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C-Terminal Region of Bfl-1 Induces Cell Death That Accompanies Caspase Activation When Fused With GFP

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Abstract Previously, we reported that anti-apoptotic Bfl-1 is converted to a pro-apoptotic protein following fusion at its N-terminus with green fluorescent protein (GFP) (GFP-Bfl-1). In this study, we performed a Bfl-1 deletion study in order to elucidate the underlying mechanism of GFP-Bfl-1-induced cell death. We found that the Bcl-2 homology (BH) domains in Bfl-1 are dispensable with respect to cell death and that GFP fusion with the 29 amino acids of the C-terminal region of Bfl-1 (GFP-BC) is sufficient to induce cell death. Moreover, when BC was fused with other tagging partners like GST or MBP, little cell death was observed, implying that the GFP region is as important as the BC region for GFP-BC-induced cell death. Further deletion analysis defined a region of GFP as a determinant of GFP-BC-induced cell death. Confocal microscopic analysis showed that GFP-chimeras containing the BC region of Bfl-1 are located mainly in mitochondria. The GFP-BC-induced cell death accompanied cellular caspase activation, and treatment with the pan-caspase inhibitor, Boc-D-FMK, partially inhibited GFP-BC-induced cell death. However, the over-expression of anti-apoptotic molecules, such as Bcl-x_L and CrmA, did not block GFP-BC-induced cell death. In summary, GFP-BC induces cell death with caspase activation through mitochondria dependent process. J. Cell. Biochem. 94: 1234–1247, 2005.

Key words: GFP; Bfl-1; cell death

Members of the Bcl-2 protein family can be functionally divided into pro-apoptotic and anti-apoptotic proteins [Adams and Cory, 1998]. They share conserved Bcl-2 homology (BH) domains that are involved in homo- and hetero-typic interactions among Bcl-2 family proteins [Kelekar and Thompson, 1998]. Pro-apoptotic Bcl-2 family proteins can be further divided into Bax-like molecules and BH3-only molecules. Bax-like molecules include Bax, Bak, and Bok and have BH1, BH2, and BH3 domains [Oltvai et al., 1993; Chittenden et al., 1995; Hsu et al., 1997]. The BH3-only molecules include Bik, Blk, Hrk, BNIP3, Bim_L, Bad, Bid, and EGL-1 and have only the BH3 domain [Yang et al., 1995; Han et al., 1996; Wang et al., 1996; Inohara et al., 1997; Conradt and Horvitz, 1998; Hegde et al., 1998; O'Connor et al., 1998; Yasuda et al., 1998]. Similarly, anti-apoptotic proteins possess combination of BH1, BH2, and BH3 domains, but important members of this family, such as Bcl-2, Bcl-x_L, Bcl-w, and Ced-9, also have an additional BH4 domain [Hengartner and Horvitz, 1994; Gibson et al., 1996; Reed et al., 1996; Huang et al., 1998]. However, several anti-apoptotic Bcl-2 family proteins do not contain the BH4 domain.

The Bfl-1 protein belongs to this BH4 non-coding anti-apoptotic Bcl-2 family proteins. Moreover, Bfl-1 has only the BH1 and BH2 domains and lacks any defined membrane-anchoring region [Choi et al., 1995]. However, its anti-apoptotic function has been clearly

Abbreviations used: GFP, green fluorescent protein; MBP, maltose binding protein; GST, glutathione-S-transferase; Boc-D-FMK, Boc-Asp(OMe)-fluoromethyl ketone.

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demonstrated in several reports. For example, Bfl-1 can inhibit Bax- or etoposide-induced cell death [Wang et al., 1999; Zhang et al., 2000]. More importantly, Bfl-1 can also inhibit TNF-induced apoptosis [Zong et al., 1999]. The promoter region of the Bfl-1 gene has binding sites for the potent anti-apoptotic nuclear factor NF-κB that is activated upon TNF ligand–receptor interaction [Edelstein et al., 2003]. Consequently, Bfl-1 is thought of as a mediator of tumor cell resistance to TNF treatment and the up-regulation of Bfl-1 expression in cancer cell lines and tumor tissues has been well documented [Choi et al., 1995; Park et al., 1997].

The classification of Bcl-2 family members is based on their effects on apoptosis. However, several reports have described pro-apoptotic functions for known anti-apoptotic member proteins and vice versa. For example, Bcl-2, a representative anti-apoptotic protein, was found to induce cell death when transiently expressed in several cell lines [Uhlmann et al., 1998; Wang et al., 2001]. Bcl-2 and Bax share BH1, BH2, BH3, and transmembrane (TM) domains, though Bcl-2 has an additional BH4 domain in its N-terminus. If the N-terminal BH4 region of Bcl-2 is removed by protease, the remaining BH1, BH2, BH3, and TM domains could function as a Bax-like molecule, as demonstrated by Cheng et al. [1997]. However, point mutations of cleavage sites in Bcl-2 did not change the cell death inducing property of Bcl-2, indicating that there exist other pathways through which over-expressed Bcl-2 mediates cell death [Wang et al., 2001]. Similarly, the strong pro-apoptotic factor, Bax, was found to accelerate staurosporine-induced apoptosis, but it protected dopaminergic neuronal cells from nigericin-induced cell death [Oh et al., 1997]. Bak also protected Epstein–Barr virus transformed WI-L2 cells from serum withdrawal or menadione-induced apoptosis [Kiefer et al., 1995].

Our previous studies also showed that the anti-apoptotic function of Bfl-1 protein was changed into a pro-apoptotic when fused with GFP [Ko et al., 2003]. Here, we report that the over-expression of GFP fused with only the 29 amino acids of the C-terminal region of Bfl-1 (GFP-BC) strongly induces cell death in the SV40 large T antigen-containing human embryonic kidney cell line, 293T. In addition, we defined a region in GFP to be responsible for GFP-BC-induced cell death, and found that both the high expression and the mitochondrial localization of GFP-BC are important for this GFP-BC-induced cell death. The characteristics of GFP-BC-induced cell death are described and the possible mechanism of GFP-BC-induced cell death is discussed.

MATERIALS AND METHODS

DNA Constructs

The cDNA fragments of human Bfl-1 corresponding to amino acids 1–175, 1–158, 1–118, 1–67, 62–175, 98–175, and 147–175 were amplified by PCR using ExTaq. Polymerase (Takara bio, Japan) and subcloned into pEGFPC vector (Clontech, Palo Alto, CA) to generate GFP-Bfl-1, GFP-ΔBC, GFP-Δ2BC, GFP-Δ12BC, GFP-ΔN, GFP-ΔN1, and GFP-BC, respectively. The cDNAs of gluthathione-S-transferase (GST) and maltose binding protein (MBP) were amplified by PCR and subcloned into GFP-BC expression plasmid to generate GST-BC and MBP-BC. The cDNA fragments encoding amino acids 11–239, 101–239, and 151–239 of GFP were amplified by PCR and subcloned into GFP-BC expression plasmid to generate ΔN10-BC, ΔN100-BC, and ΔN150-BC, respectively. To express GFP-BC at low levels, the GFP-BC encoding cDNA was amplified by PCR and subcloned into pRet-On vector (Clontech), which has a minimal cytomegalovirus (CMV) immediate early promoter, resulting in a GFP-BC^{low} construct. cDNA fragments encoding human Bcl-x_L or CrmA were amplified by PCR and subcloned into pFlag-CMV2 vector (Sigma, St. Louis, MO) to generate FL-Bcl-x_L and FL-CrmA, respectively. The cDNA fragment of mouse Bax was amplified by PCR and subcloned into pEGFPC vector to generate GFP-Bax, and the GFP-Bcl-x_L construct was produced as described previously [Ko et al., 2003]. GFP-Caspase-8 was kindly provided by Dr. Miyashita (NRICHD, Japan). All the inserts of the plasmids were verified by dideoxynucleotide sequencing. Detailed information on the primer sequences used in the PCR reactions during this study is shown in Table I.

Cell Death Assay

293T cells were grown in DMEM supplemented with 10% FBS (Invitrogen, Groningen, The Netherlands). The day before transfection, 5×10^5 cells were plated on cover slips in a 6-well culture plate (Nalgen Nunc International, Naperville, IL). The next day, the indicated

TABLE I. Primer Sequences Used in PCR

Clone name	Amino acids region	Oligonucleotide sequence (5′–3′)
GFP-Bfl-1	1–175 of Bfl-1	5′-GAATTCGATGACAGACTGTGAATTTGGATAT-3′ 5′-GGATCCTCAACAGTATTGCTTCAGGAGAGA-3′
GFP-ΔBC	1–158 of Bfl-1	5′-GAATTCGATGACAGACTGTGAATTTGGATAT-3′ 5′-TCTAGAAAAGTCATCCAGCCAGATTTAGGTT-3′
GFP-Δ2BC	1–118 of Bfl-1	5′-GAATTCGATGACAGACTGTGAATTTGGATAT-3′ 5′-AAGAATTCAAATCTCCTTATAGGTATCCAC-3′
GFP-Δ12BC	1–67 of Bfl-1	5′-GAATTCGATGACAGACTGTGAATTTGGATAT-3′ 5′-AAGAATTCAGGCAGTGTCTACGGACAC-3′
GFP-ΔN	62–175 of Bfl-1	5′-AAGAATTCGATGGTGTCCGTAGACACTGCCAG-3′ 5′-GGATCCTCAACAGTATTGCTTCAGGAGAGA-3′
GFP-ΔN1	98–175 of Bfl-1	5′-GAATTCTCATCAAGAAACTTCTACGAC-3′ 5′-GGATCCTCAACAGTATTGCTTCAGGAGAGA-3′
GFP-BC	147–175 of Bfl-1	5′-GATCCCGAATTCTGATGAAGTTTGAACCTAAATCTGGC-3′ 5′-GATCCCGGATCCTCAACAGTATTGCTTCAGGAGAGA-3′
GST-BC	1–232 of GST	5′-GATCCCGCTAGCATGTCCCCTATACTAGGTTATTG-3′ 5′-GATCCCGAATTCCCACGCGGAACCAGATCCG-3′
MBP-BC	1–367 of MBP	5′-GATCCCGCTAGCATGAAAATCGAAGAAGGTAAACTG-3′ 5′-GATCCCGAATTCGAATTAGTCTGCGCGTCTTTCA-3′
ΔN10-TM	11–239 of GFP	5′-GATCCCTCTAGAATGACCGGGGTGGTGCCCATCC-3′ 5′-GATCCCAAGCTTCGGACTTGTACAGCTCGTCC-3′
ΔN100-TM	101–239 of GFP	5′-GATCCCGCTAGCATGTTCTTCAAGGACGACGGCAAC-3′ 5′-GATCCCGAATTCGACTTGTACAGCTCGTCCATG-3′
ΔN150-TM	151–239 of GFP	5′-GATCCCTCTAGAATGGTCTATATCATGGCCGACAAG-3′ 5′-GATCCCAAGCTTCGGACTTGTACAGCTCGTCC-3′
FL-Bcl-xL	1–233 of Bcl-xL	5′-GATCCCGAATTCATGTCTCAGAGCAACCGGGAG-3′ 5′-GATCCCAGATCTTCATTTCCGACTGAAGAGTGAGC-3′
FL-CrmA	1–489 of CrmA	5′-GATCCCAAGCTTATGGATATCTTCAGGGAAATCG-3′ 5′-GATCCCAGATCTTTAATTAGTTGTTGGAGAGCAATATC-3′

amounts of DNA constructs were transferred to 293T cells using Lipofectamine plus reagent according to the manufacturer's prtocol (Invitrogen). After 18–24 h, cover slips were mounted on a glass slides and cell morphologies were observed under blue light excitation using an Axovert 100 inverted epifluorescence microscope (Carl Zeiss, Thornwood, NY). At least three pictures were taken per sample and the resulting photographs were analyzed for dying/dead cells. These micrographs contained an average of a hundred cells; disintegrated faint green cells were counted as dying/dead cells. For trypan blue assays, cells were seeded in a 6-well culture plate, and then transfected with DNA constructs. After 18–24 h of transfection, cells were stained with 0.4% trypan blue solution (Invitrogen), and viable cells were counted according to the manufacturer's protocol. For DNA fragmentation analysis, cells were harvested and centrifuged at 450*g* for 10 min at 4°C. Cell pellets obtained were incubated with 0.2 mg/ml proteinase K in 500 μl of buffer (100 mM Tris-HCl, pH 8.5, 5 mM EDTA, 200 mM NaCl, 0.2% SDS) for at least 4 h at 55°C. DNA was precipitated using an equal volume of isopropanol, treated with 0.1 mg/ml RNase A at 37°C, analyzed in a 2% agarose gel, and detected by ethidium bromide staining. When appropriate, the pan-caspase inhibitor, Boc-D-fmk (Calbiochem, San Diego, CA), was added to culture to a final concentration of 100 μM.

Fluorescent Microscopy

Cells grown on cover slips were transfected with DNA constructs using Lipofectamine plus reagent (Invitrogen). After 18–24 h, culture media was removed and cells were fixed with 2% paraformaldehyde for 10 min. Cover slips were then removed and 20 μl of 1 μg/ml DAPI solution (Calbiochem) in 50% glycerol was added to each cell monolayer. For mitochondria staining, culture media was removed, and serum free media containing 1 μM Mitotracker Red CMXRos (Molecular Probes, Eugene, OR) was added and incubated at 37°C for 15 min. Cover slips were then mounted on slide glasses and images were obtained using an LSM510 (Carl Zeiss).

Immunoblot

Cells were harvested and centrifuged at 500*g* for 10 min at 4°C. Cell pellets obtained were washed once with 1 ml phosphate buffered

saline (PBS) and lysed with 100 μl 2× sample buffer (20 mM Tris pH 8.0, 2 mM EDTA, 2% SDS, 20 mM DTT, 1 mM Na_3VO_4, 20% glycerol). Lysates were sonicated in pulse mode and boiled for 5 min. Subsequently, suspensions were centrifuged at 10,000*g* for 10 min at 4°C and supernatants were used as whole cell lysates. Protein quantification assays were performed using MicroBCA reagent (Pierce, Rockford, IL) according to the manufacturer's protocol. Typically, 50 μg of total cellular proteins were separated by SDS–PAGE and the separated proteins were then transferred to nitrocellulose membranes. Membrane were then subjected to standard Western blot using mouse anti-GFP (Santa Cruz, CA), mouse anti-MBP (Santa Cruz), mouse anti-M2 (Sigma), rabbit anti-caspase-3 (Cell Signaling, Beverly, MA), mouse anti-GST (Santa Cruz), or rabbit anti-poly (ADP-ribose) polymerase (Upstate, Charlottesville, VA), and horseradish peroxidase conjugated secondary antibodies (Santa Cruz). All blotting membranes were stained with Amido-Black (Sigma) to ensure that equal amounts of proteins had been loaded into each well.

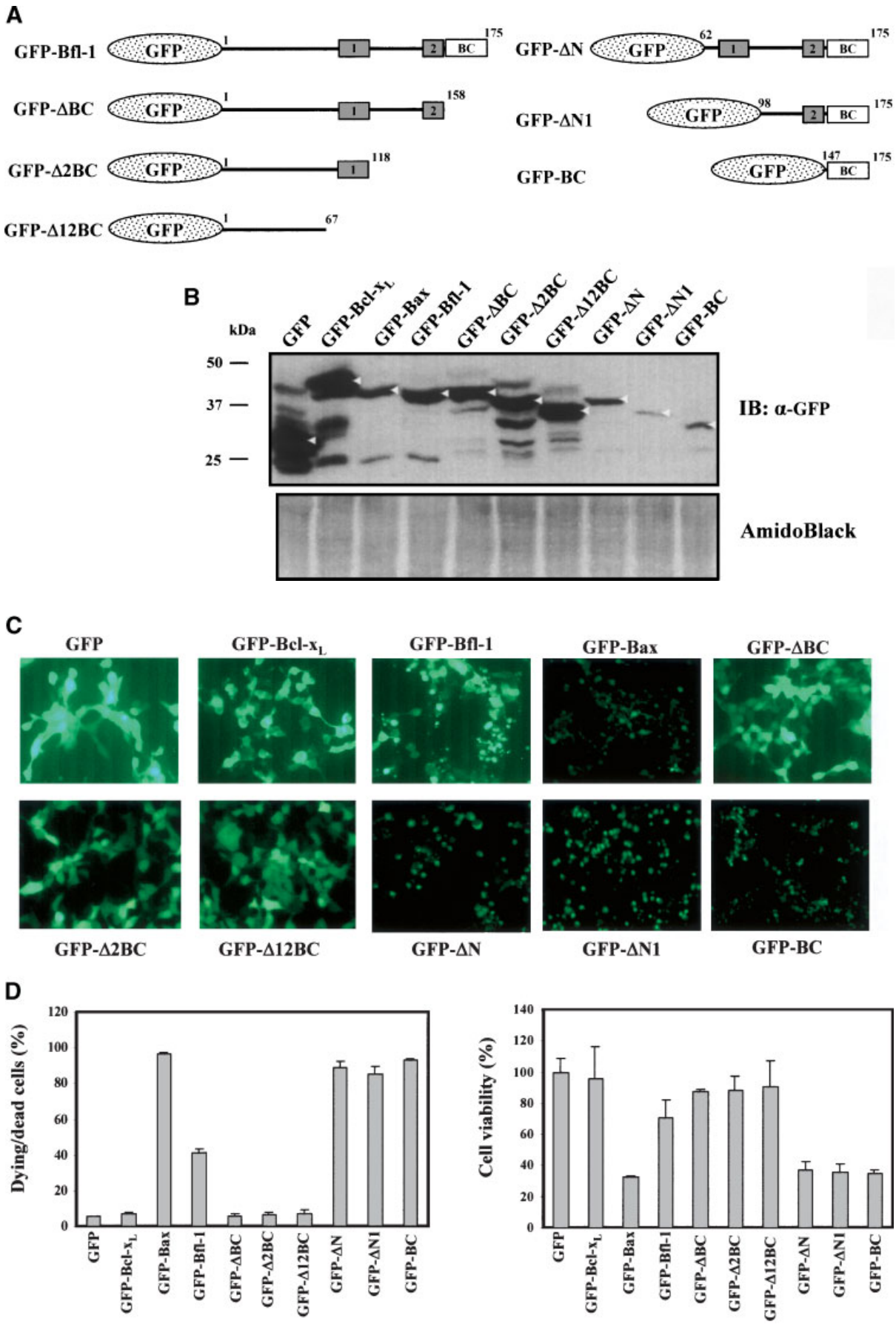
Caspase Activity Assay

Monolayer cells were harvested, and then centrifuged at 450*g* for 10 min at 4°C. After removing supernatant, cell pellets were resuspended in 100 μl of cell lysis buffer (50 mM HEPES pH 7.5, 1 mM DTT, 0.1 mM EDTA, 0.1% CHAPS). Cells were lysed by repeated freezing at −70°C and thawing on ice. Cell lysates were cleared by centrifugation at 15,000*g* for 20 min at 4°C, and the resulting supernatants were used as cell extracts. Typically, 100 μg of cell extract was added to caspase assay buffer (100 mM HEPES pH 7.5, 10% sucrose, 0.1% CHAPS, 10 mM DTT, 200 μM DEVD-pNA) with or without 100 μM Boc-D. The mixture was then incubated at 37°C for 4 h and the yellowish color caused by the release of pNA was quantified using an ELISA reader at 405 nm.

RESULTS

BC Region of Bfl-1 Is Necessary for Conversion to a Pro-Apoptotic Molecule

Bfl-1 is an anti-apoptotic member of the Bcl-2 protein family, and its expression is directly regulated by NF-κB [Zong et al., 1999; Edelstein et al., 2003]. However, we previously demonstrated that the over-expression of GFP-Bfl-1 induced cell death in 293T cells [Ko et al., 2003]. The primary structure of Bfl-1 consists of conserved BH1 and BH2 domains and less conserved N-terminal and C-terminal domains [Zhang et al., 2000]. We serially deleted Bfl-1 protein from its N-terminus and then from its C-terminus in order to identify the region important for GFP-Bfl-1-induced cell death (Fig. 1A, diagram). The deletion mutant constructs were transiently expressed in 293T cells and their expressions were confirmed by Western blot analysis using anti-GFP antibodies (Fig. 1B). One day after transfection, cell viability was monitored under a fluorescence microscope. Healthy cells were large, attached well to the culture plates, and emitted a bright green color, whereas dying/dead cells were small, fragmented, detached from culture plates, and emitted a faint green color. Healthy cell morphology was observed when cells were transiently transfected with constructs that lacked the BC region of Bfl-1 (i.e., GFP, GFP-Bcl-x_L, GFP-ΔBC, GFP-Δ2BC, or GFP-Δ12BC) (Fig. 1C). The morphologies of dying/dead cells were observed after cells had been transiently transfected with GFP-fusion constructs containing the BC region of Bfl-1 (GFP-ΔN, GFP-ΔN1, and GFP-BC) (Fig. 1C). We also used GFP-Bax as a positive control for apoptosis, and took fluorescence pictures of cells in at least three different fields (at least 100 green cells in each field) in culture using blue light excitation (488 nm). Photographs were analyzed and dying/dead cells counted based on altered green cell morphology (Fig. 1D, left). We also measured cell viability by trypan blue exclusion assay (Fig. 1D, right). GFP fusion constructs without the BC region (GFP, GFP-Bcl-x_L, GFP-ΔBC, GFP-Δ2BC, and GFP-Δ12BC) did not affect cell viability (Fig. 1D). However, GFP-Bax and GFP fusion constructs containing the BC region caused cell death (Fig. 1D). Because we did not discriminate between transfected and untransfected cells during the trypan blue assay, differences in the levels of cell death caused by the different constructs were not as dramatic as the differences found by green cell morphology counting (compare Fig. 1D left and right). Taken together, GFP fusion with only the BC domain of Bfl-1 was found to be sufficient to strongly induce 293T cell death. The ability of GFP-BC to induce cell death was comparable to that of the pro-apoptotic protein, GFP-Bax, in our assay.


A
GFP-Bfl-1
GFP
1
1
2
BC
175
GFP-ΔBC
158
GFP-Δ2BC
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GFP-Δ12BC
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GFP-ΔN
62
GFP-ΔN1
98
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GFP-Bcl-xL
GFP-Bfl-1
GFP-Bax
GFP-ΔBC
GFP-Δ2BC
GFP-Δ12BC
GFP-ΔN
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D
Dying/dead cells (%)
Cell viability (%)
0
20
40
60
80
100
120
140

Fig. 1.

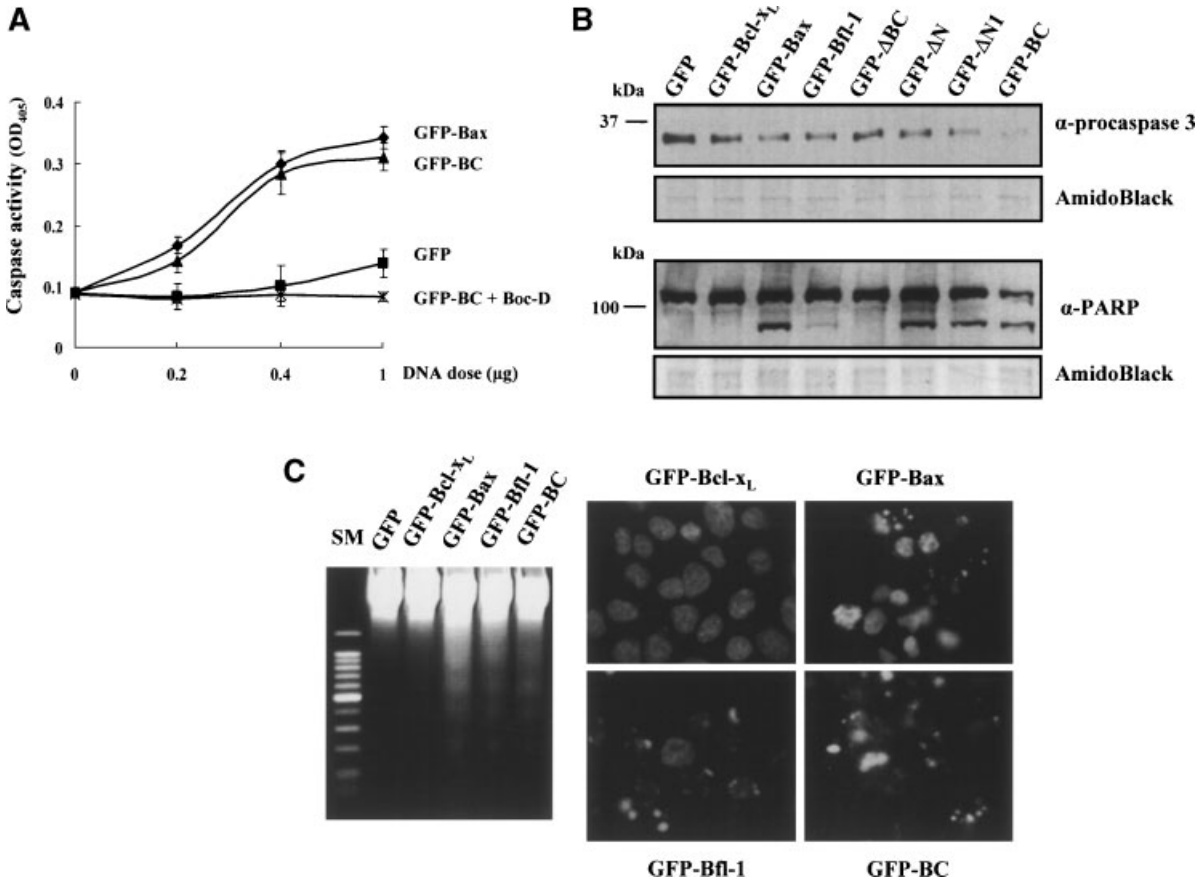


Fig. 2. GFP-BC-induced cell death is accompanied by caspase activation. **A**: Cells were transfected with the indicated amounts of each DNA construct. After 24 h, whole cell lysates were prepared and the caspase activity in 100 μg of whole cell lysate was determined using the peptide caspase substrate, DEVD-pNA. The pan-caspase inhibitor, Boc-D, was added to whole cell lysate before incubation to test the specificity of the assay. Data represent the mean (±SD) of three independent experiments. **B**: Cells were transfected with 1 μg of the indicated DNA constructs. After 24 h, whole cell lysates were prepared and 50 μg of total cellular proteins was loaded on SDS–PAGE. The pro-form of caspase-3 and a fragment of PARP were detected using anti-caspase-3 antibody and anti-PARP antibody. Blotting membranes were stained with AmidoBlack to ensure equal protein loadings. **C**: Cells were transfected with 1 μg of the indicated DNA constructs. After 24 h, genomic DNA fragmentation was analyzed by 2% agarose gel electrophoresis (**left panel**), and cell nuclei were stained with DAPI and their morphologies were monitored under a fluorescent microscope (**right panel**).

GFP-BC-Induced Cell Death Involves Caspases Activation

In order to understand the nature of GFP-BC-induced cell death, we examined whether it has apoptotic features. The transient transfection of the GFP-BC construct activated cellular caspases, as evidenced by the cleavage of the peptide caspase substrate, "DEVD-pNA" (Fig. 2A). The kinetics of caspase activation was similar to

Fig. 1. Green fluorescent protein (GFP) fusion containing the C-terminal region of Bfl-1 is sufficient to induced 293T cell death. **A**: cDNAs of Bfl-1 deletion mutants were cloned into pEGFPC vector, which resulted in various GFP-chimeras. Numbers indicate the position of the amino acids in Bfl-1. We defined the region spanning amino acids 145–175 of Bfl-1 as Bfl-1 C-terminus (BC). **B**: GFP-chimeras were expressed in 293T cells using 1 μg of DNA and Lipofectamine Plus reagent. After 18 h of transfection, whole cell lysates were prepared and the expression level of each GFP-chimera was monitored by Western blot using mouse anti-GFP antibody. Arrowheads indicate the GFP-chimera protein bands. Blotting membranes were stained with AmidoBlack to ensure equal protein loading. **C**: The morphologies of transfected cells were observed using a fluorescent microscope under blue light excitation. Healthy cells were large and emitted a bright green color while dying/dead cells were small and emitted a faint green color. The transfection was performed as described in B. **D**: The cell morphologies were observed as in C and the numbers of dying/dead cells were counted (**left panel**). The amount of cell death was further analyzed by trypan blue assay (**right panel**). Data represent the mean (±SD) of three independent experiments.

that of GFP-Bax and the addition of the caspase specific inhibitor, Boc-D, depressed caspase activity. Immunoblot analysis using antibodies specific to the precursor of caspase-3 showed that pro-caspase-3 was readily processed into active caspase-3 when death promoting GFP-chimeras were over-expressed (Fig. 2B, upper). The activation of caspase-3 was accompanied by the cleavage of its substrate, poly (ADP-ribose) polymerase (Fig. 2B, lower). Caspases activation fragmented nuclear DNAs into multiples of 200 bp nucleosome units, as demonstrated by the agarose gel DNA laddering pattern (Fig. 2C, left). The nuclei of GFP-BC transfected cells also fragmented and condensed, as visualized by DAPI staining (Fig. 2C, right). Therefore, the over-expression of GFP-BC was found to activate cellular caspases and drive cells to apoptotic cell death.

GFP Is Required for GFP-BC-Induced Cell Death

By database searching, we found that the BC region of Bfl-1 matches a region in the candidate tumor suppressor, HCCS-1, which has pro-apoptotic properties [Kim et al., 2002]. Moreover, HCCS-1 mRNA has been shown to be present in many normal tissues but the not in cancer cell lines. To investigate the possibility that the BC region of Bfl-1 is a novel pro-apoptotic domain, we also expressed only this region; however, cell viability was unaffected (data not shown). It is possible that BC protein itself is unstable, because it is a relatively short peptide consisting of 29 amino acids with an approximate molecular weight of 3.3 kDa. Thus, we constructed other chimeric proteins using GST or MBP as fusion partners in order to stabilize the BC protein. However, these fusion proteins failed to induce cell death, suggesting either that GFP is required to induce cell death or that the BC region cannot exert a cytotoxic effect alone (Fig. 3B).

GFP has a unique 3D structure called "beta-can," which is a feature of all known fluorescent proteins [Yang et al., 1996; Yarbrough et al., 2001]. This structure is highly compact as was demonstrated by a GFP deletion study [Li et al., 1997]. The deletion of only 8 N-terminal amino acids or 9 C-terminal amino acids of full-length GFP protein abolished green fluorescence; it is believed that these deletion proteins lack the beta-can structure of GFP [Li et al., 1997]. Since GFP is also critical in GFP-BC-induced cell death, we serially deleted portions of GFP in GFP-BC and monitored 293T cells transfected with these chimeras (Fig. 3A). The deletion of 10 amino acids or more from the N-terminus of GFP prevented green fluorescence, which is consistent with a previous report (data not shown) [Li et al., 1997]. But even though the fluorescence activity was abrogated by these deletions, the pro-apoptotic ability of serially deleted chimeras remained intact until more than 100 amino acids were deleted from the N-terminus of GFP (Fig. 3B, see ΔN100-BC). Therefore, it is unlikely that the integrity of the "beta-can" structure of GFP is important for GFP-BC-induced cell death since it is difficult to believe that ΔN100-BC can maintain the "beta-can" structure. However, when 150 N-terminal amino acids of GFP were deleted from GFP-BC protein (ΔN150-BC), no cell death was observed indicating the existence of a determinant in this region for GFP-BC-induced cell death (Fig. 3B). The expressions of these deletion mutants were monitored by Western blot analysis using anti-GFP, anti-GST and anti-MBP antibodies (Fig. 3C).

Cell Death Inducing GFP-Chimeras Localized to Mitochondria

To understand the underlying mechanism of GFP-BC-induced cell death, we examined the cellular localization of GFP-BC by confocal microscopy. Since Bfl-1 protein has been reported to localize to both mitochondria and the cytosol, Mitotracker dye was used to investigate whether the GFP-chimeras also localized to mitochondria [Duriez et al., 2000; Werner et al., 2002]. However, the over-expressions of GFP-ΔN, GFP-ΔN1, and GFP-BC induced rapid 293T cell death, and we failed to obtain a definitive cell images. Nevertheless, the addition of the pan-caspase inhibitor, Boc-D, delayed cell death and allowed us to observe the locations of death-inducing GFP-chimeras. When GFP was expressed alone, the green color was dispersed evenly throughout cells, showing little localization (Fig. 4A). However, GFP-Bax showed a regular dot-like pattern and appeared to be localized to mitochondria (Fig. 4A). GFP-ΔN, GFP-ΔN1, and GFP-BC also showed a similar dot-like pattern, although it was not as distinctive as that of GFP-Bax (Fig. 4A). The BC region of Bfl-1 contains a stretch of hydrophobic amino acids, but its overall hydrophobicity is insufficient to allow this region to be char-

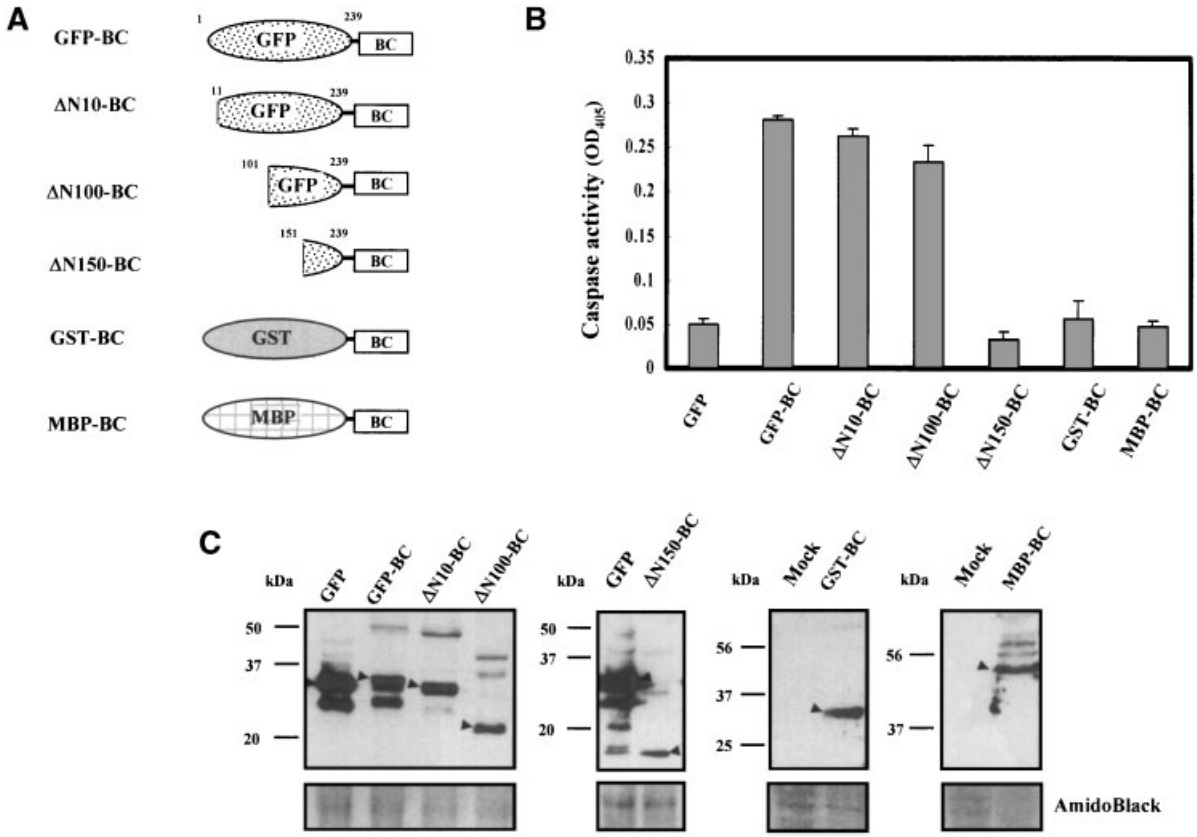


Fig. 3. A region spanning amino acids 101–150 of GFP was found to be responsible for GFP-BC-induced cell death. **A**: The GFP region in GFP-BC was replaced with GST, MBP, or various deleted forms of GFP. All of the expression plasmids had the same vector backbone, pEGFPC, to minimize discrepancies in expression levels. Numbers indicate the positions of amino acids in GFP. **B**: 293T cells were transfected with 1 μg of DNA constructs as described in A and the amounts of caspase activation in transfected cells was assayed using the peptide caspase substrate DEVD-pNA as described in ''Materials and Methods.'' Data represent the mean (±SD) of three independent experiments. **C**: Expression levels of each chimeric protein were determined by Western blot analysis using anti-GFP, anti-GST, and anti-MBP antibodies. Arrowheads indicate expressed protein bands of the correct size; portions of membranes were stained with Amido-Black as loading controls.

acterized as a transmembrane domain (Grease Program, FASTA Server at University of Virginia). Moreover, there are four-charged amino acids in the BC region (K^{151}, E^{159}, K^{163}, E^{166}) that are absent from the C-terminal regions of other Bcl-2 family members. These differences may explain the less distinctive mitochondrial localizations observed for GFP-ΔN, GFP-ΔN1, and GFP-BC. The mitochondrial locations of GFP-ΔN, GFP-ΔN1, and GFP-BC were confirmed by overlapping green images with red Mitotracker-stained images (Fig. 4A).

During the fluorescent microscopic analysis, we observed a small number of cells that were resistant to GFP-BC-induced cell death. These resistant cells emitted relatively weak green fluorescence. To test if there is a connection between GFP-BC expression level and apoptosis, we subcloned GFP-BC cDNA into a mammalian expression vector containing the minimal immediate early promoter of cytomegalovirus (P_{minCMV}). P_{minCMV} lacks the strong enhancer elements normally associated with the CMV immediate early promoter. As shown in Figure 4B, cell viability was not affected in cells expressing low levels of GFP-BC, indicating that a certain expression threshold is necessary to induce GFP-BC-mediated cell death. Moreover, this low expression of GFP-BC did not caused changes in cell morphology. Consequently, we were able to observe the cytoplasmic/mitochondrial location of the GFP-BC more clearly (Fig. 4B, lower).

Co-Expression of Anti-Apoptotic Molecules can not Prevent GFP-BC-Induced Cell Death

As GFP-BC-induced cell death showed an apoptotic character, we tried to inhibit GFP-BC induced cell death by co-expressing anti-apoptotic molecules. Two representative signal

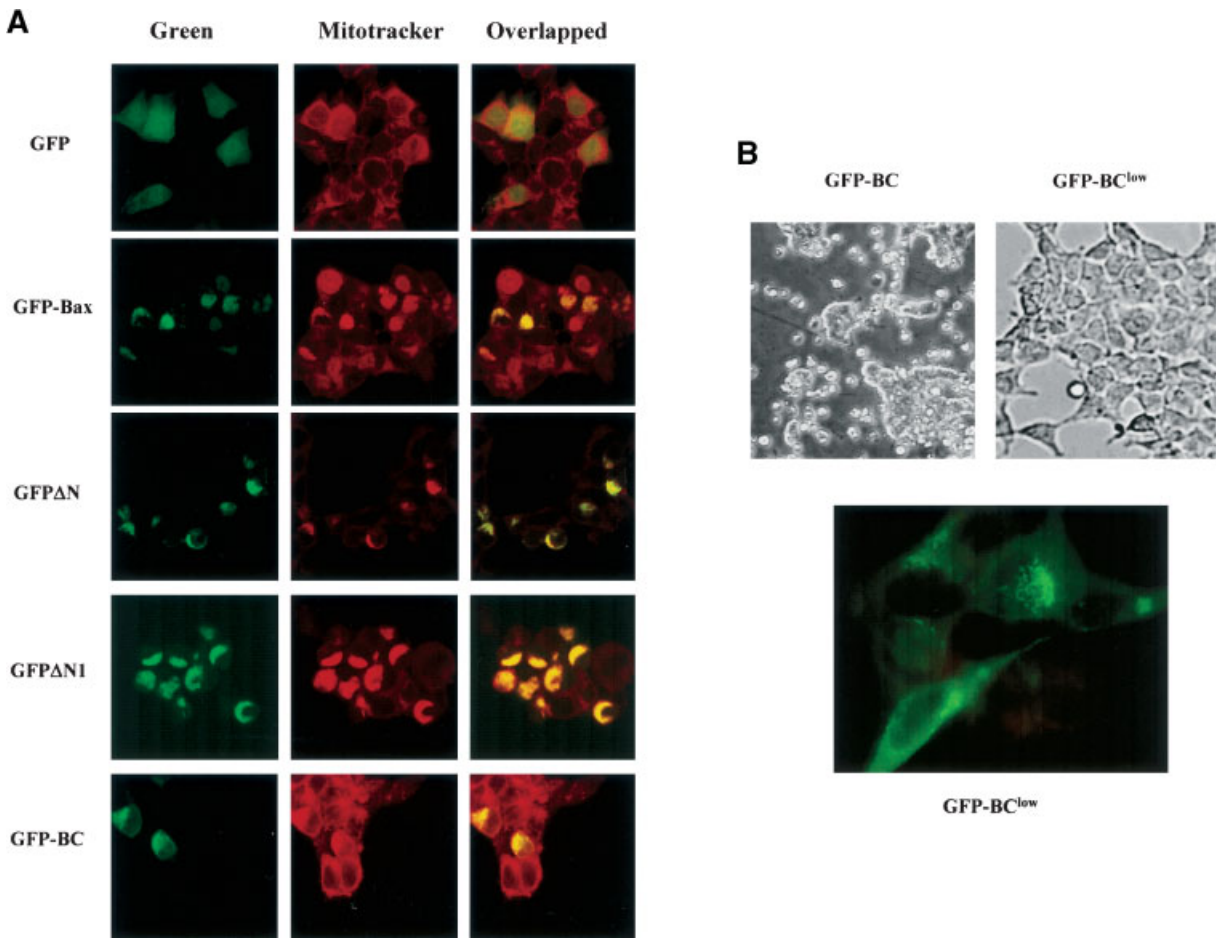
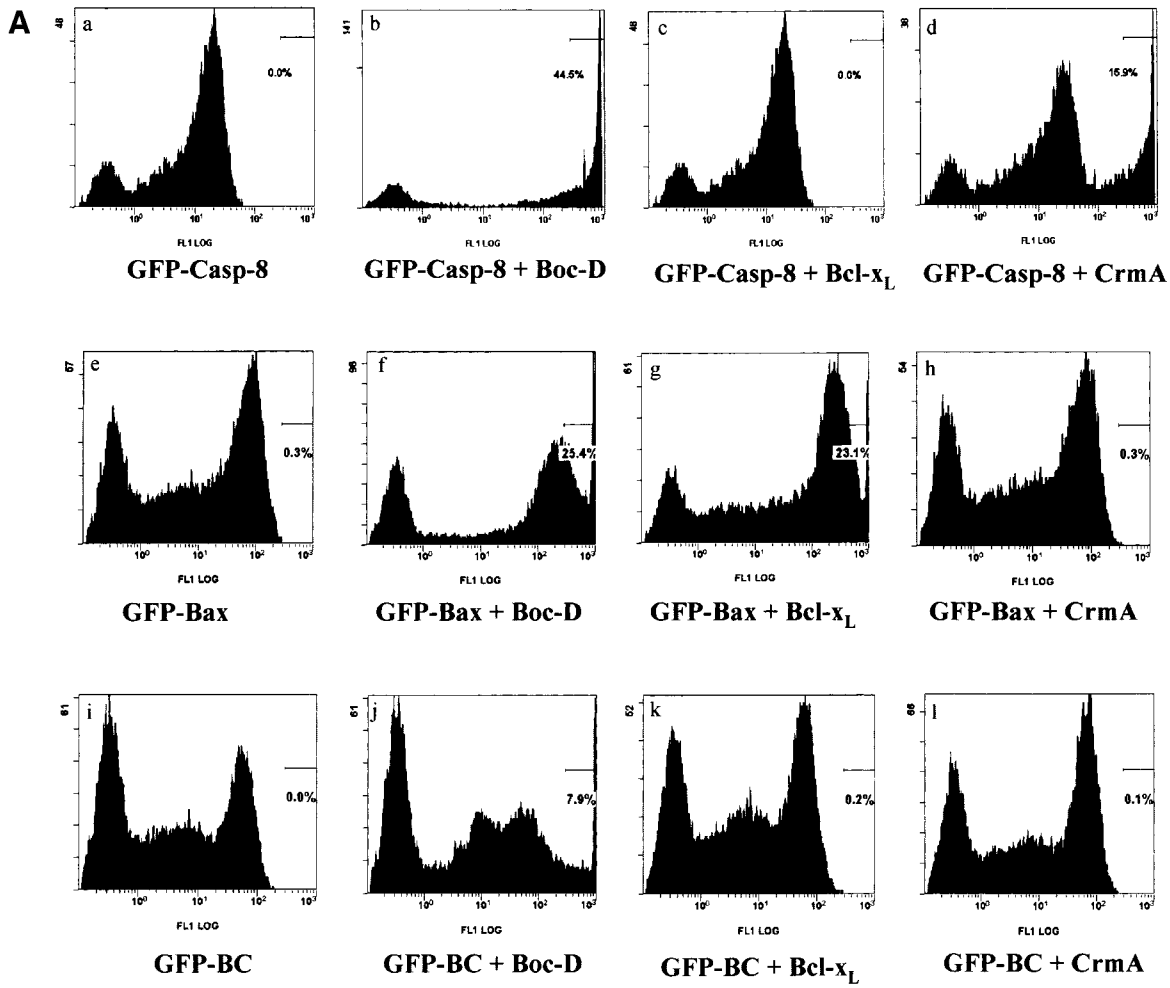


Fig. 4. Cytotoxic GFP-chimeras were located mainly in mitochondria. **A**: Cells on a cover slip were transfected with 500 ng of the DNA constructs and incubated for 24 h. The culture media was then replaced with serum free media containing 1 μg/ml of Mitotracker and incubated for a further 20 min. Cover slips were mounted on slides, and the locations of GFP-chimeras (**left column**) and mitochondria (**middle column**) were monitored using a confocal microscope. The degree of co-localization was determined by examining overlapping green and red images (**right column**). **B**: GFP-BC was expressed at a lower level using CMV promoter, which lacked the enhancer element, and the morphologies of the transfected cells were observed. A high-level GFP-BC expression strongly induced cell death (**upper left**), whereas low-level expression had no effect on cell viability (**upper right**). The mitochondrial localization of GFP-BC was further confirmed in GFP-BC^{low} expressing cells (**lower**).

pathways lead to the activations of cellular caspases: the apoptosome pathway and the "death inducing signaling complex" (DISC) pathway [reviewed in Adams and Cory, 2002; Peter and Krammer, 2003]. Bcl-2 family proteins, including Bfl-1, directly regulate the apoptosome pathway and indirectly regulate the DISC pathway.

We expressed GFP-caspase-8 or GFP-Bax to induce DISC-mediated cell death or apopto-

Fig. 5. GFP-BC-induced cell death is not inhibited by the over-expression of Bcl-x_L or CrmA. **A**: Cells were transfected with 500 ng of the indicated DNA constructs with or without Boc-D. After 24 h, cells were harvested and the level of green fluorescence was analyzed by FACS. Dead cells that over-expressed GFP-BC, GFP-Bax, and GFP-Caspase-8, were detected by a leftward-shift in the green (FL1) peak, however, surviving cells over-expressing anti-apoptotic molecules were detected by rightward-shift in the FL1 peak. **B**: The percentages of survived cells by over-expression of anti-apoptotic molecules are represented graphically. Data represent the mean (±SD) of three independent experiments. **C**: The expression levels of each protein were monitored by Western blotting using anti-Flag (**left panel**) and anti-GFP antibody (**right panel**). Arrowheads indicate expressed protein bands and corrected sizes and the asterisk indicates the processed form of GFP-Casp-8, resulting from autocatalysis. Portions of membranes were stained with AmidoBlack as loading controls.


A
a
0.0%
GFP-Casp-8
b
44.5%
GFP-Casp-8 + Boc-D
c
0.0%
GFP-Casp-8 + Bcl-x$_L$
d
16.9%
GFP-Casp-8 + CrmA
e
0.3%
GFP-Bax
f
25.4%
GFP-Bax + Boc-D
g
23.1%
GFP-Bax + Bcl-x$_L$
h
0.3%
GFP-Bax + CrmA
i
0.0%
GFP-BC
j
7.9%
GFP-BC + Boc-D
k
0.2%
GFP-BC + Bcl-x$_L$
l
0.1%
GFP-BC + CrmA
FL1 LOG

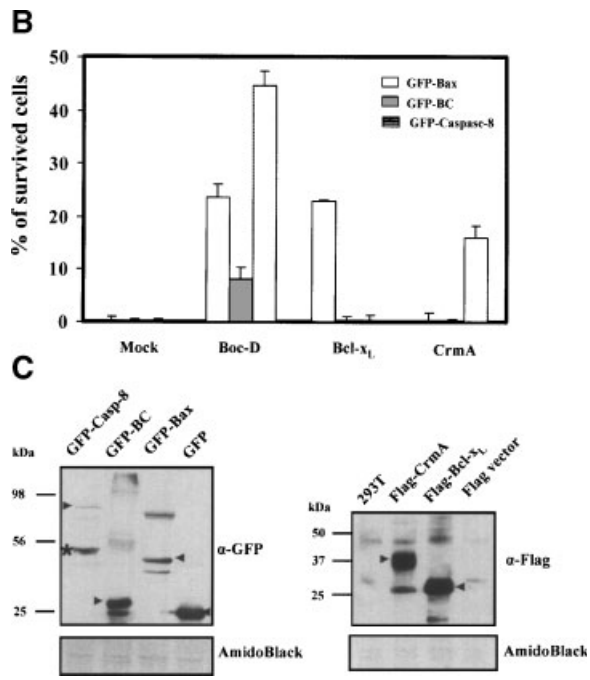

B
% of survived cells
GFP-Bax
GFP-BC
GFP-Caspase-8
Mock
Boc-D
Bcl-x$_L$
CrmA
C
kDa
GFP-Casp-8
GFP-BC
GFP-Bax
GFP
α-GFP
AmidoBlack
293T
Flag-CrmA
Flag-Bcl-x$_L$
Flag vector
α-Flag

Fig. 5.

some-mediated cell death, respectively, in 293T cells. Because all cell death-inducing proteins were fused to GFP, a simple FACS assay was used to quantify cell death, by measuring the intensity of the green fluorescence caused by GFP [Harvey et al., 2001; Strebel et al., 2001]. This assay could not be used to compare the degree of cell death induced by different constructs. However, for a given construct, this method allows us to reliably monitor the inhibition or promotion of cell death induced by various agents. Dying/dead cells emit a faint green color thus they are typically associated with a left-shifted area in the $10^0 \sim 10^2$ region on the FL1 axis (e.g., GFP-Casp-8 alone in Fig. 5A; a). Healthy live cells emit a bright green color and are associated with a right-shifted peak near 10^3 on the FL1 axis (e.g., GFP-Casp-8 plus Boc-D in Fig. 5A; b). Transfected cells expressing death-inducing GFP-chimeras, namely, GFP-Casp-8, GFP-Bax, and GFP-BC, were detected in the left-shifted region by FACS analysis (Fig. 5A; a,e,i). When GFP-Casp-8 was co-expressed with CrmA, a viral inhibitor of caspase-8, cell death was efficiently blocked, as determined by green fluorescence recovery by FACS analysis and by cell morphology (compare Fig. 5A; a,d). GFP-Bax-induced cell death was inhibited effectively by co-expression with Bcl-x_L (compare Fig. 5A; e,g). However, the co-expression of GFP-BC with CrmA or Bcl-x_L failed to inhibit GFP-BC-induced cell death, indicating that GFP-BC induces cell death via a non-classical apoptotic pathway (compare Fig. 5A; i,k,l). The pan-caspase inhibitor, Boc-D, only partially blocked GFP-BC-induced cell death, thus emphasizing the involvement of cellular caspases in GFP-BC-induced cell death (compare Fig. 5A; i,j). The FACS data from Figure 5A is represented graphically in Figure 5B. Bcl-x_L and CrmA were effective at blocking apoptosis by GFP-Bax and GFP-Caspase-8, respectively. However, GFP-BC induced cell death was inhibited only by Boc-D treatment (Fig. 5B). The expression levels of all DNA constructs were monitored by Western blotting (Fig. 5C).

DISCUSSION

Bfl-1 was considered as an anti-apoptotic factor but we found that fusion of the N-terminus of Bfl-1 with GFP induced cell death [Ko et al., 2003]. Bfl-1 contains BH1 and BH2 domains and thus we initially postulated that GFP fusion changed the conformation of Bfl-1, which result in changes in its binding partner among Bcl-2 family members. In this study, we found that the C-terminal 29 amino acids region of Bfl-1 (BC) is responsible for death induction (Fig. 1), which was unexpected because BC lacks any BH domain that has been considered to be important for both pro- and anti-apoptotic function of Bcl-2 family members. In our preliminary data, we observed a high level of GFP-BC-induced cell death in Bax/Bak double knockout cells (data not shown). This observation further supports the viewpoint that GFP-BC induces cell death via a novel pathway that does not involve interaction with other Bcl-2 family proteins.

Previous studies have demonstrated that certain truncated proteins can be more functionally potent than their parent molecules. For example, the structural analyses of Bcl-x_L and Bax revealed the existence of a pore-forming domain in the region spanning the BH1 and BH2 domains. The expression of truncated Bax containing this pore-forming domain killed bacterial and tumor cells more effectively than wild type Bax [Ishibashi et al., 1998]. As Bfl-1 has a similar core structure as Bcl-x_L consisting of seven alpha helices [Zhang et al., 2000], we created several constructs of GFP-Bfl-1 to test if our truncated Bfl-1 proteins functioned similarly to pore forming proteins. The GFP-ΔN construct resembles the potent pro-apoptotic truncated version of Bax, as it consists of BH1, BH2, the pore-forming region between BH1 and BH2, and putative transmembrane domains. Like truncated Bax, it was able to effectively induce apoptosis. However, the presence of the pore-forming domain cannot fully account for the ability of Bfl-1 to act as a pro-apoptotic molecule as both GFP-ΔN1, which lacks the complete pore-forming domain, and GFP-BC, which lacks the entire pore-forming domain are able to induce apoptosis to the same extent as GFP-ΔN.

The targeting of GFP-BC may be an important factor of cell death, since GFP-BC appeared to be localized to mitochondria (Fig. 4A,B). It is interesting to compare our results with works done by Wang et al. [2001]. They showed that mitochondria- but not ER-targeted Bcl-2 could induce cell death when transiently expressed in 293T cells either by the transient expression of whole Bcl-2 protein, or by GFP fusion with the TM domain of Bcl-2 [Wang et al.,

2001]. According to their results, cell death was induced by the transient expression of whole Bcl-2 protein, GFP fusion with only the TM domain of Bcl-2, or by GFP fusion with the mitochondrial targeting sequence of monoamine oxidase B. Therefore, it is likely that the over-expression and mitochondrial location of GFP is responsible for the observed cell death. Fusion of BC with other epitope tags like GST or MBP did not affect cell viability (Fig. 3), which indicates that a special relationship exists between GFP and the BC region of Bfl-1 in terms of inducing cell death. Further deletion analysis revealed that a region spanning amino acids 100–150 in GFP is necessary for GFP-BC-induced cell death. This region may also be a determinant of the cytotoxic effect of GFP expression, which has been sporadically reported upon, and which is still being debated [Hanazono et al., 1997; Liu et al., 1999].

GFP-BC was found to be a much more potent pro-apoptotic molecule than GFP-Bfl-1 (Fig. 1), which suggests either additional interactions with other anti-apoptotic factors at the BH domain or some other domain of Bfl-1 confers added resistance to apoptosis, or that a portion of the Bfl-1 molecule itself partially counters the effect of the BC region. Treatment with the broad-spectrum caspase inhibitor Boc-D only partially inhibited GFP-BC-induced cell death, indicating that GFP-BC may induce apoptosis through both caspase-dependent and -independent pathways.

Although we located the regions of both Bfl-1 and GFP that are responsible for the strong pro-apoptotic ability of this fusion protein, the nature of GFP-BC-induced cell death is difficult to understand, as we were unable to block GFP-BC-induced cell death with anti-apoptotic molecules. The partial inhibition of GFP-BC-induced cell death by Boc-D treatment emphasizes the involvement of cellular caspases, however, the initial cues that activate downstream caspase cascades remain unclear.

Snapp et al. [2003] demonstrated that the modulation of membrane structure by over-expressed GFP-chimeras. GFP-fusion with the transmembrane domain of cytochrome b(5) changed the membrane structure of the ER from a branching network into compact whorls or a sinusoidal structure by forming organized smooth ER (OSER). But this was found to have no effect on the viability of transfected cells. In our experiments, GFP-BC localized in mitochondria and it is possible that the mitochondrial membrane structure was also changed due to high local concentrations of GFP-BC. As mitochondria play a central role in cellular survival and death, structural changes in mitochondrial membranes could result in both the impairment of mitochondria respiratory function and the secretion of apoptogenic factors, and thus cause cell death. In support of this hypothesis, we previously observed dramatic changes in the membrane potential of mitochondria and reported the release of cytochrome c from mitochondria during GFP-Bfl-1-induced cell death [Ko et al., 2003].

In conclusion, we identified the responsible regions from both GFP and Bfl-1 for the conversion of anti-apoptotic to pro-apoptotic protein after fusion with GFP. The GFP-BC, a truncated version of GFP-Bfl-1 containing both determinants, was found to localize to mitochondria and to induce cell death, which was accompanied by caspase activation. We believe that our GFP-BC system offers a model system for characterization of a novel type of cell death that occurs via a mitochondria dependent process.

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