



The *C. elegans* orthologue ceBNIP3 interacts with CED-9 and CED-3 but kills through a BH3- and caspase-independent mechanism

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We have studied ceBNIP3, the orthologue of BNIP3 in *C. elegans*. Sequence analysis reveals that the different domains of BNIP3 have been conserved throughout evolution. ceBNIP3 contains a C-terminal transmembrane (TM) domain, a conserved domain (CD) of 19 amino acids, a BCL-2 homology-3 (BH3)-like domain and a PEST sequence. ceBNIP3 is expressed primarily as a 25 kDa monomer and a 50 kDa homodimer. After transfection, ceBNIP3 protein is rapidly degraded through a ubiquitin-dependent pathway by the proteasome. Like BNIP3, the TM domain of ceBNIP3 mediates the localization of the protein to mitochondria and is also necessary for homodimerization and cell death in mammalian cells. Neither the putative BH3 domain nor conserved domain is necessary for killing. ceBNIP3 protein interacts with CED-9 and BCL-X_L, but unlike other pro-apoptotic BCL-2 family members, the BH3-like domain does not participate in dimerization. The ceBNIP3 TM domain mediates interaction with both CED-9 and BCL-X_L. ceBNIP3 interacts with CED-3 but co-expression of CED-3 and ceBNIP3 does not significantly enhance induction of cell death in the presence or absence of CED-4. ceBNIP3 kills mammalian cells by a caspase-independent mechanism. In conclusion, we find that although ceBNIP3 interacts with CED-9 and CED-3 it kills by a BH3- and caspase-independent mechanism. *Oncogene* (2000) 19, 5453–5463.

Keywords: ceBNIP3; BNIP3; cell death; CED-9; BH3 domain

Introduction

Apoptosis or programmed cell death is an evolutionarily conserved process in multicellular organisms that plays an essential role in development and tissue homeostasis (Vaux and Korsmeyer, 1999). Genetic analysis of programmed cell death in the nematode *Caenorhabditis elegans* has identified several components of the apoptotic pathway (Horvitz *et al.*, 1994). Mutations in *ced-9*, *ced-3*, *ced-4* and *egl-1* affect the death of 131 somatic cells that occurs during normal development. Loss-of-function mutations of *ced-3*, *ced-4* and *egl-1* result in the survival of the cells that would

normally die, thereby involving these genes in the killing process (Ellis and Horvitz, 1986). In contrast, the *ced-9* gene protects cells that normally survive from undergoing apoptosis (Hengartner *et al.*, 1992). Mammalian homologs have been identified for all three genes. *Ced-3* encodes a protein similar to the cysteine proteases family called caspases (Alnemri *et al.*, 1996) whereas *ced-4* shares homology with *Apaf-1* and *CARD/Nod1* (Zou *et al.*, 1997; Bertin *et al.*, 1999; Inohara *et al.*, 1999). The mammalian counterpart of *ced-9* is *Bcl-2* and it has been shown that *Bcl-2* can partially replace *ced-9* in *C. elegans* (Vaux *et al.*, 1992; Hengartner and Horvitz, 1994). The genetic studies and physical interactions between these three genes have provided a model to explain the regulation of apoptosis in *C. elegans*. First, the genetic studies in the worm have demonstrated that *ced-9* acts upstream of *ced-4* and *ced-3* (Hengartner *et al.*, 1992). Using the yeast two hybrid system, it has been shown that CED-9 interacts with CED-4 (Wu *et al.*, 1997; Spector *et al.*, 1997), which also binds the CED-3 protein (Chinnaiyan *et al.*, 1997) and prevents the activation of CED-3 by CED-4 (Seshagiri and Miller, 1997). Egl-1, which interacts with CED-9 through its BH3 domain, has been shown to induce apoptosis in *C. elegans* (Conradt and Horvitz, 1998). The proposed model is that the interaction of Egl-1 with CED-9 releases CED-9 from the complex and consequently permits oligomerization of CED-4, thereby bringing together multiple CED-3 proteins (Yang *et al.*, 1998). Recently, both CED-9 and CED-4 proteins have been localized to the mitochondria in wild type embryos. After activation of Egl-1, CED-4 translocates to the nucleus and this event precedes any CED-3 processing (Chen *et al.*, 2000).

BNIP3 (BCL-2 and Nineteen kDa interacting protein-3) and NIX (also called BNIP3 α /BNIP3L/B5) form a unique subfamily of death inducing mitochondrial proteins (Chen *et al.*, 1997, 1999; Yasuda *et al.*, 1999; Matsushima *et al.*, 1998; Ohi *et al.*, 1999). Both mammalian proteins have the same features: a C-terminal TM domain, a putative BH3 domain and a PEST sequence (Chen *et al.*, 1999; Yasuda *et al.*, 1999). When overexpressed, BNIP3 and NIX localize to the mitochondria and induce cell death. However, a TM deletion mutant of both proteins is expressed primarily in the cytosol and has no apoptotic activity (Chen *et al.*, 1999). *In vivo*, BNIP3 and NIX are expressed as dimers and for BNIP3, the TM domain also mediates homodimerization (Chen *et al.*, 1999; Ray *et al.*, 2000). Both proteins are also rapidly degraded *in vivo* and the degradation is blocked by lactacystin treatment, a proteasome inhibitor (Chen *et al.*, 1999). BNIP3 and NIX can overcome BCL-2 or BCL-X_L suppression of apoptosis although a high level of BCL-X_L expression

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will inhibit killing activity. BNIP3 co-immunoprecipitates with BCL-2 or BCL-X_L and deletion mapping studies have identified the NH-2 terminus or the TM domain as critical for the interaction and have excluded the BH3 domain (Ray *et al.*, 2000). In another study, using the yeast two hybrid system, the ability of BNIP3 without the BH3 domain to heterodimerize with BCL-X_L is somewhat reduced (Yasuda *et al.*, 1998a). Deletion of the BH3 domain of BNIP3 has no effect on cell death (Ray *et al.*, 2000).

Overexpression of BNIP3 induces cell death as a consequence of early Δψ_m loss and ROS production which is blocked by cyclosporin A or bongkreic acid, inhibitors PT pore opening (Vande Velde *et al.*, 2000). In contrast, BNIP3 overexpression does not induce cytochrome *c* release and the broad spectrum caspase inhibitor Ac-zVAD.fmk was ineffective in blocking cell death, suggesting that BNIP3-mediated cell death is caspase-independent (Vande Velde *et al.*, 2000).

ceBNIP3, an orthologue of BNIP3 in *C. elegans*, shows 21% amino acid sequence identity with BNIP3 and shares similar structural features to mammalian BNIP3 (Yasuda *et al.*, 1998b). Overexpression of ceBNIP3 induces apoptosis in mammalian cells and ceBNIP3 interacts with CED-9 and CED-3 as a ternary complex (Yasuda *et al.*, 1998b). The interaction of CED-3 and ceBNIP3 increases the processing of CED-3 into an active form and consequently cell death leading the authors to propose that ceBNIP3 may function as a CED-4-like protein.

In this report, we have characterized the domains of ceBNIP3 that are required for cell death and interaction with CED-9. ceBNIP3 contains a TM domain, a conserved domain of 19 amino acids, a putative BH3 domain and a PEST sequence. After transient transfection, ceBNIP3 induces cell death and only the TM domain is critical for localization to the mitochondria and killing function. ceBNIP3 is also capable of homodimerization and heterodimerization with CED-9 and BCL-X_L through its TM domain, and cell death is partially suppressed by BCL-X_L. We find that ceBNIP3 heterodimerizes with CED-3 but does not enhance CED-3 killing activity and induces cell death in a caspase-independent manner.

Results

Homology between ceBNIP3 and BNIP3

We searched the *C. elegans* database for homologous sequences using segments of the human BNIP3 amino acid sequence. Only one stretch of 19 amino acid residues within an open reading frame (ORF) of 210 amino acids was found at locus CEC14F5 on chromosome III of *C. elegans* (Accession number U29082) as reported by Yasuda *et al.*, 1998b. Sequence analysis revealed two possible start codons 33 nucleotides apart containing an imperfect Kozak sequence. So in our studies, we used the first ATG and obtained an ORF encoding a protein of 221 amino acids (Accession number AF133832) in contrast to the sequence reported by Yasuda *et al.*, 1998b. Human BNIP3 and the potential ceBNIP3 showed 21% identity over their entire amino acid sequences. Further analysis of the BNIP3 subfamily sequences revealed

that BNIP3, NIX and ceBNIP3 share several common structural features (Figure 1a). The three proteins have a C-terminal α-helical hydrophobic TM domain. They each contain a putative BH3 domain with some key amino acids (Figure 1b). The N-terminus of BNIP3, NIX and ceBNIP3 contain PEST sequences, which are found in proteins that are rapidly degraded in the proteasome. The three proteins also share a span of conserved amino acids, 17 of 19 for mouse mBNIP3 and 14 of 19 for mouse mNIX.

ceBNIP3 protein expression

We compared *in vitro* transcribed and translated products of ceBNIP3 and BNIP3 by Laemmli SDS-PAGE (Figure 2a). The predicted molecular weight of BNIP3 is approximately 21.5 kDa, however BNIP3 runs as a 60 kDa dimer and a 30 kDa monomer. ceBNIP3 has a predicted molecular weight of 24.8 kDa and on SDS-PAGE runs as a major band of 27 kDa corresponding to the T7 tagged-monomer. A minor band running slightly faster is probably due to translation from the second initiation codon. Weak bands at approximately 50 kDa correspond to a putative ceBNIP3 dimer. Deletion of the TM domain results in a 25 kDa translated product with no dimeric form, suggesting that the homodimerization of ceBNIP3 is probably mediated by the TM domain, similar to BNIP3 and NIX (Chen *et al.*, 1997, 1999; Ray *et al.*, 2000). To confirm this result, we used the yeast two hybrid assay to determine whether ceBNIP3 homodimerizes through the TM domain. Figure 2b shows

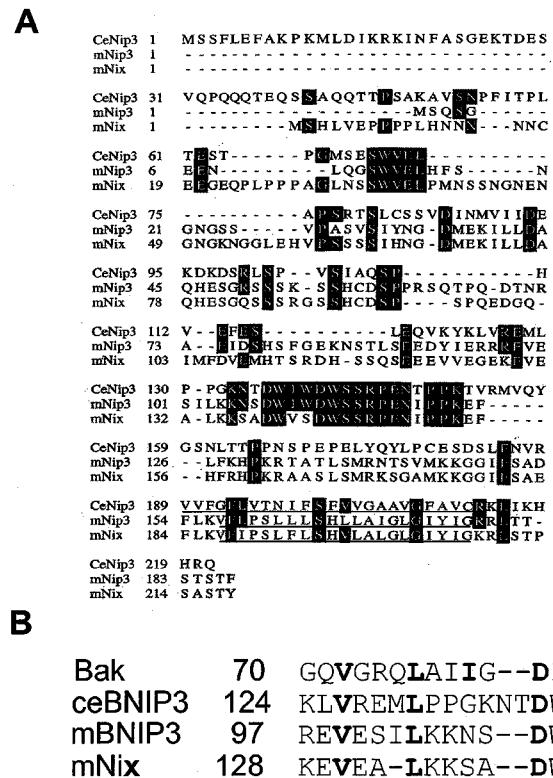


Figure 1 (a) Alignment of ceBNIP3, mouse BNIP3 (mBNIP3) and mouse NIX (mNIX) proteins. The potential transmembrane domain of ceBNIP3, mBNIP3 and mNIX is underlined. (b) Alignment of the putative BH3 domain of ceBNIP3, mBNIP3 and mNIX with the BAK BH3 domain. The key amino acids are in bold

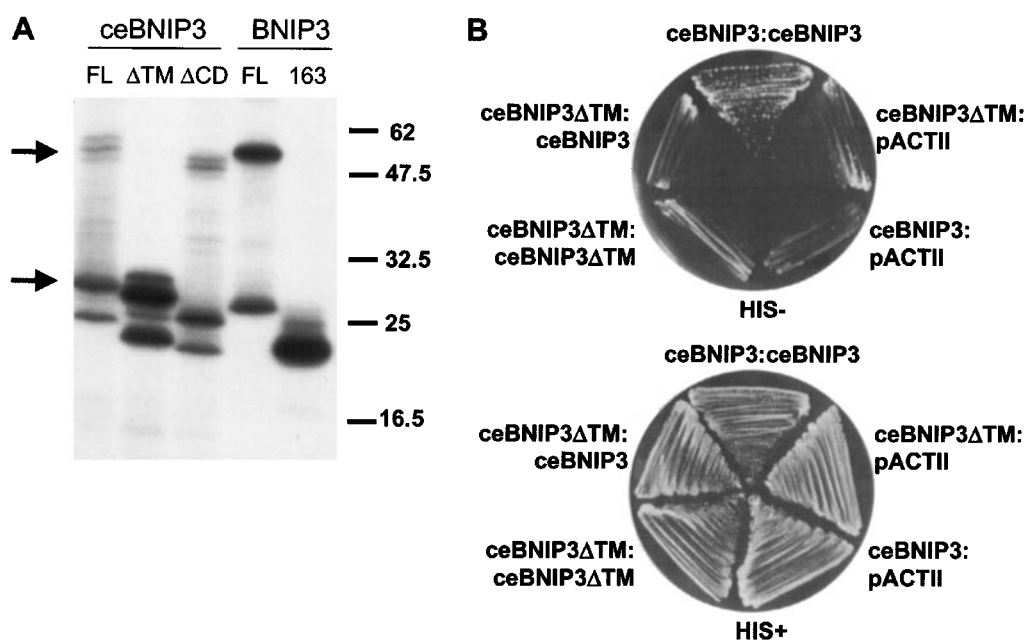


Figure 2 (a) *In vitro* expression of ceBNIP3 full length (FL), deletion of the potential transmembrane domain ceBNIP3 Δ TM (Δ TM), deletion of the conserved domain ceBNIP3 Δ CD (Δ CD) and BNIP3 full length (FL) and the BNIP3 Δ 164–194 (163). The arrows indicate the monomer and the homodimer of ceBNIP3. (b) Yeast GAL4 binding domain and activating domain plasmids encoding ceBNIP3 and ceBNIP3 Δ TM were co-transformed into yeast strain KGY37. Protein–protein interaction was determined by growth on selection media. Growth in presence of His is shown as a control. ceBNIP3 was observed to homodimerize and the removal of the TM prevented homodimerization

that ceBNIP3 is able to interact with itself but not with the ceBNIP3 Δ TM mutant, supporting the hypothesis that this region is critical for homodimerization.

Subcellular localization of ceBNIP3

The presence of a potential C-terminal TM domain in ceBNIP3 prompted us to examine the subcellular localization of ceBNIP3. BNIP3 and NIX are found to co-localize with mitochondrial matrix protein HSP60 (Chen *et al.*, 1997, 1999) and deletion of the C-terminal results in diffuse cytoplasmic staining (Chen *et al.*, 1997, 1999). MCF-7 cells were transiently transfected with N-terminal T7-tagged ceBNIP3. After 24 h, cells expressing ceBNIP3 showed a punctate pattern characteristic of intracellular membrane localization, possibly mitochondrial. By simultaneously staining with mouse monoclonal anti-T7 and rabbit anti-HSP60, and goat anti-mouse Cy3-conjugated secondary antibody and goat anti-rabbit FITC-conjugated secondary antibody, distribution of the proteins could be visualized by confocal fluorescent microscopy. The ceBNIP3 and HSP60 fluorescence had a similar punctate pattern (Figure 3a,b) and overlays of the images illustrated extensive but not total overlap (not shown). In contrast, ceBNIP3 Δ TM was diffusely stained throughout the cell (Figure 3c,d). These results suggest that ceBNIP3, like BNIP3 and NIX is localized to the mitochondria via the C-terminal TM domain.

Overexpression of ceBNIP3 in mammalian cells induces cell death

Since BNIP3 and NIX have been shown to induce apoptosis, we transiently transfected 293T or 10T1/2 cells with T7 epitope-tagged ceBNIP3. At the indicated

times, cells were stained with mouse anti-T7 antibody to identify transfected cells and Hoechst dye to assess apoptosis by nuclear chromatin condensation. 293T cells became apoptotic at 12 h after ceBNIP3 transfection and reached a peak of 45% at 48 h, compared to 60% for human BNIP3 (Figure 4a). After 36 h, 80% of the 10T1/2 cells expressing BNIP3 or ceBNIP3 were apoptotic (Figure 4b). These results confirm that ceBNIP3 is able to induce apoptosis in mammalian cell lines nearly as efficiently as BNIP3.

The same experiment was repeated using T7 epitope-tagged ceBNIP3 Δ TM. Previously, we had shown that BNIP3 or NIX without a TM domain was not targeted to the mitochondria and was unable to induce apoptosis, implying that mitochondrial localization was necessary (Chen *et al.*, 1997, 1999). Similarly, when 293T or 10T1/2 cells were transiently transfected with the T7 epitope-tagged ceBNIP3 Δ TM, no cell death was evident after 48 h (Figure 4a,b). ceBNIP3 has a putative BH3 domain (amino acids 124–138) and a conserved domain (amino acids 132–151). To examine the potential role of these domains in cell death, the deletion mutants T7-ceBNIP3 Δ BH3 and T7-ceBNIP3 Δ CD were transiently transfected into 293T or 10T1/2 cells. Figure 4a,b shows that the deletion of either of these domains had no effect on the ability of the protein to induce cell death despite similar levels of protein expression to wild type ceBNIP3 (Figure 6a).

After transient co-transfection of the 293T cells with the full length or various deletion mutants of ceBNIP3 and the β -galactosidase reporter gene, cell death was measured by counting the blue rounded cells after staining. Only the deletion of the TM domain could abrogate cell death induced by ceBNIP3 (Figure 4c). Cell death measured by the β -galactosidase assay is much more rapid with near maximum killing by 12 h,

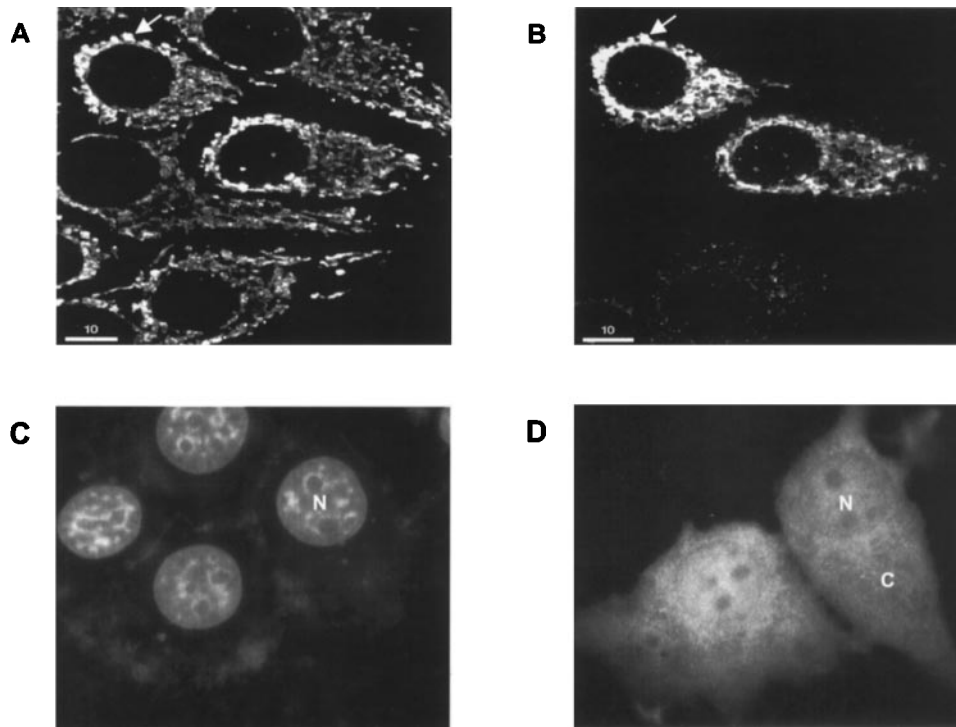


Figure 3 Subcellular localization of ceBNIP3. MCF-7 cells were transfected with T7-ceBNIP3 and stained with anti-HSP60 antibody using FITC (a) and with anti-T7 antibody using Cy3 (b). The arrow indicates region of co-localization. For the subcellular localization of ceBNIP3 Δ TM, MCF-7 cells were transfected with T7-ceBNIP3 Δ TM and stained with Hoechst (c) and mouse monoclonal anti T7-antibody using FITC (d). The nucleus (N) and cytoplasm (C) are indicated

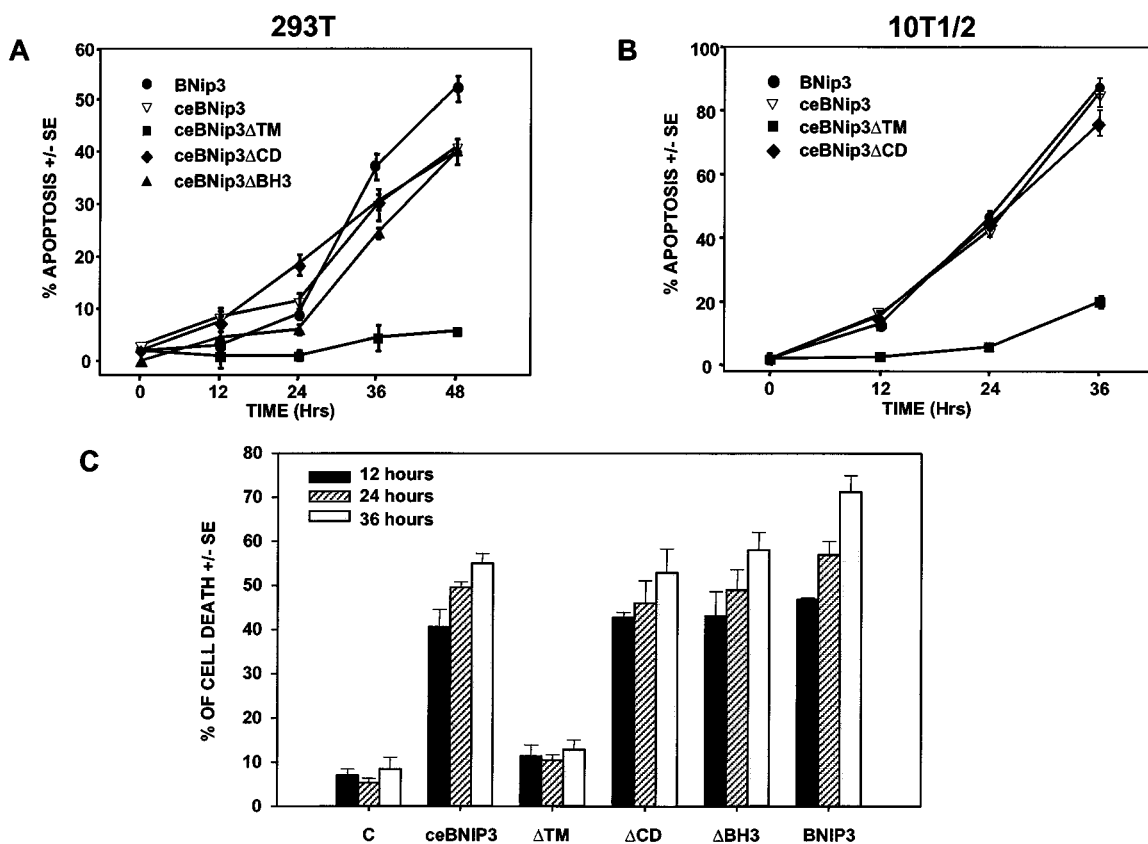


Figure 4 Overexpression of ceBNIP3 induces apoptosis. 293T cells (a) or 10T1/2 (b) were transfected with T7-ceBNIP3 (▽), T7-ceBNIP3 Δ TM (■), T7-ceBNIP3 Δ CD (◆), T7-ceBNIP3 Δ BH3 (▲) or T7-BNIP3 (●). At the indicated times, the cells were fixed and stained with mouse monoclonal anti-T7 antibody using FITC. The frequency of apoptotic cells was quantitated by Hoechst dye staining. The curves represent the mean \pm s.e. of three independent experiments. (c) 293T were transiently co-transfected with the reporter plasmid pcDNA3- β -gal and either ceBNIP3, ceBNIP3 Δ TM, ceBNIP3 Δ CD, ceBNIP3 Δ BH3 or BNIP3. At the indicated time, cells were fixed, stained and evaluated for cell death. The data represent the mean \pm s.e. of three independent experiments

while apoptosis as measured by chromatin condensation is absent at 12 h and peaks between 36 and 48 h (Figure 4c).

BCL-X_L suppression of ceBNIP3 apoptosis

It has been shown that BNIP3 and NIX-induced apoptosis are partially suppressed by BCL-2 and BCL-X_L (Chen *et al.*, 1997, 1999). In this report, we used two 10T1/2 cell lines that express a high level and a moderate level of BCL-X_L (Figure 5c). After transient transfection, BCL-X_L suppression in TX22 cell line, the moderate expressing line, partially suppresses ceBNIP3 and to about the same extent as BNIP3 (Figure 5a,b). In the TX5 cell line, the high expressing line, BCL-X_L almost completely suppresses both ceBNIP3 and BNIP3 (Figure 5a,b).

Ac-zVAD.fmk cannot block ceBNIP3-induced cell death

BNIP3-induced cell death is caspase-independent (Vande Velde *et al.*, 2000). The effect of the broad spectrum caspase inhibitor Ac-zVAD.fmk was examined after transient transfection of 293T cells with ceBNIP3 or BNIP3, using BID as a caspase-dependent pro-apoptotic positive control. Ac-zVAD.fmk (50 μM) did not prevent cell death induced by ceBNIP3 or BNIP3 (Figure 5d). In contrast, BID-induced cell death was inhibited by over 50% (Figure 5d). Another experiment using 100 μM Ac-zVAD.fmk yielded the same results (data not shown).

ceBNIP3 protein expression is regulated by the proteasome

The expression of ceBNIP3 *in vivo* following 293T cell transfection was measured by Western blotting of post nuclear lysates with anti-T7 antibody. ceBNIP3 reached peak expression between 12 and 24 h but was followed by progressive decrease over time (Figure 6a). The decrease was not correlated with increased apoptosis as ceBNIP3ΔTM was also degraded in a similar manner without cell death (Figure 6a). Removal of the CD domain did not affect protein turn over (Figure 6a). The expression of BNIP3 or NIX was similar, reaching a maximum by 24 h and then rapidly decreasing over time (Chen *et al.*, 1997, 1999).

ceBNIP3, BNIP3 and NIX contain PEST sequences that are found on proteins targeted to the proteasome. 293T cells were transiently transfected with T7-ceBNIP3 and treated with 5 μM lactacystin, an inhibitor of the threonine protease of the proteasome (Fenteany *et al.*, 1995). ceBNIP3 protein accumulated in the presence of lactacystin even after 48 h, whereas without treatment, most of the protein is degraded (Figure 6b). To assess the role of ubiquitination in the degradation of ceBNIP3 through the proteasome, we used the TS20 cell line (Chowdary *et al.*, 1994) which contains a temperature sensitive mutation in the E1 enzyme that is necessary for the ubiquitination of proteins targeted for degradation. At 34°C, the E1 enzyme is active, but becomes inactive at 39°C. TS20

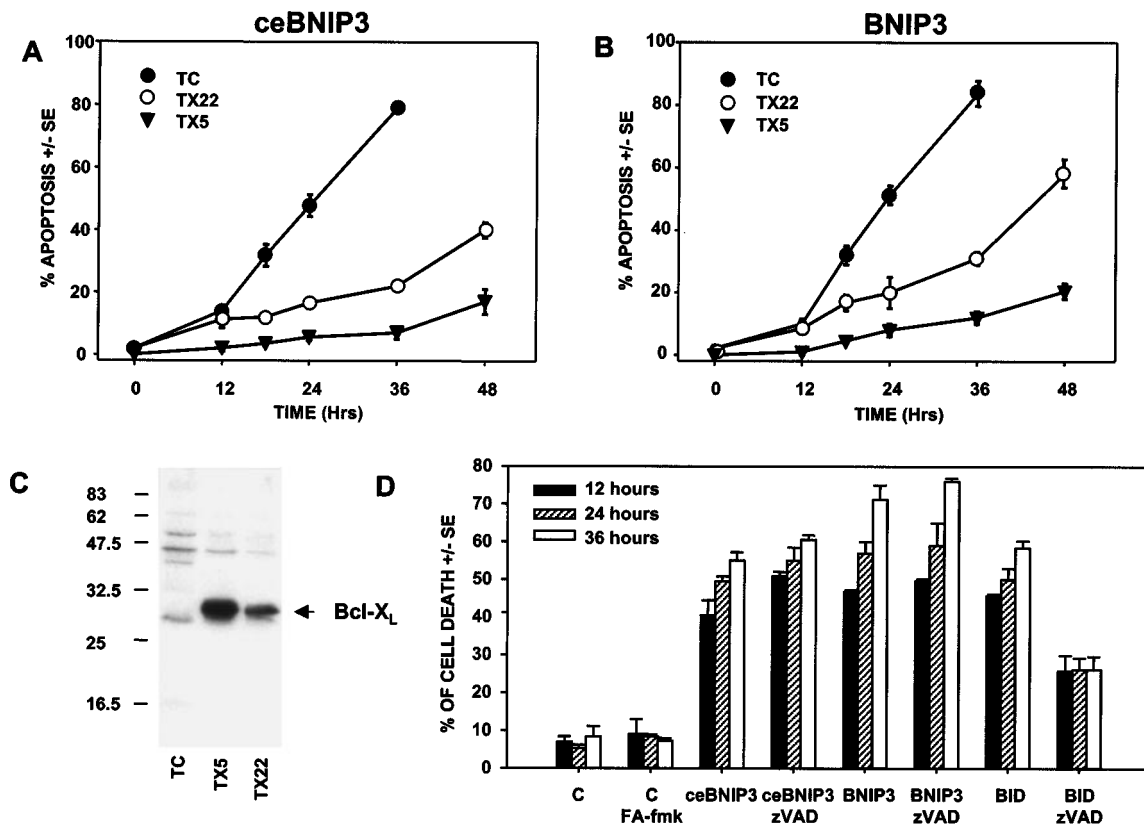


Figure 5 BCL-X_L and ceBNIP3 apoptosis. TC (parental 10T1/2 cell line) (●) and TX5 (○) or TX22 (▼) cells overexpressing BCL-X_L were transfected with T7-ceBNIP3 (a) or T7-BNIP3 (b) and apoptotic cells were scored as above. (c) Western blot of BCL-X_L (arrow) expression in cell lines TC, TX5 and TX22. Relative molecular weights (kDa) are shown on the left. (d) 293T were transiently co-transfected with the reporter plasmid pcDNA3-β-gal and either ceBNIP3, BNIP3 or BID and treated with 50 μM of Ac-zVAD.fmk. At the indicated time, cells were fixed, stained and evaluated for cell death

and its parental cell line, H38 were transiently transfected with T7-ceBNIP3 and following 12 h post-transfection, the temperature was shifted to 39°C. The cells were collected every 8 h and the lysates Western blotted for ceBNIP3 expression. As shown in Figure 7, there was an accumulation of ceBNIP3 in TS20 cells at 39°C compared to H38 or TS20 cells at 34°C, suggesting that a ubiquitination step is necessary for the proteasome processing of ceBNIP3.

Heterodimerization of ceBNIP3 with BNIP3, CED-9 and BCL-X_L

The observation that BNIP3 physically interacts with adenovirus E1B 19K, a BCL-2 functional orthologue,

as well as BCL-2 (Boyd *et al.*, 1994; Ray *et al.*, 2000) prompted us to examine the heterodimerization of ceBNIP3 with CED-9, BCL-X_L and as well BNIP3. In the yeast two hybrid assay, ceBNIP3 interacts with CED-9, BCL-X_L and BNIP3 (Figure 8a). To determine whether ceBNIP3 bound to CED-9 *in vivo*, co-transfections were performed in 293T cells. We found that following immunoprecipitation (IP) of HA-CED-9, we detected T7-ceBNIP3 by Western blot as both a monomer and a dimer (Figure 8b). Similarly, following IP of ceBNIP3, we detected CED-9 by Western blot (Figure 8b, bottom). To determine the domain necessary for the ceBNIP3 binding, 293T cells were co-transfected with CED-9 and ceBNIP3 or mutants without BH3, TM or conserved domains. As shown in Figure 8b, CED-9 immunoprecipitated with ceBNIP3ΔCD and ceBNIP3ΔBH3 as efficiently as the full length ceBNIP3. However, CED-9 co-immunoprecipitation of ceBNIP3ΔTM or ceBNIP3ΔBH3TM was virtually undetectable (Figure 8b) leaving only a faint band still seen after over exposure of the blot (not shown). The same pattern was obtained in reciprocal co-immunoprecipitation experiment of CED-9 with ceBNIP3 and the BH3, TM and CD mutants (Figure 8b, bottom). As a control, 293T were co-transfected with T7-ceBNIP3 and FLAG-DR3 and after immunoprecipitation of ceBNIP3, no DR3 was detectable and *vice versa* (not shown). The co-immunoprecipitations of BCL-X_L and ceBNIP3 deletion mutants showed exactly the same pattern as CED-9 co-immunoprecipitation (Figure 8c). These results were confirmed using yeast two hybrid system. No interactions were detected between ceBNIP3ΔTM and CED-9 or BCL-X_L in contrast to ceBNIP3 (Table 1).

Interaction of ceBNIP3 and CED-3

Yasuda *et al.*, 1998b reported that ceBNIP3 interacts with CED-3 (Figure 9a). To determine the region of interaction between ceBNIP3 and CED-3, 293T cells were co-transfected with CED-3 and ceBNIP3, ceBNIP3ΔTM, ceBNIP3ΔCD, ceBNIP3ΔN1 (deletion of the first 26 amino acids), ceBNIP3ΔN2 (deletion of the first 54 amino acids). CED-3 immunoprecipitated with all ceBNIP3 deletion mutants, thus we were unable to define any single domain or region that accounted for the interaction (Figure 9a).

293T cells were then co-transfected with ceBNIP3, CED-3 and CED-4 alone, with combinations of ceBNIP3/CED3, CED3/CED-4, ceBNIP3/CED-3/CED-4 at concentrations where any synergistic effects of the proteins could be detected. ceBNIP3 has low killing activity at this concentration and CED-3 induces around 20–30% of cell death (Figure 9b). When transfected together or in presence of CED-4, no increase other than a small additive effect was detected after 6, 9 and 12 h post-transfection. In contrast, CED-4 greatly enhanced CED-3 activity, which was efficiently blocked by CED-9.

Discussion

In this report, we have studied the *C. elegans* ortholog of BNIP3 and showed that ceBNIP3 shares common features with mammalian BNIP3 and NIX in its

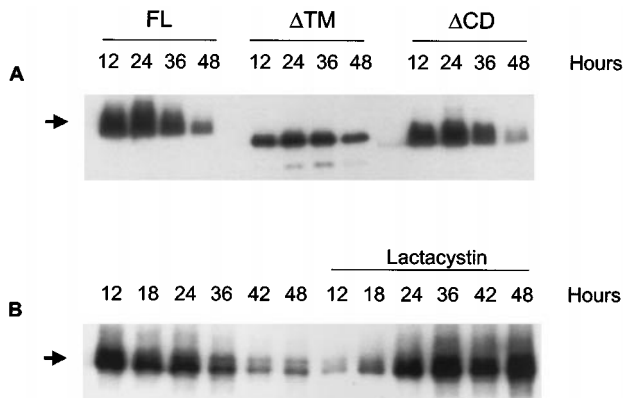


Figure 6 ceBNIP3 expression *in vivo*. (a) 293T cells were transfected with T7-ceBNIP3 (FL), T7-ceBNIP3ΔTM and T7-ceBNIP3ΔCD. Cells were harvested at the indicated times and the lysates were electrophoresed by SDS-PAGE in Tris Tricine buffer. Western blots were developed with mouse monoclonal anti-T7 antibody. (b) 293T cells were transfected with T7-ceBNIP3 and treated with 5 μM of lactacystin. At the indicated times, cells were collected and the lysates were analysed as above

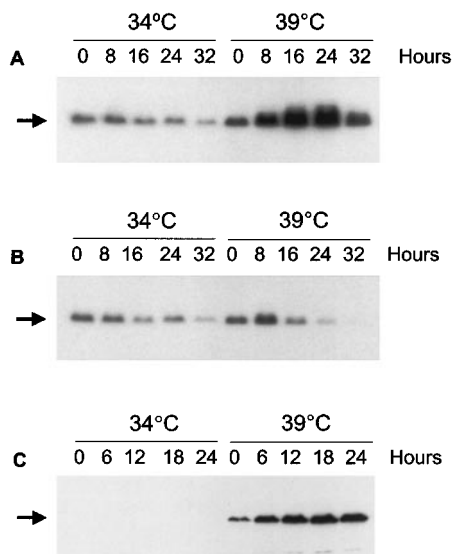


Figure 7 Regulation of ceBNIP3 protein expression by the proteasome. TS20 (a) and H38 parental cells (b) were transfected with T7-ceBNIP3. After 12 h post transfection, cells were shifted to 39°C, harvested at the indicated times and Western blots were developed with mouse monoclonal anti-T7 antibody. TS20 cell lysates (c) were also immunoblotted with a p53 antibody to show that ubiquitination was efficiently blocked as it was only detectable at 39°C

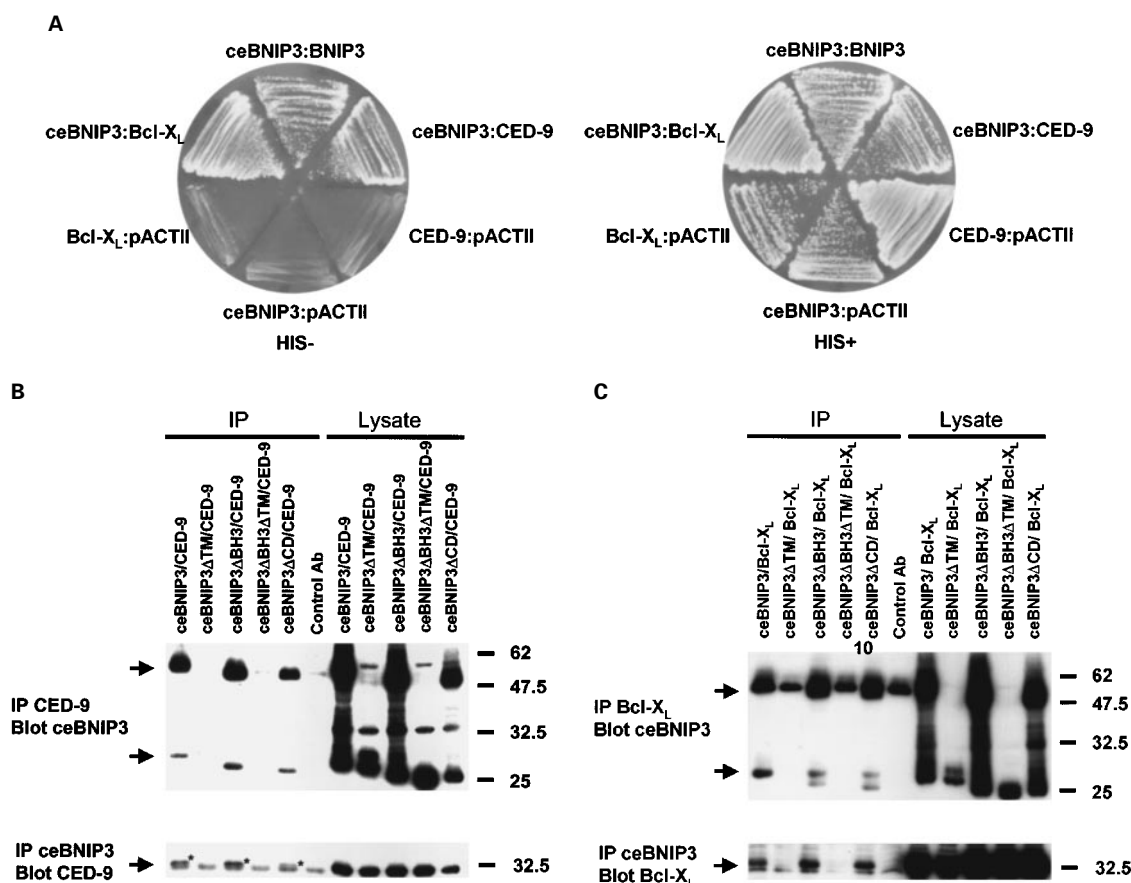


Figure 8 (a) Heterodimerization of ceBNIP3 with CED-9, BCL-X_L and BNIP3. ceBNIP3 fused to the GAL4 DNA-binding domain was co-transformed with plasmids encoding CED-9, BCL-X_L and BNIP3 fused to the GAL4 transcriptional activating domain. Protein–protein interactions were determined by growth of transformants in the absence of histidine (His⁻). Growth in the presence of histidine is shown as a control. (b) Co-immunoprecipitation of ceBNIP3 and CED-9 or BCL-X_L. 293T cells were co-transfected with T7-ceBNIP3 or different deletion mutants and HA-CED-9. After 15 h post transfection, lysates were prepared and immunoprecipitated with mouse monoclonal anti-T7 or rabbit anti-HA antibody. The presence of ceBNIP3 or the deletion mutants and CED-9 were detected by immunoblotting with mouse monoclonal anti-T7 or mouse monoclonal anti-HA antibody. The arrows indicate the homodimer and the monomer, respectively. On the bottom panel, the arrow and the symbol (*) indicate CED-9 protein. (c) 293T cells were co-transfected with the indicated plasmids for T7-ceBNIP3 and Myc-BCL-X_L. After 15 h post transfection, lysates were prepared and immunoprecipitated with mouse monoclonal anti-T7 or mouse monoclonal anti-Myc antibody. After immunoblotting, ceBNIP3 and the deletion mutants were detected with mouse monoclonal anti-T7 and BCL-X_L with rabbit anti-BCL-X_L antibody. The arrows indicate the monomer and the homodimer of ceBNIP3 and the BCL-X_L protein

Table 1 Interaction of ceBNIP3 with CED-9 and BCL-X_L in the yeast two hybrid system

Transformation (BD : AD)	HIS3 activation
CED-9 : ceBNIP3	+++
CED-9 : ceBNIP3ΔTM	-
BCL-X _L : ceBNIP3	+++
BCL-X _L : ceBNIP3ΔTM	-
CED-9 : pACTII	-
BCL-X _L : pACTII	-
pGBT9 : ceBNIP3	-
pGBT9 : ceBNIP3ΔTM	-

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

primary amino acid sequence, localization, ability to induce apoptosis, homodimerization, heterodimerization and protein degradation. The amino acid identity between these proteins is only 21%, however the homology between other *C. elegans* and mammalian

cell death proteins such as CED-9 and BCL-2, and CED-3 and caspase 1 is only 23 and 29% identity, respectively.

BNIP3, NIX and ceBNIP3 proteins contain PEST sequences and are rapidly degraded by the proteasome when overexpressed. In mammalian cells, two mechanisms target proteins to the proteasome, the first is based on the PEST sequence that is characterized by a stretch of amino acids rich in proline, glutamic acid, serine and threonine flanked between charged amino acids (Rogers *et al.*, 1986). The second is protein ubiquitination (Hochstrasser, 1996). Ubiquitination and proteasome could be involved in the regulation of apoptosis by promoting the turn over of anti-apoptotic proteins or by degradation of pro-apoptotic proteins. For example after induction of apoptosis in thymocyte, the IAP anti-apoptotic proteins are degraded by the proteasome (Yang *et al.*, 2000). The degradation is dependent on the RING domain, which catalyses an auto-ubiquitination event. In TS20 cells, defective ubiquitination has been shown to induce apoptosis through a caspase independent pathway and can be suppressed by BCL-2 (Monney *et al.*, 1998).

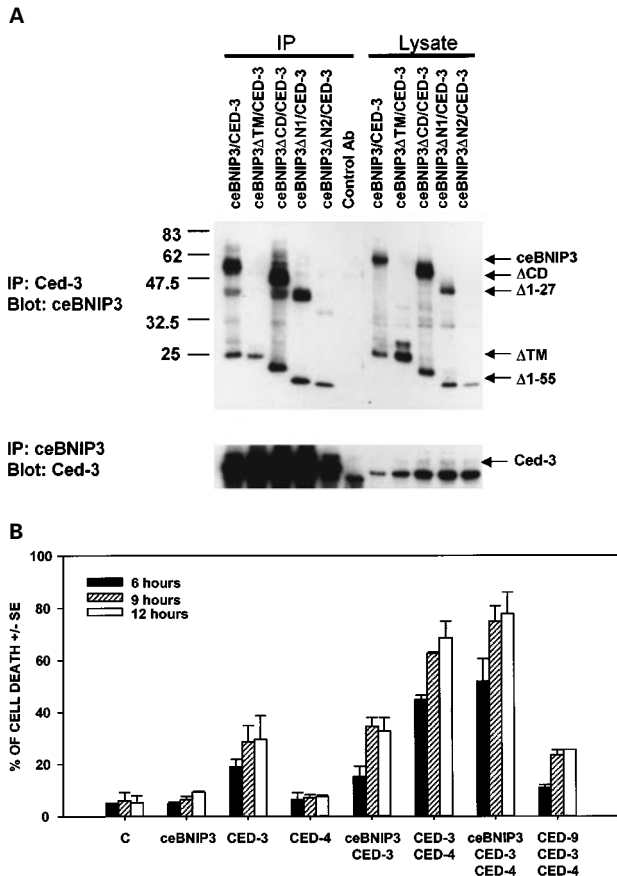


Figure 9 (a) Heterodimerization of ceBNIP3 and CED-3. 293T cells were co-transfected with the indicated plasmids for T7-ceBNIP3 and FLAG-CED-3. After 15 h post transfection, lysates were prepared and immunoprecipitated with mouse monoclonal anti-T7 antibody or rabbit anti-FLAG antibody. After immunoblotting, ceBNIP3 and the deletion mutants were detected with mouse monoclonal anti-T7 antibody and CED-3 with mouse monoclonal anti-FLAG antibody. The arrows indicate ceBNIP3 and the different deletion mutants. (b) 293T cells were transiently co-transfected with the reporter plasmid pcDNA3- β -gal and a combination of the different plasmids ceBNIP3, CED-4, CED-3 and CED-9. At the indicated time, cells were fixed, stained and evaluated for cell death

Another study showed that BAX could be degraded by a ubiquitin proteasome-dependent pathway that correlates with an increase in the survival human cancer cells (Li and Dou, 2000). The data obtained in the present study using the TS20 cells and lactacystin show that ceBNIP3 is degraded through an ubiquitin-dependent step and requires an active proteasome. The significance of this event in relation to apoptosis is unknown. It is possible that the direct ubiquitination of ceBNIP3, BNIP3 and NIX is responsible for its activation by allowing the protein to be cleaved by the proteasome, or ubiquitination modifies the conformation of the protein. The ubiquitination of an unknown ceBNIP3 binding protein could also be regulatory, possibly by permitting the displacement of ceBNIP3 from an inactive complex.

ceBNIP3, BNIP3 and NIX share a highly conserved domain of 19 amino acids, however deletion of this region has no effect on ceBNIP3-inducing cell death. Because of its evolutionary conservation, we suspect that the domain must contribute to ceBNIP3 regulation. It is possible that ceBNIP3, BNIP3 and NIX may

interact with other proteins or can be post-translationally modified through the conserved domain. However, this region is not important for interaction with CED-9 or BCL-X_L. Deletion of the conserved domain also creates a partial deletion of the putative BH3 domain. The BH3 domain of pro-apoptotic proteins forms an amphipathic α -helix that binds in the hydrophobic pocket created by the BH1, BH2 and BH3 domains of BCL-X_L (Kelekar and Thompson, 1998). The deletion of the BH3 domain of BNIP3 has no effect (Ray *et al.*, 2000) or only partially blocks apoptosis (Yasuda *et al.*, 1998a) suggesting that another portion of the protein may also be involved. The data presented in this study demonstrated that deletion of the ceBNIP3 BH3 domain had no effect on the apoptotic activity of the protein, indicating that it is not a classical pro-apoptotic BH3-only protein. Alignment of the BH3 domains of different pro-apoptotic BCL-2 family members shows that the core has a leucine at position 1 and an aspartic acid at position 6 (Kelekar and Thompson, 1998). However, the putative BH3 domain of ceBNIP3 contains the conserved leucine but the conserved aspartic acid is at position 8 (Figure 1b) due to the insertion of two proline residues at positions 2 and 3, therefore making it unlikely that this region could form an α -helix.

Sequence analysis of ceBNIP3 revealed a α -helical hydrophobic domain in the C-terminus of the protein that could be a TM domain. Like BNIP3 and NIX, the TM domain is required for homodimerization in yeast two hybrid system (Chen *et al.*, 1997; Ray *et al.*, 2000). Interestingly, ceBNIP3 and BNIP3 also heterodimerize through the TM domain despite only a few conserved amino acids suggesting that the functional specificity of the region has been conserved. Transiently transfected ceBNIP3 localized to the mitochondria and the cells underwent apoptosis, but not ceBNIP3 Δ TM indicating that its activity depends on its mitochondrial localization, similar to BNIP3 and NIX (Chen *et al.*, 1997, 1999). The β -galactosidase assay showed that overexpression of ceBNIP3 in mammalian cells induced cell death almost as efficiently as BNIP3 with 40% at 12 h and reaching 60% by 48 h. In contrast, chromatin condensation detected by Hoechst staining was under 10% after 12 h for ceBNIP3 and BNIP3. These data show that chromatin condensation is a late event compared to the global morphological changes induced by ceBNIP3 overexpression.

EGL-1 has been shown to interact with CED-9 through its BH3 domain and allows the release of CED-4 and CED-3 from the complex with CED-9. In this report, we found that ceBNIP3 interacts with CED-9 and BCL-X_L. Deletion mapping showed that the putative BH3 and conserved domains did not suppress this binding. In contrast, the deletion of the TM domain significantly reduced the interaction with CED-9 and BCL-X_L. The presence of a faint band after over exposure suggests that ceBNIP3 may also weakly bind CED-9 and BCL-X_L through another domain. The same type of interaction has been described between BCL-2 or BCL-X_L and Bap31. Bap31 is a pro-apoptotic protein associated with the endoplasmic reticulum that interacts with BCL-2 or BCL-X_L, a CED-4 like protein and pro-caspase 8 (Ng *et al.*, 1997). *In vitro* and *in vivo* studies have shown that BCL-2 or BCL-X_L bind directly Bap31 to the N-

terminal domain that contains three TM domains (Ng *et al.*, 1997; Ng and Shore, 1998). The binding domain of ceBNIP3 with CED-9 is therefore distinct from EGL-1. ceBNIP3 is the first *C. elegans* pro-apoptotic protein that has been shown to localize to the mitochondria in mammalian cells. In response to an apoptotic signal, the role of the mitochondria in *C. elegans* is still obscure but in mammalian cells, it plays an important role by releasing cytochrome *c* and $\Delta\psi_m$ disruption (Green and Reed, 1998), subsequently activating the caspase cascade. We have shown that overexpression of BNIP3 induces opening of the PT pore, which leads to a loss of $\Delta\psi_m$ and ROS production (Vande Velde *et al.*, 2000). Therefore, ceBNIP3 may also disrupt the function of the mitochondria in a similar manner. In contrast to other inducers of cell death, BNIP3 does not induce the release of cytochrome *c* and cell death is not blocked by the broad spectrum of caspase inhibitor Ac-zVAD.fmk (Vande Velde *et al.*, 2000). Ac-zVAD.fmk also has no effect on ceBNIP3, implying that it uses a caspase-independent cell death pathway. This lack of participation of caspases in cell death is also seen in ceBNIP3 and CED-3 co-transfection. Despite evidence of interaction between ceBNIP3 and CED-3 by co-immunoprecipitation, expression of both proteins does not significantly increase cell death compared to co-transfection of CED-3 and CED-4. So ceBNIP3 may act as a docking protein allowing the proper positioning of CED-3 *in vivo*, but it does not act as a CED-4-like protein to promote CED-3 activation.

In conclusion, we have characterized the orthologue of BNIP3 in *C. elegans*, ceBNIP3. ceBNIP3 is capable of rapidly activating cell death and interacting with CED-9 and CED-3. However, it kills through a BH3- and caspase-independent mechanism.

Materials and methods

Cell cultures and reagents

MCF-7 breast carcinoma cells, 10T1/2 fibroblast cells, and the BALB/c 3T3 TS20 and H38 cell lines were cultured in α -minimal essential medium (α -MEM) (GIBCO, BRL) supplemented with 10% fetal bovine serum (GIBCO, BRL), 1% glutamine (GIBCO, BRL), 1% HEPES (GIBCO, BRL) and 1% MEM sodium pyruvate (GIBCO, BRL). 293T human embryonic kidney cells were cultured in Dulbecco's Modified Eagle Medium (D-MEM) (GIBCO, BRL) supplemented with 10% fetal bovine serum. Anti-rabbit HSP60 antibody was kindly provided by Dr Radhey Gupta (McMaster University, Hamilton, ON, Canada).

Cloning and construction of expression plasmids

The ceBNIP3 cDNA was obtained by polymerase chain reaction (PCR) from a *C. elegans* cDNA library generously donated by Dr Pete Okkema (University of Illinois, Chicago, IL, USA) using the primer pair: 5'-ACAATGTCCT-CATTTCTTGAG-3' and 5'-TCATTGACGATGATGTTT-TAG-3'. To construct the T7 epitope-tagged ceBNIP3 in pcDNA3 (Invitrogen Corp.), a cDNA fragment encoding T7 was inserted into the *Hind*III and *Kpn*I sites of the vector. Restriction sites *Kpn*I and *Bam*HI were introduced by PCR at the 5' and 3' ends of the insert respectively. The fragment was then cloned into *Kpn*I and *Bam*HI sites of pcDNA3-T7.

Deletion mutants of the TM (amino acids 189–210), ceBNIP3 Δ TM, the putative BH3 domain (amino acids 124–142) ceBNIP3 Δ BH3, the double deletion mutant Δ TMBH3 and the conserved domain (amino acids 132–151), ceBNIP3 Δ CD, ceBNIP3 Δ N1 deletion of the first 26 amino acids, ceBNIP3 Δ N2 deletion of the first 54 amino acids were introduced into ceBNIP3 by site directed mutagenesis using PCR. HA-CED-9 and FLAG-CED-3 expression plasmids were kindly provided by Dr Gabriel Nunez (University of Michigan, Ann Harbor, MI, USA) and Myc-BCL-X_L by Dr Gordon Shore (McGill University, Montreal, PQ, Canada).

For yeast transformations, plasmids encoding ceBNIP3 and ceBNIP3 Δ TM with suitable restriction sites were generated by PCR and then ligated in frame into the GAL4 binding domain of pGBT9 and the GAL4 transcription activating domain of pACTII (Gietz *et al.*, 1997). The pGBT9 or pACTII constructs containing BNIP3 have been described previously (Chen *et al.*, 1997; Ray *et al.*, 2000). The nucleotide sequence of all constructs was confirmed using an ABI 310 Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

Yeast transformations

The pGBT9 and pACTII constructs were co-transformed into yeast strain KGY37 and plated on synthetic complete (SC) selection media lacking leucine and tryptophan. Protein–protein interactions were determined by growth on SC selection medium lacking leucine, tryptophan and histidine in the presence of 1 mM 3-amino-1,2,4, triazole (3AT).

In vitro translation

³⁵S-labeled ceBNIP3, BNIP3 and deletion mutants of both proteins were prepared by *in vitro* transcription and translation of cDNAs cloned into pcDNA3-T7 vector using the TnT coupled Reticulocyte Lysate System (Promega Corp., Madison, WI, USA), according to the manufacturer's instructions.

Transient transfection and cell death assays

Aliquots of 1×10^5 293T cells were seeded in 6-well plates and then transfected by the calcium phosphate precipitation method using 2 μ g of expression plasmid. MCF-7 (1×10^5), 10T1/2 (1×10^5) cells stably transfected with Bcl-X_L and 2.5×10^4 10T1/2 cells were transfected with 1 μ g of expression plasmid using 7 μ l of LipofectAMINE Reagent (GIBCO, BRL). Cells were plated on coverslips in 6-well plates and were incubated with the DNA-LipofectAMINE mixture for 5 h and then fixed with 4% formaldehyde in PBS at the indicated time. Cells were stained with mouse monoclonal anti-T7 antibody (Novagen, Madison, WI, USA) and visualized with FITC-conjugated goat anti-mouse IgG secondary antibody (Sigma Chemical Co, St. Louis, MO, USA). The nuclei were stained with Hoechst dye. Approximately 200 transfected cells were scored as apoptotic or non-apoptotic using a Zeiss axiophot microscope.

For the β -galactosidase assay, 293T cells were co-transfected with 0.05 μ g of reporter plasmid, pcDNA3- β -galactosidase, plus 0.5 μ g of the indicated plasmid for 4 h using LipofectAMINE reagent. Ac-zVAD.fmk or Ac-FA.fmk (Enzyme System Product, Dublin, CA, USA) was added to a final concentration of 50 or 100 μ M and the cells were stained at the indicated time as described previously (Miura *et al.*, 1993). Similar assay was repeated to measure the ceBNIP3, CED-3 and CED-4 killing activity. The reporter gene, pcDNA3- β -galactosidase, was used at 0.01 μ g and the various plasmids at 0.025 μ g. pcDNA3 was added to keep the concentration of DNA equal between different transfection experiments.

For subcellular localization, MCF-7 cells were transfected with expression plasmids using LipofectAMINE as described above. The cells were fixed and double stained with mouse monoclonal anti-T7 (Novagen, Madison, WI, USA) and rabbit polyclonal anti-HSP60 antibody 24 h post-transfection, and visualized with goat anti-mouse Cy3-conjugated antibody (Chemicon, Temecula, CA, USA) and goat anti-rabbit FITC-conjugated antibody (Sigma Chemical Co, St. Louis, MO, USA) respectively. Samples were analysed using a confocal fluorescence microscope equipped with an argon laser and dual detectors (Molecular Dynamics, Sunnyvale, CA, USA). The images were acquired with Image Space software using a 60 objective lens and then transferred into Adobe Photoshop for printing.

Co-immunoprecipitation

293T cells ($2-5 \times 10^6$) were transfected with 4 μg of indicated plasmids by the calcium phosphate method. After 15 h post transfection, cells were collected and resuspended in lysis buffer containing 100 mM Tris pH 8, 2 mM EDTA, 100 mM NaCl, 0.2% NP-40 and protease inhibitors. The lysates were sonicated for 10 s three times, centrifuged for 10 min and precleared with protein A-sepharose 4B (Zymed, Laboratories Inc.) for 30 min at 4°C. Equal volumes of binding buffer containing 200 mM NaCl, 20% glycerol and 0.2% NP-40 were added with 1 μg of antibody and then incubated for 2 h at 4°C. Mouse monoclonal anti-T7 antibody was purchased from Novagen (Madison, WI, USA), rabbit anti-HA antibody from Santa Cruz (San Diego, CA, USA) and the mouse monoclonal anti-Myc antibody was a gift from Dr J Wright (Manitoba Institute of Cell Biology, Winnipeg, MB, Canada), the mouse monoclonal anti-FLAG antibody from

Sigma Chemical Co. (St. Louis, MO, USA) and the polyclonal rabbit anti-FLAG antibody from Zymed (San Francisco, CA, USA). Immune complexes were captured with protein A-sepharose 4B. Immunoprecipitates were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with mouse monoclonal anti-T7, mouse monoclonal anti-HA (Boehringer Mannheim, Indianapolis, IN, USA) or rabbit anti-BCL-X_L antibody (PharMingen, Mississauga, ON, Canada).

Western blotting

Aliquots of 5×10^5 transfected cells were resuspended in SDS sample buffer and electrophoresed by the Laemmli SDS-PAGE method or SDS-PAGE using Tris-Tricine buffer and PVDF membrane (Horton *et al.*, 1993) and were immunoblotted with anti-T7 antibody using an ECL detection kit according to the manufacturer's instructions (Amersham, Amersham, UK).

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