Conversion of Bfl-1, an anti-apoptotic Bcl-2 family protein, to a potent pro-apoptotic protein by fusion with green fluorescent protein (GFP)

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Abstract **Human Bfl-1 is an anti-apoptotic Bcl-2 family member. Here, we found that Bfl-1 was converted into a potent death-promoting protein by green fluorescent protein (GFP) fusion with its N-terminus. The transient expression of GFP-Bfl-1 induced cytochrome *c* release and triggered apoptosis in 293T cells, which depended on the mitochondrial localization of GFP-Bfl-1. Apoptosis induced by GFP-Bfl-1 was significantly blocked by the pan-caspase inhibitor carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone, but was not blocked by either Bcl-xL or Bfl-1. Our findings provide a useful model for understanding the structural basis of Bcl-2 family proteins that act in an opposite way despite sharing structural similarity between anti-apoptotic and pro-apoptotic proteins.**

Key words: Bfl-1; Apoptosis; Green fluorescent protein; Bcl-2 family

1. Introduction

It is widely believed that Bcl-2 family proteins, characterized by the Bcl-2 homology (BH) domain, are central regulators of apoptosis. The family can be subdivided into two groups according to effects on cell survival. One group, including Bax, Bak, Bok, Bcl-xS, Bid, Bad, Bik, and Bim, promotes apoptosis, whereas the other group, including Bcl-2, Bcl-xL, Bfl-1, Bcl-w, Mcl-1, E1B-19K, and Ced-9, inhibits apoptosis [1–3]. Interestingly, available evidence demonstrates that the three-dimensional structures of the anti-apoptotic and pro-apoptotic proteins are strikingly similar [4–8]. However, it is still unclear how they function in opposing ways in the regulation of apoptosis despite their structural similarities.

The activity of Bcl-2 family proteins can be diversely regulated by post-translational modifications, such as by phosphorylation or by cleavage by proteases. For example in the case of phosphorylation, the loop region of Bcl-2 is phosphorylated during apoptosis induced by treatment with paclitaxel, a microtubule-stabilizing agent, and this results in the disruption of the anti-apoptotic activity of Bcl-2 [9]. Pro-apoptotic Bad is also phosphorylated by mitochondria-anchored protein kinase A, in the presence of survival factors. This causes Bad to be released from mitochondria into the cytosol thereby losing its pro-apoptotic activity [10–12].

Recent works have also demonstrated that various proteases cleave several Bcl-2 family proteins producing large C-terminal fragments with potent pro-apoptotic activity [13–18]. For example, activated caspase-8 cleaves inactive cytosolic Bid into an active pro-apoptotic truncated Bid (tBid) that translocates to mitochondria, induces the release of cytochrome *c* and then apoptosis [16,17]. Potent anti-apoptotic Bcl-2 and Bcl-xL are also cleaved by caspase-3 under apoptotic situations [13,14]. Interestingly, by removing the N-terminal region, these proteins are converted into Bax-like pro-apoptotic proteins that further activate downstream caspases and promote the release of cytochrome *c* [13,14,18]. Therefore, post-translational modifications might explain the diverse activity of the Bcl-2 family proteins despite their similar structures.

Here, we report that another type of modification can reverse the activity of Bcl-2 family proteins. We found that Bfl-1, an anti-apoptotic Bcl-2 family protein, is converted into a pro-apoptotic protein by green fluorescent protein (GFP) fusion with its N-terminus. The transient expression of GFP-Bfl-1 fusion protein remarkably induced the cell death of HEK 293T cells, which showed apoptotic features, such as the formation of apoptotic bodies, condensation of chromatin, and DNA fragmentation. However, the transient expression of GFP or Bfl-1 alone had no effect on cell death. The apoptosis induced by GFP-Bfl-1 was characterized by its mitochondrial targeting, cytochrome *c* release and by a caspase-dependent pathway. Our findings provide a new model for furthering our understanding of the structural relation between anti-apoptotic and pro-apoptotic Bcl-2 family proteins.

2. Materials and methods

2.1. Chemicals and antibodies

Pan-caspase inhibitor carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk, 100 μM) was purchased from Calbiochem (San Diego, CA, USA). Monoclonal anti-GFP antibody and anti-goat horseradish peroxidase (HRP) antibody were obtained from Santa

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Abbreviations: GFP, green fluorescence protein; BH, Bcl-2 homology; z-VAD-fmk, carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone; TM, transmembrane

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Cruz Biotechnology (Santa Cruz, CA, USA), anti-β-actin antibody from Sigma (St. Louis, MO, USA), the monoclonal anti-*myc* 9E10.2 antibody from Invitrogen (Groningen, The Netherlands), the monoclonal anti-cytochrome *c* 7H8.2C-12 antibody from PharMingen (San Diego, CA, USA), and anti-mouse HRP antibody was purchased from Amersham Pharmacia (Amersham, UK).

2.2. Plasmid constructions

Parental vectors, pEGFP-C1 (Clontech, Palo Alto, CA, USA) and pcDNA3.1 Myc, His(−)B (Invitrogen) were used to produce GFP fusion proteins and *myc*-tagged proteins. Expression vectors for GFP-Bfl-1, GFP-Bcl-xL, Bfl-1-myc, Bcl-xL-myc, and Bax-myc were as previously described [9]. To construct expression vector for GFP-Bfl-1ΔTM, the coding sequence for Bfl-1 amino acids 1–158, lacking the C-terminal 17 amino acids, was amplified by polymerase chain reaction (PCR) using pEGFP-Bfl-1 plasmid as a template, and the following primers, 5′-GAATTCGATGACAGACTGTGAATTTG-GATAT (sense) and 5′-TCTAGAAAAGTCATCCAGCCAGATT-TAGGTT (antisense). The PCR product was purified, digested with *Eco*RI and *Xba*I restriction enzymes, and subcloned into *Eco*RI and *Xba*I sites in pEGFP-C1. All the constructs were confirmed by DNA sequencing.

2.3. Transfection and apoptosis assay

Apoptosis of 293T cells was monitored following transfection with expression plasmids. 293T cells (2×10^5 cells in 35-mm well) were cultured in Dulbecco's modified Eagle's medium/F-12 supplemented with 10% fetal bovine serum. After 24 h of incubation, 293T cells were transfected using the Lipofectamine procedure (Life Technologies, Gaithersburg, MD, USA), with 1.0 μg of the indicated expression plasmids. For the morphological assessment of cell death, 293T cells were plated onto LabTek II chamber slides (Nalgen Nunc International, Naperville, IL, USA) at densities of 5×10^4 cells per well. Twenty-four hours after transfection, cells were washed with phosphate-buffered saline (PBS) prior to fixation with 4% formaldehyde. Subsequently, cells were stained with a solution containing 1 μg/ml of DAPI (Calbiochem) and visualized under an Axiovert 100 inverted epifluorescence microscope (Carl Zeiss, Thornwood, NY, USA). Nuclei with rippled contours and chromatin condensation were considered to represent the apoptotic stage of 293T cells. To analyze DNA fragmentation by agarose gel electrophoresis, cells, 24 h after transfection, were scraped out and collected by centrifugation. The cell pellet was 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% sodium dodecyl sulfate) for at least 4 h at 55°C. DNA was precipitated with an equal volume of isopropanol, treated with 0.1 mg/ml RNase A at 37°C, analyzed on a 2% agarose gel, and detected by ethidium bromide staining. If necessary, z-VAD-fmk (100 μM), a pan-caspase inhibitor, was added to the cell culture immediately following transfection.

2.4. Confocal laser scanning microscopy

Transfected 293T cells were prepared for microcopy by staining with 50 nM of a mitochondria-specific dye, MitoTracker Red CMXRos (Molecular Probes, Eugene, OR, USA) for 10 min in the cell culture medium. The cells were then washed twice with PBS and fixed with 4% paraformaldehyde. Fixed cells were counterstained with 1 μg/ml of DAPI (Calbiochem), and placed on microscope slides. Images were obtained using an LSM510 (Carl Zeiss, Jena, Germany) confocal microscope.

2.5. Analysis of cytochrome c release

Mitochondria-free cytosol was prepared as previously described [19] with some modifications. Briefly, 24 h after transfection, 293T cells were collected by scraping, washed twice with ice-cold PBS, suspended in 100 μl of extraction buffer (50 mM PIPES–KOH, pH 7.4, 200 mM mannitol, 70 mM sucrose, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol and protease inhibitors), and incubated on ice for 30 min. Cells were lysed by Dounce homogenization and homogenates were centrifuged at $100\,000\times g$ for 15 min at 4°C. Supernatants were harvested and 20 μg of protein was analyzed by Western blotting using monoclonal anti-cytochrome *c* antibody. Western blot analysis was performed as described previously [20].

3. Results

3.1. Transient expression of GFP-Bfl-1 induces the apoptotic death of 293T cells

Previously, we reported on the anti-apoptotic activity of Bfl-1 against anti-cancer drugs and Bax, a pro-apoptotic Bcl-2 family protein [20,21]. However, we found that the transient expression of GFP-Bfl-1 fusion protein in 293T cells promoted cell death. Twenty-four hours after transfection of GFP-Bfl-1, a large number of 293T cells showed several of the morphologic features of apoptotic cells, such as cell shrinkage and the formation of cytoplasmic blebs and apoptotic bodies. Finally, cells detached from the culture plate. However, Bfl-1 co-transfected with GFP had no effect on cell death (Fig. 1A). The transient expressions of Bcl-xL, another anti-apoptotic Bcl-2 family homologue, or of GFP-Bcl-xL were also examined, but they had no pro-apoptotic effect on 293T cells (Fig. 1A). The dead cells resulting from the transient expression of GFP-Bfl-1 also showed chromatin condensation by fluorescence microscopy (Fig. 1B), and chromosomal DNA fragmentation (Fig. 1C), hallmarks of apoptotic cell death. In order to estimate the levels of apoptotic cells induced by GFP-Bfl-1, we counted apoptotic nuclei stained with DNA-specific DAPI among GFP-positive 293T cells. The transient expression of GFP-Bfl-1 resulted in the marked apoptosis of 293T cells in a time-dependent manner (Fig. 1D). Twenty-four hours after transfection, 50% of 293T cells were apoptotic, and more than 70% of cells were dead 48 h after transfection. However, transfection with the other control plasmids has no significant effects on 293T cell death (Fig. 1D).

3.2. GFP-Bfl-1 induces apoptosis by targeting mitochondria

The activities of pro-apoptotic Bcl-2 family members, such as Bax, Bid, and Bak, have been associated with their regulated targeting to the mitochondrial membranes [16,17,22,23]. To investigate whether the pro-apoptotic activity of GFP-Bfl-1 involves mitochondrial targeting, we analyzed its subcellular localization by confocal laser scanning microscopy. To visualize mitochondria, cells were stained with MitoTracker, a red fluorescent dye that binds specifically to the mitochondrial membrane. Microscopic observations revealed that both at an early stage (healthy cell) and at a late stage (apoptotic cell) after transfection, GFP-Bfl-1 was co-localized with MitoTracker in 293T cells (Fig. 2). The GFP fusion protein of Bcl-xL, which is known to localize to the mitochondrial membrane [24], showed a similar localization (Fig. 2). Several anti-apoptotic Bcl-2 family proteins, including Bfl-1, contain a hydrophobic transmembrane (TM) domain in their C-termini, which localizes them to specific intracellular membranes. To further investigate the effect of the mitochondrial targeting of GFP-Bfl-1 on its pro-apoptotic activity, we expressed GFP-Bfl-1ΔTM (1–158), lacking a C-terminal hydrophobic stretch of 17 amino acids. We found that GFP-Bfl-1ΔTM diffusively localized throughout the cytoplasm and had no pro-apoptotic activity (Fig. 3A). Thus, these results suggested that GFP-Bfl-1 induces apoptosis in 293T cells after targeting the mitochondrial membrane. The expression levels of GFP and GFP-Bcl-xL protein were slightly higher than GFP-Bfl-1 and GFP-Bfl-1ΔTM although the cytotoxicity was most prominent in GFP-Bfl-1 protein (Fig. 3B).

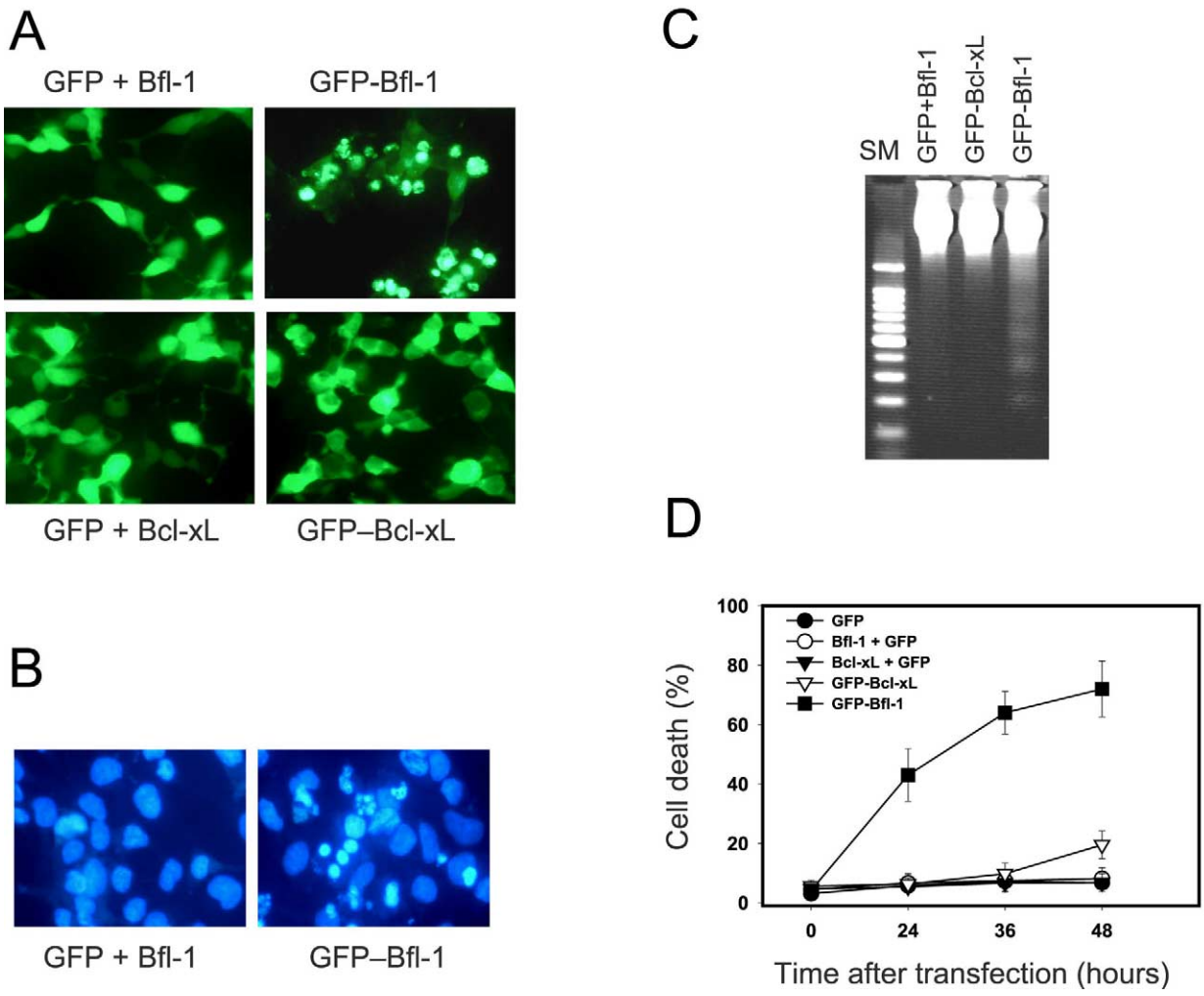

Fig. 1. The transient expression of GFP-Bfl-1 induces apoptotic cell death in 293T cells. A: Fluorescence observation showing the cell morphology of 293T cells expressing GFP fusion proteins or GFP. 293T cells were co-transfected with pEGFP-Bfl-1 (1 μg), pEGFP-Bcl-xL (1 μg), pcDNA-Bfl-1 (1 μg)+pEGFP-C1 (0.1 μg), or pcDNA-Bcl-xL (1 μg)+pEGFP-C1 (0.1 μg) plasmids. At 24 h after transfection, the effect of GFP-Bfl-1 on the cells was examined by fluorescence microscopy. B: Nuclear morphology in 293T cells transfected with pEGFP-Bfl-1 (1 μg) or pcDNA-Bfl-1 (1 μg)+pEGFP-C1 (0.1 μg) was observed after staining with DNA-specific DAPI (1 μg/ml). C: DNA fragmentation was analyzed in cells transfected with 1 μg of pEGFP-Bfl-1, pEGFP-Bcl-xL, or pcDNA-Bfl-1 plasmids. Twenty-four hours after transfection, DNA was extracted, electrophoresed on 2% agarose gel and visualized by ethidium bromide staining. D: Time course effect of GFP-Bfl-1 on 293T cell death. To quantify cell death, 293T cells were transfected with 1 μg of plasmids and stained with DAPI at the indicated time points. Fluorescence microphotographs were taken of three different fields each containing about 100 cells expressing GFP. Cells showing apoptotic nuclei were considered dead. The percentages of apoptotic cells are expressed as means ± S.D.; similar results were obtained from two other independent experiments.

3.3. *GFP-Bfl-1 induced the release of cytochrome* c *from mitochondria*

Cumulative evidence shows that the pro-apoptotic activity of Bcl-2 family proteins appears to depend on their ability to cause cytochrome *c* release from mitochondria. Thus, we examined the effect of GFP-Bfl-1 on cytochrome *c* release in 293T cells. Interestingly, the transient expression of GFP-Bfl-1 as well as of Bax, a pro-apoptotic control, induced cytochrome *c* release, whereas GFP had no such effect (Fig. 4A). Therefore, these results suggested that like other pro-apoptotic Bcl-2 family proteins, GFP-Bfl-1 acts directly on mitochondria to induce cytochrome *c* release.

3.4. *GFP-Bfl-1 induced apoptosis through a caspase-dependent pathway*

Cytosolic cytochrome *c* released from mitochondria is known to activate Apaf-1, which in turn activates caspase-9 and a downstream caspase cascade [25,26]. We examined whether z-VAD-fmk, a general caspase inhibitor, could inhibit the pro-apoptotic activity of GFP-Bfl-1. Most cells transfected with Bax expression plasmid, as a control, and cultured in the presence of 100 μM of z-VAD-fmk did not undergo apoptosis without morphological changes (Fig. 4B,C). Similarly, z-VAD-fmk also significantly blocked the cell death induced by GFP-Bfl-1 (Fig. 4B,C), indicating that GFP-Bfl-1 induced cell death through a caspase-dependent pathway, in part, in a manner similar to that of the pro-apoptotic Bax.

3.5. *Apoptosis induced by GFP-Bfl-1 is not inhibited by anti-apoptotic Bcl-xL or Bfl-1*

Next, we examined whether the anti-apoptotic members Bcl-xL and Bfl-1 inhibit the cell death induced by GFP-

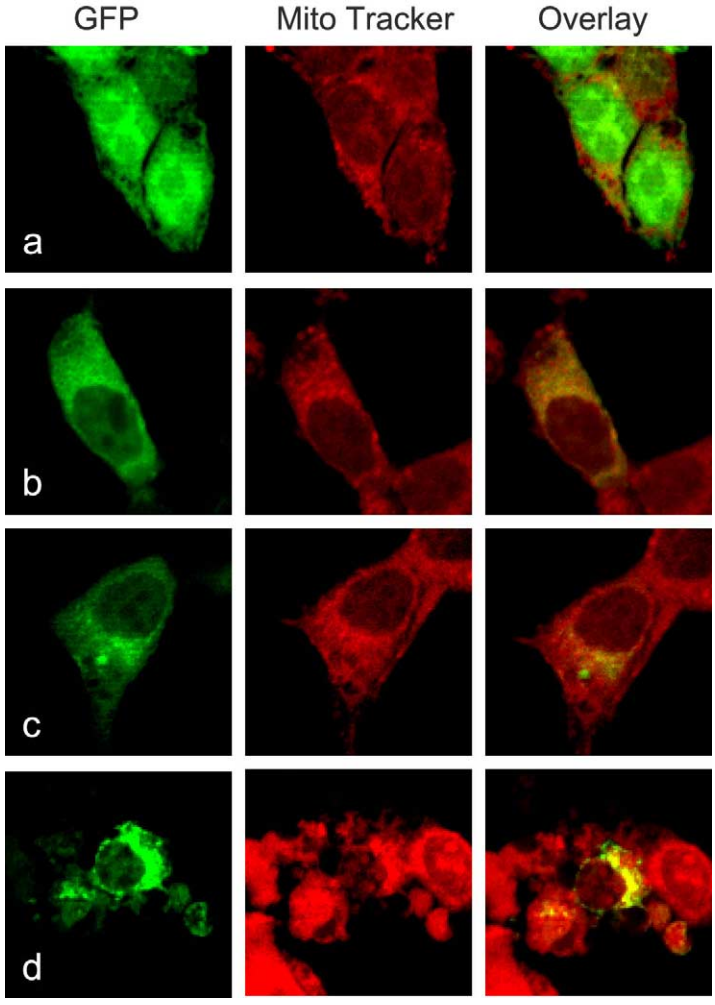

Fig. 2. GFP-Bfl-1 fusion proteins localize at mitochondria in both healthy and apoptotic 293T cells. 293T cells were transfected with 1 μg of pEGFP-Bfl-1, pEGFP-Bcl-xL, or pEGFP-C1. Twenty-four hours after transfection, mitochondria were stained for 10 min with 50 nM of Mito-Tracker, a mitochondria-specific dye, and cells were analyzed by confocal laser microscopy. a: Cells expressing GFP. b: Cells expressing GFP-Bcl-xL. c: Healthy cells expressing GFP-Bfl-1. d: Apoptotic cells expressing GFP-Bfl-1.

Bfl-1. Both proteins are known to inhibit cell death induced by various apoptotic stimuli, such as treatment with anti-cancer drugs or by the expression of pro-apoptotic proteins like Bax or Bid [27–30]. Our results showed that Bcl-xL and Bfl-1 are capable of suppressing Bax-induced cell death; however, they failed to protect 293T cells from the apoptotic cell death induced by GFP-Bfl-1 (Fig. 4D,E).

4. Discussion

Bfl-1, which consists of 175 amino acids and four conserved BH domains (BH1–4), is known to protect cells from various apoptotic stimuli. Bfl-1 suppressed tumor necrosis factor-α-induced cell death in HeLa-derived HtAT cells [31] and in human microvascular endothelial cells ([32]. It was also found to suppress the cell death induced by chemotherapeutic drugs, such as etoposide, cisplatin or adriamycin [27,28]. In addition, we previously demonstrated that Bfl-1 suppresses staurosporine-induced apoptosis in Reh human B-lymphoblastic cells [21] and in Molt-4 human T-leukemia cells [20]. In Molt-4 T cells, Bfl-1 was found to prevent the activation of Bid and of caspase-3, 8 and 9. Bfl-1 was also found to be localized at mitochondria and to prevent mitochondrial transmembrane potential loss when apoptosis was induced by staurosporine [20].

However, surprisingly we found that GFP-Bfl-1 fusion protein strongly induced apoptotic cell death in 293T cells (Fig. 1). Previously, there were several reports that GFP has a cytotoxic effect on cells [33,34]. However, the pro-apoptotic activity of GFP-Bfl-1 fusion protein was not the result of GFP itself because the transient expression of GFP, GFP with Bfl-1, or of GFP-Bcl-xL fusion protein had no effect on cell death.

Although the pro-apoptotic mechanism of GFP-Bfl-1 is not clear, our results suggest that mitochondrial targeting is critical for its pro-apoptotic activity. GFP-Bfl-1 was predominantly localized to the mitochondria of early healthy cells or late apoptotic cells (Fig. 2), induced cytochrome *c* release (Fig. 4A), and triggered apoptotic cell death mediated by caspase-dependent mechanisms (Fig. 4B,C). Therefore, it is possible that GFP-Bfl-1, like Bax or Bak (multi-domain pro-apoptotic Bcl-2 family proteins), might directly damage the mitochondrial structure resulting in cytochrome *c* release, and subsequent caspase activation [1–3].

The Bcl-2 family proteins are capable of physically interacting with each other, to form a complex network of homo- and

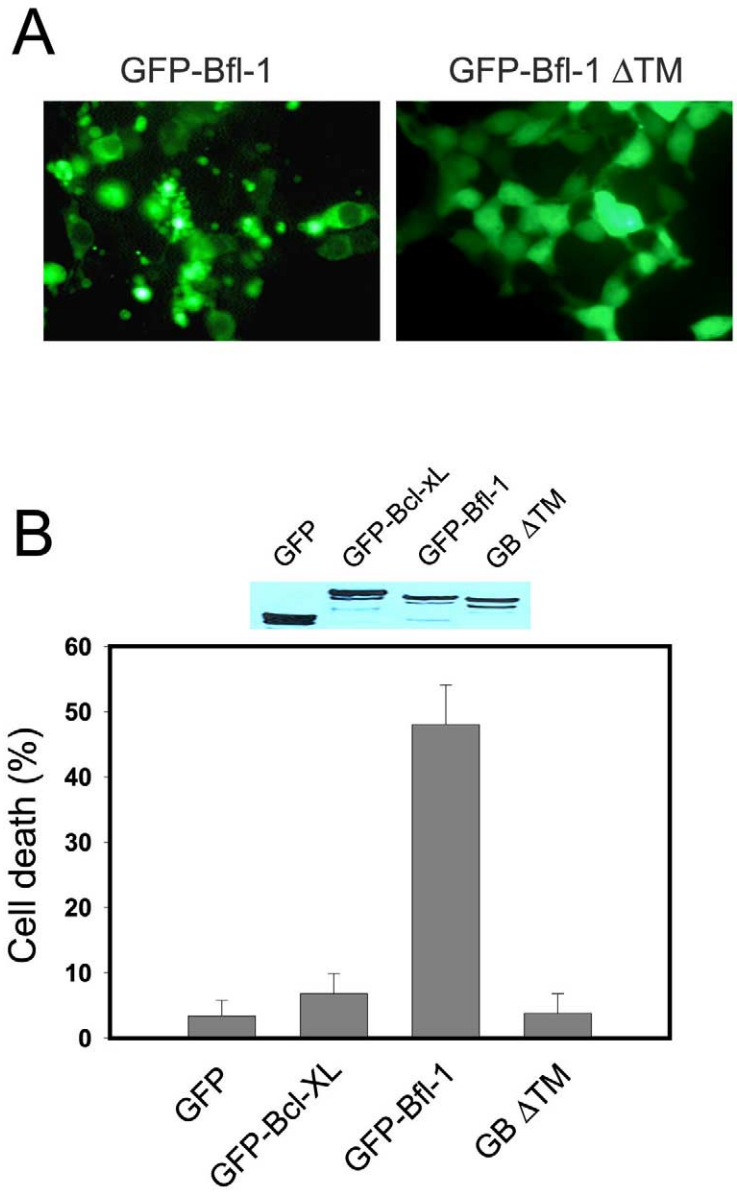

Fig. 3. The C-terminal TM domain of Bfl-1 is essential for the mitochondrial localization and pro-apoptotic activity of GFP-Bfl-1. 293T cells were transfected with 1 μg of pEGFP-C1, pEGFP-Bcl-xL, pEGFP-Bfl-1 or pEGFP-Bfl-1ΔTM (GB ΔTM) plasmids. Twenty-four hours after transfection, the cells were observed under a fluorescence microscope (A) and cell death was quantified as described in Section 2 (B). The expression levels of GFP fusion proteins were examined by Western blotting with anti-GFP antibody. The percentages of apoptotic cells are expressed as means ± S.D.; similar results were obtained from three independent experiments.

heterodimers, via molecular regions, like the BH domains. Moreover, these physical interactions sometimes play important roles in the net effects of pro- and anti-apoptotic members of the family [22,35–37]. For example, cytosolic and monomeric Bax, an inactive form, translocates to mitochondria where it becomes an integral membrane protein, in a homodimer form, that actively triggers apoptosis [22,35]. However, the coexpression of Bcl-2 or Bcl-xL with Bax was found to neutralize the pro-apoptotic effect of Bax by forming heterodimers with Bax [36]. Recently, it was proposed that pro-apoptotic Bid, which possesses only the BH3 domain, cooperates with Bax to cause mitochondrial dysfunction [37]. Previously, it was shown that Bfl-1, which has a Bcl-xL-like core structure, interacted strongly with Bax and neutralizes the pro-apoptotic effect of Bax in yeast cells [29]. In addition, recent evidence revealed that Bfl-1 selectively and tightly binds to tBid and blocks the collaboration between Bid and Bax [19].

Therefore, we initially postulated that GFP-Bfl-1 might express its pro-apoptotic effect by interacting with other pro-apoptotic proteins such as Bax or Bid, based on the interactive property of Bfl-1. If GFP-Bfl-1 like Bfl-1 binds with Bax, Bid or other unknown pro-apoptotic factors, the overexpression of Bfl-1 or of Bcl-xL could compete with GFP-Bfl-1, and might inhibit the apoptotic cell death induced by GFP-Bfl-1. Thus, we examined the antagonizing effects of Bcl-xL or Bfl-1 on the apoptosis induced by GFP-Bfl-1. However, our results showed that neither inhibited the apoptosis induced by GFP-Bfl-1 in spite of their excess overexpression relative to GFP-Bfl-1 (five-fold transfection of expression plasmids), whereas

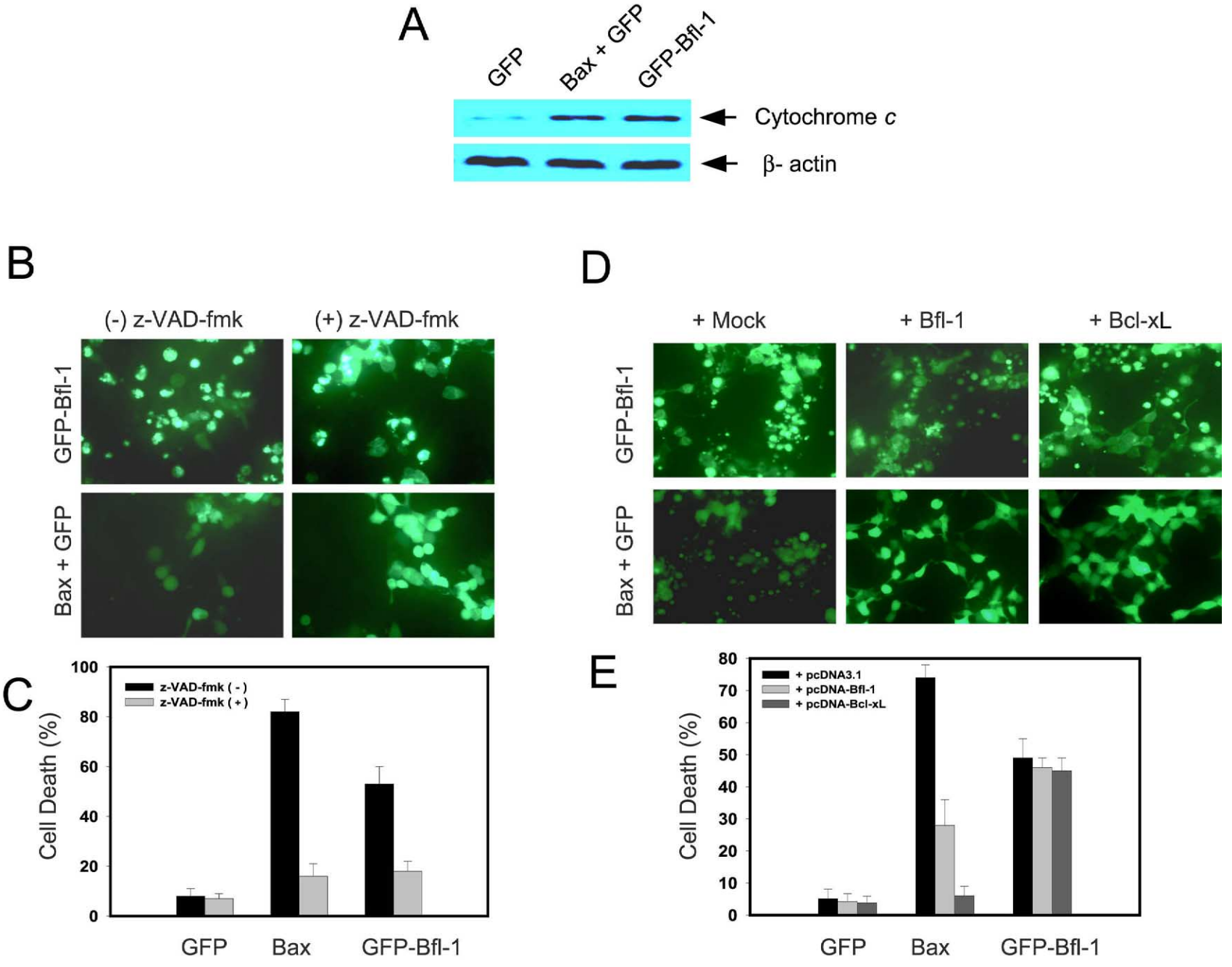
A
GFP
Bax + GFP
GFP-Bfl-1
Cytochrome c
β- actin
B
(-) z-VAD-fmk
(+) z-VAD-fmk
GFP-Bfl-1
Bax + GFP
C
z-VAD-fmk (-)
z-VAD-fmk (+)
Cell Death (%)
100
80
60
40
20
0
GFP
Bax
GFP-Bfl-1
D
+ Mock
+ Bfl-1
+ Bcl-xL
GFP-Bfl-1
Bax + GFP
E
+ pcDNA3.1
+ pcDNA-Bfl-1
+ pcDNA-Bcl-xL
Cell Death (%)
80
70
60
50
40
30
20
10
0
GFP
Bax
GFP-Bfl-1

Fig. 4. The pan-caspase inhibitor, z-VAD-fmk, inhibited the 293T cell death induced by GFP-Bfl-1. A: The transient expression of GFP-Bfl-1 induces cytochrome *c* release from mitochondria. 293T cells were transfected with 1 μg of pEGFP-Bfl-1, pcDNA-Bax or pEGFP-C1. Twenty-four hours after transfection, the cells were collected and lysed. Mitochondria-free cytosol was prepared as described in Section 2. 20 μg of protein was analyzed by Western blotting using anti-cytochrome *c* antibody; anti-β-actin antibody was used as a loading control. B,C: The pan-caspase inhibitor, z-VAD-fmk, suppresses the 293T cell death induced by GFP-Bfl-1. 293T cells were transfected with pEGFP-Bfl-1 (1 μg), pcDNA-Bax (1 μg)+pEGFP-C1 (0.1 μg), or pEGFP-C1 (1 μg) and cultured in the presence of 100 μM of z-VAD-fmk for 24 h. Transfected cells were examined by fluorescence microscopy (B) and apoptotic cells were counted (C). D,E: Anti-apoptotic Bcl-xL and Bfl-1 have no inhibitory effect on 293T cell death induced by GFP-Bfl-1. 293T cells were co-transfected with 0.3 μg plasmid of pEGFP-C1, pcDNA-Bax or pEGFP-Bfl-1 plasmid together with 1.5 μg of pcDNA3.1 (mock), pcDNA-Bfl-1 or pcDNA-Bcl-xL. Twenty-four hours after transfection, the cells were observed by fluorescence microscopy (D) and apoptotic cells were counted (E). Quantitative apoptotic cell death was measured as described in Section 2. The percentages of apoptotic cells are expressed as means ± S.D.; similar results were obtained from three independent experiments.
←

Bax-induced cell death was significantly blocked by Bcl-xL or Bfl-1, which is consistent with previous observations [29,30] (Fig. 4D,E). Thus, these results suggest that GFP fusion at the N-terminus of Bfl-1 might induce a Bfl-1 conformational change, which modifies its interactions with other proteins. Therefore, it would be interesting in the future to examine whether Bfl-1 and GFP-Bfl-1 interact with other pro-apoptotic proteins in the mitochondrial membrane in a similar manner.

During apoptosis, caspases have been shown to cleave two representative anti-apoptotic proteins, Bcl-2 and Bcl-xL, and to produce C-terminal fragments with potent pro-apoptotic activity [13,14]. However, little is known about the pro-apoptotic mechanisms of these cleaved proteins. Recently, it was revealed that the pro-apoptotic cleavage products of Bcl-xL, but not full form Bcl-xL, formed cytochrome *c*-conducting pores in the mitochondrial membrane in vivo and in vitro, and destabilized lipid bilayer membranes [38]. These results are consistent with previous observations that the three-dimensional structure of Bcl-xL shows structural similarities with the pore-forming domains of diphtheria toxin and bacterial colicins [4]. Since molecular modeling showed that the core structure of Bcl-xL, which consists of seven α-helices, is similar to that of Bfl-1, and that both proteins share the conserved BH1 and BH2 domains [29], Bfl-1 might be able to form a cytochrome *c*-permeable pore in the mitochondrial membrane when fused with GFP. Therefore, it would be interesting to determine whether GFP-Bfl-1 can form a pore complex in the mitochondrial membrane.

Further observations, including mitochondrial apoptotic events, protein interactions between GFP-Bfl-1 and other proteins, and the analysis of the conformational change induced in Bfl-1 by GFP fusion, may reveal the detailed mechanisms involved, which would provide information on the functions of the Bcl-2 family proteins.

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