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The Bax Inhibitor-1 needs a functional electron transport chain for cell death suppression

Reiko Oshima[a,1], Keiko Yoshinaga[a,b,1], Yuri Ihara-Ohori[a], Ryouichi Fukuda[c], Akinori Ohta[c], Hirofumi Uchimiya[a,d], Maki Kawai-Yamada[a,e,*]

[a] *Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan*
[b] *Laboratory of Plant Molecular Genetics, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan*
[c] *Department of Biotechnology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan*
[d] *Iwate Biotechnology Research Center, Kitakami, Iwate 024-0003, Japan*
[e] *Japan Science and Technology Agency (JST), Core Research for Evolutional Science and Technology (CREST), Japan*

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Abstract Bax inhibitor-1 (BI-1) is an evolutionarily conserved cell death suppresser in animals, yeast, and plants. In this study, yeast strains carrying single-gene deletions were screened for factors related to cell death suppression by *Arabidopsis* BI-1 (AtBI-1). Our screen identified mutants that failed to survive Bax-induced lethality even with AtBI-1 coexpression (Bax suppressor). The Δcox16 strain was isolated as a BI-1-inactive mutant; it was disrupted in a component of the mitochondrial cytochrome *c* oxidase. Other mutants defective in mitochondrial electron transport showed a similar phenotype. ATP levels were markedly decreased in all these mutants, suggesting that BI-1 requires normal electron transport activity to suppress cell death in yeast.

Keywords: Cell death; Bax inhibitor; Mutant; COX; Yeast; Apoptosis

1. Introduction

Apoptosis, or programmed cell death, is a part of normal development and homeostasis in metazoans. Apoptosis is co-regulated by the conserved family of Bcl-2-related proteins, which includes both anti-apoptotic (e.g., Bcl-2 and Bcl-xL) and pro-apoptotic (e.g., Bax and Bak) members [1]. Bcl-2 family members function mainly at the mitochondria, and are pivotal in deciding the fate of a cell.

In mammalian cells following a death signal, Bax translocates to the mitochondria and promotes release of cytochrome *c* from the mitochondrial intermembranous space to the cytosol. This cytochrome *c* is then available to associate with Apaf-1 following activation of caspases, a protease family that induces apoptotic changes such as chromatin condensation and DNA laddering. The Bcl-2 and caspase family are highly conserved in the animal kingdom, but have no obvious homologues in yeast or plant genomes. Nonetheless, expression of Bax in yeast and plants causes cell death accompanying DNA laddering [2,3]. Moreover, expression of anti-apoptotic Bcl-2-related proteins, such as Bcl-2, Bcl-xL, and Ced-9, repress Bax- and oxidative stress-induced cell death in yeast and plants [4–6].

A human Bax inhibitor-1 (BI-1) gene was identified originally as a suppresser of Bax-mediated lethality in yeast [7]. Unlike Bcl-2 family members, BI-1 is an evolutionarily conserved endoplasmic reticulum (ER) protein that suppresses cell death in animal, yeast, and plant cells [8,9]. *Arabidopsis* BI-1 homologue, AtBI-1, suppresses Bax- and H_2O_2-mediated cell death in yeast, animals and plants [3,10,11]. AtBI-1 contains six or seven potential transmembrane helices, and a highly conserved C-terminal domain that contains a binding region for calmodulin and is essential for the suppression of cell death [11,12]. Expression of AtBI-1 is rapidly upregulated in plants during wounding or pathogen challenge [13] and downregulated during elicitor-induced hypersensitive response (HR) [14]. These data indicated that BI-1 controls cell death mechanisms conserved in animals, yeast, and plants, however, the molecular machineries underlying these mechanisms remain unclear.

Traditional genetic approaches were successfully applied in yeast to identify possible apoptosis-regulating genes [7,15]. This study aimed to identify functional partners of BI-1 in *Saccharomyces cerevisiae* strains carrying single-gene deletions. Yeast mutants coexpressing AtBI-1 and Bax were screened systematically to reveal BI-1-inactive mutants, which do not survive from Bax-induced lethality despite them coexpressing BI-1. Isolated BI-1-inactive mutants demonstrated ATP-less phenotype, suggesting possible involvement of ATP in the survival mechanism of AtBI-1.

2. Materials and methods

2.1. Yeast strains and transformation

S. cerevisiae single-gene disruption mutants were purchased from EUROSCARF (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/index.html). Yeast strains were cultured in YPD (1% yeast extract,

*Corresponding author. Address: Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan. Fax: +81 3 5841 8466.
E-mail address: mkawai@iam.u-tokyo.ac.jp (M. Kawai-Yamada).

[1]These authors equally contributed to the work.

Abbreviations: BI-1, Bax inhibitor-1; ER, endoplasmic reticulum; HR, hypersensitive response; SD, synthetic dextrose

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Table 1
Saccharomyces cerevisiae strains used in this study

Strain	Systematic name	Genotype
WT	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0
Δndi1	YML120c	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YML120c::kanMX4
Δsdh2	YLL041c	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YLL041c::kanMX4
Δsdh4	YDR529c	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YDR178w::kanMX4
Δqcr7	YDR178w	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δqcr7::kanMX4
Δqcr6	YFR033c	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YDR529c::kanMX4
Δqcr8	YJL166w	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YJL166w::kanMX4
Δcyt2	YKL087c	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YAL039c::kanMX4
Δcyc2	YOR037w	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δcyc2::kanMX4
Δcox5a	YNL052w	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YOR037w::kanMX4
Δcox6	YHR051w	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δcox6::kanMX4
Δcox7	YMR256c	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YHR051w::kanMX4
Δcox8	YLR395c	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δcox8::kanMX4
Δcox11	YPL132w	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YPL132w::kanMX4
Δcox15	YJL003w	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YER141w::kanMX4
Δcox16	YER141w	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YJL003w::kanMX4
Δcox18	YGR062c	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YGR062c::kanMX4
Δatp1	YBL099w	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YBL099w::kanMX4
Δatp2	YJR121w	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YJR121w::kanMX4
Δatp15	YPL271w	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YPL271w::kanMX4
Δatp17	YDR377w	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YDR377w::kanMX4

2% peptone, and 2% glucose), YPGal (2% galactose, 1% yeast extract, and 2% peptone) or synthetic dextrose (SD) medium with appropriate supplements at 30 °C. Yeast transformation was performed by the lithium acetate method. Mutant genotypes used in this study are listed in Table 1.

2.2. Plasmid construction

The expression vector pYX112-AtBI-1 has been described previously [16]. The pYX112-mBcl-2 vector was constructed by ligating *Eco*RI-digested pYX112 (*TPI*-promoter, *Ura*-marked, 2-μm replicon) plasmid and *Eco*RI-tagged mouse *Bcl-2* gene (a kind gift from Dr. Fujita, University of Tokyo, Japan). YEp51-Bax was kindly provided by Dr. J.C. Reed (The Burnham Institute, La Jolla, CA, USA). To obtain expression vector NMV3-COX16, the *cox16* gene tagged with *Eco*RI and *Sal*I recognition sequences was amplified by the PCR method, and then introduced into the *Eco*RI- and *Sal*I-digested NMV3 expression vector (*GAL10* promoter, *Trp*-marked; a kind gift from Dr. Nakano and Dr. Matsuda, RIKEN, Japan).

2.3. Spot assay

Yeast cell suspensions grown in SD–glucose medium for 1 day were adjusted to an OD_{660} of 0.1 or 0.2, and diluted to various concentrations. Aliquots (5 μl) from each dilution were spotted onto SD–glucose or SD–galactose medium containing appropriate supplements, and incubated for 2 days (glucose medium) or 4 days (galactose medium) at 30 °C.

2.4. RT-PCR

Yeast total RNA was extracted using the RNeasy plant miniprep kit (Qiagen, Hilden, Germany). Two micrograms of this RNA constituted the template for RT-PCR using specific primers for AtBI-1 (5′-GGAATTCATGAGCATCCTTATCACTGCATT-3′ and 5′-GGTACCTCAGTTTCTCCTTTTCTTCTTC-3′) and Bax (5′-GGCATGCTCAGCCCATCTTCTTCCAGAT-3′ and 5′-CCTCGAGATGGACGGGTCCGGGGAGCAG-3′). Direct amplification with Ready-to Go RT-PCR Beads (Amersham Pharmacia Biotech Inc., Piscataway, NJ) was conducted with 1 cycle at 42 °C for 20 min and 1 cycle at 95 °C for 5 min; then 35 cycles at 95 °C for 30 s and at 72 °C for 1 min.

2.5. Measurement of ATP content

Yeast strains were grown in YPD medium for 1 day then subjected to ATP extraction using the LL100-2 kit (TOYO B-Net; Japan). An ATP luminescence kit (LL100-1; TOYO B-Net; Japan) measured the amount of ATP in yeast cells of the same OD.

3. Results

3.1. AtBI-1 does not inhibit Bax-induced cell death in Δcox16 mutants

Yeast strains carrying single-gene deletions were used to screen for genes related to BI-1 function. Each mutated gene was disrupted by homologous recombination with the kanMX marker conferring resistance to G418. We randomly selected 300 of the disrupted strains and transformed them with YEp51-Bax, which produces Bax under control of the galactose-inducible *GAL10* promoter. The cells were further transformed with pYX112-AtBI-1 (*TP1* promoter) and plated on glucose-containing medium. Coexpression of anti-apoptotic protein AtBI-1 from the constitutive TPI promoter abrogated the effects of Bax on cell viability. Thus, cells harboring both Yep51-Bax and pYX112-AtBI-1 survive on galactose-containing (Gal) medium [16]. To obtain AtBI-1-inactive mutants, strains not recovered on Gal-medium, in spite of AtBI-1 coexpression, were isolated from the randomly selected yeast mutants. Yeast colonies possessing Bax and AtBI-1 plated on SD–glucose medium were replicated onto the SD–galactose plates to induce Bax expression. After 2 days of incubation at 30 °C, strains showing inhibited growth on Gal-medium were picked up from the replica plate on glucose medium. Successive screening of ~300 strains isolated an AtBI-1-inactive single-deletion mutant, Δcox16. As shown in Fig. 1A, the wild type (WT) strain possessing Bax and BI-1 showed recovered growth on Gal-medium, whereas the Δcox16 strain failed to survive. For the complementation test, we constructed the NMV3-COX16 plasmid, which expresses *cox16* under control of the *Gal1* promoter. On the Gal-medium, Δcox16 strains transformed with YEp51-Bax, pYX112-AtBI-1, and NMV3 (as a vector control; indicated as – cox16 in Fig. 1B) did not grow, however, those transformed with YEp51-Bax, pYX112-AtBI-1, and NMV3-COX16 showed recovered growth (Fig. 1B).

AtBI-1 protein has been localized at ER membranes in yeast and plant cells [3]. To confirm the presence of AtBI-1 protein in the Δcox16 mutants, pYX112-AtBI-GFP (AtBI-GFP) and