Evaluation of the Roles of Apoptosis, Autophagy, and Mitophagy in the Loss of Plating Efficiency Induced by Bax Expression in Yeast

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We found recently that, in yeast cells, the heterologous expression of Bax induces a loss of plating efficiency different from that induced by acute stress because it is associated with the maintenance of plasma membrane integrity (Camougrand, N., Grelaud-Coq, A., Marza, E., Priault, M., Bessoule, J. J., and Manon, S. (2003) Mol. Microbiol. 47, 495–506). Bax effects were neither dependent on the presence of the yeast metacaspase Yca1p and the apoptosis-inducing factor homolog nor associated with the appearance of typical apoptotic markers such as metacaspase activation, annexin V binding, and DNA cleavage. Yeast cells expressing Bax instead displayed autophagic features, including increased accumulation of Atg8p, activation of vacuolar alkaline phosphatase, and the presence of autophagosomes and autophagic bodies. However, the inactivation of autophagy did not prevent and actually slightly accelerated Bax-induced loss of plating efficiency. On the other hand, Bax expression induced a fragmentation of the mitochondrial network, which retained, however, some level of organization in wild-type cells. However, when expressed in cells inactivated for the gene UTH1, previously shown to be involved in mitophagy, Bax induced a complete disorganization of the mitochondrial network. Interestingly, although mitochondrialy targeted green fluorescent protein was slowly degraded in the wild-type strain, it remained unaffected in the mutant. Furthermore, the slow loss of plating efficiency in the mutant strain correlated with a loss of plasma membrane integrity. These data suggest that Bax-induced loss of growth capacity is associated with maintenance of plasma membrane integrity dependent on UTH1, suggesting that selective degradation of altered mitochondria is required for a regulated loss of growth capacity.

Recent data have provided evidence for the existence of different forms of programmed cell death. In addition to the long time established existence of apoptosis in the worm Caenorhabditis elegans (1), apoptotic markers associated with loss of viability have been observed in various organisms, including Dictyostelium discoideum (2), Leishmania sp. (3), and Saccharomyces cerevisiae (4–7). However, apoptosis might not be the only process leading to programmed cell death. Autophagy, an intracellular degradation process normally responsible for cell survival under starvation conditions, has been observed frequently as accompanying the loss of growth capacity in mammalian cells (8–11). Moreover, in mammalian cells, data have suggested a co-regulation of apoptosis and autophagy, which could both participate in cell death (12, 13). These observations support the occurrence of cell death associated with autophagy, although certain authors consider autophagy to be a cellular attempt to survive apoptotic cell death (10, 14–17). The simpler eukaryotic models D. discoideum and Podospora anserina have provided the opportunity to demonstrate that autophagy inactivation stimulates rather than inhibits the kinetics of physiological programmed cell death (18, 19). It has therefore been proposed that, if a low level of autophagy is actually responsible for better survival (including apoptosis-deficient mammalian cells), an abnormally high level of autophagy might be responsible for cell death (9, 13, 20). Up to now, the deregulation events responsible for uncontrolled autophagic activity have not been characterized. The actual role of autophagy in programmed cell death thus remains unclear, and investigators have proposed to describe “programmed cell death with autophagy” rather than “autophagic programmed cell death” (21, 22).

The yeast S. cerevisiae is the organism in which autophagy has been best genetically characterized (23–25). Of note, proteins involved in autophagy (encoded by ATG genes) are widely conserved in mammals and plants, suggesting that their function is part of the basic processes of life (26, 27). This situation contrasts with apoptosis, for which key regulators, including proteins of the Bcl-2 family, are lacking in yeast cells. This
absence has made yeast a useful tool to investigate the function of some of these proteins because, when expressed in yeast, they conserve their molecular effects at the level of mitochondria, making it possible to test their function independently of each other (28). This utilization of yeast as a “living test tube” has provided new information concerning the molecular aspects of pro-apoptotic Bax protein function, further confirmed by experiments in mammalian cells (29–32). In yeast, the pro-apoptotic Bax protein can be translocated to the outer mitochondrial membrane to form a high conductance channel (33) and to allow cytochrome c release (32, 34). Other Bcl-2 family members such as anti-apoptotic Bcl-xL and BH3 (Bcl-2 homology 3) domain-only truncated Bid also retain their regulatory effect over Bax function (29, 32, 35–37).

The expression of the human pro-apoptotic Bax protein in yeast induces growth arrest and loss of plating efficiency (35, 36). This observation supports the hypothesis that a primitive form of apoptosis exists in yeast (38–40). However, the correlation between Bax-induced release of cytochrome c and Bax-induced loss of plating efficiency is unclear. Indeed, the expression of Bax-c-myc induces a loss of plating efficiency in a cytochrome c-less mutant, although more slowly than in the parental strain (35) and in a strain in which cytochrome c had been replaced with the larger and unreleased cytochrome c-GFP4 fusion protein (41). For this reason, the existence of Bax-induced cytochrome c release is not sufficient to assert that Bax induces apoptosis in yeast, so further evidence is needed.

We previously identified Uth1p, a protein that localizes to the outer mitochondrial membrane and that participates in selective mitochondrial autophagy (also called mitophagy) (42). One of the phenotypes of a mutant strain carrying an inactivation of UTH1 is a delayed loss of plating efficiency following Bax expression (43). This suggests that autophagy (and more precisely, mitophagy) is part of the Bax effects in yeast. In this study, our aim was to investigate the role(s) of apoptosis, autophagy, and mitophagy as a consequence of Bax expression in yeast.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**—The construction of plasmids pCM189/Bax-c-myc and pCM184/Bax-c-myc, expressing c-myc-tagged human Bax under the control of a Tet-Off promoter (repressed by the addition of doxycycline), has been described previously (35). The mutant Δuth1 was constructed from the wild-type strain W303-1A by disrupting UTH1 with TRP1 (43). Mutants Δaca1, Δafj1, and Δatg5 (constructed from the wild-type strain BY4742 by disrupting the respective genes with kanMX4) and mutants Δatg4, Δatg5, Δatg7, Δatg8, Δatg15, and Δatg16 (similarly constructed from BY4741) were provided by EUROSCARF. The Δatg5 mutant was also constructed by transformation of W303-1A with the PCR-amplified atg5::kanMX4 cassette and selection for Genetecin resistance. Bax repression was obtained by including 10 μg/ml doxycycline in the appropriate culture medium. Bax expression was triggered by three washes, followed by resuspension in fresh medium without doxycycline. Western blotting showed that the Bax expression level reached a plateau after ~8 h, followed by immediate growth arrest and loss of plating efficiency (see Fig. 1). The LIR43 plasmid pCBL-GFP, encoding GFP fused to the mitochondrial presequence of citrate synthase under the control of the GAL1/10 promoter, was described by Okamoto et al. (44). Its HIS3 counterpart pNC-GFP was constructed by swapping the PCR-amplified GAL1/10-mtGFP cassette from pCBL-GFP with pRS313. Replacement of PHO8 with pho8Δ60 was done by transforming the cells with a HindIII fragment from plasmid pTN9 and by selecting the colonies as described by Noda et al. (45). The construction of plasmids pDP34/Bcl-2 and pFL39/Bcl-xL, expressing full-length human Bcl-2 and Bcl-xL, respectively, under the control of the GAL1/10 promoter, has been described previously (36, 46).

**Culture Media**—Cells were grown aerobically in YNB medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 0.1% potassium phosphate, 0.2% Drop-Mix, and 0.01% auxotrophic requirements, pH 5.5) supplemented with 2% Dl-lactate as a carbon source. For mtGFP expression, 0.5% galactose was added to the medium overnight. Doxycycline (sulubilized in ethanol; Sigma) was added at 10 μg/ml except where indicated. The nitrogen starvation medium was deprived of amino acids and ammonium sulfate.

**Plating Efficiency**—Cells were routinely grown in the presence of 10 μg/ml doxycycline for Bax repression. At time 0, they were harvested, washed three times with water, and resuspended at a cell concentration of 10⁶ cells/ml in fresh medium without doxycycline (to induce Bax expression). They were counted at different time intervals and plated onto YPD plates (500 cells/plate) supplemented with doxycycline to repress Bax expression. The number of colonies was counted after 48 h at 28 °C. Alternatively, serial dilutions were plated, and the number of living cells in the culture was estimated by counting the colonies that had grown after 48 h. Data are presented as the percentage of living cells at time t divided by living cells at time 0.

**Flow Cytometry**—Caspase activity was measured on whole cells with the CaspACE™ kit (Promega Corp.) as described by Mado et al. (6). Labeling with propidium iodide (PI) was done by incubating the cells (5 × 10⁶ cells/ml) in 50 mM citrate/ phosphate buffer, pH 7.2, containing 1 μg/ml PI for 15 min in the dark. Annexin V/PI co-labeling was done with the ApoAlert™ kit (Clontech) on intact cells or zymolyase-treated cells (spheroplasts) as described by Mado et al. (5). TUNEL assay was performed on cells permeabilized and fixed in 70% ethanol overnight at 4 °C with a tetramethylrhodamine red in situ cell death detection kit (Roche Applied Science).

Fluorescence was measured in a Galaxy flow cytometer (Partec). At least 30,000 cells were counted per run, and all experiments were reproduced independently at least five times.

**Plasma Membrane Permeability**—In addition to the PI assay (see above), plasma membrane permeability was measured as the capacity of the cells to accumulate erythrosin B (47). Cells were incubated at 28 °C for 45 min in the presence of 75 μM...
erythrosin B and observed under a light microscope. Cells with altered plasma membrane properties accumulated erythrosin B and were pink, whereas unaltered cells remained white. Parallel experiments with PI accumulation and erythrosin B staining gave similar results.

In Vitro Assays—DEVDase activity was measured in cell extracts (prepared by incubating zymolyase-treated cells with 0.1% Triton X-100 to solubilize cell membranes) using the EnzCheck™ caspase-3 kit (Molecular Probes) in a Xenius spectrophotometer (SAFAS S.A.) according to the manufacturer’s instructions. Alkaline phosphatase activity was measured as described previously (42) in 1% toluene and 4% ethanol-treated yeast extracts (prepared as described previously (42), separated by SDS-PAGE, and blotted on ProBlott™ (Applied Biosystems) with primary antibodies directed against yeast phosphoglycerate kinase (mouse monoclonal, 1:5000; Molecular Probes), human Bax (clone N20, rabbit polyclonal, 1:5000; Santa Cruz Biotechnology, Inc.), yeast Atg8p (goat polyclonal, 1:250; Santa Cruz Biotechnology, Inc.), human Bcl-2 (rabbit polyclonal, 1:1000; Calbiochem), and human Bcl-x (rabbit polyclonal, 1:1000; Santa Cruz Biotechnology, Inc.). Peroxidase-coupled secondary antibodies were from Jackson ImmunoResearch Laboratories and were used at a 1:5000 dilution. Peroxidase activity was revealed with ECL Plus™ (Amersham Biosciences). Different exposure times were used for quantification of the blots with ImageJ software.

Fluorescence and Electron Microscopy—When used, the yeast vacuolar membrane autofluorescent probe FM4-64 (N-(3-triethylammoniumpropyl)4-(p-diethylaminophenylhexatrienyl)pyridinium dibromide; 40 μM) was added 4 h prior to observation. Cells were observed with a Leica Microsystems DM-LB epifluorescence microscope. For electron microscopy, yeast pellets were placed on the surface of Formvar-coated copper electron microscope grids (400 mesh). Each loop was quickly submerged in liquid propane (−180 °C) and transferred to a precooled solution of 4% osmium tetroxide in dry acetone at −82 °C for 48 h for substitution/fixation. Samples were gradually warmed up to room temperature and washed with dry acetone. Specimens were stained for 1 h in 1% uranyl acetate in acetone at 4 °C, rinsed, and infiltrated with Araldite (epoxy resin; Fluka). Ultrathin sections were stained with lead citrate and viewed with a Philips Tecnai 12 Biotwin (120 kV) electron microscope.

RESULTS

Bax-induced Loss of Plating Efficiency in Yeast Is Not Associated with Apoptotic Markers—We reported previously that, in addition to immediate growth inhibition, the expression of Bax in yeast is followed by a loss of plating efficiency, i.e., Bax-expressing cells are not able to restart growth even after Bax expression has been stopped (Fig. 1, B and C) (35, 36).

This loss of growth capacity occurs without any alteration of the plasma membrane permeability properties as measured with the widely used DNA-binding probe PI, which penetrates altered cells only. In contrast with the loss of plating efficiency induced by ethanol treatment, heat shock, or acute oxidative stress (Fig. 2A), Bax-induced loss of plating efficiency was not associated with any increase in PI permeability, even after long Bax expression (Fig. 2B). These data suggest that Bax-induced loss of growth capacity is a regulated event, unlike the one induced by acute stress, which is associated with a dramatic loss of membrane integrity.

To investigate the putative roles of apoptosis in Bax effects, Bax was expressed in two strains with impairment of the apoptotic program: one lacking metacaspase (Δycal strain) and the other lacking the apoptosis-inducing factor homolog (Δaif1 strain). Of note, the identification of a metacaspase encoded by the gene YCA1, which is activated by different stimuli (6), remains the most compelling argument supporting the existence of a primitive form of apoptosis in yeast. The inactivation of YCA1 also leads to a decrease in the kinetics of the loss of growth capacity induced by hydrogen peroxide and other stimuli (6, 48–50).

Compared with wild-type cells, the absence of the metacaspase did not change the extent and kinetics of Bax-induced loss of plating efficiency (Fig. 3A), thus confirming data from a recent study (51). However, the absence of the apoptosis-induc-
Bax-induced Cell Death in Yeast

A

Control cells

60°C

ethanol 6%

H₂O₂ 100mM

+doxycycline (-bax)

4hrs

4hrs

14hrs

14hrs

26hrs

26hrs

Number of cells

PI fluorescence

B

-doxycycline (+bax)

FIGURE 2. Bax expression does not induce alterations of the plasma membrane. A, wild-type W303-1A cells were grown aerobically at 28 °C in YNB/lactate medium to 1 A₅₀₆₀₅₀ unit and treated as follows: 15-min incubation at 60 °C, addition of 6% (v/v) ethanol for 30 min at 28 °C, or addition of 100 mM H₂O₂ for 30 min at 28 °C. They were further washed, resuspended in fresh medium in the presence of 0.1 μg/ml PI, and incubated at 28 °C for 15 min in the dark before flow cytometry analysis. Data are representative of 10 independent experiments. The three treatments led to plating efficiencies below 10%. B, Bax expression was induced as described under “Experimental Procedures.” At the indicated times, PI permeability was measured as described for A. Bax expression led to plating efficiencies of 50 and 15% after 14 and 26 h, respectively (Fig. 1B). The slight increase in PI permeability observed in control cells was related to the entry of the cells in the stationary phase of growth (77).

Bax-induced Cell Death in Yeast

Aging factor homolog, encoded by the gene AIF1, stimulated rather than inhibited the kinetics of Bax-induced loss of plating efficiency (Fig. 3A). Measurement of DEVDase activity in cell extracts did not reveal any activity (data not shown). Measurement of caspase activity with the permeant substrate valyl-alanyl-aspartyl-(O-methyl)-fluoromethyl ketone-fluorescein isothiocyanate in whole cells showed a basal signal in control cells, which decreased following Bax expression (Fig. 3B). Measurement of phosphatidylserine exposure with annexin V was also done in both intact whole cells (Fig. 3C) and spheroplasts (data not shown): no annexin V-positive cells appeared in control or Bax-expressing cells. As positive controls, significant increases in both caspase activity and annexin V binding were found in aged cells (data not shown), as described by Laun et al. (52). Although no degradation of DNA following Bax expression could be evidenced by TUNEL assay, this degradation was observed in H₂O₂-treated cells (Fig. 3D). These data unequivocally show that typical characteristics of apoptosis cannot be evidenced in yeast following Bax expression under conditions in which Bax hampers the capacity of yeast to grow after Bax expression has been stopped. Finally, in the absence of Yca1p or Aif1p, Bax-induced loss of plating efficiency was still associated with maintenance of plasma membrane integrity (Fig. 3E), showing that the apoptotic program is not responsible for this maintenance.

Bax Expression Induces the Appearance of Autophagic Characteristics—We reported previously that the kinetics of loss of plating efficiency following Bax expression are slower in a yeast mutant inactivated for the gene LITH1 than in its wild-type counterpart (43). The product of this gene was shown to be involved in mitochondrial autophagy (42). Because a possible death function of autophagy has been suggested in mammalian cells unable to achieve apoptosis (12), we reasoned that autophagy might be involved in Bax-induced loss of growth capacity in yeast.

Implementation of autophagy involves the transcriptional regulation of several genes. For example, an increased amount of the ATG8 product is considered to be one of the typical markers of autophagy that have been observed following nitrogen starvation or rapamycin treatment (53). We found that Bax expression correlated with an increase in Atg8p (Fig. 4A, similar to that induced by nitrogen starvation (Fig. 4B, panel c)). Induction of autophagy upon Bax expression was further confirmed by the widely used alkaline phosphatase assay (45). Bax expression correlated with an increase in alkaline phosphatase activity, similar to that observed in nitrogen-starved cells (Fig. 4B, panel a), showing that Bax expression is accompanied by nonselective autophagy. The coexpression of the anti-apoptotic protein Bcl-xL or Bcl-2, which prevents Bax-induced loss of growth capacity in yeast (35), also prevented Bax-induced activation of alkaline phosphatase, but not the activation of alkaline phosphatase induced by the autophagy-inducing drug rapamycin (Fig. 4B, panel a). Both Bcl-2 and Bcl-xL expression actually prevented the Bax-induced increase in Atg8p expression (Fig. 4B, panel c), in accordance with the fact that they abolished all Bax effects in yeast (34–36). Treatment of cells with low concentrations of H₂O₂, (1 or 5 mM) or with 180 mM acetic acid has been identified as a typical inducer of yeast apoptosis (5, 54). These treatments did not trigger any activation of alkaline phosphatase (data not shown; for acetic acid, see Ref. 55), showing that yeast apoptosis is not associated with the activation of autophagy. Moreover, treatment with 100 mM H₂O₂, which induced a massive loss of membrane integrity (Fig. 2A), did not induce any activation of alkaline phosphatase (data not shown).

Electron microscopy also confirmed the presence of autophagy in Bax-expressing cells, viz. microautophagy (direct engulfment of cytosolic material by the vacuoles), macroautophagy (presence of autophagosomes), and the resulting presence of autophagic bodies within the vacuoles (Fig. 4C).

Given that autophagic characteristics appeared following Bax expression, we determined whether the inactivation of sev-
eral genes involved in autophagy altered the characteristics of Bax-induced loss of plating efficiency. None of the different mutants assayed exhibited a significantly better plating efficiency following Bax expression compared with the wild-type cells (Fig. 5A). However, two of them, \( \Delta \text{atg5} \) and \( \Delta \text{atg7} \), did exhibit a significantly lower plating efficiency. This was further confirmed for the well characterized \( \Delta \text{atg5} \) mutant by accumulating data from seven independent experiments performed at different times and with different levels of Bax expression obtained with 0, 0.25, or 0.5 \( \mu \text{g/ml doxycycline} \). For all times and all doxycycline concentrations, the plating efficiency in \( \Delta \text{atg5} \) cells was slightly but reproducibly lower than that in wild-type cells (Fig. 5B).

We also checked whether the inhibition of autophagy altered the properties of Bax-expressing cells, viz. their capacity to maintain their plasma membrane integrity. \( \text{ATG5} \) inactivation did not alter the behavior of the cells (Fig. 5C). Taken together, these data suggest that, although autophagy is activated following Bax expression, it is not responsible for the loss of plating efficiency. Rather unexpectedly, it play a marginal role in the capacity of the cells to maintain growth capacity.

\textbf{UTH1-dependent Mitophagy Is Required for Regulated Loss of Growth Capacity—} The disruption of \( \text{UTH1} \) delays Bax-induced loss of plating efficiency (43), indicating that the product of this gene likely participates in this process. Moreover, Uth1p was shown to be required for efficient mitophagy (42). Therefore, we reasoned that mitophagy might be involved in the process of Bax-induced loss of viability. To address this question, mitochondrial behavior was followed \textit{in vivo} in cells expressing mtGFP. Fragmentation of the mitochondrial network was observed following Bax expression (Fig. 6A). How-
ever, even after prolonged Bax expression, mitochondrial spots remained visible, and almost no diffuse GFP appeared within the vacuoles, contrasting with what was observed when autophagy was induced by nitrogen starvation. This was confirmed by the observation that Bax expression did not induce any colocalization of both mtGFP and the vacuolar probe FM4-64 (data not shown). As expected, the coexpression of the anti-apoptotic protein Bcl-xL, which prevents the mitochondrial localization of Bax (32, 35, 36), hampered the Bax-induced fragmentation of mitochondria (Fig. 6A).

The inhibition of autophagy caused by the inactivation of ATG5 did not modify the mitochondrial network fragmentation process (Fig. 6B). The inhibition of mitophagy caused by the inactivation of UTH1 did not prevent the fragmentation of mitochondria; however, unlike the wild-type and Δatg5 strains, mitochondria appeared disorganized, with an unordered localization of spots of different sizes and intensities (Fig. 6C). Quantification of the three types of mitochondria; organization clearly showed the difference between the Δuth1 strain and the wild-type and Δatg5 strains (Fig. 6D).

Flow cytometry of mtGFP was performed to evaluate the possible degradation of mitochondria. At the time of initiation of Bax expression (removal of doxycycline), the synthesis of mtGFP and the vacuolar probe FM4-64 (data not shown). As expected, the coexpression of the anti-apoptotic protein Bcl-xL, which prevents the mitochondrial localization of Bax (32, 35, 36), hampered the Bax-induced fragmentation of mitochondria (Fig. 6A).

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cells decreased. In Δuth1, both populations remained roughly constant, suggesting that the absence of UTH1 and the resulting inhibition of mitophagy prevent the degradation of mitochondria altered by Bax expression, leading to disorganization of the chondriome. 

The altered behavior of mitochondrial degradation in the Δuth1 mutant was associated with an alteration of the characteristics of Bax-induced loss of viability. Not only was this loss delayed (43), but, in contrast to both wild-type and Δatg5 strains, Δuth1 cells were not able to maintain plasma membrane integrity; after 14 h of Bax expression, a significant increase in PI-permeable cells was observed in Bax-expressing Δuth1 cells, but not in wild-type and Δatg5 cells (Fig. 8A). The difference was even more spectacular after prolonged Bax expression (30 and 44 h); after 44 h of Bax expression, >90% of the cells from both wild-type and Δuth1 strains had lost growth capacity, and <15% of the wild-type cells and >90% of the Δuth1 cells were PI-permeable (Fig. 8B).

DISCUSSION

Since the first demonstration that fusion proteins of Bax used in a two-hybrid system are able to kill yeast (56, 57), the heterologous expression of Bcl-2 family members in yeast has been used as a tool to understand several molecular aspects of the function of these proteins (28). Despite several discrepancies in the literature caused mainly by the utilization of different variants of Bax, parallel experiments done in yeast and mammalian cells showed that the effects of Bax on mitochondria (addressing insertion, high conductance channel formation, and the capacity to promote cytochrome c release) are conserved in yeast (29–33).

However, few studies addressed the nature of Bax-induced loss of plating efficiency in yeast. The possible existence of an ancestral apoptosis-like program in yeast led several investigators to propose that Bax-induced loss of growth capacity is caused by apoptotic cell death (39, 40). In contrast with different acute stresses, Bax–induced loss of plating efficiency was not related to any alteration of plasma membrane properties (Fig. 2), suggesting that it is a form of “clean” cell death that allows cells to die without releasing their content into the medium. The identification of two molecular components of the yeast apoptotic program, the metacaspase Yca1p/Mca1p (6) and the apoptosis-inducing factor homolog Aif1p (7), offered the possibility to test this hypothesis. The data reported here show that this program is not involved in Bax-induced yeast cell death because the inactivation of these proteins did not modify its characteristics. Furthermore, typical markers of yeast apoptosis such as the appearance of DEVDase activity, annexin V binding, and DNA fragmentation did not occur following Bax expression (Fig. 3).

On the other hand, Bax expression was also associated with the appearance of autophagic characteristics. In accordance with data from different groups, this suggests that Bax expression in yeast interferes with processes linked to vesicular trafficking (58–60). Recent data have suggested that, in the absence of an active apoptotic program, autophagy might be an “alternative” mode of cell death in mammalian cells (12). Autophagy has often been observed simultaneously with apoptosis, but data suggest that it might correspond to a survival attempt in apoptotic cells because the inhibition of autophagy induces a stimulation of cell death kinetics (17). Processes of cell death with autophagy have been reported previously in two models of physiological cell death: DIF-induced death in D. discoideum (61) and genetic incompatibility in P. anserina (62). In these two models, death is clearly associated with autophagic markers,
but the inhibition of autophagy associated with the inactivation of an ATG gene does not prevent and actually accelerates cell death (18, 19). A similar situation emerged from our study, in which the inactivation of ATG5 or ATG7 accelerated Bax-induced loss of plating efficiency. However, the inactivation of several other ATG genes did not appear to have any significant effect. It should be noted, however, that the inactivation of only one gene (ATG1) was reported previously (18, 19).

In a previous study, a genetic screen to identify Bax-resistant yeast mutants allowed the identification of UTH1 (43). The further characterization of this gene indicated its requirement for mitochondrial autophagy (mitophagy), but not for cytosolic protein autophagy (42). It is now well established that the expression of c-myc-tagged Bax in yeast results in a mitochondrial localization (28, 29, 35, 36), with subsequent alterations of the outer mitochondrial membrane. The absence of UTH1 does not change this mitochondrial localization of Bax-c-myc (43). It has been hypothesized that the putative function of mitophagy would be the elimination of altered mitochondria (20, 63, 64). Furthermore, it has been shown that alteration of mitochondrial biogenesis is a trigger of autophagy (65). We therefore reasoned that Bax-induced alterations of mitochondria might be a trigger of mitophagy.

Conversely, it might be envisaged that moderate defects in mitochondrial biogenesis would be responsible for the altered response of Δuth1 to Bax expression. It should be noted, however, that the absence of Uth1p leads to a fully respiratory-competent phenotype and that only detailed bioenergetic analysis illustrated a 25% decrease in respiratory enzymes (66). Also, the absence of Uth1p does not prevent Bax-c-myc-induced alterations of mitochondria, viz. the permeabilization of the outer mitochondrial membrane (43). Furthermore, mutants of mitochondrial proteins that exhibit much stronger deficiencies in mitochondrial functions compared with Δuth1 have been tested; none of them shows a response to Bax significantly different from that of the wild-type strain (35, 67, 68). Further investigations will be required to draw a link between mitochondrial biogenesis and mitophagy.

The fragmentation of the mitochondrial network is an early step of mammalian apoptosis, although its actual function, if any, remains unclear (69). It has also been reported that the
mitochondrial fission machinery is involved in yeast programmed cell death (70). We have reported here that this process of mitochondrial fission also follows Bax expression. However, the mitochondrial network remained relatively organized in the form of spots that did not appear to be randomly distributed within the cells. Conversely, in the \( \Delta \text{uth1} \) mutant, fragmented mitochondria were disorganized, with a random distribution of spots of different sizes. Furthermore, the degradation of mtGFP observed in wild-type cells (and in \( \Delta \text{atg5} \) cells) did not appear in \( \Delta \text{uth1} \) cells, suggesting that the inactivation of mitophagy in the \( \Delta \text{uth1} \) strain is responsible for the disorganization of the mitochondrial network. This had a major consequence on the nature of the loss of growth capacity because, unlike wild-type and \( \Delta \text{atg5} \) cells, \( \Delta \text{uth1} \) cells were not able to maintain the integrity of the plasma membrane; the slow loss of plating efficiency observed in \( \Delta \text{uth1} \) cells thus resembled a necrotic form of cell death such as that observed following acute stress. It should be noted that the apparent resistance of \( \Delta \text{uth1} \) to the effects of Bax expression (i.e. the slower kinetics of the loss of growth capacity) was actually caused by the fact that this necrosis-like form of cell death in \( \Delta \text{uth1} \) was slower than the regulated loss of growth capacity in the wild-type and \( \Delta \text{atg5} \) strains.

Base on these data, mitophagy is clearly not the cause of Bax-induced loss of growth capacity, but is required for a rapid and regulated form of cell death (by opposition to the necrotic form of cell death observed in the \( \Delta \text{uth1} \) mutant) (Fig. 9). General autophagy, dependent on \( \text{ATG5} \), also appears to be activated as a protective phenomenon, but its function seems to be less crucial and modifies the kinetics of cell death only, not its nature.

This study raises several questions about the nature of the mammal-like apoptotic program in yeast. Bax is a major player in mammalian apoptosis because its absence strongly impairs the process, and the double inactivation of Bax and its closely related homolog Bak completely blocks apoptosis (71). Although human Bax retains its most important function in yeast, i.e. permeabilization of the outer mitochondrial membrane to cytochrome c, it is not, however, able to induce the yeast apoptosis-like program. However, Bax is still able to induce a "regulated" form of cell death because of the activation of autophagy and, more particularly, of \( \text{UTH1} \)-dependent mito-

![FIGURE 7. Bax induces mitophagy-dependent mtGFP degradation.](image)
Bax-induced Cell Death in Yeast

Recent reports have questioned the involvement of the yeast apoptotic program in several forms of programmed cell death, viz. the role of reactive oxygen species (72) and of the metacaspase Yca1p (73). Furthermore, the proposal that apoptosis is required for the response to α-mating factor (74) has been discussed rigorously (75). Data reported on non-animal organisms support the existence of a much wider variety of programmed cell death than the apoptotic program alone in mammalian cells (21, 76), and it seems that each organism has developed its own strategy for a clean cell death, when needed. Even when considering mammalian cells, the concept that programmed cell death is limited to apoptosis has been re-evaluated because autophagy, necrosis, and probably still unknown processes can participate in programmed cell death (21). This study supports the view that activation of mitophagy as a process that protects against mitochondrial alterations is a means to convert a necrotic form of death into a regulated form of death.

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REFERENCES

1. Hengartner, M. O., and Horvitz, H. R. (1994) Curr. Opin. Genet. Dev. 4, 581–586
2. Arnoult, D., Tatischeff, I., Estaquier, J., Girard, M., Sureau, F., Tissier, J. P., Grodet, A., Dellinger, M., Traincard, F., Kahn, A., Ameisen, J. C., and Petit, P. X. (2001) Mol. Biol. Cell 12, 3016–3030
3. Arnoult, D., Akard, K., Grodet, A., Petit, P. X., Estaquier, J., and Ameisen, J. C. (2002) Cell Death Differ. 9, 65–81
4. Madeo, F., Frohlich, E., and Frohlich, K. U. (1997) J. Cell Biol. 139, 729–734
5. Madeo, F., Frohlich, E., Ligr, M., Grey, M., Sigrist, S. J., Wolf, D. H., and Frohlich, K. U. (1999) J. Cell Biol. 145, 757–767
6. Madeo, F., Herker, E., Maldener, C., Wissing, S., Lachelt, S., Herlan, M., Fehr, M., Lauber, K., Sigrist, S. J., Wesselborg, S., and Frohlich, K. U. (2002) Mol. Cell 9, 911–917
7. Wissing, S., Ludovico, P., Herker, E., Buttner, S., Engelhardt, S. M., Decker, T., Link, A., Proksch, A., Rodrigues, F., Corte-Real, M., Frohlich, K. U., Manns, J., Cande, C., Sigrist, S. J., Kroemer, G., and Madeo, F. (2004) J. Cell Biol. 166, 969–974
8. Edinger, A. L., and Thompson, C. B. (2004) Curr. Opin. Cell Biol. 16, 663–669
9. Levine, B., and Yuan, J. (2005) J. Clin. Investig. 115, 2679–2688
10. Codogno, P., and Meijer, A. J. (2005) Cell Death Differ. 12, Suppl. 2, 1509–1518
11. Tsujimoto, Y., and Shimizu, S. (2005) Cell Death Differ. 12, Suppl. 2, 1528–1534
12. Shimizu, S., Kanaseki, T., Mizushima, N., Mizuta, T., Arakawa-Kobayashi, S., Thompson, C. B., and Tsujimoto, Y. (2004) Nat. Cell Biol. 6, 1221–1228
13. Pattingre, S., Tassa, A., Qu, X., Garutti, R., Liang, X. H., Mizushima, N., Park, M., Schneider, M. D., and Levine, B. (2005) Cell 122, 927–939
14. Petiot, A., Ogier-Denis, E., Blommaart, E. F., Meijer, A. J., and Codogno, P. (2000) J. Biol. Chem. 275, 992–998
15. Arico, S., Petiot, A., Bauvy, C., Dubbelhuis, P. F., Meijer, A. J., Codogno, P., and Ogier-Denis, E. (2001) J. Biol. Chem. 276, 35243–35246
16. Meijer, A. J., and Codogno, P. (2004) Int. J. Biochem. Cell Biol. 36, 2445–2462
17. Lavieu, G., Scarlatti, F., Sala, G., Carpentier, S., Levade, T., Ghidoni, R., Botti, I., and Codogno, P. (2006) J. Biol. Chem. 281, 8518–8527
18. Kosta, A., Roisin-Bouffay, C., Luciani, M. F., Otto, G. P., Kessin, R. H., and Golstein, P. (2004) J. Biol. Chem. 279, 48404–48409
19. Pinan-Lucarré, B., Balguerie, A., and Clavé, C. (2005) Eukaryot. Cell 4, 1765–1774
