIP1-TM fusion proteins, the fluorescence was primarily associated with the nuclear envelope and cytoplasmic punctate structures (Fig. 7, B and C). Cells expressing the GFP/BCL2-TM or GFP/BNIP1-TM fusion proteins, the fluorescence was primarily associated with the nuclear envelope and cytoplasmic punctate structures (Fig. 7D). The transfected cells were also stained with the MitoTracker Red dye to determine if the TM domain fusion proteins are localized to the mitochondria. In cells expressing GFP, the observed cytoplasmic punctate fluorescence did not correspond to the GFP fluorescence (Fig. 7E). In cells expressing the GFP/
BNIP3-TM fusion protein, clear punctate cytoplasmic fluorescence that coincides with the GFP fluorescence was observed (Fig. 7H), suggesting that most of this fusion protein localizes primarily in mitochondria. In cells expressing GFP-BCL2-TM or GFP/BNIP1-TM, significant punctate fluorescence coincident with the MitoTracker dye was also observed (Fig. 7, F and G). These results suggest that the TM domains of BCL-2 and BNIP1 can target a heterologous protein to both nuclear envelope/endoplasmic reticulum regions as well as to mitochondria, whereas the TM domain of BNIP3 primarily targets to mitochondria.

**DISCUSSION**

We have shown that the cellular protein, BNIP3, which binds to the BCL-2 family of anti-apoptosis proteins, contains a BH3 domain. We have demonstrated that the BH3 domain of BNIP3 is functional since substitution of the corresponding sequences in a different pro-apoptotic protein BAX restores two activities of BAX, induction of cell death and heterodimerization with anti-apoptosis proteins, both of which are ascribed to the BH3 domain. The BH3 domain of BNIP3 more closely resembles the BH3 domain of BAD (Fig. 1B). Thus, BNIP3 appears to have an overall resemblance to several BH3-alone-containing BCL-2 family proteins such as BIK, BID, HRK, and BAD. The BH3 domain of these proteins plays a crucial role in their pro-apoptotic activity. Recent *in vitro* studies indicate that BH3 domains of BAX and BAK alone are sufficient to induce apoptosis (34). Although BNIP3 does not possess a potent pro-apoptotic activity like other BH3-containing pro-apoptotic proteins, the BH3 domain appears to play some role in the pro-apoptotic activity of BNIP3. However, the BNIP3 BH3 domain is as efficient as the BAX BH3 domain in eliciting apoptosis under the context of BAX. It is possible that the pro-apoptotic activity dictated by the BH3 domain is suboptimal in the context of the BH3 domain, and such activity may be enhanced by exposure to certain apoptotic stimuli or by other protein interactions. Our results raise the possibility that the cell death activity dictated by the BH3 domain is also influenced by the other sequences within the cognate protein. Sequence comparisons of BNIP3 indicate that there are several human homologs of this protein. Interestingly, there is also a BNIP3 homolog in *Caenorhabditis elegans*. The possibility that BNIP3 or its homologs may function cooperatively with other apoptotic factors remains to be investigated.

We have shown that coexpression of BNIP3 results in suppression of the anti-apoptosis activity of BCL-x<sub>L</sub>. Since the suppressive effect of BNIP3 is relieved by the deletion of the BH3 domain, it appears that BH3 may directly or indirectly modulate the activity of survival proteins. Since the BH3 domain also mediates heterodimerization with survival proteins, the simplest explanation would be that BNIP3 and the survival proteins may form inactive dimers. However, we note that others have observed certain BH3 mutants of BAX and BAK...
retain their ability to antagonize the activity of BCL-2/BCL-xL (35, 36). Similarly, BCL-x\textsubscript{S} that does not appear to complex with BCL-xL also suppresses the activity of BCL-xL (37). It is possible that physical interaction between the BNIP3 and anti-apoptosis proteins may modulate the activity of the latter proteins by altering the conformation and/or accessibility to other cofactors. It is possible that BH3 mutants of BAX and BAK may suppress the activity of anti-apoptosis proteins by competing for certain cofactors or simply clogging or displacing anti-apoptosis proteins from subcellular sites where these proteins are active.

We have provided evidence that BNIP3 may primarily function in mitochondria where most other BCL-2 family proteins appear to function. Here, we have shown that the C-terminal TM domain of BNIP3 can target a BCL-2 family protein (BHFRP1) and a heterologous protein (GFP) to mitochondria. Although the cellular protein BNIP1 is not characterized here, based on the subcellular localization dictated by the C-terminal trans-membrane domain, we believe that it may also function at locales (nuclear envelope and mitochondria) where other BCL-2 family proteins function. Among the two TM domains we have studied here, it appears that the TM domain of BNIP3 is efficient in mitochondrial targeting, whereas the TM domains of BCL-2 and BNIP1 appear to target GFP to both the nuclear envelope/endoplasmic reticulum regions and mitochondria. It appears that in addition to the TM domains of certain BCL-2 family proteins, other internal sequences may also play a role in subcellular targeting. For example, BCL-2 mutants lacking the TM domain appear to be fully functional under certain assay conditions while a comparable mutant that also lacks the TM domain appears to be fully functional under certain assay conditions while a comparable mutant that also lacks the internal non-conserved region has been shown to be functionally defective (20). Since the GFP/BNIP3-TM and GFP/BNIP1-TM exhibit similar subcellular localization patterns as the native BNIP1 and BNIP3 proteins (3), it appears that the TM domains of these proteins may alone be sufficient for subcellular targeting. Several recent studies have revealed that both pro-apoptotic and anti-apoptotic BCL-2 family proteins modulate mitochondrial functions (for review, see Ref. 38). It is possible that BNIP3, either autonomously or in concert with BCL-2, may also be involved in regulating mitochondrial functions.

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Motoaki Yasuda, Paul Theodorakis, T. Subramanian and G. Chinnadurai

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