Letter to the Editor

Yeast mutants resistant to Bax lethality reveal distinct vacuolar and mitochondrial alterations

Cell Death and Differentiation (2004) 11, 946–948. doi:10.1038/sj.cdd.4401422

Published online 19 March 2004

Dear Editor,

The Bcl-2 family of proteins is at the core of the metazoan cell death machinery. Bax, a proapoptotic member of this family, is thought to regulate critical control points of cell death in the endoplasmic reticulum (ER) and mitochondria.¹ In mammalian cells, Bax normally resides in the cytoplasm, and only upon apoptotic stimulation does the protein translocate and insert into the mitochondrial membrane and the ER. The regulated insertion of Bax into the outer mitochondrial membrane elicits a number of changes in mitochondrial physiology that include membrane depolarization and the release of apoptosis-promoting factors, such as cytochrome *c*.²

Despite the fact that no Bcl-2 family member exists in *Saccharomyces cerevisiae*, cell death caused by Bax or Bak appears relevant to its function in a physiological setting. Bax-expressing yeast cells exhibited depletion of intracellular glutathione levels, transient increases in reactive oxygen species, alterations of the mitochondrial membrane potential, and mitochondrial lipid oxidation.^{3,4} The antiapoptotic Bcl-2 protein can rescue yeast from Bax lethality, while mutants of Bcl-2 and Bcl-X_L that fail to protect mammalian cells from Bax-induced cell death are also inactive in yeast. Moreover, mutations in Bax and Bak that abolish their function in mammalian cells, also render these proteins inactive in yeast.⁵

The whole family of Bcl-2 proteins appears to have an impact on survival pathways in heterologous systems that do not contain endogenous Bcl-2 members. Plants expressing Bcl-2 and Bcl-X_L or CED-9 displayed resistance to several necrotrophic fungal pathogens and to a necrogenic virus.^{6–8} In yeast, Bcl-2 and CED-9 both inhibited oxidative stress-induced PCD.⁹ Conversely, Bax-expression in plants caused localized tissue collapse in a manner resembling the hypersensitive response, a PCD response of plants in defense against pathogens.¹⁰

To further understand the nature of the interactions of Bax with the yeast intracellular environment in eliciting cell death, we undertook the generation and characterization of a set of methane sulfonic acid ethyl ester (EMS)-mutagenized yeast strains that are tolerant to Bax expression. One of them was used in a genetic screen to isolate yeast genes restoring Bax sensitivity. The results presented show the presence of distinct pathways of cell damage involving the mitochondria and the vacuole.

To identify *S. cerevisiae* mutants that would retain viability in the presence of the LexA-Bax protein, EGY48 cells carrying the pGILDA/Bax plasmid and a lacZ reporter plasmid

(pJK101), both under the control of the galactose promoter, were treated with EMS and plated on galactose-based restrictive media. A total of 70 blue colonies were selected for further characterization. Blue-white selection facilitates identification of false-positive clones that carry a mutation affecting expression from the galactose promoter. In all, 12 mutant strains that reproducibly exhibited tolerance to Bax lethality were isolated (Figure 1a). The involvement of mitochondria in Bax-induced cell death has been well documented. To assess whether Bax tolerance was due to respiratory inhibition, the strains were streaked on YPG, a medium that contains glycerol as carbon source. All mutants were able to grow, exhibiting respiratory competence (data not shown).

It has recently been reported that progression of Bax-induced cell death in yeast was inhibited by a defect in vacuole formation.¹¹ This was attributed to aberrant membrane traffic and defects in autophagic degradation. Autophagy is a conserved process of controlled self-digestion involving the vacuole/lysosome encountered in yeast, plant and animal cells. It is dramatically enhanced under conditions of stress or nitrogen starvation.¹² To assess potential alterations in the autophagic process in the Bax-resistant mutants, cells were incubated in nitrogen-deprived media, aliquots were removed at regular intervals for a period of 7 days, and enumerated by plating on nutrient-rich plates. Wild-type EGY48 cells exhibited a large drop in cell viability on the third day of incubation. The viable cell count, subsequently, recovered and stabilized to approximately 60% viability. Most of the isolated mutants (R2, R4, R5, R7, R8, R13, R21, R25) exhibited an attenuated drop in viability on the third day, which ranged between 30 and 40%, compared to 5% for the parental wild-type strain. Mutant strain R6 exhibited a rapid drop in viability, but unlike the rest of the strains never recovered in cell count (Figure 1b). These results suggest that nutrient recycling processes associated with autophagy have been compromised for most mutants.

Mitochondrial functionality was probed with the Mitotracker fluorophore, which localizes in the mitochondria as a consequence of the mitochondrial membrane potential ($\Delta\Psi_m$), while mitochondrial morphology was assayed using a GFP (fused to the mitochondrial presequence of subunit 9 of the F₀-ATPase from *Neurospora crassa*) which translocates to the mitochondrial matrix and allows microscopic visualization of the organellar changes in size and shape.¹³ Expression

of Bax in wild-type cells caused a decrease in the number of observed mitochondria per cell, as well as a swollen appearance, suggesting fusion of the organelles. This coincided with a hyperpolarization of the mitochondrial membrane, as it has previously been observed by FACS analysis.⁴ Prolonged expression of Bax caused loss of Mitotracker stain and $\Delta\Psi_m$.³ In contrast, most mutant strains did not exhibit such alterations upon Bax expression (no hyperpolarization and subsequent $\Delta\Psi_m$ collapse was evident). However, three strains (R5, R13, R21) failed to localize the mitochondria-targeted GFP, irrespective of Bax expression (Figure 1c). Since all cells stained with Mitotracker, indicating functional mitochondria, the block in GFP localization could be attributed to defects in the mitochondria protein-import machinery. Recent studies indicate that no common mechanism exists for import of all mitochondrial precursor proteins, but a multitude of mechanisms exist for the recognition and translocation of the various precursor proteins.¹⁴ The defects observed in mitochondrial import in the three Bax-tolerant yeast mutants could either be due to direct involvement of a component of the import machinery in the insertion of Bax on the outer mitochondrial membrane, or to the selective inhibition of import of mitochondrial proteins which are important for Bax insertion.

Vacuolar morphology and function were assayed using two fluorescent dyes, MDY-64, a bright lipophilic dye that preferentially labels the vacuolar membrane, and CMAC, an intense blue dye that accumulates in the lumen of acidified vacuoles in living cells. Upon expression of Bax, wild-type cells exhibit disrupted vacuoles that are eventually lost (Figure 1C). Most mutant strains exhibit minor alterations in vacuolar morphology which is not affected by Bax expression. Strain R6 revealed distinct alterations in vacuolar morphology, and strain R20 exhibited changes in both GFP localization and vacuolar morphology. Overall, the organellar alterations observed in the mutant strains suggest distinct pathways affected by Bax expression.

The loss-of-function haploid strain R6 was selected for a complementation study due to its pronounced vacuolar alterations and the inability of the cells to recover during nitrogen starvation, which are effects less characterized compared to the mitochondrial changes. A yeast genomic library was used to transform R6 cells carrying the pGILDA/Bax plasmid and screened for genes that would restore Bax sensitivity. Six library clones were isolated, and examined to confirm that did not exhibit any toxicity when expressed in EGY48 and R6 cells in the absence of Bax. Sequence analysis identified the genes TSA2, SOP4, ROM2, NUP42/RIP1, STE5 and DSS4. Three of the six isolates appear to be involved in protein transport. SOP4 is involved in regulation of

Figure 1 (a) In all, 12 EMS-mutagenized yeast strains exhibit tolerance to LexA-Bax expression when plated on galactose-based media inducing LexA-Bax expression. (b) Cells were incubated in medium lacking a nitrogen source for 7 days. Live cells were enumerated at regular intervals by plating aliquots in nutrient-rich media. (c) Mitochondrial morphology was examined by expressing a GFP localizing in the mitochondrial matrix (first column); $\Delta\Psi_m$ was visualized using the Mitotracker fluorophore (second column); vacuolar membranes were stained with the MDY-64 fluorophore (third column). (d) R6 cells harboring the pGILDA/Bax plasmid and the library clones isolated from the genetic screen, exhibit only vacuolar, but not mitochondrial alterations

quality control and transport of the Pma1 protein from the ER to Golgi.¹⁵ ROM2 is a GDP–GTP exchange protein for the Rho1 small GTP-binding protein. It is involved in cell-wall biogenesis, organization, and small GTPase signal transduction. Its null mutant exhibits temperature-sensitive growth defects.¹⁶ DSS4 is a guanyl nucleotide exchange factor in the secretory pathway. It is implicated in intracellular transport, Golgi organization, cell growth and division.¹⁷ TSA2 encodes a thioredoxin peroxidase involved in the regulation of redox homeostasis. It has a protective role in cellular defense against heat shock.¹⁸ The protein has been identified as a component of several complexes that also include proteins involved in ER to Golgi traffic.¹⁹ NUP42/RIP1 is a structural protein found in the nuclear pore and is essential for the export of heat-shock mRNAs following stress.²⁰ STE5 is a known adaptor protein involved in the pheromone response signal transduction pathway. Interestingly, it has recently been shown that pheromone induces PCD in yeast.²¹

The effects of the isolated clones to the R6 Bax-tolerant strain were examined in the presence and absence of the LexA-Bax protein. R6 cells carrying only the library isolates did not exhibit any major alterations in the morphology of the mitochondria or the vacuole (data not shown), while cells that carry the pGILDA/Bax plasmid together with the YLB clones exhibit a further disruption of the vacuole but no changes in mitochondrial morphology (Figure 1d). These results indicate that the alterations conferring Bax sensitivity in the R6 strain do not involve the mitochondrial pathway.

Being the final compartment in converging transport pathways, the vacuole receives endocytic traffic from the cell surface, biosynthetic traffic from the Golgi, and material from the cytoplasm through the process of autophagy.²² The defects in vacuolar function seen in the mutants are probably caused by aberrant membrane trafficking, which originates in the ER and the Golgi, located upstream of the mitochondrial translocation pathway in most cases. Interestingly, the ARL1 gene, which upon mutation also confers Bax tolerance, belongs to a family of highly conserved guanine nucleotide-binding proteins that participate in vesicular transport from the Golgi to the ER.²³ Our results show the participation of the

yeast protein machinery in the mode by which Bax exerts its lethal effects. They identify the presence of two distinct pathways that are affected, which may overlap at the initial steps, since the genes restoring Bax sensitivity in the R6 mutant disrupt vacuolar biogenesis but not the mitochondria. This raises the issue of whether Bax in its natural context can also disrupt steps upstream of the mitochondrial translocation from the ER, which can lead to lysosome disruption, or if a different mechanism exists in mammalian cells that would exclude such a possibility at all times.

S Belhocine¹, C Mbithe¹, I Dimitrova¹, S C Kampranis¹ and A M Makris^{*1}

¹ Mediterranean Agronomic Institute of Chania, Chania 73100, Greece

* Corresponding author: A M Makris, Tel.: + 30 28210 35050;

Fax: + 30 28210 35001; E-mail: antonios@maich.gr

1. Scorrano L *et al.* (2003) *Science* 300: 135–139
2. Zamzami N *et al.* (1996) *J. Exp. Med.* 183: 1533–1544
3. Kampranis SC *et al.* (2000) *J. Biol. Chem.* 275: 29207–29216
4. Gross A *et al.* (2000) *Mol. Cell. Biol.* 20: 3125–3136
5. Zha H *et al.* (1996) *Mol. Cell. Biol.* 16: 6494–6508
6. Dickman MB *et al.* (2001) *Proc. Natl. Acad. Sci. USA* 98: 6957–6962
7. del Pozo O and Lam E (2003) *Mol. Plant Microbe Interact.* 16: 485–494
8. Mitsuhashi I *et al.* (1999) *Curr. Biol.* 9: 775–778
9. Chen SR *et al.* (2003) *Free Radic. Biol. Med.* 34: 1315–1325
10. Lacomme C and Santa Cruz S (1999) *Proc. Natl. Acad. Sci. USA* 96: 7956–7961
11. Abudugupur A. *et al.* (2002) *Cell Death Differ.* 9: 158–168
12. Knop M *et al.* (1993) *Curr. Opin. Cell Biol.* 5: 990–996
13. Westermann B and Neupert W (2000) *Yeast* 16: 1421–1427
14. Pfanner N and Wiedemann N (2002) *Curr. Opin. Cell Biol.* 14: 400–411
15. Luo WJ *et al.* (2002) *Traffic* 3: 730–739
16. Ozaki K *et al.* (1996) *Embo J.* 15: 2196–2207
17. Moya M *et al.* (1993) *Nature* 361: 460–463
18. Lee SM and Park JW (1998) *Arch. Biochem. Biophys.* 359: 99–106
19. Ho Y *et al.* (2002) *Nature* 415: 180–183
20. Vainberg IE *et al.* (2000) *Mol. Cell Biol.* 20: 3996–4005
21. Severin FF and Hyman AA (2002) *Curr. Biol.* 12: R233–R235
22. Conibear E and Stevens TH (2002) *Methods Enzymol.* 351: 408–432
23. Poon PP *et al.* (1999) *Embo J.* 18: 555–564