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Induction of mammalian cell death by a plant Bax inhibitor

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Abstract ***Arabidopsis thaliana*** **AtBI-1 is an orthologue of mammalian Bax inhibitor-1 capable of suppressing Bax-induced cell death in yeast as well as mammalian cells. Here we investigated whether or not AtBI-1 suppresses Bax-induced cell death using human fibrosarcoma HT1080 cells. Surprisingly, AtBI-1 did not block Bax-induced cell death, but it triggered apoptotic cell death in mammalian cells. The proapoptotic effect of AtBI-1 was blocked by the X-linked caspase inhibitor XIAP, suggesting that the cell death caused by AtBI-1 is similar to that caused by Bax.** © **2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Apoptosis; AtBI-1; Bax; Chromatin condensation; Caspase inhibitor

1. Introduction

Fundamental features regulating programmed cell death (PCD) are believed to be conserved throughout metazoan and plants [1]. The PCD eliminates redundant cells during development and eliminates diseased cells. Unlike necrotic cell death which is characterized by rapid cell swelling and lysis, apoptotic cell death involves nuclear condensation, cytoskeletal disruption, membrane blebbing, and cell shrinkage [2]. When apoptosis is triggered in animal cells, components of the membrane skeleton such as spectrin are proteolysed by caspases. As a result, the plasma membranes are degenerated to form vesicular apoptotic bodies [3]. In vertebrates, apoptotic pathways are indispensable during tissue homeostasis, development of the nervous system and the regulation of the immune system.

The Bcl-2 family proteins are key regulators of mammalian PCD. Bcl-2, Bcl-X_L, Bcl-w, Bfl-1, A1, Mcl-1 and Bag-1 all suppress apoptosis, whereas Bax, Bcl-Xs, Bak, Bid, Hrk and Bad promote cell death [4]. It was reported that Bax could induce cell death in plant cells [5], whilst Bcl-X_L could inhibit cell death in tobacco plants [6]. This suggests some common elements of the cell death exist in both animals and plants. Lam et al. [7] discussed similar involvement of mitochondria in regulating PCD between animal and plant cells. Also, many of the cell death regulators that have been characterized in human, worms and flies are absent from the *Arabidopsis* genome, which suggests that plants probably use other regulators to control this process.

It has been demonstrated that the Bax inhibitor-1 (BI-1) protein inhibits Bax-induced apoptosis in mammalian cells and when ectopically expressed in yeast [8]. BI-1 contains six or seven predicted transmembrane domains. As an integrate membrane protein, the localization of BI-1 was found to be similar to Bcl-2, exhibiting a nuclear envelope and endoplasmic reticulum (ER)-associated pattern. When over-expressed in human cells, an association of BI-1 and Bcl-2 was demonstrated by both chemical cross-linking and co-immunoprecipitation experiments [8]. Moreover, BI-1 was isolated as one of the candidate suppressors of TRAIL, an apoptosis-inducing member of the tumor necrosis factor [9]. Kawai et al. [10] also isolated plant orthologues of *BI-1* genes from rice *Oryza sativa* (*OsBI-1*) and *Arabidopsis thaliana* (*AtBI-1*). When *OsBI-1* was ectopically overexpressed in the budding yeast *Saccharomyces cerevisiae*, cell death caused by Bax protein was eliminated [10]. Transgenic *Arabidopsis* plants capable of producing Bax protein under a conditional promoter system have been generated [11]. While Bax caused potent apoptosis symptoms including leaf discoloration, cytoplasmic shrinkage and DNA laddering, the induction of AtBI-1 overexpression rescued the transgenic plants from lethality [11]. Therefore, similar cross-talk can be demonstrated between the Bax and BI-1 proteins regardless of whether expressed in yeast, plants or mammalian cells.

To gain insights into the biological role of the evolutionary conserved proteins between mammals and plants, we investigated function of plant BI-1 (AtBI-1) in heterologous mammalian cells. We report here that AtBI-1 induces apoptosis in mammalian cells, suggesting that plant BI-1 may operate as a dominant inhibitor of endogenous mammalian BI-1 (mBI-1).

2. Materials and methods

2.1. Cell culture

Human fibrosarcoma HT1080 cells were maintained at 37°C in a humidified atmosphere of 5% CO_2, 95% air in RPMI 1640 medium (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Moregate, Australia), 2 mM L-glutamine, and 100 μg/ml kanamycin [12].

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Abbreviations: AtBI-1, *Arabidopsis thaliana* orthologue of Bax inhibitor-1; AtBI-1-ΔC, AtBI-1 with the carboxyl-terminal 14 residues removed; BI-1, Bax inhibitor-1; EGFP, enhanced green fluorescence protein; ER, endoplasmic reticulum; HA, hemagglutinin; OsBI-1, *Oryza sativa* orthologue of Bax inhibitor-1; PCD, programmed cell death; SDS, sodium dodecyl sulfate; XIAP, X-linked inhibitor of apoptosis protein

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2.2. Plasmid constructions

An *Eco*RI-tagged *AtBI-1* cDNA fragment [10] was cloned into pcDNA3.1 vector (Invitrogen), which contains an Xpress epitope at the 5′ end. For expression of AtBI-1:enhanced green fluorescence protein (EGFP) fusion protein, an *Eco*RI–*Apa*I fragment of *AtBI-1* cDNA without the stop codon, was fused to the coding region of pEGFP-N2. With the same strategy, an *AtBI-1* truncate lack of the 3′ 45 nucleotides was also fused to pEGFP-N2 [8]. The sense direction of the *AtBI-1* gene in the plasmids and the in-frame constructions were verified by nucleotide sequencing.

2.3. Transfection

The cells were seeded into 12-well tissue culture dishes at 1.2×10^5 cells/well. After overnight incubation, the transfection of plasmids was carried out with a FuGENE reagent, according to the manufacturer's instruction (Roche, Mannheim, Germany). The pcDNA-EGFP was co-transfected with the targeted DNA each time as a monitor of the transfection success, except in the case when the target gene was constructed in the pEGFP-N2 vector. The cells were cultured for additional 24 h before harvest. The number of dead cells floating in a medium was recorded through FACScan (Becton Dickinson).

2.4. Western blot analysis

Western blot analysis was performed as Hirotani et al. [12], with some modifications. Cells were solubilized with 0.1 M Tris–HCl (pH 8.0), containing 1% sodium dodecyl sulfate (SDS) and 10% glycerol. The cell lysates were subjected to SDS–polyacrylamide gel electrophoresis, followed by immunohybridization to the Immobilon™ transfer membrane (Millipore). An anti-rat HA (hemagglutinin) antibody (Boehringer Mannheim) was used to detect the HA epitope-tagged Bax protein.

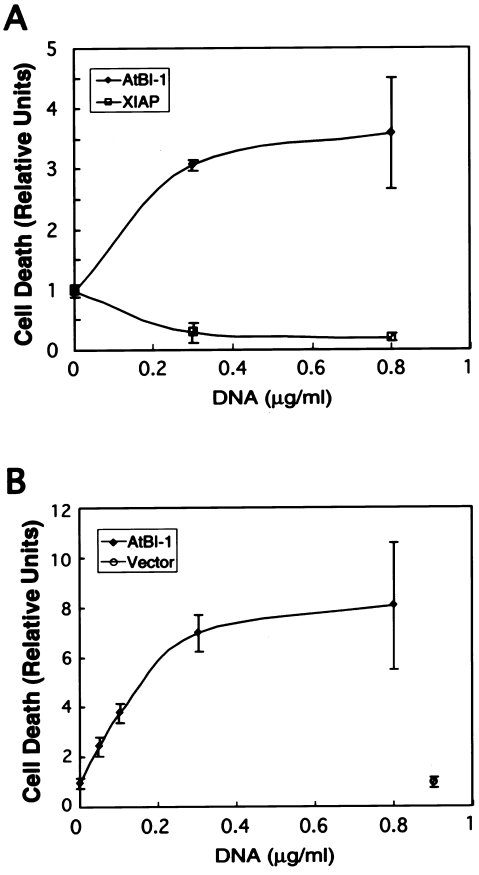

Fig. 1. AtBI-1 caused death in human fibrosarcoma HT1080 cells. A: Effects of AtBI-1 or XIAP on Bax-induced death in human fibrosarcoma HT1080 cells. The cells were co-transfected either with *Bax*/*AtBI-1* or *Bax*/*XIAP*, followed by the 24 h culture for the observation of dead cells. The following vectors were used: pcDNA3.1-AtBI-1, pcDNA3-XIAP, pcDNA-Bax. For comparison, dead cell number obtained by treatment of pcDNA3-Bax was set as relative unit 1. B: Plasmid encoding *AtBI-1* (pcDNA3.1-AtBI-1) was added to RPMI medium at different concentrations (0, 0.05, 0.1, 0.3, and 0.8 μg/ml). Dead cells floating at 24 h after DNA treatment were counted, and expressed as a relative unit. An open circle shows data from the control plasmid pcDNA3.1 at 0.9 μg/ml. Means ± S.D. of triplicate experiments are presented.

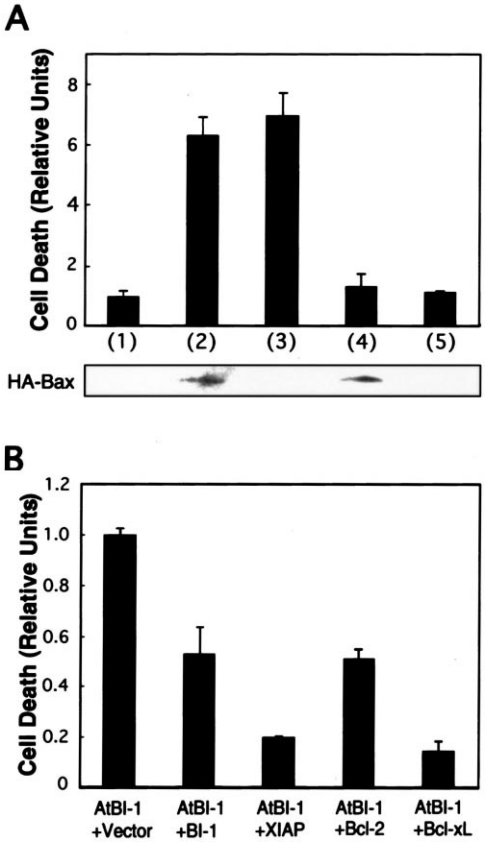

Fig. 2. Interactions between AtBI-1 and the apoptosis inhibitors. A: Effects of XIAP on Bax- or AtBI-1- induced cell death. (1) Control vector pcDNA3.1 alone (0.9 μg/ml); (2) pcDNA3-Bax (0.1 μg/ml)+pcDNA3.1 (0.8 μg/ml); (3) pcDNA3.1-AtBI-1 (0.3 μg/ml)+pcDNA3.1 (0.6 μg/ml); (4) pcDNA3-Bax (0.1 μg/ml)+pcDNA3-XIAP (0.8 μg/ml); (5) pcDNA3.1-AtBI-1 (0.3 μg/ml)+pcDNA3-XIAP (0.6 μg/ml). The lower panel shows Western blot analysis using an anti-HA antibody for detection of Bax-HA protein. After 24 h of treatment, crude proteins were extracted from total cells and subjected to SDS–polyacrylamide gel electrophoresis, followed by immunological detection of Bax protein. B: Effect of apoptosis suppressors on AtBI-1-induced mammalian cell death. The cells were co-transfected with plasmid pcDNA3.1-AtBI-1 (0.3 μg/ml)+pcDNA3-BI-1 (0.6 μg/ml), or pcDNA3-XIAP (0.6 μg/ml), or pRc/CMV-Bcl-2 (0.6 μg/ml), or pcDNA3-Bcl-X_L (0.6 μg/ml). As a control, cell death induced by pcDNA3.1-AtBI-1 alone (0.3 μg/ml) is expressed as cell death unit 1. Means ± S.D. of triplicate experiments are presented.

2.5. Microscopic observation

To observe chromatin condensation, cells were treated with Hoechst 33342 (Sigma) and observed at a 343 nm excitation wavelength under a fluorescence microscope (DMRD, Leica, Germany). The EGFP fluorescence was examined at a 488 nm excitation wavelength.

3. Results

3.1. AtBI-1 causes death in human fibrosarcoma cells

We demonstrated previously that AtBI-1 is a suppressor of

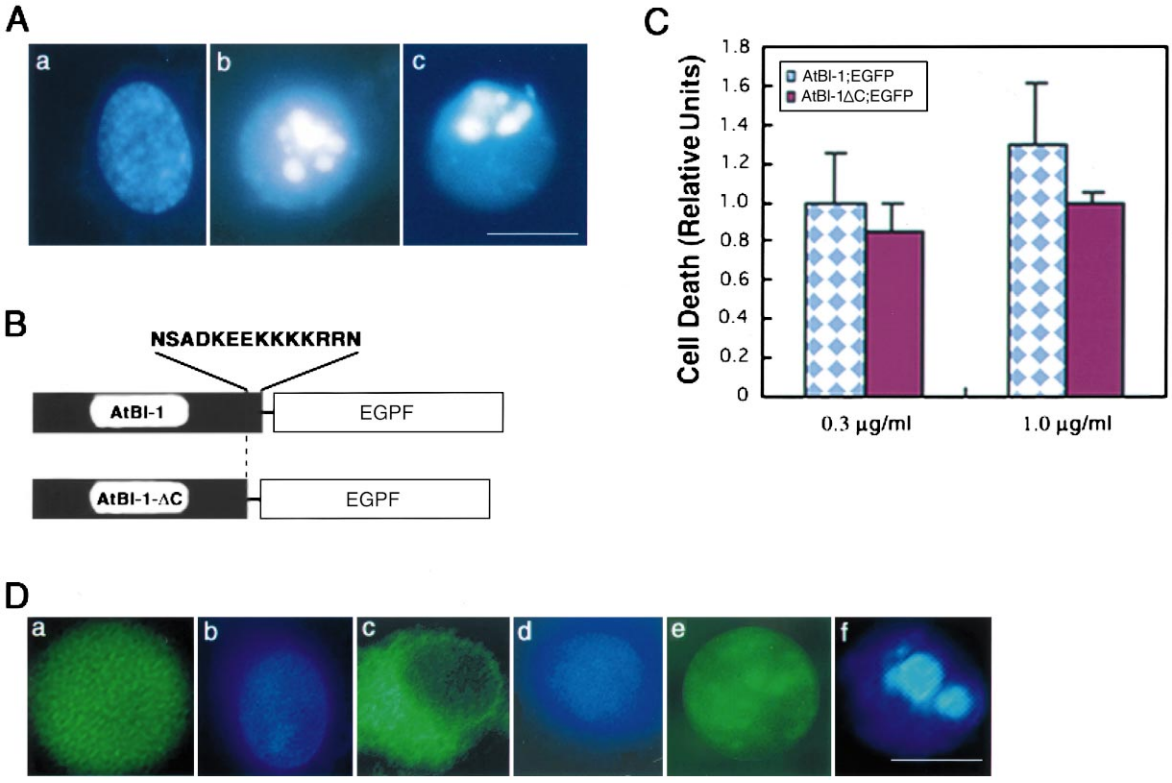

Fig. 3. Microscopic observation of mammalian cells treated with Bax and AtBI-1. A: Nuclear morphology in human fibrosarcoma HT1080 cells transfected with either *Bax* or *AtBI-1*. Cultured cells were treated with vector pcDNA3.1 (a), pcDNA3-Bax (b), or pcDNA3.1-AtBI-1 (c), respectively, followed by treatment of cells with Hoechst 33342, and then photographs were taken under UV emission light as described in Section 2.5. The bar indicates 10 μm. B: A sketch map showing fusion proteins AtBI-1:EGFP and AtBI-1-ΔC:EGFP. C: Both plasmids caused death in HT1080 cells. The cells were treated with either pEGFP-AtBI-1 or pEGFP-AtBI-1-ΔC at the concentrations of 0.3 and 1.0 μg/ml, respectively. The dead cell number under the treatment of 0.3 μg/ml pEGFP-AtBI-1 was set as relative unit 1. Means ± S.D. of triplicate experiments are presented. D: Fluorescence observation of HT1080 cells expressing AtBI-1:EGFP fusion protein. Cultured cells were treated with pEGFP-N2 empty vector; photographs were taken for EGFP (a) and Hoechst (b), respectively. Cultured cells treated with pEGFP-AtBI-1; photographs were taken 18 h after transfection for EGFP (c) and Hoechst (d), and 24 h after transfection for EGFP (e) and Hoechst (f), respectively. The bar indicates 10 μm.

Bax-induced cell death in yeast [10]. We also demonstrated that transgenic *Arabidopsis* plants over-expressing AtBI-1 are resistant to the lethal action of Bax in whole plants [11]. Thus, we were prompted to investigate the biological action of AtBI-1 in the mammalian cell system, asking whether or not AtBI-1 mimics the functional properties of mBI-1.

To our surprise, when HT1080 cells were co-transfected with both pcDNA-Bax (0.1 μg/ml) and pcDNA3.1-AtBI-1 (0.3 or 0.8 μg/ml), we did not see suppression of Bax-induced cell death by AtBI-1. In contrast, co-transfection of cells with pcDNA-Bax and pcDNA-XIAP (X-linked inhibitor of apoptosis protein) caused dramatic reduction of dead cells (Fig. 1A). Moreover, transfection of HT1080 cells with pcDNA3.1-AtBI-1 induced cell death. This AtBI-1-induced cell death was proportional to the DNA transfected, up to 0.3 μg/ml, whereas little cell death was observed with the vector alone (Fig. 1B). Following DNA transfection, cells were treated with Hoechst 33342 and examined by UV microscopy. Chromatin condensation typical of apoptosis was observed in AtBI-1-treated cells, which was similar to the apoptotic morphology of Bax-treated cells (Fig. 3A).

3.2. Suppression of AtBI-1-induced cell death by XIAP

To explore some of the steps downstream of AtBI-1 in mammalian cells, we analyzed the effect of XIAP, a potent caspase inactivator [13,14]. As shown in Fig. 2A, AtBI-1 caused prominent cell death similar to the effect of Bax. When *XIAP* was co-transfected with *AtBI-1*, cell death was greatly reduced. This reduction of cell death was comparable to that obtained for co-transfected *Bax*/*XIAP* cells (Fig. 2A). Thus, AtBI-1 caused apoptosis through a caspase-dependent pathway.

3.3. BI-1 and Bcl-2 family suppress AtBI-1-induced cell death

To explore the possibility that AtBI-1 might inhibit endogenous mBI-1, we co-transfected cells with *AtBI-1* and *mBI-1*. The results indicated that mBI-1 suppresses cell death induced by AtBI-1. Furthermore, Bcl-2 and Bcl-X_L were also capable of reducing the amounts of mammalian cell death induced by AtBI-1 (Fig. 2B).

3.4. Localization of AtBI-1 in transfected cells

To determine the subcellular localization of AtBI-1 in mammalian cells, we created plasmids encoding full-length AtBI-1:EGFP or mutant AtBI-1-ΔC (14 residues of the carboxyl-terminal were removed): EGFP fusion proteins (Fig. 3B). Both fusion proteins showed similar lethal effects on HT1080 cells (Fig. 3C). Microscopic observation revealed that at an early stage after transfection when the chromatin condensation had not occurred, AtBI-1:EGFP was located in

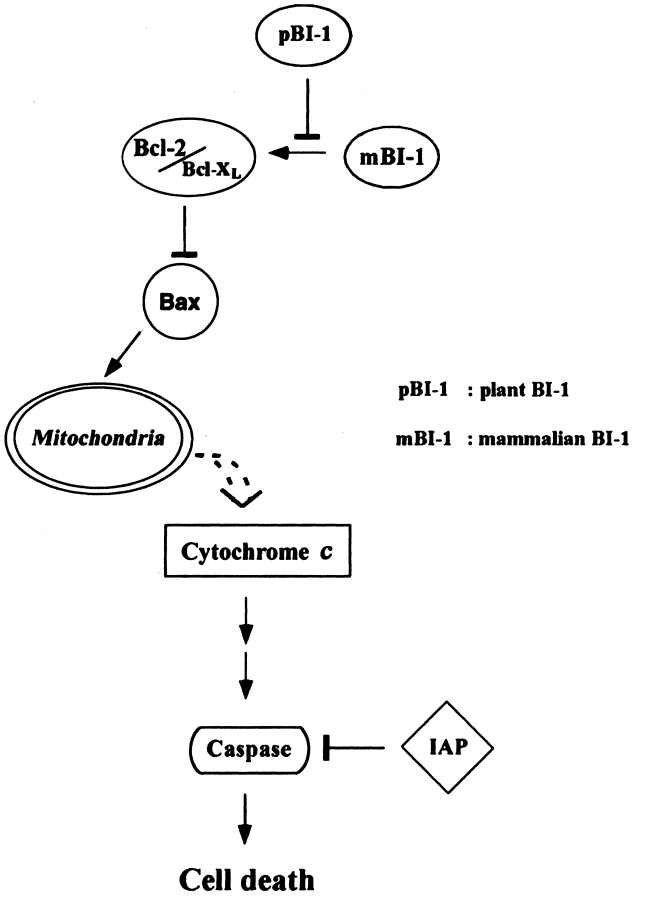

Fig. 4. Proposed model illustrating induction of cell death by AtBI-1 in mammalian cells. pBI-1: plant BI-1; IAP: inhibitor of apoptosis protein. Plant orthologue of mammalian Bax inhibitor may compete with endogenous Bax inhibitor protein by an unknown mechanism, which in turn changes the balance of Bcl-2 and Bax proteins. Consequently, the cell death pathway can be activated as a Bax-induced apoptotic event.

cytosol and perinuclear compartments, possibly in the ER network (Fig. 3D, c). The localization of AtBI-1-ΔC:EGFP was similar to the full-length AtBI-1:EGFP (data not shown). At late stages after transfection, diffusion of EGFP fluorescence and chromatin condensation were noted (Fig. 3D, e and f).

4. Discussion

The death-suppressing action of BI-1 was first discovered in screening in which transformed yeast cells containing a galactose-inducible Bax plasmid were transformed with a human cDNA library under a constitutively active yeast promoter and cDNAs were isolated that prevented Bax-induced lethality in response to galactose [8]. We have previously cloned plant *BI-1* cDNAs (*AtBI-1*, and rice *OsBI-1*) [10]. Interestingly, Sanchez et al. [15] reported that *AtBI-1*, the same gene isolated by our group, was obtained by differential screening of genes from plants challenged with the phytopathogen *Pseudomonas syringae*, suggesting that BI-1 may be involved in pathogen-resistance mechanisms.

Due to our interest in evolutionary conservation of cell death in plants and metazoans, we investigated the function of AtBI-1 in a mammalian cell system. Since AtBI-1 shares 41% amino acid identity with mBI-1, we anticipated that AtBI-1 might prevent cell death induced by Bax in mammalian cells. Surprisingly, AtBI-1 did not suppress Bax-induced cell death in human fibrosarcoma HT1080 cells. In fact, AtBI-1 induced cell death, comparable to Bax. Moreover, chromatin condensation typical of Bax-induced apoptotic cell death was also confirmed in cells transfected with AtBI-1. Also, apoptosis induced by AtBI-1 was inhibited by XIAP, similar to Bax, implying a caspase-dependent mechanism. Bcl-X_L also prevented AtBI-1-induced cell death in mammalian cells. Therefore, AtBI-1-induced cell death may mimic Bax-induced apoptosis in mammalian cells.

Using GFP tagging, the AtBI-1 proteins seem to be located at the nuclear envelope and ER network in mammalian cells, in a pattern that resembled the subcellular distribution pattern of AtBI-1 in both yeast and tobacco cells [11]. mBI-1 was also previously reported in a similar localization in human cells [8].

Because of the similarity of BI-1 proteins, we presume that the pro-apoptotic action of AtBI-1 in human cells can be explained by competition with endogenous mBI-1. Although plant-type BI-1 proteins possess high homology with mammalian counterparts, the carboxyl-terminals of plant BI-1 proteins are subtly longer than those of mBI-1 proteins [10]. Our unpublished results indicated that removal of the carboxyl 14 amino acid residues from AtBI-1 depleted the protein's suppressive effect on Bax-induced cell death in yeast. Nevertheless, as demonstrated in this study, an apparent increase of dead cell numbers was noted when this truncated mutant protein was transfected into human HT1080 cells. Thus, AtBI-1 may act via a different mechanism in mammalian cells from that in yeast. While the carboxyl-terminal region of AtBI-1 is crucial for suppressing Bax-induced death in yeast, it is not needed for dominant-negative activity against endogenous mBI-1.

The possibility exists that AtBI-1 might directly damage the mitochondrial structure causing cytochrome *c* release. However, co-transfection of HT1080 cells with both human BI-1 and AtBI-1 crippled cell death, suggesting preferably a dominant-negative mechanism, in which AtBI-1-induced apoptosis is minimized by over-expressed mBI-1.

Based on the observation that Bcl-2 and Bcl-X_L suppressed AtBI-1-induced cell death, AtBI-1 may act at the early stage of the cell death pathway. As shown in Fig. 4, we speculate that AtBI-1 competitively interacts with endogenous mBI-1 or with a BI-1 target protein, interfering with its function and thereby triggering cell death. In this regard, Xu and Reed [8] demonstrated in vitro binding of BI-1 with Bcl-2, but not with Bax. Thus, it remains unclear how BI-1 suppresses Bax's function, given that yeast and plants contain no obvious Bcl-2 homologues. Further comparisons of the mechanisms of BI-1 in plant and animal cells therefore may reveal detailed examples of molecules that regulate cell survival throughout evolution.

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