ENDOGENOUS PROGRAMMED CELL DEATH IN **SACCHAROMYCES CEREVISIAE**

DECLARATION

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“Dissertação apresentada à Escola de Ciências da Universidade do Minho para prestação de provas de Doutoramento em Ciências.”

AGRADECIMENTOS

O trabalho de investigação desenvolvido e apresentado nesta tese de doutoramento foi realizado no Departamento de Biologia da Universidade do Minho, no laboratório de Imunobiologia do Instituto de Biologia Molecular e Celular (IBMC) da Universidade do Porto e no Department of Biological Sciences of Colombia University em Nova Iorque. À Professora Doutora Célia Pais e aos Professores Doutores A. Quintanilha e A. Tzagoloff na qualidade de responsáveis pelos respectivos laboratórios agradeço o apoio e colaboração concedidos no desenvolvimento do trabalho.

À Professora Doutora Manuela Côrte-Real, quero expressar o meu profundo agradecimento pela orientação e constante estímulo com que me acompanhou ao longo destes anos. O meu reconhecimento pela sua amizade e confiança.

Ao"Professor", Doutor Manuel Teixeira da Silva, quero expressar o meu profundo agradecimento pela sua amizade, pela disponibilidade pessoal e pelo afecto e carinho que me dispensou. A forma como me acolheu e encorajou, inspirou e enriqueceu o trabalho científico de forma inestimável. Obrigada por tudo.

À Professora Doutora Cecília Leão quero agradecer, muito em especial, a amizade, o carinho e a confiança que sempre me dedicou.

Ao Professor Doutor Enrique O'Connor gostaria de agradecer a sua amizade e disponibilidade. Apesar de Valencia ser tão longe, muito obrigada.

I would like to thank Doctor Frank Madeo for his helpful suggestions, discussion and encouragement.

Ao Professor Doutor João Ferreira um agradecimento muito especial pelos conhecimentos científicos que me transmitiu na área da espectrofluorimetria e que foram relevantes para a minha formação.

Ao Dr. Filipe Sansonetty um agradecimento muito especial, pelos conhecimentos que me transmitiu, decisivos para a minha formação na área da citometria de fluxo, bem como pela amizade demonstrados ao longo deste trabalho.

A todos os funcionários e colegas do Departamento de Biologia o meu agradecimento pela amizade e pelo excelente ambiente de trabalho que proporcionaram.

À Sandra e ao Fernando agradeço a amizade que me concederam ao longo de todos estes anos de percurso em comum. Fernando tem sido bom trabalhar contigo, Obrigada.

A special word to Toni. Thank you for the opportunity that you gave me. My stay in New York was the experience of a life both under a personal and a scientifical point of view.

Gostaria de endereçar uma palavra muito especial à Paula Macedo e à Ana do Vale pela amizade e carinho com que me receberam no IBMC e por tudo o que aprendi com elas. Obrigada.

A todos os que de alguma forma contribuíram para a realização deste trabalho, o meu muito obrigada.

Por último, mas em primeiro lugar queria agradecer a toda a minha Família o apoio e carinho em especial à "Pititinha" pela companhia e amizade, as nossas aventuras não acabam aqui.... Aos Meus Pais por terem dedicado toda a sua vida ao sucesso da minha. O Vosso amor é o meu porto seguro.

Tia, a saudade por vezes sufoca mas a doce lembrança permanecerá para sempre comigo. Obrigada.

A autora usufruiu de uma bolsa de Doutoramento do Programa PRAXIS XXI.

A autora agradece o apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio.

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Abstract

Programmed cell death (PCD), of which apoptosis is the most common morphological expression, is an orchestrated collapse of the cell resulting from the activation of a widely described cascade of molecular events. This process plays an important role in the normal development and homeostasis mechanisms of multicellular organisms. The occurrence of PCD in unicellular organisms was initially discarded based on the altruistic role attributed to such a cell death process in multicellular organisms. However, the number of reports concerning the PCD occurrence either in bacteria or in yeast had increased and nowadays this is an accepted concept. In this thesis an endogenous PCD process induced by acetic acid in *Saccharomyces cerevisiae* is described. The death induced by 20-80 mM acetic acid is inhibited by cycloheximide and is accompanied by the following alterations typical of apoptosis: (i) chromatin condensation, seen by transmission electron microscopy; (ii) exposure of phosphatidylserine on the surface of the cytoplasmic membrane, revealed by the FITC–annexin V reaction; and (iii) the occurrence of DNA strand breaks, demonstrated by the TUNEL assay. These results show that a PCD process sharing common features with a mammalian apoptotic phenotype can be induced by acetic acid in *S. cerevisiae*. At least two major apoptotic pathways have been described in mammalian cells, one requiring the participation of mitochondria and another in which mitochondria are bypassed and caspases are activated directly. To address the presence of a mitochondrial apoptotic pathway in the PCD process induced by acetic acid in *S. cerevisiae* a flow cytometry protocol using the fluorescent dye Rhodamine 123 was optimised to assess mitochondrial membrane potential ($\Delta\Psi m$), a mitochondrial functional apoptotic marker. This method proved to be very efficient in the characterization of the cell death induced by acetic acid in *S. cerevisiae* and in *Zygosaccharomyces bailii*. Although for higher concentrations, acetic acid also induces in *Z. bailii* a PCD process that is inhibited by cycloheximide and accompanied by alterations typical of apoptosis, namely chromatin condensation, occurrence of DNA strand breaks, and decrease of $\Delta\Psi m$. Moreover, several mitochondrial ultrastructural changes, namely decrease of the cristae number, formation of myelinic bodies, solute accumulation and swelling, with preservation of mitochondrial membrane integrity, were seen in *Z. bailii*. That mitochondria are implicated in the PCD process induced by acetic acid in *S. cerevisiae* is concluded from the finding of translocation of

cytochrome *c* (CytC) to the cytosol and of reactive oxygen species production. Associated to these events, reduction in O_2 consumption and of $\Delta\Psi m$ was found. Enzymatic assays showed that cytochrome *c* oxidase (COX) was strongly decreased in accordance with the observed reduction in the amounts of COX II subunit. The inability of *S. cerevisiae* mutant strains (lacking mitochondrial DNA, heme lyase or ATPase) to undergo acetic acid-induced PCD and, in the case of the ATPase mutant (knockout in *ATP10*), the absence of CytC release, provides further evidence that the process is mediated by a Mitochondria-Dependent Apoptotic Pathway (MDAP). A caspase-dependent and a caspase-independent pathways have been described in mammalian cells as major mitochondrial-dependent apoptotic pathways. The latter requires the involvement of an Apoptotic-Inducing Factor (AIF). In the present work, we present evidence that Ynr074c protein of *S. cerevisiae* has a high similarity with the human AIF and is localized in the mitochondria. In addition, the Δ*YNR074*c strain showed a phenotype of resistance to death induced by acetic acid and camptothecin two known apoptotic inducers. Translocation of the Ynr074c-GFP from the mitochondria to the cytoplasm and to the nucleus was observed during camptothecin treatment and cell ageing. Moreover, overexpression of *YNR074c* resulted in an increased susceptibility to killing by acetic acid and by camptothecin. Altogether the results indicate that, besides CytC, Ynr074cp could be another putative mitochondrial effector of *S. cerevisiae* apoptotic cell death, suggesting that two distinct pathways leading to nuclear apoptosis could be present in yeast similarly with what has been reported for mammalian cells. The use of yeast-based functional analysis of human proteins implicated in apoptosis will provide powerful systems for the efficient screening for drugs that may result in the finding of universal activators or inhibitors of apoptosis, which would be helpful for basic research, and potentially for medical applications. Exploiting these assays for compounds that are able to alter apoptotic proteins in different clinically useful ways, may become one of the greatest contributions that yeast make to humans besides bread and wine.

Resumo

A morte celular programada (MCP), de que a apoptose é a expressão morfológica mais comum, é o colapso organizado da célula como resultado da activação de uma cascata de eventos moleculares. Este processo desempenha um papel crucial no desenvolvimento normal e nos mecanismos de homeostase de organismos multicelulares. A ocorrência de MCP em organismos unicelulares foi inicialmente rejeitada dado o papel altruísta atribuído a este processo em organismos multicelulares. No entanto, o crescente número de referências à ocorrência de MCP quer em bactérias quer em leveduras faz com que actualmente este conceito seja aceite. Nesta tese descreve-se e caracteriza-se o processo de MCP endógeno induzido pelo ácido acético em *Saccharomyces cerevisiae*. A morte induzida por tratamento com 20-80 mM de ácido acético é inibida pela cicloheximida e é acompanhada pelas seguintes alterações morfológicas típicas da apoptose: (i) condensação de cromatina, detectada por microscopia electrónica; (ii) exposição de fosfatidilserina na superfície externa da membrana citoplasmática, demonstrado por reacção com FITC–anexina V e (iii) ocorrência de fragmentação de DNA, observada por TUNEL. Estes resultados mostram que o processo de MCP induzido pelo ácido acético em *S. cerevisiae* partilha aspectos comuns com o fenótipo apoptótico de células animais. Pelo menos duas grandes vias apoptóticas têm sido descritas em células animais, uma que requer a participação da mitocôndria e outra, em que há um desvio da mitocôndria e activação directa das caspases. Para estudar o envolvimento da mitocôndria no proceso de MCP induzido pelo ácido acético em *S. cerevisiae*, foi optimizado um protocolo para citometria de fluxo recorrendo ao uso da sonda fluorescente Rhodamina 123, para determinação do potencial de membrana mitocondrial ($\Delta\Psi m$), um conhecido marcador funcional da apoptose. Este método mostrou ser bastante eficiente na caracterização da morte celular induzida pelo ácido acético em *S. cerevisiae* e em *Zygosaccharomyces bailii*. Apesar de requerer concentrações mais elevadas, o ácido acético também induz um processo de MCP em *Z. bailii* que é inibido pela cicloheximida e acompanhado por alterações típicas da apoptose, tal como condensação de cromatina, ocorrência de fragmentação de DNA e diminuição do $\Delta\Psi m$. Para além destas alterações, foi ainda possível observar em *Z. bailii* diversas modificações ultraestruturais da mitocôndria, tais como diminuição do número de cristas, formação de corpos mielínicos, acumulação de solutos e tumefacção com preservação da integridade da membrana

mitocondrial. A descoberta de que o processo de MCP induzido pelo ácido acético em *S. cerevisiae* é acompanhado pela translocação de citocromo *c* (CitC) para o citoplasma e pela produção de espécies reactivas de oxigénio permitiu concluir que a mitocôndria está envolvida neste processo de morte celular. Estes eventos moleculares estão ainda associados à redução do consumo de O_2 e do $\Delta\Psi m$. Ensaios enzimáticos mostraram que a actividade do complexo citocromo *c* oxidase (COX) se encontrava fortemente diminuída, de acordo com a observada diminuição da quantidade da COXII. A incapacidade que estirpes mutantes de *S. cerevisiae* (sem DNA mitocondrial, heme liase ou ATPase) têm para desencadear um processo de MCP induzido pelo ácido acético e, no caso do mutante na ATPase ("knockout" no *ATP10*), a ausência de libertação de CitC, fornece dados adicionais para a evidência de que o processo de morte é mediado por uma via apoptótica dependente da mitocôndria. Em células animais estão descritas duas grandes vias apoptóticas mitocondriais, dependente e independente de caspases. Esta última requer o envolvimento de um factor indutor da apoptose (FIA). No presente trabalho, são apresentadas evidências de que a proteína Ynr074c de *S. cerevisiae* tem uma elevada similaridade com o FIA humano e que se localiza na mitocôndria. Adicionalmente, a estirpe mutante no gene *YNR074*c demonstrou um fenótipo de resistência à morte induzida pelo ácido acético e pela camptotecina dois conhecidos indutores apoptóticos. A translocação da proteína quimérica Ynr074c-GFP da mitocôndria para o citoplasma e depois para o núcleo foi observada durante o tratamento com a camptotecina e durante o envelhecimento celular. A sobre-expressão do gene *YNR074c* resultou no aumento da sensibilidade ao ácido acético e à camptotecina. No seu conjunto estes resultados indicam que para além do CitC, a proteína Ynr074c poderá ser outro activador mitocondrial da morte celular apoptótica em *S. cerevisiae*, sugerindo que duas vias distintas conduzem à apoptose nuclear em *S. cerevisiae* à semelhança do que acontece em células animais. O uso das leveduras para a análise funcional de proteínas humanas envolvidas na apoptose é um modelo poderoso para o rastreio de drogas que poderão ser activadores ou inibidores universais da apoptose, utéis para a investigação fundamental e com potenciais aplicações médicas. A exploração destes ensaios com vista à obtenção de compostos capazes de modificar proteínas apoptóticas para diferentes usos clínicos poderá ser uma das grandes contribuições das leveduras para o Homem para além do pão e do vinho.

Scope and outline of the thesis

The observation that yeast as a unicellular organism can perform programmed cell death with a close phenotypical (and potentially close evolutionary) relation to metazoan apoptosis was quite emerging at the time the work included in this thesis started and had only been described in two reports (Madeo *et al*., 1997, 1999). Thus, the work developed in the scope of the PhD research programme aimed at elucidating the pathways underlying cell death in yeast. Although *Saccharomyces cerevisiae* was used in most part of the work, another yeast *Zygosaccharomyces bailii* was also investigated due to its higher resistance to acetic acid, one of the lethal agents studied.
The experimental work selected for the present thesis is included in chapters (2 to 6) and regards the following topics:
Chapter 2 - Characterization of the PCD process induced by particular concentrations of acetic acid in *S. cerevisiae*, using classical markers of apoptosis.
Chapter 3 - Development of a method to assess mitochondrial functionality in yeast. This method was used to investigate the role of mitochondria in the PCD process induced by acetic acid in yeast.
Chapter 4 - Demonstration that *Z. bailii* also dies by apoptosis when exposed to selected concentrations of acetic acid.
Chapter 5 - Demonstration that mitochondria are involved in the PCD process due to exposure of *S. cerevisiae* to particular concentrations of acetic acid. This study included the observation that cytochrome *c* (CytC) is translocated from mitochondria to the cytosol during that PCD process.
Chapter 6 - Demonstration that an Apoptosis-Inducing Factor (AIF)-equivalent is present in *S. cerevisiae* mitochondria and could be involved in the PCD process.
The final chapter is devoted to a general discussion focusing on the main contributions of the present work to the understanding of programmed cell death processes in yeast and on the use of these organisms as a powerful tool in apoptosis research.

Chapter 1

Death mechanisms in Saccharomyces cerevisiae – *General Introduction*

Chapter 1 – Death mechanisms in **Saccharomyces cerevisiae** *– General Introduction*

1 - Necrosis and apoptosis: two contrasting modes of cell death

2 - Apoptosis in yeast

- 2.1 - Apoptosis in yeast induced by heterologous expression of Bcl-2 family proteins
  - 2.1.1 - The Bcl-2 family of proteins
  - 2.1.2 - Yeast as a tool for research in metazoan apoptosis
  - 2.1.3 - Yeast cell death induced by Bax-expression shows an apoptotic phenotype
- 2.2 - Endogenous apoptosis in yeast

3 - Acetic acid as a lethal agent in yeast and its cytotoxic effects: a brief review

1 – Necrosis and apoptosis: two contrasting modes of cell death

Cell death is a biological process that culminates with cessation of biological activity. It has been generally accepted that cell death can be due to one of two distinct, mutually exclusive, processes, namely necrosis and apoptosis (see for review Darzynkiewicz *et al*., 1997). The term "necrosis" is nowadays reserved for a passive form of cell death. On the other hand, the term "apoptosis", frequently referred as "programmed cell death", is used for the cell-autonomous active process.

As discussed by Wyllie *et al*. (1980) in their classical review on cell death processes, necrosis can be described as a death process accompanied by cell swelling with rupture of nuclear, cytoplasmic and organelle membranes and dissolution of organised structure. It results from injury by physical or chemical agents and affects cells in groups rather than single cells and leads to an inflammatory response. In contrast, apoptosis (Kerr *et al*., 1972) is a death process with shrinkage of the cell, maintenance of organelle integrity and extensive cytoplasmic membrane blebbing leading to the shedding of sub-cellular structures called apoptotic bodies. In metazoans, apoptotic cells and apoptotic bodies are phagocytosed and eliminated by adjacent cells (usually resident macrophages) so that no important inflammatory response ensues. This form of cell death is analogous to "cell suicide", because death is the consequence of molecular signals contained, and triggered, within individual cells.

Apoptosis-associated morphological changes have provided the background for the definition of apoptosis-inducing stimuli and to guide the investigation of the biochemistry and signal transduction of apoptosis (Häcker, 2000). Therefore, the accurate detection of apoptosis has become crucial and several assays for detection of apoptosis were developed during the last decade, some of which are applicable to cytochemical analysis. Regardless of which situation is investigated or which agent is used to induce apoptosis experimentally, the appearance of cell death always has some similar features (Table 1).

Table 1 – Categories of cellular changes that form the basis of apoptosis assays (adapted from Willingham, 1999)

Cellular change		Assays
Surface morphology and composition	Alterations in plasma membrane permeability	Staining with DNA binding dyes: impermeable (propidium iodide) and permeable (DAPI, Hoescht)
	Phosphatidylserine externalization	Fluorescent labelled annexin V binding (presented in chapter 2)
Nuclear events and DNA cleavage	Changes in nuclear morphology	By light or electron microscopy to analyse chromatin condensation (presented in chapter 2)
	DNA cleavage	Evaluation by gel electrophoresis of large segments and internucleosomal cleavage
	DNA cleavage/detection of strand breaks	In situ nick-translation (ISNT); TUNEL (terminal transferase) (presented in chapter 2); anti-single-stranded DNA antibody
Cell fragmentation	Shedding of apoptotic bodies containing DNA	Detection of sub-G1 peak Electron microscopy
Cytoplasmic biochemical activation events	Caspase cleavage	Fluorescence detection of the cleavage products
	Induction of caspase activity	Detection by caged fluorophores, FRET
	Induction of PARP activity	Detection with antibodies conjugated with fluorophores
Mitochondrial function and integrity	Transitory permeability	Evaluated by staining with vital dyes
	Changes in mitochondrial membrane potential	Using specific fluorescent dyes such as Rhodamine 123 (as described in chapter 3) or $DiOC_6$
	ROS production	Using specific fluorescent dyes such as Mitotracker or Dihiidrorhodamine (see chapter 5)
	Maintenance of mitochondrial integrity	Analysed by the accessibility of mitochondrial antigens or by staining with fluorescent dyes such as Rh123 (see chapter 4)
	Cytochrome *c* release	Immunodetection of cytochrome *c* in cytoplasm (see chapter 5)

While death of yeast cells by necrosis has been known for a long time, the history of apoptosis in yeast started very recently, now being an emergent area with potential medical impact.

2 – Apoptosis in yeast

2.1 – Apoptosis in yeast induced by heterologous expression of Bcl-2 family proteins

…yeast cells have been extensively used as tools for the study of fundamental aspects of cell and molecular biology. Advances in the knowledge of mechanisms of death occurring in yeast cells will be useful in areas like food industry, medicine and basic biology…

Pioneer studies on cell death signalling/effector pathways were conducted in *Caenorhabditis elegans* (Miura and Yuan, 1996). However, the great facility with which yeast can be grown quickly and the possibility to easily manipulate yeast genetics make these single cell organisms ideal for applying genetic approaches to many biological questions. The exploitation of yeast as a tool for studying human apoptosis-regulatory proteins has yielded novel insights into cell death mechanisms (Matsuyama *et al.,* 1999). As elegantly pointed out by Fröhlich and Madeo (2000), these advances were obtained considering the yeast *S. cerevisiae* as a "clean room" system to study interactions between heterologous expressed regulatory components of apoptotic pathways.

2.1.1 – The Bcl-2 family of proteins

Before considering the advances made using yeast as a tool for the study of metazoan apoptosis, the relevant aspects of the biology of the Bcl-2 family of proteins will be briefly reviewed.

As described above, programmed cell death (PCD), of which apoptosis is the most common morphological expression, is an orchestrated collapse of the cell. This process plays an important role in the normal development and homeostasis mechanisms of multicellular organisms and it is highly regulated involving interactions between extracellular molecules, intracellular signal transduction pathways and intrinsic

suicide/rescue programs (Williams and Smith, 1993; Hale *et al.,* 1996; Nagata*,* 1997). Disregulation of cell death can contribute to developmental defects, immune dysfunction, neurodegenerative disorders and cancer (Thompson, 1995).

Among the more prominent regulators of cell death are members of the Bcl-2 family (Adams and Cory, 1998). This family of proteins consists of both cell death promoters (e.g., Bax and Bak) and cell death preventers (e.g., Bcl-2 and Bcl-xL). Several lines of evidence suggest that cell death regulating activity of Bcl-2 family members depends on their ability to modulate mitochondrial function (Green and Reed, 1998; Gross *et al.,* 1999; Mignotte and Vayssiere, 1998; Reed *et al.,* 1998; Susin *et al.,* 1998). Many of these proteins reside in the outer mitochondrial membrane, oriented towards the cytosol. Though the mitochondrial location has usually been emphasized, significant portions of the Bcl-2 family proteins are also integrated into the membranes of the endoplasmic reticulum and nuclear envelope (Reed *et al.,* 1998).

While the biochemical mechanisms underlying the death regulator properties of the Bcl-2 family of proteins remain unclear, Bax and other apoptosis-inducing Bcl-2 family proteins are known to have two ways to induce mammalian cell death. One is through heterodimerization with anti-apoptotic Bcl-2 family proteins suppressing their cytoprotective activity. The other is through its own intrinsic cytotoxic activity, probably by forming ion-channels or pores in membranes (Matsuyama *et al.,* 1999). The structural similarity of Bcl-2 family proteins to channel-forming proteins of some types of bacterial toxins, like diphtheria toxin and the colicins (Muchmore *et al.,* 1996; Stroud *et al.,* 1998), used by bacteria as weapons to kill competing bacteria, is most intriguing. According to the endosymbiotic theory, mitochondria are originated from eubacteria that formed a symbiotic relation with host cells, and a reasonable speculation is that some aspects of cell death mechanisms employed by Bax evolved from bacterial systems and were adapted for cell suicide responses by higher eukaryotes. Several investigators have hypothesized that the endosymbiotic origins of mitochondria and the evolution of aerobic metabolism in eukaryotes formed the basis for the evolution of active cell death, which manifests predominantly as apoptosis in

metazoans (Green and Reed, 1998). This theory emphasises that the apoptosis machinery is based on several ancient domains that have not been 'invented' for this function but, rather, have been recruited from proteins that, in unicellular organisms, perform other regulatory functions (Aravind *et al.,* 1999).

2.1.2 - Yeast as a tool for research in metazoan apoptosis

Yeast can be modified so to express cell death promoters of the Bcl-2 family, and Bax expression in *S. cerevisiae* can result in two different responses: growth arrest and cell death (reviewed in Gross *et al.,* 2000). The ability of Bax to confer a lethal phenotype in yeast (discussed below) has permitted to take advantage of yeast genetics for gaining improved understanding of the apoptotic pathway. Additionally, given that yeast are readily capable of fermentative metabolism under anaerobic growth (unlike mammalian cells), a wide variety of nuclear genes encoding mitochondrial proteins can be knocked-out without lethality hence making the use of yeast more advantageous to study mitochondrial involvement in cell death.

Examples of the different approaches using yeast as a tool to the study of metazoan apoptosis are described below:

1- Structure-function analysis by mutagenesis of apoptosis-regulating proteins when a phenotype is available. Zha *et al.* (1996) found that the dimerization-mediating BH3 domain and targeting to mitochondrial membranes appear to be essential for the cytotoxic function of Bax in both yeast and mammalian cells. Ink and coworkers (1997) described that the pro-apoptotic protein Bak requires the same critical BH3 domain to induce lethality in *Schizosaccharomyces pombe* as is required for killing in mammalian cells.

2- Two-hybrid and related protein interaction cloning strategies have been developed. This approach allowed the demonstration that BFL-1 (the smallest member of the Bcl-2 family, that retards apoptosis in various cell lines) strongly interacts with human Bax

but is not able to form homodimers nor to interact with human Bcl-2 or Bcl-xL. BFL-1 has also the ability to neutralize Bax lethality in yeast (Zhang *et al.*, 2000).

3- Functional screens have been used for identification of cDNAs that suppress apoptotic, protein-induced, lethality (Kampranis *et al*., 2000; Levine *et al*., 2001; Pan *et al*., 2001).

4- Cloning by classic complementation has identified yeast genes required for phenotypes conferred by ectopic expression of animal apoptosis regulators. Matsuyama *et al.* (1998) discovered that components of the mitochondrial F0F1-ATPase, which are conserved from yeast to humans, are required for Bax-lethality in yeast.

5- Genetic analysis of interactions of apoptosis-regulating proteins with host proteins. Since mammalian cells lacking the voltage-dependent anion channel (VDAC) are not viable, a yeast mutant strain deficient in VDAC was used by Shimizu and coworkers (1999). Using this strategy and that referred in 4, these authors demonstrated the requirement of the VDAC and adenine nucleotide translocator (ANT) for Bax-lethality in yeast, thus corroborating biochemical data suggesting physical and functional interactions of Bcl-2 family proteins with these mitochondrial proteins (Shimizu *et al.,* 1999; Marzo *et al.,* 1998; Vander Heiden *et al.,* 1999).

Yeast-based functional assays for human apoptosis proteins could also provide powerful systems for drug screening. Additionally, Bax-expressing yeast cells have been used for the isolation of plant anti-apoptotic genes. Kampranis *et al.* (2000) followed this strategy and transformed yeast cells expressing Bax with a tomato cDNA library and selected the cells surviving after the induction of Bax. They found a novel plant glutathione S-transferase/peroxidase that suppresses Bax lethality in yeast. Pan *et al.* (2001) used the same strategy but with an *Arabidopsis* cDNA library and obtained an *Arabidopsis thaliana* ethylene-responsive element binding protein that can function as a dominant suppressor of Bax-induced cell death of yeast.

Levine *et al.* (2001) also described that vesicle-associated membrane protein of *Arabidopsis* suppresses Bax-induced cell death in yeast downstream of oxidative burst.

As mentioned above, apoptosis is a physiological process that, when inappropriately controlled, causes diseases. The exploitation of yeast as a tool to study apoptosis-regulator proteins has already yielded new insights into PCD in mammalian cells as will be discussed later, and may in the near future contribute to the identification of genes and drugs that modulate the functions of proteins involved in apoptosis control.

2.1.3- Yeast cell death induced by Bax expression shows an apoptotic phenotype

As already stated above, Bax-expression in *S. cerevisiae* can result in two different responses: growth arrest or cell death (see for review Gross *et al.,* 2000). Bax-induced growth arrest was found to be independent of VDAC, F0F1-ATPase, mitochondrial cyclophilin, cytochrome *c* and of proteins encoded by the mitochondrial genome as revealed by ρ^0 cells (Gross *et al.,* 2000). However, Bax killing is dependent on F0F1-ATPase and on proteins encoded by the mitochondrial genome (Gross *et al.,* 2000). Thus, Bax induces two cellular processes in yeast, which can be abrogated by co-expression of Bcl-xL, namely cell arrest, which does not require participation of mitochondrial biochemistry, and cell killing, which does. In addition, Bax-expressed in yeast localizes to mitochondria and induces a hyperpolarization of the mitochondrial membrane potential similar to the initial effects observed in mammalian cells (Minn *et al*., 1999; Priault *et al*., 1999a; Zha *et al*., 1996; Gross *et al*., 2000). Reactive oxygen species (ROS) production is also involved in Bax yeast lethality (Gross *et al*., 2000). The Bax-effects are different depending on the type of metabolism (Priault *et al.,* 1999a). Under respiratory conditions, there is a strict correlation between Bax-induced growth arrest and death. However, under fermentative conditions, although growth arrest still occurs, Bax-induced lethality is markedly delayed (Priault *et al.,* 1999a). Priault *et al.* (1999a) proposed that these behaviours might be related to the different intracellular ATP/ADP ratio under fermentative (value of 2) and respiratory conditions

(value of 10). A high ATP/ADP ratio is not necessary for Bax-induced cell growth arrest but is required for a rapid Bax-induced cell death.

Manon *et al.* (1997) have found that Bax-induced growth arrest of yeast cells is related to two defects in the respiratory chain: i) a decrease in the amount of cytochrome *c* oxidase and ii) a dramatic increase in the release of cytochrome *c* (CytC) to the cytosol. Surprisingly, these results obtained in yeast parallel similar observations reported in mammalian cells dying by apoptosis. In an attempt to get clues of the mechanisms underlying CytC release, Priault and coworkers (1999b) described that Bax-induced release of CytC in yeast does not involve any permeability transition of the inner mitochondrial membrane but involves a general alteration of the permeability to macromolecules of the outer mitochondrial membrane. Recently, a channel named Mitochondrial Apoptosis-induced Channel (MAC) was proposed by Pavlov *et al.* (2001) as a candidate for the outer-membrane pore through which CytC and possibly other apoptotic factors exit mitochondria during apoptosis both in mammalian and in Bax-expressing yeast cells. Even though the above mentioned reports pointed to a crucial role of CytC in Bax-induced cell death, Roucou and coworkers (2000) reported that Bax-expression in yeast cells containing only a CytC-GFP fusion, which is not released upon Bax-expression, is causing cell death as well, indicating that mitochondrial CytC release is not essential for killing.

Recently, Manon *et al.* (2001) reported that the decrease of cytochrome *c* oxidase observed upon Bax-expression in yeast is due to the activation of mitochondrial protease Yme1p, of which cytochrome *c* oxidase subunit 2 is a substrate. The absence of Yme1p slightly delays Bax-induced cell death, suggesting a role of this protease in yeast cell death and thus of its mammalian homologue in apoptosis. Although all these findings are in agreement with the necessity of an active respiratory chain in the suicide mechanism, the question whether mitochondrial function is crucial for Bax-induced cell death in yeast is still controversial. In fact, Kissova *et al.* (2000), contradicting Greenhalf *et al.* (1996), have found that Bax induces death of a

respiratory deficient mutant lacking mitochondrial DNA as well as of the wild type strain either undergoing a fermentative or respiratory metabolism.

Interestingly, the biochemical alterations just reviewed due to Bax-expression in yeast namely CytC release, change of mitochondrial membrane potential, formation of the Mitochondrial Apoptosis-induced Channel (MAC), and ROS production, resemble those occurring in metazoan cells dying by apoptosis (see for example: Willigham, 1999; Darzynkwietz *et al*., 1997). Moreover, as in metazoan apoptosis, the alterations induced in yeast by Bax-expression are prevented by Bcl-xL protein. These and a variety of other studies in yeast strongly indicate that Bcl-2 family proteins are acting upon highly conserved mitochondrial components that correspond directly to their substrates in mammalian cells. This agrees with the report by Ink and coworkers (Ink *et al*., 1997) which shows that the phenotype of the Bak-induced death in yeast have morphological features characteristic of mammalian apoptosis including cytoplasm vacuolisation, chromatin condensation, disappearance of nuclear envelope and nuclear fragmentation and specific DNA cleavage into high-molecular-weight fragments. Ligr *et al*. (1998) presented evidence that mammalian Bax triggers apoptotic changes in yeast, and found that several hallmarks characteristic of apoptotic death (Wyllie, 1992) were present in yeast dying due to Bax-expression, namely chromatin condensation, DNA fragmentation, exposition of phosphatidylserine and plasma blebbing, indicating, for the first time, that a PCD pathway evolutionarily conserved in metazoans is also present in unicellular yeast.

The fact that Bax-expression in yeast induces a death process with biochemical alterations similar to those occurring in metazoan cells and accompanied by typical markers (Table 2) points to the presence of a common pathway responsible for the execution of cell death at the different levels of biological organization.

Table 2 – Biochemical changes and markers common to metazoan (see for example Willingham, 1999) and yeast cells under PCD.

PCD in yeast due to Bax-expression	
Exposition of phosphatidylserine on the outer surface of the plasma membrane	Ligr *et al.,* 1998
Chromatin condensation	Ligr *et al.,* 1998
DNA fragmentation	Ligr *et al.,* 1998
Mitochondrial membrane-potential hyperpolarization	Gross *et al*., 2000
Release of cytochrome *c*	Manon *et al.,* 1997
ROS production	Gross *et al*., 2000
Decrease of cytochrome *c* oxidase	Manon *et al.,* 1997
Mitochondrial apoptosis-induced channel (MAC) activity	Pavlov *et al.,* 2001

2.2 – Endogenous apoptosis in yeast

Apoptosis was firstly described and morphologically defined by Kerr, Wyllie and Currie in 1972 as a mammalian phenomenon utilized to remove, quickly and without an inflammatory response, cells that are either unwanted or potentially harmful to the organism. Apoptosis has therefore often been assumed to be a product of attaining multicellularity (Fraser and James, 1998). However, cell suicide responses have been documented in the last decade in unicellular organisms including bacteria and some eukayotic cells (Ameisen, 1996; Jacobson *et al.,* 1997). Examples of cell suicide among unicellular organisms include PCD of bacterial plasmid-free segregants, avoiding in this way the survival of bacteria unable to retain the plasmid (Yarmolinsky, 1995; Naito *et al.,* 1995), and PCD of phage-infected *Escherichia coli*, with the prevention of virus spreading among the bacterial population (Matsuyama *et al*., 1999; Skulachev, 1999; Skulachev, 2000). Moreover, PCD in bacteria also plays an important role in developmental processes, such as lysis of the mother cell during sporulation of *Bacillus subtilis* and lysis of vegetative cells in fruiting body formation of *Myxococcus xanthus* (Lewis, 2000).

The report by Longo *et al.* (1997) that stationary cells of *S. cerevisiae* survive for long periods in pure water but quickly lose viability in nutrient-depleted synthetic media, although not recognized as such, probably represents the first description of an endogenous apoptotic death process in yeast. Death of majority of the cells may spare resources for the best adapted, healthier or younger cells. Albeit at the time it was not generally accepted that yeast do have an endogenous PCD process, the reports discussed in the previous section that Bax-expression in yeast leads to a cell death process with markers typical of apoptosis showed that the biochemical machinery involved in the apoptotic death process is actually present in yeast. Within this scenario, an endogenous yeast cell death process with features typical of the mammalian apoptosis phenotype was firstly described in 1997 by Madeo *et al.* (1997). These authors reported that the *S. cerevisiae* mutant in cell division cycle gene $cdc48^{S565G}$ dies by a process that shows a number of morphological and biochemical apoptotic markers: exposure of phosphatidylserine on the outer leaflet of the plasma membrane (demonstrated by annexin V reaction), DNA breakage (demonstrated by TUNEL assay) and chromatin condensation and fragmentation. The same research team reported two years later (Madeo *et al.,* 1999) that exposing *S. cerevisiae* cells to low exogenous H_2O_2 concentrations or growing a *gsh1* deletion mutant in the absence of glutathione resulted in apoptotic cell death. Most interesting were the observations made by those authors that cycloheximide prevents chromatin condensation and DNA fragmentation and enhances the survival rate of yeast cells treated with low H_2O_2 concentrations, indicating that an active cell death process was involved. Madeo and co-workers (1999) also described reactive oxygen species as the first regulators of apoptosis shared between yeast and higher eukaryotes.

Although the *S. cerevisiae* genome sequence lacks any obvious homologue of the major apoptotic regulators described in metazoan organisms (Bcl-2 family of proteins, Apaf-1/CED-4, p53), the co-ordinate occurrence of the events described above indicates the presence of a machinery performing the basic steps of apoptosis in yeast

(Fröhlich and Madeo, 2000), in keeping with the initial reports, discussed in section 3.1, that showed that Bax-expression in yeast results in a PCD process.

3 - Acetic acid as a lethal agent in yeast and its cytotoxic effect: a brief review

Our group at the University of Minho has been involved for several years in the study of the responses of yeast to several stress agents in particular to weak carboxylic acids. The work developed in this thesis focus on the elucidation of the pathways underlying the cell death induced by acetic acid in Saccharomyces cerevisiae *and* Zygosaccharomyces bailii

Weak carboxylic acids, such as acetic, sorbic and benzoic acids are generally regarded as safe antimicrobial additives and have wide application as preservatives in acidic foods and beverages (Thomas and Davenport, 1985; Malfeito-Ferreira *et al.,* 1990; Fleet, 1992). Their antimicrobial effects have often been attributed to the intracellular acidification and anion accumulation (Krebs *et al.,* 1983; Russel, 1991; Salmond *et al.,* 1984). Weak organic acids when in solution dissociate and a dynamic equilibrium between the undissociated form (uncharged molecule) and their respective charged ions (protons and anions) is established. This equilibrium is pH dependent being the undissociated form the predominant one in acidic environments. The uncharged molecule usually can freely diffuse through the plasma membrane (Leão and van Uden, 1986; Stratford and Rose, 1986) and once in the cytoplasm, whose pH is near the neutrality, rapidly dissociates into the respective ions. Since the plasma membrane is impermeable to dissociated form of the acid, the anion will accumulate in the cell unless they can be metabolised or pumped out by a transport system. In addition, the dissociation of the acid induces an acidification of the cytoplasm (Pampulha and Loureiro-Dias, 1990; Cardoso and Leão, 1992). Although the cytotoxic effects of the acids have not been thoroughly understood several hypothesis have been postulated, namely: i) the accumulation of the charged anion; ii) the decrease of the intracellular

pH; iii) the uncoupling between catabolism and anabolism and the iv) the decrease of cellular ATP by the active pumping out of anions and protons. It has been recognised that cytoplasm acidification might prevent yeast growth by inhibiting glycolysis, active transport of nutrients, cell signalling and DNA/RNA synthesis (for references see for example Rodrigues, 2001). In addition, glycolytic enzymes, especially enolase can be inhibited by the accumulation of the anion (Pampulha and Loureiro-Dias, 1990). Inhibition of glycolysis ultimately leads to the inhibition of respiration and fermentation (Pampulha, 1989; Fernandes *et al*., 1997).

Zygosaccharomyces bailii is a food and beverage spoilage yeast that is typically characterized by its tolerance to high acid concentrations and low pH (Thomas and Davenport, 1985), where *Saccharomyces cerevisiae* can not survive. The different susceptibility of yeast to weak carboxylic acids has been mainly attributed to differences in plasma membrane permeability (Sousa *et al*., 1998) to the undissociated form of the acid, as a consequence of different chemical compositions and, at least in the case of acetic acid (Rodrigues, 2001) and of sorbic or benzoic acids (Mollapour and Piper, 2001), to the capacity to co-metabolize the acid in the presence of glucose under aerobic conditions.

Pinto *et al*. (1989) reported that in *S. cerevisiae* acetic acid induces two different types of death; a high enthalpy death, predominant at high temperatures and low acetic acid concentrations, and a low enthalpy death, predominant at intermediate and low temperatures and high acetic acid concentrations. Fernandes *et al*. (1999) have found that acetic acid enhanced death of glucose-grown *Z. bailii* cells but, unlike with what happens with *S. cerevisiae*, only induces death characterized by high enthalpy.

Some of the functional and structural alterations induced by acetic acid in those two yeast species using three different fluorescent dyes associated to flow cytometry have also been detected (Prudêncio *et al*., 1998).

In summary, several aspects regarding the negative effects of acetic acid on the metabolic activity of *S. cerevisiae* and *Z. bailii* have been reported. However, the

pathways underlying death induced by that acid in the two yeast species remain unknown. Therefore, in the present thesis, studies aiming to ascertain the possible participation of the two basic mechanisms of cell death- necrosis and apoptosis- on the killing action of acetic acid on yeast, were performed.

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Chapter 2

Saccharomyces cerevisiae *commits to a programmed cell death process in response to acetic acid*

Microbiology, *147: 2409-2415 (2001)*

Microbiology (2001), **147**, 2409–2415 Printed in Great Britain

Saccharomyces cerevisiae commits to a programmed cell death process in response to acetic acid

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Recent evidence has revealed the occurrence of an apoptotic phenotype in *Saccharomyces cerevisiae* that is inducible with oxidative stress. Here, exposure of *S. cerevisiae* to 20–200 mM acetic acid for 200 min at pH 3·0 resulted in cell death. Yeast mortality induced by 120–200 mM acid was not inhibited by cycloheximide and was accompanied by ultrastructural alterations typical of necrosis. In contrast, alterations associated with cell death induced by 20–80 mM acetic acid included: (i) cycloheximide-inhibitable chromatin condensation along the nuclear envelope; (ii) exposure of phosphatidylserine on the surface of the cytoplasmic membrane, revealed by the FITC–annexin V reaction; and (iii) the occurrence of DNA strand breaks, demonstrated by the TUNEL assay. These results show that a programmed cell death process sharing common features with an apoptotic phenotype can be induced by acetic acid in *S. cerevisiae*. This observation raises the possibility of this mode of cell death being more generalized in yeasts than previously considered and extended to cell death induced by other stress agents.

Keywords: active cell death, apoptosis, yeast, acid stress

INTRODUCTION

In animal cells, apoptosis, necrosis and atypical cell death have been identified as distinct forms of cell death (Darzynkiewicz *et al.*, 1997). A cell triggered to undergo apoptosis activates a widely described cascade of molecular events, which results in a programmed cell suicide. The occurrence of programmed cell death in unicellular organisms was initially discarded based on the altruistic role attributed to such a cell death process in multicellular organisms. However, cell suicide responses have already been reported in prokaryotic and unicellular eukaryotic cells (Matsuyama *et al.*, 1999). It has also been recognized that the sacrifice of individuals of a population of unicellular organisms can be advantageous to the rest of the population. Hence, by analogy with apoptosis, the term 'phenoptosis' has been proposed to designate programmed cell death of an organism, either multi- or unicellular (Skulachev, 1999a, 2000).

Abbreviations: PI, propidium iodide; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl transferase mediated dUTP nick end labelling.

The reported mechanisms underlying programmed cell death in prokaryotes are different from those involved in apoptosis of animal cells. Examples include mechanisms involved in programmed cell death of plasmid-free segregants, avoiding in this way the survival of bacteria unable to retain the plasmid (Yarmolinsky, 1995; Naito *et al.*, 1995), and programmed cell death of phage-infected *Escherichia coli*, with the prevention of virus spreading among the bacterial population (Matsuyama *et al.*, 1999; Skulachev, 1999a, 2000). Moreover, programmed cell death in bacteria also plays an important role in developmental processes, such as lysis of the mother cell during sporulation of *Bacillus subtilis* and lysis of vegetative cells in fruiting body formation of *Myxococcus xanthus* (Lewis, 2000). In addition, programmed cell death has been reported in unicellular eukaryotic organisms (Ameisen, 1996). The yeast *Saccharomyces cerevisiae* has been used mainly as a tool to study human apoptosis regulatory proteins and it is not generally accepted to have an endogenous programmed cell death process. However, evidence indicating the presence of some basic features characteristic of an apoptotic phenotype in *S. cerevisiae* was recently reported (Madeo *et al.*, 1997; Ligr *et al.*, 1998). The

expression of the anti-apoptotic protein Bcl-2 in *S. cerevisiae* provides antioxidant protection and delays natural death (Longo *et al.*, 1997). Furthermore, it was recently shown in *S. cerevisiae* that depletion of glutathione or exposure to low external doses of H_2O_2 triggered the cell into apoptosis, whereas depletion of reactive oxygen species (ROS) or hypoxia prevented apoptosis (Madeo *et al.*, 1999). In addition, an intracellular accumulation of ROS was detected in the cell cycle mutant *cdc48*^{S565G} of *S. cerevisiae* and in yeast cells expressing mammalian Bax. These results allowed the identification of ROS production as a key cellular event common to the known scenarios of apoptosis in yeast and animal cells (Madeo *et al.*, 1999). Recently, it was suggested that the Rad9 protein of *Schizosaccharomyces pombe* could be a member of the Bcl-2 protein family, being the first protein of this family identified in yeast (Komatsu *et al.*, 2000). The above evidence has suggested that a programmed cell death process occurs in yeasts and shares some conserved aspects with metazoan apoptosis. Nevertheless, yeast cell death processes are far from being clarified. Characteristic features of cell death patterns in yeast need to be identified in order to provide some insights into the cell death mode(s) in this unicellular eukaryote.

Acetic acid is a normal end product of the alcoholic fermentation carried out by *S. cerevisiae*. This compound is not metabolized by glucose-repressed yeast cells and enters the cell in the undissociated form by simple diffusion. Inside the cell, the acid dissociates and if the extracellular pH is lower than the intracellular pH, this will lead to intracellular acidification, anion accumulation and inhibition of the metabolic cell activity, namely fermentation/respiration (Leão & van Uden, 1986; Cássio *et al.*, 1987; Pampulha & Loureiro, 1989). Moreover, it was shown that in *S. cerevisiae*, under certain conditions, acetic acid compromises cell viability and ultimately results in two types of cell death, high and low enthalpy (Pinto *et al.*, 1989). Previous studies from our laboratory have focused on the assessment by flow cytometry of cell structural and functional changes induced by acetic acid in *S. cerevisiae* and *Zygosaccharomyces bailii* (Ludovico, 1999; Prudêncio *et al.*, 1998). However, the process by which the yeast cell dies when injured by acetic acid is unknown.

The aim of the present study was to identify morphological, structural and functional cellular markers that would allow the characterization of the mode(s) of cell death induced by acetic acid in *S. cerevisiae*.

METHODS

Micro-organism and growth conditions. The yeast *Saccharomyces cerevisiae* W303-1A (*MAT*a *ade2 leu2 his3 trp1 ura3*) was used. This organism was maintained on YEPD agar slants containing glucose (2%, w/v), yeast extract (1%, w/v), peptone (2%, w/v) and agar (2%, w/v). In experiments, the yeast cells were subcultured in liquid YEPD medium. The growth experiments were performed in 250 ml flasks containing a 2:1 ratio of air to liquid phase, and incubated on a mechanical shaker (150 r.p.m.) at 26 °C. Cells were grown to mid-exponential phase.

Treatments with acetic acid and inhibition of protein synthesis. Exponential-phase cells were harvested and suspended (10^7 cells ml^{-1}) in YEPD (pH 3·0, set with HCl) containing 0, 20, 40, 80, 120, 160 and 200 mM acetic acid. The treatments were carried out for 200 min at 26 °C with magnetic stirring (100 r.p.m.). The inhibition of protein synthesis was performed by adding 100 μg cycloheximide (Merck) ml^{-1} (Barnett *et al.*, 2000) to the cell suspensions at the same time as the different acetic acid concentrations tested. Cycloheximide at this concentration was not cytotoxic after 200 min incubation, as assessed by c.f.u. counts. Viability was determined by c.f.u. counts after 2 d incubation at 26 °C on YEPD agar plates. No further colonies appeared after that incubation period. In all the above experiments, the extracellular pH did not change during the incubations.

Transmission electron microscopy analysis. Cells from different treatment conditions were washed with phosphate/magnesium buffer (40 mM K_2HPO_4/KH_2PO_4, pH 6·5, 0·5 mM $MgCl_2$), suspended in 2·5% (v/v) glutaraldehyde in 40 mM phosphate/magnesium buffer, pH 6·5, and fixed overnight at 4 °C. After fixation, the cells were rinsed twice in 0·1 M phosphate/citrate buffer (pH 5·8) and suspended in this buffer containing 10 U lyticase (Boehringer Mannheim) ml^{-1} for about 90 min at 37 °C, to digest the cell wall. After cell wall digestion of the pre-fixed yeast cells, protoplasts were washed and postfixed with 2% (w/v) osmium tetroxide (2 h) followed by 30 min incubation with 1% (w/v) aqueous uranyl acetate. Dehydration was performed as described by Byers & Goetsch (1991) for embedding vegetatively grown yeast cells. After 100% ethanol washes, the samples were transferred to 100% propylene oxide, and infiltrated with 50% (v/v) propylene oxide and 50% (v/v) Epon (TAAB Laboratories) for 30 min and with 100% Epon overnight. Cells were transferred to gelatin capsules with 100% Epon and incubated at 60 °C for 48 h before cutting thin sections and staining with uranyl acetate and lead acetate. Micrographs were taken with a Zeiss EM 10C electron microscope. For quantitative assessment of yeast cells with condensed chromatin, at least 50 yeast cells with nuclear profiles were counted per sample.

Terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) and propidium iodide staining. DNA strand breaks were demonstrated by TUNEL with the '*In Situ* Cell Death Detection Kit, Fluorescein', from Boehringer Mannheim. This technique labels free 3′-OH termini with FITC-labelled deoxyuridine triphosphate (dUTP), which was detected by epifluorescence microscopy. Yeast cells were fixed with 3·7% (v/v) formaldehyde as described by Madeo *et al.* (1999) and cell walls were digested with lyticase (as above, for 105 min). Cytospins of the cell suspensions were made using a Shandon Cytospin 2 cytocentrifuge, operating at 1500 r.p.m. for 5 min. The slides were rinsed with PBS, incubated in permeabilization solution (0·1%, v/v, Triton X-100 and 0·1%, w/v, sodium citrate) for 2 min on ice, rinsed twice with PBS and incubated with 10 μl TUNEL reaction mixture, containing terminal deoxynucleotidyl transferase and FITC-dUTP, for 60 min at 37 °C. Finally the slides were rinsed three times with PBS and a coverslip was mounted with a drop of anti-fading agent Vectashield (Molecular Probes) and with 2 μl of a propidium iodide (PI; Molecular Probes) working solution (50 μg ml^{-1}) in Tris buffer (10 mM, pH 7·0) with $MgCl_2$ (5 mM) and RNase (0·5 μg ml^{-1}). Observations were carried out using a Zeiss Axioskop epifluorescence microscope equipped with a HBO-100 mercury lamp, filter set 40 (BP360/51, BP485/17, BP560/18) from Zeiss, excitation filter

BP 450-490, beam splitter FT510 and emission filter LP520. Images were acquired with a Spot 2 camera (Diagnostic Instruments). For the quantitative assessment of TUNEL staining, 50–700 cells were counted per sample.

Annexin V staining. Phosphatidylserine exposure was detected by an FITC-coupled annexin V reaction with the ApoAlert Annexin V Apoptosis kit (CLONTECH Laboratories), essentially as described by Madeo *et al.* (1999). Cells were harvested and washed with sorbitol buffer (1·2 M sorbitol, 0·5 mM $MgCl_2$, 35 mM K_2HPO_4, pH 6·8). Cell walls were digested with 15 U lyticase (Sigma) ml^{-1} and 2% (v/v) glusulase (NEE-154 Glusulase; NEN) in sorbitol buffer for about 30 min at 28 °C; digestion with the two enzymes was monitored by phase-contrast microscopy in order to prevent damage to the unfixed protoplasts. Cells were then washed twice with binding buffer (10 mM HEPES/NaOH, pH 7·4, 140 mM NaCl, 2·5 mM $CaCl_2$; CLONTECH Laboratories) containing 1·2 M sorbitol. To 38 µl cell suspension in binding/sorbitol buffer were added 2 µl annexin V (20 µg ml^{-1}) and 2 µl of a PI working solution (as described for the TUNEL reaction) and incubated for 20 min at room temperature. The cells were then washed and resuspended in binding/sorbitol buffer. Finally the slides with coverslips were mounted with the cell suspensions. Microscope settings and image acquisitions were as described above for the TUNEL technique. For quantitative assessment of annexin V–PI staining, at least 150 yeast cells were counted per sample.

Reproducibility of the results. All the experiments were repeated at least three times. The data reported for c.f.u. counts are mean values and standard deviations. Quantitative data for annexin V and TUNEL staining and for chromatin condensation are from one representative experiment; while absolute data were not comparable in these experiments performed on different days, the trends described were fully consistent in the independent experiments.

RESULTS

Exposure of *S. cerevisiae* strain W303-1A to acetic acid at different concentrations (20–200 mM), pH 3·0, resulted in cell death (Fig. 1). The percentage of survival, estimated by c.f.u. counts after 200 min treatment, decreased with increasing concentrations of the acid in the extracellular medium. In order to address whether cell death induced by acetic acid treatment was an active process, the same experiments were carried out in the presence of cycloheximide. The presence of this inhibitor of protein synthesis attenuated the toxic effects of acetic acid and enhanced the survival percentage (Fig. 1). However, the protective effect of cycloheximide was

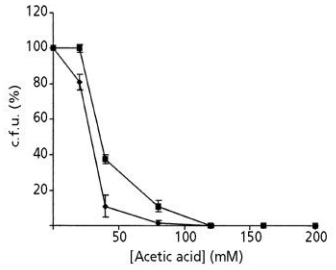


Fig. 1. Relative survival (% of c.f.u. on YEPD agar plates; 100% corresponds to the number of c.f.u. at time zero) of *S. cerevisiae* incubated for 200 min with acetic acid in the absence (◆) or presence (■) of cycloheximide. Vertical error bars represent standard deviations.

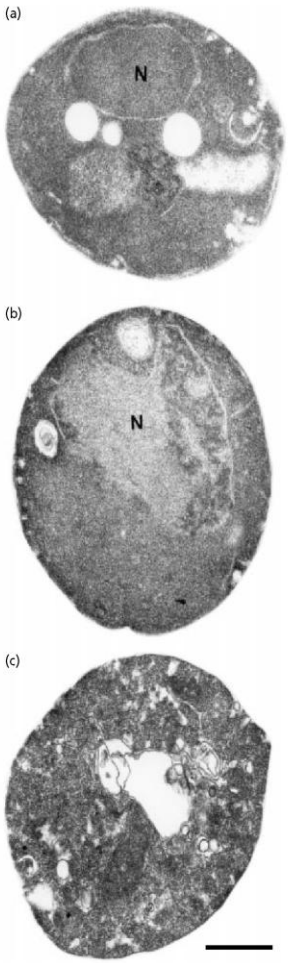


Fig. 2. Electron micrographs of exponentially growing cells of *S. cerevisiae*. (a) Untreated control, (b) cell treated with 40 mM and (c) cell treated with 160 mM acetic acid for 200 min. N, Nucleus. Bar, 1 µm.

Table 1. Cycloheximide inhibits chromatin condensation induced in *S. cerevisiae* by exposure to 20–120 mM acetic acid for 200 min

The percentage of yeast cell profiles with chromatin condensation along the nuclear envelope was determined by counting at least 50 cell profiles per sample. The values in samples exposed to acetic acid in the presence of 100 μg cycloheximide ml^{-1} are given in parentheses. ND, Not determined.

[Acetic acid] (mM)	Chromatin condensation (%)
0	0 (0)
20	ND
40	78·0 (4·8)
80	73·6 (30·0)
120	9·7 (1·2)
160	0 (0)
200	0 (0)

observed only for the lower acid concentrations; for concentrations higher than 120 mM the survival percentages were not significantly increased by the drug. In fact, a very high mortality was seen in yeasts exposed to 120 mM acid regardless of the presence of cycloheximide (99·86 and 99·90%, with and without the inhibitor, respectively). This indicates that the cell death process induced at the higher concentrations was not dependent on protein synthesis.

Subsequently, it was attempted to characterize further the type of cycloheximide-inhibitable cell death induced by acetic acid at the lower acid concentrations. Thus, samples of cell suspension treated with the acid were harvested and assayed for different apoptotic markers, namely chromatin condensation along the nuclear envelope, exposure of phosphatidylserine at the outer surface of the plasma membrane and formation of DNA strand breaks.

Extensive chromatin condensation along the nuclear envelope in cells treated with 20–80 mM acetic acid (shown in Fig. 2b for 40 mM) was revealed by quantitative transmission electron microscopy analysis (Table 1), whereas nuclei of untreated cells were homogeneous in shape and density (Fig. 2a). Only a low

Table 2. Characterization of *S. cerevisiae* cells (%) according to FITC-coupled annexin V and PI staining after exposure to 20–120 mM acetic acid for 200 min

Cell category*	[Acetic acid] (mM)			
	20	40	80	120
Annexin V (−), PI (−)	86·6	47·1	10·1	0·0
Annexin V (+), PI (−)	12·5	40·4	40·7	14·7
Annexin V (+), PI (+)	0·9	12·5	43·5	41·1
Annexin V (−), PI (+)	0·0	0·0	5·7	44·2

* Cell category as defined in Results.

percentage of cells treated with 120 mM acid, and no cells treated with 160 or 200 mM, exhibited chromatin condensation (Table 1). Consistent with the enhancement by cycloheximide of the survival of *S. cerevisiae* to 40–80 mM acetic acid treatment, a decrease in the number of cells with chromatin condensation along the nuclear envelope was evident in the presence of cycloheximide (Table 1).

However, in many yeast cells treated with 120 mM acetic acid and in most cells treated with 160 mM, alterations typical of necrosis were observed by electron microscopy, namely an extensive cell disorganization with most intracellular structures destroyed (shown in Fig. 2c for 160 mM).

In yeasts, as in mammalian cells, phosphatidylserine has an asymmetric distribution in the lipid bilayer of the cytoplasmic membrane (Cerbón & Calderón, 1991). The exposure of phosphatidylserine at the outer surface of the cytoplasmic membrane occurs at the early stages of apoptosis (Martin *et al.*, 1995) when membrane integrity is still retained. Therefore, with the technique used in the present study, which includes simultaneous staining with FITC-coupled annexin V and the membrane impermeant fluorochrome PI, those early apoptotic cells only stain green, from FITC, indicating the presence of phosphatidylserine at the outer surface of the plasma membrane [annexin V (+), PI (−), shown in Fig. 3a for 20 mM acetic acid]. Numbers of annexin V (+), PI (−) cells were maximal at 40–80 mM acetic acid (Table 2). Yeast cells in more advanced apoptotic stages, or necrotic cells, stain green and red due to the inability

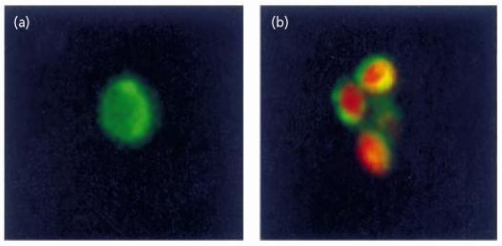


Fig. 3. Annexin V–PI staining of *S. cerevisiae* exponentially growing cells exposed to 20 (a) or 120 (b) mM acetic acid for 200 min. The cell in Fig. 3(a) is stained only in green by annexin V [annexin V (+)], and not in red [PI (−)], indicating that it is in an early stage of apoptosis. In contrast, the cells in Fig. 3(b) are stained in both green [annexin V (+)] and red [PI (+)], indicating that they are in advanced apoptosis or in necrosis.

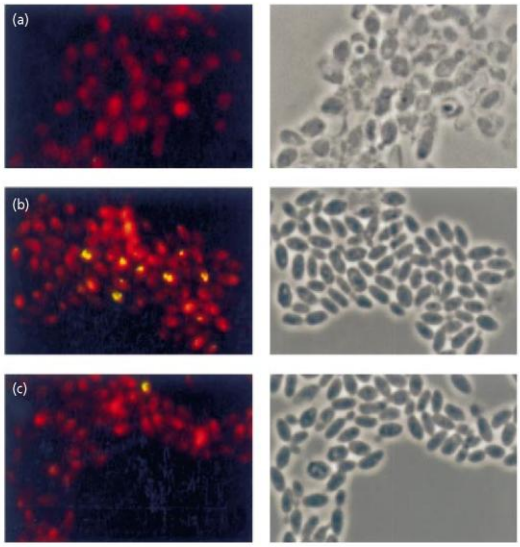


Fig. 4. Left panel: TUNEL reaction after 200 min of treatment of exponentially growing *S. cerevisiae* cells with 0 (a), 40 (b) or 120 (c) mM acetic acid. Right panel: phase-contrast of the fields in the left panel.

of the cell membrane to exclude PI [annexin V (+), PI (+), shown in Fig. 3b for 120 mM acid]. Numbers of annexin V (+), PI (+) cells were maximal at 80 and 120 mM acetic acid (Table 2). In the 120 mM sample, most of the annexin-V-negative cells were PI-positive [annexin V (−), PI (+)]. Untreated cells did not exhibit a positive annexin V reaction or PI staining [annexin V (−), PI (−)].

Treatment of *S. cerevisiae* with 20, 40 and 80 mM acetic acid resulted in a TUNEL-positive phenotype (yellow nuclear fluorescence as the result of superimposition of green and red fluorescence due to simultaneous staining with TUNEL and PI, as shown in Fig. 4b for 40 mM), indicating the occurrence of DNA strand breaks. The percentage of cells displaying a positive TUNEL staining increased with the acetic acid concentration (19·0% and 51·5% of TUNEL-positive cells were counted in preparations treated with 40 and 80 mM acid, respectively). With 120 mM acetic acid, a few cells (5·7%) showed a positive TUNEL reaction (Fig. 4c). Incubation with acetic acid concentrations from 160 to 200 mM resulted in no detectable TUNEL staining. Control cells maintained in YEPD medium, pH 3·0, for 200 min in the absence of acetic acid showed a negative TUNEL staining (Fig. 4a).

For reasons not yet completely understood, cells exposed to cycloheximide (with or without acetic acid) and then stained by the annexin V or TUNEL reactions displayed a bright yellow fluorescent background that made it impossible to analyse the effect of the inhibitor on the two apoptotic markers.

Preliminary results have shown that the apoptotic markers referred to above are present, in a concentration-dependent way, in samples exposed to 20–120 mM acetic acid for time periods shorter than the 200 min used in the present study.

DISCUSSION

Although inhibitors of protein synthesis may, in some cases, prevent the anti-apoptotic cascade (Skulachev, 1999b), it has been reported that in many cell types apoptosis can be prevented by the inhibition of protein synthesis (Hiraoka *et al.*, 1997; Sánchez *et al.*, 1997). Recently, it was described in *S. cerevisiae* that cycloheximide prevented the apoptotic effects of H_2O_2 (Madeo *et al.*, 1999). In the present study, it is shown that, in this species, exposure to 20–80 mM acetic acid for 200 min induces a programmed cell death pathway that requires the functionality of the protein synthesis machinery. This conclusion was based on the observation that cycloheximide inhibited cell death induced by acetic acid at that low concentration range but not at the higher acid concentrations. Although with 120 mM it was still possible to detect this inhibition, it occurred in a very small sub-population, more than 99·86% of the cells remaining non-viable regardless of the presence of cycloheximide.

The mode of programmed cell death under acetic acid stress conditions was further characterized by the examination of cell death markers that are typical of apoptosis. The evidence obtained indicates that acetic

acid, like H_2O_2 (Madeo *et al.*, 1999), can trigger an apoptotic phenotype in *S. cerevisiae*. The following concentration-dependent changes were observed after treatment with acetic acid: chromatin condensation along the nuclear envelope, exposure of phosphatidylserine at the outer surface of the yeast cytoplasmic membrane and formation of DNA strand breaks. The nuclear ultrastructural changes were significantly reduced when the treatment was carried out in the presence of cycloheximide, reinforcing our interpretation about the active nature of cell death induced by the lower concentration of acetic acid.

Even though a very small population with apoptotic markers was detected at 120 mM, the present results suggest that a non-active (necrotic) process is the predominant cell death mechanism in samples exposed for 200 min to acetic acid at concentrations above 80 mM. This interpretation is based on the following points: (i) mortality at 120 mM (or more) is not significantly inhibited by cycloheximide; (ii) the percentage of cells with apoptotic markers, induced by increasing concentrations of acid, increases with treatments up to 80 mM acid and then decreases; (iii) the percentage of cells with ultrastructural alterations typical of necrosis is highest and very extensive in samples treated with 120–160 mM acid. An apoptotic or necrotic phenotype was also reported for *S. cerevisiae* exposed for 200 min to low or high concentrations of H_2O_2, respectively (Madeo *et al.*, 1999).

Our data regarding the structural/morphological markers of apoptosis showed that the proportion of yeast cells with chromatin condensation induced by 40 mM acetic acid was higher than the proportion of cells exhibiting surface exposure of phosphatidylserine [annexin V (+), PI (−)] and DNA cleavage (TUNEL-positive). This observation indicates that, in our model, nuclear chromatin alteration precedes both phosphatidylserine exposures at the cytoplasmic membrane surface and significant endonuclease DNA cleavage. Similar observations have been reported in other models of apoptosis, i.e. chromatin condensation preceding DNA cleavage (Sun *et al.*, 1994) and phosphatidylserine exposure (Darzynkiewicz *et al.*, 1997).

The yeast *S. cerevisiae* has been used as a host to express proteins of the Bcl-2 family and hence to study the mechanisms underlying apoptosis in higher eukaryotes. Up to now, only one instance of an active cell death process in yeasts, independent of heterologous expression of pro-apoptotic proteins, has been reported, namely programmed cell death due to exposure of *S. cerevisiae* to H_2O_2 (Madeo *et al.*, 1999). In the present work, we have shown that the apoptotic phenotype in *S. cerevisiae* can also be induced by acetic acid, pointing to the possibility that this mode of cell death may be more generalized in yeasts and extended to other stress agents. It is known that ROS play a central role in the regulation of apoptosis at various levels (Madeo *et al.*, 1999). Further studies are in progress in order to evaluate ROS production in *S. cerevisiae* under the conditions where programmed cell death induced by acetic acid was detected. This would show if commitment of *S. cerevisiae* to a programmed cell death process in response to acetic acid is also mediated through a ROS-dependent apoptotic pathway.

ACKNOWLEDGEMENTS

The authors are grateful to Mrs Paula Macedo and Professor Jorge Pedrosa for expert assistance in the electron microscopy analysis and in the TUNEL assay, respectively. P.L. has a fellowship from PRAXIS XXI (Fundação da Ciência e Tecnologia, Portugal).

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Received 29 January 2001; revised 26 March 2001; accepted 14 May 2001.

Chapter 3

Assessment of mitochondrial membrane potential in yeast cell populations by flow cytometry

Microbiology, 147: 3335-3343 (2001)

Microbiology (2001), **147**, 3335–3343 Printed in Great Britain

Assessment of mitochondrial membrane potential in yeast cell populations by flow cytometry

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In yeast the use of rhodamine 123 (Rh123) has been restricted to the evaluation of mitochondrial respiratory function including the discrimination between respiratory-competent and -deficient cells. This study describes the optimization and validation of a low-concentration Rh123 staining protocol for the flow-cytometric assessment of mitochondrial membrane potential ($\Delta\Psi m$) changes in whole yeast cells. The optimized protocol was validated by the use of compounds that specifically affect mitochondrial energetics. Epifluorescence microscopy was used to monitor Rh123 distribution within the cell. Incubation of yeast cell suspensions with Rh123 (50 nM, 10 min) gave minimal non-specific binding and cytotoxicity of the dye. The ratio (R) between the green fluorescence and forward scatter (both measured as log values) was used to measure $\Delta\Psi m$ with only little dependence on cell 'volume' and mitochondrial concentration. Cells treated with mitochondrial membrane de- or hyper-polarizing agents displayed a decrease and an increase of R values respectively, indicating that changes of the Rh123 distribution in cells indicate variations in the $\Delta\Psi m$. Live and dead cells also displayed significantly different R values. The method described here allows assessment of $\Delta\Psi m$ changes in whole yeast cells in response to a given drug. Moreover, the relationship between drug effects and disorders of mitochondrial energetics might be addressed.

Keywords: mitochondrial functionality, flow cytometry, rhodamine 123, whole yeast cells

INTRODUCTION

In the eukaryotic cell ATP is provided mainly through mitochondrial oxidative phosphorylation. Such a mechanism is achieved by the respiratory electron-transport chain and by the ATP synthase complex, both localized at the mitochondrial inner membrane. In mitochondria, the energy released by the transfer of electrons is used to pump out H^+. Therefore, H^+ electrochemical potential (protonmotive force; Δp) is formed by a proton gradient (ΔpH) and an electrical gradient ($\Delta\Psi m$). To produce ATP, the ATP synthase complex mediates the H^+ flow back to the matrix and the energy is conserved in the phosphorylation of ADP to ATP. In fact, the Δp is one of the most important physiological parameters that reflects energy status and mitochondrial membrane transport. Direct assessment of Δp is not possible; thus it is necessary to evaluate separately the mitochondrial electrical potential ($\Delta\Psi m$) and the proton chemical gradient (ΔpH) (Chen, 1989).

In recent years, many probes for monitoring $\Delta\Psi m$ have been developed and described with increasing emphasis on membrane-permeable lipophilic cations. Mitochondria are the only organelles known to have a significant membrane potential, with a negative charge inside. Hence lipophilic compounds with delocalized positive charge may be accumulated by mitochondria to a much greater extent than by other organelles (Grinius *et al.*, 1970). These compounds can freely enter the cell by simple diffusion and although plasma membrane potential contributes to some accumulation into the cytoplasm, the dye concentration there would be much

Abbreviations: EFM, epifluorescence microscopy; FCM, flow cytometric microscopy; FS, forward scatter; R, ratio; Rh123, rhodamine 123; SS, side scatter.

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lower than that in the mitochondria. In fact, the Nernst equation predicts a $\Delta\Psi p$ of -60 mV and a $\Delta\Psi m$ of -180 mV with accumulation ratios of 10 and 1000 in the cytoplasm and mitochondria, respectively. Therefore, the probe concentration inside mitochondria will be 10000-fold higher than that in the extracellular medium (Chen, 1988).

Among the different fluorescent dyes available, rhodamine 123 (Rh123) is the one most frequently used for the assessment of $\Delta\Psi m$ in mammalian cells (Emaus *et al.*, 1986; Johnson *et al.*, 1981; Goldstein & Korczac, 1981; O'Connor *et al.*, 1988; Petit *et al.*, 1990; Juan *et al.*, 1994). Rh123 stains mitochondria directly, without passage through endocytotic vesicles and lysosomes (Chen *et al.*, 1981), and distributes electrophoretically into the mitochondrial matrix in response to $\Delta\Psi m$ (Emaus *et al.*, 1986). In yeast the use of that probe has been restricted to the evaluation of the mitochondrial (respiratory) function (Skowronek *et al.*, 1990; Lloyd *et al.*, 1996; Lloyd, 1999) and the detection of efflux pumps (Prudêncio *et al.*, 2000; Kolaczkowski *et al.*, 1996). The success of this cationic dye in probing $\Delta\Psi m$ in mammalian living cells is closely related to its lower toxicity than other fluorescent cations. However, depending on the concentration and the cell type, it can also be cytotoxic. So far Rh123 has been used at concentrations up to 10 μg ml^{-1} (Emaus *et al.*, 1986; Johnson *et al.*, 1981; Goldstein & Korczac, 1981; O'Connor *et al.*, 1988; Petit *et al.*, 1990; Juan *et al.*, 1994; Lloyd *et al.*, 1996).

The aim of the present work was to optimize a Rh123 staining protocol to assess qualitative changes of $\Delta\Psi m$ in whole yeast cells by flow cytometry. Flow cytometry provides a sophisticated technique for research on heterogeneous microbial populations to determine physiological characteristics on a single-cell basis.

METHODS

Micro-organisms and culture conditions. *Zygosaccharomyces bailii* ISA 1307, originally isolated from a continuous production plant of sparkling wine (Wium *et al.*, 1990), was used. *Saccharomyces cerevisiae* IGC 4072 was originally isolated from a sample of Fremivin, an industrial wine yeast distributed by Rapidase. *Saccharomyces cerevisiae* J-1-3 (*MAT α ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 aac1::LEU2 aac2::HIS3 aac3::URA3*) was kindly provided by Professor Jordan Kolarov (Department of Biochemistry, Faculty of Sciences, Comenius University, Bratislava, Slovakia). All strains were maintained on slants of YEPD medium containing yeast extract (0·5 %, w/v), peptone (1 %, w/v), glucose (2 %, w/v) and agar (2 %, w/v). Cells were grown in 250 ml flasks containing 100 ml synthetic medium (van Uden, 1967) with vitamins, oligoelements and 2 % (w/v) glucose (MGV). The cells were grown to early and late exponential phase in a water bath, with magnetic stirring (250 r.p.m.), at 26 °C.

Fluorochrome solutions. Concentrated stock solutions of Rh123 (500 μM; Molecular Probes) were prepared in 100 % ethanol and stored at −20 °C. Working solutions (25 μM) were prepared by diluting the stock solutions in 100 % ethanol and kept on ice in the dark to minimize degradation.

Preparation of yeast suspensions, flow cytometric (FCM) and epifluorescence microscopy (EFM) analysis. In control assays, cell suspensions were prepared from cultures at early and late exponential phase. Cells were harvested, centrifuged (1600 *g* for 4 min at 4 °C) and washed twice with ice-cold distilled water, then resuspended (about 1×10^6 cells ml^{-1}) in sterile commercial water (PARACELSIA), pH 6·0. It is generally accepted that this cell concentration allows an adequate data acquisition by flow cytometry. Control suspensions of killed cells were prepared by boiling the cell suspensions, prepared as above, for 10 min.

The Rh123 staining protocol was developed as described in Results. FCM analysis was performed on a EPICS XL-MCL (Beckman-Coulter) flow cytometer, equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. The green fluorescence was collected through a 488 nm blocking filter, a 550 nm long-pass dichroic and a 525 nm band-pass. Twenty thousand cells per sample were analysed. An acquisition protocol was defined to measure forward scatter (FS), side scatter (SS) and green fluorescence (FL1) on a four decades logarithmic scale. Green fluorescence was gated in a scattergram of log SS × log FS in order to include the subpopulation with the highest frequency and homogeneity in the fluorescence measurements. The data were analysed with the Multigraph software included in the system II acquisition software for the EPICS XL/XL-MCL version 1.0. The ratios between green fluorescence and FS were performed offline with Multiparameter Data Analysis Software, MULTIPLUS AV (Phoenix Flow Systems).

The kinetics of Rh123 uptake by the yeast cells was followed by flow cytometry. For this, green fluorescence acquisition over time of an unstained yeast cell suspension, was started and paused after 1 min. As quickly as possible, Rh123 was added to the cell suspension; the acquisition continued again and was followed for 30 min.

The EFM analyses were performed on a Leitz Laborlux S epifluorescence microscope equipped with a 50 W mercury lamp and a filter set (excitation filter BP 450–490, a beam splitter FT510 and an emission filter LP520). Samples of stained cell suspensions (20 μl) were placed between a slide and a cover slip after mixing with an equal volume of antifading reagent (Vectashield Mounting Medium for fluorescence H-1000; Vector Laboratories). The digital images were acquired with a 3CCD Colour Video Camera (SONY, DXC-9100P), a frame grabber (IMAGRAPH, IMASCAN/Chroma-P) and software for image archival and management (HOSPITRANS, Fotoscope version 1.0). In the EFM analysis the concentration of Rh123 used was 500 nM instead of 50 nM, due to the lower sensitivity of EFM compared with FCM. However, the dye to cell concentration ratio was the same as that used in FCM analysis.

Cytotoxicity assays of Rh123. Cytotoxicity of Rh123 was determined by estimating its effect on cell viability assessed by the plate count method. The c.f.u. were counted after 2 d incubation at 26 °C on YEPD agar plates.

Modification of mitochondrial membrane potential. To induce a decrease in the mitochondrial membrane potential ($\Delta\Psi m$), cells were treated for 10 min at room temperature prior to the Rh123 staining, with one of the following chemical agents (all from Sigma): the uncoupler of oxidative phosphorylation carbonyl cyanide *p*-(tri-fluoromethoxy)-phenyl-hydrazone (FCCP; 5, 10 and 15 μM), the electron-transport inhibitor sodium azide (NaN_3; 20 mM) or the potassium ionophore, valinomycin (8 μM). A decrease of $\Delta\Psi m$

was also achieved by cell starvation. For this purpose, cells from an overnight culture, prepared as described above, were resuspended in culture medium without glucose and incubated for 48 h in a water bath, with magnetic stirring (250 r.p.m.), at 26 °C.

Incubation of the cells with 2-deoxy-D-glucose (2-DOG; Sigma) was also carried out. This compound is a glucose analogue that competitively inhibits glucose transport and depletes cells of ATP. Cells were also incubated with 2-DOG (100 mM) at room temperature, for 10 min before Rh123 staining.

The ΔΨm was also evaluated in the presence of other agents that disrupt mitochondrial metabolism, namely oligomycin (0·4 μg ml^{-1}), an F_0-ATP-synthase inhibitor, and nigericin (5 μM), a dissipater of ΔpH (both from Sigma). These agents were added 10 min before Rh123 staining.

Stimulation of ΔΨm was achieved by incubation at room temperature with 100 or 150 mM glucose, or 5 mM succinate, 10 min before Rh123 staining. Incubation with glucose was carried out at pH 6·0 and incubation with succinate at pH 3·0. Both substrates were from Merck.

Reproducibility of the results. All experiments were repeated at least three times. The data reported are from one representative experiment.

RESULTS

The aim of the present work was to optimize a Rh123 staining protocol for the assessment of mitochondrial membrane potential (ΔΨm) in yeast. This protocol was designed to ensure that the total fluorescence level obtained reflected a relative measure of that functional parameter. It was taken into account that the flow cytometer measures, in each individual cell, the total amount of fluorescence and not the actual concentration of the dye. Consequently, cells with the same fluorochrome concentration but different relative volumes will show different levels of fluorescence and could be misinterpreted as cells displaying different ΔΨm values. Therefore, it was necessary to correlate the data on fluorescence intensity from individual cells with the relative cell volume estimated from the forward light scatter (Shapiro, 1994; Seligmann & Gallin, 1986). In addition, we attempted to i) identify other factors that could lead to different levels of fluorescence independent of changes in ΔΨm and ii) select the minimum concentration of the dye to minimize both non-specific binding and cytotoxicity.

Normalized Rh123 fluorescence

The analysis of biparametric histograms, plotting log SS against log FS, of cell suspensions of *Zygosaccharomyces bailii* or *Saccharomyces cerevisiae* revealed a marked heterogeneity, in both relative complexity and size. In the scattergram of a *Z. bailii* cell suspension (Fig. 1a) several subpopulations can be visualized. The subpopulation at the top right consists mainly of cell aggregates, since it disappeared after a brief ultrasonication of the cell suspension (data not shown). The subpopulation with the lowest scatter most probably corresponds to young cells that have been recently separated from their mother cells. The most numerous subpopulation is present in the middle of the scattergram. The monoparametric histogram of the total fluorescence of this subpopulation has a great coefficient of variation (Fig. 1b), and three different gated regions (A, B and C) can be defined in the corresponding biparametric histogram (Fig. 1a). Previous results on DNA distributions (Howlett & Avery, 1999; Fortuna *et al.*, 2000) showed that these regions are related to the different phases of the cell cycle; the A, B

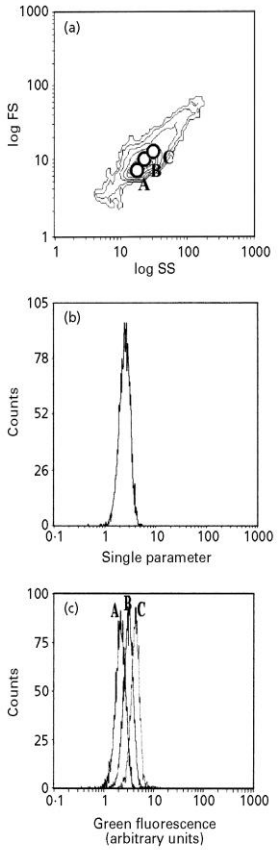


Fig. 1. (a) Scattergram (contour plot) for *Z. bailii* after Rh123 staining. (b) Green fluorescence histogram of *Z. bailii* after Rh123 staining of non-gated population. (c) Overlay of green fluorescence histograms of the same cell suspension of gated regions A, B and C in (a).

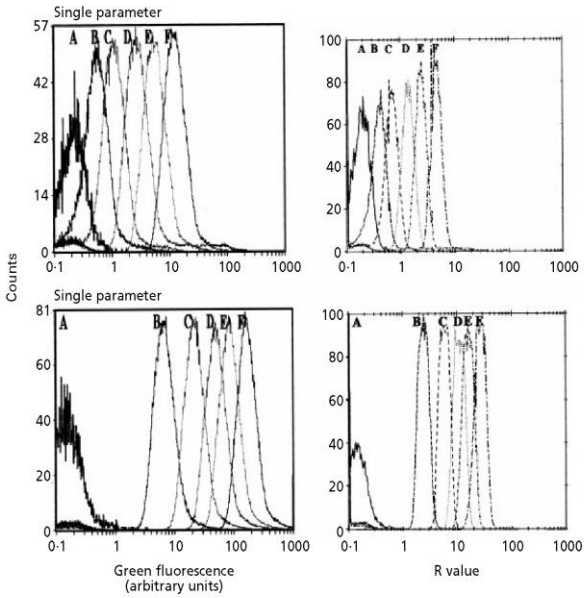


Fig. 2. Overlay of green fluorescence histograms (left panels) and respective R values (right panels) of *Z. bailii* before (A; autofluorescence) or after staining with different Rh123 concentrations (B, 10 nM; C, 25 nM; D, 50 nM; E, 100 nM and F, 200 nM) in a live cell control suspension (top panels) and a dead cell control suspension (bottom panels).

and C gated regions correspond to cells in phases G0/G1, S and G2/M, respectively. The fluorescence obtained in each gated region gives rise to three different distributions (Fig. 1c), pointing to the necessity to normalize the Rh123 fluorescence. Actually, cells with the same fluorochrome concentration but different relative volumes showed different levels of fluorescence. Normalization was achieved by representing in a monoparametric histogram the log of the green Rh123 fluorescence intensity, divided by the log of the FS signal. This ratio (R) was calculated by MULTIPLUS software (Phoenix Flow Systems).

Optimization of the staining conditions with Rh123: dye to cell concentration ratio, pH, temperature, incubation time and composition of the staining medium

To find the optimal ratio between the dye and cell concentration, the Rh123 concentration was varied from 10 to 200 nM while the cell concentration was kept constant (about 1×10^6 cells ml^{-1}). For the range of Rh123 concentration tested the mean fluorescence intensity increased stepwise with the dye concentration (Fig. 2, top panels) and no saturating kinetics were observed in the concentration range tested. This indicates that changes in the concentration of the probe produced distinct fluorescence levels for a given cell concentration. Thus, the maintenance of a constant ratio between dye and cell concentration, when comparing $\Delta\Psi m$ from different cell samples, turned out to be a critical step in the staining protocol. In addition, the mean fluorescence intensity of dead cells was seen to be much higher than for live cells, when a heat-killed cell suspension was stained with the same dye concentration (Fig. 2, bottom panels). The higher mean fluorescence level observed in dead yeast cells was also evident on EFM (Fig. 3). In fact, dead cells displayed a diffuse fluorescence in the cytoplasm whereas live cells displayed mitochondrial staining together with some slight non-specific fluorescence at the cell envelope.

The R values between green fluorescence and FS (both measured as log signals) for the live and dead cell suspensions are shown in Fig. 2. This estimation increased the homogeneity of the fluorescence and confirmed the advantage of normalizing this value.

Following these results, the Rh123 concentration selected was 50 nM rather than 25 or 10 nM. The former concentration value, although yielding a cell

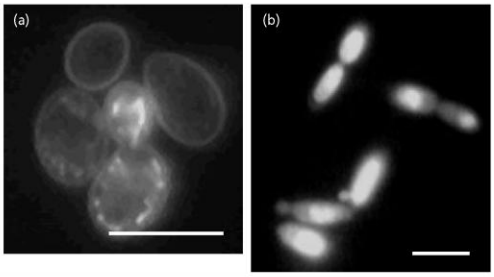


Fig. 3. Photomicrographs of *Z. bailii* live (a) and dead (b) cells, stained with Rh123 (500 nM). Bars, 10 µm.

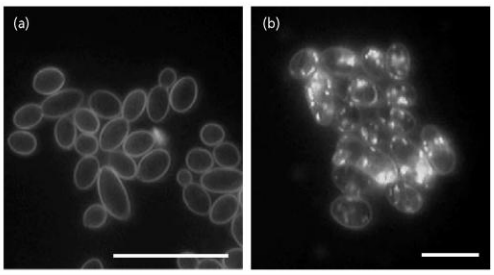


Fig. 4. Photomicrographs of *Z. bailii* cells stained with Rh123 (50 nM) after (a) 5 min incubation with Rh123 (b) 10 min incubation with Rh123. Bars, 10 µm.

fluorescence level distinct from autofluorescence, would allow a more sensitive detection of decreases in the mean fluorescence intensity and hence in $\Delta\Psi m$. Moreover, 50 nM Rh123 was not cytotoxic for either of the yeast species tested when assessed by counts of c.f.u. (data not shown).

The kinetics of Rh123 uptake by *S. cerevisiae* and *Z. bailii* was studied by FCM (data not shown). For both yeast species the uptake of the probe was very fast and the steady level of fluorescence was reached in about 10 min. Rh123 uptake was also followed by EFM (Fig. 4). By observation of the image obtained after 5 min incubation it was concluded that the observed rapid initial uptake corresponded to non-specific binding of the dye to the cell envelope. However, after 10 min Rh123 was electrophoretically accumulated into the mitochondria and gave a specific staining of this organelle. The non-specific binding of the dye, associated with the cell envelope, still observed at this incubation time is most probably negligible for the dye concentration used for FCM.

The mean fluorescence intensity of cells stained with Rh123 was constant for 30 min (data not shown). However, a decrease in fluorescence intensity was observed when cells were transferred to a dye-free medium. The observed leakage of the probe probably occurs due to the establishment of a new steady state distribution between the extra- and intra-cellular compartments.

The mean fluorescence intensity of cells stained with Rh123 at room temperature or at 37 °C was identical. Therefore, incubation at room temperature was selected.

Most of the buffers used in staining media have a complex ionic composition and different pH values that could directly interfere with Rh123 uptake. However, similar levels of fluorescence were observed (data not shown) for yeast cell suspensions from both species stained with Rh123 at pH 3·0 or 6·0, indicating that the mitochondrial accumulation of the dye is independent of the extracellular pH. Furthermore, based on the dependence of the plasma membrane potential ($\Delta\Psi p$) on the extracellular pH (van den Broeck, 1982), we concluded that mitochondrial accumulation of Rh123, under the optimized staining conditions is hardly affected by $\Delta\Psi p$.

To evaluate the influence of the medium composition on Rh123 staining, the fluorescence intensity of cells stained in mineral medium (pH 4·5, MGV medium), PBS (pH 7·0) or sterile water (pH 6·0) was compared. Higher levels of fluorescence were achieved when staining with Rh123 was carried out in sterile water (Fig. 5).

Effect of yeast cell growth phase on Rh123 staining

To evaluate the effect of cell growth phase on Rh123 staining, *Z. bailii* and *S. cerevisiae* cell suspensions prepared from cultures at early and late exponential

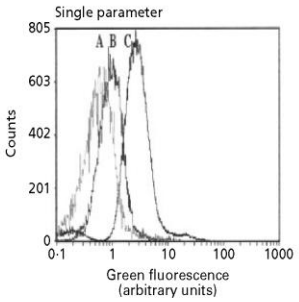


Fig. 5. Green fluorescence histograms after staining with Rh123 (50 nM) of *Z. bailii* cell suspensions in mineral medium (curve A), PBS buffer (curve B) and sterile water pH 6·0 (curve C).

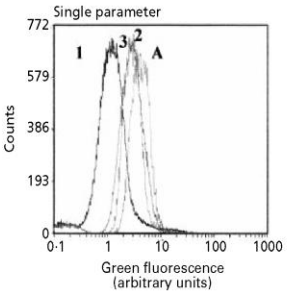


Fig. 6. Overlay of green fluorescence histograms obtained for *Z. bailii* cells stained with Rh123 (50 nM). Cells were untreated (curve A) or treated with sodium azide (curve 1) or 2-deoxy-D-glucose (curve 2) or starved (curve 3).

phase were stained under the conditions referred to above. The results obtained indicate that, whereas Rh123 staining of S. *cerevisiae* cells is growth-phase dependent, the same was not observed for *Z. bailii* cells. In *S. cerevisiae*, a concentration of 10 nM Rh123 is enough to produce the same fluorescence intensity in late exponential phase cells as 50 nM in early exponential phase cells. However, in *Z. bailii* a concentration of 50 nM of Rh123 appeared to be necessary to stain cells both from early and late exponential phases.

Definition of a simple staining protocol with Rh123

In summary, the conditions selected for the optimal staining with Rh123 and for assessment of mitochondrial membrane potential included the use of: (i) yeast cell suspensions in sterile water at a final cell concentration of 1×10^6 cells ml^{-1}; (ii) Rh123 at a final concentration of 10 nM for late exponential phase *S. cerevisiae* cells, and 50 nM for early exponential phase *S. cerevisiae* cells and *Z. bailii*, independent of the growth phase; (iii) staining for 10 min at room temperature in the dark.

Validation of the staining protocol with Rh123 for the assessment of $\Delta\Psi m$

To ascertain whether Rh123 accumulation in whole yeast cells was correlated to $\Delta\Psi m$, the effects of ionophores, specific inhibitors and substrates of mitochondrial respiration were tested. Valinomycin and FCCP [carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone] at the concentrations tested had no effect on Rh123 accumulation in yeast cells (data not shown). The same result was obtained by other authors and attributed to the possible formation of complexes between putative adducts and components of the inner mitochondrial membrane. Some of these complexes are freely accessible whatever the energy status of mitochondria but others are hidden in the energized state and freely accessible only in the de-energized state of the organelle (Salvioli *et al.*, 1997).

Cells exposed to sodium azide or 2-deoxy-D-glucose, or starved cells displayed a decrease in the Rh123 accumulation compatible with a decrease in $\Delta\Psi m$ (Fig. 6). Furthermore, the two former conditions did not affect the dye accumulation in heat-killed yeast cells (data not shown). Yeast cells treated with oligomycin and nigericin showed increased Rh123 staining (data not shown). These results are consistent with previous reports by other authors. Actually, oligomycin was used by Macouillard-Poulletier de Gannes *et al.* (1998) to mimic a decrease in cellular ATP demand (transition to state 4). In a similar way, these authors observed an increase in $\Delta\Psi m$ linked to the inhibition of ATP synthase by oligomycin associated with a significant increase in $DiOC_6(3)$ fluorescence.

Nigericin collapses ΔpH and hyperpolarizes (Johnson *et al.*, 1980, 1981) to a value close to that of the expected total electrochemical gradient (Nicholls, 1982). Petit *et al.* (1990) also observed an increase in the mean fluorescence value of isolated mitochondria stained with Rh123, under those conditions.

The addition of succinate, which provides reducing equivalents to the respiratory chain through succinate dehydrogenase, increased the accumulation of Rh123 in living cells.

Strain J-1-3 of *S. cerevisiae* is a null mutant for the ADP/ATP translocator and compared with the parental strain, produced a small but significant decrease of $\Delta\Psi m$ when assessed by Rh123 staining. Incubation of this mutant strain with sodium azide should abolish the mitochondrial respiratory function: $\Delta\Psi m$ should collapse and Rh123 should not accumulate in the mitochondria. Actually, under these conditions the J-1-3

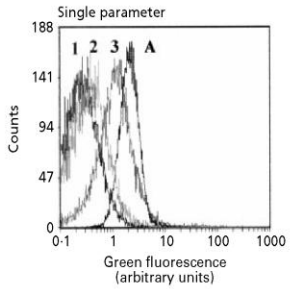


Fig. 7. Overlay of green fluorescence histograms of *S. cerevisiae* J-1-3 cells. Curve 1 shows autofluorescence. Cells were stained with Rh123 (50 nM) after (curve 2) or before (curve 3) treatment with sodium azide. Curve A shows *S. cerevisiae* wild-type cells stained under the same conditions.

mutant strain displayed a mean fluorescence intensity quite similar to the autofluorescence (Fig. 7).

DISCUSSION

In the present work, flow cytometry was exploited for the assessment, in whole yeast cells, of mitochondrial function as expressed by the membrane potential ($\Delta\Psi$m). The results showed that the relative cell volume affects the fluorescence measurements without revealing a real difference in $\Delta\Psi$m. Therefore, all the subsequent cytometric data were expressed as a ratio (R) value in an attempt to normalize the fluorescence intensity for the relative cell volume as estimated by the FS signal.

Most of the methods recently described for the assessment of $\Delta\Psi$m were derived from the protocol of Johnson *et al.* (1980), in which the Rh123 concentration used was 25 µM. More recently, Juan *et al.* (1994) used a Rh123 concentration of 250 nM to assess $\Delta\Psi$m in mouse liver cells. The Rh123 concentration selected in the present work (50 nM) is much lower than those described in the literature. At this concentration the cytotoxic effects of the probe and its non-specific binding to other intracellular organelles were eliminated or at least became negligible. The results obtained with the ADP/ATP null mutant strain of *S. cerevisiae* in the presence of sodium azide further confirmed this. In fact, in this strain under conditions where $\Delta\Psi$m is collapsed, Rh123 accumulation in the mitochondria was prevented and the level of staining corresponded almost to that of autofluorescence.

In both *S. cerevisiae* and *Z. bailii*, comparable levels of fluorescence intensity were obtained regardless of the incubation temperature (room temperature or 37 °C). In addition, and consistent with the results reported by other authors (Ronot *et al.*, 1986), the uptake of Rh123 (50 nM) at room temperature is rapid and equilibrium is reached after 10 min.

In the two yeast species tested under the optimized staining conditions, an increase or decrease in the R value of stained yeast cell suspensions was observed after treatment with hyperpolarizing (substrate of mitochondrial respiration) or depolarizing (ionophores or specific inhibitors of respiration) agents, respectively. This confirmed that Rh123 fluorescence changes reflect changes of $\Delta\Psi$m, as has been described in intact animal cells (Juan *et al.*, 1994).

It has been discussed in previous work that alterations of $\Delta\Psi$m may not be the only cause of Rh123 fluorescence changes. In fact, changes in mitochondrial Rh123 accumulation can be independent of $\Delta\Psi$m and reflect changes in either mitochondrial mass or the cytoplasmic concentration of the probe. An example of the former situation has been reported for an ADP/ATP translocator mutant (Petit *et al.*, 1996). Such a mutant has a nearly normal $\Delta\Psi$m; however, the decrease observed in $\Delta\Psi$m by Rh123 staining is associated with a decrease in mitochondrial mass, assessed by cardiolipin staining (by nonyl acridine orange dye). This aspect further reinforces the need to complement $\Delta\Psi$m studies with a mitochondrial structure probe when changes in the cellular content of mitochondria are suspected to occur. Moreover, alterations of the Rh123 cytoplasmic concentration might cause fluorescence alterations independently of $\Delta\Psi$m (Bernardi *et al.*, 2001). These alterations can be elicited either by the activity of multidrug resistance pumps for Rh123 or changes in the plasma membrane potential ($\Delta\Psi$p). Elimination of the interference of active efflux pumps on $\Delta\Psi$m determinations by the optimized Rh123 staining protocol is difficult since it would imply the knowledge for all the active systems, that use Rh123 as substrate, of the respective affinity constants. Actually, these data are only available in *S. cerevisiae*, and there only for specific active efflux systems (Kolaczkowski *et al.*, 1996). That mitochondrial accumulation of Rh123 was independent of the extracellular pH eliminates $\Delta\Psi$p interference on $\Delta\Psi$m determinations.

Salvioli *et al.* (2000) observed that in a given cell only some mitochondria were polarized, indicating heterogeneous behaviour at the mitochondrial level. If observed in yeast, this further complicates $\Delta\Psi$m measurements.

The different Rh123 concentrations required to stain optimally *S. cerevisiae* glucose-grown cells from early or late-exponential phase is most probably due to the well known shift from fermentative to respiratory metabolism (Pronk *et al.*, 1996), and to inherent and significant changes in the mitochondrial compartment (Visser, 1995) associated with the growth phase. The same is not found in *Z. bailii*. This observation is consistent with the higher relative proportion of respiration to fermentation (1:3) in *Z. bailii* (Fernandes *et al.*, 1997) as compared with *S. cerevisiae* (1:20) (Lagunas, 1986). In addition, and in contrast to the pro-mitochondria described in glucose-grown *S. cerevisiae* cells, *Z. bailii* displayed a higher proportion of mature mitochondria (P. Ludovico, F. Sansonetty, M. T. Silva, & M. Côrte-Real,

unpublished results). The results referred to above indicate that the application of the Rh123 staining protocol to other species should take into account possible differences or changes in the mass of the mitochondrial compartment with growth conditions.

The data obtained with heat-killed yeast suspensions suggest that Rh123 can be used as a probe to discriminate live from dead cells. This is similar to what has been reported by Juan *et al.* (1994) with animal cells. Actually, heat-killed yeast cells showed a diffuse and more intense fluorescence than untreated cells. It should be expected that in the former cells, Rh123 staining would produce a lower fluorescence level. A possible explanation could be attributed to the loss of mitochondrial membrane integrity in the dead cells and consequently to a non-localized distribution of Rh123. The observed behaviour might be determined by different fluorescence-quenching effects of the probe when interacting with the denatured and precipitated cell constituents.

It has been recently reported that *S. cerevisiae* becomes committed to a programmed cell death process in response to acetic acid (Ludovico *et al.*, 2001). The protocol described here will be crucial in the further evaluation of the involvement of mitochondrial function, namely of $\Delta\Psi$m changes in triggering such active cell death processes.

Presently one of the most generalized probes to assess mitochondrial membrane potential in mammalian cells is JC-1 (Seligmann & Gallin, 1986; Cossarizza *et al.*, 1993). In the presence of a high $\Delta\Psi$m, JC-1 forms J-aggregates that are associated with a large shift in the wavelength of fluorescence emission. However, one disadvantage of JC-1 compared with Rh123 is related to the speed of its responses to $\Delta\Psi$m changes, which are slower than those seen under equivalent conditions when using Rh123 (Haugland, 1996). This suggests that the slower redistribution of JC-1 could result in an underestimation of the actual rate of change of $\Delta\Psi$m.

In summary, optimization of the Rh123 staining conditions described here allowed the definition of a flow cytometric protocol for the qualitative assessment of rapid changes in $\Delta\Psi$m of yeasts. However, it should be emphasized that when the Rh123 staining protocol is to be applied to a new yeast species, preliminary experiments should be carried out to establish the optimal staining conditions.

ACKNOWLEDGEMENTS

We thank Professor Manuel T. Silva for his critical reading of the manuscript and all the helpful comments and suggestions. We thank Professor Jordan Kolarov who kindly provided us with the *S. cerevisiae* mutant strain. We thank Mr Alexandre Salvador and Ms Cristina Freitas for their helpful assistance in the acquisition of flow cytometric data.

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Received 1 June 2001; revised 13 July 2001; accepted 26 July 2001.

Chapter 4

Acetic acid induces a programmed cell death process in the food spoilage yeast Zygosaccharomyces bailii

Submitted (Appl. Environ. Microbiol.)

Acetic acid induces a programmed cell death process in the food spoilage yeast Zygosaccharomyces bailii

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Abstract

Recently we reported the occurrence of a programmed cell death (PCD) process, with an apoptotic phenotype in *Saccharomyces cerevisiae* cells exposed to particular concentrations of acetic acid. Herein, evidence is presented that acetic acid, although at higher concentrations, also induces in the food spoilage yeast *Zygosaccharomyces bailii* a PCD process that is inhibited by cycloheximide and that is accompanied by the following alterations typical of apoptosis: i) chromatin condensation along the nuclear envelope; ii) occurrence of DNA strand breaks, demonstrated by the TUNEL assay, and iii) decrease of mitochondrial membrane potential. These structural and biochemical alterations were observed in cells with preserved mitochondrial and plasma membrane integrity as revealed by Rh123 and PI staining, respectively. Transmission electron microscopy showed that *Z. bailii* cells under acid-induced PCD have mitochondrial ultrastructural changes, namely decrease of the cristae number, formation of myelinic bodies and swelling, but with structural integrity of their outer and inner membranes. TUNEL staining showed that exposure to acetic acid above 800 mM resulted in *Z. bailii* killing by a necrotic process. The observation of an active cell death process in *Z. bailii* further reinforces the concept of a physiological role of the PCD process in the normal yeast life cycle and rises the possibility of this mode of cell death being more generalized in yeast than previously considered. Moreover, advances in the understanding of mechanisms of death induced by acid stress in yeast will allow the development of a new categories of preservatives for food and beverages with the consequent reduction of economic losses in the food industry.

Introduction

Weak carboxylic acids, such as acetic, sorbic and benzoic acids, are generally regarded as safe anti-microbial additives, and have wide application as preservatives in acidic foods and beverages (Thomas and Davenport, 1985; Malfeito-Ferreira *et al.,* 1990; Fleet, 1992). However, many yeast are able to survive, adapt and even grow in the presence of the maximum levels of these preservatives permitted for use in foods. When compared with other fungi and bacteria, yeasts are more resistant to weak carboxylic acids. The elucidation of the cytotoxic effects induced by weak acids in yeast, which may compromise cell viability and ultimately result in cell death, can provide further insights into the mechanisms that determine different susceptibilities of yeast to weak carboxylic acids comparatively to other microorganisms and will allow the improvement or design of new strategies for food and beverage preservation. *Zygosaccharomyces bailii* is a food and beverage spoilage yeast that is typically characterized by a high tolerance to stressful acidic environments with ethanol (Thomas and Davenport, 1985; Sousa *et al*., 1998; Piper *et al*., 2001), where *Saccharomyces cerevisiae* cannot survive. Thus, these two yeast species have frequently been selected to serve as models for the study of yeast response to acid stress. In *S. cerevisiae* (Pinto *et al*., 1989) and in *Z. bailii* (Fernandes *et al*., 1999) acetic acid enhances cell death. However, in *Z. bailii* this effect is observed at much higher concentrations of acetic acid. In both species the enhancement of cell death was related with an intracellular acidification (Cardoso and Leão, 1992; Fernandes *et al.*, 1999). In *Z. bailii*, the mechanism (s) of death due to exposure to weak acids remains to be clarified.

Recently, we have shown that acetic acid in concentrations between 20 and 120 mM induce in exponentially growing *S. cerevisiae* cells a programmed cell death (PCD) process that displays the most common apoptotic hallmarks such as chromatin condensation along the nuclear envelope, exposure of phosphatidylserine on the surface of the cytoplasmic membrane and occurrence of internucleosomal DNA

fragmentation (Ludovico *et al.,* 2001a). Our previous study also showed that acetic acid, at concentrations above those that induce the PCD process, kill *S. cerevisiae* by a necrotic death process (Ludovico *et al.,* 2001a).

In the present work, that extends our previous studies on the modes of cell death induced by acetic acid in *S. cerevisiae*, we show that, like in this yeast, acetic acid also kills *Z. bailii* by a necrotic (higher acid concentrations) or by an apoptotic process (lower concentrations).

Methods

Microorganisms and Growth Conditions

Zygosaccharomyces bailii ISA 1307, originally isolated from a continuous production plant of sparkling wine (Wium *et al*., 1990) was used. The strain was maintained on slants of YEPD medium containing yeast extract (0.5 %, w/v), peptone (1 %, w/v), glucose (2 %, w/v) and agar (2 %, w/v). The cells were grown to mid-exponential phase in a water bath, with magnetic stirring (250 r.p.m.), at 26ºC, in 250 ml flasks containing 100 ml of synthetic medium (van Uden, 1967) with vitamins, oligoelements and 2 % (w/v) glucose (MGV).

Treatments with Acetic Acid and Preparation of Yeast Suspensions

Exponential phase cells were harvested, washed twice in ice-cold distilled sterile water and suspended (10^6 cells/ml) in sterile commercial water (PARACÉLSIA), (pH 3.0, set with HCl) with glucose 2 % (w/v) and in the absence or presence of 85, 170, 320, 515, 600, 800 or 1000 mM acetic acid. The treatments were carried out for 130 min at 26 °C with magnetic stirring (150 rpm). At time intervals, samples were taken, washed twice with ice-cold destilled water and processed for the different assays. Viability was determined by colony forming units (cfu) counts after 2 days of incubation at 26 ºC on YEPD agar plates.

Inhibition of Protein Synthesis

The inhibition of protein synthesis was performed by adding 50 μg/ml cycloheximide to the cell suspensions (10^7 cells/ml) at the same time as the different acetic acid

concentrations tested. Cycloheximide at this concentration was not cytotoxic after 130 minutes incubation, as assessed by colony forming units (cfu).

Transmission Electron Microscopy Analysis

Cells from control conditions (untreated) and treated with 320 mM acetic acid were washed with sodium cacodilate buffer (0.1 M, pH 7.2), and processed for transmission electron microscopy of ultrathin sections as described before (Ludovico *et al*., 2001a). Micrographs were taken with a Zeiss EM 10C electron microscope.

Terminal deoxynucleotidyl Transferase-mediated dUTP Nick End Labelling (TUNEL) and propidium iodide staining

DNA strand breaks were demonstrated by Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labelling (TUNEL) with the "In Situ Cell Death Detection Kit, Fluorescein", from Boehringer Mannheim (Indianapolis, IN) as described before (Ludovico *et al*., 2001a).

Fluorochrome Solutions and Staining Protocols

Assessment of mitochondrial membrane potential ($\Delta\Psi m$) in whole cells was performed by flow cytometry as described before by Ludovico *et al*. (2001b). A ratio (R value) between green fluorescence (due to mitochondrial Rh123 accumulation) and forward scatter (correlated with cell size) was used to detect depolarisation or hiperpolarisation of $\Delta\Psi m$ (Ludovico *et al.*, 2001b). Calculation of R values also allows the discrimination between live and dead cells since dead cells display a very high R value (Ludovico *et al.*, 2001b). Therefore, in cytotoxic assays of lethal

treatments of yeast, like acetic acid treatment, when cell death occurs, two subpopulations (corresponding to live and dead cells) appear with Rh123 staining. The evaluation of the ΔΨm, expressed by the R value, was done in the cell subpopulation with the lowest R value characteristic of live cells (Ludovico *et al.*, 2001b).

Plasma membrane integrity was assessed by propidium iodide (PI) staining. Cells with injured membranes incorporate PI, which then stains double strand nucleic acids (Haugland, 2000). The PI staining protocol we used was modified from de previous described protocol by De la Fuente *et al*. (1992). PI was added from a working solution (0.5 mg PI, Molecular Probes, in 10 ml of Tris-$MgCl_2$ buffer) to a final concentration of 6.7 μg/ml. The incubation was carried out at room temperature for 10 min in the dark.

Flow Cytometric Assays

Flow cytometric (FCM) analysis was performed on a EPICS XL-MCL (Beckman-Coulter Corporation, Hialeah, FL) flow cytometer, equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. The green and red fluorescences were collected through a 488 nm blocking filter, a 550 nm/long-pass dichroic with a 525 nm/band-pass and a 590nm/long-pass with a 610 nm/band-pass, respectively. Twenty thousand cells per sample were analysed. An acquisition protocol was defined to measure forward scatter (FS Log), side scatter (SS Log), green fluorescence (FL1 Log) and red fluorescence (FL3 Log) on a four decades logarithmic scale. The data were analysed with the Multigraph software included in the system II acquisition software for the EPICS XL/XL-MCL version 1.0. Ratios between green fluorescence and forward scatter were performed off line with Multiparameter Data Analysis Software, MULTIPLUS AV (Phoenix Flow Systems) as described by Ludovico *et al*., (2001b).

Reproducibility of the results

All the experiments were repeated at least three times independently, and the data reported are mean values.

Results

Acetic acid induces cell death at concentrations above 170 mM in Z. bailii

Exposure of *Zygosaccharomyces bailii* ISA 1307 to acetic acid concentrations above 170 mM, pH 3.0, resulted in cell death as revealed by counting colony forming units (Fig.1). These results are in accordance with previous reports (Cardoso, 1994; Pinto *et al*., 1989; Prudêncio *et al*., 1998).

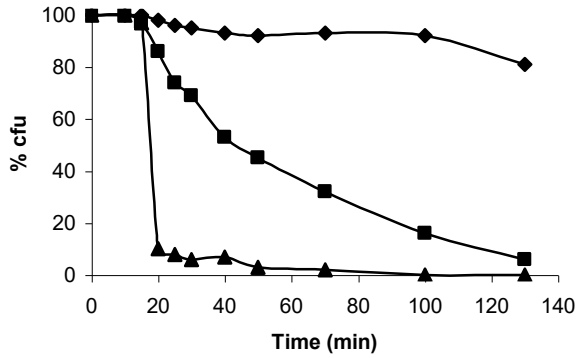


Figure 1. Relative survival (% of colony forming units, cfu, on YEPD agar plates; 100 % corresponds to the number of cfu at time zero) of *Zygosaccharomyces bailii* cells in the absence (◆) or presence of 320 mM (■) or 515 mM (▲) of acetic acid, pH3.0.

Acetic acid in the range of 320-800 mM induces a TUNEL-positive phenotype in Z. bailii

Following our recent observation (Ludovico *et al*., 2001a) that acetic acid at particular concentrations induces a PCD process in *Saccharomyces cerevisiae* we used the TUNEL reaction to investigate whether such a process also occurs with *Z. bailii*. TUNEL-positive cells (see for example Fig. 2 for 320 mM acetic acid) were seen after exposure to acetic acid at concentrations between 320-800 mM, suggesting that death due to those concentrations of acid would be due to a PCD process. On the other hand, in the samples of yeast cells killed by acetic acid above 800 mM, no TUNEL-positive cells were present, indicating that the killing process was by necrosis.

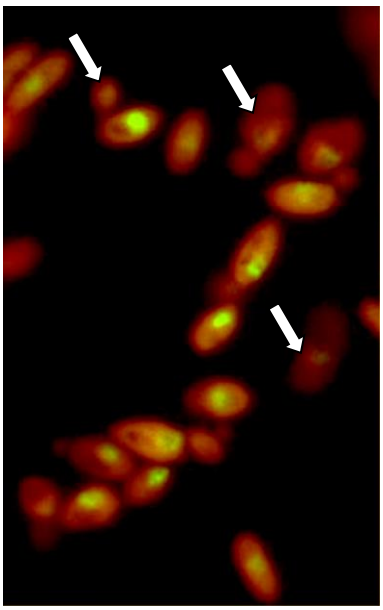


Figure 2. TUNEL reaction after 130 min treatment of exponentially growing *Zygosaccharomyces bailii* cells with 320 mM acetic acid. TUNEL-negative cells are indicated by arrows.

To further investigate the PCD process induced by acetic acid in *Z. bailii* the following studies were carried out.

Cycloheximide inhibits Z. bailii *death induced by 170-515 mM acetic acid*

In order to address if the cell death process induced in *Z. bailii* by acetic acid was an active process, which is characteristic of apoptotic death, we analysed the dependence of the acid-induced yeast killing on the *novo* protein synthesis. The presence of the inhibitor of protein synthesis, cycloheximide, attenuated the toxic effects of 170-515 mM acid, enhancing the survival percentage (Table 1), an indication that such a cell death is an active process.

Table 1 - Relative survival (% of colony forming units, cfu, on YEPD agar plates; 100 % corresponds to the number of cfu at time zero) of *Zygosaccharomyces bailii* incubated for 20 min with acetic acid in the absence or presence of cycloheximide (50 μg/ml). The values reported are the average of the two assays.

	Cycloheximide	
Acetic acid (mM)	absence	presence
0	100	100
175	93	100
320	80	95
515	10	30

Acetic acid induces mitochondrial dysfunction in Z. bailii

Since we had previously found that the PCD process induced by acetic acid in *S. cerevisiae* is mediated by a mitochondria-dependent pathway (Ludovico *et al.*, 2001c),

we assessed the mitochondrial function in *Z. bailii* cells exposed to acetic acid. This study was done by determination of the mitochondrial membrane potential (ΔΨm) using flow cytometry associated to the staining with a fluorescent dye, rhodamine (Rh123), as described before (Ludovico *et al.*, 2001b). Acetic acid treatment with 320 and 515 mM rapidly induced in *Z. bailii* cells a depolarisation of mitochondria. The decrease of ΔΨm was maintained from 10 to 130 minutes of treatment with acetic acid (data not shown).

Damage to cytoplasmic and mitochondrial membrane integrity induced by acetic acid is dose-dependent

Rh123 is a cationic dye that accumulates in yeast mitochondria with preserved membrane integrity according to its electrical potential (Ludovico *et al*., 2001b). When mitochondrial membrane integrity is affected, Rh123 is not able to accumulate in mitochondria producing a diffuse, high level, cytoplasmic green fluorescence (Ludovico *et al*., 2001b). On the other hand, cells with injured plasma membranes incorporate propidium iodide (PI (+) cells), which stains double strand nucleic acids (Haugland, 2000). Thus, these two fluorescent dyes were used to assess by flow cytometry the integrity of cytoplasmic and mitochondrial membranes, two cell structures that are not changed during the early phase of the apoptotic process.
Treatment of *Z. bailii* with 320 or 515 mM of acetic acid for 130 minutes did not induce loss of mitochondria membrane integrity as revealed by Rh123 staining (data not shown). Additionally, PI staining indicated that treatment of *Z. bailii* with 320 mM acetic acid for 130 minutes does not greatly affect the plasma membrane integrity, since only about 20 % of the cells were PI (+) (Fig. 3). The damaging action of acetic acid to plasma membrane integrity increased with the increase of acetic acid concentration. As shown in Fig. 3, the treatment of *Z. bailii* with 515 mM acetic acid for 130 minutes resulted in 50 % of PI (+) cells.

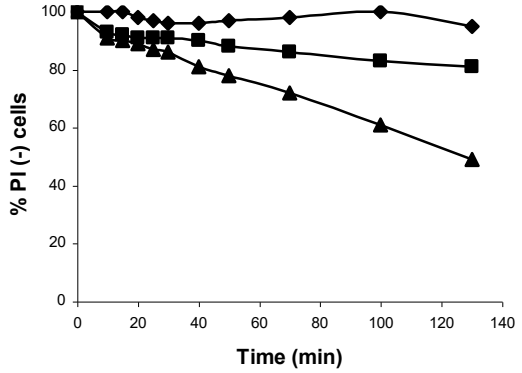


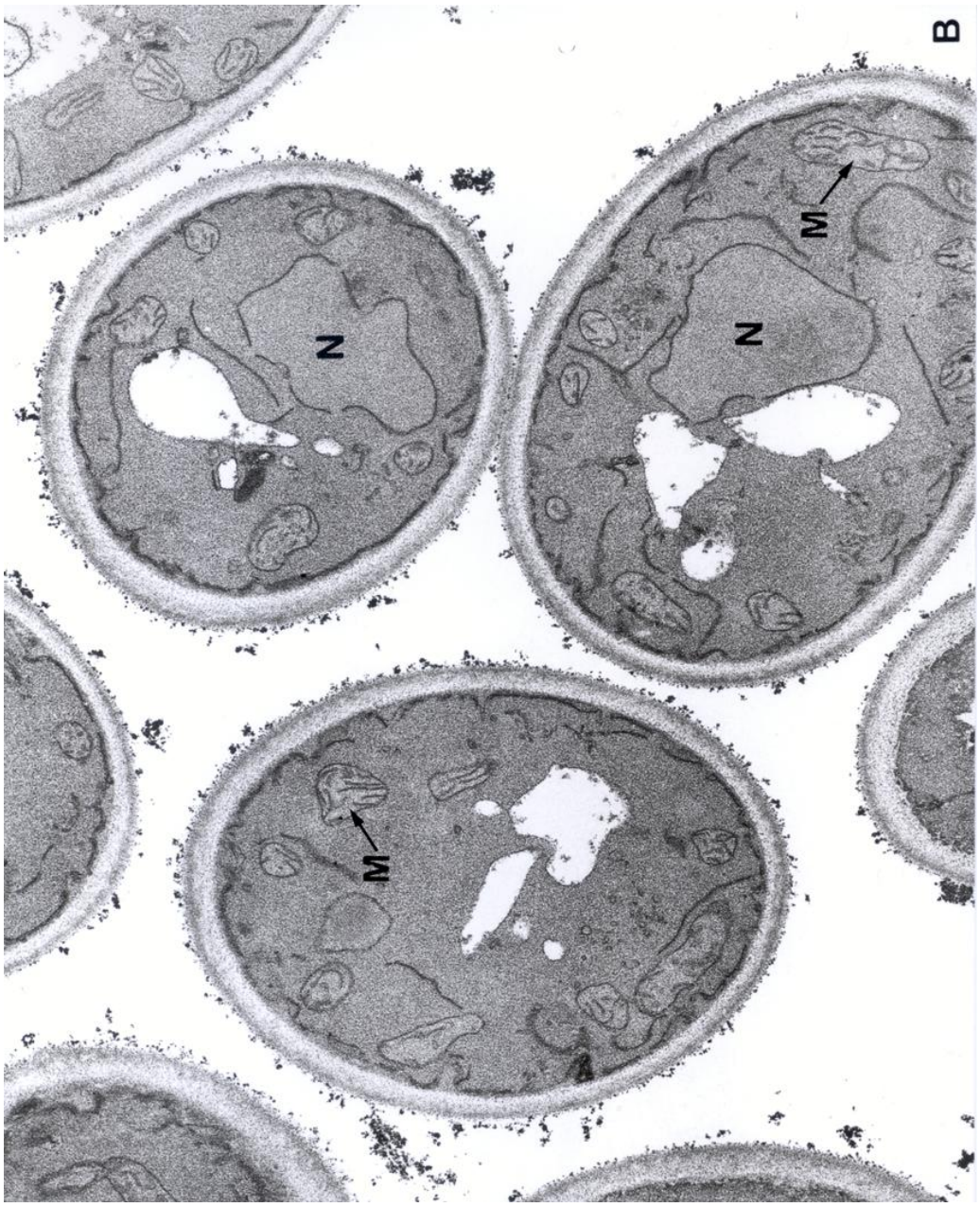
Figure 3. Percentage of PI (-) *Zygosaccharomyces bailii* cells after exposure to 0 mM (control) (◆), 320 mM (■) or 515 mM (▲) acetic acid.

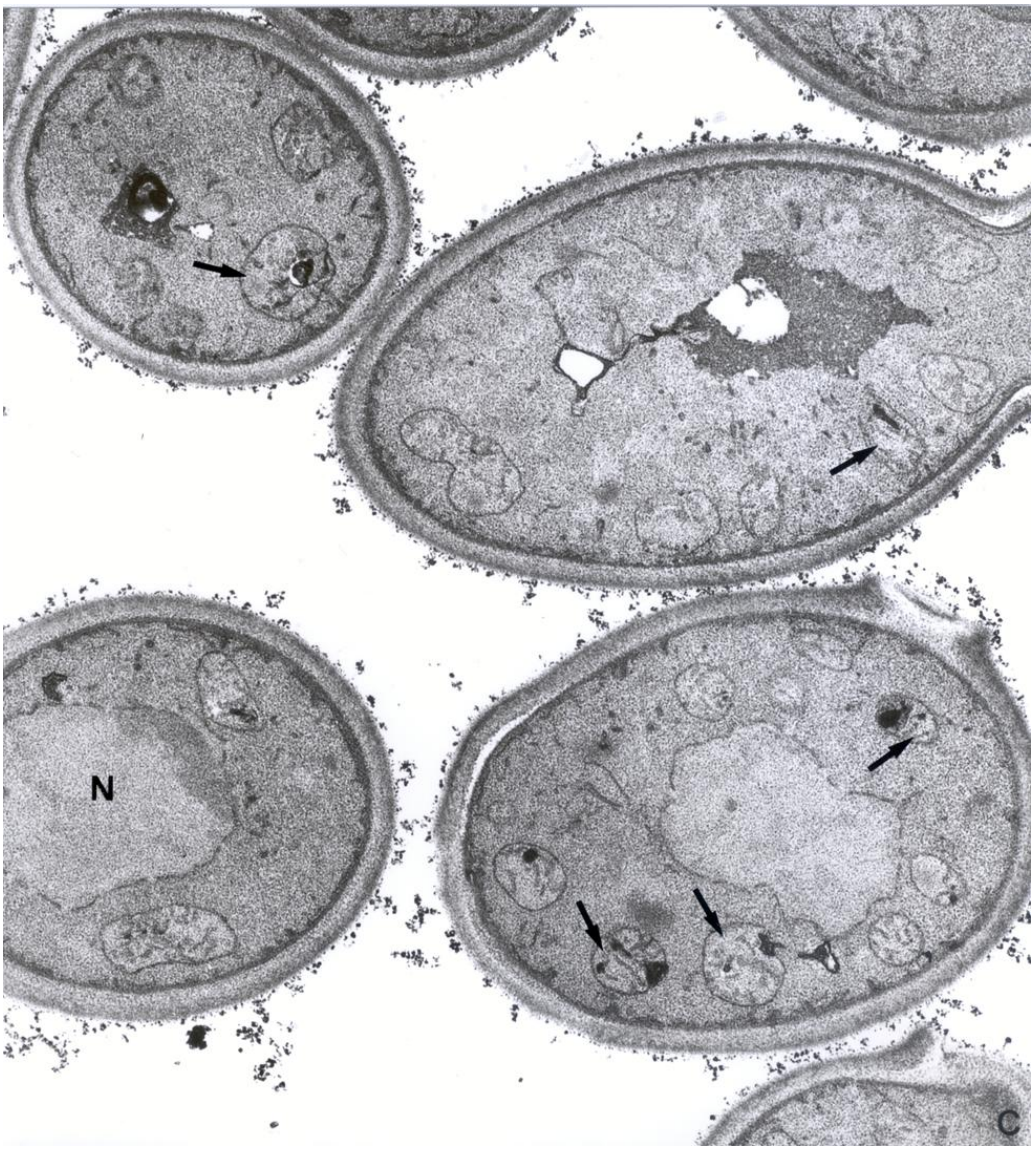
Acetic acid induces ultrastructural changes in Z. bailii *cells*

Treatment of *Z. bailii* exponentially-growing cells with acetic acid resulted in several utrastructural alterations. As soon as after 10 minutes of treatment with 320 mM acid, nuclei showed chromatin condensation along the nuclear envelope (Fig. 4 C, D and E). Interestingly, many mitochondria displayed ultrastructural alterations, namely a reduction of the cristae number, solute accumulation, the presence of myelinic bodies and swelling. However, mitochondrial membranes seem to maintain their structural integrity (Fig. 4 C, D, E and F) being in accordance with our Rh123 results. After 130 minutes of treatment the ultrastructural changes affected a higher percentage of cells, and with nuclei showing extensive chromatin condensation.

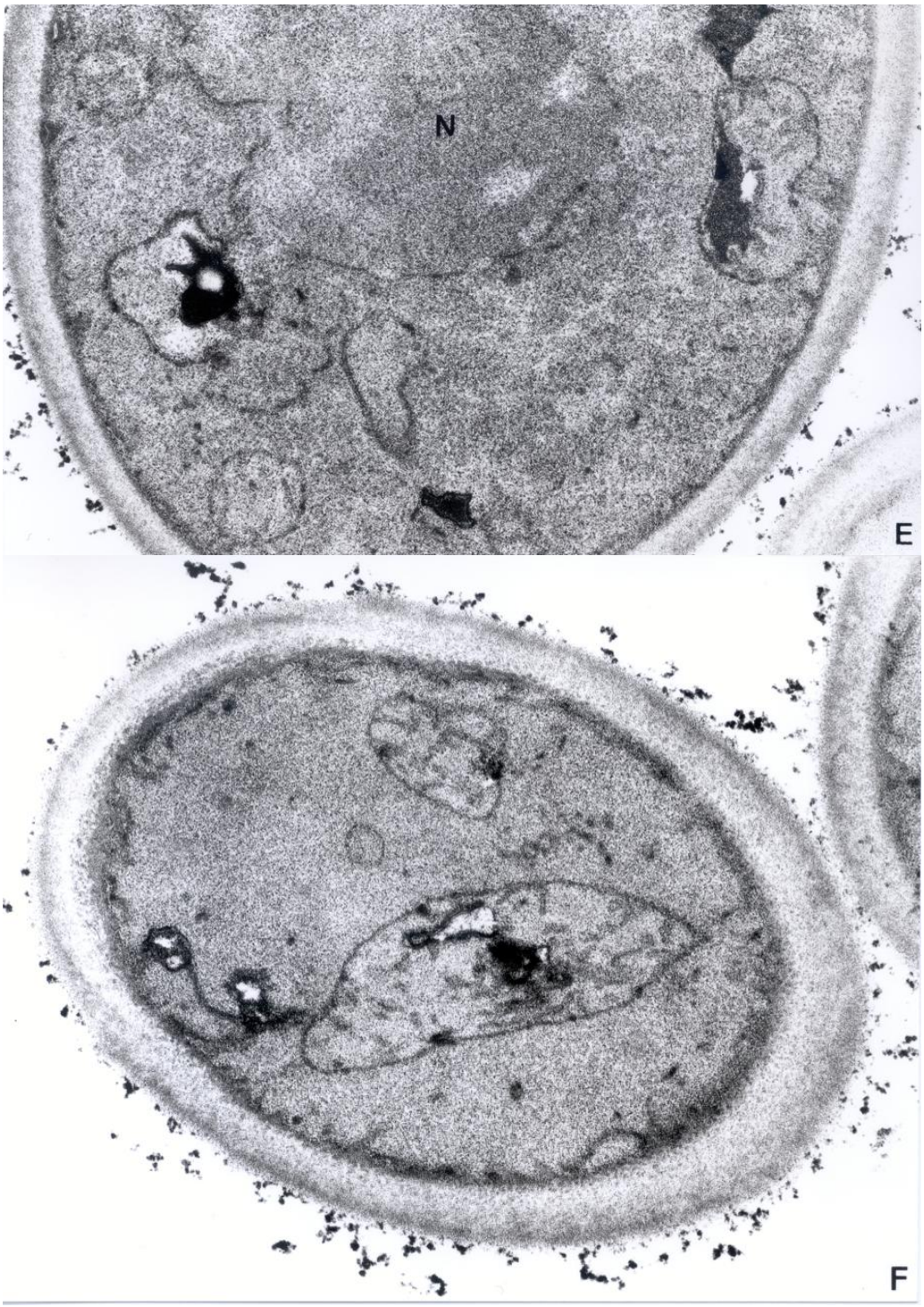
Figure 4. Electron micrographs of exponentially growing cells of *Zygosaccharomyces bailii.* A and B untreated control cells. Notice nuclei (N) and mitochondria (M) with a normal fine structure. C-F cells treated with 320 mM acetic acid for 10 min. C and D: Some nuclei (N) show chromatin condensation and most mitochondria are altered (arrows), with swelling, reduction of number of cristae and with myelinic bodies. E and F: details of altered mitochondria. Note in E chromatin condensation in the nucleus (N). A: X22000.B: X15800.C: X17000.D: X158000. E: X38000. F: X38000.


A
M
N


B
N
N
M
M


N
C


D
N
N


N
E
F

Discussion

Our recent report on the commitment of *S. cerevisiae* to a PCD process in response to exposure to specific concentrations of acetic acid raised the question if the more acid-resistant yeast *Z. bailii* could also commit suicide and recognize acetic acid as an apoptotic stimulus. Two alternative modes of cell death can be distinguished, namely programmed cell death and accidental cell death, generally defined as apoptosis and necrosis, respectively. Numerous methods have been developed to identify apoptotic and necrotic cells based on changes in cell morphology, plasma membrane structure and transport function, function of cell organelles and endonucleolytic DNA degradation (Darzynkiewicz *et al*., 1997). A cell triggered to undergo apoptosis activates a characteristic cascade of biochemical and molecular events including (see for e.g. Darzynkiewicz *et al*., 1997): i)- condensation of nuclear chromatin; ii)- activation of endonuclease(s) which preferentially cleave internucleosomal DNA; iii)- exposure of phosphatidylserine at the outer surface of cytoplasmic membrane. These alterations occur together with the preservation, at least during the initial phase of cell death, of plasma membrane structural integrity and function, and of mitochondrial and lysosomal integrity, although the mitochondrial membrane potential is markedly decreased (Castedo *et al.*, 1996; Darzynkiewicz *et al*., 1982; Lizard *et al*., 1995). In the present work data is presenting showing some of the above mentioned apoptotic features were found to occur in *Z. bailii* treated with particular concentrations of acetic acid. As referred to above, one of the most characteristic PCD hallmarks is internucleosomal DNA cleavage, which can be detected by the TUNEL reaction. This assay revealed that acetic acid induces in *Z. bailii*, similarly to *S. cerevisiae*, internucleosomal DNA fragmentation. The results point to the occurrence of a PCD process in *Z. bailii* as a response to particular concentrations of acetic acid, similarly with what recently have been reported for *S. cerevisiae* (Ludovico *et al*., 2001a). However, the process was found to occur for higher acetic acid concentrations (320-800 mM, as compared to 20-120 mM for *S. cerevisiae*), which is consistent with the

known higher resistance of *Z. bailii* comparatively to *S. cerevisiae*. Similarly to what was found for *S. cerevisiae* (Ludovico *et al*., 2001a), there is a concentration above which no TUNEL-positive cells were detected indicating the occurrence of a necrotic process.

Several additional observations confirmed that acetic acid in the range 320-800 mM induces a PCD process in *Z. bailii*, as follows:

i- Treatment with 170-515 mM acetic acid in the presence of cycloheximide showed that the cell death is dependent of the *novo* protein synthesis, being therefore an active process.

ii- Mitochondria integrity is preserved, as revealed by Rh123 staining, although with a marked decreased of $\Delta\Psi m$, during killing by 320 or 515 mM acid.

iii- Plasma membrane integrity, assayed by PI staining, is preserved in most of the cells for long periods of treatment with acid in concentrations in the range inducing PCD.

iv- The ultrastructural analysis of *Z. bailii* cells treated with 320 mM acetic acid revealed nuclear chromatin condensation and preservation of cellular organisation.

Although the nuclear chromatin condensation has already been reported in *S. cerevisiae* under PCD processes (Madeo *et al.,* 1997; Madeo *et al.,* 1999; Ludovico *et al.,* 2001a), the mitochondrial ultrastructural alterations detected in the present study were not found in those reports regarding *S. cerevisiae*, due probably to the low respiratory capacity of this yeast. Our recent results (Ludovico *et al*., 2001a) showed that, in the PCD process induced by acetic acid in *S. cerevisiae,* important molecular events occur in mitochondria, namely loss of cytochrome *c* and production of reactive oxygen species. Since it is conceivable that also in the PCD process induced by acetic acid in *Z. bailii* such mitochondrial molecular events are likely to occur, the mitochondrial ultrastructural alterations now seen in *Z. bailii* may well represent the morphological counterpart of these biochemical events.

The finding of the occurrence of a PCD process in *Z. bailii*, a food spoilage yeast that is implicated in a wide range of problems with heavy economic losses in food and beverages industries, will allow the improvement of new food preservation strategies.

Moreover, the observation of a PCD process in a yeast species other than *S. cerevisiae,* reinforces the previous suggestions for a likely physiological role of this process in the normal life cycle of yeast.

Acknowledgements

The authors are grateful to Mrs. Paula Macedo for expert assistance in the electron microscopy analysis. Paula Ludovico has a fellowship from PRAXIS XXI (Fundação da Ciência e Tecnologia, Portugal).

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Chapter 5

Cytochrome c release and mitochondria involvement in programmed cell death induced by acetic acid in Saccharomyces cerevisiae

Mol. Biol. Cell (in press)

Cytochrome c release and mitochondria involvement in programmed cell death induced by acetic acid in Saccharomyces cerevisiae

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Running title: Cytochrome c release in yeast apoptosis

Footnotes

1. Abbreviations used in this paper: COX, Cytochrome c oxidase; CytC, cytochrome c; MDAP, mitochondria-dependent apoptotic pathway; ΔΨm, mitochondria membrane potential; PCD, programmed cell death; PMS, post-mitochondrial supernatant; ROS, reactive oxygen species; TUNEL, TdT-mediated dUTP nick end labelling.

Summary

Evidence is presented that mitochondria are implicated in the previously described programmed cell death (PCD) process induced by acetic acid in *Saccharomyces cerevisiae*. In yeast cells undergoing a PCD process induced by acetic acid, translocation of cytochrome *c* (CytC) to the cytosol and reactive oxygen species production, two events known to be pro-apoptotic in mammals, were observed. Associated to these events, reduction in oxygen consumption and in mitochondrial membrane potential was found. Enzymatic assays showed that the activity of complex bc_1 was normal whereas that of cytochrome *c* oxidase (COX) was strongly decreased. This decrease is in accordance with the observed reduction in the amounts of COX II subunit and of cytochromes $a+a_3$. The acetic acid-induced PCD process was found to be independent of oxidative phosphorylation since it was not inhibited by oligomycin treatment. The inability of *S. cerevisiae* mutant strains (lacking mitochondrial DNA, heme lyase or ATPase) to undergo acetic acid-induced PCD and in the case of the ATPase mutant (knockout in *ATP10*) the absence of CytC release provides further evidence that the process is mediated by a Mitochondria-Dependent Apoptotic Pathway (MDAP). The understanding of the involvement of a MDAP in *S. cerevisiae* PCD process will be most useful in the further elucidation of an ancestral pathway common to PCD in metazoans.

Introduction

Programmed cell death (PCD), of which apoptosis is the most common morphological expression, is described as an orchestrated collapse of the cell. This process plays an important role in the normal development and homeostasis mechanisms of multicellular organisms. At least two major apoptotic pathways have been described in mammalian cells. One requiring the participation of mitochondria, called "intrinsic pathway", and another one in which mitochondria are bypassed and caspases are activated directly, called "extrinsic pathway" (Matsuyama *et al*., 2000; Hengartner, 2000). Regarding the mitochondrial pathway, two main events have been proposed as integral control elements in the cell's decision to dye, namely, the release of apoptogenic factors such as cytochrome *c* (CytC) and the production of reactive oxygen species (Liu *et al.*, 1996; Kluck *et al*., 1997; Pham *et al*., 2000). Release of CytC to the cytosol drives the assembly of a high-molecular weight complex, the mitochondrial apoptosome that activates caspases (Adrain and Martin, 2001). Translocation of CytC to the cytosol is, therefore, a pivotal event in apoptosis. CytC is a soluble protein loosely bound to the outer face of the inner mitochondrial membrane, and its release is associated with an interruption of the normal electron flow at the complex III site of the respiratory chain, that could divert electron transfer to the generation of superoxide (Cai and Jones, 1998). The mechanism by which CytC is released from mitochondria during apoptosis remains unknown. However, two competing models have been proposed, a volume-dependent mechanism, involving mitochondrial swelling and the rupture of the outer membrane, and a volume-independent mechanism, in which the permeability of the outer membrane is selectively altered (Matsuyama *et al*., 2000). Pavlov *et al.* (2001) reported, very recently, a new high conductance channel named mitochondrial apoptosis-induced channel (MAC) linked to apoptosis in mammalian cells and Bax expression in yeast, which is in agreement with the latter model. The authors propose this channel as a

candidate for the outer mitochondrial membrane pore through which CytC and possible other factors exit mitochondria during apoptosis.

Recently, we have shown that acetic acid (20-80 mM) induces death in exponential cells of *Saccharomyces cerevisiae* which displays the most common PCD hallmarks such as chromatin condensation along the nuclear envelope; exposure of phosphatidylserine on the outer surface of the cytoplasmic membrane and occurrence of DNA fragmentation (Ludovico *et al.,* 2001a). It was also shown that similar to hydrogen peroxide (Madeo *et al.,* 1999), acetic acid at high doses (above 120 mM) induces cell morphological changes typical of necrosis without apoptotic markers such as TUNEL-positive phenotype. Acetic acid is a normal end-product of fermentation carried out by *S. cerevisiae* and may be produced by contaminating acetic acid bacteria being therefore quite familiar to the yeast environment. In this context the occurrence of acetic acid-induced PCD points to a physiological role for such a cell death in yeast.

In the last few years the occurrence of a PCD process independent of Bax-expression and with an apoptotic phenotype in *S. cerevisiae* has been reported (Madeo *et al.,* 1997; Madeo *et al.,* 1999; Laun *et al.,* 2001; Ludovico *et al.,* 2001a; Yamaki *et al.,* 2001; Carratore *et al.,* 2002). However, only Yamaki and coworkers (2001) studied the involvement of mitochondria in such a cell death pathway. The authors observed that cell death mediated by deletion of histone chaperone *ASF1/CIA1* is associated to a decrease of mitochondrial membrane potential, dysfunction of mitochondrial ATPase and release of CytC to the cytoplasm, a phenotype that largely resembles mammalian apoptosis. Although controversial, there is a interpretation that mitochondria seem to be involved in Bax-induced cell death in yeast. Matusyama and coworkers (1998) reported that *ATP4*, which is a nuclear gene encoding subunit 4 of the yeast mitochondrial ATPase complex, is required for yeast death induced by Bax-expression. Additionally, as in mammalian cells, Bax-expression leads to CytC release from yeast mitochondria (Manon *et al.,* 1997; Kluck *et al.,* 2000). Manon and coworkers (1997) described that Bax-expression in yeast cells stimulates the release of

CytC to the cytosol and causes a concomitant decrease in the amount of COX complex.

In the present paper a large body of evidence supporting the involvement of mitochondria in the *S. cerevisiae* PCD process triggered by acetic acid is presented, indicating that, like in mammalian cells, the PCD in yeast can be mediated by a Mitochondria-Dependent Apoptotic Pathway (MDAP).

Materials and Methods

Microorganisms and growth conditions

The yeast *Saccharomyces cerevisiae* W303-1A (*MAT* a *ade*2 *leu*2 *his*3 *trp*1 *ura*3), the respective knockout in *ATP10* and *CYC3* genes and the isogenic derivative ρ^0 strain (lacking mitochondrial DNA) were used. These organisms were maintained on YEPD agar slants containing glucose (2 %, w/v), yeast extract (1 %, w/v), peptone (2 %, w/v) and agar (2 %, w/v). In experiments, the yeast cells were sub-cultured in liquid YEPD medium. The growth experiments were performed in 250 ml flasks containing a ratio 2:1 of air to liquid phase, and incubated on a mechanical shaker (150 rpm) at 26 °C.

Treatments with acetic acid and inhibition of protein synthesis or oxidative phosphorylation

Stationary phase cells were harvested and suspended (10^7cells/ml) in treatment medium (YEPD, pH 3.0, set with HCl) containing 0, 120, 140, 180, 200 and 240 mM acetic acid. The treatments were carried out for 200 minutes at 26 °C with magnetic stirring (150 rpm). Inhibition of protein synthesis or oxidative phophorylation was performed by adding 50 μg/ml cycloheximide (Merck) or 10 μM oligomycin (Matsuyama *et al*., 1998), respectively, at the same time as the different acetic acid concentrations tested. At these concentrations cycloheximide and oligomycin were not cytotoxic after 200 minutes incubation, as assessed by counting colony forming units (cfu). Viability was determined by cfu counts after 2 days of incubation at 26 ºC on YEPD agar plates. No further colonies appeared after that incubation period. In all the above experiments, the extracellular pH did not change during the incubations.

Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labelling (TUNEL) and propidium iodide staining

DNA strand breaks were demonstrated by Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labelling (TUNEL) with the "In Situ Cell Death Detection Kit, Fluorescein", from Boehringer Mannheim (Indianapolis, IN) as described before (Ludovico *et al*., 2001a).

Isolation of yeast mitochondria and mitochondrial respiratory chain activity measurements

Mitochondria were isolated from *S. cerevisiae* (W303-1A and *ATP10* mutant) cells grown to stationary phase, harvested and resuspended in YEPD, pH 3.0 in the absence or presence of 140 mM acetic acid for 200 min. Mitochondria were prepared essentially as described by Faye *et al.* (1974) except that Zymolyase 20,000 instead of Glusulase was used to digest the cell wall. The post-mitochondrial supernatant (supernatant after the first 12,000 rpm centrifugation) was kept for the detection of cytochrome *c* (CytC). Mitochondria were washed twice with 0.5 M sorbitol at 12,000 rpm for 15 min and suspended in 0.5 M sorbitol. Protein concentration was ascertained by the method published by Lowry *et al.* (1951).

Oxygen utilization in isolated mitochondria was measured polarographically in 1 ml of standard medium (0.5 M sorbitol, 20 mM K_2PO_4 pH 7.4, 1 mM EDTA) with a Clark oxygen electrode (model 5300: Yellow Springs Instruments Co., Yellow Springs Ohio), in a water-jacketed cell, magnetically stirred at room temperature. Twenty µg of mitochondrial protein were used to assay for NADH oxidase activity. Oxygen consumption was monitored using NADH (0.8 mM) as a substrate. In a set of reactions, the effect of exogenous CytC on the oxidative rate was checked out by

adding 10 µM horse CytC (Sigma, St Louis, MO) to the chamber. Respiration was inhibited by addition of 700 µM potassium cyanide (KCN).

Measurement of respiratory chain enzymatic activities in isolated mitochondria was performed essentially as described by Tzagoloff *et al.* (1975). Cytochrome *c* oxidase (COX) activity was measured at room temperature in 20 mM K_2HPO_4, pH 7.5; containing 65 µM reduced CytC. COX activity was assayed in mitochondria (10 µg protein) permeabilized with potassium deoxycholate (KDOC) to maximize the access of the substrate to the enzyme, by measuring oxidation of ferrocytochrome *c* at 550 nm. NADH-Cytochrome *c* reductase activity was measured in KDOC permeabilized mitochondria at room temperature in 10 mM K_2HPO_4, pH 7.5, containing 100 µM KCN by following the reduction of CytC (65 µM) at 550 nm in the presence of 1 M NADH.

Cytochromes spectra

Optical absorption spectra of mitochondrial cytochromes were obtained on an Aminco DW-2A dual wavelength scanning spectrophotometer. The spectrophotometer was operated in the split beam mode with 1 nm band pass. Mitochondria were extracted at a protein concentration of 5 mg/ml with potassium deoxycholate under conditions that quantitatively solubilize all the cytochromes (Tzagoloff *et al*., 1975). Difference spectra of the reduced (sodium dithionite) versus oxidized (potassium ferricyanide) extracts were recorded at room temperature. The α absorption bands corresponding to cytochromes *a* and a_3 have maxima at 603 nm. The maxima for cytochrome *b* and for cytochrome *c* and c_1 are 560 nm and 550 nm, respectively.

Cytochrome c distribution

The release of CytC from mitochondria to cytoplasm when *S. cerevisiae* (W303-1A and *ATP10* mutant) cells were grown in the presence or absence of acetic acid was checked out by Western blot detection of CytC in the subcellular compartments. Total

mitochondrial proteins (5, 10 or 20 µg) and the post-mitochondrial supernatant (PMS) were electrophoretically separated on 12.0 % SDS-polyacrylamide gels (Laemmli, 1970) and transferred to 0.2 µm nitrocellulose membranes. The membranes were blocked with 5 % (w/v) milk powder for at least 1 hour followed by the incubation for another hour with anti-yeast CytC polyclonal antibody (kind gift from Dr. R. Lill). The blots were then incubated in goat anti-rabbit IgG conjugated to horseradish peroxidase (Sigma). The SuperSignal chemiluminescent substrate kit (Pierce, Il, USA) was used for the final detection.

Steady-state levels of bc_1, complex COX and ATPase subunits

The detection of cytochrome *b*, COX subunit II and V, and ATPase subunit 6 were performed by Western blotting as described above for CytC detection, and using primary polyclonal antibodies against the yeast forms of the mentioned subunits. All the antibodies used were a kind gift of Dr. Alexander Tzagoloff (Columbia University, New York).

Assessment of mitochondrial membrane potential (ΔΨm) and of Reactive Oxygen Species (ROS) production

Assessment of ΔΨm in whole cells was performed by flow cytometry and epifluorescence microscopy as described by Ludovico *et al.* (2001b). The control suspensions of killed cells used both in the assessment of ΔΨm and of ROS production were prepared by boiling for 10 min the cell suspensions as referred by Ludovico *et al.* (2001b).

ROS production by mitochondria, was monitored by using the MitoTracker® Red CM-H_2XRos staining. The reduced version of MitoTracker Red CMXRos does not fluoresce until entering an actively respiring cell, where it is oxidized by ROS to a red fluorescent compound, which is sequestered in the mitochondria. Cells were harvested and then suspended in the treatment medium (10^7cells/ml). Before the addition of

acetic acid, cells were pre-loaded with 50 μg/ml of the dye for 20 minutes at 37 ºC. After the pre-loading the different acetic acid concentrations were added and the fluorescence was detected by flow cytometry and epifluorescence microscopy.

Flow cytometric analysis was performed in a basic FACS® Calibur (Beckton Dickinson) flow cytometer equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. Red fluorescence was collected through a 560 nm short-pass dichroic, a 640 nm long-pass and another 670 nm long-pass. Twenty thousand cells per sample were analysed at low flow rate. An acquisition protocol was defined to measure forward scatter (FS Log), side scatter (SS Log) and red fluorescence (FL3 Log) on a four decades logarithmic scale. Data were acquired and analysed with CELLQuest PRO 3.3 (Beckton Dickinson).

Results

Wild-type S. cerevisiae *stationary cells undergo a PCD process induced by acetic acid*

The study was focused on the role of mitochondria in the previously reported acetic acid induced programmed cell death (PCD) process in *Saccharomyces cerevisiae* W303-1A (Ludovico *et al.*, 2001a). Stationary cells rather than exponential cells were used for that purpose. Cells from stationary growth phase are more advantageous since they possess fully active mitochondria and display a higher mitochondrial mass. When exposed to different acetic acid concentrations at pH 3.0, and in the presence of glucose, *S. cerevisiae* stationary cells also committed to a PCD process. This conclusion is based on the detection of cell death accompanied by DNA strand breaks evaluated by the TUNEL assay (data not shown), an evident apoptotic marker, and by the fact that cycloheximide inhibited cell death (Fig. 1). Consistently with the recognized higher resistance of stationary cells to different stress agents, the effect was only observed for higher acetic acid concentrations. Actually, while acetic acid concentrations above 120 mM are necrotic for exponential cells, they induce a PCD process in stationary cells. In fact, 140 mM acetic acid concentration induces, after 200 min, about 50 % of loss of cell viability evaluated by cfu and about 30 % of TUNEL-positive cells. This concentration (140 mM) was selected to perform mitochondrial function analysis. Incubation with acetic acid concentrations above to 200 mM resulted in no detectable TUNEL staining (data not shown).

In order to evaluate whether the acetic acid-induced PCD in *S. cerevisiae* is affected by the inhibition of oxidative phosphorylation, the treatment with acetic acid was carried out in the presence of oligomycin. Figure 1 shows that cell death was not affected by the drug.

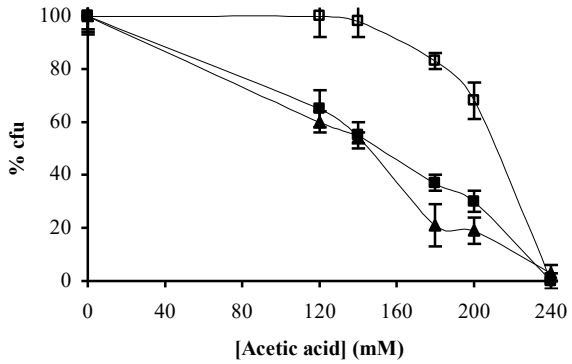


Figure 1. Programmed cell death of *S. cerevisiae* W303-1A stationary cells induced by acetic acid is partially inhibited by cycloheximide and is independent of oxidative phosphorylation. Relative survival (% of colony forming units, cfu, on YEPD agar plates; 100 % corresponds to the number of cfu at time zero) of cells incubated for 200 min with 140 mM acetic acid in the absence (■) or presence of cycloheximide (□) or oligomycin (▲).

Cytochrome c is translocated from mitochondria to the cytosol during the acetic-acid induced PCD process

To check if acetic acid-induced PCD process was accompanied by a release of CytC from mitochondria to cytosol, the levels of CytC in mitochondria and in the post-mitochondrial supernatant (PMS, containing soluble cytosolic proteins), from *S. cerevisiae* W303-1A cells undergoing acid-induced PCD, were detected by Western blot analysis. The amount of CytC present in mitochondria of cells treated with 140 mM acetic acid was decreased by a 2-3 fold compared with the mitochondria from untreated cells (Fig. 2). This portion of "lost" CytC in mitochondria from acetic acid treated cells was detected in its PMS, while no CytC was detected in the PMS of untreated control cells (Fig. 2). Levels of other mitochondrial proteins, such as COX subunits II and V were not detected in PMS (data not shown), although COX II was

found to be reduced in mitochondria (described hereafter). Decrease of the CytC amount in mitochondria was confirmed by cytochrome spectra analysis of mitochondria from treated and untreated cells. As shown in Figure 3A, there was some decrease in the amount of cytochromes $c+c_1$ extracted from the mitochondrial membranes.

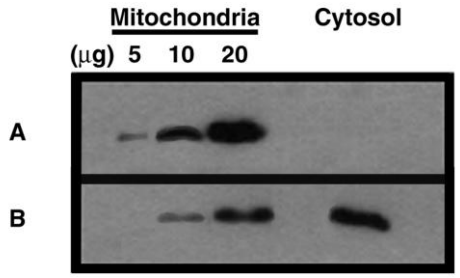


Figure 2. In *S. cerevisiae* W303-1A cytochrome *c* is released from mitochondria to the cytosol during PCD induced by acetic acid. CytC was detected by immunodetection in mitochondria (5, 10 and 20 μg of protein) and post-mitochondrial supernatants obtained from *S. cerevisiae* stationary untreated cells (A) or cells treated with 140 mM acetic acid (B).

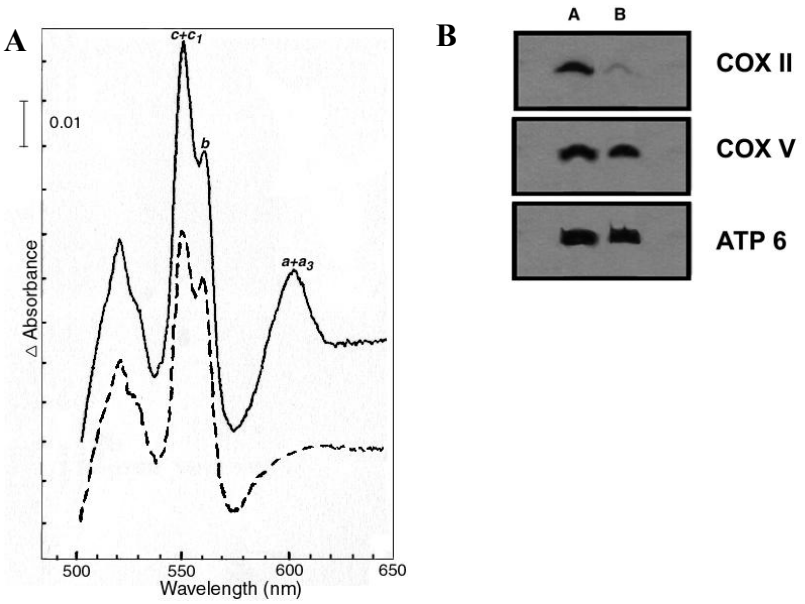


Figure 3. The release of CytC parallels a reduction of cytochrome *c* oxidase in acid treated cells. A. Cytochrome spectra. Cytochrome spectra of *S. cerevisiae* mitochondria isolated from untreated cells (full line) or from cells treated with 140 mM acetic acid (dashed line). The α absorption bands corresponding to cytochromes $a+a_3$ have maxima at 603 nm. The corresponding maximum for cytochrome *b* is 560 nm and for cytochrome $c+c_1$, 550 nm. **B. Steady-state levels of COX II subunit are decreased in mitochondria from acid-treated cells.** Immunodetection of COX II, V and ATP 6 subunits in mitochondrial membranes of *S. cerevisiae* untreated (A) or treated cells with 140 mM acetic acid (B).

The levels of CytC were also evaluated in mitochondria and in the PMS from *S. cerevisiae ATP10* mutant cells which do not undergo a PCD process induced by acetic acid as shown hereafter. CytC was not detected in PMS and it was found to be at a normal level in mitochondria, comparatively to untreated cells (data not shown).
These results indicate that CytC is specifically translocated from mitochondria to the cytosol during the acid-induced PCD.

Reactive Oxygen Species (ROS) are produced in mitochondria during the acetic acid-induced PCD process

S. cerevisiae W303-1A stationary cells stained with MitoTracker® Red CM-H_2Xros, and treated with 140 mM acetic acid, were analysed by flow cytometry. An increase in the red fluorescence indicative of cells with an increased mitochondrial ROS production could only be detected after 100 min of treatment (data not shown). The results obtained after 200 min of treatment showed a heterogeneous population and a second sub-population (about 45 %) with a higher mean fluorescence intensity (Fig. 4). The epifluorescence analysis showed that this sub-population displays a bright red fluorescence localized in mitochondria (data not shown). Moreover, killed cells displayed a higher red fluorescence corresponding to an unspecific cell staining (data not shown).

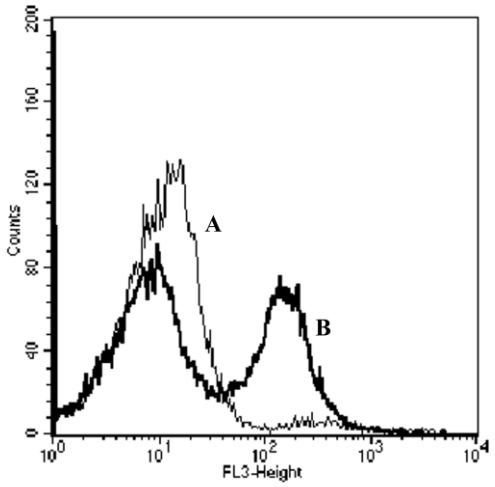


Figure 4. ROS are produced in mitochondria of *S. cerevisiae* W303-1A cells treated with acetic acid. Overlay of red fluorescence histograms obtained for cells stained with MitoTracker Red CM-H_2XRos. (A) untreated or (B) cells treated with 140 mM acetic acid.

Acetic acid-induced PCD is accompanied by mitochondrial alterations

The respiratory capacity of mitochondria isolated from control and acetic acid-treated cells was assayed polarographically by measuring oxygen uptake using NADH as substrate. The effect on respiration of exogenously added CytC was evaluated in a set of experiments. Results presented in Table I show that mitochondria isolated from cells treated with 140 mM acetic acid have a dramatically reduced oxygen consumption, with a decrease of nearly 75 % in NADH oxidase activity. Although an increase of about 2.8 fold of the oxygen consumption was observed after addition of CytC, the inability to fully restore the respiration rate with this addition suggested that an enzymatic portion of the respiratory chain could be intrinsically affected.

Table I

Respiratory activities of mitochondria from control and acetic acid treated cells. Mitochondria were assayed polarographically for NADH oxidase. The specific activities (expressed as nmol O_2 per min per mg of protein) were corrected for KCN insensitive respiration. Cytochrome oxidase activity (expressed as μmol cytochrome *c* oxidized per min per mg of mitochondrial protein) was assayed spectrophotometrically in mitochondria permeabilized with potassium deoxycholate (COX/DOC) by measuring oxidation of ferrocytochrome *c* at 550 nm. NADH cytochrome *c* reductase (expressed as μmol cytochrome *c* reduced per min per mg of mitochondrial protein) was also measured spectrophotometrically as described under "Experimental Procedures." The rates measured in at least two independent assays did not differ by more than 10%. The values reported are the average of the two assays.

Acetic Acid Treatment (mM)	NADH oxidase		NADH CytC Reductase	Cytochrome Oxidase
	-	+ CytC		
0	**367**	**550**	**0.602**	**2.925**
140	**68**	**192**	**0.645**	**1.552**

To establish the biochemical basis for the decrease in the respiratory activity of mitochondria, the NADH-CytC reductase and COX activities of isolated mitochondria from untreated and acid-treated cells, were measured. As shown in Table I, the treatment with 140 mM acetic acid resulted in a decrease of 50 % of COX activity in mitochondrial membranes, while NADH-CytC reductase activity was essentially identical to that obtained with mitochondria from untreated cells. These results confirm that COX complex was affected whereas complex bc_1 was unaffected by the treatment. Cytochromes spectra were recorded in isolated mitochondria (Fig. 3A) to clarify the observed respiratory chain alterations (Table I). In agreement with the observed lower COX activity, a decrease in the amount of cytochromes a+a_3 in mitochondrial membranes of cells treated with acetic acid was observed while the levels of cytochrome *b* were not affected (Fig. 3A).

When the COX subunits II and V were analysed by immunodetection of mitochondrial proteins obtained from cells treated with 140 mM acetic acid, the amount of COX II protein but not COX V was found to be lower than in the control (Fig. 3B). Additionally, the amount of cytochrome *b* from bc_1 complex and of subunit 6 of ATPase remained identical to control (Fig. 3B).

Some studies on mammalian apoptosis reported an increase in mitochondrial membrane potential (ΔΨm) after a lethal stimulus, with ΔΨm decreasing later in the death process (Vander Heiden *et al*., 1997; Vander Heiden *et al.*, 1999). Consistently with such observations, in our study, acetic acid treatment induced a transient slight hyperpolarization followed by a depolarisation (data not shown). However, although

with a lower ΔΨm, cells maintained the specific mitochondria staining indicating that mitochondria membrane integrity is still preserved (data not shown).

Mitochondrial respiration is essential for* S. cerevisiae *to undergo a PCD process induced by acetic acid

The requirement of mitochondria function in the PCD process induced by acetic acid was analysed by the study of three *S. cerevisiae* W303-1A mutant strains, namely the ρ^0, lacking mitochondrial DNA, the null *ATP10* mutant, deleted in an assembly factor of mitochondrial ATPase, and the null *CYC3* mutant, deleted in the gene encoding a heme lyase, essential for the covalent binding of the heme group to isoform 1 and 2 of apocytochrome *c* (Pearce and Sherman, 1995). It was observed that these mutant strains were more resistant to death induced by acetic acid, comparatively to the wild type strain. In addition, cycloheximide had no effect on survival (Fig. 5) and no TUNEL-positive cells were found, for any of the acetic acid concentrations tested, even for concentrations inducing a percentage of dead cells identical to that obtained for the wild-type (data not shown).

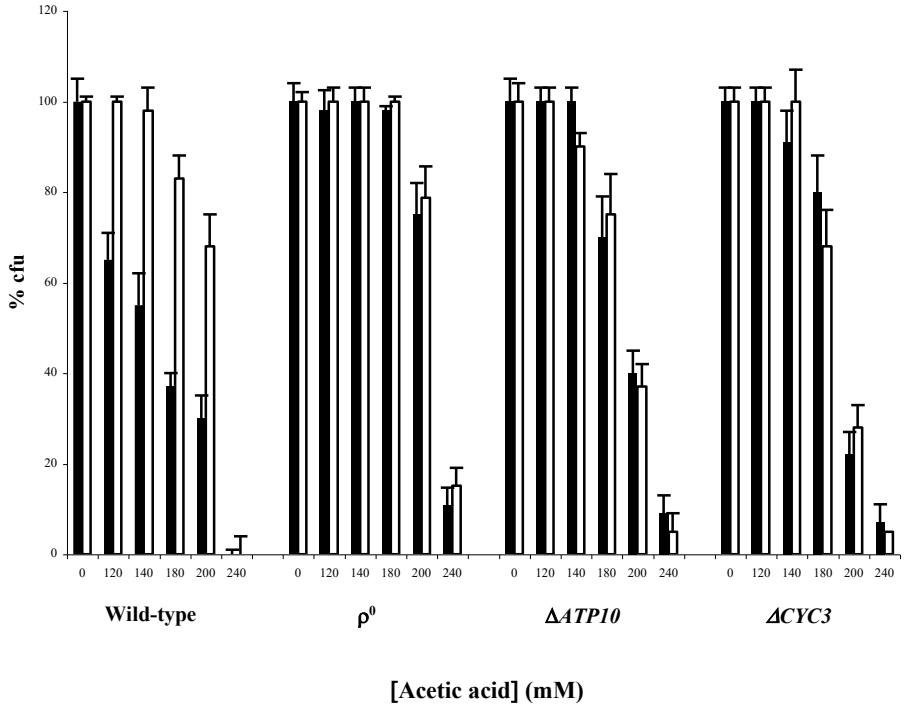


Figure 5. Respiratory deficient mutant cells of *S. cerevisiae* W303-1A are more resistant to acetic acid and acid-induced death is not inhibited by cycloheximide. Relative survival (% of colony forming units, cfu, on YEPD agar plates; 100 % corresponds to the number of cfu at time zero) of cells of wild-type, ρ^0 (lacking mitochondrial DNA), Δ*ATP10* (depleted of ATPase) and of Δ*CYC3* (depleted of CytC) cells of *S. cerevisiae* incubated for 200 min with acetic acid in the absence (black bars) or presence (white bars) of cycloheximide.

Discussion

Mitochondria have been implicated in mammalian PCD processes (Skulachev, 1996, 1998, 2000, 2001; Green and Reed, 1998; Yang *et al*., 1997; Kluck *et al*., 1997) being called the "cell death organelle". Data reported in this paper allowed us to conclude that mitochondria are, as well, implicated in the active cell death of *Saccharomyces cerevisiae* W303-1A induced by acetic acid. The strategy followed to analyse the possible role of mitochondria in that PCD process involved the use of stationary cells of *S. cerevisiae* which have a high mass of fully active mitochondria, and of selected *S. cerevisiae* strains with mutations affecting mitochondria. Regarding the first strategy, we found that *S. cerevisiae* W303-1A stationary cells, like exponential cells (Ludovico *et al*., 2001a), can organise its own death in response to acetic acid. Using those cells, the possibility was addressed that CytC could be in this unicellular eukaryont, as in mammalian cells, a direct or indirect apoptogenic factor. The analysis of CytC localization by immunodetection revealed that acetic acid triggers CytC release from mitochondria to the cytosol in *S. cerevisiae* W303-1A under PCD. CytC extrusion from mitochondria was also concluded from the results of cytochromes spectra analysis. Recently, Yamaki *et al.* (2001) observed a release of CytC to the cytoplasm associated to a phenotype that largely resembles mammalian apoptosis in *S. cerevisiae* cells lacking the histone chaperone *ASF1/CIA1*. The human homologue of this chaperone appears to be involved in the regulation of apoptosis. Manon and coworkers (1997), using heterologous expression of the pro-apoptotic protein Bax, had previously described CytC release in *S. cerevisiae*. In this context, since CytC release was found in the apoptosis-like cell death induced by Bax-expression, deletion of the histone chaperone *ASF1/CIA1* and acetic acid treatment (Ligr *et al.*, 1998; Ludovico *et al.*, 2001a; Yamaki *et al.,* 2001), we have an explanation why the apoptotic phenotype is identical in those three situations. It is conceivable that, when in the cytosol after its translocation from mitochondria, yeast CytC could work as an apoptogenic factor, as

reported for mammalian cells. In these cells the pro-apoptotic activity of cytoplasmic CytC is due to the activation of a caspase cascade. Very recently, Madeo and coworkers (2002) reported a caspase-related protease that regulates yeast apoptosis, named Yeast Caspase-1 (YCA-1). These authors showed that the disruption of *YCA-1* gene resulted in a higher survival to acetic acid treatment comparatively to the wild-type, whereas its overexpression led to the enhancement of cell death. In this context, when in cytosol, CytC may also work as an apoptogenic factor in *S. cerevisiae* through the activation of YCA-1, but further research is needed to get a complete frame of the interaction between these two proteins.

On the other hand, the release of CytC from mitochondria to the cytosol, being accompanied by the loss of the molecule at the mitochondria, leads to the occurrence of another pro-apoptotic event, namely the production of reactive oxygen species (ROS). Indeed, the observed increase in ROS production in *S. cerevisiae* W303-1A cells exposed to acetic acid may well be the consequence of the reduction in COX activity due to the loss of CytC from mitochondria. Whether loss of COX activity is a consequence of CytC release or a primary effect of acetic acid on the assembly of the enzyme remains to be studied although we favour the first possibility. The observed deficiency in COX activity could be responsible for an inhibition of the electron transfer with the accumulation of reducing equivalents in the middle portion of the electron transfer chain, and thus directing one-electron transfer to O_2, resulting in the production of superoxide (Cai and Jones, 1998). ROS production appears as an event common to all the instances of *S. cerevisiae* apoptotic phenotypes reported so far (Madeo *et al.,* 1997; Madeo *et al*., 1999; Fröhlich and Madeo 2000; Laun *et al*., 2001; Levine *et al.,* 2001; Narasimhan *et al.,* 2001; present results).

In mitochondria, CytC is an essential component of the respiratory chain acting as an electron carrier between complex bc_1 and COX complex. Therefore, the decrease in the amount of CytC in mitochondria of *S. cerevisiae* exposed to acetic acid must have other functional implications. The biochemical basis for the observed decrease in oxygen consumption and in $\Delta\Psi m$ is the important reduction in COX activity. Such a

reduction could be due to an inefficient COX assembly in the absence of CytC. The decrease of steady-state levels of COX II protein observed in mitochondria from acetic acid treated cells that have lost CytC and the impossibility to detect it in PMS indicate that, probably a degradation of the subunit occurred, as has been reported in mutants lacking CytC (Pearce and Sherman, 1995). In fact, COX II participates in CytC binding (Bisson *et al*., 1977) and the absence of CytC appears to destabilise COX II, making it susceptible to degradation (Pearce and Sherman, 1995). Manon *et al.,* (2001) reported very recently that Bax-induced CytC release is directly involved in the decrease of COX activity by activating the mitochondrial AAA-type protease Yme1p, which leads to COX II degradation.

A pattern of mitochondrial dysfunction identical to that above discussed was observed in *S. cerevisiae* cells expressing Bax (Manon *et al.,* 1997). With this new insight, a link between major alterations in the respiratory chain, namely decrease in the amount of CytC and reduction of the COX activity, and the PCD process in yeast, can be envisaged, being the first time that the apoptotic mitochondrial pathway was found to be activated in yeast wild type cells independent of the heterologous expression of Bax.

The comparison between main structural and functional cellular changes, including mitochocondrial changes, associated to *S. cerevisiae* programmed cell death induced by Bax-expression, by deletion of histone chaperone *ASF1/CIA1* or by acetic acid treatment is outlined in Table II.

Table II

Involvement of mitochondria in yeast programmed cell death. Differences between *S. cerevisiae* programmed cell death induced by Bax-expression, mediated by deletion of histone chaperone *ASF1/CIA1* (Yamaki *et al.*, 2001) and induced by acetic acid treatment (Ludovico *et al.*, 2001a; present results).

Cellular changes	**Bax-expression**	***asf1/cia1***	**Acetic acid**
Exposition of phosphatidylserine on the outer surface of the plasma membrane	+ (Ligr *et al.,* 1998)	nd	+
Chromatin condensation	+ (Ligr *et al.,* 1998)	+	+
DNA fragmentation	+ (Ligr *et al.,* 1998)	nd	+
Fragmentation of nuclei	nd	+	-
Fragmentation of chromatin	nd	+	+
Mitochondrial membrane-potential	Hyperpolarization (Gross *et al*., 2000)	Depolarization	Hyperpolarization followed by depolarization
Dysfunction of mitochondrial proton pump	nd	+	nd
Release of cytochrome *c*	+ (Manon *et al.,* 1997)	+	+
ROS production	+ (Gross *et al*., 2000)	nd	+
Decrease of cytochrome *c* oxidase	+ (Manon *et al.,* 1997)	nd	+
Mitochondrial apoptosis-induced channel (MAC) activity	+ (Pavlov *et al.,* 2001)	nd	nd

+, present; -, absent; nd, not determined.

The above discussed results, obtained with experiments using stationary cells of *S. cerevisiae* W303-1A, indicated that mitochondria would be involved in the PCD process induced by acetic acid through the release of CytC and ROS production. The use of three *S. cerevisiae* strains with mutations affecting mitochondrial respiratory chain function led to results that further indicate that mitochondria are required for that process. Although the literature concerning the mitochondria involvement in Bax-induced cell death is controversial (Greenhalf *et al*., 1996; Kissova *et al*., 2000), our results clearly demonstrate that a yeast cell depleted of mitochondrial DNA does not undergo a PCD process in response to acetic acid.

Supporting the interpretation that CytC exhibits apoptogenic properties in *S. cerevisiae*, the mutant in *CYC3* gene, which encodes a heme lyase essential for the heme binding to apocytochrome *c* (isoform 1 and 2) (Pearce and Sherman, 1995), did not display PCD when exposed to acetic acid. It is interesting to notice that in ρ^o strains, the *CYC1* gene (coding for iso-1-CytC) is 4-6 fold repressed, probably due to the lack of mitochondrial DNA and of mitochondrial respiratory activity (Epstein *et al.*, 2001), perhaps explaining why those strains do not undergo a PCD induced by acetic acid.

Matsuyama *et al.* (1998) related cell death induced by Bax-expression with the mitochondrial ATPase complex, namely with ATP subunit 4. Our results regarding the inability of *ATP10* mutant [*ATP10* codes a mitochondrial protein encoded by nuclear DNA that acts as an ATPase assembly factor (Paul *et al*., 2000)] to undergo PCD by exposure to acetic acid indicate that a fully assembled F_0F_1-ATPase is required for the PCD process. Moreover, and because it has been proposed that ATPase complex could be involved in the mechanism of CytC release in apoptotic mammalian cells (Matsuyama *et al*., 2000), our results with *ATP10* null mutant showing that treatment with 140 mM acetic acid does not result in PCD nor CytC release suggest that the ATPase complex would also be involved in the mechanism of CytC release from mitochondria during PCD induced by acetic acid in *S. cerevisiae*. Because of the absence of oxidative phosphorylation in the ρ^0 and *ATP10* mutant strains, it could be argued that the inability of those strains to develop the acid-induced PCD process

could be due to the failure in mitochondrial ATP generation. Nevertheless, this can not be the explanation since cells of wild-type strain are able to induce PCD even when mitochondrial ATP synthesis is inhibited by oligomycin.

In summary, the experimental evidence herein presented strongly supports that the PCD process induced by acetic acid in *S. cerevisiae* is mediated by a Mitochondria-Dependent Apoptotic Pathway (MDAP). Therefore, in yeast the pivotal role of mitochondria is related not only to the exogeneous cell death induced by Bax-expression but also, and more relevantly, to the endogenous PCD induced with acetic acid. These advances surpass the contemporary knowledge on PCD in unicellular eukaryotes and raise new questions regarding PCD in yeast, such as: i) is CytC an activating factor of YCA-1; ii) is CytC release an event common to all endogenous PCD processes? The answers to these questions will be most useful in the further elucidation of ancestral pathway(s) common to PCD in metazoans and will validate the yeast cell as the tool of choice in apoptosis research.

Acknowledgements

The stay of Paula Ludovico in Department of Biology at Columbia University, New York, was supported in part by "Fundação Luso-Americana para o Desenvolvimento". Paula Ludovico has a fellowship from PRAXIS XXI (Fundação da Ciência e Tecnologia, Portugal). Antoni Barrientos is a recipient of a grant MDACU01991001 from the Muscular Dystrophy Association. The authors would like to thank Prof. Vladimir Skulachev and Dr. Frank Madeo for their critical reading of the manuscript and all the helpful comments and suggestions.

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Chapter 6

A mitochondrial Apoptotic-Inducing Factor (AIF) equivalent is present in Saccharomyces cerevisiae *and is involved in apoptotic death*

Original manuscript

A mitochondrial Apoptotic-Inducing Factor (AIF) equivalent is present in Saccharomyces cerevisiae *and is involved in apoptotic death*

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Footnotes

1. Abbreviations used in this paper: AIF, apoptotic-inducing factor; CytC, cytochrome c; MDAP, mitochondria-dependent apoptotic pathway; ΔΨm, mitochondria membrane potential; PCD, programmed cell death; ROS, reactive oxygen species; TUNEL, TdT-mediated dUTP nick end labelling.

Abstract

Two major mitochondrial-dependent apoptotic pathways have been described in mammalian cells: a dependent- and an independent-caspase pathway. The latter requires the involvement of an Apoptotic-Inducing Factor (AIF). Recently, two distinct reports showed the involvement of cytochrome *c* release and of yeast caspase-1 (Yca1) in the programmed cell death in *Saccharomyces cerevisiae* indicating the presence, in this microorganism, of a caspase-dependent pathway. Here we show that Ynr074cp of *S. cerevisiae* has high similarity with the human AIF. Using the chimeric protein Ynr074c-GFPp, we show that Ynr074cp is localized in *S. cerevisiae* mitochondria. In addition, the *YNR074*c mutant strain showed a phenotype of resistance to death induced by acetic acid and camptothecin, two known apoptotic inducers. Translocation of the Ynr074c-GFP from the mitochondria to the cytoplasm and to the nucleus (labelled with DsRed-NLS protein) was observed during camptothecin treatment and cell ageing. Moreover, the *YNR074c* overexpression led to increased susceptibility to death induced by acetic acid and by camptothecin. Altogether the results indicate that besides cytochrome c, Ynr074cp could be another mitochondrial effector of *S. cerevisiae* apoptotic cell death. Our findings suggest that two distinct pathways leading to nuclear apoptosis could be present in yeast similarly with what has been reported for mammalian cells.

Introduction

Programmed cell death (PCD), of which apoptosis is the most common morphological expression plays an important role in the normal development and homeostasis mechanisms of multicellular organisms. Mitochondria play a key part in the regulation of mammalian apoptosis (Kroemer, 1997; Green and Reed, 1998) being both essential for a cell's life and the reservoir of key components involved in its death. Several key death factors, such as cytochrome *c* (CytC) (Liu *et al*., 1996; Kluck *et al*., 1997) and pro-caspases 2, 3 and 9 (Mancini *et al*., 1998; Susin *et al*., 1999a), normally reside in the intermembrane mitochondrial space and are released through the outer membrane in order to participate in the degradation phase of apoptosis (Zamzami *et al*., 1996; Liu *et al*., 1996; Susin *et al*., 1996; Kluck *et al*., 1997; Vander Heiden *et al*., 1997; Susin *et al*., 1999b).

CytC is the most well known mitochondrial death-promoting factor and upon its translocation to cytoplasm forms a protein complex with pro-caspase 9 and apoptotic protease activating factor 1 (Apaf-1), named apoptosome. Although the CytC/Apaf-1/caspase-9 apoptosome is essential for several mitochondria-dependent apoptotic pathways, cells deficient in these molecules can still die by apoptosis (Green and Reed, 1998). It has also been shown that Bcl-2 preserves the integrity of mitochondrial membranes and protects cells from death independently of Apaf-1 and caspases, implying that Bcl-2 interferes with two different mitochondria-dependent death effector cascades (Amarante-Mendes *et al*., 1998; Haraguchi *et al*., 2000). These observations suggested the existence of another mitochondrial death-promoting factor, which was actually described by Susin *et al*. (1999b). These authors showed that purified mitochondria could release another factor besides CytC that induces apoptotic morphological changes in nuclei *in vitro*, such as chromatin condensation and cleavage of chromosomal DNA into "domain-sized" fragments, but not an oligonucleosomal ladder, indicating that no Caspase-Activating DNAses (CAD)-like nuclease were involved. Those authors have termed this factor apoptosis-inducing factor (AIF) and

found that, similarly to other death-promoting factors, it resides in the mitochondrial intermembrane space, being released from mitochondria by apoptosis-inducing stimuli. In these conditions and upon opening of the permeability transition pores by atractyloside (Atr), Ca^{2+} or *tert*-butylhydroperoxide, AIF is translocated to, and accumulate in, the nucleus in a caspase-independent fashion. Susin *et al*. (1999b) have shown that injection of AIF into cytoplasm induced several signs of apoptosis, including the release of CytC, loss of mitochondrial membrane potential ($\Delta\Psi m$), exposure of phosphotidylserine on the cell surface and chromatin condensation. On the other hand, injection of anti-AIF antibodies into cells blocked chromatin condensation although not decreasing of $\Delta\Psi m$ (Susin *et al*., 1999b). Experiments with inhibitors indicated that AIF does not act downstream of caspases or other mitochondrial factors. When added to purified nuclei, the recombinant AIF causes caspase-independent large scale (~50 Kb) DNA fragmentation and a type of peripheral chromatin condensation that resembles the first stage of nuclear apoptosis observed in intact cells undergoing apoptosis (Susin *et al*., 1999b; Daugas *et al*., 2000).

AIF also affects mitochondrial membrane(s) function, indicating that it can engage in a self-amplification loop whereby AIF released from some mitochondria acts on other mitochondria to compromise their membrane function, thus providing a new molecular link between mitochondrial membrane permeabilization and nuclear apoptosis (Susin *et al*., 1999b). Susin and colleagues (1999b) suggested that caspases and AIF are probably engaged in cooperative or redundant pathways that lead to nuclear apoptosis.

Joza *et al*., (2001) have shown that the genetic inactivation of AIF abolishes the first wave of developmental cell death occurring during early mouse embryogenesis. According to these authors, and assuming that ontogenesis recapitulates phylogeny, it is tempting to speculate that AIF represents a pathway of apoptosis that predates the caspase pathway. In fact, AIF is a phylogenetically old, bifunctional flavoprotein with an electron acceptor-donor function and a second, independent apoptogenic function, that could have been recruited to the executionary pathway earlier than caspases (Lorenzo *et al*., 1999). AIF shares significant homology with several ferredoxin or

NADH-oxidoreductases found in all metazoan phyla (Lorenzo *et al*., 1999; Susin *et al*., 1999b). In fact, Arnoult *et al*. (2001) recently cloned and characterized a homolog of mammalian AIF being one of the putative effectors involved in DNA degradation during cell death of the unicellular eukaryote *Dyctyostelium discoideum* (DdAIF). These authors found that DdAIF is released into the cytosol and targeted to the nucleus during cell death mediated by a protoporphyrin and induced by differentiation-inducing factor-1 (DIF-1).

Recently we have shown that mitochondria, like in metazoans, play an important role in the programmed cell death of *Saccharomyces cerevisiae,* through the release of CytC and reactive oxygen species production (Ludovico *et al*., 2002). However, in that study complete inhibition of cell death was not achieved in *CYC3* mutants, lacking CytC, indicating that, also in *S. cerevisiae*, mitochondria could possess another death-promoting factor besides CytC. Therefore, we investigated whether an AIF-like molecule, involved in apoptotic death, is present in *S. cerevisiae*.

Material and Methods

Microorganisms and growth conditions

The yeast *Saccharomyces cerevisiae* BY7242 (*MATa his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*; EUROSCARF, acc. number Y10000) and its isogenic strain deleted in the gene *YNR074c* (Mat α *his3Δ1*, *leu2Δ0*, *lys2Δ0*, *ura3Δ0*; *YNR074c::kanMX4*, EUROSCARF, acc. number Y16233) were used. Yeast strains were grown in YPD (Ludovico *et al*., 2001) and for selective purposes, a synthetic medium (YNB, Difco) was used, supplemented when required with the respective bases and amino acids. *Escherichia coli* XL1-Blue was used as the bacterial host for plasmids and was grown in Luria-Bertani medium (LB) at 37 ºC supplemented with 100 μg/ml ampiciline when required (Bullock *et al*., 1987).

DNA manipulations and transformation procedures

For plasmid isolation from *E. coli* and *S. cerevisiae* a Qiaprep spin miniprep kit (Qiagen) was used (Rodrigues *et al*., 2001). Yeast chromosomal DNA was isolated as described previously (Holm *et al.*, 1986). Agarose gel electrophoresis, DNA sub-cloning and restriction site mapping were performed according to standard procedures (Sambrook *et al*., 1998). The *YNR074c* gene was amplified by polimerase chain reaction (PCR) on the cognate clone plasmid pYCG_YNR074c (EUROSCARF, acc. number P20348) with the use of gene-specific primers (sense primer, 5'-CGG GAT CCC GGA GCA GAG AAA GGA AGA AAG- 3'; antisense primer, 5' –CGG AAT TCC GCG GAG AAA AGA TTC TTC GAC- 3'). PCR was performed using PfuTurbo DNA polymerase (Stratagene), an enzyme with processing and proof reading activity. The amplification was performed using the following conditions: 94ºC, 3'; 94ºC, 1'; 55ºC, 1'; 72ºC, 2'; 72ºC, 10' followed by thirty cycles of 94ºC, 1'; 55ºC, 1';

72ºC, 2'. A fragment of the expected molecular mass 1.1 kb, was purified and cloned into the BamHI- and EcoRI-digested plasmid pUG35 (http://www.mips.biochem.mpg.de/proj/yeast/info/tools/hegemann/gfp.html). The resulting plasmid, pPF 1 contained an in frame fusion of the gene *YNR074c* and *GFP* (green fluorescent protein), under the control of the MET25 promoter. Competent cells of *E. coli* XL1-Blue and *S. cerevisiae* were prepared as described elsewhere (Gietz and Schiestl, 1995; 1998; Bullock *et al*., 1987).

Staining procedures and epifluorescence microscopy analysis

Chimeric Ynr074c-GFPp fusion protein was co-localized in mitochondria by the staining of live cells with the mitochondrial membrane potential (ΔΨm)-sensitive dye CMXRos (50 μg/ml) or with the mitochondrial structural dye nonyl-acridine orange (50 μg/ml). For co-localization of the fusion protein in the nuclei, cells were transformed with the vector pUR36NLS (Rodrigues *et al*., 2001) and therefore the nuclei were *in vivo* labelled with DsRed-NLS protein.

A Leica standard epifluorescence microscope equipped with filter wheels to control excitation and emission wavelength, with a cooled charge-coupled device (CCD) video camera (SONY, DXC-9100P), a frame grabber (IMAGRAPH, IMASCAN/ chroma-P) and software for image archival and management (AXIOVISION) was used for examination of cell populations. The excitation source was a 50 W Hg vapour arc lamp. In each case the appropriate fluorescence filter was used. Selected panels were composed using the Adobe Photoshop 5.0 program.

Acetic acid and camptothecin treatments and cell ageing

Acetic acid treatment was performed in order to induce a programmed cell death process as described elsewhere (Ludovico *et al*., 2002). For camptothecin treatment,

stationary phase cells were harvested and suspended (10^7cells/ml) in synthetic medium containing 0, 50, 100, 120, 140, 180, 200 and 250 μg/ml of camptothecin (Sigma). The camptothecin treatments were carried out for 180 minutes at 26 °C with magnetic stirring (150 rpm). Viability was determined by counting colony forming units (cfu) after 2 days of incubation at 26 ºC on YPD agar plates.

For cell ageing analysis, cultures were maintained for 8 days in synthetic medium (YNB) at 26 ºC with magnetic stirring.

Results

Saccharomyces cerevisiae *Ynr074c protein shows similarity with the human Apoptotic-Inducing Factor (hAIF)*

The identification of similar proteins among different organisms is a proven strategy to establish common biological pathways. The genome of *S. cerevisiae* is completely sequenced, and therefore this type of studies can be performed using a genome wide search. Using the BLASTP 2.1.1 program (NCBI), we found that the ORF YNR074c (acc. number NP_014472) of *S. cerevisiae* encodes a putative protein with a sequence producing significant alignment with the hAIF and with the recently reported *Dictyostelium discoideum* AIF (DdAIF) (Figure 1). The 382 amino acid sequence of Ynr074cp displayed an identity of 22 % and a similarity of 41 % with the hAIF. This protein is described in Yeast Proteome Database (YPD^{TM}) as a putative reductase with a FAD and a NADH domain with similarity to FAD-dependent pyridine nucleotide reductases (FADPNR). Its mature form has a predicted molecular mass of 41.2 kDa, where the first methionine residue is cleaved. In addition, previous studies by Ververidis and coworkers have shown that the resistance of *S. cerevisiae* to cercosporin is dependent on the *YNR074c* copy number (Ververidis *et al*., 2001). Cercosporin is a lipid-soluble red polyketide produced by filamentous fungi of the genus *Cercospora*. This toxin acts as a non-host-specific photoactivated drug (Daub 1982, 1987) that interacts with molecular oxygen producing reactive oxygen species (ROS), principally singlet oxygen (Spikes, 1989). Therefore, Ververidis and coworkers have designated the Ynr074cp as *CPD1* (cercosporin and photosensitizer detoxification) and have described it as a putative transmembrane reductase by an *in silico* analysis. Even though a putative cellular role of this protein was described, the similarity with hAIF and with DdAIF together with the FAD and NADH domains we

found are strongly indicative of an *S. cerevisiae* equivalent-AIF. Therefore, our studies were developed in order to further characterize the cellular role of this protein.

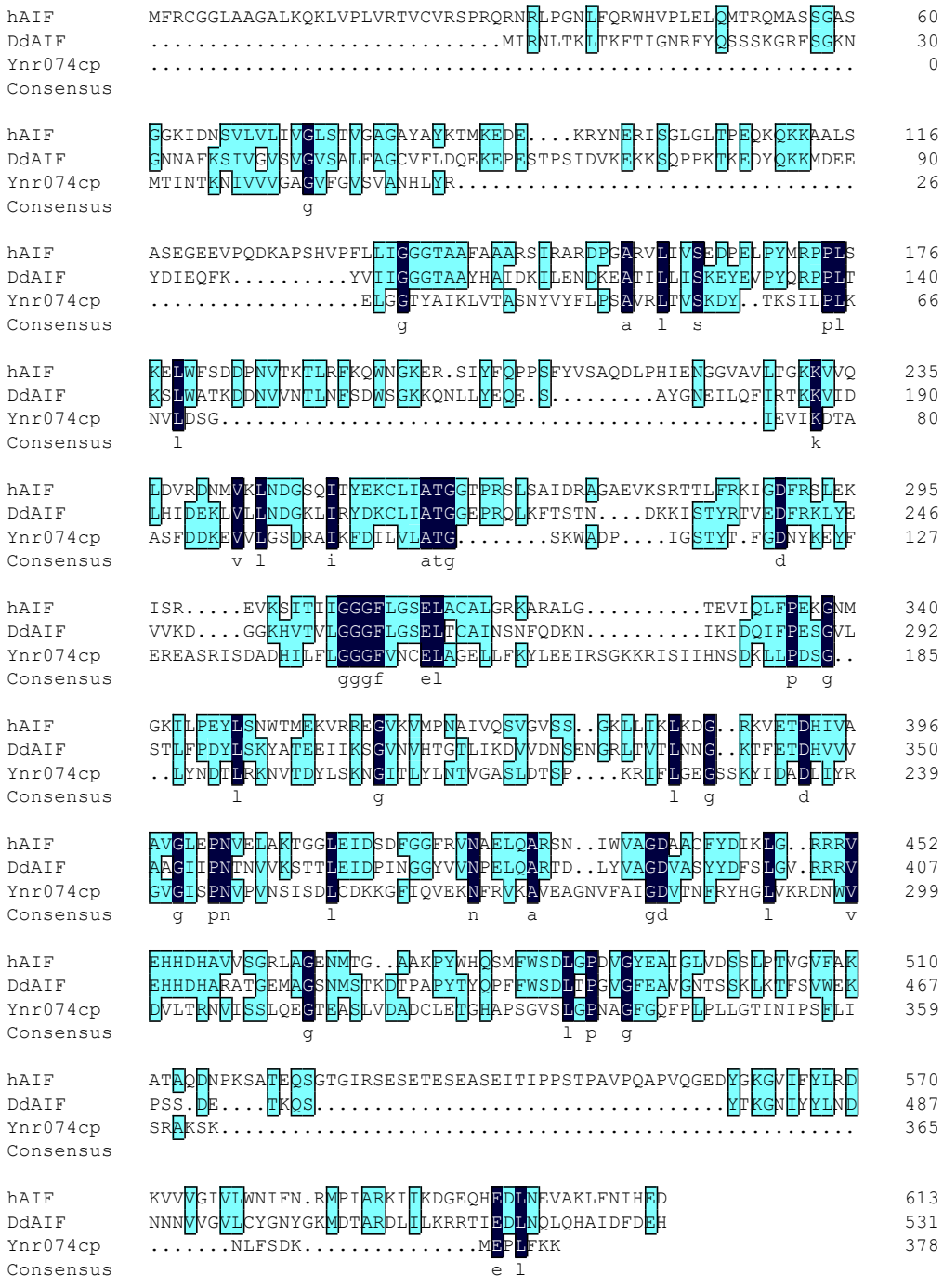

hAIF MFRCGGLAAGALKQKLVPLVRTVCVRSPRQRNRLPGNLFQRWHVPLELQMTRQMASSGAS 60
DdAIF .....MIRNLTKLTKFTIGNRFYQSSSKGRFSGKN 30
Ynr074cp 0
Consensus
hAIF GGKIDNSVLVLIVGLSTVGAGAYAYKTMKEDE....KRYNERISGLGLIPEQKQKKAALS 116
DdAIF GNNAFKSIVGVSVGVSALFAGCVFLDQEKEPESTPSIDVKEKKSQPPKTKEDYQKKMDEE 90
Ynr074cp MTINTKNIVVVGAGVFGVSVANHLYR..... 26
Consensus g
hAIF ASEGEEVPQDKAPSHVPFLLIGGGTAAFAAARSIRARDPGARVLIVSEDPELPYMRPPLS 176
DdAIF YDIEQFK.....YVIIGGGTAAYHAIDKILENDKEATILLISKEYEVPYQRPPLT 140
Ynr074cp .....ELGGTYAIKLVTASNYVYFLPSAVRLTVSKDY..TKSILPLK 66
Consensus g a l s pl
hAIF KELWFSDDPNVTKTLRFKQWNGKER.SIYFQPPSFYVSAQDLPHIENGGVAVLTGKKVVQ 235
DdAIF KSLWATKDDNVVNTLNFSDWSGKKQNLLYEQE.S.....AYGNEILQFIRTKKVID 190
Ynr074cp NVLDSG.....IEVIKDTA 80
Consensus l k
hAIF LDVRDNMVKLNDGSQITYEKCLIATGGTPRSLSAIDRAGAEVKSRTTLFRKIGDFRSLEK 295
DdAIF LHIDEKLVLLNDGKLIRYDKCLIATGGEPRQLKFTSTN....DKKISTYRTVEDFRKLYE 246
Ynr074cp ASFDDKEVVLGSDRAIKFDILVLATG.....SKWADP....IGSTYT.FGDNYKEYF 127
Consensus v l i atg d
hAIF ISR.....EVKSITIIGGGFLGSELACALGRKARALG.....TEVIQLFPEKGNM 340
DdAIF VVKD....GGKHVTVLGGGFLGSELTCAINSNFQDKN.....IKIDQIFPESGVL 292
Ynr074cp EREASRISDADHILFLGGGFVNCELAGELLFKYLEEIRSGKKRISIIHNSDKLLPDSG.. 185
Consensus gggf el p g
hAIF GKILPEYLSNWTMEKVRREGVKVMPNAIVQSVGVSS..GKLLIKLKDG..RKVETDHIVA 396
DdAIF STLFPDYLSKYATEEIIKSGVNVHTGTLIKDVVDNSENGRLTVTLNNG..KTFETDHVVV 350
Ynr074cp ..LYNDTLRKNVTDYLSKNGITLYLNTVGASLDTSP....KRIFLGEGSSKYIDADLIYR 239
Consensus l g l g d
hAIF AVGLEPNVELAKTGGIEIDSDFGGFRVNAELQARSN..IWVAGDAACFYDIKLG..RRRV 452
DdAIF AAGIIPNTNVVKSTTLEIDPINGGYVVNPELQARTD..LYVAGDVASYYDFSLGV.RRRV 407
Ynr074cp GVGISPNVPVNSISDLCDKKGFIQVEKNFRVKAVEAGNVFAIGDVTNFRYHGLVKRDNWV 299
Consensus g pn l n a gd l v
hAIF EHHDHAVVSGRLAGENMTG..AAKPYWHQSMFWSDLGPDVGYEAIGLVDSSLPTVGVFAK 510
DdAIF EHHDHARATGEMAGSNMSTKDTPAPYTYQPFFWSDLTPGVGFEAVGNTSSKLKTFSVWEK 467
Ynr074cp DVLTRNVTSSLQEGTFASLVDADCLETGHAPSGVSLGPNAGFGQFPLPLLGTINIPSFLI 359
Consensus g l p g
hAIF ATAQDNPKSATEQSGTGIRSESETESEASEITIPPSTPAVPQAPVQGEDYGKGVIFYLRD 570
DdAIF PSS.DE....TKQS.....YTKGNIYYLND 487
Ynr074cp SRAKSK..... 365
Consensus
hAIF KVVVGIVLWNIFN.RMPIARKIIKDGEQHEDLNEVAKLFNIHED 613
DdAIF NNNVVGVLCYGNYGKMDTARDLILKRRTIEDLNQLQHAIDFDEH 531
Ynr074cp .....NLFSDK.....MEPLFKK 378
Consensus e l

Figure 1 – CLUSTAL-W was used to generate an alignment of the predicted amino acid of Ynr074cp with DdAIF and hAIF. Black boxes indicate amino-acid identity and green boxes indicate amino acid similarity. Ynr074cp is ~22 % identical and ~41% similar to DdAIF and hAIF.

Ynr074cp is involved in acetic acid induced PCD

In order to assess the putative role of Ynr074cp in the yeast PCD process(es), we tested whether the *YNR074c* null mutant cells could also undergo apoptosis by treatment with either acetic acid or hydrogen peroxide. As shown in figure 2, the *YNR074c* null mutant of *S. cerevisiae* was more resistant to death induced by acetic acid, comparatively to the wild-type strain, and cycloheximide had no effect on the survival. In contrast, when cell death was induced by hydrogen peroxide the *YNR074c* null mutant did not display an increased resistance. Madeo and coworkers (2002), have recently described a caspase-like protease in *S. cerevisiae.* They have showed that cells deleted in the correspondent gene display an increased resistance both to hydrogen peroxide and to acetic acid. However, this effect was not so evident for the acid, suggesting the presence of different apoptotic pathways induced by the two stress agents. A protein probably involved in the yeast mitochondria-dependent apoptotic pathway could be the AIF, a protein that in human cells is localized in mitochondria.

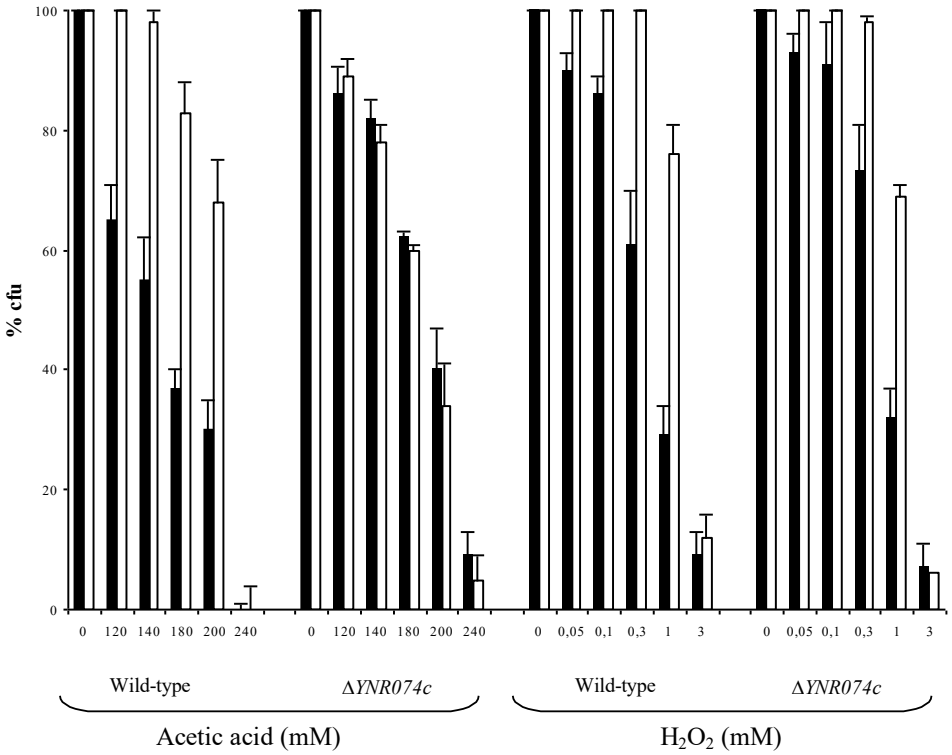


Figure 2 - Relative survival (% of colony forming units, cfu, on YEPD agar plates; 100 % corresponds to the number of cfu at time zero) of *S. cerevisiae* cells of wild-type strain and of Δ*YNR074c* incubated for 200 min with acetic acid or with hydrogen peroxide in the absence (black bars) or presence (white bars) of cycloheximide.

Ynr074cp is a mitochondrial protein

In order the access the cellular localization of Ynr074cp we constructed a C-terminal in frame fusion with green fluorescent protein (GFP). Cells of *S. cerevisiae* expressing this chimeric fusion in a centromeric vector display an intense green fluorescence localised in intracellular organelles. The staining of mitochondria of live cells with the

structural dye nonyl-acridine orange or with the ΔΨm-sensitive dye MitoTracker CMXRos showed that the green fluorescence from GFP was colocalized with the orange or red fluorescence from nonyl-acridine orange or from MitoTracker Red CMXRos, respectively (see Fig. 3 for colocalization with MitoTracker Red CMXRos). These results are indicative that Ynr074cp has a mitochondrial localization in accordance with its putative AIF function. To further establish the functionality of the chimeric protein, the null mutant of *S. cerevisiae* on *YNR074c* was transformed with a low copy number plasmid containing the fusion *YNR074c-GFP* and we tested its ability to functionally complement the *YNR074c* null mutant of *S. cerevisiae*. As described above, the mutant, in contrast with the wild-type strain, does not display a cycloheximide inhibitable death induced by acetic acid. However, this resistant phenotype was reverted by the expression of the fusion *YNR074c-GFP*. This result indicates that the chimeric protein is able to functionally complement the mutation indicating that it maintains its functional cell activity.

Although Ververidis and coworkers (2001) have suggested, by an *in silico* analysis, that Ynr074p is most probably a plasma membrane protein, herein we show that this is not the case and that Ynr074p is a mitochondrial protein.

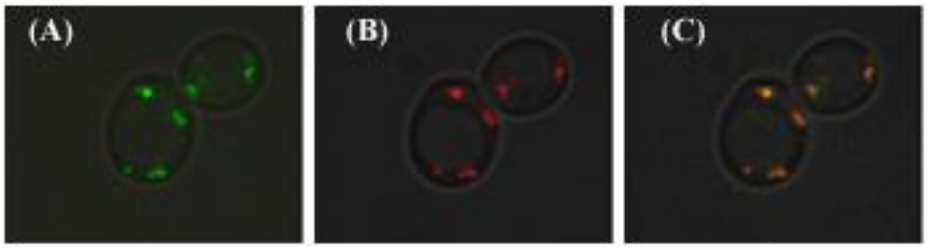


Figure 3 – Cells of *S. cerevisiae* expressing YNR074c-GFP gene stained with MitoTracker red CMXRos. (A) cellular localization of green fluorescence; (B) mitochondrial red fluorescence of MitoTracker red CMXRos and (C) Colocalization of green fluorescence with red fluorescence in the mitochondria.

During ageing of* S. cerevisiae *cells, Ynr074c-GFPp is translocated from mitochondria to the nucleus

In human cells AIF protein, after a specific apoptotic stimulus, suffers a translocation from mitochondria to the cytosol and then to the nucleus (Susin *et al*., 1999a). To further understand if Ynr074cp could be the *S. cerevisiae* equivalent of hAIF we followed the fluorescence of GFP during induction of PCD by acetic acid treatment. During acetic acid treatment the intense mitochondrial green fluorescence of the cells was lost. This observation is in agreement with the high sensitivity of GFP to pH changes (Tsien, 1998). In fact, acetic acid induces in *S. cerevisiae* an intracellular acidification (Cardoso and Leão, 1992). To surround this technical problem and since aged yeast cells die with an apoptotic phenotype (Laun *et al*., 2001), we tested if in these cells Ynr074c-GFP chimeric protein was translocated from mitochondria to the nucleus. For such purpose, cells expressing Ynr074c-GFP chimeric protein and transformed with the vector pUR36NLS, being able to express the red fluorescent protein targeted into the nuclei (DsRed-NLS) (Rodrigues *et al*., 2001), were maintained in synthetic medium for 7-8 days. Epifluorescence microscopy analysis revealed three different types of cells, namely: i) cells with red-labelled nuclei due to the expression of DsRed-NLS protein and with green fluorescence, from Ynr074c-GFPp, localized in mitochondria; ii) cells with orange-stained nuclei and green cytoplasm most probably due to a release of the Ynr074c-GFPp from mitochondria to the cytosol and then to the nucleus (Fig. 4); iii) cells with the nuclei labelled orange probably due to the relocalization of Ynr074c-GFPp in the nuclei (Fig. 4). The translocation of Ynr074c-GFPp was also observed in yeast cell populations treated with camptothecin as it will be described hereafter.

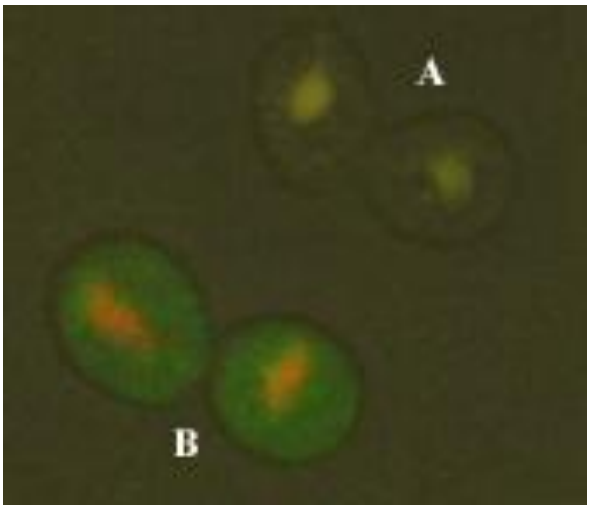


Figure 4 – *S. cerevisiae* cells expressing DsRed-NLS and YNR074c-GFP after 8 days of incubation. Cells showing colocalization of green and red fluorescence in the nuclei (A) and during translocation of Ynr074c-GFPp from cytoplasm to the nuclei (B).

Ynr074cp confers resistance to the death induced by camptothecin in S. cerevisiae

Camptothecin is a S-phase-specific anticancer drug used in chemotherapy that inhibits the action of the enzyme DNA topoisomerase-I (Morris and Geller 1996). Campothecin is described to induce apoptosis in different human cell types (see for example Kaufmann, 1998) and in *S. cerevisiae* it was also described as an agent that induces inhibition of cell growth and enhances cell death in *S. cerevisiae* (Nitiss and Wang, 1998). We found that treatment with camptothecin (50-250 μg/ml) induces death of wild-type *S. cerevisiae* strain. However, the null mutant in *YNR074c* gene displayed a higher resistance to the death induced by this drug. Moreover, when *YNR074c* gene was over-expressed under the control of MET25 promotor, the cells showed a higher sensitivity to camptothecin comparatively with the wild-type strain (Fig. 5).

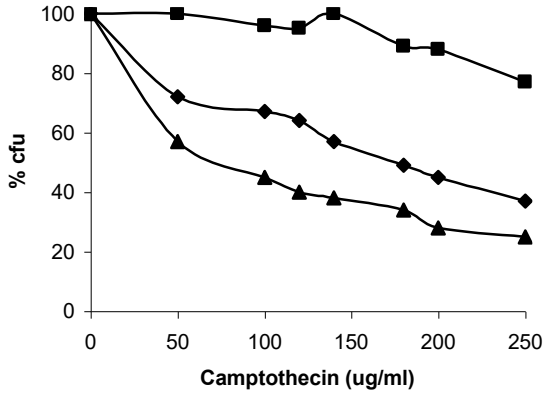


Figure 5 - Relative survival (% of colony forming units, cfu, on YEPD agar plates; 100 % corresponds to the number of cfu at time zero) of *S. cerevisiae* cells incubated with different concentrations of camptothecin for 180 minutes. (■) *YNR074c* mutant strain; (♦) wild-type strain and (▲) YNR074c over-expressing strain.

The translocation of Ynr074c-GFPp from mitochondria to the nucleus (data not shown) was observed in *YNR074c* mutant cells transformed with YNR074c-GFP and treated with camptothecin, similarly with what was observed in aged cells.

Conclusions

Taken together, the results presented above suggest that Ynr074cp is the AIF-equivalent in *S. cerevisiae*. The Ynr074cp homology, localization and phenotype of null mutant and of strain overexpressing Ynr074cp, undoubtedly indicate the crucial role of this protein in a caspase-independent pathway present in *S. cerevisiae*. In fact, we found that Ynr074c protein has a high similarity with hAIF and DdAIF possessing the FAD and NADH domains characteristics of AIF proteins. Moreover, and in opposition with what was proposed by Ververidis and coworkers (2001), Ynr074cp has a mitochondrial localization and is able to translocate to the nucleus after an apoptotic stimulus. The reporting in Yeast Proteome Database (YPD^{TM}) of a physical interaction of Ynr074cp with Nup116p, which is a subunit of a nuclear pore, further points to a possible involvement of Nup116p in the entrance of Ynr074cp in the nucleus.

To further strengthen the role of Ynr074cp in *S. cerevisiae* apoptosis and to demonstrate its functional homology with hAIF, additional studies should be performed, namely: i) isolation of Ynr074cp (which could be technical performed using a His-tag construction) and *in vivo* treatment of nuclei isolated from *S. cerevisiae* and from a human cell line, to look for the typical chromatin condensation; ii) treatment of S. *cerevisiae* isolated nuclei with cytosolic extracts isolated from cells undergoing apoptosis induced by acetic acid or camptothecin or other apoptotic stimulus in which Ynr074p has been proved to play an important role; iii) induction of apoptosis with acetic acid or camptothecin in the presence of a caspase inhibitor to prove that this pathway is independence of caspase activity; iv) measurement of Yca1p activity under conditions inducing a caspase-independent apoptotic pathway; v) in order to prove a functional similarity between Ynr074cp and hAIF, a functional complementation of the acetic acid and camptothecin phenotype of the YNR074c null mutant should be achieved by the expression of hAIF and vi) study of all parameters

concerning the mitochondria function such as evaluation of ΔΨm changes, ROS production, expression of Bcl-2 proteins and release of cytochrome c.

It is expectable that the finding of this putative *S. cerevisiae* AIF-equivalent will be of extreme importance to the study of apoptosis in human cells. In fact, the biological aspects of the hAIF function are still scarce and, once more, the use of yeast, particularly *S. cerevisiae*, as a model could open doors to the understanding of the cellular role of AIF. Indeed, biological relevant aspects such as the AIF primary cellular function, the protein interactions and the mechanism(s) involved in the cross-talk between the two mitochondria-dependent apoptotic pathways could be underlined.

Acknowledgements

The authors would like to thank Prof. Manuel Teixeira da Silva and Doctor Frank Madeo for their helpful comments and suggestions and to Dr. Agostinho Almeida for his technical assistance.

Paula Ludovico has a fellowship from PRAXIS XXI (Fundação da Ciência e Tecnologia, Portugal).

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Chapter 7

General Discussion

1 - Programmed cell death in unicellular organisms

2 - Genetic basis of the PCD process in yeast

3 - Contributions of the present work to the understanding of the apoptotic process in yeast

3.1 - Acetic acid-induced apoptosis in yeast: a PCD process with physiological relevance

3.2 - PCD in yeast can be mediated by a Mitochondria-Dependent Apoptotic Pathway (MDAP)

4 - Yeast as a powerful tool in apoptosis research

The studies described in this thesis aimed to contribute to the understanding of the mechanisms of cell death in yeast. Our results regarding the cytotoxic effects induced by acetic acid in *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* showed that, while high concentrations of the acid kill the yeast cells by a necrotic process, lower concentrations induce a programmed cell death (PCD) process with features common to metazoan apoptosis. The occurrence of an apoptotic process in unicellular organisms was at the time of our observations still controversial. On the other hand, confirmation of the occurrence of such a process in yeast would be of great biological relevance, because yeast can be a very powerful tool in the study of metazoan apoptosis. Therefore, our efforts were centered in the deeper analysis of the apoptotic process in *S. cerevisiae*, including the investigation of the role of mitochondria in that process.

1 - Programmed cell death in unicellular organisms

The occurrence of a PCD process with an apoptotic phenotype in unicellular organisms has been the subject of a great controversy. PCD has been described as a highly regulated active form of cell death, of which apoptosis is the most common morphological expression, and, as already discussed in chapter 1, it is utilized to remove, quickly and without an inflammatory response, cells that are either unwanted or potentially harmful to the organisms. Apoptosis has therefore often been assumed to be a product of attaining multicellularity, acquired shortly after the multicellular state arose (Matsuyama *et al*., 1999). Although the number of reports concerning the occurrence of a PCD process in unicellular prokaryotes and eukaryotes had increased in the last five years, some still questioned: Why would it be useful for unicellular organisms to possess a suicide program? Indeed, it is not immediately apparent what would be the benefit of a defective unicellular organism committing suicide. However, the answer came from the recognition that unicellular organisms live and die in

complex communities that give rise to colonies or populations of cells, all possessing the same genome derived from a common ancestor, and could, in many ways, resemble a multicellular organism. From this point of view, PCD of damaged cells may be valuable to a multicellular community of unicellular individuals. For example, suicide could limit the spread of a viral infection or, in the case of serious damage by toxic agents, cell suicide would allow that damaged cells donate their nutrients to their neighbours instead of exhausting resources from the community in a futile attempt to repair themselves. Moreover, the elimination of cells with damaged DNA would contribute to the maintenance of a low mutation rate and would lower the probability that takeover mutants will arise. Thus, cellular behaviours that sacrifice some of the members of a population so that others can ultimately survive, ensure propagation of the original genome to future generations (Matsuyama *et al.,* 1999).

As discussed before, in spite of substantial morphological similarities between apoptotic yeast and metazoan cells, the complete genome of *S. cerevisiae* lacks any obvious homologue of the major apoptotic regulators described in metazoan organisms (Bcl-2 family, caspases, Apaf-1 and p53) (Fröhlich and Madeo, 2000). However, it has been observed that the expression of several mammalian pro-apoptotic proteins in yeast cells causes cell death with a characteristic apoptotic morphology. Some authors suggest that the action of pro-apoptotic proteins in yeast cells derives from the known ability of pro-apoptotic proteins to induce the formation of reactive oxygen species (ROS), while Bcl-2 suppresses it. In fact, ROS production appears as an event common to all the instances of *S. cerevisiae* apoptotic phenotypes reported so far (Madeo *et al.,* 1997; Madeo *et al*., 1999; Laun *et al*., 2001; Levine *et al.,* 2001; Narasimhan *et al.,* 2001; Ludovico *et al*., 2002). However, the presence in *S. cerevisiae* of a PCD morphologically similar to metazoan apoptosis in the apparent absence of homologous apoptotic proteins is mystifying. After the pioneer works of Madeo and his team (Madeo *et al*., 1997; 1999) and besides our own contributions (Ludovico *et al*., 2001; 2002) an increasing number of different reports concerning the

occurrence of a PCD in *S. cerevisiae* with an apoptotic phenotype came out in the literature:

- Laun *et al.* (2001) observed that aged mother cells of *S. cerevisiae* show markers of oxidative stress and apoptosis. Although as referred to above it had been previously shown by Madeo *et al.* (1997, 1999), that *S. cerevisiae* could commit to an active cell death process, the reported occurrence of apoptosis in unstressed but aged wild-type yeast mother cells points to a physiological role for this phenomenon.
- Yamaki *et al.* (2001) used *S. cerevisiae* cells lacking the histone chaperon asf1/cia1 to study apoptosis in yeast since its human homologue appears to be involved in the regulation of apoptosis. As expected, the authors observed that cell death occurs with a phenotype (nuclei fragmentation, chromatin condensation and fragmentation, decrease of mitochondrial membrane potential, dysfunction of mitochondrial ATPase and cytochrome *c* release) that largely resembles mammalian apoptosis. Those authors also proposed that yeast undergoes a 'prototypical' active cell death that retains some characteristics of passive cell death (necrosis). In their opinion the active cell death is ubiquitously an essential attribute of life.
- Carratore *et al.* (2002) evaluated whether UV irradiation could induce apoptosis in yeast. These authors showed that UV treated cells display a TUNEL-positive phenotype and found by flow cytometry a sub-G1 population. The sub-G1 population was isolated and electron microscopy analysis revealed that these cells have chromatin condensation.

Very recently, Madeo and coworkers (Madeo *et al*., 2002) reported a caspase-related protease that regulates yeast apoptosis, named Yeast Caspase-1 (*YCA-1*). This important new observation will be discussed in more detail in section 3.2.

The comparison of the apoptotic features (independent of heterologous expression) observed in yeast and mammalian cells is summarized in the table I.

Table I - Comparison of the apoptotic features (independent of heterologous expression) observed in yeast and mammalian cells

Event	Yeast	Mammalian
Chromatin condensation	+	+
DNA fragmentation	+	+
Cytochrome c release	+	+
DNA ladder	-[a]	+
Exposition of phosphatidylserine	+	+
Decrease of mitochondrial membrane potential	+	+
Dysfunction of mitochondrial proton pump	+	+
Decrease of vacuolar membrane potential	+	-[b]
Dysfunction of vacuolar proton pump	+	-[b]
Authophagic body	+	+
ROS production	+	+
Presence of caspase	+	+

[a] It is not possible to observe in yeast due to the small internucleosomal space

[b] Vacuole is absent in animal cells

2 - Genetic basis of the PCD process in yeast

As referred in chapter 1, Madeo *et al.* (1997) found that a point-mutation in *S. cerevisiae cdc48*^{S565G} gene results in death with a typical apoptotic phenotype. Recently, the mammalian orthologue of Cdc48p, p97/VCP (Shirogane *et al.,* 1999) and the related Mac-1 of *Caenorhabditis elegans* (Wu *et al.,* 1999) have also been identified as anti-apoptotic factors, making the Cdc48p the first identified regulator of yeast apoptosis.

In mammalian cells, inhibition of proteasome-dependent proteolysis leads to either induction or repression of apoptosis, depending on the proliferation status of the cells (Frölich and Madeo, 2000). To characterize the role of proteasome in yeast apoptosis, Ligr *et al.* (2001) screened for proteasomal substrates and found six genes, which, by overexpression in a proteasome-deficient background, triggered apoptosis. One of the genes is *STM1*, a DNA-binding protein involved in DNA repair. Those authors

suggested that Stm1p is an activator of apoptosis that can be triggered by exposure of cells to low H_2O_2 concentrations.

In a search for Bcl-2 family protein regulators in yeast, Komatsu *et al.* (2000) found in *Schizosaccharomyces pombe* an orthologue of Rad9p, the SpRad9p. The SpRad9p, like the correspondent human Rad9p, also contains a region with similarity to the BH3 domains of pro-apoptotic Bcl-2 family proteins, but lacks critical residues found in BH1, BH2 and BH4 domains. However, overproduction of this fission yeast protein in human cells induced cell death, which can be blocked by co-expression of Bcl-2 or Bcl-xL indicating that SpRad9p may represent the first member of the BH3-only family of apoptosis promoting proteins identified in yeast (Komatsu *et al.,* 2000).

A gene coding for an IAP protein was another interesting gene found in yeast by Uren *et al.* (1999). IAP proteins are a family of cell death inhibitors identified in baculoviruses, which prevent defensive apoptosis of the host cell and thereby promote viral replication (Crook *et al.,* 1993). Uren *et al.* (1998) identified homologs of IAP proteins in *S. cerevisiae* (*BIR1* gene) and *Sch. pombe* (*bir1* gene) and found that yeast IAPs inhibit apoptosis in mammalian cells indicating that the function of IAPs is conserved. Uren *et al.* (1999) proposed, at that time, that *BIR1* and *bir1* are unlikely to function by inhibiting cell death mechanisms resembling those in metazoan because it appeared that *S. cerevisiae* and *Sch. pombe* had no caspases or cell suicide programs. However, a PSI-BLAST search allowed the identification of a distantly related family of caspase-like proteins (metacaspases), members of which are present in *Arabidopsis thaliana* and *S. cerevisiae* (Uren *et al.,* 2000). As mentioned in chapter 1 and discussed in more detail below in section 3.2, Madeo *et al*., (2002) studied the functionality of the found metacaspase gene and reported it as a caspase-related protease (named YCA-1) that regulates yeast apoptosis.

Recently a putative yeast ortholog of DAP-3 (mammalian mitochondrial mediator of apoptosis), Ygl129c, was described (Berger *et al.,* 2000). Madeo and coworkers (Madeo *et al*., 2002) found that mutation of this yeast gene completely prevented the induction of apoptosis by *YCA-1* overexpression. These authors suggest that the

function of DAP-3 is evolutionary conserved from yeast to mammals and places the molecule downstream of caspase function.

All the results discussed above regarding the so-called death-defying genes of *S. cerevisiae*, point to the advantage of searching apoptotic regulator proteins by analysing database for putative functional protein domains rather than by looking for genetic homology. This approach had allowed the identification of regulators and effectors involved in yeast apoptosis that are functionally equivalent to mammalian regulators but that were believed not to exist in yeast until very recently.

3 – Contributions of the present work to the understanding of the apoptotic process in yeast

3.1 – Acetic acid-induces apoptosis in yeast: a PCD process with physiological relevance

Apart from the proposal of PCD as a mechanism of eliminating yeast aged cells (Sinclair *et al*., 1998; Laun *et al*., 2001), the work described in the chapter 2 reports for the first time a PCD process inducible in yeast as a response to a physiological event, bringing new insights into the yeast cell death pathway. The demonstration of the occurrence of a PCD induced by acetic acid, which is a normal sub-product of alcoholic fermentation, further reinforces the physiological role of this active cell death process in normal yeast life cycle. Moreover, our results raise the possibility of PCD being more generalized in yeast than previously considered and extended to cell death induced by other stress agents besides those mentioned in this thesis.

3.2 – PCD in yeast can be mediated by a Mitochondria-Dependent Apoptotic Pathway (MDAP)

Mitochondria are well known to have a central role in energy metabolism and in cell life. However, damage to the mitochondria has been linked to cell death (Green and Reed, 1998; Yang *et al*., 1997; Kluck *et al*., 1997). Mitochondria have been implicated in mammalian PCD processes (Skulachev, 1996, 1998, 2000, 2001; Green and Reed, 1998; Yang *et al*., 1997; Kluck *et al*., 1997) being called the "cell death organelle". Results described in chapter 5 show that mitochondria participate in the PCD process induced by acetic acid in *S. cerevisiae*. We found that in yeast cells undergoing a PCD process induced by acetic acid, there is translocation of cytochrome *c* (CytC) to the cytosol and reactive oxygen species (ROS) production, two events known to be pro-apoptotic in mammals. Associated to these events, reduction in oxygen consumption and in mitochondrial membrane potential was found. Moreover, enzymatic assays showed that the activity of complex bc_1 was normal whereas that of cytochrome *c* oxidase (COX) was strongly decreased in accordance with the observed reduction in the amounts of COX II subunit and of cytochromes $a+a_3$. The acetic acid-induced PCD was found to be independent of oxidative phosphorylation since it was not inhibited by oligomycin treatment. Further evidence that the PCD process induced by acetic acid in *S. cerevisiae* is mediated by a Mitochondria-Dependent Apoptotic Pathway (MDAP) was obtained by the observation that three *S. cerevisiae* mutant strains lacking mitochondrial DNA, heme lyase or mitochondrial ATPase, respectively, fail to undergo acetic acid-induced PCD.

The results described in chapter 5 obtained with *S. cerevisiae* cells mutated in *ATP10* gene (coding for an ATPase assembly factor) suggest a role of mitochondrial ATPase complex in the PCD process under discussion. We found that the lack of this protein that inhibits the assembly of ATPase (Paul *et al*., 2000) prevents yeast apoptotic killing by acetic acid. We speculate that, considering the involvement of ATPase complex in the mammalian apoptosis (Matsuyama *et al*., 1998), the structural integrity of the

ATPase is required for that killing to occur probably because the ATPase complex would participate in the exit of apoptogenic factors out of mitochondria. The relevance of this interpretation would be extended further if we consider the advance it would bring to the understanding of the translocation process of mitochondrial apoptogenic factors to the cytoplasm in metazoan cells.

Mitochondria were called “the Pandora’s box” since they contain a series of proteins that, due to local post-translational modification, are not found anywhere else in the cells (Loeffler and Kroemer, 2000). CytC is one of these proteins, existing in cytoplasm as an immature apo-CytC and only after its import to mitochondria the attachment of the heme group occurs. Similarly, some flavoproteins such as the Apoptotic-Inducing Factor (AIF) are synthetized as precursors and, only after the import to the mitochondria, the FAD group is incorporated.
The release of CytC and of AIF has been described as keys events in several different mammalian apoptotic models (Loeffler and Kroemer, 2000). The possibility of those two mitochondrial proteins being in *S. cerevisiae*, as in mammalian cells, direct or indirect apoptogenic factors was successful addressed for the first time with our work.
Our finding (chapter 5) that acetic acid induces the release of CytC from yeast mitochondria, similarly with what was reported for Bax-expression (Manon *et al*., 1997) and for cell death mediated by deletion of histone chaperone *ASF1/CIA1* (Yamaki *et al*., 2001), raised the possibility that, when in the cytosol after its translocation from mitochondria, yeast CytC could work as an apoptogenic factor, as reported for mammalian cells. In these cells the pro-apoptotic activity of cytoplasmic CytC is due to the activation of a caspase cascade (Adrain and Martin, 2001). Therefore, the very recent report (Madeo *et al*., 2002) on the presence in *S. cerevisiae* of a caspase-related protease (Yeast Caspase-1 or YAC-1) that regulates yeast apoptosis, is very relevant. Those authors showed that the disruption of the YAC-1 gene results in a higher survival of *S. cerevisiae* cells to exposure to acetic acid comparatively to the wild type strain, whereas its overexpression leads to the

enhancement of acid-induced cell death. Therefore, these observations reinforce the interpretation of cytoplasmic CytC having, also in yeast, an apoptogenic activity.
On the other hand, since Kluck and collaborators (2000) had previously found that *S. cerevisiae* CytC fails to activate the caspases (DEVDases) in mammalian cytosols, it follows that the interaction between yeast CytC and yeast caspase YAC-1 would be specific, a finding that, if confirmed, would have interesting implications as will be discussed later.

Our studies described in chapter 5 on the release of CytC during the PCD induced by acetic acid in *S. cerevisiae* led to the consideration of another important event in yeast apoptosis. In fact, we found that the release of CytC from mitochondria to the cytosol is accompanied by the occurrence of another pro-apoptotic event, namely the production of ROS. The observed increase in ROS production in *S. cerevisiae* cells dying by apoptosis upon exposure to some concentrations of acetic acid is likely to be due to the loss of CytC at the mitochondria and to the reduction in cytochrome *c* oxidase (COX) activity. Whether loss of COX activity is a consequence of CytC release or a primary effect of acetic acid on the assembly of the enzyme remains to be studied although we favour the first possibility. The observed deficiency in COX activity could be responsible for an inhibition of the electron transfer with the accumulation of reducing equivalents in the middle portion of the electron transfer chain, and thus directing one-electron transfer to O_2, resulting in the production of superoxide (Cai and Jones, 1998). It is interesting that ROS production appears, therefore, as an event common to all the instances of *S. cerevisiae* apoptotic phenotypes reported so far (Madeo *et al.,* 1997; Madeo *et al*., 1999; Fröhlich and Madeo 2000; Laun *et al*., 2001; Levine *et al.,* 2001; Narasimhan *et al.,* 2001; Ludovico *et al*., 2002).

ROS may induce cell damage by modifying and affecting proteins, lipids and nucleic acids. However, radicals act as signal molecules and do not simply cause lethal damage to DNA, lipids or proteins (Madeo *et al*., 1999). In fact, ROS, such as $O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet-}$, are a well characterized class of apoptotic regulators, which are not proteins, that can trigger apoptosis in numerous mammalian cells (Kane *et al.,* 1993; Hockenbery *et al.,* 1993; Greenlund *et al.,* 1995; Slater *et al.,* 1995). ROS are byproducts of respiration and occur in every aerobic organism. A specific modulation of the respiratory chain may have been developed to increase the output of ROS as needed and CytC release may be a result of that modulation. In fact, the mitochondrial electron transport chain can be effectively blocked by removing CytC, becoming then the complex III highly reduced and reactive to oxygen. Further, the presence of mitochondrial CytC in the cytoplasm has a synergistic effect on ROS production either by formation of superoxide (reaction of oxygen with complex III) or of hydrogen peroxide from dismutation of superoxide that can then participate in Fenton reactions (Brennan *et al.,* 1994) catalysed by CytC (Blackstone and Green, 1999). During a further refinement in the regulation of apoptosis, release of CytC itself became used as an apoptotic signal, perhaps in order to make the regulatory cascade less dependent on the redox state of the cell (Blackstone and Green, 1999).

It has been demonstrated that ROS have a central role in yeast apoptosis (Madeo *et al.,* 1997; Madeo *et al*., 1999; Laun *et al*., 2001; Levine *et al.,* 2001; Narasimhan *et al.,* 2001; Ludovico *et al*., 2002). Madeo *et al.* (1999) also showed that radical trapping or growth under strictly anaerobic conditions prevent yeast cell death and the accompanying apoptotic effects, demonstrating that the radicals are not only a byproduct but also a promoter of the apoptotic-like features in yeast. Recently Laun *et al.* (2001) found that mitochondria are the source of ROS in senescent yeast cells. Mitochondria producing ROS were found only in these senescent cells but not in virgin cells.

These observations of radical induced apoptosis under several conditions including in senescent yeast cells, further strengthens the oxidative stress theory of apoptosis and

confirms the similarity between yeast and higher eukaryotic cells both in cell ageing and in apoptotic processes.

We have results further implicating mitochondria in yeast PCD processes besides CytC release and ROS production. As described in chapter 5, complete inhibition of the PCD due to acetic acid in *S. cerevisiae* was not achieved in mutant cells lacking CytC, suggesting the existence of another apotogenic factor besides CytC.
The results reported in chapter 6 indicate that, indeed, an equivalent to the Apoptosis-Inducing Factor (AIF), is also present in *S. cerevisiae* mitochondria and would be involved in yeast PCD processes. Those results show that a protein (Ynr074cp) with significant similarity with AIF of human and *Dyctiostelium discoideum* is located in *S. cerevisiae* mitochondria and, when *S. cerevisiae* cells were treated with camptothecin or were aged, it is translocated to the nucleus. Moreover, the level of Ynr074cp in yeast cells affects their susceptibility to the apoptotic killing by the chemotherapeutic agent camptothecin: mutant cells lacking YNR074c are more resistant while, conversely, *S. cerevisiae* overexpressing it are more susceptible.
In conclusion, the work reported in chapters 5 and 6 represent a significant contribution to the understanding of the role of mitochondria in the life and death of yeast cells and support the concept of the existence of a conserved cell death pathway.

In spite of all the progress in the understanding of the mechanism of cell death in yeast, several important questions remain unanswered, namely:

- Would CytC physically interact with the YCA-1 protein described by Madeo *et al*. (2002) leading to the formation of a yeast apoptosome?
- Which is (are) the mechanism(s) underlying the release of CytC and other apoptotic factors from mitochondria?

The recently reported channel, named mitochondrial apoptosis-induced channel (MAC) that was found in yeast mitochondria upon Bax expression (Pavlov *et al*.,

2001) could be a good candidate for the outer membrane pore through which CytC and other apoptotic factors exit mitochondria during apoptosis;

- How would AIF be involved in the mechanism of CytC release? Is CytC release partially controlled by the AIF translocation to the nucleus?
- Could the putative metacaspase-independent pathway (AIF) function as an amplification-loop of the death signal?
- Which is(are) the apoptotic signal(s) that "order" mitochondria to kill the cell? Is there any enzymatic complex, or specific mitochondria function, that, once affected, triggers the downstream death pathway?

The answer to these questions requires further understanding of the role of the mitochondria in yeast life and death. Although we cannot at this time answer the questions mentioned above, Figure 1 shows a model for the PCD cascade in *S. cerevisiae*, which deals with the information available until the present date.

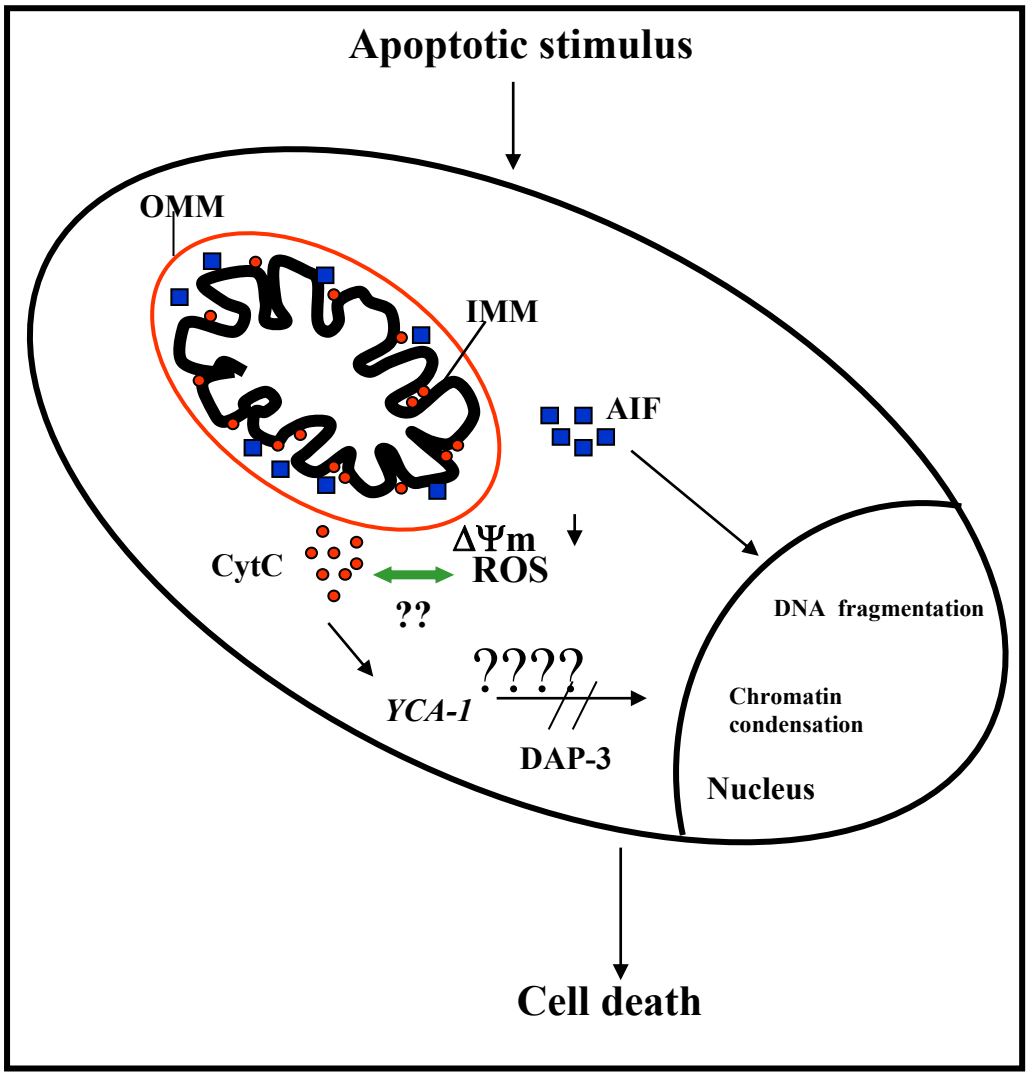


Figure 1- A model for the programmed cell death cascade in *S. cerevisiae*. OMM-outer mitochondrial membrane; IMM- inner mitochondrial membrane; AIF-Apoptotic-Inducing Factor; CytC-cytochrome *c*; YCA-1-Yeast-Caspase 1; DAP-3 – mitochondrial mediator of apoptosis; ROS-Reactive Oxygen Species and ΔΨm-mitochondrial membrane potential.

4 - Yeast as a powerful tool in apoptosis research

Recent results reported in the present thesis and elsewhere show that yeast represent a valuable model for apoptosis research since they possess the machinery for PCD and its genetics and molecular biology are powerful tools to identify the components of complex mammalian physiological pathways. Yeast provide the basis for efficient screens to determine how a specific protein kills the cell (via the isolation of resistant mutant cells) and for identification of novel mammalian regulators of cell death (see chapter 1). However, the new insights brought with the present work, and other recently published works, into the mechanisms of endogenous PCD process of yeast, that is in part homologous to the metazoan PCD, will contribute to the use of yeast as a tool in apoptosis research in a different perspective. As discussed before, both metazoan and yeast death machinery would presumably have descended from a hypothetical ancestral death programme. However, although the PCD processes in yeast and metazoans seem similar, they are different enough to allow their independent manipulation. This may have strong implications in the development of novel treatment approaches of some human diseases like mycoses. The reported role of CytC in the yeast PCD process is a striking example. In fact, yeast CytC, despite extensive sequence similarity with mammalian CytC, was found to be unable to activate the caspases DEVDases (Kluck *et al*., 2000), even though it has an important role in the PCD process in *S. cerevisiae* as discussed in 3.2. These observations suggest that the induction of apoptotic cell death in pathogenic yeast would be possible by the manipulation of part of the PCD pathway, without consequences for human cells, replacing the classical antimicrobial therapies. On the other hand, yeast-based strategies for studying apoptotic genes will certainly continue to provide new insights into conserved cell death machinery and would allow in the near future the identification of genes and molecules that modulate the functions of apoptosis-regulator proteins, leading to the design of apoptosis-modulatory drugs. The use of yeast-based functional analysis for human proteins implicated in apoptosis will provide

powerful systems for the efficient screening for drugs and may result in the finding of universal activators or inhibitors of apoptosis, that would be helpful for basic research, and potentially for medical applications. Exploiting these assays for compounds that are able to alter apoptotic proteins in different clinically useful ways, may become one of the greatest contributions that yeast make to humans besides bread and wine.

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