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Involvement of mitochondrial proteins in yeast apoptosis

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Thesis for Doctoral degree in Sciences

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and
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Involvement of mitochondrial proteins in yeast apoptosis

Abstract

In the yeast *Saccharomyces cerevisiae,* acetic acid triggers a mitochondria-mediated death pathway with apoptotic characteristics. In mammalian cells, the mitochondrial outer membrane permeabilization (MOMP), necessary for the release of pro-apoptotic proteins, is a pivotal event for the activation of the apoptotic cascade in numerous cell death pathways. MOMP is thought to be mediated by a complex of proteins that constitute the permeability transition pore (PTP). Since *S. cerevisiae* possesses orthologues of the components believed to be involved in mammalian PTP composition/regulation, it was used herein to study such proteins both concerning their role in mitochondrial permeabilization and involvement in the course of cell death. The proteins studied were Por1p (yeast voltage-dependent anion channel, VDAC), Cpr3p (mitochondrial cyclophilin) and Aac1/2/3p (ADP/ATP carrier, AAC). We found that during apoptosis triggered by acetic acid deletion of *CPR3* has no effect. Absence of Por1p enhances and absence of AAC proteins decreases acetic acid-induced apoptosis indicating an anti- and pro-apoptotic role, respectively, for these proteins. Moreover, the pro-death role of AAC does not require the ADP/ATP translocase activity. Absence of AAC proteins impairs MOMP and release of cytochrome *c*, which is degraded along with other mitochondrial inner membrane proteins. We observe that, during acetic acid-induced apoptosis, caspase activation is independent of AAC proteins but strongly dependent on the growth phase of the culture. In addition, a strain deleted for the yeast metacaspase *YCA1* shows decreased overall caspase activation but still died exhibiting apoptotic features, supporting the existence of an Yca1p-independent apoptotic pathway.

Fragmentation and degradation of mitochondria are common events in mammalian apoptosis. Both fragmentation and degradation are able to strongly affect the course of cell death and a relation has been proposed between these events and MOMP/cytochrome *c* release. Interestingly, *por1*Δ cells exhibit fragmented mitochondrial reticulum in the absence of any death stimulus. This phenotype however, does not contribute to the apoptosis stimulation observed in *por1*Δ mutant. We observe that during acetic acid-induced apoptosis the absence of AAC proteins leads to aggregation of fragmented mitochondria and a slower degradation of these organelles. Degradation of mitochondria in response to acetic acid is not due to classical autophagy or mediated by Uth1p-dependent mitophagy. We show that mitochondrial degradation during acetic acid-induced apoptosis is

dependent on the protease Pep4p that is released from the vacuole to the cytosol. *pep4*Δ cells, which are strongly impaired in mitochondria degradation, are sensitized to acetic acid and mainly die by necrosis. This suggests that mitochondria degradation in response to acetic acid helps to sustain the apoptotic process. Taken together, the results show that vacuolar and mitochondrial proteins interfere with mitochondria morphological remodelling and subsequent degradation, suggesting that there is a complex interplay between these organelles in the regulation of yeast cell death.

In conclusion, we were able exploring the distinctive ability of yeast to survive without respiration-competent mitochondria to study the involvement of mitochondria and mitochondria-interacting proteins in cell death. Additional studies using this model will undoubtedly further our understanding of the complex cell death processes.

Envolvimento de proteínas mitocondriais na apoptose em leveduras

Resumo

Na levedura *Saccharomyces cerevisiae*, o ácido acético desencadeia uma via de morte dependente da mitocôndria com características apoptóticas. Nos mamíferos, a permeabilização da membrana mitocondrial externa (PMME), necessária para a libertação de proteínas mitocondriais pró-apoptóticas, constitui um passo crítico na activação do processo de morte apoptótico. Pensa-se que a PMME seja mediada por um complexo proteico que constitui o poro de transição de permeabilidade (PTP). Uma vez que *S. cerevisiae* possui ortólogos de algumas das proteínas que se pressupõe compor/regular o PTP de mamíferos, o principal objectivo desta tese foi estudar essas proteínas, tanto ao nível do seu papel na permeabilização mitocondrial bem como do seu envolvimento na execução do processo de morte apoptótico. As proteínas estudadas incluem o canal Por1p (canal de aniões dependente da voltagem, VDAC), a ciclofilina mitocondrial Cpr3p e as três isoformas do transportador Aac1/2/3p (antiportador mitocondrial de ATP/ADP, AAC). A interrupção do gene *CPR3* não afecta a morte induzida pelo ácido acético. A interrupção do gene *POR1* estimula e a ausência dos genes *AAC1/2/3* protege as células da morte apoptótica induzida pelo ácido acético indicando um papel anti- e pró-apoptótico, respectivamente, para estas proteínas. A função das proteínas AAC na morte celular apoptótica não depende da actividade de antiporte. Em células tratadas com ácido acético a ausência das proteínas AAC afecta negativamente a PMME e a libertação de citocromo *c*, o qual juntamente com outra proteína da membrana mitocondrial interna sofre degradação. Observamos que durante o processo apoptótico induzido pelo ácido acético ocorre a activação de caspases e que esta activação é independente das proteínas AAC e fortemente dependente da fase de crescimento da cultura. Adicionalmente, uma estirpe interrompida na metacaspase de levedura, *YCA1*, que exibe um decréscimo na activação total de caspases, desencadeia uma morte celular apoptótica. Esta observação suporta a existência de uma via de morte apoptótica independente de Yca1p.

A fragmentação e a degradação mitocondrial são eventos comuns no processo apoptótico em mamíferos. Estes eventos podem ter um forte impacto no decurso da morte celular tendo sido proposta uma relação entre estes e a PMME/libertação de citocromo *c*. Curiosamente, a ausência da Por1p origina uma elevada percentagem de células com o retículo mitocondrial fragmentado mesmo na ausência de qualquer estímulo externo. Este

fenótipo, contudo, não parece contribuir para a estimulação da apoptose exibida pela estirpe *por1*Δ. Durante o processo de morte apoptótico induzido pelo ácido acético, a ausência das proteínas AAC leva à formação de agregados mitocondriais associada a uma menor degradação destes organelos. A degradação mitocondrial durante a apoptose induzida pelo ácido acético não é devida à activação da autofagia clássica, nem mediada pela mitofagia dependente de Uth1p. A degradação mitocondrial induzida pelo ácido acético é dependente da protease Pep4p, que é libertada do vacúolo para o citosol. A estirpe *pep4*Δ que exibe uma degradação mitocondrial manifestamente diminuída é sensível ao ácido acético, e direcciona o processo de morte para uma forma necrótica. Estes dados sugerem que a degradação mitocondrial favorece a manutenção do processo apoptótico. Globalmente, os resultados evidenciam que proteínas vacuolares e mitocondriais interferem com a morfologia mitocondrial e subsequente degradação e sugerem uma interacção e regulação complexa entre estes organelos durante o processo apoptótico em leveduras.

Concluindo, a capacidade de sobrevivência da levedura em condições em que a respiração mitocondrial está afectada ou ausente permitiu a sua utilização como um excelente modelo para a pesquisa do papel de proteínas mitocondriais, ou de proteínas que interactuam com a mitocôndria no processo de morte celular. Estudos adicionais utilizando este modelo irão seguramente contribuir para uma melhor compreensão dos processos de morte celular.

Index

Abbreviations

AAC	ADP/ATP carrier
AK	adenylate kinase
ANT	adenine nucleotide translocator
BA	bongkrekic acid
CATR	carboxyatractyloside
CS	citrate synthase
CsA	cyclosporin A
CypD	cyclophillin D
DAPI	4,6-diamidino-2-phenylindole dihydrocloride
Diamide	diazenedicarboxylic acid bis 5N,N-dimethylamide
D_2R	$(Asp)_2$-Rhodamine 110
FITC-VAD-fmk	fluorescein isothiocyanate conjugate of z-VAD-fmk
MOMP	mitochondrial outer membrane permeabilization
OMM	outer mitochondrial membrane
PGK	phosphoglycerate kinase
Pi	phosphate
PI	propidium iodide
PiC	mitochondrial phosphate carrier
PT	permeability transition
PTP	permeability transition pore
ROS	reactive oxygen species
SCFA	short chain fatty acids
VDAC	voltage-dependent anion channel
YMUC	yeast mitochondrial unselective channel
z-VAD-fmk	carbobenzoxy-valyl-alanyl-aspartyl-fluoromethylketone
$\Delta\Psi_m$	mitochondrial membrane potential

Chapter 1

Introduction

- Mitochondria in apoptosis
- Mitochondrial outer membrane permeabilization
- Mitochondria morphological remodelling in apoptosis

This chapter comprises parts from the following publication:

Pereira, C., R.D. Silva, L. Saraiva, B. Johansson, M.J. Sousa, and M. Corte-Real. 2008. Mitochondria-dependent apoptosis in yeast. Biochimica et Biophysica Acta [Epub ahead of print].

Introduction

Around 1850, microscopists used the Greek word “chondros” to mean “granule” and describe distinct sub-cellular structures seen in the light microscope. Improved staining techniques yielded more accurate descriptions and grains were seen as “threads”, “mitos” in Greek. Thus, those highly dynamic and variable structures were called mitochondria (Benda, 1898). In the following years, many people speculated on the role of mitochondria in the cell, until Warburg (Warburg, 1913) discovered the association of mitochondria with respiration. Another forty years of biochemical studies led to the characterization of mitochondria as the sites of energy conversion and cellular respiration. It was only at the end of the XX century, with the unexpected discovery of the mitochondrial localization of the anti-apoptotic protein Bcl-2 (Monaghan et al., 1992; Krajewski et al., 1993; de Jong et al., 1994) that the “powerhouses” of the cell were suggested to play a role in cell death. A large amount of rapidly accumulating evidence, including the involvement of cytochrome *c* release from mitochondria in cell death (Liu et al., 1996), led to the acknowledgment of these organelles as major players in cell death regulation. The role of mitochondria in cell death is wide-ranging. After its initial recognition in mammalians, it was soon reported for diverse organisms such as plants or even unicellular yeast, which constitute the object of our study.

The foregoing introduction intends to provide background information that is not present in the individual chapters concerning the role of mitochondria in yeast cell death. However, since the yeast field is strongly influenced by hypotheses driven by our understanding of the mammalian system, on which the majority of the studies have been performed, this model will also be addressed.

Mitochondria in apoptosis

Apoptosis is probably the best characterized form of programmed cell death. It was originally defined based on morphological features found in dying mammalian cells like nuclear condensation, nuclear fragmentation, membrane blebbing, cellular fragmentation into membrane-bound bodies, phagocytosis of the dying cell, and lack of an ensuing inflammatory response (Kerr, 1971; Wyllie et al., 1980). Some of these features were recently observed in yeast, although others, reasonably due to its unicellular nature, do not occur. As such, the classification of cell death in yeast as apoptosis is still a matter of debate.

Apoptosis may be elicited through several molecular pathways. These pathways have been best characterized in higher eukaryotes, and the most prominent ones are the extrinsic and intrinsic pathways (Hengartner, 2000) (Fig. 1). The extrinsic pathway (or the death receptor pathway) is defined as mitochondria-independent, although mitochondria can be involved in the amplification of the death signal. It involves the activation of receptors in the plasma membrane through binding of ligands that trigger a proteolytic chain responsible for the characteristic morphological features of apoptosis. The intrinsic pathway (or mitochondrial pathway) involves the permeabilization of the mitochondrial outer membrane allowing the release into the cytosol of a variety of pro-apoptotic proteins. The release of mitochondrial factors is widely accepted as the initiating event of the intrinsic pathway of apoptosis, but it can also serve as an amplification mechanism during the extrinsic pathway of cell death. The proteins released can either activate a group of enzymes of the cysteine protease family known as caspases or act in a caspase-independent fashion to bring about cell death. Factors activating caspase-dependent pathways include cytochrome *c*, Smac/DIABLO (Second mitocondria-derived activator of caspases/Direct IAP-binding protein with low pI) and possibly HtrA2/Omi (high temperature requirement protein A2), while caspase-independent factors include apoptosis inducing factor (AIF) and endonuclease G (Endo G) (van Loo et al., 2002; Kuwana and Newmeyer, 2003).

Among the released factors, cytochrome *c* plays an essential role in mitochondria-dependent apoptotic death. Upon its release, it induces the apoptosis protease-activating factor 1 (APAF-1) and ATP/dATP to assemble the apoptosome, a molecular platform which promotes the proteolytic maturation of caspase-9 (Cain et al.,

2002). Active caspase-9, in turn, cleaves and activates the effector caspases, which finally lead to the apoptotic phenotype.

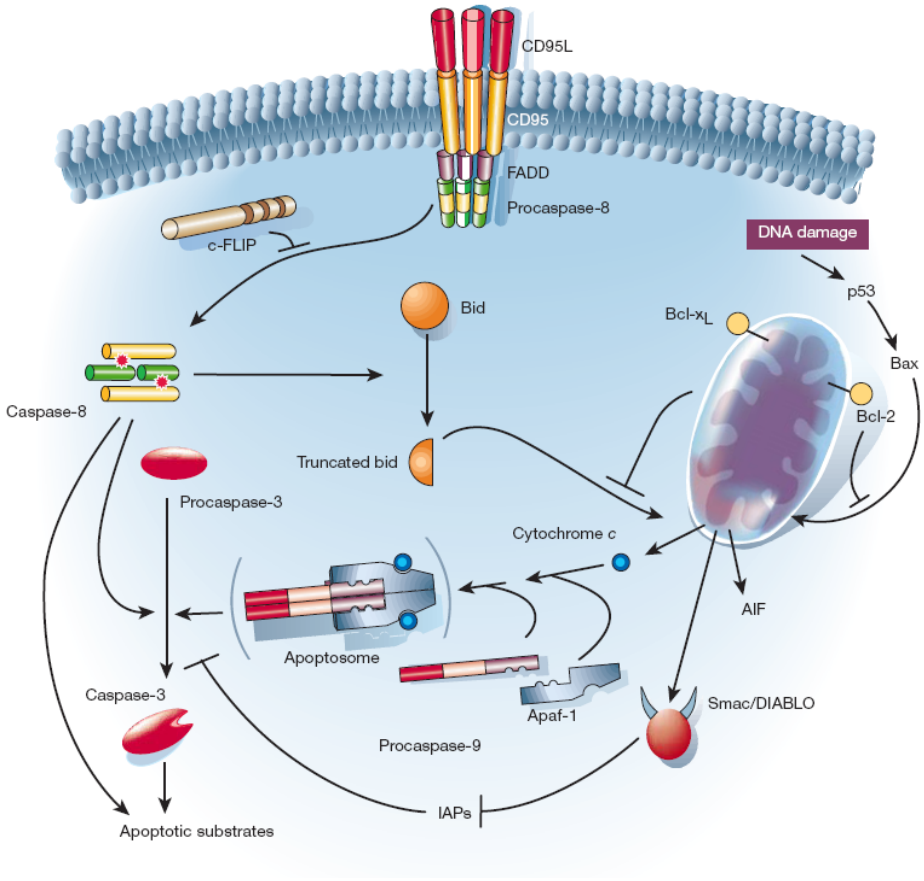

Figure 1. The two major apoptotic pathways in mammalian cells are shown: the extrinsic death receptor pathway and the intrinsic mitochondrial pathway. A crosstalk can occur between the two pathways in mammalian cells, mediated by the pro-apoptotic Bcl-2 family member Bid (scheme from (Hengartner, 2000)).

Besides caspases, other proteases have emerged as mediators of apoptosis (Bursch, 2001). These proteases include the cysteine and aspartate cathepsins that normally reside in the lysosome and are released in an early phase of apoptosis (Roberg et al., 1999). However, the mechanisms by which cathepsins are released from lysosomes and contribute to apoptosis are largely unspecified. There is evidence indicating that these lysosomal proteases can trigger a mitochondrion-dependent pathway (reviewed in Boya et al., 2003; Minarowska et al., 2007). It was reported that the release of cathepsin D, the major intracellular aspartate protease, precedes translocation of cytochrome *c* and that this translocation can be prevented by inhibiting

cathepsin D, delaying the course of cell death (Roberg et al., 1999; Johansson et al., 2003; Emert-Sedlak et al., 2005). This protease was also implicated in the selective release of AIF through activation of the pro-apoptotic protein Bax in a Bid-independent manner (Bidere et al., 2003). Other authors showed that cathepsin D cleaves Bid *in vitro* and that Bid is no longer activated in cathepsin-deficient fibroblasts, indicating that Bid may be a direct downstream target of cathepsin D, leading to cytochrome *c* release (Stoka et al., 2001; Heinrich et al., 2004). Conflicting data, however, attribute Bid cleavage to members of the cathepsin family other than cathepsin D (Cirman et al., 2004). While these data provide evidence for cathepsin D as a positive regulator in the death process, this protease has an opposing role in cancer progression. Cathepsin D, like other cathepsins, is overexpressed in a number of cancers, and the magnitude of expression often has prognostic significance (Wu et al., 1998; Rochefort et al., 2000; Guicciardi et al., 2004).

Mitochondrial involvement in yeast apoptosis is probably the most unifying feature in this recent field. It was only in 1997 that death with apoptotic features was described for the first time in yeast (Madeo et al., 1997), and since then the involvement of mitochondria has been shown for different death stimuli. It is still not clear if a mitochondria-independent pathway exists, since mitochondria are recurrently associated with the different death scenarios studied (reviewed in Eisenberg et al., 2007; Pereira et al., 2008).

In yeast, homologues of cytochrome *c*, AIF, EndoG and HtrA2/Omi were uncovered and their role in cell death has been assessed. Aif1p and Nuc1p, likewise cytochrome *c*, were shown to have a mitochondrial localization from where they escape upon apoptotic induction (Ludovico et al., 2002a; Wissing et al., 2004a; Buttner et al., 2007). However, the yeast homologue of HtrA2, Nma111p (Fahrenkrog et al., 2004), is not predominatly localized in mitochondria like its mammalian orthologue but in the nucleus, where it remains upon apoptotic induction. A caspase-related protease, Yca1p, was described as a mediator of apoptosis in yeast (Madeo et al., 2002). However, requirement for Yca1p to the course of apoptosis heavily relies on the trigger used (reviewed in Liang et al., 2008; Mazzoni and Falcone, 2008). While, under osmotic stress induced apoptosis, a partial dependence of Yca1p activation on cytochrome *c* was described (Silva et al., 2005), in other experimental conditions the metacaspase seems to act upstream of mitochondria (Fahrenkrog et al., 2004; Mazzoni et al., 2005). The

involvement of other proteases in yeast apoptosis has been reported in only few cases. Nma111p, refered above, is a serine protease described to have a role in promoting yeast apoptosis (Fahrenkrog et al., 2004). Esp1p is a cysteine protease that was shown to be the responsible for the cleavage of Mcd1p, the yeast orthologue of the human cohesin Rad21 (Yang et al., 2008). The C-terminal fragment of Mcd1p is translocated from the nucleus into mitochondria, causing the amplification of cell death in a cytochrome *c* dependent manner (Yang et al., 2008). An aspartate protease, Pep4p, like its mammalian akin cathepsin D, was shown to translocate from the vacuole to the cytosol during apoptosis induced by hydrogen peroxide (Mason et al., 2005) and actin cytoskeleton stabilization (Gourlay and Ayscough, 2006).

Mitochondrial outer membrane permeabilization

An increase in the permeability of the outer mitochondrial membrane (OMM) is a central event in many cell death processes, and is mandatory for the release of proteins with key functions in cell dismantling. As already discussed, this membrane sequesters a variety of putative pro-apoptotic proteins in the mitochondrial intermembrane space. When this membrane is permeabilized or its integrity is lost, these proteins are released into the cytosol and contribute to bring about cell death. How OMM permeabilization is achieved is still a matter of intensive research and will be further discussed.

Clues from mammals

Several models have been put forward to explain the permeabilization of mitochondrial membranes during apoptosis. On the whole, the main mechanisms proposed are the direct OMM permeabilization model and the permeability transition (PT) model (Fig. 2). According to the former model, pro-apoptotic Bcl-2 family proteins such as Bax and Bak promote, directly or indirectly, the opening of pores on the OMM that are large enough to allow the channelling of apoptogenic proteins (reviewed in Adams and Cory, 2007). Although the importance of this family of proteins for mammalian apoptosis is unarguable, they will not be further discussed, since these proteins do not have obvious orthologues in yeast and as such are beyond the scope of this thesis. In the latter model, rupture of the OMM and the release of the intermembrane

space components follow the opening of a voltage-dependent, high-conductance multi-protein complex known as the permeability transition pore (PTP). The term 'permeability transition' was introduced by Haworth and Hunter (1979) and is defined as a sudden increase of the inner membrane permeability to solutes with a molecular mass below ≈ 1500 Da. The hypothesis that mitochondrial PTP opening could be a factor in cell death was put forth 20 years ago (Crompton et al., 1988; Armstrong, 2006). Mitochondrial PTP opening causes a collapse of the mitochondrial membrane potential and the osmotically-driven matrix swelling, leading to cristae unfolding and, subsequently, to breaches in the OMM (Jayadev et al., 1994; Crompton, 1999; Rasola and Bernardi, 2007). It is important to stress that PTP openings can induce rupture of the OMM only as a result of matrix swelling, and therefore apoptotic proteins do not exit mitochondria through the PTP itself.

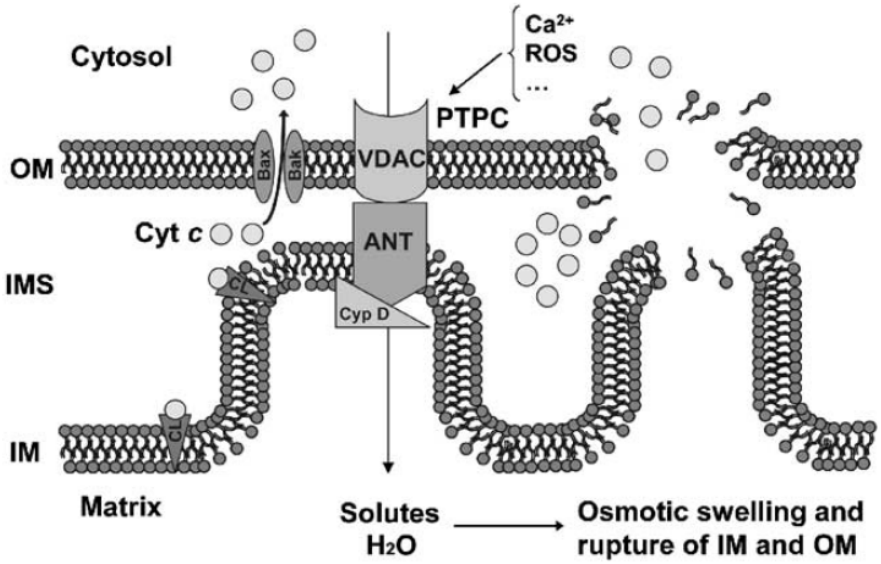

Figure 2. MOMP has been proposed to occur by several mechanisms. The two most prominent are depicted in the scheme. 1) Activated pro-apoptotic proteins of the Bcl-2 family assemble into large multimers, allowing the release of inner membrane space proteins 2) Opening of the PTP channel (PTPC), which may be regulated by CypD, leads to an osmotic imbalance that induces the swelling of the mitochondrial matrix, causing outer mitochondrial rupture. Other theories (not depicted in the scheme) propose interactions between PTP components and pro-apoptotic members of the Bcl-2 family to provide a pathway for release of inner membrane proteins or, alternatively, formation of lipidic pores by alterations in the mitochondrial membrane (Kroemer et al., 2007). Scheme from (Garrido et al., 2006).

Besides the release of apoptogenic proteins as a result of the outer mitochondrial membrane rupture, an important consequence of the PTP opening is the uncoupling of oxidative phosphorylation. Not only is the synthesis of mitochondrial ATP prevented but there is also rapid hydrolysis of the ATP produced by glycolysis through reversal activity of the mitochondrial ATPsynthase (Di Lisa and Bernardi, 1998). An increase in cytosolic calcium (Macho et al., 1997) and decrease in pH (Furlong et al., 1998) also seems to follow the PT. The PTP is inhibited by cyclosporin A (CsA) (Crompton et al., 1988) through its binding to cyclophillin D (CypD) and by ADP and Mg^{2+} (Broekemeier et al., 1998). High mitochondrial membrane potential ($\Delta\Psi_m$), cyclosporin A, ADP, H^+, and bongkrekic acid (BA) stabilize the closed conformation of the PTP, whereas low $\Delta\Psi m$, increased intramitochondrial Ca^{2+}, low Pi, and carboxyatractyloside (CATR) promote the open conformation (Chernyak and Bernardi, 1996; Bernardi, 1999). Interestingly, evidence suggests that in the presence of ATP (necessary for apoptosis) the mitochondrial PT leads to apoptosis, while in the absence of ATP the mitochondrial PT leads to necrosis (Tsujimoto, 1997; Qian et al., 1999).

The molecular components of the PTP remain an unsolved riddle, despite detailed functional characterization. In 1998, Halestrap and colleagues showed that PTP activation required the specific interaction between the mitochondrial matrix protein CypD, a member of a family of highly homologous peptidylprolyl cistrans isomerases (Walsh et al., 1992), and the adenine nucleotide translocator (ANT) from the inner membrane (Woodfield et al., 1998). CATR and BA, effectors of the mitochondrial permeability transition as referred above, are specific ligands of ANT. In the same year, the involvement of the voltage dependent anion channel (VDAC) in the outer mitochondrial membrane was also proposed (Crompton et al., 1998). Further research led to the idea that these PTP proteins interacted at specialized contact sites between the mitochondrial outer and inner membranes and form a polyprotein structure spanning both membrane systems (Crompton, 2000). Other proteins such as the benzodiazepine receptor, hexokinase, and creatine kinase have been appointed as regulators of the PTP (Armstrong, 2006). Yet, the validity of this model has been challenged, since ablation of the suspected core components of the PTP, i.e., ANT, VDAC and CypD showed that none of them is essential for pore formation (Kokoszka et al., 2004; Basso et al., 2005; Nakagawa et al., 2005; Krauskopf et al., 2006a; Baines et al., 2007). Instead, ANT and CypD were proposed by some groups to have a regulatory role on PTP (Kokoszka et al., 2004; Azarashvili et al., 2007; Chiara et al., 2008; Leung and Halestrap, 2008). Among

those were Halestrap and colleagues, who reformulated their hypothesis of ANT as a structural pore component and suggested that the protein fulfilling the latter role is the mitochondrial phosphate carrier (PiC). A conformational change of the PiC, facilitated by ANT and CypD, induces pore opening (Leung and Halestrap, 2008). In the absence of some or all of the three VDAC isoforms, the basic properties of the PTP did not change (Krauskopf et al., 2006b; Baines et al., 2007; Chiara et al., 2008). However, sensitivity to cell death has been reported (Cheng et al., 2003; Baines et al., 2007; Chiara et al., 2008), indicating VDAC proteins, or some specific isoform, may also play a regulatory role (Lemasters, 2007).

Another model emerged to explain the observation that the mitochondrial PT was not always blocked by CsA (He and Lemasters, 2002). These authors propose PTP is not a pre-formed pore but the result of oxidative damage of mitochondrial inner membrane proteins. Aggregation of misfolded proteins due to thiol crosslinking at hydrophilic surfaces defines aqueous transmembrane channels that result in membrane permeabilization. He and Lemasters (He and Lemasters, 2002; Lemasters, 2007) describe two possible functional modes for this PTP model, one activated by Ca^{2+} and inhibited by CsA and the other unregulated (Fig. 3). Chaperone-like proteins bind to the amphipathic protein clusters to block pore conductance. CypD also binds to the clusters, perhaps to refold proteins to their native state. Increased Ca^{2+} causes CypD to perturb the protein cluster/chaperone complex that opens into a regulated PTP, an effect antagonized by CsA. When nascent pores formed by misfolded protein clusters exceed the number of chaperones available to regulate conductance, unregulated pore opening occurs. Such unregulated pores no longer require Ca^{2+} for opening and are insensitive to CsA inhibition. The protein misfolding model of PTP formation accounts for the occurrence of PT in the absence of the putative PTP components, like ANT, because other proteins can misfold in their absence to form PT pores. However, because ANT is the most abundant protein in the mitochondrial inner membrane, ANT would likely contributes to the pore formation.

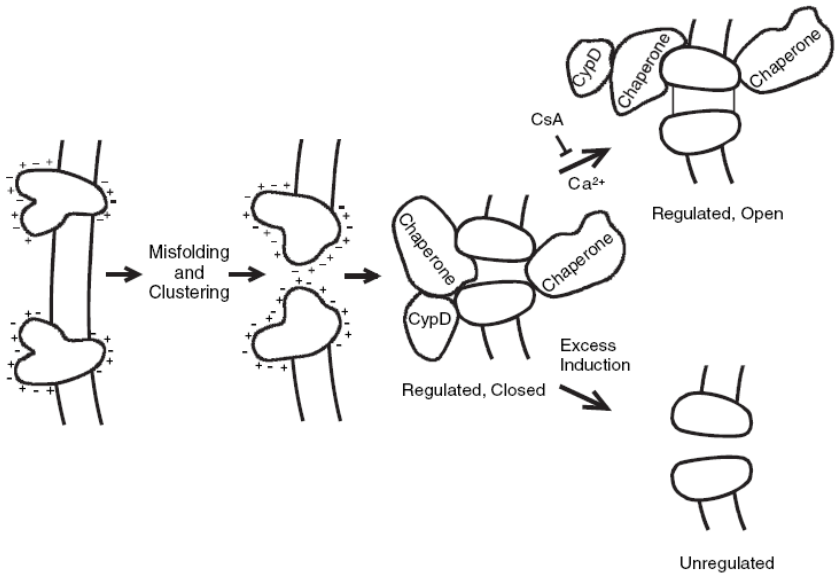

Figure 3. Model of the PTP formed by misfolding and clustering of proteins due to oxidative damage. This model proposes two modes for the PTP, one regulated and exhibiting the classical features like Ca^{2+} regulation and CsA inhibition, and another, unregulated, formed when oxidative damage reaches an extent beyond the control of chaperones. Scheme from (Lemasters, 2007).

Insights from yeast

Yeast does not possess obvious orthologues of Bcl-2 family members, but the core components ascribed to the mammalian PTP, (ANT, Adenine Nucleotide Translocator; VDAC, Voltage Dependent Annion Channel; and cyclophilin D) are highly conserved among eukaryotic organisms. In the yeast *S. cerevisiae* there are three isoforms of the ADP/ATP carrier, orthologues of ANT, encoded by the genes *AAC1*, *AAC2*, *AAC3*, whose expression is differentially regulated by environmental conditions (Adrian et al., 1986; Lawson and Douglas, 1988; Kolarov et al., 1990). Yeast also possesses two VDAC genes, *POR1* and *POR2*, with *POR1* coding for the major isoform, and a mitochondrial cyclophilin, Cpr3p, known to be involved in the protein folding process (Matouschek et al., 1995).

Most of the studies concerning the role of putative PTP components in death and mitochondrial permeabilization in yeast have been performed in cells expressing the pro-apoptotic protein Bax. Heterologous expression of Bax causes growth arrest and cell death in *S. cerevisiae*, accompanied by mitochondrial membrane permeabilization, cytochrome *c* release and ROS production (Greenhalf et al., 1996; Ligr et al., 1998; Xu

et al., 1999; Polcic and Forte, 2003; Priault et al., 2003a; Priault et al., 2003b; Kissova et al., 2006). The role of PTP in Bax-induced cytochrome *c* release and cell death was studied by examining the involvement of putative components of PTP, namely the Por1p, AAC and cyclophilin. The results obtained were quite contradictory, some showing a role for Por1p and AAC in Bax-induced death (Marzo et al., 1998; Shimizu et al., 2000) and others showing that Bax is able to induce cytochrome *c* release and/or cell death independently of the putative PTP components Por1p, AAC and cyclophilin (Priault et al., 1999b; Priault et al., 1999d; Gross et al., 2000; Kissova et al., 2000; Sanjuan Szklarz et al., 2007). In mammalian systems Bax, alone or together with other pro-apoptotic members of the Bcl-2 family, has proven to be sufficient to induce mitochondrial membrane permeabilization (Jurgensmeier et al., 1998; Kuwana et al., 2002). Therefore, heterologous expression of Bax does not appear suitable to study components of alternative modes of mitochondrial membrane permeabilization. Instead, the use of stimuli that elicit activation of the yeast apoptotic machinery should be more adequate to find such alternative components.

Like mammalian mitochondria, yeast mitochondria have the ability to form an inner membrane large-conductance unselective channel similar in size to its mammalian counterpart. However, the different regulatory properties of the yeast mitochondrial unselective channel (YMUC), also called yeast PTP (Jung et al., 1997), make it a controversial counterpart of the mammalian PTP (reviewed in Manon et al., 1998). While the mammalian PTP is opened in the presence of Ca^{2+} and Pi and closed by ATP and CsA (Halestrap and Davidson, 1990), the YMUC is induced by ATP and respiratory substrates and closed by high phosphate (Pi) concentrations (Velours et al., 1977; Roucou et al., 1995). Additionally, YMUC is insensitive to CsA (Jung et al., 1997; Kowaltowski, 2000; Gutierrez-Aguilar et al., 2007) although yeast possess a CsA inhibitable mitochondrial cyclophilin (Matouschek et al., 1995). The effect of Ca^{2+} on YMUC is more complicated, since all the possible scenarios (no effect, inhibition or stimulation) have been reported. It has been described that high Ca^{2+} levels have no effect in yeast PTP induction (Guerin et al., 1994; Jung et al., 1997). However, *S. cerevisiae* does not have a mitochondrial Ca^{2+} uniporter equivalent to the mammalian one (Carafoli et al., 1970) and this could account for Ca^{2+} resistance. This hypothesis was convincingly discarded by Jung and colleagues (Jung et al., 1997), who showed that large amounts of Ca^{2+} can be accumulated in brewer yeast mitochondria in the presence of the Ca^{2+} ionophore ETH129 and Pi, without PTP induction. Additionally, in contrast

to *S. cerevisiae*, *Endomyces magnusii* possess mitochondrial systems for Ca^{2+} influx and efflux effectively regulated and do not exhibit the classical Ca^{2+} dependent PTP-induction either (Deryabina et al., 2004).

At the moment, both the functionality of the YMUC and its molecular composition are uncertain. The participation of the inner membrane AAC (Lohret et al., 1996) or VDAC in the Ca^{2+}-insensitive pore has been dismissed (Ballarin and Sorgato, 1995). Nevertheless, carboxyatractyloside, an ANT specific ligand, is able to inhibit pore formation (Roucou et al., 1997) and its voltage dependence is altered in *por1*Δ cells, although the large conductance channel is still detected (Lohret and Kinnally, 1995). The fact that YMUC is not sensitive to CsA even though yeast possess a CsA inhibitable mitochondrial cyclophilin makes this protein an unlikely component of YMUC. Other authors have found an inhibitory effect of Ca^{2+} on YMUC (Perez-Vazquez et al., 2003). Later on, VDAC was appointed as the site of Ca^{2+} action since Ca^{2+} was no longer inhibitory in a *por1*Δ strain (Gutierrez-Aguilar et al., 2007). The sensing of Ca^{2+} by an outer mitochondrial membrane protein would override the problem of Ca^{2+} uptake by *S. cerevisiae* mitochondria. Despite all the aforementioned data, a Ca^{2+} and Pi-dependent, CsA insensitive pore did arise in *S. cerevisiae* in response to ATP (Kowaltowski et al., 2000). Formation of this pore, whose molecular composition was not investigated, required the presence of the cross-linking agent phenylarsine oxide (PhAsO) or inhibitors of catalase, and was facilitated in mutants lacking the thioredoxin peroxidase gene (Kowaltowski et al., 2000). This yeast PTP induction was accompanied by enhanced H_2O_2 production and decreased cell viability. The Ca^{2+}-induced yeast PTP seems to require overcoming the yeast highly active antioxidant system. The potential involvement of this pore in yeast apoptotic scenarios can easily be conciliated with the observation that oxidative stress is a common point in all the yeast apoptotic scenarios. An increase in mitochondrial ROS is often accompanied by cytochrome *c* release, indicating the occurrence of mitochondria outer membrane permeabilization in yeast, even in the absence of heterologous expression of Bcl-2 family members (Yamaki et al., 2001; Ludovico et al., 2002b; Pozniakovsky et al., 2005; Braun et al., 2006). Pozniakovsky and colleagues reported a rise in cytosolic Ca^{2+} in cells undergoing amiodarone-induced apoptosis (Pozniakovsky et al., 2005) and a rise was observed under acetic acid-induced apoptosis (our unpublished results). In addition, it was shown that overexpression of *Arabidopsis thaliana* BI-1 (Bax inhibitor-1) does not suppress Bax-induced cell death in *S. cerevisiae* mutants lacking endoplasmic reticulum Ca^{2+}-

ATPase (Pmr1 or Spf1), supporting a role for Ca^{2+} in yeast cell death (Ihara-Ohori et al., 2007).

While the lack of an effect of CsA on yeast PTP was consensual (Jung et al., 1997; Kowaltowski et al., 2000; Gutierrez-Aguilar et al., 2007) the effect of this compound on cell death is less clear (Severin and Hyman, 2002; Wissing et al., 2004a). The role of Cpr3p, the mitochondrial target of CsA, in apoptosis is also uncertain. Mutation of *CDC48* ($cdc48^{S565G}$), a gene essential in the endoplasmic reticulum (ER)-associated protein degradation (ERAD) pathway, leads to apoptosis accompanied by cytochrome *c* release and mitochondrial enlargement (Braun et al., 2006). Authors found that Cpr3p was depleted in mitochondrial extracts of $cdc48^{S565G}$ cells, suggesting Cpr3p plays a role in apoptosis, at least in this scenario. Very recently it was shown that deletion of Cpr3p abrogated copper-, but not manganese-induced apoptosis (Liang and Zhou, 2007). The involvement of AAC and VDAC proteins in yeast apoptosis is also not clear. Absence of AAC or VDAC proteins did not affected apoptosis induced by NaCl (Huh et al., 2002). In contrast, strains lacking the AAC's or VDAC proteins were shown to be highly resistant to cell death induced by heterologous expression of the viral protein R encoded by HIV-1 (Jacotot et al., 2000). Recently, Buttner and colleagues (Buttner et al., 2007) observed that Nuc1p, a yeast EndoG orthologue, usually localizes to the inner mitochondrial membrane but, under apoptosis induction, translocates to the nucleus, leading to cell death. The same authors showed that Nuc1p physically interacts with both Aac2p and Por1p. Deletion of AAC proteins reduced the sensitizing effect of *NUC1* overexpression in response to hydrogen peroxide, suggesting that AAC proteins may have a role in Nuc1p-facilitated killing and possibly in its release from mitochondria (Buttner et al., 2007).

Mitochondria morphological remodelling in apoptosis

Mitochondria are morphologically dynamic organelles that continuously fuse and divide to form interconnected tubular networks or small round units within the cell (Bereiter-Hahn, 1990). The equilibrium between these two shapes is achieved by regulating the relative rates of organelle fusion and fission (Nunnari et al., 1997). Mitochondria associates with the cytoskeleton (actin in yeast, microtubules in mammals)

and thus mitochondrial morphology is also affected by cytoskeleton movements (Hermann and Shaw, 1998; Okamoto and Shaw, 2005).

Clues from mammals

It was observed that the interconnected mitochondrial tubular network early converts to small round units during a variety of apoptotic scenarios in mammalian cells (reviewed in Scorrano, 2005; Youle and Karbowski, 2005; Parone and Martinou, 2006). The molecular machinery responsible for the mitochondrial fission under apoptotic conditions remained obscure until Frank and colleagues (Frank et al., 2001) hypothesized that it might result from the activation of the machinery involved in physiological fission. Inhibition of mammalian Drp1 (yeast Dnm1), which normally regulates mitochondrial morphology in healthy cells, inhibits the fission that occurs during apoptosis and actually delays the process of cell death (Frank et al., 2001). In cells expressing dominant mutations of Drp1 there was no loss of mitochondrial membrane potential, release of cytochrome *c* or cell death (Frank et al., 2001; Lee et al., 2004). Since then, several other proteins from the fission and fusion machinery have been identified as participants in apoptotic mitochondria remodelling. Fis1p impairs apoptotic fragmentation of the mitochondrial network as well as cytochrome *c* release and the death process itself (James et al., 2003). Another protein, Opa1, localizes to the inner mitochondrial membrane, and participates in the mitochondrial fusion machinery. It also regulates inner membrane cristae reorganization soon after MOMP in order to facilitate the complete release of cytochrome *c* during apoptosis (Arnoult et al., 2005a; Frezza et al., 2006).

The relationship between MOMP/cytochrome *c* release and mitochondrial fragmentation is not clear (reviewed in Arnoult, 2007). Some authors proposed that mitochondrial fragmentation (involving at least Drp1-mediated mitochondrial fission) is required for MOMP, cytochrome *c* release and the execution of apoptosis (Frank et al., 2001; Youle and Karbowski, 2005). However, it is unclear if these events are coincident rather than interrelated; cytochrome *c* release can occur before fragmentation of the network (Arnoult et al., 2005a; Perfettini et al., 2005) and fragmentation of the mitochondrial network does not always imply cytochrome *c* release (Lee et al., 2004; Delivani and Martin, 2006). An emerging consensus in this area suggests that mitochondrial fission is not required for apoptotic MOMP, yet it may contribute to

MOMP induction in some circumstances, as an accelerating factor (Cereghetti and Scorrano, 2006).

It is now clear that proteins that control mitochondrial morphology can also participate in apoptosis. Likewise, proteins associated with mammalian apoptosis have also been shown to affect mitochondrial ultrastructure. The pro-apoptotic protein Bax was shown to co-localize with Drp1 at the tips of mitochondrial tubules and at mitochondrial constriction sites (Karbowski et al., 2002). Surprisingly, the Bcl-2 family members can also affect mitochondrial ultrastructure under physiological conditions (Delivani et al., 2006; Karbowski et al., 2006).

Drp1p affects not only fragmentation but also the subsequent degradation of mitochondria, leading to the assumption that fragmentation is an important step before degradation (Skulachev et al., 2004; Arnoult et al., 2005b). Degradation of mitochondria during apoptosis induction seems to be a widespread phenomenon (Tolkovsky et al., 2002). Mitochondrial removal is mainly attributed to autophagy activation, with PTP being a possible trigger (reviewed in Mijaljica et al., 2007). However, mitochondria degradation proceeded normally in a strain inactivated for *ATG5*, coding for an autophagy essential protein, during the lens and erythroid differentiation (Matsui et al., 2006). Therefore, degradation system(s) other than autophagy may account for mitochondrial degradation in distinct cellular settings.

Insights from yeast

Several known factors control mitochondrial dynamics in healthy yeast cells. The proteins Dnm1p, Mdv1/Net2p and Fis1p form a complex involved in fission, and Fzo1p and Mgm1p are required for mitochondrial fusion (Mozdy et al., 2000; Tieu and Nunnari, 2000; Shaw and Nunnari, 2002; Cerveny and Jensen, 2003).

In yeast apoptosis induced by different compounds (Fannjiang et al., 2004; Mazzoni et al., 2005; Pozniakovsky et al., 2005; Kitagaki et al., 2007) or upon heterologous Bax expression (Kissova et al., 2006), mitochondria undergo extensive fragmentation, suggesting it is a general feature of yeast apoptosis. While deletion of *DNM1* or *MDV1/NET2* inhibits cell death, deletion of *FIS1* increases H_2O_2, acetic acid and heat-shock induced apoptosis (Fannjiang et al., 2004). In agreement with these results, *dnm1*Δ cells had an increased life span (Scheckhuber et al., 2007). Interestingly, Dnm1p-deficiency protected cells from death more efficiently than from mitochondrial fragmentation. This suggests that the absence of Dnm1p in yeast might confer protection

against cell death by mechanisms other than those related to mitochondrial fission. In *S. cerevisiae*, Fis1p is evenly distributed along the mitochondrial surface, where it functions as a receptor to recruit Dnm1p from the cytosol to mitochondria (Mozdy et al., 2000). The fact that the *dnm1*Δ*fis1*Δ double mutant behaved like *dnm1*Δ led Fannjiang and colleagues (Fannjiang et al., 2004) to propose that Fis1p inhibits the action of Dnm1p, thus promoting cell survival. The pro-survival role of Fis1p in yeast was surprising, since cells overexpression of *FIS1* in mammalian cells triggers not only mitochondrial fission but also cytochrome *c* release and apoptosis (James et al., 2003). This pro-survival role in yeast was further supported by the sensitivity of *fis1*Δ mutant to death triggered by a viral pore-forming toxin (Ivanovska and Hardwick, 2005) and ethanol treatment (Kitagaki et al., 2007). In contrast with the results obtained for acetic acid, H_2O_2 and heat shock treatment (Fannjiang et al., 2004), lack of *DNM1* or *MDV1* did not significantly alter ethanol induced death (Kitagaki et al., 2007), suggesting different stimuli may involve different components of the fission machinery.

The yeast metacaspase Yca1p has been implied in apoptotic mitochondrial fragmentation, presumably as an upstream regulator of this process. Absence of *YCA1* decreased fragmentation of the mitochondrial network caused both by H_2O_2 treatment and by expression of a truncated form of *Kluyveromyces lactis* Lsm4p. This protein is involved in messenger RNA decapping and cells expressing the truncated form exhibit markers of apoptosis (Mazzoni et al., 2003; Mazzoni et al., 2005).

Two novel proteins that affect the fragmentation of the mitochondrial reticulum were identified in genetic screens searching for proteins involved in pheromone or amiodarone-induced cell death (Pozniakovsky et al., 2005; Sokolov et al., 2006). These proteins were named yeast suicide protein 1 (Ysp1), and yeast suicide protein 2 (Ysp2), and are both localized to mitochondria and necessary for mitochondrial fragmentation in the course of apoptosis (Pozniakovsky et al., 2005; Sokolov et al., 2006). Mitochondrial fragmentation *per se* does not result in cell death either in mammals (Lee et al., 2004) or yeast (Fannjiang et al., 2004). However, the multiple components of the mitochondrial morphogenesis machinery seem to be able to regulate apoptosis both positively and negatively.

Mitochondrial fragmentation, as referred above for mammalian cells, seems to also facilitate mitochondrial disposal in yeast. In fact, mitochondrial degradation triggered by acetic acid (Fannjiang et al., 2004) is impaired in the absence of Dnm1p

and, unlike mitochondrial fragmentation, loss of mitochondria seems to mark the commitment point to cell death (Fannjiang et al., 2004).

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Main goals and thesis overview

It was recently shown that during acetic acid-induced apoptosis cytochrome *c* is translocated from mitochondria to the cytosol. In mammalian cells, the permeabilization of the mitochondrial outer membrane allows the release of several pro-apoptotic proteins and therefore, is a critical step in the apoptotic cascade. As such, it became important to study the mechanisms and the proteins involved in the permeabilization of the mitochondrial membrane during yeast apoptosis. Since the budding yeast *Saccharomyces cerevisiae* possesses orthologues of the putative core components of the PTP, the main goal of this thesis was to study these proteins, both concerning their role in mitochondrial permeabilization and their involvement in the course of cell death. The elucidation of the endogenous yeast cell death pathway(s) will be of great importance from the point of view of the importance of yeast in human life but also as a valuable model for investigating the death mechanisms of metazoan cells.

The study concerning the yeast orthologues of the core components of the mammalian PTP in yeast apoptosis is shown in Chapter 2. Among the proteins studied, ADP/ATP carrier was shown to be required for mitochondrial outer membrane permeabilization and cytochrome *c* release. It was however, not required for caspase activation. In Chapter 3 the role of caspase activation and the yeast metacaspase Yca1p during acetic acid-induced apoptosis is further explored. In Chapter 4 the degradation of mitochondria in response to acetic acid was characterized. The possible involvement of AAC proteins in degradation of mitochondria was also studied. Finally in Chapter 5, we show preliminary data on the role of Por1p in mitochondrial fusion and a possible relation to the apoptotic process. The last chapter includes a discussion highlighting the main outcomes of the present work and the future prospects as well on the exploitation of yeast to unravel novel attractive targets for the development of apoptosis-based therapies.

Chapter 2

ADP/ATP carrier is required for mitochondrial outer membrane permeabilization and cytochrome *c* release in yeast apoptosis

This chapter comprises parts from the following publication:

Pereira C., Camougrand N., Manon S., Sousa M.J. e Côrte-Real M. (2007). ADP/ATP carrier-dependent cytochrome *c* release in yeast apoptosis. Molecular Microbiology 66 (3), 571-582.

ADP/ATP carrier is required for mitochondrial outer membrane permeabilization and cytochrome *c* release in yeast apoptosis

Abstract

Adenine nucleotide translocator (ANT) is a mitochondrial inner membrane protein involved in the ADP/ATP exchange and is a component of the mitochondrial permeability transition pore (PTP). In mammalian apoptosis, the PTP can mediate mitochondrial outer membrane permeabilization (MOMP), which is suspected to be responsible for the release of apoptogenic factors including cytochrome *c*. Though release of cytochrome *c* in yeast apoptosis has previously been reported it is not known how it occurs. Herein we use yeast genetics to investigate whether depletion of proteins putatively involved in MOMP and cytochrome *c* release affects these processes in yeast. While deletion of *POR1* (yeast voltage-dependent anion channel, VDAC*)* enhances apoptosis triggered by acetic acid, H_2O_2 and diamide, *CPR3* (mitochondrial cyclophilin) deletion had no effect. Absence of ADP/ATP carrier (AAC) proteins, yeast orthologs of ANT, protects cells exposed to acetic acid and diamide but not to H_2O_2. Expression of a mutated form of Aac2p (op1) exhibiting very low ADP/ATP translocase activity indicates AAC`s pro-death role does not require translocase activity. Absence of AAC proteins impairs MOMP and release of cytochrome *c* which*,* together with other mitochondrial inner membrane proteins, is degraded. Our findings point to a crucial role of AAC in yeast apoptosis.

Introduction

Mitochondrial outer membrane permeabilization (MOMP) is a crucial step in the intrinsic, or mitochondrial, apoptotic pathway. This permeabilization triggers the release of apoptogenic factors, such as cytochrome *c*, from the mitochondrial intermembrane space into the cytosol where these factors ensure propagation of the apoptotic cascade and execution of cell death. However, the mechanisms underlying permeabilization of the outer membrane remain controversial (Kinnally and Antonsson, 2007). Opening of a mitochondrial pore called the permeability transition pore (PTP), which leads to the swelling of mitochondria and rupture of the mitochondrial outer membrane, has been considered as one of the key mechanisms underlying mammalian MOMP. Though the molecular composition of the pore is not completely defined, it has been proposed that its major components are the adenine nucleotide transporter (ANT), the voltage-dependent anion channel (VDAC) and cyclophilin D (Crompton, 1999; Martinou et al., 2000; Bras et al., 2005). The proteins that may constitute the PTP and which are also engaged in energy metabolism are well conserved in eukaryotes. Therefore, the involvement of the components of the PTP in apoptotic death has been studied in yeast after heterologous expression of the human pro-apoptotic protein Bax (Manon et al., 1997). However, contradictory results have been reported concerning the roles of *AAC1/2/3* and *POR1* participation (yeast orthologs of mammalian ANT and VDAC, respectively) in Bax-induced cell death, depending both on growth and Bax induction conditions (Marzo et al., 1998; Priault et al., 1999a; Shimizu et al., 1999; Gross et al., 2000; Harris et al., 2000; Kissova et al., 2000).

Cytochrome *c* release has also been reported to occur in a yeast strain lacking the histone chaperone *ASF1/CIA1* (Yamaki et al., 2001), during cell death induced by acetic acid and amiodarone (Ludovico et al., 2002a; Pozniakovsky et al., 2005) and more recently in a strain with a mutation in *CDC48* ($cdc48^{S565G}$) (Braun et al., 2006). In mammalian cells, cytochrome *c* release contributes to the activation of the apoptotic machinery by forming a cytosolic complex with Apaf-1 and procaspase-9, in the presence of dATP or ATP. In yeast, very few studies address the question whether release of cytochrome *c* is essential for the organism to undergo cell death and whether, in common with mammalian cells, it activates a downstream pathway. Cytochrome *c* release in yeast was first reported for strains expressing the human pro-apoptotic protein Bax (Manon et al., 1997). Cytochrome *c* release may be less important for this kind of death, since a yeast strain expressing a functional cytochrome *c*-GFP fusion that is not

re-localised into the cytosol still induces cell death (Roucou et al., 2000). However, deletion of the genes encoding the two isoforms of cytochrome *c*, although not abolishing death, leads to a delay in response to several death stimuli, including Bax (Priault et al., 1999a; Gross et al., 2000; Harris et al., 2000; Severin and Hyman, 2002; Pozniakovsky et al., 2005; Silva et al., 2005). The involvement of cytochrome *c* in metacaspase activation has also been suggested after exposing yeast cells to hyperosmotic stress (Silva et al., 2005).

In this work we aimed to study the involvement of yeast protein orthologs of the putative components of the mammalian PTP in the death process triggered by two well studied yeast apoptosis inducers, acetic acid and H_2O_2, and by diamide, rather than by heterologous expression of pro-apoptotic effectors. We demonstrate that while the absence of Cpr3p had no effect, yeast cells devoid of Por1p were more susceptible to apoptosis suggesting that Por1p may promote cell survival. Notoriously, absence of AAC proteins protects cells exposed to acetic acid and diamide, but not to H_2O_2, and hampers MOMP and cytochrome *c* release. We also showed for the first time that in the absence of AAC proteins the retained cytochrome *c,* together with other proteins of the inner mitochondrial membrane, is degraded. Furthermore, we showed that absence of AAC proteins did not completely prevent apoptosis suggesting that alternative, redundant pathways are involved in apoptosis some of which do not entail MOMP and cytochrome *c* release.

Experimental procedures

Strains and plasmids

W303-1B (*MATa, ade2, his3, leu2, trp1, ura3, can1*) was used as the wild-type strain. Strains *Δcpr3*, *Δatp2*, and *Δpor1* were constructed from W303-1B by direct transformation with PCR products amplified from genomic DNA from the Euroscarf deletion strains *Δcpr3*::kanMX4, *Δatp2*::kanMX4 or *Δpor1*::kanMX4 with subsequent selection in geneticin media. The deletion was confirmed by PCR using primers that bind upstream and downstream of the insertion, plus additional primers binding within the kanamycin gene. Strain JL-1-3 (*MATa, ade2, his3, leu2, trp1, ura3, can1, aac1::LEU2, aac2::HIS3, aac3::URA3*) which is a derivative of W303-1B was a kind

gift from Prof. Jordan Kolarov (Kissova et al., 2000). Strain JL1-3Δ2Δ3 (*MATa, ade2, his3, leu2, trp1, ura3, can1, aac1*Δ*::LEU2, aac2*Δ*::HIS3, aac3*Δ*::URA3*) which is a derivative of JL-1-3, was used for transformation with plasmid pRSop1 expressing a R96H mutated form of Aac2p (op1) (Postis et al., 2005). This was done in order to avoid homologous recombination with the JL-1-3 *aac2*Δ. The W303-1B and JL1-3Δ2Δ3 strains were transformed with the empty vector pRS314 using the lithium acetate method (Ito et al., 1983). The resulting transformants were grown in selective media (lacking tryptophan) and the presence of the plasmid was verified by gel electrophoresis. Strain JL1-3Δ2Δ3 transformed with pRSop1 and the vector pRS314 were kindly provided by Dr. Véronique Trézéguet and Prof. Guy Lauquin.

Growth conditions and treatments

Yeast cells were maintained and grown in rich (YEPD; 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose) or synthetic complete media (SC; 0.67% (w/v) Bacto-yeast nitrogen base w/o amino acids (Difco), 2% (w/v) glucose and 0.2% (w/v) Dropout mix). The wild-type and disrupted strains were harvested at approximately the same cell density ($O.D._{640\ nm}$, 1.0), and then were suspended (10^7 cells/ml) in the treatment medium. For acetic acid, treatment medium consisted on YEPD, pH 3.0 (set with HCl), containing 0-200 mM acetic acid. Inhibition of respiration and oxidative phosphorylation was performed by adding oligomycin (Merck; 5 μg/ml final concentration) and antimycin A (Sigma; 5 μg/ml final concentration) both to the growth and treatment medium. Control cells for these assays were grown and treated with ethanol (0.25%, v/v) and acetone (0.5%, v/v) used as solvents of oligomycin and antimycin A, respectively. When used, cyclosporin A (Sigma) was added to the treatment medium at a final concentration of 240 μg/ml. For the H_2O_2 and diamide assay the treatment media consisted on YEPD containing 0-5 mM H_2O_2 or 0-16 mM diamide. The treatments were performed in 50 ml flasks containing a 5:1 ratio of air to liquid phase, and incubated on a mechanical shaker (160 rpm, 26 ˚C) for 200 min.

For mitochondria isolation (see below), cells were grown in YEPGal and treatments were performed in 5 L flasks containing 5:2 ratio of air to liquid phase, and incubated under the same conditions as described above. Galactose like glucose can be respired and fermented but is not so effective in repressing respiratory metabolism

(Herrero et al., 1985). Viability (clonogenicity) was determined by counting colony forming units (cfu) in YEPD agar (YEPDA) plates after 2-3 days incubation at 30 ˚C.

Detection of apoptotic markers

Mitochondria dysfunction monitored by reactive oxygen species (ROS) production was assessed using the MitoTracker Red CM-H_2XRos staining (Molecular Probes). Cells were incubated with 0.4 µg/ml MitoTracker for 20 min at 30 °C. Nuclear staining was performed with 4,6-diamido-2-phenyl-indole (DAPI, Sigma) according to Silva *et al*. (Silva et al., 2005). For propidium iodide (PI, Sigma) staining cells were incubated with 5 µg/ml of PI for 10 min at room temperature. DNA strand breaks were assessed by TUNEL with the "In Situ Cell Death Detection Kit, Fluorescein", (Roche Applied Science) as described previously (Ludovico et al., 2001a). To determine the frequency of apoptotic phenotypes at least 300 cells of three independent experiments were evaluated. The samples were observed in a Leitz Laborlux S Microscopic with accessory apparatus for fluorescence (Ploemopak Filter 12). 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 (Axio-Vision 3.0, Carl Zeiss Vision GmbH).

Preparation of mitochondria and redox spectra analysis

Isolation of mitochondria was carried out by differential centrifugation of homogenized spheroplasts essentially as previously described (Daum et al., 1982), except for the homogenization procedure. Spheroplasts were suspended in 0.5 M sorbitol, 20 mM Tris/HCl pH 7.5, 1 mM EDTA, and a Dounce homogenizer was used to obtain an even suspension.

Assessement of cytochrome *c* content was measured by redox spectra of isolated mitochondria essentially as described previously (Manon et al., 1997). Differential spectra of the reduced (sodium dithionite) minus oxidized (potassium ferricyanide) extracts were recorded on a microplate spectrophotometer (Spectra Max 340pc, Molecular Devices). The maxima absorption for cytochrome *b* and for cytochromes *c+c1* used were 561 and 550 nm, respectively. The ratio cytochrome *c*/ cytochrome *b* was always used to normalize the total protein content from the different samples.

Western blot analysis

Preparation of protein samples, SDS-PAGE, and Western blots was done as described previously (Camougrand et al., 2003b). The primary antibodies used were mouse monoclonal anti yeast phosphoglycerate kinase (PGK1) antibody (1:5000, Molecular Probes), mouse monoclonal anti yeast porin (POR1) antibody (1:5000, Molecular Probes) rabbit polyclonal anti yeast cytochrome *c* (CYC1) antibody (1:1000, custom-made by Millegen), rabbit polyclonal anti yeast subunit β of ATP synthase (ATP2) antibody (1:10000, gift from Prof. Jean Velours, IBGC, France). Secondary antibodies against mouse or rabbit IgGs, coupled to horse radish peroxidase (Jackson Laboratories) were used at 1:5000 and revealed by chemiluminescence (ECL+Amersham).

Adenylate kinase and citrate synthase activity

Adenylate kinase and citrate synthase activities were determined as described previously (Priault et al., 1999c). Briefly, adenylate kinase was measured as the formation of ATP from ADP, in the presence of oligomycin. Mitochondria were suspended in 10 mM Tris/maleate buffer (pH 6.7) that contained 0.1 M KCl, 5 mM $MgCl_2$ and 10 µg/mL oligomycin at 28 °C. The reaction was started by the addition of 1 mM ADP and aliquots were withdrawn at different time points in a solution that contained 7% perchloric acid and 25 mM EDTA. The samples were then neutralized and centrifuged. ATP was measured in the supernatant with a luciferin/luciferase kit (BioOrbit) in a LKB bioluminometer. Citrate synthase was measured as the formation of CoASH in the presence of 5,5`-dithiobis (2-nitrobenzoic acid) (Nbs_2). An aliquot of the samples (containing 0.2 mg of protein) was added to 0.1 mL Tris/HCl buffer that contained Nbs_2 and acetyl-CoA, and the reaction was started by the addition of oxaloacetate. The reaction was followed at 412 nm in a Secomam S1000 spectrophotometer.

Detection of caspase activation

Caspase activation was assessed using the fluorescent caspase inhibitor "CaspACE, FITC-VAD-fmk In Situ Marker" (Promega). Briefly, 2.10^6 cells were

washed in PBS, suspended in 100 µl staining solution containing 50 µM of FITC-VAD-FMK and incubated for 30 min at 30 ºC in the dark. Cells were then washed once and suspended in PBS. For double staining, cells were subsequently incubated with 5 µg/ml of PI for 10 min at room temperature. Sample analysis was performed in an Epics® XL-MCL™ (Beckman Coulter) flow cytometer, equipped with an argon-ion laser emitting a 488-nm beam at 15mW. Twenty thousand cells were analyzed per sample using FL-1 channel (Ex/Em =488/525 nm) for FITC fluorescence and FL-3 (Ex/Em =488/620 nm) for PI fluorescence. Signal compensation to eliminate optical overlap between both dyes was of 30%. Data were analyzed using WinMDI 2.8 software.

Results

Deletions in POR1 *and* AAC1/2/3*, encoding mitochondrial proteins, affect apoptosis*

To elucidate the role of yeast orthologs of putative mammalian permeability transition pore components in *Saccharomyces cerevisiae* apoptotic death, three *S. cerevisiae* deleted strains were studied, namely: *por1*Δ, lacking YVDAC1; JL-1-3 (*aac1/2/3*Δ), lacking the three ADP/ATP carrier isoforms and *cpr3*Δ, lacking the mitochondrial cyclophilin. In what regards respitation competence, strains lacking *POR1* have impaired exchange of metabolites and nucleotides across the outer mitochondrial membrane and as a result have a low oxidative phosphorylation rate (Dihanich et al., 1987). Strains lacking the three *AAC* genes (*AAC1*, *AAC2* and *AAC3*) are defective in oxidative phosphorylation, have reduced respiration rates and ATP levels (Muller et al., 1996; Trancikova et al., 2004), and are unable to grow on nonfermentable carbon sources. Strains lacking mitochondrial cyclophilin are respiratory competent (Davis et al., 1992). Death was induced with acetic acid (up to 200 mM) for 200 min. While *por1*Δ was found to be more sensitive than the wild-type, the *aac1/2/3*Δ strain was more resistant (Fig. 1A). In an attempt to evaluate the possible effect of reduced activity of the electron transport chain and low levels of ATP on the observed higher resistance of *aac1/2/3*Δ strain to acetic acid, wild-type cells were grown in the presence of antimycin A and oligomycin and subsequently treated with acetic acid in the presence of these compounds. Antimycin A is an inhibitor of the enzyme ubiquinol-cytochrome *c* oxidoreductase (complex III) within the electron transport chain, and oligomycin is an

inhibitor of the F0 portion of *F0F1* ATP synthase. Cells grown under such conditions were unable to grow when plated in medium containing glycerol as the sole carbon source. A higher sensitivity, instead of a higher resistance to acetic acid-induced death was observed in the presence of these inhibitors (Fig. 1B). Moreover, the strain *atp2*Δ, lacking subunit β of ATP synthase, included as an additional control for the absence of oxidative phosphorylation and low ATP levels, also displayed a high sensitivity to the acid (Fig. 1A). Together these results further rule out the possibility of attributing the resistance of *aac1/2/3*Δ strain to its defects in oxidative phosphorylation.

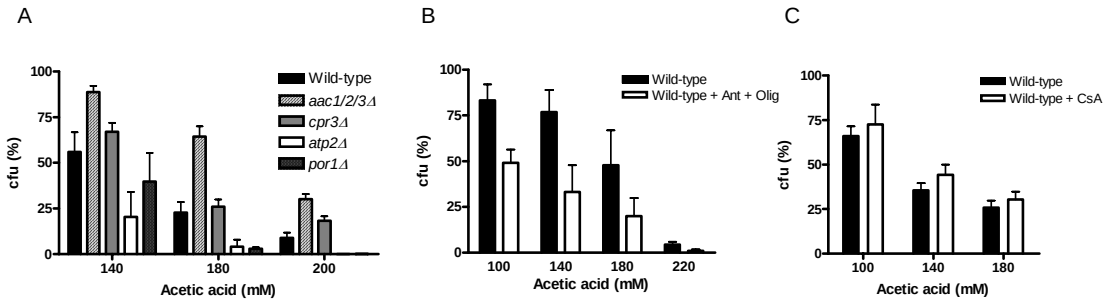

Figure 1. *Absence or inhibition of mitochondrial proteins affects cell survival in response to acetic acid.* Relative survival of *S. cerevisiae* cells incubated for 200 min with acetic acid (percentage of cfu on YEPDA plates; 100% corresponds to the number of cfu at time 0): (A) wild-type (W303-1B), *aac1/2/3*Δ (JL-1-3), *cpr3*Δ, *atp2*Δ, and *por1*Δ; (B) Wild-type grown and treated in the presence of antimycin A plus oligomycin or in the presence of ethanol (0.25%, v/v) and acetone (0.5%, v/v) used as solvents of oligomycin and antimycin A, respectively; (C) Wild-type treated in the absence or in the presence of cyclosporin A. Values represent the mean of four independent experiments and the vertical error bars represent SD. Statistical analysis was performed using a two-way ANOVA test. The effect of the deletions on acetic acid-induced death was significant for *por1*Δ (p= 0.04), very significant (p = 0.004) for strain *atp2*Δ, and extremely significant (p < 0.001) for *aac1/2/3*Δ, while *cpr3* deletion had no significant effect on death (p= 0.1). The differences between wild-type and wild-type treated with oligomycin plus antimycin A were significant (p< 0.05). The differences between wild-type and wild-type treated with cyclosporin A were not significant (p= 0.37).

The strain *cpr3*Δ behaved identically to the wild-type (Fig. 1A). Consistently, cyclosporin A, described to inhibit the mitochondrial cyclophilin (Matouschek et al., 1995), did not significantly affect cell death induced by acetic acid (Fig. 1C).

Time-course appearance of apoptotic markers is delayed in the aac1/2/3Δ strain

We previously reported that death induced by acetic acid is accompanied by typical markers of apoptosis like chromatin condensation along the nuclear envelope, exposure of phosphatidylserine on the outer surface of the plasma membrane, occurrence of DNA strand breaks and mitochondrial dysfunction with release of cytochrome *c* (Ludovico et al., 2001a; Ludovico et al., 2002a).

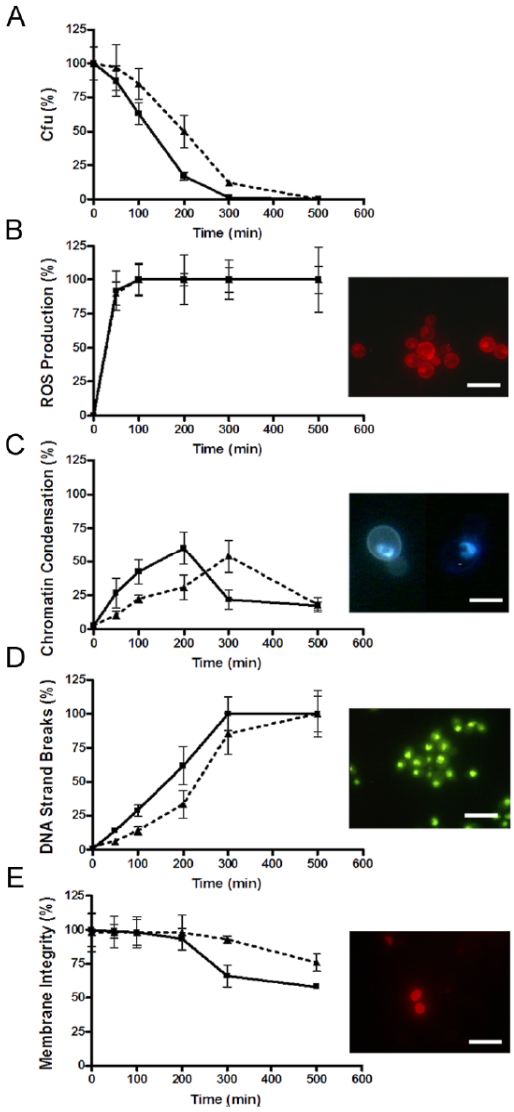

Figure 2. *The appearance of apoptotic markers is delayed in the* aac1/2/3*Δ mutant*. Kinetics of cellular changes induced by acetic acid (180 mM) in *S. cerevisiae* wild-type (W303-1B; full line) and *aac1/2/3*Δ (JL-1-3; dotted line) cells during a 500 min time-course together with photomicrographs of fluorescence images illustrative of positive staining of *aac1/2/3Δ* cells at 500 min. (A) Cell viability was determined by cfu counts on YEPDA plates (100% corresponds to the number of cfu at time 0); (B) ROS production was monitored using MitoTracker Red CM-H2XRos staining, bar, 10 μm; (C)

Chromatin condensation was visualised after DAPI staining, bar, 5 μm; (D) DNA strand breaks were detected using the TUNEL assay, bar, 10 μm; and (E) membrane integrity was visualised by PI exclusion, bar, 10 μm. Values represent the mean of four independent experiments and the vertical error bars represent SD.

Since *aac1/2/3*Δ strain displayed a higher resistance to acetic acid, we assessed if, like the wild-type strain, it still exhibited apoptotic markers. A kinetic analysis of cellular events associated with acetic acid-induced death, including loss of cell viability, ROS production, chromatin condensation, occurrence of DNA strand breaks and preservation of plasma membrane integrity, was carried out over 500 min in the *aac1/2/3Δ* and wild-type, after exposure to 180 mM acetic acid (Fig. 2). Mitochondrial ROS production, evidenced by MitoTracker Red CM-H2Xros staining, was detected from the beginning of acid treatment and before any significant loss of cell viability occurred in both the wild-type and *aac1/2/3*Δ strain (Fig. 2A, B). Following this marker, kidney shaped condensed chromatin characteristic of apoptotic cells, was visualized after 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining in both wild-type and *aac1/2/3*Δ, albeit delayed in the mutant. Both strains displayed a maximum percentage of cells with condensed chromatin of about 60% that decreased approximately 5 fold at the end of the treatment (Fig. 2C). TUNEL-positive cells, displaying nuclear green fluorescence, were also detected for wild-type and *aac1/2/3*Δ strains indicating the occurrence of DNA strand breaks. The percentage of cells displaying TUNEL-positive staining increased over time reaching 100% at 300 min for the wild-type strain, and at 500 min for the *aac1/2/3*Δ strain (Fig. 2D). Finally, preservation of plasma membrane integrity, monitored by propidium iodide (PI) exclusion, was essentially observed for both strains until 200 min treatment. Afterwards, the percentage of cells with compromised membranes unable to exclude PI, indicative of the progress of apoptosis to secondary necrosis (Ludovico et al., 2005), increased up to 35% and 12% in the wild-type and *aac1/2/3*Δ strains, respectively (Fig. 2E). Thus the higher resistance of *aac1/2/3*Δ strain is associated with the delayed emergence of apoptotic markers compared to the wild-type strain. Even though *por1*Δ, *atp2*Δ, and *cpr3*Δ strains did not display higher resistance to acetic acid-induced death we checked whether the deletions affected the outcome of acetic acid induced-cellular events. For such purpose, ROS production and chromatin condensation were monitored in the deletion strains after treatment with 180 mM acetic acid. All the three strains displayed these apoptotic features. Furthermore, chromatin condensation, DNA strand breaks and loss of plasma

membrane integrity occurred earlier in *por1*Δ compared to the wild-type strain (Fig. 2). The rapid formation of DNA strand breaks followed by the disappearance of TUNEL staining, probably due to DNA washout, was the most impressive feature displayed by *por1*Δ (Fig. 2D).

AAC is required for MOMP and cytochrome c release in acetic acid-induced apoptosis

Acetic acid treatment has been shown to induce the translocation of cytochrome *c* from the mitochondrion to the cytosol. In addition, it has been reported that the higher resistance of the *ATP10*-deleted mutant was associated with the absence of cytochrome *c* release, suggesting a possible role of this event in acetic acid-induced apoptosis (Ludovico et al., 2002a). The mechanism of cytochrome *c* release from yeast mitochondria remains unknown. The resistance of *aac1/2/3*Δ strain to acetic-acid treatment suggested that AAC might be involved in this process. To investigate this hypothesis, redox spectra of mitochondria isolated from wild-type and *aac1/2/3*Δ strains untreated and treated with acetic acid were recorded (Fig. 3A). For mitochondria isolation, cells were grown in YPGal instead of YPD in order to obtain a higher mitochondrial mass since galactose is less effective in the repression of the respiratory metabolism (Herrero et al., 1985). The kinetics of death and of morphological markers appearance in cells grown in this medium was similar to that of glucose grown cells (not shown). The extent of cytochrome *c* release was evaluated by the ratio between residual cytochrome *c* over residual cytochrome *b*, a membrane protein that is not released. For untreated cells of the wild-type strain a value of 2.48 ± 0.08 for the ratio cytochrome *c*/cytochrome *b* (cyt *c*/cyt *b*) was obtained (Fig. 3A). This value decreased to 1.50 ± 0.12 (40% reduction) in cells treated with acetic acid (180 mM, 200 min). Concerning the *aac1/2/3*Δ mutant, we obtained, for untreated cells, a cyt *c*/cyt *b* ratio of 1.77 ± 0.42 (Fig. 3A). This lower cytochrome *c* content of the *aac1/2/3*Δ mutant is consistent with previous reports for the *aac2*Δ strain (Gawaz et al., 1990). This ratio was not significantly decreased (1.72 ± 0.34) after acetic acid treatment (180 mM or 200 mM, 200 min). Cytochrome *c* was also monitored by western blot in isolated mitochondria and in the corresponding cytosolic fraction, in both the wild-type and *aac1/2/3*Δ mutant (Fig. 3B). In the wild-type we could confirm that, according to the spectra analysis and as previously reported (Ludovico et al., 2002a), there was a decrease in the

mitochondrial cytochrome *c* content upon acetic acid treatment which was accompanied by an increase in the cytosolic fraction. In the *aac1/2/3*Δ mutant we could verify that there was not a significant increase of cytochrome *c* in the cytosolic fraction (Fig. 3B). However, in contrast to what was suggested by spectra analysis we observed that there was a drastic decrease in the mitochondrial cytochrome *c* content. This difference suggests that the maintenance of the ratio cyt *c*/cyt *b* reflects the degradation of both proteins. This interpretation was further supported by the observation that Atp2p, another protein of oxidative phosphorylation, was also degraded (Fig. 3B).

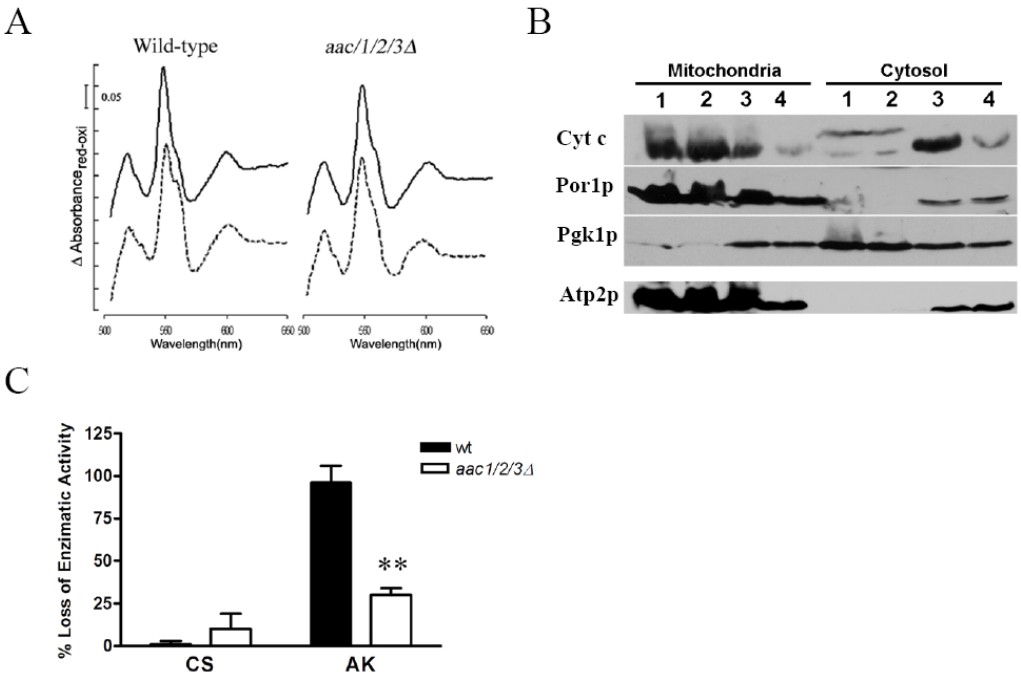

Figure 3. *Absence of AAC suppresses MOMP and cytochrome* c *release in acetic acid-induced cell death.* Cytochrome *c* release and enzymatic activities were measured in mitochondria isolated from untreated cells and acetic acid (180 mM, 200 min) treated cells of wild-type (W303-1B) and *aac1/2/3*Δ (JL-1-3). (A) Cytochrome *c* content in mitochondria assessed by redox spectra analysis of isolated mitochondria from untreated cells (full lines) and acetic acid-treated cells (dotted lines). (B) Cytochrome *c* detected by western blot in mitochondria and postmitochondrial supernatants obtained under the same conditions as described above. Porin (Por1p) from outer mitochondrial membrane and cytosolic phosphoglycerate kinase (Pgk1p) were also assessed together with Atp2p from the inner mitochondrial membrane. Lanes: 1, untreated wild-type cells; 2, untreated *aac1/2/3*Δ cells; 3, acetic acid-treated wild-type cells; and 4, acetic acid-treated *aac1/2/3*Δ cells. (C) Enzymatic assays of matricial citrate synthase (CS) and intermembrane space adenylate kinase (AK). Results are given as the percentage of decrease in activity found in mitochondria isolated from acetic acid treated cells considering 0% loss, the activity found in mitochondria isolated from untreated cells. The activity values of citrate synthase and adenylate kinase for mitochondria iolated from untreated cells of the wild-type were 34 ± 1.4 nmol.min^{-1}.mg^{-1} protein and 973 ± 270 pmol. min^{-1}. mg^{-1} protein, respectively. Values represent the mean of three independent experiments and the vertical error bars represent SD. ***p*<0.001; Unpaired t test.

Mitochondrial content of citrate synthase (CS) and adenylate kinase (AK) were used to monitor inner and outer membrane permeability of isolated mitochondria since CS is a protein from the mitochondrial matrix, and AK is localized in the mitochondrial intermembrane space. We verified that there was no decrease in CS activity in isolated mitochondria from both wild-type and *aac1/2/3*Δ cells treated with acetic acid (Fig. 3C). This observation indicates that permeability of the inner mitochondrial membrane remains unaltered after acetic acid treatment and subsequent isolation procedure, allowing ruling out the possibility that cytochrome *c* release was caused by some unspecific mitochondrial rupture. Regarding mitochondrial AK activity, a decrease of about 95% was observed upon acid treatment for the wild-type strain, indicating a loss of this intermembrane protein. In contrast, in the *aac1/2/3*Δ strain, the AK release (Fig. 3C) was considerably lower (only about 30% loss of activity).

aac1/2/3*Δ mutant resistance to acetic acid-induced death is not related to the loss of ADP/ATP translocation activity*

We next addressed whether the resistance phenotype of the *aac1/2/3*Δ mutant to death induced by acetic acid was dependent on the ADP/ATP carrier activity. For such purpose we expressed a mutated form of Aac2p (op1) that carries a R96H mutation and displays a highly reduced ADP/ATP translocation activity (Gawaz et al., 1990). Op1 is present in the mitochondrial inner membrane in amounts equivalent to the wild-type Aac2p. The resistance phenotype observed for the *aac1/2/3*Δ mutant (strain JL1-3Δ2Δ3 transformed with empty vector) was reversed by the expression of op1 with a decrease in survival from a value of 98% to about 70% (Fig. 4). This survival percentage was very close to the one observed for the wild-type strain transformed with the empty vector (Fig. 4). These results suggest that AAC-mediated death does not depend on the ADP/ATP translocase activity.

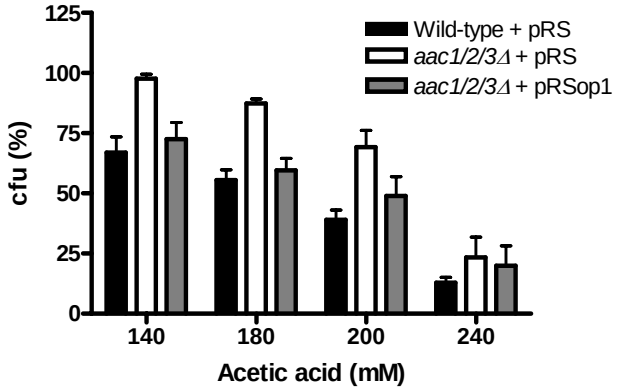

Figure 4. *Resistance phenotype of the* aac1/2/3*Δ mutant is independent of the loss of ADP/ATP translocation activity*. Relative survival of wild-type and JL1-3Δ2Δ3 strains transformed with the empty vector (pRS314) and pRSop1, respectively, incubated for 200 min with acetic acid (percentage of cfu on YEPDA plates; 100% corresponds to the number of cfu at time 0). Values represent the mean of five independent experiments and the vertical error bars represent SD. Statistical analysis was performed using a two-way ANOVA test. The differences between the wild-type strain transformed with the empty vector pRS314, and JL1-3Δ2Δ3 strain transformed with pRSop1 were not significant (p=0.46).

Caspase activation is not associated with the release of cytochrome c under the conditions used in this study

A previous study with a yeast caspase-1 (*YCA1*) deletion and with a strain overexpressing *YCA1* indicated the involvement of the product of this gene in acetic acid-induced apoptotic pathway in *S. cerevisiae* (Madeo et al., 2002c). In addition, we recently reported that caspase activation in *S. cerevisiae* cells undergoing hyperosmotic stress-induced apoptosis is partially dependent on cytochrome *c* (Silva et al., 2005). Since acetic acid-induced apoptosis in wild-type and *aac1/2/3*Δ strain differ in the release of cytochrome *c*, the possibility of detecting a relationship between caspase activation and release of cytochrome *c* using these strains was investigated. To do so, caspase activation was assessed by flow cytometry using the "CaspACE, FITC-VAD-fmk In Situ Marker" together with PI staining. We observed that caspase activation in response to acetic acid was strongly dependent on the growth phase of the culture used to perform the assays (Fig. 5). Actually, when wild-type cells were harvested at OD ~1.0, as used throughout this study, low caspase activation (~6%) was observed after acid treatment and for a survival rate of 37%. However, higher activation levels (~32%) were detected after acid treatment for cells harvested at lower OD (~0.05) and displaying a survival rate of ~37%. These observations are in

line with the decrease of *YCA1* expression accompanying growth progression (DeRisi et al., 1997). Most of the cells displaying caspase activation exhibited compromised membrane integrity (Fig. 5A) in agreement with a previous report (Guaragnella et al., 2006).

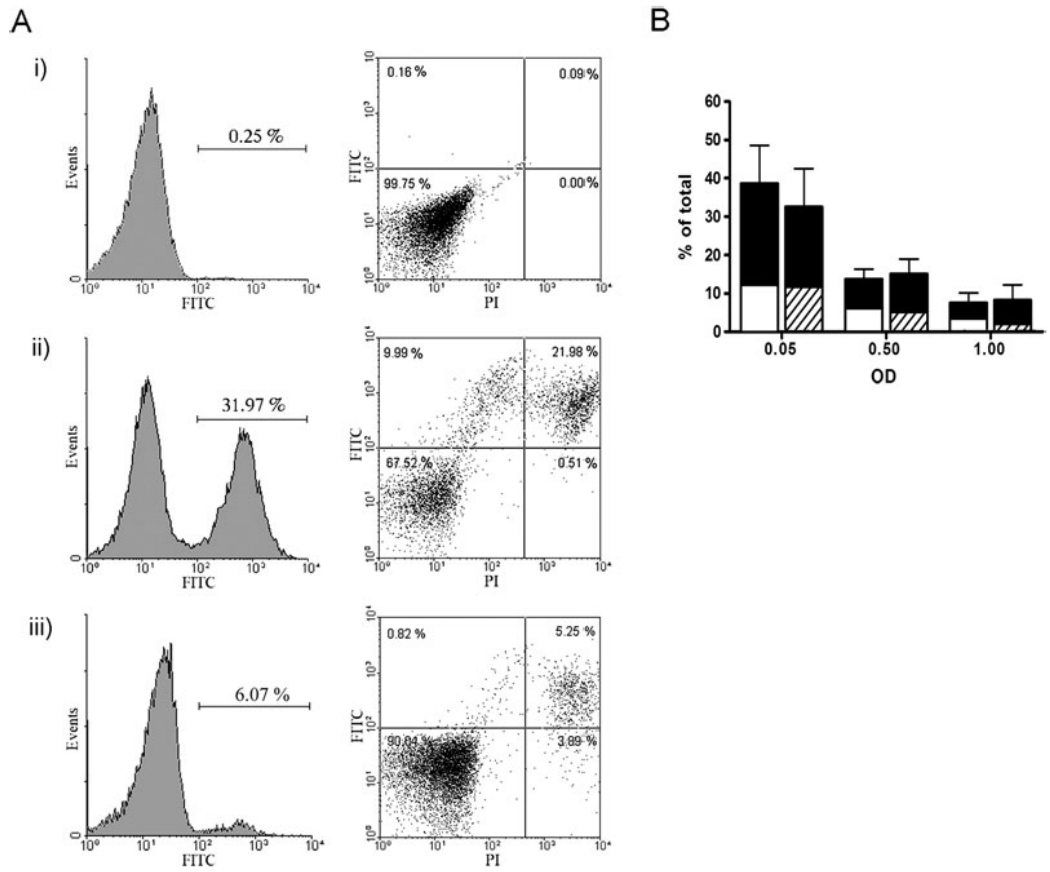

Figure 5. *Caspase activation in response to acetic acid is strongly dependent on the growth phase.* (A) Monoparametric histograms of FITC (in the left) and corresponding biparametric histograms of FITC and PI fluorescences (in the right) are shown for: i) control untreated cells; ii) cells harvested at OD-0.05, and treated with acetic acid and iii) cells harvested at OD-1.0 and treated with acetic acid. Results for untreated cells were independent of culture OD. Double staining FITC/PI allows discriminating between cells with caspase activation and preserved membrane integrity from those with caspase activation and compromised plasma membrane. Data represent one of six independent experiments. (B) Quantification of caspase activation measured by flow cytometry analysis. Wild-type and *aac1/2/3*Δ (JL1-3 strain) cells displaying FITC and PI staining (full bars) are shown together with wild-type (clear bars) and *aac1/2/3*Δ cells (clear striped bars) only displaying FITC staining. Cells harvested at OD-0.05, and OD-1.0 were treated for 200 min, with 80 and 180 mM acetic acid, respectively. Different concentrations of acetic acid were used in order to obtain similar survival percentages when assessed by cfu counts. Results represent the mean of six independent experiments and the vertical error bars represent SD. A two-way ANOVA analysis indicate difference between strains is not significant ($p = 0.89$) but that the effect of the OD is extremely significant ($p < 0.001$).

The *aac1/2/3*Δ strain similarly to the wild-type, exhibited higher levels of caspase activation upon acid treatment when cells were harvested at lower OD (Fig. 5B), suggesting that caspase activation is not associated with the release of cytochrome *c*.

AAC-dependent cytochrome c release and MOMP also occur in H_2O_2-induced apoptosis

In addition to acetic acid we tested the response of the mutants to hydrogen peroxide which is also known to trigger apoptosis in yeast (Madeo et al., 1999). With the exception of *cpr3*Δ, the mutants tested exhibited a higher sensitivity to H_2O_2 comparing to the wild-type (Fig. 6A) which is consistent with the reported sensitivity to H_2O_2 of mutants affected in mitochondrial function (Thorpe et al., 2004). As for acetic acid, death of all mutant strains in response to H_2O_2 dispalyed apoptotic features (data not shown).

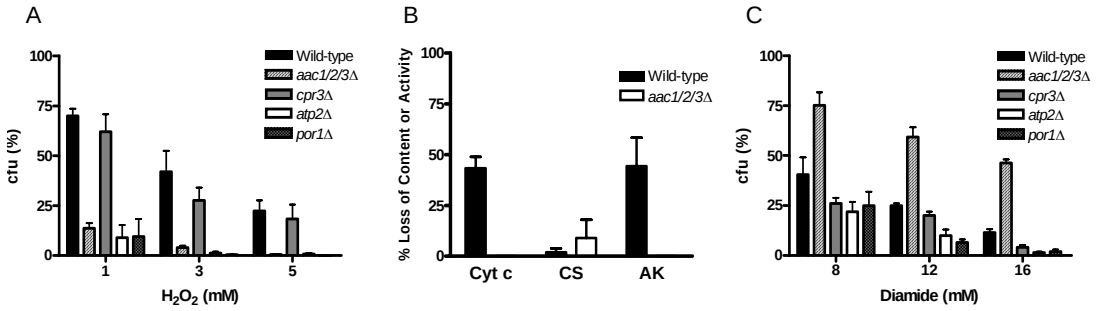

Figure 6. *Role of AAC, Por1p and Cpr3p in apoptosis induced by H_2O_2 and diamide.* Relative survival of *S. cerevisiae* wild-type (W303-1B), *aac1/2/3*Δ (JL-1-3), *Δcpr3*, *atp2*Δ, and *por1*Δ cells, incubated for 200 min with (A) H_2O_2 and (B) diamide (percentage of cfu on YEPDA plates; 100% corresponds to the number of cfu at time 0). Values represent the mean of four independent experiments and the vertical error bars represent SD. A two-way ANOVA was employed to compare the wild-type *versus* deleted strains for each treatment tested. The effect of the deletions on H_2O_2- and diamide-induced death was extremely significant ($p < 0.001$) for *aac1/2/3*Δ, *atp2*Δ and *por1*Δ and not significant for *cpr3* deletion ($p = 0.17$ and $p = 0.07$, respectively). (C) Cytochrome *c* release and enzymatic activities were measured in mitochondria isolated from untreated cells and from H_2O_2 (5 mM, 200 min) treated cells, by redox spectra analysis and enzymatic assays, respectively. CS stands for citrate synthase and AK stands for adenylate kinase. Results are given as the percentage of decrease in content/activity found in mitochondria isolated from H_2O_2 treated cells considering 0% loss, the content/activity found in mitochondria isolated from untreated cells. Values represent the mean of three independent experiments and the vertical error bars represent SD. *$p<0.05$; **$p<0.001$; Unpaired t test.

We next tested whether H_2O_2 mediated-death was also accompanied by MOMP and cytochrome *c* release and if these events were affected in the

*aac 1/2/3*Δ mutant. Redox spectra of mitochondria isolated from wild-type and *aac 1/2/3*Δ strains after treatment with 5 mM H_2O_2 for 200 min, were obtained. The cyt *c*/cyt *b* ratio for the wild-type strain after treatment with H_2O_2, decreased from 2.48 ± 0.08 to 1.43 ± 0.18 (Fig. 6B). A cyt *c*/ cyt *b* ratio of 1.83 ± 0.17 was obtained in the *aac 1/2/3*Δ strain after H_2O_2 treatment, identical to the value measured in untreated cells (Fig. 6B). The redox spectra obtained for both strains after H_2O_2 treatment were therefore comparable to the ones obtained for acetic acid treatment. CS and AK activities were again used to monitor permeabilization of mitochondrial membranes, as described above. Like acetic acid, H_2O_2 induced a decrease in mitochondrial AK activity for the wild-type strain but not for the *aac 1/2/3*Δ, whereas CS activity remained unaltered in both strains (Fig. 6B).

In ligth of these results we tested whether treatment of the mutants with diazenedicarboxylic acid bis 5N,N-dimethylamide (diamide), another pro-oxidant compound, resulted in a phenotype similar to the one observed with H_2O_2. This compound acts as a thiol crosslinking agent and has been reported to induce apoptosis by direct modulation of the mitochondrial PTP in mammalian cells (Zamzami et al., 1998).

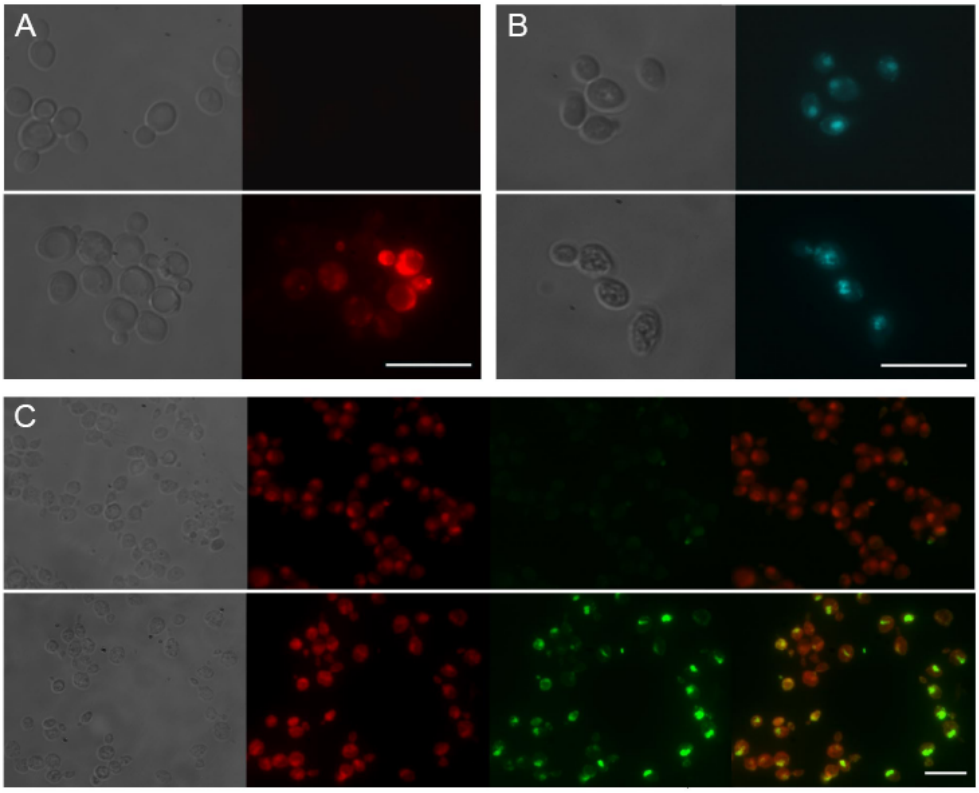

Figure 7. *Diamide treatment induces death with apoptotic-like features.* *S. cerevisiae* wild-type cells were assessed for (A) ROS production, (B) chromatin condensation and (C) occurrence of DNA strand breaks both in untreated (upper panels) and treated cells with 16 mM diamide for 200 min

(lower panels). ROS production was monitored using MitoTracker Red CM-H2XRos staining, chromatin condensation was visualised after DAPI staining and DNA strand breaks were detected using the TUNEL assay. Bar, 10 μm.

We observed that a 200 min-exposure of wild-type strain to diamide (up to 16 mM) led to cell death associated with the production of mitochondrial ROS, chromatin condensation and DNA fragmentation (Fig. 7), indicating that, as in mammalian cells, diamide triggers an active cell death process in yeast. The resistance/sensitivity phenotypes of deleted mutants upon diamide treatment were similar to those observed for acetic acid (Fig. 6C), that is, *aac1/2/3*Δ was more resistant, *por1*Δ and *atp2*Δ were more sensitive, and *cpr3*Δ showed no change in resistance.

Discussion

In mammalian apoptosis, the PTP can mediate mitochondrial membrane permeabilization leading to the release of apoptogenic factors including cytochrome *c* (Crompton, 1999; Zamzami et al., 2005; Garrido et al., 2006). Although the involvement of cytochrome *c* release in yeast apoptosis has been reported (Ludovico et al., 2002a), no information is available regarding how this process occurs and whether mitochondrial outer membrane permeabilization takes place in a system independent of the heterologous expression of the pro-apoptotic members of the Bcl-2 family. In an attempt to gain further insight in this matter, yeast strains deleted in *POR1*, *CPR3* and *AAC1/2/*3 were studied after treatment with acetic acid, H_2O_2 and diamide.

So far, most of the studies concerning the role of Por1p in yeast cell death involved heterologous expression of Bax. However, the conclusions drawn either in what regards the contribution of Por1p to cytochrome *c* release or to cell survival have been contradictory (Priault et al., 1999b; Shimizu et al., 1999; Harris et al., 2000). Our results show that absence of Por1p sensitized cells to death in response to the three different death stimuli studied, and hence suggest that Por1p may work as a negative regulator of the death process rather than a pro-death molecule.

It was recently reported that the mammalian ortholog of yeast Cpr3p, cyclophilin D, is involved in necrotic, but not apoptotic, cell death (Baines et al., 2005; Nakagawa et al., 2005). Accordingly, we found that mitochondrial cyclophilin (*CPR3*) does not play a role in acetic acid-induced apoptosis. This conclusion is supported by the lack of effect

of cyclosporin A as well as by the observation that *CPR3* deletion does not affect loss of cell viability and the emergence of apoptotic markers or cytochrome *c* release (not shown).

We found that absence of AAC proteins protects yeast cells against death induced by acetic acid. Since the respiratory deficient mutants ρ^0, *atp10*Δ and *cyc3*Δ strains were previously shown to be resistant to acetic acid (Ludovico et al., 2002a) we asked if respiratory deficiency could be the cause of the resistance of *aac1/2/3*Δ mutant. Several lines of evidence allow us to discard this hypothesis. *por1*Δ and *atp2*Δ, which both have impaired respiration, were more sensitive to acetic acid and the same was observed for wild-type cells grown and treated with antimycin A and oligomycin. Furthermore, op1 expression clearly demonstrates that the resistance phenotype of *aac1/2/3*Δ mutant cannot be attributed to its respiratory deficiency since op1 has a reduced respiration capacity similar to the *aac1/2/3*Δ mutant (Trancikova et al., 2004) but loses its death resistance.

The *aac1/2/3*Δ strain exhibited a delay in cell death and in the outcome of the cellular events associated with acetic acid-induced apoptosis except for ROS production. ROS production has been described as a common event in most of the yeast apoptotic scenarios (reviewed in Ludovico et al., 2005). Although ROS removal has proven beneficial for cell survival in several cases, including acetic acid (Madeo et al., 1999; Chen et al., 2003; Granot et al., 2003; Saraiva et al., 2006b), the fact that ROS levels in *aac1/2/3*Δ and wild-type strain are similar, indicate that ROS production does not always correlate with viability loss, as described in other studies (Poliakova et al., 2002; Severin and Hyman, 2002; Trancikova et al., 2004; Pozniakovsky et al., 2005). This observation implies that in the *aac1/2/3*Δ mutant the apoptotic signal that proceeds from the mitochondria to the nucleus must be impaired downstream of ROS production.

When the mitochondrial mutants referred above were assessed regarding their response to H_2O_2 or diamide, they showed a similar response as to acetic acid, except for *aac1/2/3*Δ, which displayed a higher sensitivity when treated with H_2O_2. Because we found that H_2O_2-induced apoptosis also triggered AAC-dependent cytochrome *c* release and MOMP, we expected that *aac1/2/3*Δ would display a resistant phenotype. However, Thorpe *et al.* (Thorpe et al., 2004) observed that mutants affected in mitochondrial respiration function were particularly sensitive to H_2O_2. They propose that this is due to the simultaneous presence of superoxide, originating from the impaired electron transport chain, and H_2O_2 leading to the enhanced production of the reactive hydroxyl

radical by the Fenton reaction. This effect can overcome the possible protection provided by the absence of AAC proteins against H_2O_2- induced death.

By monitoring the enzymatic activities of mitochondrial citrate synthase (CS, matrix) and adenylated kinase (AK, intermembrane space) we conclude that acetic acid, while preserving inner membrane integrity, disturbs mitochondrial outer membrane permeability depending on the presence of AAC proteins. When assessed by western blot, it was observed for the wild-type strain, a decrease in mitochondrial cytochrome *c* and a correspondent increase of this protein in the cytosolic fraction during acetic acid treatment. In the absence of AAC proteins, this enrichment in cytochrome *c* in the cytosolic fraction is much more reduced, and in agreement with the maintenance in AK activity suggests that AAC is necessary for the release of mitochondrial proteins. Unexpectedly, cytochrome *c* and β subunit of ATP synthase disappear also from the mitochondrial fraction. In acetic acid–treated *aac1/2/3*Δ cells, while AK and CS activities are maintained, the mitochondrial outer membrane Por1p is degraded in a similar extent as observed for the wild-type cells. Therefore, it seems that in the absence of AAC proteins, acetic acid triggers a selective degradation of some proteins located at the inner mitochondrial membrane, presumably through the activation of ATP-dependent AAA proteases, responsible for the protein quality surveillance of mitochondrial inner membrane proteins (reviewed in Koppen and Langer, 2007).

The possibility that AAC proteins are involved in the release of proteins from mitochondria, through formation of an unselective channel that would cause an increase of mitochondrial volume, is advanced. In fact, expression of a mutated form of Aac2p (A128P), which is equivalent to the human protein responsible for ophthalmoplegia ($Ant1p^{A114P}$) results in depolarization, structural swelling and disintegration of mitochondria, and ultimately leads to an arrest of cell growth (Chen, 2002). It was proposed that $Aac2^{A128P}$ might induce an unregulated channel allowing free passage of solutes across the inner mitochondrial membrane. We also show that the expression of another mutated form of Aac2p (op1) exhibiting very low ADP/ATP translocase activity in the *aac1/2/3*Δ background reverted its resistance phenotype. This observation indicates that AAC pro-death role does not require translocase activity and supports that acetic acid might induce AAC to form an unregulated channel. Moreover, our working model is strongly supported by previous observations that in mammalian cells lines short chain fatty acids (SCFA) or acetic acid alone, act on mitochondria, having AAC as a

probable cell target since SCFA specifically permeabilize liposomes containing AAC (Hague et al., 1995; Jan et al., 2002).

The present study does not allow us to conclude on the ensuing role of cytochrome *c* in acetic acid-induced apoptosis since the observed delay in apoptosis observed for the *aac1/2/3*Δ mutant might be a consequence of cytochrome *c* not being released, but could result from the non-release of other mitochondrial proteins, like for example Aif1p and EndoG, or even of the absence of MOMP *per se*. Translocation of Aif1p and EndoG from the yeast mitochondria to the nucleus in response to acetic acid has been reported (Wissing et al., 2004a; Buttner et al., 2007) Buttner *et al*., 2007) and EndoG-mediated apoptosis was abolished in *aac1/2/3*Δ cells. Since in the absence of AAC acetic acid- and H_2O_2-induced death still exhibited apoptotic markers, it is likely that yeast possess alternative death pathways, which do not involve MOMP and cytochrome *c* release.

In conclusion, we show that AAC proteins are required for stability and release of cytochrome *c* to the cytosol and that Por1p contributes to the resistance of yeast cells to apoptotic death, bringing a new perspective on the role of PTP components in cell death. Our finding offers the possibility to use the simpler yeast eukaryotic system to further elucidate the mechanisms underlying mitochondrial release of apoptogenic regulators, and to screen for drugs interfering with such a process. This can be mostly relevant because of the current increasing interest on the pore as a pharmacological target (Rasola and Bernardi, 2007).

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Chapter 3

Acetic acid triggers *Sacharomyces cerevisiae* into *YCA1* dependent and independent apoptotic pathways

This chapter comprises parts from the following publication:

Pereira C., Guaragnella N., Sousa, M. J., Antonacci L., Passarella S., Côrte-Real M., Marra, E., and Giannattasio, S. (2006). *YCA1* participates in the acetic acid induced yeast programmed cell death also in a manner unrelated to its caspase-like activity. FEBS Letters 580, 6880-6884.

Acetic acid triggers *Sacharomyces cerevisiae* into *YCA1* dependent and independent apoptotic pathways

Abstract

Acetic acid-induced cell death in yeast includes *bona fide* apoptotic features such as externalization of phosphatidylserine or chromatin condensation, prerequisite for apoptosis in mammalian cells. However, the molecular mechanisms underlying acetic acid-induced death have not been completely elucidated. Here, we show that deletion of *YCA1* gene delays acetic acid-triggered programmed cell death in *Saccharomyces cerevisiae*, but that *yca1*Δ strain still dies exhibiting apoptotic markers. Flow cytometry measurements showed that the percentage of FITC-VAD-fmk positive cells produced during acetic acid treatment is reduced in *yca1*Δ strain comparatively to the wild-type and that in the previous the positive staining is partially inhibited by the pan-caspase inhibitor z-VAD-fmk. Thus, Yca1p contributes to, but is dispensable for, acetic acid-induced apoptosis indicating that Yca1p-independent death pathways are able to kill the cells.

Introduction

In the last decade it has been shown that *Saccharomyces cerevisiae* may undergo a form of cell death similar to apoptosis with its own physiological significance (Skulachev, 2002; Herker et al., 2004a; Knorre et al., 2005). In fact, in response to different stimuli *S. cerevisiae* commits to cell death showing typical hallmarks of metazoan apoptosis (Madeo et al., 2004). Although several yeast orthologues of key apoptotic regulators have already been identified (Ligr et al., 2001; Madeo et al., 2002a; Fahrenkrog et al., 2004; Fannjiang et al., 2004; Wissing et al., 2004a; Ahn et al., 2005), how they work in yeast apoptosis remains to be fully established.

Among the variety of proteins involved in cell death, caspases play a major role as central effectors in the execution of almost all form of apoptosis in metazoan cells (Earnshaw et al., 1999; Los et al., 2001; Shi, 2002). Yet, there is now accumulating evidence indicating that cell death can also occur in a programmed fashion independent of caspase activation (Leist and Jaattela, 2001; Broker et al., 2005a). In yeast, *YCA1* codes for a protein belonging to the superfamily of caspase-related proteases, termed metacaspases (Uren et al., 2000). *YCA1* is involved in apoptosis in *S. cerevisiae* triggered by different stimuli (Madeo et al., 2002b; Herker et al., 2004b; Wadskog et al., 2004; Mazzoni et al., 2005; Mitsui et al., 2005; Silva et al., 2005). In addition, disruption of *YCA1* attenuated stimulation of apoptosis under Aifp overexpressing conditions (Wissing et al., 2004b) or hydrogen peroxide (Khan et al., 2005).

Nevertheless, no endogenous substrate of Yca1p has been identified so far in yeast cells and examples of metacaspase-independent apoptosis have also been reported (Ivanovska and Hardwick, 2005; Hauptmann et al., 2006).

We have shown that *S. cerevisiae* commits to apoptosis upon treatment with acetic acid (Ludovico et al., 2001a). Previous genetic evidence pointed to the yeast caspase-related protease (Yca1p) as an executor of acetic acid-induced apoptosis in *S. cerevisiae* (Madeo et al., 2002b). More recently, it was proposed that the role of yeast caspase in acetic acid-induced apoptosis is less relevant (Saraiva et al., 2006a) than in other stimuli (Madeo et al., 2002b; Silva et al., 2005). To further investigate the role of Yca1p in acetic acid-induced apoptosis we studied the effect of *YCA1* disruption on *S. cerevisiae* cell death in response to such stimulus.

Experimental procedures

Strains and growth conditions

Saccharomyces cerevisiae strain used in this study was W303-1B (*MAT*a *ade2 leu2 his3 trp1 ura3*) and cells were grown at 30°C in rich medium (1% yeast extract and 2% Bacto-peptone) containing 2% dextrose (YPD). To delete *YCA1* gene genomic DNA was isolated from BY4743 (*MAT*a/*MAT*α *leu2/leu2 his3/his3 ura3/ura3 lys2/LYS2 MET15/met15 yca1*::*kan*MX4/*YCA1*) cells, kindly provided by Prof. R. A. Butow, and amplified using the oligonucleotides couple 5'-TTA TTG GCC GAG TTG CGC T-3'and 5'-GGA AGA ACA GGA AGA GTC TG-3'. *yca1*Δ::KanMX4 cassette-containing PCR product was purified from agarose gel and used to replace the *YCA1* gene in W303-1B cells. Recombinant clones were selected for resistance to antibiotic G418 disulphate (Sigma Aldrich). Gene disruption was verified by PCR analysis.

Acetic acid treatment

Exponential phase (OD_{600} = 0.5-0.8) wild type and *yca1*Δ cells were grown in YPD medium at 26 °C, 160 r.p.m and were suspended (10^7 cells/ml) in fresh YPD medium. For acetic acid treatment this medium containing acetic acid was adjusted to pH 3.0 (set with HCl). Cells were incubated for different times at 26 °C as described previously (Ludovico et al., 2001b). As a positive control of caspase activation, wild-type cells were transferred to medium containing 60% (w/w) of glucose as described [18] and incubated for 300 min at 26 °C.

Cycloheximide (Sigma Aldrich) dissolved in water or z-VAD-fmk (Calbiochem) dissolved in dimethylsulphoxide, were added to yeast cell cultures grown to an OD_{600} of 0.6-0.7 at a final concentration of 100 μg/ml or 20 μM, respectively, and the cultures incubated for 30 min before acetic acid treatment. Cell viability was determined by measuring colony-forming units (cfu) after 2 days of growth on YPDA plates at 30°C.

Fluorescence microscopy analysis

Exponential cells were treated with acetic acid in YPD pH 3.0 medium as described in the previous section. At different times, 10^8 cells were collected and suspended in water in the presence of 0.25 μg/ml PI and 50 μg/ml DAPI at 37°C for 15 min. After incubation,

live cells were observed on an Axioplan 2 microscope (Zeiss) equipped with a UV HBO 50/AC lamp and a 100X objective. Digital images were acquired with CCD camera Axiocam using Axio Vision software.

Detection of caspase activation

Detection of caspase activation was performed using the "CaspACE, FITC-VAD-fmk In Situ Marker" (Promega). Briefly, for the CaspACE kit 1.10^6 cells were washed in PBS, suspended in 100 µl staining solution containing 50 µM of FITC-VAD-fmk and incubated for 20 min at 30 ºC in the dark. After incubation cells were washed once and suspended in PBS. For double staining with PI, cells were subsequently incubated with 2 µg/ml of PI for 10 min at room temperature. For the assays with the broad caspase inhibitor z-VAD-fmk, cells were prepared as described above, washed with PBS and incubated with 20 µM z-VAD-fmk for 30 min at 30 ºC before incubation with FITC-VAD-fmk.

Flow cytometric analysis was performed in an Epics® XL-MCL™ (Beckman Coulter) flow cytometer, equipped with an argon-ion laser emitting a 488-nm beam at 15 mW, and biparametric detection of FITC fluorescence (488/525 nm; FL1; log units) and PI fluorescence (488/620 nm; FL3; log units). Signal compensation that eliminates optical overlap between both dyes was of 30%. Twenty thousand events were acquired for each analysis. Data were analyzed using WinMDI 2.8 software.

Results

Deletion of YCA1 *gene delays but does not prevent acetic acid-triggered apoptosis in* S. cerevisiae

W303-1B wild type and *yca1*Δ cells in early exponential phase exposed to 80 mM acetic acid in YPD pH 3.0 medium were compared with respect to their viability up to 200 min after after apoptotic induction (Fig. 1A). In both cases the yeast cells died but the cell death patterns were different. After 30 min of acetic acid treatment, wild-type and *yca1*Δ cells showed about 74% and 83% cell viability, respectively. This progressively decreased to 0% for wild-type and 7% for *yca1*Δ cells at 200 min. *yca1*Δ cell viability was significantly higher ($P < 0.001$) than that of wild-type cells from 60 to 120 min after acetic

acid challenge with death rates of ld = 0.015 ± 0.0021 min^{-1} and 0.0074 ± 0.0002 min^{-1} for wild-type and *yca1*Δ cells, respectively.

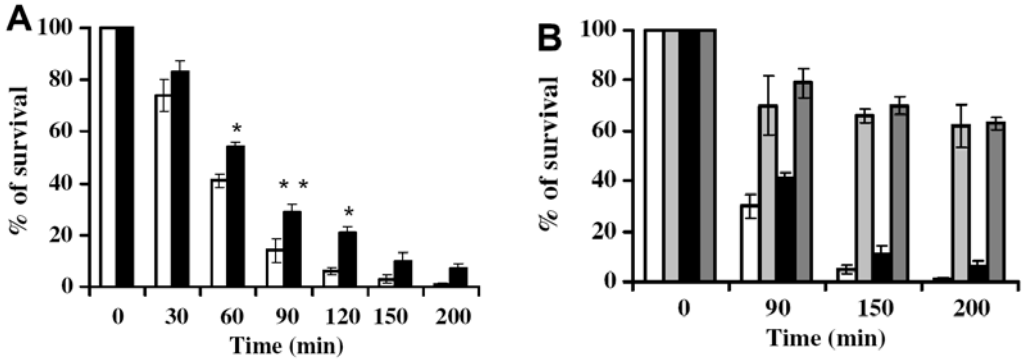

Figure 1. *Effect of YCA1 deletion and/or cycloheximide on viability of* S. cerevisiae *W303-1B exponential cells exposed to acetic acid.* wild-type (white bars) and *yca1*Δ (black bars) cell death was induced with 80 mM acetic acid in the absence (A and B, white and black bars, respectively) or in the presence of cycloheximide (B, light and dark grey bars, respectively) and cell viability analyzed at indicated times. Cell survival (100%) corresponds to cfu at time zero. Reported values are the mean of three experiments with standard deviations. ANOVA and Bonferroni test: statistically significantly different with (*) $P < 0.05$, (**) $P < 0.001$ when comparing wild-type and *yca1*Δ cells. Death rate μd (see text) was calculated by GraFit 3.0 software as the slope of the linear part of the semilogarithmic plot of the number of cfu as a function of incubation time.

To determine whether death of *yca1*Δ cells occurs via apoptosis, given that acetic acid-induced apoptosis is dependent on *de novo* protein synthesis (Ludovico et al., 2001a), the effect of cycloheximide on survival of *yca1*Δ and wild-type cells was investigated. In both cases cycloheximide prevented cell death in a similar way (Fig. 1B). Chromatin condensation, another apoptotic hallmark, was also analyzed during acetic acid induced-apoptosis in both wild-type and *yca1*Δ cells, through analysis of nuclear morphology and plasma membrane integrity by co-staining cells with DAPI and PI (Fig. 2). With both cell types chromatin condensation along the nuclear envelope was detectable after 60, 120 and 200 min of acetic acid treatment in cells with an integral plasma membrane as shown by lack of staining with PI. Thus, exponential yeast cells lacking the metacaspase *YCA1* gene undergo the process of acetic acid-induced apoptosis in a manner similar to that of the wild-type cells but at a lower rate. In stationary phase cells however, deletion of *YCA1* has no effect in the rate of cell death (data not shown).

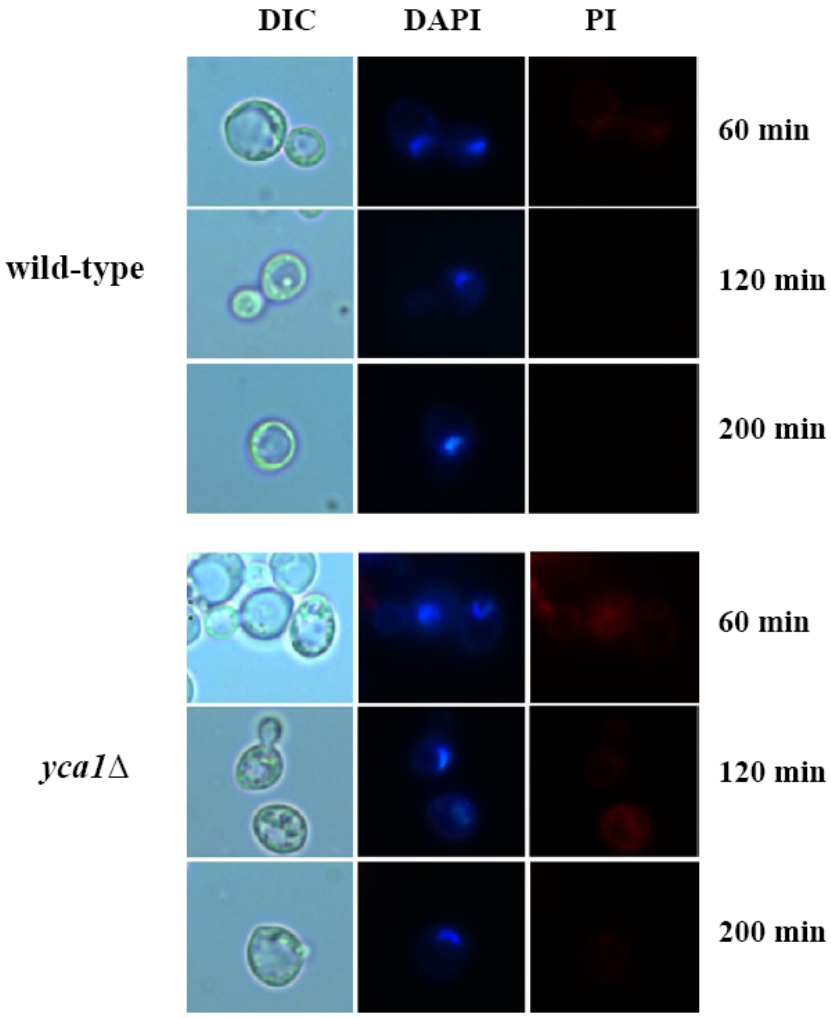

Figure 2. *DAPI/PI staining of wild type and yca1Δ* S. cerevisiae *W303-1B exponential cells exposed to 80 mM acetic acid, pH 3.0.* Wild type and *yca1*Δ yeast cells were exposed to 80 mM acetic acid. At indicated times cells were collected, stained with DAPI and PI and observed with a fluorescence microscope. Representative pictures of wild type and *Δyca1* cells are shown. DIC, differential interference contrast. As a control, wild type and mutant cells incubated in the same conditions, but in the absence of acetic acid, did not show either chromatin condensation or PI-positive cells at any time analysed (not shown).

z-VAD-fmk does not prevent acetic acid-induced apoptosis even though it partially inhibits caspase-like activity

In the light of the above results, to ascertain whether and how caspase activity is involved in acetic acid-induced apoptosis, we monitored caspase-like activity by simultaneous staining of cells with FITC-VAD-fmk (Madeo et al., 2002b; Silva et al., 2005) and with PI to differentiate between FITC-VAD-fmk specific and unspecific staining (Vachova and Palkova, 2005).

Cells treated with 60% (w/w) of glucose were used as a control for positive staining with FITC-VAD-fmk (Silva et al., 2005). Only about one third of the wild type FITC-VAD-fmk-stained cells displayed a PI negative staining (specific staining) after treatment with acetic acid (Fig. 3A). This fraction increased to about two thirds for the positive control. Moreover, pre-incubation with the pan-caspase inhibitor z-VAD-fmk before staining with FITC-VAD-fmk, caused a reduction in the percentage of FITC-VAD-fmk positive cells (Fig. 3A). Caspase activation could not be detected in wild-type cells treated under the same conditions (pH 3.0) but without acetic acid (Fig. 3A) and was found to increase progressively up to about 20% at 200 min after induction of apoptosis (Fig. 3B). It should be pointed out that acetic acid-induced caspase activation was strongly influenced by the growth phase of the culture, decreasing with the increase of the culture optical density (data not shown). By contrast, with *yca1*Δ cells the percentage of FITC-VAD-fmk staining remained virtually constant at about 5% between 60 and 200 min of cell death. These results are consistent with *YCA1* acting as a zVAD-fmk sensitive caspase-like protease and/or as a protease activator. In parallel, cell survival was monitored either in the absence or presence of z-VAD-fmk. We found that either in the presence or in the absence of z-VAD-fmk there were no differences in cell death between wild-type and *yca1*Δ cells (Fig. 3C).

We attempted to use the caspase substrate $(Asp)_2$-Rhodamine 110 (D_2R) to monitor caspase activity as reported (Hug et al., 1999; Vachova and Palkova, 2005). Because acetic acid interfered with the green fluorescence produced from the intracellular cleavage of the caspase substrate D_2R (data not shown) probably due to intracellular acidification, we conclude that D_2R staining is not adequate to monitor caspase activity in cells committed to dye in response to acetic acid challenge.

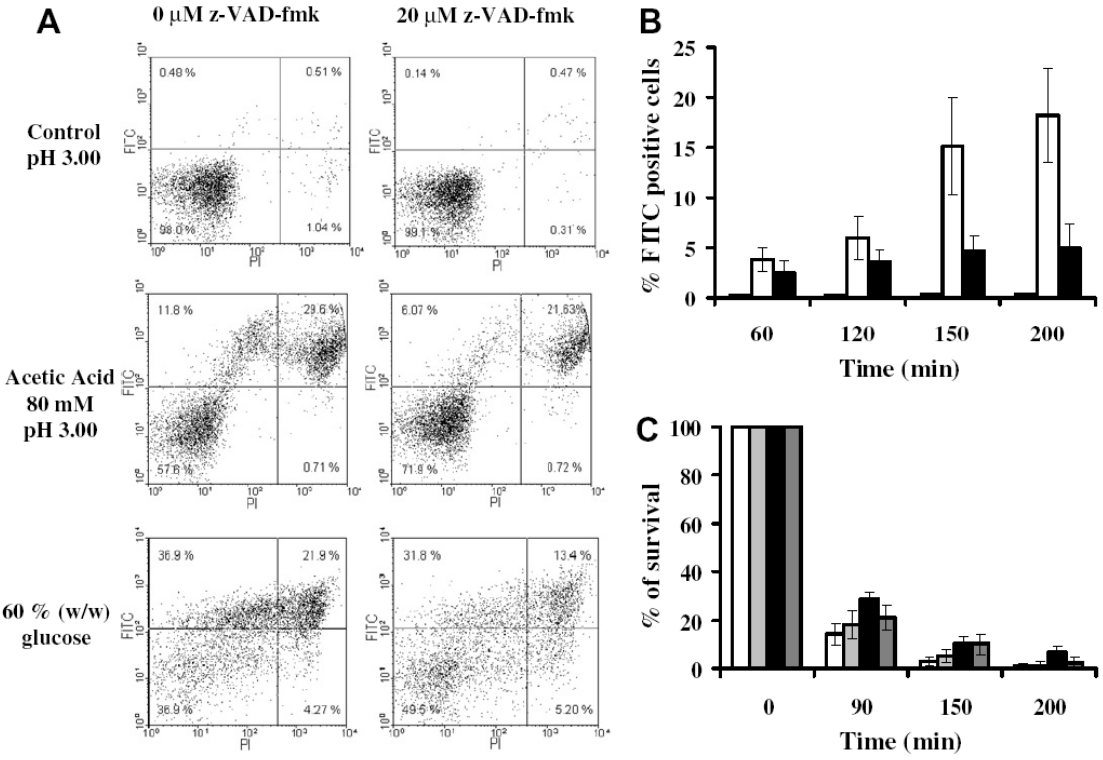

Figure 3. *Caspase-like activity in* S. cerevisiae *W303-1B exponential cells upon treatment with acetic acid and effect of z-VAD-fmk on cell viability.*

A. Flow cytometric analysis using bi-parametric detection of FITC fluorescence (FL1) and PI fluorescence (FL3) of: control non-treated cells with or without pre-incubation with 20 μM z-VAD-fmk; cells treated with 80 mM acetic acid for 200 min with or without pre-incubation with 20 μM z-VAD-fmk; cells treated with 60% (w/w) glucose for 300 min with or without pre-incubation with 20 μM z-VAD-fmk. Percentages of cells exhibiting only FITC fluorescence (upper left quadrant), both FITC and PI fluorescences (upper right quadrant), only PI fluorescence (lower right quadrant) or absence of both fluorescences (lower left quadrant) are depicted in the respective quadrants. Data represent one of three independent experiments.

B. Results presented are percentages of caspase activation detected by FITC-VAD-fmk in wild-type cells incubated at pH 3.0 (grey bars, near zero); wild-type cells treated with 80 mM acetic acid (black bars) and *YCA1*-deleted cells treated with 80mM acetic acid (white bars), along time. A two-way ANOVA was employed to compare wild type untreated *versus* acetic acid-treated cells and these latter *versus* acetic acid-treated *yca1Δ* cells. In both cases the difference in the % of FITC positive cells was considered significant ($p < 0.05$). Values are mean of three independent experiments with 20,000 cells, counted by flow cytometry, per each time point.

C. Wild type and *yca1*Δ cell death was induced with 80 mM acetic acid in the absence (white and black bars, respectively) or in the presence of z-VAD-fmk (light and dark grey bars, respectively). Cell viability was analysed at indicated times. 100% of cell survival corresponds to the cfu at time zero. Reported values are the mean of three experiments with standard deviations.

Discussion

This study was aimed at giving an insight into the role of *YCA1* gene in acetic acid-induced apoptosis in *Saccharomyces cerevisiae*. We showed that deletion of *YCA1* significantly delays death of exponential phase yeast cells treated with acetic acid. Yca1p -independent cell death was found associated with chromatin condensation and was inhibited by cycloheximide, showing that Yca1p is dispensable for acetic acid-triggered apoptosis. Moreover, we show that apoptosis in wild-type and *yca1*Δ cells the characteristics of the death process are the same for both cell types except that in *YCA1* disruption there is a reduced death rate.

We also used the pancaspase inhibitor z-VAD-fmk to investigate further the role of the yeast metacaspase. We showed that in wild-type cells treated with acetic acid there is a time-dependent increase in caspase-like activity, as revealed by flow cytometry with FITC-VAD-fmk, which is inhibited by zVAD-fmk. This does not occur in *yca1*Δ cells, thus strongly suggesting a role for Yca1p in promoting caspase-like activity. Nonetheless, z-VAD-fmk addition did not result in death prevention. Given that this substance can inhibit caspase-like activity, as indicated by the experiments with flow cytometry, we conclude that yeast can die in a manner independent of caspase activity and consequently that Yca1p participates in acetic acid-induced apoptosis in a manner unrelated to its putative caspase-like activity.

In this regard, acetic acid-induced apoptosis differs significantly from that due to defective N-glycosylation. In the latter case both FITC-VAD-fmk cell-staining activity as well as loss of cell viability have been shown to be inhibited by z-VADfmk and this caspase-like activity is independent of Yca1p (Hauptmann et al., 2006). At present the explanation of the reduced rate of death in yeast cells lacking *YCA1* can only be speculative. In addition to the possibility that Yca1p is a caspase-like protein, as suggested by the fact that deletion of *YCA1* inhibits FITC-VAD-fmk cell-staining, the possibility that Yca1p could be associated with other function different from caspase activity, perhaps protease activity must be taken into consideration. This is supported by recent findings which show that recombinant Yca1p exhibits arginine/lysine-specific cysteine endopeptidase activity, which is not inhibited by z-VAD-fmk (Watanabe and Lam, 2005). Thus, it cannot be excluded that Yca1p, acting as a protease, activates other caspase-like activities.

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Chapter 4

Mitochondrial degradation in acetic acid-induced yeast apoptosis: the role of Pep4p and ADP/ATP carrier

This chapter comprises parts from the following publication:

Pereira C., Camougrand N., Manon S., Sousa M.J. e Côrte-Real M. (2008) Mitochondrial degradation in acetic acid-induced yeast apoptosis: the role of Pep4p and ADP/ATP carrier. Submitted manuscript.

Mitochondrial degradation in acetic acid-induced yeast apoptosis: the role of Pep4p and ADP/ATP carrier

Abstract

We have previously shown that acetic acid activates a mitochondrial-dependent death process in *Saccharomyces cerevisiae* and that ADP/ATP carrier (AAC) was required for mitochondrial outer membrane permeabilization and cytochrome *c* release. Mitochondrial fragmentation and degradation in cells undergoing programmed cell death was also shown by other authors. Herein we proposed to study the degradation of mitochondria and the role of AAC proteins/mitochondrial permeabilization in such a process. We show that the autophagic pathway is not induced during acetic acid-triggered apoptosis and thus is not responsible for mitochondrial degradation. Conversely, the vacuolar protease Pep4p, orthologue of human cathepsin D, is released from the vacuole to the cytosol in response to acetic acid and is involved in mitochondrial degradation. AAC-deleted cells also show a decrease in mitochondria degradation in response to acetic acid that, like for *PEP4*-deleted cells, results in formation of mitochondria aggregates. In AAC-deleted cells, Pep4p is released from the vacuole rather normally indicating that AAC proteins affect degradation at a step subsequent to Pep4p release, probably in signalling the doomed mitochondria. Together the results suggest that the removal of potentially harmful mitochondria depends on both on mitochondrial and vacuolar proteins and seems to be used as a protective mechanism directing death through a programmed process, rather than by a necrotic process.

Introduction

Yeast mitochondria were found to undergo both structural and functional changes and to play a key role in the apoptotic death process activated by acetic acid (Ludovico et al., 2002b; Ludovico et al., 2003). Acetic acid induces different ultrastructural mitochondrial alterations in yeast, including mitochondrial swelling, reduction in cristae number and formation of myelinic bodies. Additionally, *S. cerevisiae* cells committed to apoptosis in response to acetic acid display mitochondrial ROS accumulation, a transient hyperpolarization followed by depolarization, and a decrease in the activity of COX affecting mitochondrial respiration. The uncoupling effect of acetic acid (Lawford and Rousseau, 1993) is expected to result in an ATP consuming futile cycle aggravated by the loss of respiratory chain components. Mitochondria become permeabilized in response to acetic acid allowing the release of lethal factors like cytochrome *c* (Ludovico et al., 2002b) and yeast AIF (Wissing et al., 2004a) and hence contributing to the death process. Other mitochondrial proteins also affect the final cell fate including those involved in fission/fusion (Fannjiang et al., 2004) as well as homologues of the components of mammalian permeability transition pore (PTP) (Pereira et al., 2007). Mitochondrial degradation in yeast cells undergoing apoptosis was previously reported (Fannjiang et al., 2004).

Mitochondrial degradation following apoptotic induction is a common feature in mammalian cells (reviewed in Tolkovsky *et al*., 2002). This event usually occurs through an autophagic process that shows selectivity for mitochondria, and therefore, was termed mitophagy (Lemasters, 2005). How the damaged mitochondria in a cell are selectively removed is not known, but recent evidence supports the view that mitochondria permeability transition (MPT) could be the trigger for this removal (Rodriguez-Enriquez et al., 2006; Kim et al., 2007). The role of mitophagy in the cell death process is still unclear. It has been proposed to have a cytoprotective role since it allows removal of deleterious ROS-producing mitochondria and hinders the release of pro-apoptotic proteins to the cytosol. On the other hand, the catabolism of dysfunctional organelles could facilitate the apoptotic process by preventing an energy drain caused by defective chemiosmotic coupling (when the MPT pore opens, ions equilibrate between the matrix and cytosol), while providing at the same time a source of ATP generation (Wallace, 2005; Maiuri et al., 2007; Terman et al., 2007).

The accumulation of damaged mitochondria is considered to underlie a wide range of age-related disorders, various forms of cancer and of late-onset genetic diseases (Zeviani and Carelli, 2007; Zhang and Qi, 2008). Thus the understanding of this phenomenon can unravel attractive targets for the development of therapeutics for different diseases.

In yeast, selective removal of mitochondria was reported following heterologous expression of Bax (Kissova et al., 2006), mitochondrial dysfunctions (Priault et al., 2005), osmotic swelling (Nowikovsky et al., 2007) and in stationary phase cells (Tal et al., 2007). Removal of mitochondria is not always dependent on the autophagic machinery (Kissova et al., 2006) and the outcome in terms of survival obtained by blocking mitochondrial degradation varied with the stimulus used (Tal et al., 2007).

In this work, we proposed to study the mechanisms responsible for mitochondrial degradation during acetic acid-induced apoptosis and to assess the role of AAC proteins/mitochondrial permeabilization in this process. We found that neither autophagy nor Uth1p-dependent mitophagy are active during acetic acid-induced apoptosis, and that the vacuolar protease Pep4p is translocated to the cytosol playing a crucial role in mitochondrial degradation. Moreover, we show that though mitochondrial degradation is decreased in the absence of AAC proteins, Pep4p release is not affected. Together we propose that in yeast undergoing apoptosis mitochondria removal can occur through an autophagic-independent pathway yet involving the vacuolar protease Pep4p and the mitochondrial AAC proteins. Moreover, we propose that these latter proteins might signal dysfunctional mitochondria for degradation.

Experimental procedures

Strains and plasmids

The following *Saccharomyces cerevisia*e strains were used in this study: W303-1A (*MATα, ade2, his3, leu2, trp1, ura3, can1*); W303 *PHO8*Δ*60* (Kissova et al., 2004); W303 *atg5*Δ::*KanMX4* (Kissova et al., 2004); W303 *atg5*Δ::*KanMX4 PHO8*Δ*60* (Kissova et al., 2004); W303 *uth1*Δ::*TRP1* (Camougrand et al., 2000); W303 *atp2*Δ::*KanMX4* (Pereira et al., 2007), JL1-3Δ2Δ3 (W303; *aac1::LEU2,* Δ*aac2::HIS3,* Δ*aac3::URA3*) (Postis et al., 2005) and *pep4*Δ*::HIS3* (Marques et al., 2006). Plasmids

used: pDP34 and pDP34-*PEP4* (Rupp and Wolf, 1995) for *PEP4* overexpression, pGAL-CLbGFP (Okamoto et al., 1998) and YX232-mtGFP (Westermann and Neupert, 2000) for mitochondria visualization, p416ADH and p416ADH-PEP4-EGFP (Mason et al., 2005) for Pep4p tagging.

Whenever strains were transformed the lithium acetate method (Ito et al., 1983) was used and the resulting transformants were grown in selective media lacking the appropriate aminoacids.

Growth conditions

Yeast cells were grown in rich media [YEPD; 1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (w/v) glucose] or synthetic complete media [SC; 0.67 % (w/v) Bacto-yeast nitrogen base w/o amino acids, 2% (w/v) galactose and 0.2% (w/v) Dropout mix] lacking the appropriate amino acids until exponential phase in an orbital shaker, at 26 ˚C, 160 rpm. The strains were harvested and suspended (10^7 cells/ml) in the treatment medium consisting on YEPD or SC at pH 3.0 (set with HCl), containing the appropriate amounts of acetic acid or H_2O_2 and incubated as described for the growth conditions. For nitrogen starvation assays cells were grown in rich media overnight, washed twice with distilled sterile water and transferred to nitrogen starvation media. Nitrogen starvation media consisted on 0.67% (w/v) Bacto-yeast nitrogen base w/o amino acids and ammonium sulphate (Difco) and 2% (w/v) glucose. Cells were then incubated at 26 ˚C, 160 rpm, for 15 hours.

Alkaline Phosphatase assay

ALP activity assays using α-naphtyl phosphate (Sigma) as a substrate were performed as described previously (Nothwehr et al., 1996). Fluorescence intensity was measured at 472 nm (excitation at 345 nm) in a Xenius spectrofluorimeter (Safas). Protein was quantified by the Biuret method and the ALP activities are expressed as arbitrary fluorescence units/min/mg protein. ALP activity was measured in non treated cells; cells treated with 180 mM acetic acid (1 or 3 hours) and in nitrogen starved cells (15 hours).

Western blot analyses

Preparation of protein samples, SDS-PAGE and Western blots were performed as described previously (Camougrand et al., 2003a). The primary antibodies used were mouse monoclonal anti-yeast phosphoglycerate kinase (PGK1) antibody (1:5000, Molecular Probes), mouse monoclonal anti-yeast porin (POR1) antibody (1:5000, Molecular Probes), goat polyclonal anti-yeast Atg8p (1:250, Santa Cruz Biotechnology), and anti-yeast Tom22 (1:5000, Santa-Cruz Biotechnology).

Secondary antibodies against mouse or rabbit IgGs, coupled to horseradish peroxidase (Jackson Laboratories), were used at 1:5000 and revealed by chemiluminescence (ECL, Amersham). Quantification of protein amounts was done using the ImageJ program (NCBI).

Fluorescence microscopy

Mitochondria were visualized using a matrix-targeted GFP, either pGAL-CLbGFP or YX232-mtGFP. For Pep4p labelling cells expressing the construction p416ADH-PEP4-EGFP were used. Cells were grown and treated as described in the growth conditions section, and immobilized in the slides by adding 0.5% (w/v) agar prior to microscopy.

For staining with propidium iodide (PI) cells were incubated with 5 µg/ml of PI for 10 min at room temperature. When used, 0.4 µg/ml MitoTracker Red CMXRos (Molecular Probes) was added in culture medium and incubated for 20 min at 37 º C.

Overnight cultures were analyzed on a Leica Microsystems DM-5000B epifluorescence microscope with appropriate filter settings using a 100× oil-immersion objective. Images were acquired with a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software.

Flow cytometry

Mitochondrial degradation was assessed by quantification of loss of mitochondrial GFP fluorescence. Plasmids used were pGAL-CLbGFP (Okamoto et al., 1998) for strains W303, JL1-3Δ2Δ3 and *pep4*Δ, and YX232-mtGFP (Westermann and Neupert, 2000)for strains W303 pDP34 and W303 pDP34-PEP4. For quantification of loss of plasma membrane integrity, cells were collected and suspended at a concentration

of 5.10^6 cells/ml in PBS and incubated with 5 μg/ml of PI for 10 min at room temperature.

For assessment of mitochondrial potential the probe 3,3'dihexyloxacarbocyanine iodide ($DiOC_6(3)$) was used. Cells were collected and suspended at a concentration of 5.10^6 cells/ml in PBS and incubated with 1nM $DiOC_6(3)$ at 30 ºC in the dark.

Sample analysis was performed in an Epics® XL™ (Beckman Coulter) flow cytometer, equipped with an argon-ion laser emitting a 488-nm beam at 15mW. Monoparametric detection of PI fluorescence was performed using FL-3 (488/620 nm) and detection of $DiOC_6(3)$ or GFP fluorescence was performed using FL-1 (488/525 nm).

Thirty thousand cells were analyzed per sample and experiments were reproduced independently at least four times. Data were analyzed using WinMDI 2.8 software.

Results

Autophagy is not activated during acetic acid-induced programmed cell death

Mitochondria were found to play a key role in the apoptotic death activated by acetic acid (for a revision see (Eisenberg et al., 2007; Pereira et al., 2008) being fragmented and subsequently removed in a late phase of the death process (Fannjiang et al., 2004).

It was our purpose to assess the contribution of autophagy to the process of mitochondrial degradation that occurs during acetic acid-induced apoptosis. To this end, we used an established biochemical method that consists in following the vacuolar delivery of Pho8Δ60p, a truncated form of the yeast vacuolar alkaline phosphatase (ALP) lacking the N-terminal 60 amino acids (Noda et al., 1995). This protein, which was shown to accumulate in the cytosol as a zymogen, is delivered to the vacuole exclusively by an autophagic process, where the active form of the enzyme is generated by proteolytic cleavage (Noda et al., 1995). Therefore, in cells only expressing this truncated form of the enzyme, the level of APL activity reflects the level of autophagy, either if it is macroautophagy, requiring autophagosomes for the delivery of the cell material, or microautophagy, implying direct uptake of cell material by the vacuole (Kissova et al., 2007).

A *S. cerevisiae* W303 strain where the chromosomal *PHO8* gene was replaced with *pho8Δ60* was incubated in the presence of 180 mM acetic acid and the ALP activity was determined after 0, 1 and 3 hours treatment. We observed that there was no increase in ALP activity following acetic acid treatment indicating the absence of transport of material into the vacuole (Fig. 1A). Nitrogen starved cells were used as a positive control for autophagy induction and displayed a more than twofold increase in ALP activity (Fig. 1A). Identical experiment was done in an *atg5*Δ strain also expressing the Pho8Δ60p truncated protein in replacement of the native Pho8p, and was used as a negative control for autophagy induction. Atg5p belongs to the autophagic pathway in yeast, where it takes part of a cytosolic complex essential for the autophagosome formation/completion, and its disruption results in defective macroautophagy (Kametaka et al., 1996; George et al., 2000). As expected there was no activation of ALP for nitrogen starved cells in the absence of *ATG5*. The basal level of ALP activity found in control cells (t0) of both W303 and *atg5*Δ strains is probably due to Pho13p, a cytosolic variant of ALP that is constitutively active (Tuleva et al., 1998). Interestingly acetic acid leads to a reduction of this activity after 3h treatment.

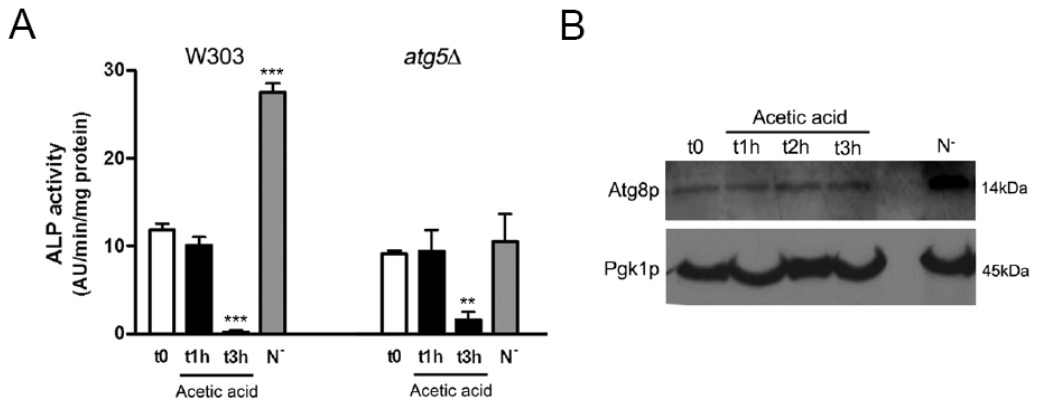

Figure 1. *Acetic acid does not induce autophagy-dependent ALP activation or increase in Atg8p.* (A) ALP activity in *S. cerevisiae* W303 or *atg5*Δ cells (negative control for autophagy induction) carrying a *pho8*Δ60 mutation in replacement of wild-type *PHO8*, after treatment with 180 mM acetic acid for 1 and 3 hours. Nitrogen starved cells (N^-) for 15 hours were used as a positive control for ALP activity. ALP activities are expressed as arbitrary units/min/mg protein. Values are means ± SD of four independent experiments. *** $P< 0.001$, ** $P < 0.01$; unpaired *t*-test. (B) The levels of Atg8p, a protein essential for autophagosome formation are not increased by acetic acid treatment (180 mM) as verified by western blot. Increased accumulation of Atg8p was observed for the nitrogen starved cells (N^-, 15 hours) used again as a positive control.

We also assessed autophagy induction by monitoring the amount of Atg8p. This protein is essential for autophagosome formation and its increase is among the diagnostic markers of autophagy (Kirisako et al., 1999). In accordance, we observed an increase in the amount of Atg8p in nitrogen-starved cells used as a positive control (Fig. 1B). Yet, consistently with the lack of ALP activity, we did not observe an increase in Atg8p during acetic acid treatment (Fig. 1B).

A typical, although not exclusive, morphological alteration of mitochondria during apoptosis, either in higher eukaryotes or yeast, is the change from a tubular network to a punctuate pattern. This mitochondria fragmentation is also observed during autophagy and in this case, it can be prevented by inactivation of *ATG5* (Kissova et al., 2004). In response to acetic acid both W303 cells and *atg5*Δ strain showed a rapid fragmentation of the network into small round units, which persisted for some time until the fluorescence started to diffuse throughout the cell (Fig. 2A). No discrete vacuolar localization was ever observed for these two strains following acetic acid treatment, oppositely to W303 nitrogen-starved cells. During nitrogen starvation W303 cells exhibited an evident green fluorescence inside the vacuoles pointing to the internalization of mitochondria due to autophagy activation (Fig. 2B).

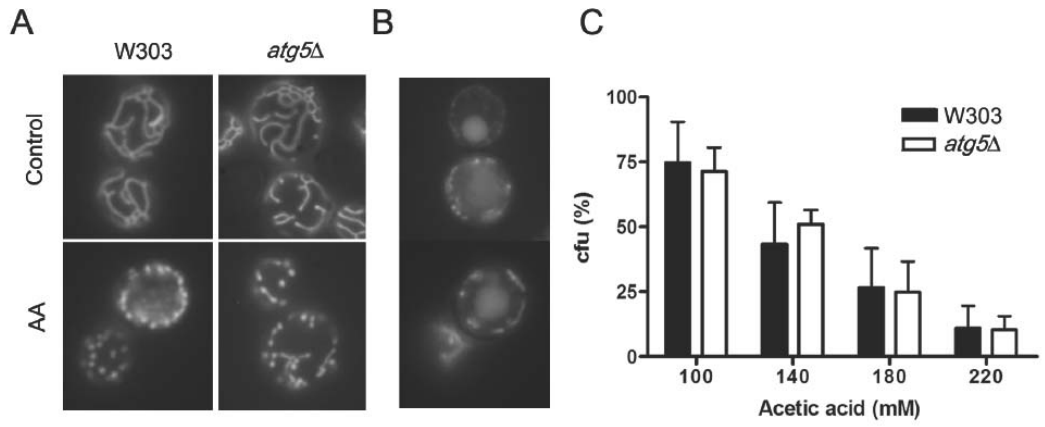

Figure 2. *Acetic acid-induced death is independent on the autophagic protein Atg5p* (A) W303 and *atg5*Δ cells expressing mitochondrial matrix targeted GFP (mtGFP) were treated with 180 mM acetic acid for 30 min. (B) W303-1B cells expressing mtGFP, under nitrogen starvation conditions for 10 h. Bar, 4 μm. (C) Cell survival of *S. cerevisiae* strains W303 and *atg5*Δ. Strains were incubated for 200 min with 100-220 mM acetic acid. Values are means ± SD of five independent experiments; $P > 0.05$ (*atg5*Δ versus W303 cells); two-way ANOVA. Viability (clonogenicity) in response to treatment with acetic acid was also not affected by *ATG5* deletion (Fig. 2C).

As a whole, these results show that the autophagic machinery is not activated during acetic acid-induced apoptosis, and hence, mitochondrial degradation must rely on other processes.

Uth1p is involved in resistance to acetic acid but not in mitochondrial degradation

Two mitochondrial proteins, Uth1p, localized at mitochondrial outer membrane (Kissova et al., 2004) and Aup1p from the intermembrane space (Tal et al., 2007) were described as being dispensable for classical macroautophagy, but required for effective mitophagy in yeast. While Aup1p-mediated degradation depends on the autophagic machinery (Tal et al., 2007), Uth1p mediated degradation can occur even when the autophagic pathway is not functioning (Kissova et al., 2006). As such, we decided to investigate the role of Uth1p on acetic acid-induced mitochondrial degradation. Prior to that, the response to acetic acid of an *uth1*Δ strain regarding loss of cell viability was assessed, and was found to be highly resistant (Fig. 3A). The strain *uth1*Δ was transformed with a mitochondrial matrix-targeted GFP and mitochondrial degradation was assessed by monitoring the loss of GFP fluorescence by flow cytometry. This method allows monitoring mitochondria degradation along time in live cells. Cells with different relative volumes show different levels of fluorescence that may not reflect changes in mitochondrial content. To account for this factor samples were normalized by calculating the ratio of the green fluorescence (FL1, log) by relative size (FS, log).

We observed a significant delay in mitochondrial degradation (Fig. 3B) which however, corresponded to the exact same delay in cell death. That is, for the same cell survival percentage in both W303 and *uth1*Δ strains we obtained the same degradation. We show in Fig. 3C that the mitochondrial degradation obtained for 50% survival for W303 and for *uth1*Δ, and strains were identical. Moreover, deletion of *UTH1* does not affect the number of PI-permeable cells in response to acetic acid (data not shown), unlike what was shown during Bax-induced death (Kissova et al., 2006). It is apparent that although Uth1p affects acetic acid-induced death, it does not influence the degradation of mitochondria.

In Fig. 3C we can also see that *ATG5*-deletion does not significantly affects degradation comparatively to W303 strain. Of note, when *uth1*Δ mutant is treated with acetic acid we observed a small percentage of cells with big mitochondrial spots, as reported before for this strain upon expression of Bax (Kissova et al., 2006) (data not

shown). The most distinct feature of *uth1*Δ strain however, is some resistance (around 20%) to fragmentation of mitochondrial filaments comparing to the W303 strain.

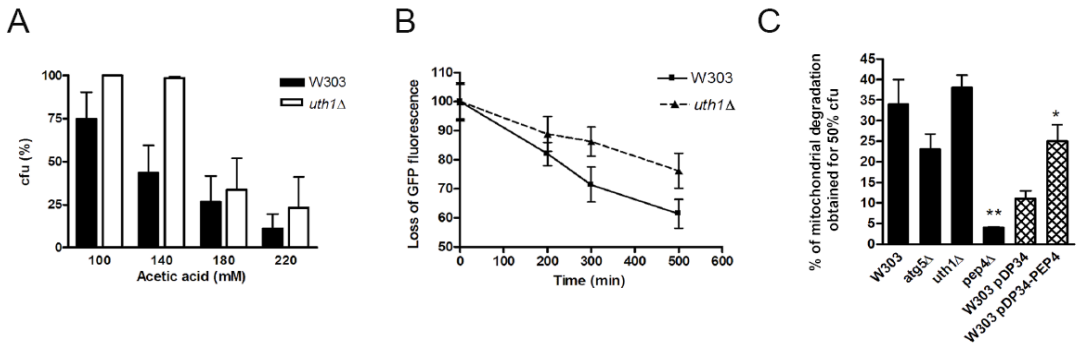

Figure 3. *Uth1p is not involved in acetic acid-induced mitochondrial degradation.* (A) Cell survival of *S. cerevisiae* strains W303 and *uth1*Δ. Strains were incubated for 200 min with 100-220 mM acetic acid. Values are means ± SD of five independent experiments; P > 0.001 (*uth1*Δ versus W303); two-way ANOVA. (B) Assessment of mitochondrial degradation by quantification of mitochondrial matrix targeted GFP disappearance by flow cytometry in strains W303 and *uth1*Δ along time. Values are means ± SD of four independent experiments. P < 0.05 (*uth1*Δ versus W303); two-way ANOVA. (C) Quantification of mitochondrial degradation (as in B) obtained at 50 % cell survival (by cfu counts) for strains W303, *atg5*Δ, *uth1*Δ and *pep4*Δ (black full bars) and W303 pDP34 and W303 pDP34-*PEP4* (white crossed bars).

Pep4p translocates from the vacuole to the cytosol in response to acetic acid

S. cerevisiae Pep4p, or proteinase A, is a pepsin-like aspartic protease found in the yeast vacuole with homology to the human cathepsin D. Pep4p was shown to translocate from the yeast vacuole to the cytosol during hydrogen peroxide- (Mason et al., 2005) and actin cytoskeleton stabilization-induced apoptosis (Gourlay and Ayscough, 2006).

We investigated whether acetic acid could also trigger the release of vacuolar Pep4p. To this end we used a Pep4-EGFP fusion (Mason et al., 2005) which allows visualizing the protein distribution in live cells (Fig. 4). As expected in non-treated healthy cells, the Pep4-EGFP fusion protein localized specifically to the vacuole (Fig. 4). After 60 min treatment with 180 mM acetic acid an extra-vacuolar fluorescence, indicative of the presence of Pep4p in the cytosol, started to be observed in a number of cells (Fig. 4). It was also detected some punctuate staining which co-localises with mitochondria (stained with Mitotracker Red). Yet, this co-localization appeared to be

unspecific, since control cells expressing only EGFP presented identical staining already from the beginning of the treatment with acetic acid (data not shown). This unspecific staining has already been reported in yeast cells undergoing hydrogen peroxide-induced apoptosis after long treatment times (Mason et al., 2005).

Removal of acetic acid after short incubation periods lead to reappearance of the vacuolar staining in cells expressing Pep4-EGFP (data not shown), suggesting that release of Pep4p is not associated to the rupture of the vacuolar membrane. Pep4p release preceded loss of plasma membrane integrity as assessed by propidium iodide (PI) co-staining (Fig. 4). Together, these observations corroborate data obtained in mammalian cells showing that the release of cathepsins from the lysosomes can be selective and reversible, and hence does not result from destruction and deacidification of the lysosomes (Brunk et al., 1995; Bidere et al., 2003).

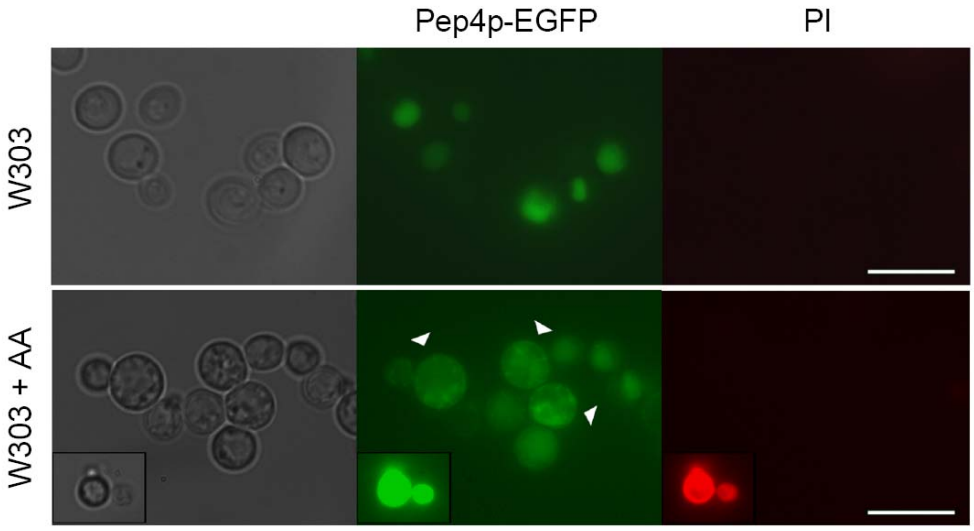

Figure 4. *Pep4p translocates from the vacuole to the cytosol during acetic acid-induced death.* Release of the protease Pep4p from the vacuole due to acetic acid treatment (AA; 180 mM, 60 min) was assessed in living cells using a W303 strain expressing a PEP4-EGFP fusion protein. Upper panels show control cells with the Pep4p-EGFP fluorescence localized in the vacuole (visible in the differential interference contrast (DIC)) and the lower panels show some cells with the PEP4-EGFP fusion already in the cytosol (indicated by the arrow heads). Release of Pep4p precedes loss of plasma membrane integrity as assessed by propidium iodide (PI) staining. In the insert we can see a dead cell with a strong bright unspecific green staining that also stains with PI. Bar, 10 μm.

Pep4p is involved in the late mitochondrial degradation induced by acetic acid

To assess the role of Pep4p in mitochondrial degradation we used a strain disrupted in the gene *PEP4* (Marques et al., 2006) and respective W303 parental strain, and a W303 strain transformed with pDP34 (empty vector, multicopy) or pDP34-*PEP4* (Rupp and Wolf, 1995) to overexpress *PEP4*. Degradation of mitochondria in these strains was assessed by monitoring the loss of matrix-targeted GFP by flow cytometry, as referred above. GFP disappearance was monitored along 500 min treatment with 180 mM acetic acid. An example of a typical histogram obtained after 500 min treatment for each strain is shown along with the quantification of cells which displayed loss of mitochondrial fluorescence (part below the cursor, Fig. 5A). We observe a delay in GFP disappearance in *PEP4*-deleted strain and a slight acceleration in GFP loss when *PEP4* was overexpressed (Fig. 5A).

When treated with acetic acid, *pep4*Δ mutant exhibited, similarly to W303, a very fast (less than 30 min) disorganization of the typical tubular network. However, in the *pep4Δ* mutant the tubular mitochondrial morphology was not replaced by the evenly distributed small spots, but instead by a fewer number of larger spots with the appearance of clustered mitochondria. Fig. 5B illustrates normal (dispersed spots) and abnormal (clustered) mitochondrial morphology. The percentage of cells with this mitochondrial morphology could reach almost 70% of the cells in the *pep4Δ* culture. In accordance, the percentage of clusters in the culture decreased when *PEP4* was overexpressed (Fig. 5C).

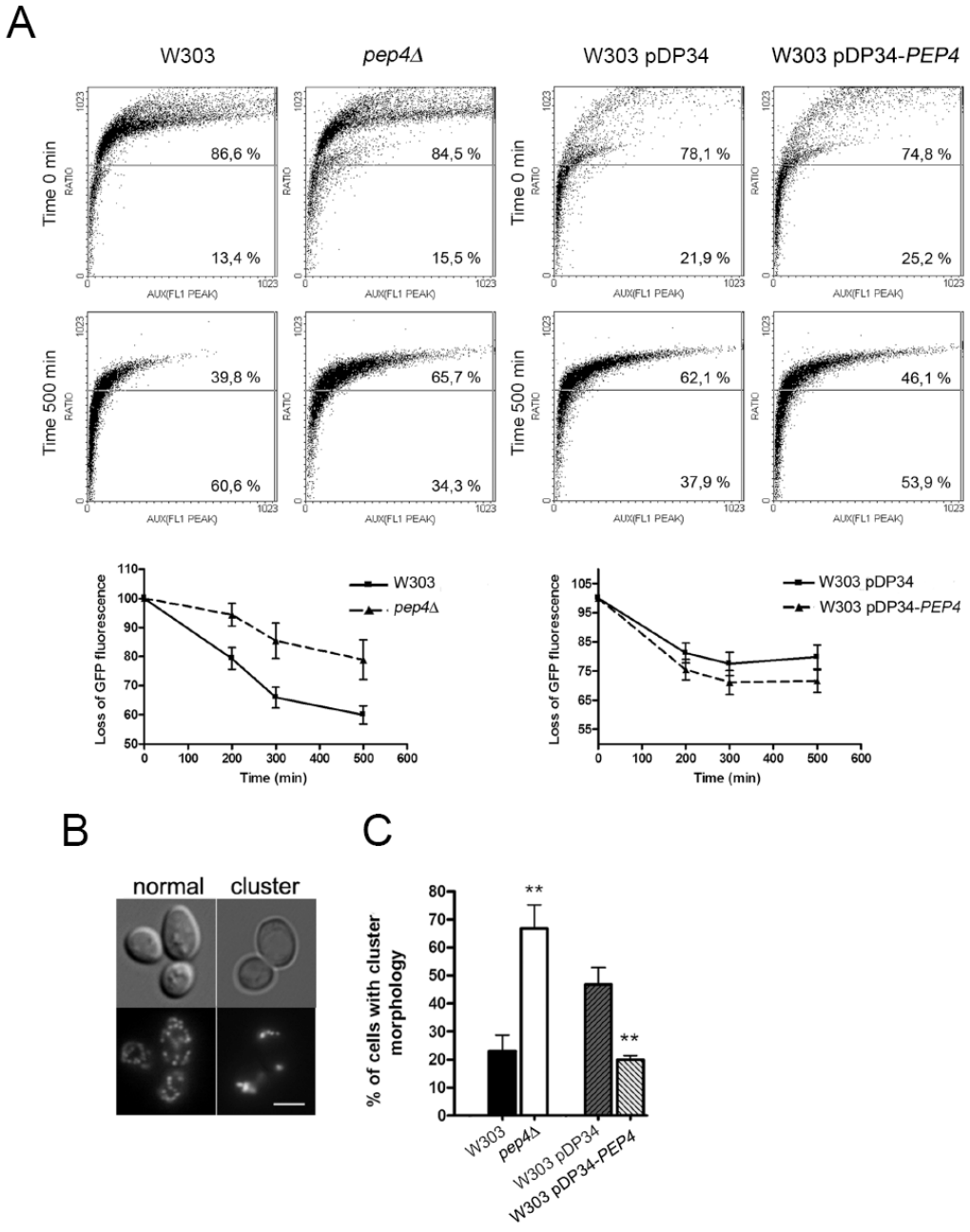

Figure 5. *Pep4p is involved in mitochondrial degradation triggered by acetic acid.* (A) Flow cytometry analysis of W303 and *pep4*Δ, W303 pDP34 and W303 pDP34-*PEP4* strains transformed with a mitochondrial matrix targeted GFP. Loss of GFP fluorescence was used as a measure of mitochondrial degradation. Images show biparametric histograms of the Ratio [FL1 area (log) / FS (log)] versus GFP fluorescence (FL1 Peak). The Ratio value was used to eliminate variations in fluorescence due to cell size. Data represent one of four independent experiments. Quantification of the GFP loss along treatment with acetic acid (180 mM) is shown together. Values are means ± SD of four independent experiments. $P < 0.001$ (*pep4*Δ versus W303), $P < 0.05$ (W303 pDP34-*PEP4* versus W303 pDP34); two-way ANOVA. (B) Absence of *PEP4* leads to an increase in the percentage of cells that after acetic acid treatment display clustered mitochondria. *PEP4* overexpression, in turn, lead to a decrease in the percentage of cells with formation of clustered mitochondria. An example illustrating what was considered normal dispersed spots and clustered morphology after acetic acid treatment (180 mM, 30 min) is shown. (C) Quantification of the percentage of cells displaying the clustered morphology for strains W303, *pep4*Δ, W303 pDP34 and W303 pDP34-*PEP4* after treatment

with acetic acid (180 mM, 60 min). Values are means ± SD of four independent experiments with at least 300 cells counted per sample. $^{**}P < 0.01$; unpaired *t*-test.

Pep4p has a pro-survival role during acetic acid-induced apoptosis

The effect of acetic acid on cell viability in the *PEP4*-deleted and *PEP4*-overexpressing strains was assessed. We observe a decrease in the cell survival in *pep4*Δ comparing to the W303 strain, and an increase in the W303 pDP34-*PEP4* strain comparing to W303 pDP34 (Fig. 6). Together these observations indicate that Pep4p and the ensuing degradation of mitochondria seem to have a pro-survival role during acetic acid-induced apoptosis. These survival data further strengthens the results described above regarding mitochondrial degradation assays. When the loss of GFP fluorescence used to monitor mitochondrial degradation in the different strains was normalised to the same cell survival percentage, the decrease in mitochondrial degradation in the acid-sensitive *pep4*Δ strain and the increase in degradation in the acid-resistant *PEP4*-overexpressing strain are even higher (see Fig. 3C above).

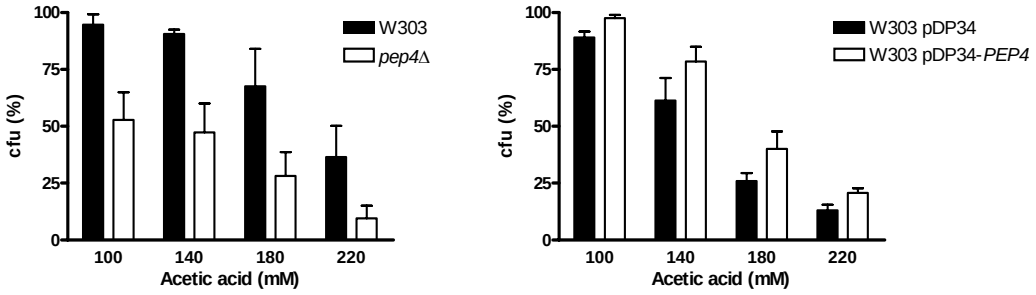

Figure 6. *Pep4p has a pro-survival role during acetic acid-induced apoptosis.* Cell survival of *S. cerevisiae* strains W303 and *pep4*Δ (upper image) and W303 pDP34 and W303 pDP34-*PEP4* (lower image). Strains were incubated for 200 min with 100-220 mM acetic acid. Values are means ± SD of four independent experiments; $P < 0.001$ (*pep4*Δ versus W303 cells), $P < 0.05$ (W303 pDP34 versus W303 pDP34-*PEP4*); two-way ANOVA.

In order to assess if *PEP4*-deletion affected the death process, we quantified by flow cytometry the permeabilization of the plasma membrane as a reliable assay to detect necrotic death. During acetic acid treatment (180 mM) *pep4*Δ strain showed an increase in the number of cells with disrupted plasma membrane, as assessed by PI staining. Fig. 7 show the histograms obtained for W303 and *pep4*Δ strains after 200 and 500 min treatment with acetic acid. No PI-permeable cells were detected for time 0 (data

not shown). The increase in the percentage of PI-permeable cells obtained for *pep4*Δ strain was not due to the fact that this mutant displays death percentages higher than W303 cells in response to acetic acid. For example, for the data presented in Fig. 7 we obtained for *pep4*Δ strain, after 200 min treatment, 34% of cell survival and 36.4% of PI-permeable cells, while for W303 at 500 min we obtained 5% survival and only 28.2% PI-permeable cells. We also observed that the number of PI-permeable cells in *pep4*Δ strain also increases when this strain enters stationary phase, even without any treatment (data not shown).

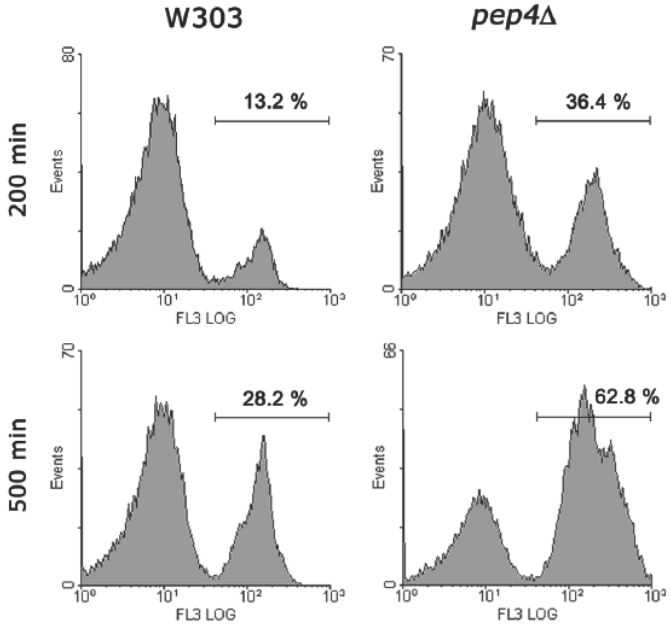

Figure 7. pep4*Δ strain shows an increase in the percentage of cells with loss of plasma membrane permeability*. Flow cytometric analysis of *S. cerevisiae* strains W303 and *pep4*Δ treated with 180 mM acetic acid for 200 and 500 min and incubated with PI. Images show biparametric histograms of PI fluorescence (FL3 area (log)). Data represent one of four independent experiments.

Absence of AAC proteins during acetic acid-induced apoptosis originates mitochondrial clusters

The selective removal of mitochondria after apoptosis induction has often been reported in mammalian cells (Tolkovsky *et al*., 2002). How mitochondria are signalized for destruction is not known so far. Yet, mitochondrial permeability transition (MPT) has been pointed as a possible trigger of such selective degradation in mammalian cells (Lemaster, 1998).

Because we have previously reported that absence of the inner mitochondrial membrane ADP/ATP carrier (AAC) impairs mitochondrial outer membrane permeabilization and cytochrome *c* release (Pereira et al., 2007) we questioned whether these proteins could have a role in the onset of mitochondrial degradation. We started to characterize mitochondrial alterations in AAC-deleted cells upon acetic acid-induced apoptosis.

When treated with acetic acid, *aac1/2/3*Δ mutant (lacking the three yeast isoforms of the ATP/ADP carrier) and transformed with mtGFP presented, like *pep4*Δ strain, a high percentage of cells displaying the mitochondrial clustered morphology (Fig. 8A). We decided to further investigate if this altered morphology in the *aac1/2/3*Δ mutant corresponded to dysfunctional mitochondria. We could observe that in most *aac1/2/3*Δ acetic acid-treated cells mitochondrial clusters were still able to accumulate the membrane potential-sensitive probe $DiOC_6(3)$ or Mitotracker Red CMXRos, indicating that clustered mitochondria were still functional. Fig. 8A shows cells stained with Mitotracker Red CMXRos, which allowed co-visualization of mitochondria and mitochondrial functionality. For longer times of treatment both strains begun to lose mitochondrial membrane potential. However, while W303 strain displayed a significant transient hyperpolarization between 100 and 150 min acetic acid treatment, the same did not occur for the *aac1/2/3*Δ mutant (Fig. 8B). Moreover, to discard the possibility that mitochondrial morphology in *aac1/2/3*Δ mutant could be a consequence of low mitochondrial energy levels (AAC proteins exchange cytosolic ADP for mitochondrial synthesized ATP across the mitochondrial inner membrane), we monitored mitochondrial morphology changes along death induced by acetic acid in an *atp2Δ* strain. This mutant is deleted for subunit β of the ATPase complex. We could confirm that the spots originated from the fragmentation of the mitochondrial network in the *atp2*Δ strain were of normal size and similar to those observed in W303 strain (Fig. 8C). Quantification along time of the mitochondrial filaments, spots and clusters observed along treatment with acetic acid in W303 and *aac1/2/3*Δ strains was performed (Fig. 8D). This figure evidences the distinct mitochondrial morphology pattern exhibited by W303 and *aac1/2/3*Δ strains and the significant increase in clustered mitochondria in the latter strain. Formation of mitochondrial aggregates in the *aac1/2/3*Δ strain was also observed during hydrogen peroxide induced-apoptosis (Fig. 8E), indicating that this peculiar morphology is not an effect restricted to acetic acid treatment. Noteworthy, the

absence of AAC proteins also hindered MOMP and cytochrome *c* release in cell undergoing apoptosis induced by H_2O_2 (Pereira et al., 2007).

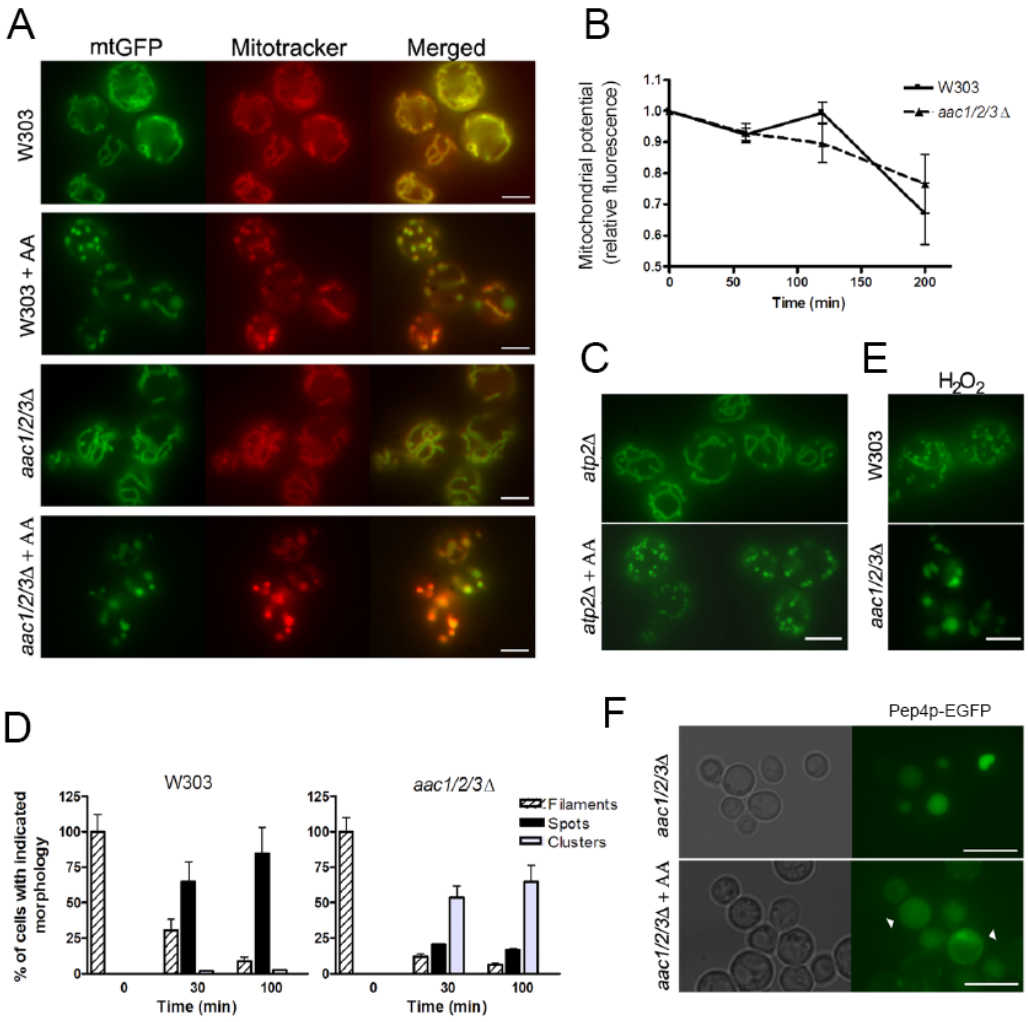

Figure 8. aac1/2/3*Δ strain shows clustered mitochondria upon acetic acid treatment, without being affected in translocation of Pep4p to the cytosol.* (A) Mitochondrial membrane potential in *S. cerevisiae* W303 and *aac1/2/3*Δ cells, non-treated and treated with acetic acid (AA; 180 mM, 30 min), was examined by Mito-Tracker Red staining (middle panels) and compared with mitochondrial matrix targeted GFP (left panels) in the merged images (right panels). At early times of treatment the majority of the clusters observed in *aac1/2/3*Δ retain the mitochondrial membrane potential similarly to W303 mitochondria. Bar, 4 μm.
(B) Quantification of mitochondrial membrane potential (relative fluorescence values) for strains W303 and *aac1/2/3*Δ along 200 min treatment with acetic acid (180 mM) by flow cytometry using the probe $DiOC_6(3)$.Fluorescence at t0 was considered as 100%. For time 120 min mitochondrial potential for W303 and *aac1/2/3*Δ strain is statistically different (**P* < 0.05; unpaired *t*-test).
(C) *atp2*Δ strain non-treated and treated with acetic acid (AA; 180 mM, 30 min). *atp2*Δ cells do not present cluster formation discarding the hypothesis that this morphology alteration is due to absence of oxidative phosporylation. Bar, 4 μm.

(D) Quantification of the percentage of cells containing the different types of mitochondrial morphology observed after acetic acid (180 mM) treatment (filaments, dispersed spots and clusters) for W303 and *aac1/2/3*Δ strain. Values are means ± SD of five independent experiments (at least 200 cells were counted for each time point).
(E) W303 and *aac1/2/3*Δ cells treated with H_2O_2 (3 mM, 30 min). H_2O_2 treatment also leads to formation of mitochondria aggregates in *aac1/2/3*Δ. Bar, 4 μm.
(F) Release of the protease Pep4p from the vacuole due to acetic acid treatment (AA; 180 mM, 60 min) was assessed in *aac1/2/3*Δ cells expressing a PEP4-EGFP fusion protein. Release of PEP4-EGFP to the cytosol (indicated by the arrow heads) was not affected by the absence of AAC proteins. Bar, 10 μm.

Because absence of Pep4p also originated clustered mitochondrial morphology identical to that observed for the *aac1/2/3*Δ mutant we investigate whether the release of Pep4p from the vacuole was affected in this strain. For such purpose the *aac1/2/3*Δ strain was transformed with the Pep4p-EGFP fusion protein. In *aac1/2/3*Δ cells without acetic acid treatment Pep4p-EGFP localizes correctly to the vacuole (Fig. 8F). Unexpectedly, upon acetic acid treatment, the Pep4p-EGFP fusion protein translocates to the cytosol in a similar extent as observed previously for the wild-type (Fig. 8F). This data indicates that mitochondrial aggregation in the *aac1/2/3*Δ strain is independent on the Pep4p release from the vacuole.

Depolymerization of actin filaments disrupts the mitochondrial clusters formed in the absence of AAC proteins

Mitochondria in yeast associate with actin filaments, and depolymerization of these filaments alters the normal mitochondrial morphology giving origin to short tubular or small spherical structures (Boldogh et al., 1998). Latrunculin-A (Lat-A) is a drug used for rapid depolymerization of F-actin (Spector et al., 1983). Mitochondrial network was broken upon 30 min (37 °C) treatment with 0.25 mM Lat-A (dissolved in DMSO) in both W303 (data not shown) and *aac1/2/3Δ* mutant cells (Fig. 9B) while the same cells with equal volume of DMSO alone presented no alterations in the mitochondrial network organization (Fig. 9A). Lat-A (Fig. 9D) or the solvent DMSO (Fig. 9C) were added to acetic acid-treated *aac1/2/3Δ* cultures (180 mM, 30 min). The percentage of *aac1/2/3*Δ cells containing clustered mitochondria decreased to about one half (46.4% decrease) while the number of cells containing small spots of mitochondria increased. In addition, incubation of the cells with Lat-A previously to acetic acid

treatment also prevented formation of the clusters. Together these results reinforce the idea that clusters are composed of aggregates of mitochondria and show that actin filaments are responsible for the formation and maintenance of such clusters.

Formation of these clusters is a reversible event. Until 30 min treatments, removal of acetic acid allowed an almost fully recovery of the tubular shape of the mitochondrial network both for the W303 and *aac1/2/3Δ* strains, though the recovery for the latter strain was slower than for the W303 strain (Fig. 9B). For longer periods of incubation with the acid, the ability of both strains to recover normal mitochondrial morphology declined, and this was even more notorious in the *aac1/2/3Δ* mutant, feasibly due to its altered mitochondrial morphology. While the localization of mitochondrial spots never coincided with the vacuole as identified by DIC field microscopy, the recovery assays allowed us to strengthen the idea that the clustered mitochondria observed for *aac1/2/3Δ* strain are not internalised by the vacuole and most likely reside in the cytosol, hence allowing recovery of their filamentous shape.

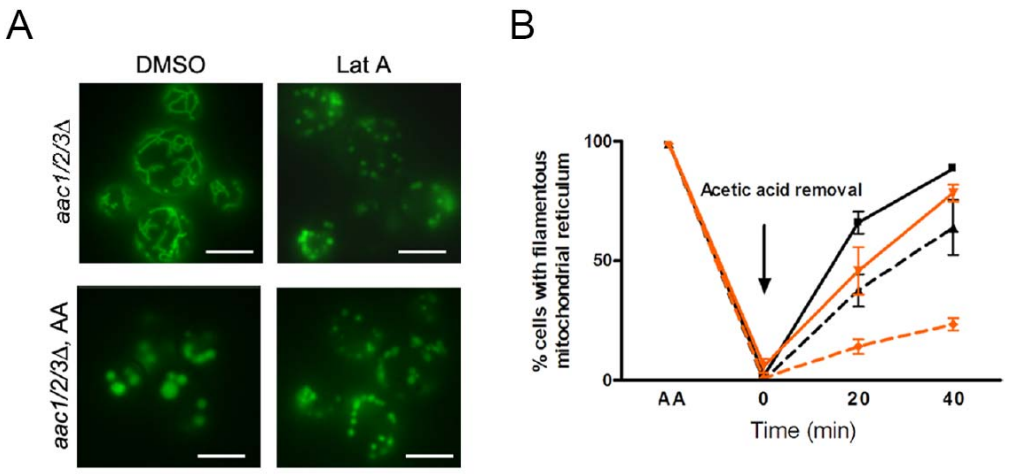

Figure 9. *Mitochondrial clusters in Δ*aac1/2/3 *mutant are disrupted by treatment with Lat A, an actin depolimerizing agent.* (A) *aac1/2/3*Δ cells with mitochondrial matrix targeted GFP were grown until mid exponential phase and non-treated or treated with acetic acid (180 mM, 30 min). DMSO alone (the vehicle) or 0.25 mM Latrunculin-A (Lat-A) were added to non treated (upper panels) or acetic acid (AA) treated cells (lower panels) and incubated for 30 min at 37 °C before visualization. Bar, 5 μm. (B) At early times of treatment with acetic acid *aac1/2/3*Δ clustered mitochondria can be reversed to the original filamentous structures after acetic acid removal, although not as efficiently as the normal fragmented wild-type mitochondria. Cells were treated with 80 mM acetic acid for 30 or 200 min, washed once and incubated in media without acetic acid. Samples were taken after 20 or 40 min of recovery in the media without the acid. W303 is represented by the black lines and *aac1/2/3*Δ by the orange lines; full lines correspond to 30 of min and dotted lines correspond to 200 min of acetic acid treatment.

Absence of AAC proteins leads to a decrease in the degradation of mitochondria

The possibility that the clustered mitochondrial morphology of the *aac1/2/3*Δ strain, like in *pep4*Δ strain, could be associated to a lower degradation of mitochondria was ascertained. Mitochondrial degradation in *aac1/2/3*Δ cells was assessed again by monitoring loss of GFP fluorescence by flow cytometry. We observed that for the same cell survival percentage *aac1/2/3*Δ mutant cells show about half of the degradation obtained for the wild-type (Fig. 10A). Still, absence of AAC proteins affected degradation less severely than *PEP4*-deletion (Fig. 3C).

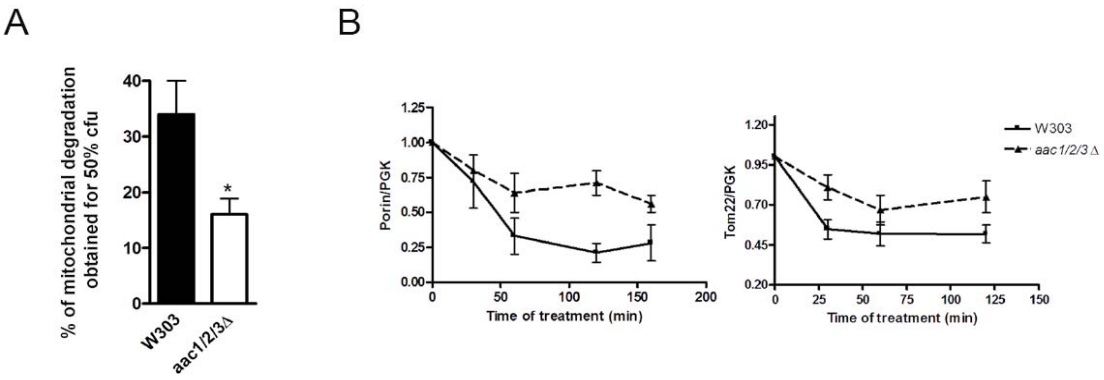

Figure 10. *Mitochondrial degradation is impaired in the absence of AAC proteins.* (A) Flow cytometric quantification of mitochondrial degradation by loss of GFP fluorescence for W303 and *aac1/2/3*Δ mutant expressing a mitochondrial matrix-targeted GFP, after min treatment with mM acetic acid. Values are means ± SD of five independent experiments. **P* < 0.05; unpaired *t*-test.
(B) Quantification of mitochondrial degradation by Western blot of W303 (full line) and *aac1/2/3*Δ mutant (dot line) in cell lysates prepared at the indicated times after treatment with 180 mM acetic acid. Cytosolic phosphoglycerate kinase (Pgk1p) level was used to normalize protein amount loaded on the gel. Values are means ± SD of three independent experiments; P < 0.001 for Porin and P < 0.05 for Tom22 (*aac1/2/3*Δ versus W303 cells); two-way ANOVA.

The GFP fluorescence method used to monitor mitochondrial degradation was performed with cells grown in synthetic media instead of rich media. Under these conditions, we observed a decrease in *aac1/2/3*Δ resistance to acetic acid (data not shown). As such, we also assessed mitochondrial degradation by western blot using cells treated with acetic acid in rich medium. In addition, this method allowed to assess specifically the degradation of outer mitochondrial proteins since we previously observed that in *aac1/2/3*Δ mutant acetic acid triggers a selective fast degradation of cytochrome *c* together with some other inner mitochondrial membrane proteins (Pereira

et al., 2007). Western blot of total protein extracts collected at increasing times following acetic acid treatment showed for *aac1/2/3*Δ strain, in comparison to W303, a delayed degradation of the outer mitochondrial membrane proteins Por1p and Tom22p (Fig. 10B). These results support our interpretation that AAC proteins, besides Pep4p, are also required for effective mitochondrial degradation. In addition, *aac1/2/3*Δ cells showed no differences, comparatively with W303, regarding plasma membrane integrity, as assessed by PI staining (Pereira et al., 2007).

Discussion

Mitochondrial degradation has been shown to occur in a number of systems following apoptotic induction and usually involves an autophagic process. We demonstrate that during acetic acid treatment, among other evidences, there is no uptake of proteins from the cytosol to the vacuole or an increase in the mature form of Atg8p, events typically observed during autophagy induction. Moreover we show that though Uth1p promotes acetic acid-induced apoptosis it has no effect on mitochondrial degradation. These data support our interpretation that autophagy or Uth1p-dependent mitophagy are not active in cells undergoing acetic acid-induced apoptosis, and hence that mitochondrial degradation must rely on other process.

Recently, Pep4p was shown to be released from the vacuole during hydrogen peroxide- (Mason et al., 2005) or actin stabilization-induced apoptosis (Gourlay and Ayscough, 2006). In the first case, it was also shown that Pep4p, like its mammalian orthologue cathepsin D (Tang and Wong, 1987), is released without major vacuolar rupture and is involved in nucleoporins degradation (Mason et al., 2005).

We observed that Pep4p is also released from the vacuole during acetic acid-induced apoptosis and has an important role in mitochondrial degradation during the apoptotic process. Whether Pep4p acts directly on mitochondria or activates other proteases remains to be elucidated. In contrast to what we observe for acetic acid treatment, *pep4*Δ strain shows a similar resistance as the wild-type strain when death is induced by H_2O_2 (Mason et al., 2005) while it exhibits a shortened lifespan during chronological ageing (Marques et al., 2006). We demonstrate that deletion of Pep4p and the consequent impairment in mitochondrial degradation enhances acetic acid-induced death, while the opposite phenotype is observed for the *PEP4* overexpression. Therefore,

it is apparent that the process of removal of damaged mitochondria has a protective role in acetic acid-treated cells. A similar protective role for mitochondrial degradation has been suggested for cells in prolonged stationary phase cultures (Tal et al., 2007). In this case although mitochondrial degradation is achieved by an autophagic-dependent process, impediment of this process (due to *AUP1* deletion) also leads to an increase in cell death. In Bax induced-death, impairment of mitochondrial uptake by the vacuole leads to a higher resistance. However, this is due to the switch from a regulated to a necrotic death occurring at a slower rate. Induction of mitophagy was also shown to occur due to osmotic swelling caused by shutdown of Mdm38p (Nowikovsky et al., 2007). In this case mitophagy has no effect in exponential cells, while in stationary phase cells results in an increase in cell death (Nowikovsky et al., 2007). Since, as referred above, in stationary phase cells there is already an ongoing process of mitochondrial removal (Tal et al., 2007), the addition of another degradation trigger may overcome a given threshold converting a process with a protective role into one with a lethal function. This concept is widely spread in mammalian cells concerning the role of autophagy in cell death (Maiuri et al., 2007). That is, a certain degree of intracellular degradation protects cells, but if it becomes too much leads to cell death. Several hypotheses have been raised sustaining the cellular protection provided by mitochondrial degradation, as already referred in the introduction. From our results we conclude as unlikely, the possibility that protection could be afforded by the removal of ROS producing mitochondria. In fact, in the degradation resistant *aac1/2/3*Δ mutant mitochondria produce a similar amount of ROS as the wild-type and yet cells do not have a higher death percentage (Pereira et al., 2007). The alternative hypothesis that mitochondrial degradation could protect from the release of pro-apoptotic proteins is also improbable since degradation occurs after mitochondrial permeabilization and cytochrome *c* release (Fig. 11). Our results sustain the view that removal of damaged mitochondria would avoid high ATP consumption by these organelles (Gellerich et al., 2004). This interpretation is supported by the observation that acetic acid treatment leads to a decrease in ATP (our unpublished data) and that the mitochondrial degradation-resistant *pep4*Δ mutant seems to switch to a less ATP demanding necrotic form of death (Tsujimoto, 1997) evidenced by the high number of PI-permeable cells in response to acetic acid.

How mitochondria are targeted for degradation is unclear. Since in mammalian cells it was reported that the selective removal of mitochondria followed MPT and was

inhibited by MPT inhibitors like cyclosporin A (Lemasters et al., 1998; Elmore et al., 2001; Xue et al., 2001; Rodriguez-Enriquez et al., 2006) it was hypothesised that in mammalian cells MPT is the, or one of the triggers. Using mitochondria-addressed GFP, we observed for *aac1/2/3*Δ mutant a similar mitochondrial aggregation pattern as for *pep4*Δ mutant, and it was also associated to a decrease in degradation of mitochondria. For the *aac1/2/3*Δ mutant the clustering of mitochondria seems to be dependent on actin since disrupting actin also partly disrupts clusters. We showed previously that in the absence of AAC proteins mitochondrial outer membrane permeabilization (MOMP) and cytochrome *c* release induced by acetic acid are impaired (Pereira et al., 2007). Thus the associated delay in degradation of *aac1/2/3*Δ mutant mitochondria may indicate that MOMP or associated event in yeast is the, or one of the trigger(s) of acetic acid-induced mitochondrial removal as suggested for the autophagic mitochondrial removal in mammals. Which is the precise event associated to MOMP that triggers degradation needs further research, but what seems clear from our data and others (Nowikovsky et al., 2007) is that mitochondrial depolarization is not the trigger. Because AAC-deleted cells do not show the transient hyperpolarization detected in the wild-type strain it may be a good trigger candidate since mitochondrial degradation starts to occur after it (Fig. 11).

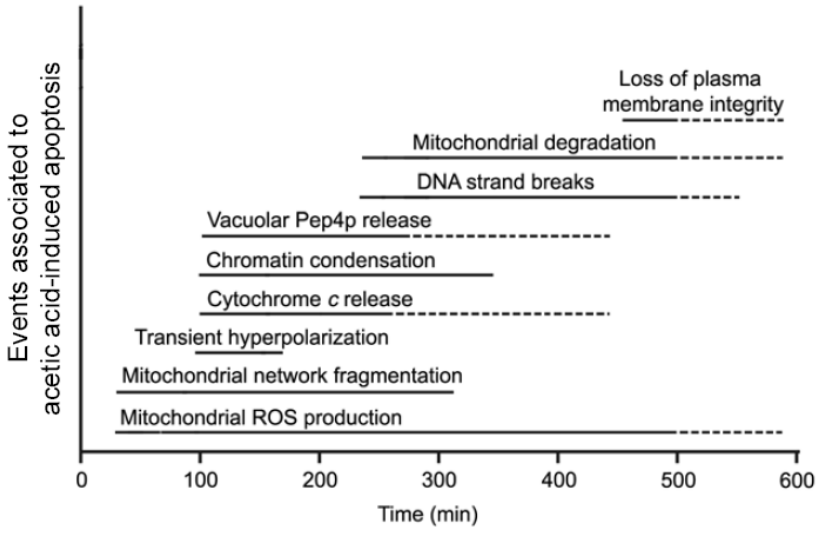

Figure 11- *Temporal order (sequence) of events occurring during acetic acid induced death in W303 strain*. Kinetics for ROS production, cytochrome *c* release, chromatin condensation, DNA strand break formation and loss of plasma membrane integrity are adapted from Pereira et al. (Pereira et al., 2007) while the remainder events result from data obtained with this work. Vacuolar Pep4p release

and mitochondrial cytochrome *c* release occur in the same time period and before mitochondrial degradation.

In addition, looking at the temporal order of the cell events occurring during acetic acid-induced death in wild-type (Fig. 11) we can observe that mitochondria and vacuole destabilization, measured by cytochrome *c* and Pep4p release, respectively, are simultaneous or very close, and that mitochondria affect their own degradation at a step posterior to Pep4p release since this protein is normally released in AAC-deleted cells.

In mammalian cells the orthologue of Pep4p, cathepsin D was shown to have a role in cell death by triggering mitochondrial dysfunction and subsequent release of mitochondrial proteins, although some studies show an inhibitory role for cathepsin D in apoptosis (Roberg et al., 1999; Bidere et al., 2003; Boya et al., 2003; Jaattela et al., 2004; Chwieralski et al., 2006; Liaudet-Coopman et al., 2006; Minarowska et al., 2007). Yet a role for cathepsin D in mitochondria degradation in mammals has not been assessed so far.

For a long time lysosomes, the mammalian organelle akin to the yeast vacuole, were considered to be only involved in necrotic cell death. Increasing evidence indicate that lysosomes communicate with mitochondria through selective leakage of proteins activating some forms of apoptotic cell death, while necrosis follows a major lysosomal breakdown (for reviews see (Kroemer et al., 2007; Terman et al., 2007)). Herein we demonstrate that, in yeast cells undergoing apoptosis, the vacuole communicates with mitochondria (Fig. 12). The crosstalk between these two organelles involves the release of a protease engaged in the removal of mitochondria rather than unspecific vacuolar permeabilization, and appears to assure the prosecution of death through an apoptotic pathway.

In summary, while relevant differences in cellular processes show to subsist between yeast and mammalian cells, our study opens new perspectives for an enhanced understanding of apoptosis by identifying a novel role for AAC proteins and Pep4p in yeast. Because of the importance of lysosomes in mammalian cell death is increasing, our study further reinforces the use of yeast as a valuable model of programmed cell death. Finally, since the accumulation of damaged mitochondria is associated to different human diseases the deeper understanding of how mitochondria degradation is activated and regulated during apoptosis can unravel novel attractive targets for the development of apoptosis-based therapies.

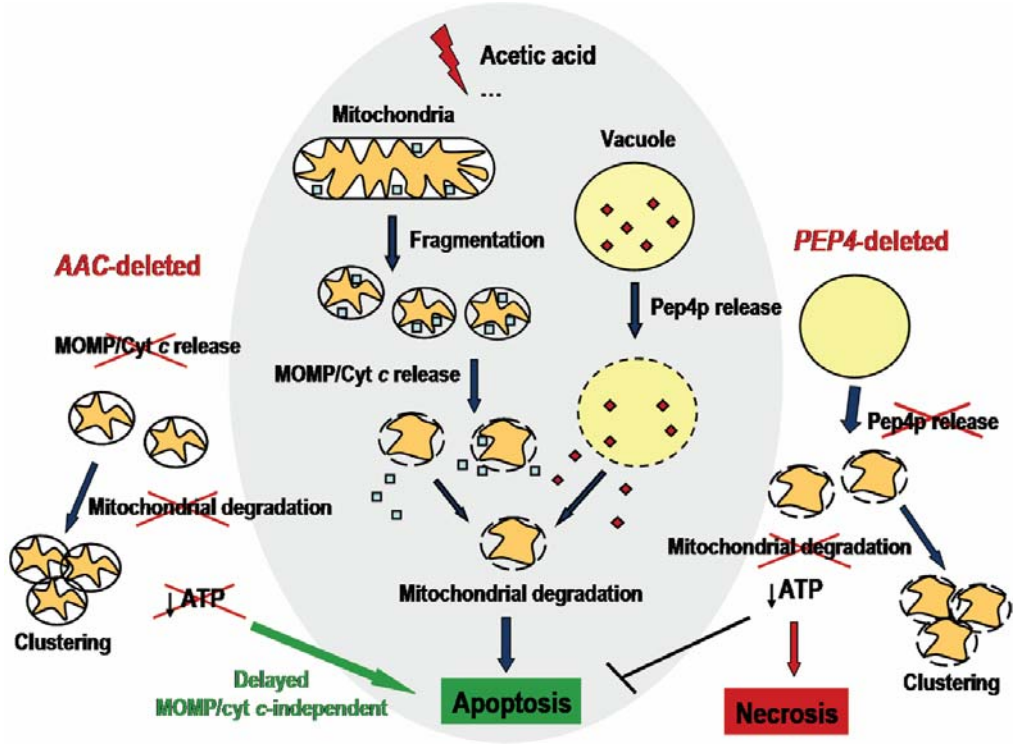

Figure 12- *Working model proposed for the involvement of mitochondrial degradation in the apoptotic death.* As depicted in the scheme MOMP/ cytochrome *c* release occur normally in the wild-type strain as part of the apoptotic cascade. Afterwards, mitochondria are degraded impeding energy depletion caused by mitochondrial dysfunction and assuring in this way the progression of death through an apoptotic pathway. For the AAC-deleted strain absence of MOMP/cytochrome *c* release (Pereira et al., 2007) hinders mitochondrial degradation suggesting the involvement of these events in the signalization of doomed mitochondria. The previous observation that cytochrome *c* and Atp2p, a subunit of the ATPsynthase are degraded in the AAC-deleted mutant (Pereira et al., 2007) may also contribute to avoid ATP consumption by the ATPsynthase predicted to reverse its activity by the collapse of the proton gradient across the inner mitochondrial membrane. Non-degraded mitochondria cluster in the cytosol, and this clustering is dependent on the actin cytoskeleton. Despite impairment of MOMP/cytochrome *c* release, apoptosis still occurs in the AAC-deleted strain, although delayed.
In the PEP4-deleted strain, the absence of this protease strongly affects mitochondrial degradation that also aggregate in the cytosol. These mitochondria clusters are ATP consumers and extinguish the ATP supplies required for apoptosis progression resulting in a necrotic form of cell death.

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Chapter 5

Por1p contributes to mitochondrial morphology and inhibits AAC-mediated apoptosis in yeast

Por1p contributes to mitochondrial morphology and inhibits AAC-mediated apoptosis in yeast

Abstract

During the early stages of apoptotic cell death mitochondrial network fragments to small roundish dispersed spots. Remodelling of mitochondrial morphology during apoptosis is controlled by the proteins responsible for this process under physiological conditions which seem to be able to positively and negatively regulate the death process both in mammals and in yeast.

Here we demonstrate that absence of Por1p, the major of the two VDAC isoforms in yeast, shown to enhance acetic acid-induced apoptosis, also affects mitochondrial morphology in non-treated cells. In the absence of Por1p mitochondria reticulum is fragmented and often aggregated. In addition, *por1*Δ mutant exhibits a high mitochondrial membrane potential ($\Delta\Psi_m$) that is however, unrelated to the fragmentation process, since an *aac1/2/3*Δ strain also shows an elevated $\Delta\Psi_m$ and displays a normal mitochondrial network. Por2p, the second isoform of VDAC, seems to have the opposite function to Por1p concerning mitochondrial morphology since its deletion slightly increases the extent of the branching of the mitochondrial network. *por2*Δ strain exhibits normal $\Delta\Psi_m$ and it does not affect the course of acetic acid-induced death. Moreover, both a ρ^0 mutation and deletion of AAC proteins rescue the sensitive phenotype of *por1*Δ mutant to apoptosis but not the mitochondrial fragmentation phenotype, dissociating these two events. Since the pro-survival role of Por1p is dependent on the presence of AAC proteins these must share the same apoptotic pathway.

Introduction

During proliferation and budding of *S. cerevisiae*, the efficient transport of mitochondria and other organelles into daughter cells is a critical process. Mutational analyses of yeast cells with aberrant distribution and morphology of organelles led to the discovery of a number of genes involved in the regulation of mitochondrial morphology. Among these, the proteins Dnm1 (Drp1 in mammals), Mdv1/Net2p and Fis1p form a complex involved in fission, and Fzo1p and Mgm1p (Opa1in mammals) are required for mitochondrial fusion (Mozdy et al., 2000; Tieu and Nunnari, 2000; Shaw and Nunnari, 2002; Cerveny and Jensen, 2003). In addition, several other proteins participate with dynamins in mitochondrial morphogenesis (reviewed in Hoppins et al., 2007).

During apoptotic cell death, mitochondrial reticulum becomes fragmented due to the action, although not exclusively, of the proteins involved in the physiological control of fission/fusion processes. Similarly to what occurs in mammalian cells, components of the mitochondrial morphogenesis machinery seem to be able to positively and negatively regulate apoptosis in yeast (Fannjiang et al., 2004; Kitagaki et al., 2007).

In mammalian cells mitochondrial outer membrane permeabilization (MOMP) is a decisive event in apoptosis leading to the release of apoptotic factors that activate caspases and cellular death. One of the mechanisms responsible for MOMP has been attributed to the opening of the permeability transition pore (PTP), an inner membrane large unselective channel that, when in the open state, should lead to large amplitude swelling of the matrix causing rupture of the outer mitochondrial membrane. The core components attributed to the mammalian PTP, (ANT, Adenine Nucleotide Translocator; VDAC, Voltage Dependent Annion Channel; and cyclophilin D) are conserved in yeast. As presented in chapter 2 absence of AAC proteins (yeast orthologues of ANT) impairs the mitochondrial outer membrane permeabilization and release of cytochrome *c* (Pereira et al., 2007). By hypothesis it can also impair Nuc1p release (orthologue of mammalian EndoG), since deletion of AAC proteins abolishes the sensitizing effect of *NUC1* overexpression in response to H_2O_2 (Buttner et al., 2007). Yeast also possesses two VDAC genes, *POR1* and *POR2*, with *POR1* coding the major isoform (Blachly-Dyson et al., 1997). Deletion of *POR1* rendered cells more sensitive to some stimuli like acetic acid or H_2O_2 (Pereira et al., 2007) and more resistant to others (Jacotot et al., 2000; Liang and Zhou, 2007).

Here, we show that Por1p and Por2p are required for the maintenance of mitochondrial morphology in normal, healthy cells. *por1*Δ mutant shows a high percentage of cells with fragmented mitochondrial reticulum while a *por2*Δ strain shows a slight increase in the extent of the branching. Whereas alterations in mitochondrial membrane potential ($\Delta\Psi_m$) in *por1*Δ mutant or in respiratory deficient strains are associated to fragmentation of the mitochondrial network, no causal relation can be established between both cell events since an *aac1/2/3*Δ strain exhibits high $\Delta\Psi_m$ and normal filamentous mitochondrial reticulum. Furthermore, absence of AAC proteins in a *por1*Δ strain leads to a reversion of the *por1*Δ sensitive phenotype to acetic acid, suggesting Por1p may act through inhibiting AAC. Yet, Por1p inhibition of the death process and contribution to mitochondrial morphology may be unrelated processes since absence of AAC proteins does not reverse the mitochondrial-fragmented morphology of *por1*Δ mutant.

Materials and Methods

Strains and plasmids

The following *Saccharomyces cerevisia*e strains were used in this study: BY4742 (*MATα his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0), *por1*Δ (BY4742 *por1*Δ::*KanMX4*) and *por2*Δ (BY4742 *por2*Δ::*KanMX4*) from the gene deletion library (EUROSCARF, Frankfurt, Germany). W303-1B (*MATa, ade2, his3, leu2, trp1, ura3, can1*); W303-1A (*MATα, ade2, his3, leu2, trp1, ura3, can1*) JL1-3Δ2Δ3 (W303-1A; *aac1::LEU2,* Δ*aac2::HIS3,* Δ*aac3::URA3*) (Postis et al., 2005); W303-1B *por1*Δ::*KanMX4* (Pereira et al., 2007); *por1*Δ*aac1/2/3*Δ (JL1-3Δ2Δ3; *por1*Δ::*KanMX4*). Strain *por1*Δ*aac1/2/3*Δ was constructed from JL1-3Δ2Δ3 by direct transformation with PCR products amplified from genomic DNA from the EUROSCARF deletion strain *por1*Δ::kanMX4 with subsequent selection in geneticin media and confirmation by PCR (Cardoso, T., unpublished results). Mitochondrial respiratory deficient (ρ^0) mutants BY4742-ρ^0 and BY4742 *por1*Δ-ρ^0 were generated by a two-step growth of the parental strains on YPD plates supplemented with ethidium bromide (40 μg/ml) (Fox et al., 1991). Cells unable to grow on medium containing a non-fermentable carbon source (2% glycerol) were identified by replica

plating and subsequently by observation of cells after 4,6-diamidino-2-phenylindole (DAPI) staining.

Growth conditions and treatments

Yeast cells were grown in rich media [YEPD: 1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (w/v) glucose] or synthetic complete media [SC: 0.67% (w/v) Bacto-yeast nitrogen base w/o amino acids, 2% (w/v) galactose and 0.2% (w/v) Dropout mix] lacking the appropriate amino acids until exponential phase in an orbital shaker, at 26 ºC, 160 rpm. The strains were harvested and suspended (10^7 cells/ml) in the treatment medium consisting on YEPD or SC at pH 3.0 (set with HCl), containing the appropriate amounts of acetic acid and incubated as described for the growth conditions.

For the assays with the VDAC inhibitor *4´-diisothiocyano-2,2´-disulfonic acid stilbene* (DIDS), cells grown overnight were harvested and incubated in the growth medium for 5 min with 0.5 mM of DIDS (Shafir et al., 1998).

Viability (clonogenicity) was determined by counting colony forming units (cfu) in YPD agar plates after 2-3 days incubation at 30 ˚C.

Fluorescence microscopy

For fluorescent microscopy experiments strains were transformed with the plasmid YX232-mtGFP (Westermann and Neupert, 2000) using the lithium acetate method (Ito et al., 1983). Cells were grown and treated as described in the growth conditions, and immobilized in the slides by adding 0.5% (w/v) agar prior to microscopy.

When used, MitoTracker Red CMXRos (Molecular Probes) was added to the culture medium at a final concentration of 0.4 µg/ml and incubated for 20 min at 37 ºC.

For DAPI staining, cells were harvested and ressuspended in 3.7% (w/v) formaldehyde for 30 min, washed three times with PBS and incubated with 2 µg/ml of DAPI. After 10 min at room temperature cells were washed with PBS and observed by fluorescence microscopy.

Overnight cultures were analyzed on a Leica Microsystems DM-5000B epifluorescence microscope with appropriate filter settings using a 100× oil-immersion objective. Images were acquired with a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software.

Flow cytometry

For the assessment of mitochondrial membrane potential the probe 3,3'dihexyloxacarbocyanine iodide ($DiOC_6(3)$) was used. Cells were collected and suspended at a concentration of 5.10^6 cells/ml in PBS and incubated with 1nM $DiOC_6(3)$ for 30 min at 30 ºC in the dark.

Sample analysis was performed in an Epics® XL™ (Beckman Coulter) flow cytometer, equipped with an argon-ion laser emitting a 488-nm beam at 15mW. Monoparametric detection of PI fluorescence was performed using FL-3 (488/620 nm) and detection of $DiOC_6(3)$ or GFP fluorescence was performed using FL-1 (488/525 nm).

Thirty thousand cells were analyzed per sample and experiments were reproduced independently at least four times. Data were analyzed using WinMDI 2.8 software.

Results

Por1p contributes to the mitochondrial morphology in healthy cells

Saccharomyces cerevisiae possesses two VDAC genes, *POR1* and *POR2*. *POR2* has 49% amino acid sequence identity to *POR1* which codes for the major VDAC isoform as referred above (Blachly-Dyson et al., 1997). A BY4742 strain and its derivatives *por1*Δ and *por2*Δ strains, were transformed with a plasmid expressing a mitochondria matrix-targeted GFP (Westermann and Neupert, 2000). In the parental strain, exponential phase cells exhibited mitochondria with a normal elongated tubular network morphology (Fig. 1A). In the absence of Por1p, a high percentage of cells presented short spherical mitochondria of different sizes and often aggregated (Fig. 1A). Identical morphology was observed in a *por1*Δ strain generated in a W303-1B background (data not shown). Treatment of the wild-type cells with DIDS, a compound known to inhibit VDAC (Shafir et al., 1998), led to a quick fragmentation of the mitochondrial network, which supports the view that VDAC contributes to mitochondrial fusion. To assess whether *POR1* deletion causes complete reticulum fragmentation or just fission/constriction of the inner membrane (Jakobs et al., 2003; Pevala et al., 2007) mitochondria were stained with Mitotracker Red. This fluorescent

probe is mitochondrion-selective and accumulates in response to negative mitochondrial membrane potential. Mitotracker staining of *por1*Δ cells showed a high percentage of cells with fragmented reticulum similar to that observed when using the mitochondrial matrix targeted-GFP. This observation indicates that a complete fragmentation of the mitochondrial reticulum occurs in *por1*Δ mutant mitochondria (Fig. 1A).

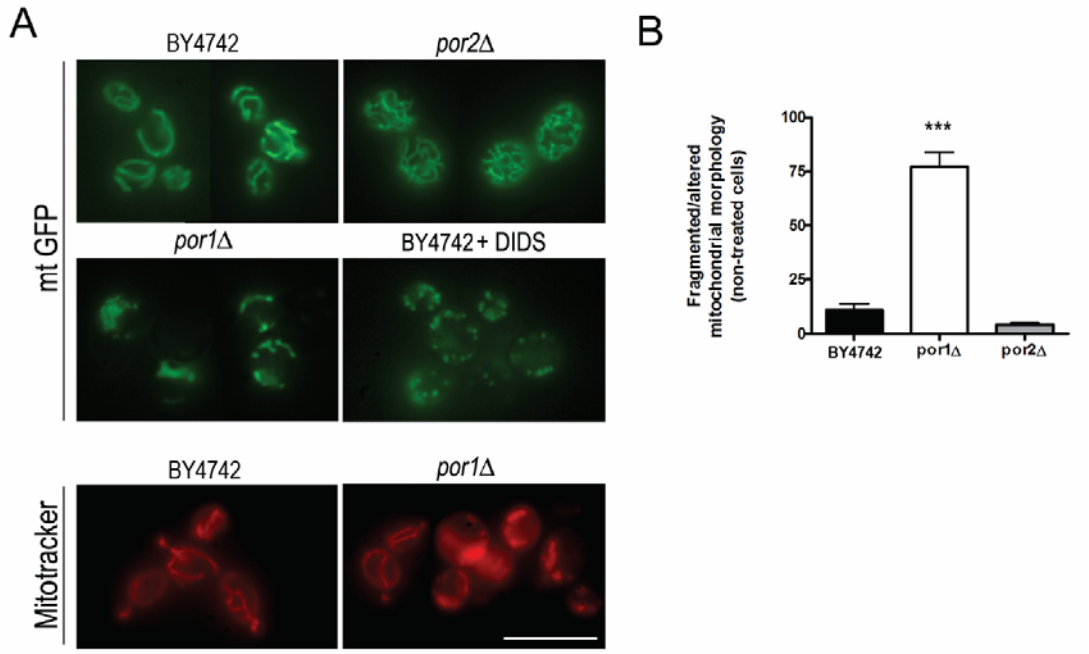

Figure 1. *Por1p contributes to fusion in non treated, healthy cells.* (A) Mitochondrial morphology of BY4742, *por1*Δ and *por2*Δ cells from overnight grown cultures of visualized by expression of a mitochondria matrix-targeted GFP (upper panels). BY4742 strain was treated for 5 min with 0.5 mM of the VDAC inhibitor, DIDS. Mitochondrial morphology in BY4742 and *por1*Δ strains was also visualized by Mitotracker Red straining (lower pannels). (B) Quantification of fragmented mitochondrial morphology for strains BY4742, *por1*Δ and *por2*Δ is shown. Values are means ± SD of four independent experiments. *** $P < 0.001$, unpaired *t*-test.

Absence of Por2p, opposite to Por1p, led to an increase in the extent of the mitochondrial branching comparing to the parental strain (Fig. 1A). This effect however is not as dramatic as in mutants impaired in mitochondrial fission like *dnm1*Δ mutant (Mozdy et al., 2000) suggesting that Por2p contributes to this process only in some extent. Quantification of the percentage of cells exhibiting fragmented morphology for strains BY4742, *por1*Δ and *por2*Δ is shown in Fig. 1B.

por1*Δ mutant exhibits a high membrane potential that is reversed by loss of mitochondrial DNA*

Changes in $\Delta\Psi_m$ were suggested to affect mitochondrial morphogenesis in mammalian cells. Conflicting data propose either mitochondrial depolarization or hyperpolarization as implicated in fission of the mitochondrial network (Legros et al., 2002; Ishihara et al., 2003; Mattenberger et al., 2003; De Vos et al., 2005; Benard et al., 2007). As such, we assessed whether *por1*Δ strain could be inducing mitochondrial fission indirectly, by affecting $\Delta\Psi_m$.

$\Delta\Psi_m$ was assessed by flow cytometry using the probe $DiOC_6(3)$. The green fluorescence intensity was normalized to cell size by dividing it by the intensity of the forward scatter signal (FL1 area (log)/ FS area (log)). In this way changes in green fluorescence intensity do not reflect changes in cell size. $\Delta\Psi_m$ in *por1*Δ cells was higher comparatively to the wild-type (Fig. 2A). This alteration may be due to decreased ADP availability as a consequence of impaired exchange of ADP from the cytosol with ATP from mitochondria (Vander Heiden et al., 1999; Vander Heiden et al., 2000). A strain disrupted in *POR2*, an isoform without obvious channel properties (Blachly-Dyson et al., 1997; Lee et al., 1998), presented a $\Delta\Psi_m$ identical to the wild-type (Fig. 2A). Unpredictably, the double mutant *por1*Δ*por2*Δ was reported to be slightly depolarized (Gross et al., 2000).

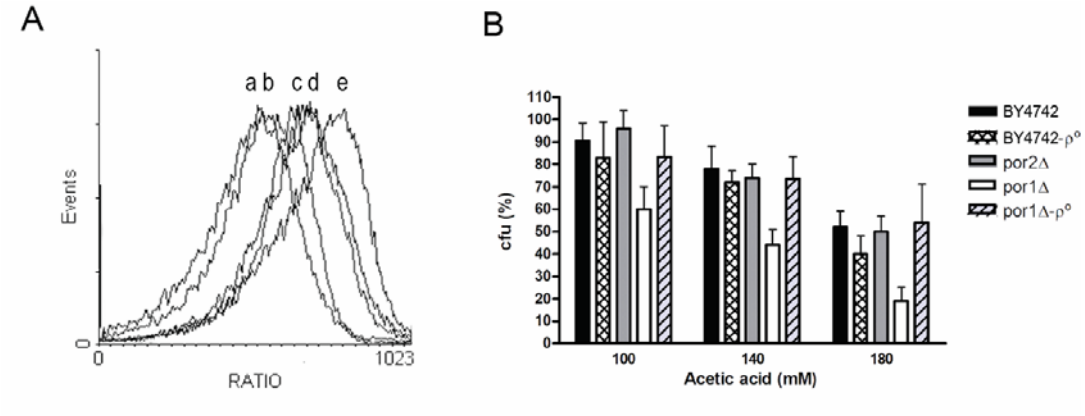

Figure 2. por1*Δ exhibits a high mitochondrial membrane potential ($\Delta\Psi_m$) and a high death rate, both reversed by loss of mitochondrial DNA.* (A) Flow cytometric analysis of $\Delta\Psi_m$ in the *S. cerevisiae* strains BY4742-ρ° (A), *por1*Δ-ρ° (B), *por2*Δ (C), BY4742 (D) and *por1*Δ (E) using the probe $DiOC_6(3)$. Images show monoparametric histograms of Ratio [FL1 area (log) / FS (log)]. The Ratio value was used to eliminate variations in fluorescence due to cell size. Data represent one of four independent experiments. (B) Cell survival of the same *S. cerevisiae* strains after 200 min treatment

with 100-180 mM acetic acid. Values are means ± SD of four independent experiments. $P < 0.01$ (*por1* versus *por1*Δ-ρ^0 cells); two-way ANOVA.

ρ^0 cells were described in mammalian cell lines (Appleby et al., 1999; Loiseau et al., 2002) and yeast (Gross et al., 2000) to have a decreased $\Delta\Psi_m$ compared with the control cells. Accordingly, we also observed a decrease in $\Delta\Psi_m$ in BY4742-ρ^0 and *por1*Δ-ρ^0 generated from the respective parental strains by ethidium bromide mutagenesis (Fig. 2A). We further assessed whether the decrease of $\Delta\Psi_m$ in the *por1*Δ mutant caused by the loss of mitochondrial DNA, was sufficient to recover the mitochondrial tubular morphology. The BY4742-ρ^0 and *por1*Δ-ρ^0 strains transformed with a plasmid expressing a matrix-targeted GFP displayed a fragmented morphology (data not shown). These results are not in agreement with previous reports on ρ^0 strains derived from other backgrounds that were not severely affected in mitochondrial morphology (Guan et al., 1993; Kawai et al., 2001). Whether the alteration in mitochondria morphology observed for the ρ^0 strains in the BY4742 background is strain dependent remains to be assessed.

Deletion of *POR1* in the BY4742 background makes this strain sensitive to acetic acid (Fig. 2B), similarly to what occurs in the W303 background (Pereira et al., 2007). Deletion of *POR2*, on the other hand, does not exhibit a significant difference from the parental BY4742 strain. Interestingly, while the BY4742-ρ^0 strain behaves like the parental strain, *por1*Δ-ρ^0 reverses the acetic acid sensitive phenotype of the *por1*Δ mutant (Fig. 2B).

por1*Δ*aac1/2/3*Δ strain reverts the sensitivity of* por1*Δ mutant to apoptosis but retains the fragmented mitochondrial reticulum*

aac1/2/3 Δ mutant showed similar to the *por1* Δ strain, and likely for the same reason (Vander Heiden et al., 1999), a relative increase in $\Delta\Psi_m$ (Fig. 3B). However, mitochondrial network in the *aac1/2/3* Δ strain is identical to the wild-type strain (Fig. 3A) despite the high $\Delta\Psi_m$ displayed, allowing dissociating high $\Delta\Psi_m$ from mitochondrial fragmentation. The time course of cellular events in the presence of 180 mM acetic acid (viability loss, ROS production, chromatin condensation and loss of membrane integrity) in the *por1*Δ*aac1/2/3*Δ mutant (Cardoso, T., unpublished results) was identical to

*aac1/2/3*Δ strain (Pereira et al., 2007) suggesting that in yeast the pro-survival role of Por1p is dependent on the presence of AAC proteins. On the contrary, the role of Por1p in mitochondrial fusion is not affected by the absence of AAC, since *por1*Δ*aac1/2/3*Δ strain still retained the fragmented mitochondrial morphology of *por1*Δ mutant.

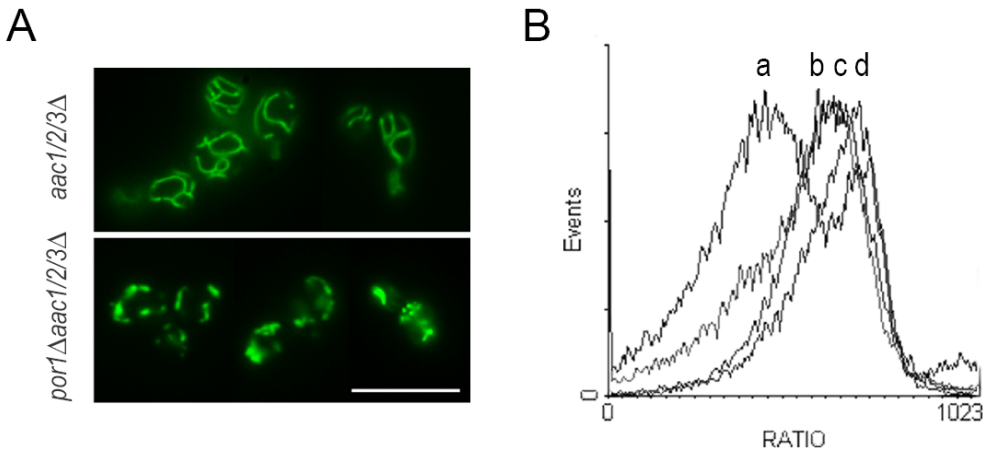

Figure 3. (A) *por1Δaac*1/2/3*Δ* strain retains the fragmented mitochondrial morphology of por1Δ mutant. Mitochondrial morphology in *aac1/2/3*Δ and *por1*Δ*aac1/2/3*Δ strains expressing a matrix-targeted GFP. (B) Flow cytometric analysis of $\Delta\Psi_m$ in *S. cerevisiae* strains W303-1A (a), *por1*Δ (b), *aac1/2/3*Δ (c) and *por1*Δ*aac1/2/3*Δ (d). Images show monoparametric histograms of Ratio [FL1 area (log) / FS area (log)]. Data represent one of four independent experiments.

Discussion

In healthy growing cells, absence of Por1p leads to a phenotype of fragmentation of the mitochondrial network while absence of Por2p leads to a slight increase in the extent of the branching. High $\Delta\Psi_m$ is also found in *por1*Δ but not in *por2*Δ strain. In addition, absence of Por1p, but not Por2p, leads to a high sensitivity to acetic acid induced-apoptosis. It was described that cells deleted for the *POR1* gene are able to grow on yeast media containing a nonfermentable carbon source except if they face an elevated temperature (37°C). *POR2* overexpression, can correct this growth defect (Blachly-Dyson et al., 1997). However, no Por2p channels were detected electrophysiologically in reconstituted systems and Por2p overexpression does not confer additional permeability to liposomes or intact mitochondria (Blachly-Dyson et al.,

1997; Lee et al., 1998) indicating that Por2p, unlike Por1p, does not normally forms a channel. Our results further strengthen the idea that the two yeast VDAC isoforms have different, specialized functions.

It has been suggested that remodelling of the mitochondrial membrane in mammalian cells may help in the release of cytochrome *c*. In fact most pro-fusion factors are anti-apoptotic while pro-fission factors are pro-apoptotic (Gazaryan and Brown, 2007; Jeong and Seol, 2008). The same seems to be true for yeast, as deletion of the pro-fission *DNM1* or *MDV1/NET2* inhibits cell death triggered by several stimuli (Fannjiang et al., 2004) and results in an increase in life span (Scheckhuber et al., 2007). Accordingly, Ysp1p (Pozniakovsky et al., 2005), Ysp2p (Sokolov et al., 2006) and Yca1p (Mazzoni et al., 2005) are all required for fission of the mitochondrial network during apoptosis and their absence leads to cell death resistance. Fis1p has a pro-fission function in normal conditions but unpredictably, seems to play a pro-fusion role during apoptosis, acting by inhibiting Dnm1p (Fannjiang et al., 2004) *FIS1* deletion increases susceptibility to apoptosis in different scenarios (Fannjiang et al., 2004; Ivanovska and Hardwick, 2005; Kitagaki et al., 2007). In mammalian cells, proteins associated to the release of cytochrome *c* were also shown to affect mitochondria morphology (Delivani et al., 2006; Karbowski et al., 2006) making a link between apoptosis, cytochrome *c* release and fragmentation of the mitochondrial network. We cannot discard that the highly fragmented mitochondrial network in the *por1*Δ strain may contribute to the increase susceptibility of this strain to apoptosis either directly or indirectly, for example by facilitating cytochrome *c* release. However, fragmentation of the mitochondrial network *per se* does not enhance cell death, since *POR1*-deletion in an *aac1/2/3*Δ background or in the absence of mitochondrial DNA (ρ^0) rescues the enhanced apoptosis phenotype of the strain but not the exacerbated mitochondrial fragmentation.

While an alteration in mitochondrial membrane potential (both hyperpolarization and depolarization) in some of the strains studied was associated to mitochondrial fragmentation, that was not the case for *aac1/2/3*Δ strain, and as such, no causal relation can be assumed between both events. There is also no relation between $\Delta\Psi_m$ of the strains and susceptibility to cell death suggesting it is not an important factor for the cell demise.

As referred above, since in *aac1/2/3*Δ strain the high $\Delta\Psi_m$ was not associated to an increased fragmentation of the mitochondrial network, it is not likely that high $\Delta\Psi_m$ is the cause of mitochondrial fragmentation in the *por1*Δ mutant. In mammalian cells

VDAC was implicated in the association of mitochondria with the cytoskeleton (Linden and Karlsson, 1996). Disruption of the interaction of mitochondria with cytoskeleton alters the normal mitochondrial morphology giving origin to a fragmented mitochondrial network (Boldogh et al., 1998). However, Blachly-Dyson and colleagues (Blachly-Dyson et al., 1997) reported that Por1p in yeast is not necessary for mitochondrial segregation into the daughter cell casting doubts on a role of Por1p in actin binding. As such, it will be important to assess if the destabilization of the mitochondrial network in the absence of Por1p is due to improper binding of mitochondria to the actin cytoskeleton or if Por1p is interfering with the organelle fission/fusion machinery.

In summary, both yeast VDAC isoforms affect mitochondrial morphology in yeast, although in opposite ways. The exacerbated mitochondrial network fragmentation and high mitochondrial membrane potential in the *por1*Δ mutant is not the responsible for the sensitive phenotype displayed when challenged with acetic acid. The pro-survival role of Por1p, unlike its function in mitochondrial fragmentation, is dependent on the presence of AAC proteins suggesting that both proteins share the same apoptotic pathway.

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Concluding remarks

Concluding remarks

The recognition of a mitochondria-mediated apoptotic pathway in *Saccharomyces cerevisiae* was of special interest to the yeast apoptosis field. The similarities to the mammalian intrinsic apoptotic pathway, along with the genetic tractability of yeast and easy manipulation of mitochondrial respiration, led to and increased interest in exploiting this simple model to unveil unknown facets of the role of mitochondria in the execution of cell death.

The studies described in this thesis aimed to contribute to a better understanding of the mitochondrial cell death pathway in the yeast *Saccharomyces cerevisiae*. Acetic acid was one of the first and better characterized inducer of apoptosis in yeast and as such was used as the main apoptotic inducer in our studies. Using this system, we showed that absence of AAC proteins impairs mitochondrial outer membrane permeabilization (MOMP) and cytochrome *c* release. Further research will be required to determine how AAC proteins influence the permeabilization of yeast mitochondria. Based on the current knowledge of the mammalian system, we hypothesise that the yeast AAC may be part of an inner membrane pore, either as a structural or regulatory component. This pore would open under apoptotic conditions, allowing inner mitochondrial membrane permeabilization, matrix swelling and outer mitochondrial membrane rupture. Whether this pore has a defined structure and a regulated mode of function or is formed by aggregation of misfolded integral membrane proteins damaged by oxidants or other stresses, as proposed for mammalian cells (He and Lemasters, 2002), is an open question. Since absence of Por1p leads to an enhancement of cell death dependent on the presence of AAC proteins, Por1p may negatively regulate this pore. In addition, in healthy non-treated cells, both yeast VDAC isoforms Por1p and Por2p affect mitochondrial morphology, although in opposite ways; absence of Por1p promotes fragmentation while absence of Por2p decreases it. The involvement of these proteins in mitochondrial morphology however, seems independent from the death function.

We show that the impairment of MOMP/cytochrome *c* release observed in the absence of AAC proteins causes a delay rather than an impediment of the apoptotic process in response to acetic acid. These results suggest the existence of alternative death pathways independent of cytochrome *c* release. In accordance, a strain deleted for heme lyase, responsible for the synthesis of holocytochrome *c,* showed a delay in acetic acid-induced apoptosis, but not a blockage (Ludovico et al., 2002b). Other proteins, like

Aif1p (Wissing et al., 2004a) and Nuc1p (Buttner et al., 2007), were shown to be released from mitochondria during yeast apoptosis and to regulate the apoptotic process. While the pro-death role of Nuc1p was shown to be dependent on the presence of AAC proteins, no data is available regarding Aif1p. Thus, in the case Aif1p release is AAC-independent, the observed acetic acid-induced apoptotic death in the absence of AAC proteins could be ascribed to Aif1p. Alternatively, mitochondrial- independent pathways may exist that can be activated upon a blockade of the mitochondrial pathway.

Cpr3p was recently identified in a genetic screen for mediators of copper-induced apoptotic cell death in yeast (Liang and Zhou, 2007). It was shown that deletion of Cpr3p abrogated copper-, but not manganese-induced apoptosis. We observed no effect on cell death induced by either acetic acid or diamide in the absence of Cpr3p. In that same study, a *por1*Δ mutant was shown to be resistant to copper-induced death, in contrast with the higher sensitivity observed for acetic acid-induced death. These data provide evidence that the death process in yeast, like in mammals, may have more alternative trigger-dependent pathways than initially assumed. Emergence of redundant death pathways is evolutionarily justified since a single and common death mechanism would have been easily hijacked by opportunistic parasitic organisms or by the emergence of spontaneous mutations.

AAC-deletion also causes impairment in mitochondrial degradation, pointing to a role for this protein in the signalling of doomed mitochondria. It is generally believed that autophagy is the main process responsible for mitochondrial degradation during an apoptotic process. Herein we show that mitochondrial degradation during acetic acid-induced apoptosis does not rely on the autophagic process. Mitochondrial degradation is also not due to Uth1p-dependent mitophagy, but is dependent on the protease Pep4p that is released from the vacuole during acetic acid-induced apoptosis. Together, our results support the view that mitochondria play a crucial role in yeast apoptosis and can affect the path of cell death not only by the release of pro-apoptotic proteins, but also at a later stage, by playing a role on its own degradation. Additionally, the assurance of degradation of damaged mitochondria appears critical for apoptotic progression, possibly by preventing a decrease in the ATP levels required for this death process.

We observe that global caspase activity and Yca1p participation in acetic acid-induced apoptosis is affected by the growth phase of the cells used in the assays. Dependence of the death process on caspase activation and on Yca1p was only detected for early exponential phase cells. We have mainly used cells grown to late exponential

phase prior to apoptosis induction. Under these conditions, overall caspase activation and Yca1p requirement for cell death are negligible. The release of Pep4p, and possibly other proteases from the vacuole, may replace the caspase proteolytic activity during acetic acid-induced apoptosis in yeast in a manner resembling some caspase-independent pathways in mammalian cells (Broker et al., 2005).

Whilst in this thesis we addressed most of the issues initially identified, some other aspects remain to be elucidated and new questions have arisen. Hereafter, we list additional studies that will clarify several unanswered issues:

- assessement of yeast Aifp release from mitochondria in response to acetic acid in the *aac1/2/3*Δ mutant would be relevant;
- electrophysiological studies on isolated mitochondria would allow a better understanding of MOMP induced by acetic acid, as well as on the role of AAC on this process;
- ROS-mediated Aac2p modifications were recently shown to underlie the reduced proliferation rates and accelerated replicative senescence observed in response to overactivation of RAS/protein kinase A (PKA) signalling (Hlavata et al., 2008). Since those functions of Aac2p were shown to be independent of its ADP/ATP exchange activity, the authors suggested that oxidative modification of the Aac2p carrier may lead to a PTP-like function in yeast mitochondria. It would be interesting to assess whether such oxidative damage to Aac2p is associated or required for MOMP/cytochrome *c* release to occur during yeast apoptosis; cysteine-less *AAC2* mutants, which still display fully active carrier activity could be used to address this aspect (Bamber et al., 2007).
- Farah and colleagues (Farah and Amberg, 2007) discovered that the oxidation state of actin is able to regulate cell death. It was suggested that the oxidoreductase Oye2p protects cells from dying by preventing oxidation of the actin cytoskeleton (Farah and Amberg, 2007). The role of actin in acetic acid-induced apoptosis could be explored, for instance, through manipulation of the oxidoreductase Oye2p.
- related to the previous issues, the role of ROS in yeast apoptosis remains to be fully clarified. Though there is evidence indicating that an increase in anti-oxidant defences decreases acetic acid toxicity, it was not clearly shown that acetic acid

can induce death in a ROS-independent pathway. Therefore, it would be relevant to assess whether ROS are mediators of acetic acid-induced apoptosis, and if so, at what level they affect this process;

- the mammalian phosphate carrier was recently implicated as a possible structural component of the PTP (Leung and Halestrap, 2008). This carrier belongs to the same mitochondrial 6-transmembrane domain family of transporters as AAC (Halestrap, 2004). Hence it would be appealing to assess the role of the yeast phosphate carrier in mitochondrial permeabilization during yeast apoptosis;
- *por1*Δ strains displayed enhanced apoptosis in response to acetic acid and altered mitochondrial network fragmentation. Still, the role of Por1p in MOMP/cytochrome *c* release needs to be assessed;
- transmission electron microscopy (TEM) would also provide important information regarding the morphological alterations of mitochondria in *aac1/2/3*Δ and *por1*Δ strains during acetic acid-induced apoptosis;
- to our knowledge, the role of Pep4p in mitochondrial degradation has never been shown for mammalian cells. It would be interesting to see whether human cathepsin D, the Pep4p orthologue, has a role in mitochondria degradation in mammalian cells; similarly, the role of Pep4p in cytochrome *c* release in yeast, described for cathepsin D in mammals, remains to be assessed.

Answering these questions will certainly improve our knowledge of mitochondrial membrane permeabilization, a key apoptotic event, as other related aspects of the apoptotic process in *S. cerevisiae*.

In summary, while there are relevant differences in cellular processes between yeast and mammalian cells, our study opens new perspectives for an enhanced understanding of apoptosis by identifying a novel role for putative components of yeast PTP (Por1p and AAC proteins) and Pep4p in yeast apoptosis. In addition, this work offers the possibility to use the simpler yeast eukaryotic system to further elucidate the mechanisms underlying mitochondrial release of apoptogenic regulators and degradation of damaged mitochondria, as well as to screen for drugs interfering with these processes. Indeed, there is a currently increasing interest on the pore as a pharmacological target (Rasola and Bernardi, 2007) and on the role of lysosomes in mammalian cell death. Finally, since mitochondrial dysfunction and accumulation of damaged mitochondria are associated with different human diseases, a deeper understanding of how mitochondria

degradation is activated and regulated during apoptosis can unravel novel attractive targets for the development of apoptosis-based therapies.

Despite the use, throughout this thesis, of the term apoptosis to refer to yeast cell death displaying some apoptotic features, this nomenclature is still controverse, mainly among mammalian apoptosis researchers. It is generally assumed that apoptosis emerged as a response to the challenges of attaining multicellularity. While the benefits of suicide to unicellular organisms are not immediately apparent, it is believed that altruistic death could be beneficial to the fitness of the population (recently reviewed in Severin et al., 2008). Yeast cells do not grow as individuals but as members of a population in liquid medium or of macrocolonies. In that sense, they face many challenges in common with multicellular organisms (limiting the spread of a viral infection, for instance). Anyway, as remarked by Hardwick and Cheng (Hardwick and Cheng, 2004) *"Whether or not yeast cell death is altruistic, apoptotic, or otherwise analogous to programmed cell death in mammals is controversial. However, growing attention to cell death mechanisms in yeast has produced several new papers that make a case for ancient origins of programmed death involving mitochondrial pathways conserved between yeast and mammals"*.

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