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Professor Doutor Hernêni Varanda Gerós
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Abstract

The vacuole is the largest organelle of yeast cells and is functionally equivalent to animal lysosome and plant vacuole. Vacuoles are the most acidic organelles of the cell, and play major roles in protein degradation, ion and metabolite storage, as well as in ion homeostasis, response to nutrient deprivation, osmotic and ionic stress, autophagy and even in apoptosis. Lysosome membrane permeabilization and the consequent release of lysosomal proteases, namely cathepsins, are now widely accepted to trigger apoptosis. Of all lysosomal cathepsins, the aspartic cathepsin D was the first identified protein with apoptogenic properties. Acetic acid was shown to induce apoptosis in yeast, associated with changes in mitochondria and release of pro-apoptotic proteins. Pep4p, a vacuolar yeast protease orthologue of the lysosomal human cathepsin D was also shown to be involved in acetic acid-induced apoptosis.

The ultimate objective of the present study was to contribute to the understanding of the crosstalk between the vacuole and mitochondria in yeast programmed cell death induced by acetic acid. For these purposes the effect of acetic acid in isolated vacuoles and whole cells with different genetic backgrounds, namely on vacuole membrane permeabilization as well on the release of Pep4p and their relation with alterations in vacuole function, was investigated. The functional characterization of isolated vacuoles as well the effect of acetic acid on vacuolar function was monitored with fluorescent probes combined with flow cytometry and fluorescence microscopy. Functional characterization of isolated vacuoles was also monitored through the activity of V-H^+-ATPase by spectrofluorimetry.

Epifluorescence microscopy imaging showed that the vacuolar membrane stains strongly with the styryl dye FM1-43 and that most of the vacuoles accumulate Ca^{2+}, as assessed with Fluo-4 AM. Flow cytometry analysis of vacuole samples incubated with these fluorescent probes confirmed a well-defined population of intact and functional vacuoles. Consistently spectrofluorimetric assays with the pH-sensitive probe ACMA suggested that the isolated vacuoles were intact and functional, the vacuolar membrane being able to generate and maintain a pH gradient through a concanamycin A-sensitive V-H^+-ATPase. The addition of acetic acid induced the release from the vacuole lumen of an EGFP-Pep4p fusion protein. Changes in fluorescence of vacuoles stained with acridine orange and Fluo-4 AM suggest that the acid induces a transient perturbation of vacuolar pH and Ca^{2+} release. It has been described that release of Ca^{2+} is an event involved in cell death, as well as the release of H^+ and consequent cytosol acidification. The main novelty of the present study with isolated vacuoles is the finding that acetic acid is able to directly induce a partial permeabilization of the vacuolar membrane, similar to LMP in mammalian cells, without the involvement of other organelles or triggering upstream pathways.

Sumário

O vacúolo é o maior organelo da levedura e é funcionalmente equivalente ao lisossoma das células animais e ao vacúolo das células vegetais. Este organelo, cujo lúmen tem o pH mais ácido, participa em processos celulares importantes tais como degradação proteica e armazenamento de iões e metabolitos, bem como na homeostase iónica, resposta à privação de nutrientes, stress osmótico e iónico, autofagia e mesmo na apoptose. Sabe-se que a permeabilização da membrana lisossomal (LMP) e a consequente libertação de proteases lisossomais, nomeadamente de catepsinas, induz apoptose. De todas as proteases lisossomais, a catepsina D foi a primeira proteína a ser identificada com propriedades apoptóticas. Foi demonstrado que o ácido acético induz apoptose em leveduras, associada a alterações mitocondriais e à libertação de proteínas pró-apoptóticas. Foi igualmente demonstrado que a protease vacuolar Pep4p, ortóloga da catepsina D humana, está envolvida na apoptose induzida por ácido acético.

O presente estudo teve como principal objectivo contribuir para a compreensão da interligação entre o vacúolo e a mitocôndria na morte celular programada induzida por ácido acético na levedura *Saccharomyces cerevisiae*. Para tal foi investigado o efeito do ácido acético em vacúolos isolados e em células inteiras de leveduras com diferentes *backgrounds* genéticos, nomeadamente no que respeita à permeabilização da membrana vacuolar bem como à libertação de Pep4p e sua relação com alterações nas funções do vacúolos. A caracterização funcional de vacúolos isolados bem como o efeito da adição de ácido acético na função vacuolar foram realizadas por citometria de fluxo e microscopia de fluorescência associada à utilização de diferentes sondas fluorescentes. A caracterização funcional dos vacúolos isolados foi complementada pela determinação da actividade da V-H^+-ATPase por espectrofluorimetria.

Os estudos de microscopia de fluorescência mostraram uma marcação forte da membrana vacuolar com a sonda lipofílica FM1-43 e que a maioria dos vacúolos acumulavam Ca^{2+} com base na marcação pela sonda Fluo-4 AM. A análise por citometria de fluxo de amostras de vacúolos incubadas com estas sondas fluorescentes evidenciou uma população bem definida de vacúolos intactos e funcionais. Consistentemente os ensaios de espectrofluorimetria com a sonda ACMA, sensível ao pH, sugeriram que os vacúolos isolados se encontravam intactos e funcionais, dado que a membrana vacuolar era capaz de gerar e manter um gradiente de pH através da actividade da bomba V-H^+-ATPase, sensível à concanamicina A. A adição de ácido acético promoveu a libertação da proteína de fusão EGFP-Pep4p em vacúolos isolados e induziu variações de fluorescência de vacúolos marcados com laranja de acridina e com Fluo-4 AM indicativas de uma perturbação transitória do pH vacuolar e de uma libertação de Ca^{2+}. Está descrito que a libertação de Ca^{2+} e de H^+, com a consequente acidificação do citosol, está envolvida na morte celular. Estes novos resultados em vacúolos isolados permitem concluir que

o ácido acético é capaz de induzir directamente uma permeabilização parcial da membrana vacuolar, semelhante à LMP em mamíferos, sem o envolvimento de outros organelos ou sem a activação de vias a montante.

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Abbreviations

$\Delta\psi_m$	Transmembrane mitochondrial potential
ACMA	9-amino-6-chloro-2-methoxyacridine
AIF	Apoptosis-inducing factor
ANT	Adenine nucleotide translocator
Apaf-1	Apoptotic protease-activating factor-1
ATP	Adenosine-5'-triphosphate
a.u.	Arbitrary units
Bcl-2	B-cell lymphoma-2
BH3	Bcl-2 homology 3
Caspase	Cysteine-dependent aspartate-specific protease
dATP	Deoxyadenosine triphosphate
DED	Death-effector domain
DD	Death domain
Diablo	Direct IAP binding protein with low pI
DISC	Death inducing signaling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EGFP	Enhanced green fluorescent protein
EGTA	Ethylene glycol-bis[β-aminoethylether]-N, N, N', N'-tetraacetic acid
ER	Endoplasmatic reticulum
FM1-43	N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl)pyridinium dibromide
FADD	Fas-associated death domain
Fas	Apoptosis stimulating fragment
FasL	Fas ligand
H_2O_2	Hydrogen peroxide
HtrA2	High temperature requirement A2
IAP	Inhibitor of apoptosis protein
IMM	Inner mitochondrial membrane
LMP	Lysosomal membrane permeabilization
MES	2-(Nmorpholino)ethanesulfonic acid
$MgCl_2$	Magnesium chloride
MOMP	Mitochondrial outer membrane permeabilization

MOPS	3-(N-morpholino)propanesulfonic acid
OMM	Outer mitochondrial membrane
PCD	Programmed cell death
PLA2	Phospholipase A2
PT	Permeability transition
PTP	Permeability transition pore
ROS	Reactive oxygen species
rpm	Rotations per minute
SC	Synthetic complete medium
siRNA	Small-interfering RNA
Smac	Second mitochondria-derived activator of caspases
SNARE	Soluble NSF attachment receptors
tBid	Truncated Bid
TNF	Tumor necrosis factor
TNFR-1	Tumor necrosis factor receptor-1
TRAIL	TNF-related apoptosis inducing ligand
TRADD	TNFR1-associated death domain protein
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet
V-H^+-ATPase	Vacuolar-type proton-translocating ATPase
VDAC	Voltage-dependent anion channel
XIAP	X-chromosome linked inhibitor of apoptosis protein
YEPD	Yeast extract/peptone/dextrose medium

1. Introduction

The yeast vacuole is the most acidic organelle and is equivalent to the lysosome of animal cells and to the vacuole of plant cells. Besides its well known degradative and storage functions, this organelle is also involved in cell death processes, namely in apoptosis. Apoptosis is a type of programmed cell death (PCD) that eliminates excess or damaged cells in a controlled way. This cell death process is vital for embryonic development, immune system and tissue homeostasis in metazoans. However, it is currently recognised that apoptosis also occurs in single-celled organisms, for instance in the budding yeast *Saccharomyces cerevisiae*. Thus this simple and genetic tractable unicellular eukaryotic organism has been used as a valuable model system for cell death research.

In this section the yeast vacuole, its characteristics and various functions as well its responses to stress stimuli will be focused. Next, the mammalian apoptotic pathways will be briefly described as well as the apoptotic components and regulators in yeast so far identified. Particular focus will be given to apoptosis induced by acetic acid since it was the apoptotic agent used in this study. Finally, an overview of the role of the vacuole and lysosome in apoptosis will be presented, as well as the release of Pep4p, the orthologue of the human lysosomal cathepsin D, which was addressed in this study.

1.1. The yeast vacuole

The vacuole is the largest and most acidic organelle of yeast cells playing major roles in protein degradation, ion and metabolite storage as well in detoxification. It also has an important function in ion homeostasis, response to nutrient deprivation, osmotic and ionic stress, autophagy and even in apoptosis (Klionsky *et al.*, 1990; Guicciardi *et al.*, 2004; Li and Kane, 2009). These features support the recent view of vacuoles as dynamic, highly sensitive and responsive organelles to environmental changes, and not only as storage compartments.

1.1.1. The role of V-H^+-ATPase in the acidification of the vacuolar lumen

Almost all vacuolar and lysosomal functions are dependent either on the acidic pH of vacuolar lumen or on the pH gradient across vacuolar membrane. It has been shown that yeast vacuolar pH varies from <5-6.5 depending mostly on growth conditions (Plant *et al.*, 1999; Brett *et al.*, 2005; Padilla-Lopez *et al.*, 2006; Martinez-Munoz and Kane, 2008). In both vacuoles and lysosomes, acidification is achieved through the action of a proton pump V-H^+-ATPase. This proton pump is responsible for the coupling the free energy of ATP hydrolysis to proton transport from the cytosol to the organelle lumen. V-H^+-ATPases are highly conserved enzymes, consisting of a complex V_1 that contains the sites for ATP hydrolysis in the cytosolic side, bound to a complex V_0 that is integrated into the vacuolar membrane (Figure 1).

The yeast V-H^+-ATPase consists of 14 subunits all encoded by a single gene, except for the largest membrane subunit *a* (Manolson *et al.*, 1994) which exists in two isoforms. Mammalian V-H^+-ATPases have a similar structure overall, but the number of isoforms is much higher (reviewed by Li and Kane, 2009), which complicates the biochemical analysis of lysosomal V-H^+-ATPases. This is one of the main reasons why yeast is an appropriate model system to study the V-H^+-ATPase.

In contrast to other organisms, genetic deletions of ubiquitously expressed V-H^+-ATPase subunits in fungi are not lethal. Thus, yeast mutants lacking V-H^+-ATPase subunits (*vma* mutants) offer important insights into the functional importance of vacuolar acidification (Kane, 2006). Genetic deletion of any of V-H^+-ATPase subunits appears to abolish organelle acidification, resulting in a phenotype characterized by sensitivity to high extracellular pH and high extracellular calcium concentrations as well to heavy metals and oxidants. Moreover these mutants are unable to grow on non-fermentative carbon sources, and are multidrug sensitive (Kane, 2006).

Calcium sensitivity of *vma* mutants results from the dependence of the vacuolar Ca^{2+}/H^+ exchanger (Vcx1p) on the proton gradient established by V-H^+-ATPase (Forster and Kane, 2000). Vacuolar sequestration of heavy metals is mediated by some transporters and by binding to polyphosphate vacuolar stores and is compromised in mutants lacking V-H^+-ATPase activity (Eide *et al.*, 1993; MacDiarmid *et al.*, 2002; Freimoser *et al.*, 2006). *Vma* mutants are also unable to grow on a medium containing low iron and calcium concentrations (Davis-Kaplan *et al.*, 2004; Yadav *et al.*, 2007) and

at high pH (Serrano *et al.*, 2004), both reflecting the important role of the vacuole as a storage compartment and in pH homeostasis, respectively.

1.1.2. Regulation of vacuolar acidification

Vacuolar acidification is regulated by distinct mechanisms. In yeast, V-H^+-ATPase is regulated at the level of assembly of its V_1 and V_0 complexes (Forgac, 2007; Kane, 2000, 2006). Disassembly is rapid and regulated post-transcriptionally (Forgac, 2007; Kane, 2006) and leads to inactivation of both ATP hydrolysis and proton translocation. Disassembly of V-H^+-ATPase is reversible and occurs in order to adjust the level of assembled enzyme to the availability of ATP and/or the need for proton pump activity. Glucose metabolism also regulates V-H^+-ATPase assembly. Fructose-6-phosphate is necessary to maintain V-H^+-ATPase assembly (Parra and Kane, 1998), as well as some glycolytic enzymes that also support its assembly by signalling glucose deficiency (Lu *et al.*, 2001, 2003, 2007). Additionally, there are mechanisms to regulate vacuolar acidification without acting on the V-H^+-ATPase itself (Paroutis *et al.*, 2004). Due to the electrochemical gradient produced by V-H^+-ATPase, counterion transport plays a critical role for continued pumping (Arai *et al.*, 1989). Hence, a number of exchangers such as Nhx1p (a Na^+/H^+ exchanger), transport H^+ against the pH gradient created by the V-H^+-ATPase, and Nhx1p has been shown to limit acidification in the compartment where it resides, the late endosome/ multivesicular body, and indirectly, to control vacuolar pH (Ali *et al.*, 2004).

Buffering systems also influence the pH of the vacuole. In yeast, polyphosphate is present at very high concentrations inside the vacuole (Beauvoit *et al.*, 1991; Thomas and O'Shea, 2005; Freimoser *et al.*, 2006), and is the major vacuolar buffering system. Recently it has been suggested that vacuolar proteins may also act as buffers (Brett *et al.*, 2006). Together, these systems operate to determine the final vacuolar pH and each one represents a possible way for regulation of the vacuolar acidification.

1.1.3. The vacuolar morphology

Vacuolar morphology depends on growth phase and undergoes alterations in response to different stress conditions. During exponential phase, active yeast cells contain medium-sized vacuoles, which fuse into one large vacuole in stationary phase or under glucose deprivation. Under conditions of osmotic stress, vacuoles also adjust their shape and number, to facilitate the release or uptake of water and ions. The exposure of vacuoles to hyper-osmotic conditions leads to fragmentation into numerous small vesicles, whereas under hypo-osmotic conditions they fuse into one large vacuole (Li and Kane, 2009). These changes in vacuole morphology rely in fusion/fission machinery and on the equilibrium between these two processes.

Vacuolar morphology is dictated by the balance between fusion and fission (Baars *et al.*, 2007). Vacuolar fusion and fission are necessary for the merging of small vacuolar vesicles and fragmentation of a large vacuole, for delivering cargo into and out of the vacuole as well for regulation of vacuolar volume in response to stress conditions.

Fusion includes four major steps namely, priming, tethering, docking and fusion. It also involves coordinated and regulated interactions between many proteins, such as soluble NSF attachment receptors (SNAREs), SNARE chaperones, GTPases and specific lipids like phosphoinositides which are widely present in the vacuolar membrane (reviewed by Li and Kane, 2009). On the other hand, fission-mediated vesicle formation during endocytosis requires coat proteins like COPI, COPII and clathrin (Peplowska and Ungermann, 2005). The molecular mechanisms underlying fission of vacuolar vesicles are hard to define comparatively to the knowledge of those associated to vesicle fission during endocytosis.

In yeast, vacuolar fission is necessary for vacuolar inheritance during cell division and in response to osmotic shock. Interestingly, V-H^+-ATPase is required for both fusion and fission processes (Baars *et al.*, 2007). A recent study has shown that fusion requires the presence of the V_0 membrane complex of V-H^+-ATPase but not its pumping activity, while proton translocation activity is required for fission (Baars *et al.*, 2007). It was observed that cells with no V-H^+-ATPase activity due either to the deletion of V-H^+-ATPase or its inhibition by concanamycin A (a specific inhibitor drug of V-H^+-ATPase) were defective for fission and possessed a large vacuole. In contrast, the deletion of a V_0 subunit (*vph1*Δ) complex that still maintained a partial acidification

led to a defective fusion and cells had fragmented vacuoles. Because cells treated with concanamycin A contained big vacuoles, defective fission caused by the lack of proton pumping activity likely dominates over defective fusion by physical absence of the total or of a subunit of the V_0 sector (Baars *et al.*, 2007). These observations support the interpretation that the equilibrium between vacuolar fusion and fission determines vacuolar morphology.

1.1.4. Vacuolar functions

As referred to above vacuoles are highly dynamic organelles, playing constitutive roles in different processes such as degradation, storage, homeostasis and detoxification (Figure 1), as well as in response to stress conditions like nutrient deprivation and osmotic shock. In the following sections the most important functions of the vacuole are detailed.

1.1.4.1. Degradation

Similarly to the lysosomes, vacuoles are the major degradation systems of the cell. Both soluble (e.g. proteinase A, Pep4p; carboxypeptidade Y, CPY; proteinase B, Prb1p; and carboxypeptidade S, CPS) and membrane-bound (e.g. dipeptidyl aminopeptidase B, DPAP-B) have been described. Usually, vacuolar proteases are relatively non-specific and are engaged in the degradation of a large number of different substrates. Vacuolar proteases are transported to the vacuole in a zymogen form and are activated in a complex cascade at low pH (Van Den Hazel *et al.*, 1996), but since vacuolar protease activity occurs in the absence of V-H^+-ATPase activity, acidic vacuolar pH does not appear to be critical for zymogen activation. For instance Pep4p activation inside the acidic vacuole can occur by two different pathways, a one-step process to release mature proteinase A, involving the intervention of proteinase B, or a step-wise pathway via the autoactivation product known as pseudo-proteinase A. Once active, *S. cerevisiae* proteinase A is essential to the activities of other yeast vacuolar hydrolases, including proteinase B and carboxypeptidase Y (Parr *et al.*, 2007).

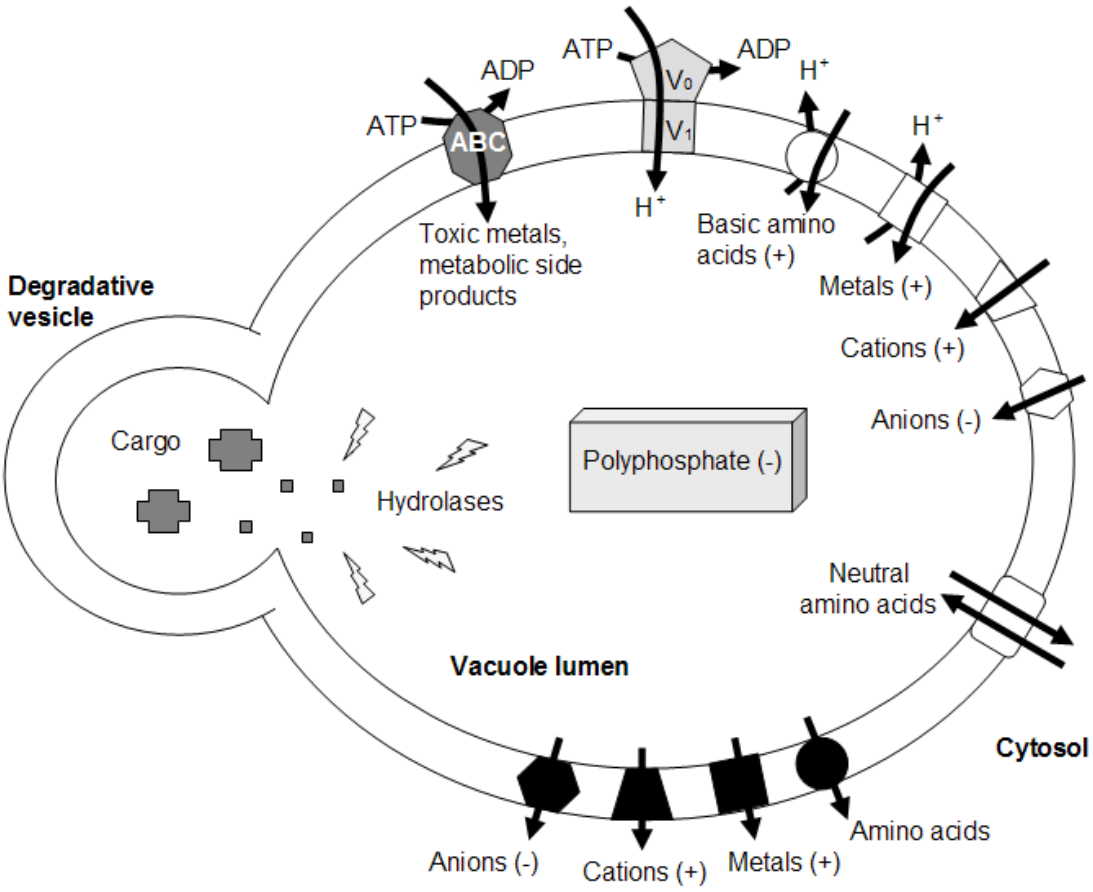

Fig. 1. The yeast vacuole is involved in many cellular processes. a) Degradation: the vacuole is highly enriched in hydrolases responsible for the breaking down of molecules and/or organelles delivered to the vacuole via different pathways. b) Storage: the vacuolar membrane contains multiple transporters that import amino acids, ions, and metals. Activity of V-H^+-ATPase is necessary for hydrolases maturation and for the generation of the proton gradient that drives many transporters. c) Buffering: the vacuolar membrane contains transporters that export amino acids, ions, and metals, which combined to the activity of importers contribute to ion homeostasis and amino acid recycling. The vacuole also accumulates polyphosphate that buffers cations. d) Detoxification: the vacuole sequesters toxic metals and potentially harmful metabolic side-products via ABC transporters (adapted from Li and Kane, 2009).

Vacuolar proteins and protein substrates are directed to the vacuole by different pathways (Bowers and Stevens, 2005). While resident vacuolar proteins are sent to the vacuole by biosynthetic pathways (from Golgi or cytosol), proteins targeted for degradation are sent via endocytosis (plasma membrane proteins) or autophagy (cytosolic and organellar proteins).

1.1.4.2. Metabolite and ion storage and homeostasis

Vacuole is the major storage compartment for amino acids, phosphate, Ca^{2+} and metal ions (Klionsky *et al.*, 1990; Van Ho *et al.*, 2002). Basic and neutral amino acids accumulate in yeast vacuole at high levels, but there is little vacuolar storage of acidic amino acids (Klionsky *et al.*, 1990), which mostly require the proton gradient for their accumulation. There are many specific amino acid transporters identified in yeast responsible for the bidirectional transport across the vacuolar membrane. Influx and efflux is highly balanced to allow storage or amino acid recycling, depending on cellular conditions and needs.

Vacuole contains very high concentrations of phosphate, stored as polyphosphate. It acts as both a macromolecular anion that facilitates the uptake and retention of positive charged ions and amino acids, and as a phosphate store, serving as a cytosolic phosphate buffer during conditions of phosphate depletion (Thomas and O'Shea, 2005).

Vacuole also serves as the main intracellular Ca^{2+} store. Yeast vacuoles membrane contains an H^+/Ca^{2+} antiporter (Vcx1p) which drives Ca^{2+} uptake at the expense of the proton gradient, and a P-type Ca^{2+}-ATPase which operates independently of the proton gradient (Miseta *et al.*, 1999). Vcx1p generates a high uptake of Ca^{2+} but with low affinity, while Pmc1p is important for Ca^{2+} uptake in absence of a functional V-H^+-ATPase or under high concentrations of extracelular Ca^{2+} (Forster and Kane, 2000). In addition, there is a transient receptor potential calcium channel, Yvc1p that allows the release of Ca^{2+} from the vacuole (Denis and Cyert, 2002; Zhou *et al.*, 2003).

The storage and release of other metal ions, like Zn^{2+} and Fe^{3+} follow a similar pattern. Yeast vacuolar membrane contains two zinc transporters, Zrc1p and Cot1p that direct zinc uptake into the vacuole when Zn^{2+} is in excess, while deficiency in zinc leads to transcription of a Zn^{2+} exporter, Zrt3p (MacDiarmid *et al.*, 2000, 2002). Regulation of iron is particularly important because it is a redox-active metal as well as a scarce and essential nutrient. Ccc1p is responsible for iron import (Li *et al.*, 2001), while Fet5p/Fth1p complex and Smf3p transporter are responsible for iron mobilization when Fe^{3+} supply is limited (Singh *et al.*, 2007).

As a storage compartment for a wide variety of ions, the vacuole is the major organelle responsible for intracellular ion homeostasis and response to ion shock. Vacuolar transporters mediate ion homeostasis by regulating ion transport between the vacuole and the cytosol, along with plasma membrane ion transporters that regulate ion

influx/efflux between the cytosol and the extracellular environment. The activity of many vacuolar transporters is dependent on the pH gradient across vacuolar membranes generated by the V-H^+-ATPase. For instance, the late endosomal transporter Nhx1p confers osmotolerance following acute hypertonic shock (Nass and Rao, 1999).

1.1.4.3. Detoxification

Besides its storage functions, vacuole also plays an important role in detoxification. Different toxic molecules are sequestered into the vacuole away from cytosol and other organelles. Some of these molecules are redox-active heavy metals or even nutrients and metabolites that become toxic when in excess. There are two different ATP binding cassette (ABC) transporters present in the vacuolar membrane responsible for detoxification: Ycf1p and Bpt1p (Ghosh *et al.*, 1999; Sharma *et al.*, 2002). These two pumps have analogous mechanisms and specificities, but different regulatory mechanisms. The toxic metals arsenite and cadmium, and accumulated side-products of adenine metabolism are some of their substrates.

1.1.4.4. Response to nutrient deprivation

Autophagy is the vesicular sequestration of cytosolic cargo for vacuolar degradation. In starvation conditions, autophagy can be triggered as a stress response, in order to provide supplementary reserves for the cell (Kim and Klionsky, 2000). There are two main types of autophagy: macroautophagy and microautophagy. Macroautophagy involves the enclosing of cytosolic components by the autophagosome, a double membrane vesicle, with subsequent fusion with the vacuole. Both the inner membrane and autophagosome luminal components are digested and the resulting macromolecules are released back into the cytosol. In microautophagy, the vacuole membrane forms invaginations and cytosolic materials are directly engulfed by the vacuole (Mizushima *et al.*, 2008).

Under starvation conditions, vacuolar hydrolases are produced at a higher rate induced by the influx of material into the vacuole through autophagy, which results in an increase of vacuolar hydrolytic activity (Teichert *et al.*, 1989; Scott *et al.*, 1996; Kim and Klionsky, 2000). The influx of material to the vacuole also leads to the accumulation of membranes. Microautophagy has been observed to occur after

macroautophagy, suggesting that this may be a mechanism for vacuolar membrane recycling (Mijaljica *et al.*, 2007).

1.1.4.5. Response to ionic and osmotic shock

Yeast cells have two different responses to osmotic shock. A long-term response that involves the HOG (high osmolarity glycerol) pathway, leading to production of high levels of glycerol, a cellular osmoprotectant (Hohmann, 2002, 2007), and in contrast, an immediate response that leads to vacuolar fragmentation and release of calcium.

Upon salt shock, vacuoles release calcium via Yvc1p, resulting in a transient increase in the cytosolic Ca^{2+} concentration (Denis and Cyert, 2002). The exact role of transient Ca^{2+} release remains unclear. Ca^{2+} is a key signalling molecule and its release probably activates other cellular responses that alleviate osmotic shock.

Vacuolar fragmentation may be a result of the release of water from vacuoles to maintain proper osmotic pressure in the cytosol. Interestingly, high cytosolic levels of Ca^{2+} also induce vacuolar fragmentation (Kellermayer *et al.*, 2003). Possibly fragmentation is a normal response to Ca^{2+} stress by increasing the surface/volume ratio of the vacuole, to enable maximal sequestration of calcium at the vacuole (Kellermayer *et al.*, 2003).

1.2. Programmed cell death

Since the mid-nineteenth century, many observations have already indicated that cell death plays considerable roles in multicellular organisms, particularly during embryogenesis and metamorphosis (Gluecksmann, 1951; Lockshin and Williams, 1964). The term programmed cell death (PCD) was introduced in 1964, proposing that cell death during development is not of accidental nature but follows a sequence of controlled steps leading to locally and temporally defined self-destruction (Lockshin and Williams, 1964). The term PCD also includes the concept that cell death has been genetically programmed, as this is the case during development and aging. The development and maintenance of multicellular biological systems depends on a complex interaction between the cells of the organism, sometimes involving an

altruistic behaviour of individual cells that trigger suicide in the benefit of the organism as a whole. For instance, during the development, several cells are produced in excess which then undergo PCD, thus contributing to the final shape of the many organs and tissues (Meier *et al.*, 2000). Balance between cell division and cell death is of utmost importance for the development and maintenance of multicellular organisms. Disorders of either process have pathological consequences and can lead for instance to disturbed embryogenesis, neurodegenerative diseases, or the development of cancer.

1.2.1. Apoptosis and its importance

The most frequent and well-defined form of PCD is apoptosis. Apoptosis is of Greek origin, meaning "falling off" or "dropping off", in analogy to leaves falling off trees. This analogy emphasizes that the death of living matter is an integral and necessary part of the life cycle of organisms. The term apoptosis has been coined in order to describe the morphological processes leading to controlled cellular self-destruction and was first introduced in a publication by Kerr and colleagues (Kerr *et al.*, 1972), but other, non-apoptotic types of cell death also might be of biological significance (Leist and Jäättelä, 2001). Therefore, it is worth noting that "programmed cell death" and "apoptosis" are not synonyms because cell death, as it occurs during physiological development, can manifest non-apoptotic features (Barkla and Gibson, 1999; Roach and Clarke, 2000; Baehrecke, 2002).

Apoptotic processes are of a widespread biological importance, being involved in the development, differentiation, proliferation, homeostasis, regulation and function of immune system and in the removal of defective cells. Therefore, defects in apoptotic processes are implicated in several pathological conditions, which may result in cancer, autoimmune diseases and spreading of viral infections, while neurodegenerative diseases, AIDS and isquemias are caused or enhanced by excessive apoptosis (Fadeel *et al.*, 1999a). Due to its importance in such biological processes, apoptosis is a very common phenomenon, occurring in all kinds of metazoans (Tittel and Steller, 2000). Moreover, it plays an important role in plant biology (Solomon *et al.*, 1999), and even unicellular organisms such as yeast exhibit apoptotic-like mechanisms, hence being

used as a simpler eukaryotic model system for apoptosis studies (Fröhlich and Madeo, 2000; Skulachev, 2002).

1.2.2. Apoptotic morphological features

Apoptotic cells can be distinguished by their typical morphological changes: cell shrinks, becomes deformed and loses contact with neighbour cells. Chromatin condenses, plasma membrane buds and finally cell fragments in membranar structures, the apoptotic bodies, which contain cytosol, cell organelles and fragments of condensed chromatin. Apoptotic bodies are then engulfed by phagocytosis by surrounding cells and removed from the tissue (in multicellular organisms), without triggering an inflammatory response (unlike during a necrotic process). All these morphological changes are a result of many biochemical and molecular events that take place in an apoptotic cell, essentially the activation of proteolytic enzymes, which eventually carry out DNA cleavage in fragments, as well as cleavage of multiple proteins involved in cytoplasm integrity and organelle's shape (Saraste and Pulkki, 2000).

1.2.3 Key components of the apoptotic machinery

During apoptosis, the cell is mainly degraded by proteases and endonucleases. The main class of proteases involved in the apoptotic process are caspases (cysteine-dependent aspartate-specific proteases), which play an important role in the triggering and execution of apoptosis. These proteases, when activated, acquire the capacity of cleaving intracellular key-substrates, resulting in morphological and biochemical modifications characteristic of apoptosis (Earnshaw *et al.*, 1999).

Another set of proteins that play a central role in apoptosis is the Bcl-2 family. Bcl-2 family can be defined by the presence of conserved sequence motifs known as Bcl-2 homology domains (BH1 to BH4). In mammals, up to 30 relatives have been described of which some belong to pro-apoptotic members and others to a group of anti-apoptotic members (Borner, 2003). Bcl-2, Bcl-X_L, Bcl-w, A1, and Mcl-1 are examples of anti-apoptotic proteins, which all possess the domains BH1, BH2, BH3, and BH4.

The pro-apoptotic group of Bcl-2 members can be divided into two subgroups: the Bax-subfamily consisting of Bax, Bak, and Bok, all possessing the domains BH1, BH2, and BH3, and the BH3-only proteins (Bid, Bim, Bik, Bad, Bmf, Hrk, Noxa, Puma, Blk, BNIP3, and Spike) which possess only the short BH3 motif, an interaction domain that is both necessary and sufficient for their killing action (Cory and Adams, 2002; Mund *et al.*, 2003).

Inhibitor apoptosis proteins (IAPs) are another class of apoptosis regulators which neutralise pro-apoptotic components and that can in turn be antagonised by other proteins.

1.2.4 Apoptotic signalling pathways

Apoptosis can be triggered by different extra- or intracellular stimuli, such as attachment of death ligands to membrane death receptors, DNA damage caused by faults in DNA repair mechanisms, treatment with cytotoxic compounds or irradiation, lack of survival signals, or development of death signals. Cell death through apoptosis is tightly controlled by changes in protein expression, protein-protein interactions, and various post-translational modifications, including proteolytic cleavage and phosphorylation. For some of these events to occur, proteins must re-localize from one sub-cellular compartment to another to gain access to their substrates or interacting partners. Thus, compartmentalization is yet an important regulatory mechanism, preventing accidental triggering of cell death signalling.

Two main signalling pathways lead to apoptosis: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Figure 2). These two pathways differ in the initiator caspases that transmit the signal but that may later converge at the level of activation of executioner caspases (caspases-3, -6, and -7) (Hengartner, 2000).

1.2.4.1. The extrinsic pathway

The extrinsic pathway is triggered upon binding of specific ligands (such as TNF-α, FasL or TRAIL) to their membrane death receptors (such as TNFR-1 or Fas), which allows the exposure of their death domain (DD) in the cytosol side. Through the DD, adaptor molecules such as FADD or TRADD are recruited to form the death inducing

signalling complex (DISC). The procaspase-8 is then recruited to the DISC by its death-effector domain (DED) and activated (Sartorius *et al.*, 2001; Denault and Salvesen, 2002). Active caspase-8 initiates a caspase cascade by processing the executioner caspases which in turn cleave different protein substrates.

1.2.4.2. The intrinsic pathway

Mitochondrial apoptotic signalling includes the release of pro-apoptotic factors from the mitochondrial intermembrane space to the cytosol such as cytochrome *c* (Salvesen and Renatus, 2002b), apoptosis inducing factor AIF (Susin *et al.*, 1999), the endonuclease endoG (Li *et al.*, 2001), Smac/Diablo (Verhagen *et al.*, 2000) and HtrA2/Omi (Verhagen *et al.*, 2002). In the cytosol, cytochrome *c* contributes to the formation of the apoptosome, a complex that consists of cytochrome *c*, Apaf-1 (an adaptor molecule) and dATP (Acehan *et al.*, 2002), which activates another initiator caspase, caspase-9 (Denault and Salvesen, 2002), hence mediating a caspase cascade by activating caspase-3 (Slee *et al.*, 1999).

1.2.4.3. Type I and type II cells

In some cells (type I cells), the signal coming from the DISC generates a caspase signalling cascade strong enough for execution of cell death on its own. Cells that have the capacity to induce such direct and mainly caspase-dependent apoptotic pathway belongs to the so-called type I cells (Scaffidi *et al.*, 1998).

In type II cells, the amount of active caspase-8 generated is limited and activation of caspase-3 can be blocked by overexpression of Bcl-2 (Scaffidi *et al.*, 1998). Thus, signal coming from the activated receptor must be amplified by the proteolytic activation of the pro-apoptotic protein Bid by caspase-8. In its truncated form (tBid), is translocated to mitochondria with subsequent induction of apoptotic events (Luo *et al.*, 1998). However, it has been recently suggested that the level of XIAP, an inhibitor of apoptosis protein, is the critical discriminator between type I and type II apoptosis signalling (Jost *et al.*, 2009).

1.2.5. The crucial role of mitochondria in apoptosis

Besides amplifying and mediating extrinsic apoptotic pathways, mitochondria also play a central role in the integration and propagation of death signals originating from inside the cell. Most of the events inducing apoptosis involve disruption of trans-membrane mitochondrial potential ($\Delta\psi_m$), as well as the so-called permeability transition (PT), a sudden increase of outer mitochondrial membrane (OMM) permeability to low molecular mass solutes. Simultaneously, an increase of mitochondrial volume takes place caused by water influx to the matrix, eventually originating the rupture of OMM and resulting in a non specific release of pro-apoptotic factors from the mitochondrial intermembrane space to the cytosol (Bernardi *et al.*, 1999; Loeffler and Kroemer, 2000).

1.2.5.1. Permeability transition pore

Several possible mechanisms for PT have been proposed. Although, there is some consensus regarding the formation of the so-called permeability transition pore (PTP), the molecular components involved have not yet been completely identified. The adenine nucleotide translocator (ANT) and the voltage-dependent anion channel (VDAC) have been proposed as their major components, since ANT is the most abundant protein in inner mitochondrial membrane (IMM), and VDAC the most common protein in OMM. Indicated by direct protein-protein interactions, VDAC-ANT complexes presumably could be able to connect IMM and OMM to the so-called "contact sites" corresponding to a close association of the two membranes and thus possibly constituting the PT pore (Beutner *et al.*, 1998). However, subsequent genetic ablation studies have unequivocally shown that neither the ANT nor any VDAC isoform are essential for the PT to occur (Kokoszka *et al.*, 2004; Baines *et al.*, 2007).

Since PT, loss of $\Delta\psi_m$, and release of mitochondrial proteins are of central importance in mediating and enhancing apoptotic pathways, those mitochondrial events must be kept under strict control of regulatory mechanisms which are in many ways dependent on members of the Bcl-2 family. How cytochrome *c* can cross the OMM is not exactly known, but many different mechanisms have been proposed (Muchmore *et al.*, 1966; Reed, 1997; Adams and Cory, 1998; Gross *et al.*, 1999; Antonsson and Martinou, 2000; Suzuki *et al.*, 2000; Basanez *et al.*, 1999, 2002; Hardwick and Polster,

2002), with none of them having been proven definitively: formation of a non specific pore, through which cytochrome *c* and other intermembrane space proteins can escape; heterodimerization between pro- and anti-apoptotic family members, since Bax and Bak display structural similarities with bacterial pore-forming toxins, leading to the hypothesis that these proteins themselves might directly form pores in the OMM; interaction with other mitochondrial proteins, either to generate a pore for cytochrome *c* exit, or to modulate mitochondrial homeostasis (for example, opening of the PTP); oligomerization to form a weakly selective ion channel; and interaction of activated Bax and Bak with OMM lipids leading to membrane bending and, ultimately, formation of transient lipid pores or inverted micelles. Additionally, it was recently suggested that protein release from mitochondria can occur through ceramide channels. The hypothesis of such channels are involved early in apoptosis is strongly supported by their regulation by the Bcl-2 family proteins. The regulation observed is in the direction concurrent with their role in apoptosis: anti-apoptotic proteins inhibit ceramide channels while the pro-apoptotic proteins enhance these channels (Ganesan and Colombini, 2010).

In addition to mitochondrial factors release, dissipation of $\Delta\psi_m$ and PT also cause changes in cell homeostasis: synthesis of ATP is interrupted, molecules involved in redox reactions oxidize, and reactive oxygen species (ROS) are generated (Kroemer and Reed, 2000; Kroemer *et al.*, 1997). High levels of ROS directly cause oxidation of macromolecules, thus contributing to $\Delta\psi_m$ disruption and acting as a positive feedback (Marchetti *et al.*, 1997).

1.2.6. Regulatory mechanisms

The pro-apoptotic factor Smac/Diablo, which is released from mitochondria, acts by inhibiting the inhibitors of apoptosis proteins (IAPs; Du *et al.*, 2000), a family of proteins with anti-apoptotic activity by directly inhibiting caspases (Salvesen and Duckett, 2002). IAP expression is upregulated in response to survival signals, hence providing a means to suppress apoptosis signalling (Raff *et al.*, 1993; Ameisen, 2002).

Members of the BH3-only subfamily are required for the activation of pro-apoptotic Bax/Bak (Bouillet and Strasser, 2002). Bax is a cytosolic monomer in viable

cells that during apoptosis changes its conformation, integrates into the OMM and oligomerizes (Nechushtan *et al.*, 2001). Although the mechanism is still controversial, Bax and Bak oligomers are believed to contribute to the mitochondrial PT, either by forming channels by themselves (Antonsson *et al.*, 2000) or by interacting with components of the PT pore such as VDAC (Tsujimoto and Shimizu, 2000).

BH3-only proteins interfere with the fine-tuned balance of homo- or hetero-oligomerization between pro-apoptotic multidomain members Bax/Bak and anti-apoptotic members Bcl-2/Bcl-X_L. BH3 domains of Bid and Bim can directly mediate Bax/Bak oligomerization, whereas Bad and Bik BH3 domains preferentially interact with anti-apoptotic Bcl-2/Bcl-X_L. As a consequence, activated Bad/Bik might be able to displace Bid/Bim from the binding pocket of anti-apoptotic Bcl-2/Bcl-X_L, and thus released Bid/Bim induce Bax/Bak oligomerization and cytochrome *c* release, even at low levels (Letai *et al.*, 2002).

The anti-apoptotic Bcl-2 family members play a central role by guarding mitochondrial integrity and by controlling the release of mitochondrial proteins into the cytoplasm, thus counteracting the action of pro-apoptotic proteins and inhibiting mitochondrial pro-apoptotic events (Cory and Adams, 2002). Anti-apoptotic Bcl-2 members sequester proapoptotic Bcl-2 members by binding to their BH3 domains and hence preventing Bax or Bak activation/ oligomerization and consequently inhibiting mitochondrial proapoptotic events. Overexpression of Bcl-2 or Bcl-X_L potently inhibits apoptosis in response to many cytotoxic insults, e.g. by suppressing the generation of ROS, stabilizing $\Delta\psi_m$, preventing PT and consequently blocking the release of cytochrome *c* (Reed, 1999).

BH3-only proteins are regulated either by post-translational modifications or by transcription. For instance, Bid is activated by cleavage as referred before, Bim and Bad are activated by dephosphorylation, and Puma and Noxa are transcriptionally regulated by the p53 transcription factor. Similar events occur with anti-apoptotic Bcl-2 proteins (Youle and Strasser, 2008). The fate of the cell is therefore determined by the balance between the intracellular levels of anti-apoptotic and pro-apoptotic proteins (Brenner and Mak, 2009).

Apoptotic signals coming from the inside of the cell frequently have their origin within the nucleus. DNA damage usually results in the activation of p53, which promotes expression of pro-apoptotic Bcl-2 members and suppresses anti-apoptotic

members Bcl-2 and Bcl-X_L. Other organelles besides mitochondria and the nucleus, such as the endoplasmatic reticulum (ER) and lysosomes also have been implicated in apoptotic signalling pathways, and it should be kept in mind that probably hundreds of proteins are part of an extremely fine-tuned regulatory network consisting of pro- and anti-apoptotic factors (Hengartner, 2000).

1.2.7. Apoptosis in yeast

The occurrence of an apoptotic process in yeast was first described in cells with a mutation in the AAA-ATPase *CDC48* gene that plays a role in cell division and vesicle trafficking (Madeo *et al.*, 1997). These cells displayed apoptotic markers such as chromatin condensation, DNA fragmentation, externalization of phosphatidylserine to the outer leaflet of plasma membrane and ROS generation. Moreover, apoptotic scenarios have been observed in yeast undergoing cell death such as mitochondrial fragmentation, cytochrome *c* release, perturbations of the cytoskeleton and modifications in the chromatin (Carmona-Gutierrez *et al.*, 2010). In addition, if not engulfed by neighboring cells or in cell culture, where phagocytosis does not usually happen, dead cells in the late stages of apoptosis may present necrotic features due to the loss of cellular energy and plasma membrane integrity. This process is called "apoptotic necrosis" or "secondary necrosis" (Majno and Joris, 1995) and constitutes the ultimate fate of the yeast apoptotic cell. Since the first description of apoptosis in yeast, a growing number of proteins homologues of apoptotic regulators in higher eukaryotes have been identified in yeast, such a caspase Yca1p/Mca1p (Madeo *et al.*, 2002), Nma111p the orthologue of HtrA2/Omi (Fahrenkrog *et al.*, 2004), Aif1p the orthologue of AIF (Wissing *et al.*, 2004), Bir1p, an yeast IAP (Walter *et al.*, 2006), and the yeast endonuclease G (Büttner *et al.*, 2007). Additionally, it has been suggested that Rad9 protein from *Schizosaccharomyces pombe* could belong to Bcl-2 family, being the first protein from this family identified in yeast (Komatsu *et al.*, 2000). These results have suggested that apoptosis occurring in yeast has many similarities to apoptosis in metazoans. A large number of external stimuli and stress conditions have also been described as apoptosis inducers in yeast, including H_2O_2 (Madeo *et al.*, 1999), acetic acid (Ludovico *et al.*, 2001), replicative ageing (Laun *et al.*, 2001), chronological

ageing (Herker *et al*., 2004), UV radiation (Del Carratore *et al*., 2002), exposure to high concentrations of mating pheromone (Severin and Hyman, 2002), salt stress (Huh *et al*., 2002), hyper-osmotic stress (Silva *et al.*, 2005), and exposure to yeast killer toxins (Reiter *et al*., 2005). Moreover yeast cells undergoing replicative ageing (Laun *et al*., 2001) or chronological ageing (Herker *et al*., 2004) also die by apoptosis.

An explanation for such a process in which a unicellular organism commits to cellular suicide, has been provided by demonstrating its role in several physiological scenarios, for example, during meiosis, ageing and mating (Büttner *et al.*, 2006; Laun *et al.*, 2001; Fabrizio *et al.*, 2004; Henker *et al.*, 2004). Budding yeast grows as multicellular colonies that are capable of simple differentiation. Each colony arises from a single cell that has undergone many divisions. Thus sacrifice of an aged cell containing high levels of ROS and potentially damage DNA acts as a selective advantage for the yeast population as a whole, protecting the integrity of the original cell's genome and allowing the spreading of the clone. Hence, in the case of *S. cerevisiae* one should consider it as a multicellular entity in which apoptosis acts as a self-preservation mechanism for the colony as a whole (Gourlay *et al.*, 2006; Carmona-Gutierrez *et al.*, 2010).

1.2.8. Apoptosis induced by acetic acid

Acetic acid is a final product of alcoholic fermentation produced by *S. cerevisiae*. Glucose-repressed yeast cannot metabolize this compound that enters the cell in its non-dissociated form by simple diffusion. Within the cell, acetic acid dissociates and promotes internal acidification, ion accumulation and inhibition of cell metabolic activity, namely fermentation/respiration (Leão and van Uden, 1986; Cássio *et al.*, 1987; Pampulha and Loureiro, 1989). Moreover, it was demonstrated that, in *S. cerevisiae*, under certain conditions acetic acid compromises cell viability, resulting in cell death (Pinto *et al.*, 1989). Nevertheless, the process by which yeast lose viability when damaged by acetic acid is not yet completely understood.

Cell death in stress conditions induced by acetic acid has been characterized in our Laboratory (Ludovico *et al.*, 2001, 2002 2003). After treatment with acetic acid, apoptotic markers such as chromatin condensation, phosphatidylserine exposure and

DNA fragmentation were observed, indicating that acetic acid, like H_2O_2 (Madeo *et al.*, 1999), is able to activate an apoptotic process in *S. cerevisiae* (Ludovico *et al.*, 2001). Moreover, high doses of acetic acid (120-200 mM) lead to a necrotic phenotype in exponential phase cells of *S. cerevisiae* whereas low doses (20-80 mM) trigger a PCD exhibiting characteristics of mammalian apoptosis (Ludovico *et al.*, 2001).

Yeast mitochondria play a key role in the apoptotic death process activated by acetic acid (Ludovico *et al.*, 2002; 2003). *S. cerevisiae* cells committed to apoptosis in response to acetic acid display mitochondrial ROS accumulation, and mitochondria become permeabilized, promoting the release of lethal factors like cytochrome *c* (Ludovico *et al.*, 2002) and Aif1p (Wissing *et al.*, 2004), hence contributing to the death process.

Yeast genetic approaches revealed that while deletion of POR1 (yeast VDAC) enhances apoptosis triggered by acetic acid, absence of ADP/ATP carrier (AAC) proteins (yeast orthologues of ANT) protects cells exposed to acetic acid (Pereira *et al.*, 2007). Absence of AAC proteins does not completely prevent acetic acid-induced apoptosis, suggesting that alternative redundant pathways are involved. One such pathway may be the translocation of Aif1p from the mitochondria to the nucleus in response to acetic acid (Wissing *et al.*, 2004). Other mitochondrial proteins have been implicated in the execution of the yeast apoptotic program induced by acetic acid, including those involved in fission/fusion, namely Fis1p, Dnm1p, Mdv1p (Fannjiang *et al.*, 2004) and Nuc1p, the yeast ortholog of the mammalian endonuclease G (Büttner *et al.*, 2007). Ysp2p is another mitochondrial protein with a direct function in mitochondria-mediated PCD, since its absence confers resistance to acetic acid-induced PCD (Sokolov *et al.*, 2006). The metacaspase Yca1p is activated in cells undergoing acetic acid-induced apoptosis (Pereira *et al.*, 2007), although cells lacking Yca1p also undergo apoptosis in response to acetic acid, which suggests the existence of a caspase-independent pathway (Guaragnella *et al.*, 2006).

The Kex1p protease, involved in PCD caused by defective N-glycosylation, also contributes to the active cell death program induced by acetic acid stress (Hauptmann *et al.*, 2006). Transient proteasome activation is also necessary for protein degradation during acetic acid-induced apoptosis (Valenti *et al.*, 2008).

1.3. Role of vacuole/lysosome in apoptosis

Over the last decade, the lysosome has appeared as an important component of the cellular death machinery. In the classic concept of cell death, lysosomes were exclusively considered involved in necrotic and autophagic cell death. The concept of lysosomal constituents participating in cellular pathology and degeneration was originally proposed by De Duve (De Duve, 1966). After the proposal that lysosomal components could be involved in programmed cellular degeneration, several studies have attempted to evidence the role of lysosomes in triggering apoptosis.

There are several lines of evidence that sustain the participation of lysosomes in apoptosis. Leakage of lysosomal proteases into the cytosol is involved in the activation of caspases (Ishisaka *et al.*, 1998). Active participation of lysosomal proteases, cathepsins, has also been observed in cell death induced by several stimuli, including TNF-α (Guicciardi *et al.*, 2000; Foghsgaard *et al.*, 2001; Guicciardi *et al.*, 2001; Werneburg *et al.*, 2002), bile salt-induced apoptosis (Roberts *et al.*, 1997; Canbay *et al.*, 2003), and chemotherapeutic drugs (Johansson *et al.*, 2003; Bröker *et al.*, 2004). Moreover, lysosomal apoptotic pathway, either cathepsin-dependent or independent, is believed to proceed through mitochondria (Boya *et al.*, 2003a; Droga-Mazovec *et al.*, 2008), suggesting a crosstalk between lysosomes and mitochondria.

1.3.1. Mechanisms underlying lysosome membrane permeabilization

Lysosome membrane permeabilization (LMP) can be triggered by an extensive range of apoptotic stimuli (reviewed by Johansson *et al.*, 2010). These include pro-apoptotic Bcl-2 family proteins, ROS, cathepsins, calpains, caspases, changes in lysosomal membrane lipid composition, proteins from pathogenic bacteria and virus, and p53.

Several studies have suggested that the magnitude of LMP and consequently the amount of proteolytic enzymes released into the cytosol are key factors in determining the type of cell death (necrosis *versus* apoptosis) mediated by lysosomal enzymes (Li *et al.*, 2000). Massive collapse of lysosomes causes necrosis, while moderate permeabilization of lysosomes leads to apoptosis (Turk *et al.*, 2002). Importantly, in

some contexts, LMP is an early event essential for apoptosis signalling to proceed, while under other circumstances it occurs late in the apoptotic process, contributing to amplification of the death signal.

The mechanism underlying LMP is not yet completely understood. Besides cathepsins, other hydrolases (Nylandsted *et al.*, 2004; Kågedal *et al.*, 2005; Blomgran *et al.*, 2007; Miao *et al.*, 2008), H^+ (causing acidification of the cytosol) (Nilsson *et al.*, 2006), Ca^{2+} (Mirnikjoo *et al.*, 2009), and many lysosomotropic dyes (Brunk *et al.*, 1997; Kågedal *et al.*, 2001b; Werneburg *et al.*, 2002) can escape into the cytosol at the onset of apoptosis. This may suggest a nonspecific release mechanism due to limited membrane damage or through a pore, rather than selective transport across the membrane. However, the release of H^+, Ca^{2+}, and lysosomotropic dyes does not necessarily reflect a change in membrane integrity, but could merely be due to a change in ion pump activity (Johansson *et al.*, 2010).

1.3.2. The crosstalk between lysosomes and mitochondria during cell death

Increasing evidence indicates that lysosomes communicate with mitochondria during cell death. This may occur through the selective leakage of proteins that activate some forms of apoptotic cell death, or through the massive lysosomal permeabilization that leads to an uncontrolled cell dismantling characteristic of necrosis. Although cells manifest large-scale autophagy shortly before or during cell death, autophagy is a pro-survival process. In response to metabolic stress, autophagy can indeed delay apoptotic cell death, and in apoptosis-defective cells it was demonstrated that inactivation of autophagy promoted cell death by necrosis (White, 2008).

Similarly to other cellular organelles, damaged mitochondria are removed by autophagy (mitophagy; Chu *et al.*, 2007). In apoptosis, this mechanism becomes particularly significant. For instance, degradation of mitochondria that undergo PT are frequently removed by mitophagy (Rodriguez-Enriquez *et al.*, 2006), therefore preventing the release of pro-apoptotic factors (Geisler *et al.*, 2010).

Lysosomes are the end point of autophagic pathway. So that if lysosomes are destabilized prior to mitochondria, the cell loses the capacity of degrade autophago-cytosed mitochondria. Thus, induction of apoptosis and LMP would therefore not only

amplify the pathway leading to MOMP but also prevent cells from rescue by autophagy (Groth-Pedersen *et al.*, 2007; Hoyer-Hansen and Jäättelä, 2008; Turk and Turk, 2009). In conclusion, LMP amplify cell death in two ways: i) amplifying MOMP by cleaving Bid and degrading other anti-apoptotic factors following the release of cathepsins, and ii) inhibiting autophagy downstream of autophagosome formation.

1.3.3. Lysosomal cathepsins

Lysosomes are equipped with more than 50 acid hydrolases, including phosphatases, nucleases, glycosidases, proteases, peptidases, sulphatases, and lipases (De Duve, 1983), thus representing the main reservoir of nonspecific proteases in mammalian cells. The family of cathepsins, which belongs to lysosomal hydrolases, is the best described. Cathepsins are subdivided, according to their active site amino acids, into cysteine (cathepsins B, C, F, H, K, L, O, S, V, W, and X), serine (cathepsins A and G), and aspartic cathepsins (cathepsins D and E; Turk and Stoka, 2007).

Cathepsins have been thought to be involved only in protein degradation within lysosomes, and their function outside lysosomes has been ignored because of their instability at neutral pH (Turk *et al.*, 2000, 2002). However, it has become evident that cathepsins can remain active at neutral pH from several minutes to several hours (Turk *et al.*, 1993), allowing temporary activity in the cytosol. Intralysosomal concentration of cathepsins can reach 1 mM (Mason, 1996), so they represent a huge destructive potential if released from lysosomes. The localization of cathepsins into the lysosome prevents the cell from their harmful action (Turk and Stoka, 2007), and allows the regulation of the release of cathepsins to the cytosol. Thus, compartmentalization is essential to prevent accidental leakage of components and triggering cell death signalling pathways (Figure 2) (Boya and Kroemer, 2008).

It is now commonly accepted that apoptosis is frequently associated with release of cathepsins into the cytosol. Accordingly, it has been reported that microinjection of cathepsins into the cytosol is enough to induce apoptosis (Roberg *et al.*, 2002; Bivik *et al.*, 2006; Schestkowa *et al.*, 2007).

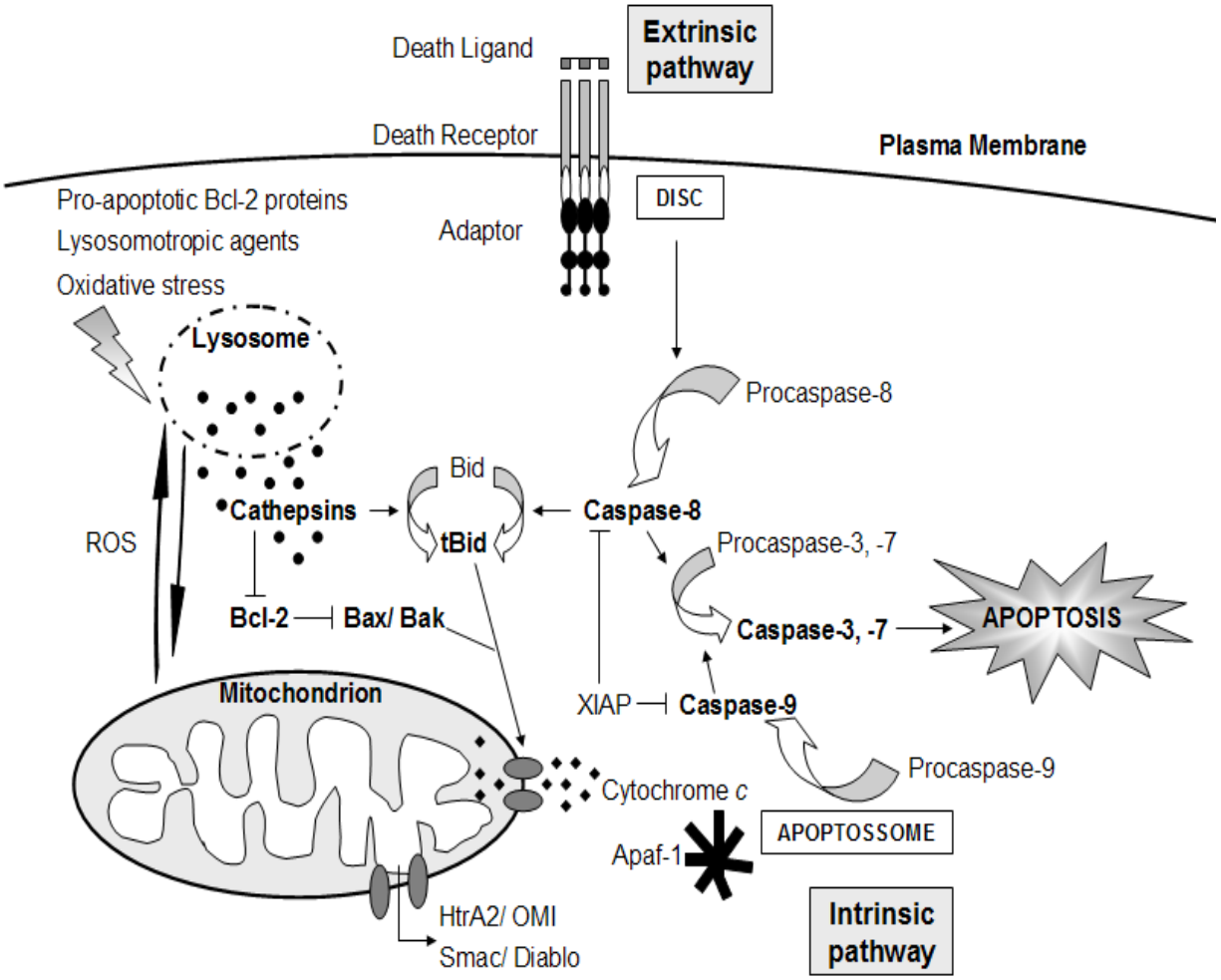

Fig. 2. The link between lysosome, mitochondria and apoptosis. Pro-apoptotic Bcl-2 proteins, lysosomotropic agents or oxidative stress induce LMP and the release of lysosomal content into the cytosol. Cathepsins cleave Bid and degrade anti-apoptotic Bcl-2 proteins. Bax and Bak are activated and OMM is permeabilized, triggering the intrinsic apoptotic pathway, with the release of cytochrome *c* and the formation of the apoptosome. Extrinsic apoptotic pathway is triggered by the binding of death ligands to the death receptors, with the recruitment of adaptor proteins and the formation of the DISC. In both pathways, executioner caspases are activated, with Bid cleavage being the linker between both. Also in both pathways, ROS and other factors are involved in the feedback between lysosomes and mitochondria, which amplifies apoptotic cell death (adapted from Repnik and Turk, 2010).

Cathepsin release may result in a caspase-dependent or -independent cell death process with or without involvement of mitochondria, depending on cellular context and the death stimulus (Boya and Kroemer, 2008; Kirkegaard and Jäättelä, 2009).

Cathepsins can act through the cleavage of Bid (Houseweart *et al.*, 2003; Cirman *et al.*, 2004; Blomgran *et al.*, 2007; Stoka *et al.*, 2001, 2007; Droga-Mazovec *et al.*, 2008) and by degradation of the anti-apoptotic Bcl-2 proteins Bcl-2, Bcl-X_L and Mcl-1 (Blomgran *et al.*, 2007; Droga-Mazovec *et al.*, 2008), but also caspase-2, phospholipase A2 (PLA2) and sphingosine kinase-1 may all be targets of cathepsins. Cathepsins can also act downstream of mitochondria by degrading XIAP, hence facilitating caspase activation (Droga-Mazovec *et al.*, 2008). Importantly, cathepsins appear to carry both the ability to act as initiator as well as effector proteases of PCD depending on the stimulus and the cellular context.

1.3.3.1. The role of cathepsin D

In 1998, Roberg and Öllinger discovered that cathepsin D was released from lysosomes to the cytosol upon oxidative stress-induced apoptosis, being the first identified lysosomal protein with apoptogenic properties (Roberg and Öllinger 1998). Of all lysosomal proteins, cysteine cathepsins and the aspartic cathepsin D are most commonly associated with cell death.

Cathepsin D (also known as proteinase A) is a glycoprotein of A1 aspartyl proteases family, which hydrolyses peptide bonds in polypeptides (Faust *et al.*, 1985). Cathepsin D is active at an optimal acidic pH ranging from 3.5 to 4, and loses its activity reversibly at a neutral pH due to deprotonation of both active site aspartate residues. However, considerable activity may exist transiently, due to acidification of the cytosol by lysosomal rupture (Conus *et al.*, 2008; Turk and Turk, 2009).

The involvement of cathepsin D in cell death has been demonstrated both in lysosomal (Conus *et al.*, 2008; Kågedal *et al.*, 2001) and death receptor apoptotic pathways (Heinrich *et al.*, 2004). Moreover, caspase-8 has been identified as a substrate of cathepsin D during apoptosis (Conus *et al.*, 2008). Several evidences suggest that an increase of cathepsin D concentration in the cytosol has a large impact on apoptosis. Unlike the cysteine lysosomal proteases, the aspartic proteases have no endogenous cytosolic inhibitors that limit its proteolytic activity (Matus and Green, 1987).

It has been observed an increase in expression and/or activity of cathepsin D in apoptotic cells after exposure to several apoptotic agents (reviewed by Repnik and Turk, 2010), such as death ligands TNF-α and Fas, p53, ROS, sphingosine, and chemotherapeutic drugs. It has been also demostrated that during apoptosis induced by oxidative stress, the release of cathepsin D from lysosomes to the cytosol preceded cytochrome *c* release and loss of $\Delta\psi_m$. When cells were treated with pepstatin A, a cathepsin D inhibitor before exposure to oxidative stress, it was not observed release of cytochrome *c* nor caspase-3 activation, and apoptosis was arrested (Öllinger, 2000; Kågedal *et al.*, 2001; Roberg *et al.*, 1999; Roberg, 2001). Using specific pharmacological inhibitors and the siRNA technique, it has been shown that cytosolic cathepsin D induces Bax activation and relocation to the mitochondria, resulting in the release of AIF and triggering of apoptosis (Bidère *et al.*, 2003). Moreover, overexpression of cathepsin D has lead to activation of both caspases-9 and -3 after treatments with etoposide (Beaujouin and Liaudet-Coopman, 2008). Together, these results suggest that translocation of cathepsin D to the cytosol plays an important role in apoptosis induction, acting as a key mediator of pro-apoptotic factors release and activation of caspases.

1.3.3.2. The role of Pep4p in yeast

A recent study demonstrated that the vacuole communicates with mitochondria in yeast cells undergoing apoptosis (Pereira *et al.*, 2010). The crosstalk between mitochondria and the vacuole when autophagy is inhibited seems to involve a partial release of Pep4p, an orthologue of human cathepsin D, engaged in the proteolytic degradation of mitochondria, rather than an extensive vacuolar permeabilization.

It has been shown that Pep4p protease has a role in mitochondrial degradation. This protease is released from the vacuole into the cytosol in response to acetic acid treatment. Depletion of Pep4p delays and accelerates apoptosis and secondary necrosis, while overexpression enhances mitochondrial degradation (Pereira *et al.*, 2010). Thus, the process of mitochondrial degradation by Pep4p appears to have a protective role in acetic acid-treated cells. The finding that the vacuolar Pep4p interferes with mitochondrial degradation suggests a complex regulation and interplay between mitochondria and the vacuole in yeast PCD.

Although cathepsin D was shown to have a role in mammalian cell death by inducing mitochondrial dysfunction and subsequent release of pro-apoptotic factors, some studies reported an inhibitory role for cathepsin D in PCD (Jäättelä *et al.*, 2004; Chwieralski *et al.*, 2006; Liaudet-Coopman *et al.*, 2006; Minarowska *et al.*, 2007).

Taken together, these observations suggest that vacuole and mitochondria destabilization, measured by Pep4p and cytochrome *c* release, respectively, are events in the yeast cell death cascade. Although a role for cathepsin D in mitochondrial degradation in mammals has not been assessed so far, it is possible that cathepsin D can be indeed associated with mitochondrial degradation and cell protection against an apoptotic stimulus. This hypothesis whether confirmed will unravel a novel process for maintenance of intracellular homoeostasis by ensuring the degradation of damaged mitochondria and highlight a pro-survival role of a specific protease when released out of the lysosome. Whether Pep4p acts directly on mitochondria or activates other proteases remains to be elucidated.

2. Aims

The present study aimed to contribute to the understanding of the role of the vacuole in yeast apoptosis, in particular the one induced by acetic acid. Taking advantage of the yeast genetic tractability and of the use of isolated intact vacuoles, the effect of acetic acid on vacuole membrane permeabilization and on the selective release of Pep4p were investigated in relation to alterations of the vacuole function. *S. cerevisiae* strains with different genetic backgrounds were used.

The preparation of spheroplasts and purification of intact vacuoles benefited from the method previously optimized in our laboratory to isolate vacuoles from other cell models. Epifluorescence microscopy and flow cytometry analysis were used to characterize the purity and integrity of intact vacuoles. Functionality was monitored by the activity of V-H^+-ATPase by spectrofluorimetry.

The effect of acetic acid on vacuolar function was tested both in whole cells and isolated vacuoles using fluorescent probes combined with flow cytometry and epifluorescence microscopy. Selective permeabilization of the vacuole membrane to Pep4p in response to acetic acid was followed in whole cells expressing the chimeric protein Pep4p-EGFP and in vacuoles isolated from this transformant strain. Further investigation of the role of the vacuole in mitochondria-dependent death pathways will offer new perspectives for an enhanced understanding of the role of lysosome in mammalian apoptosis.

3. Experimental procedures

3.1. Yeast strains and growth conditions

Saccharomyces cerevisiae wild type strain W303-1A (*MATα, ade2, his3, ura3, leu2, trp1*) was grown in YEPD medium with 1% (w/v) yeast extract, 1% (w/v) bactopeptone and 2% (w/v) glucose.

S. cerevisiae W303 1A strain transformed with the plasmid p416ADH-PEP4-EGFP (Mason *et al.*, 2005) was used to assess the release of Pep4p from the vacuole. Transformant strains were grown in SC medium (0.67%, w/v) yeast nitrogen base without amino acids, 2% (w/v) glucose, supplemented with adenine (2 g/L), histidine (2 g/L), leucine (10 g/L) and triptophan (5 g/L). Transformants were selected in supplemented selective medium lacking uracil.

All strains were kept at −80°C in micro tubes containing adequate culture medium supplemented with glycerol (30%, v/v). Frozen aliquots of yeast strains were streaked onto plates containing the adequate culture medium and incubated for 48 h at 25°C prior to each experiment. All strains were inoculated at an OD of about 0.008 and grown overnight to log phase (OD_{640} = 0.5-0.8) in 500 mL flasks with 250 mL of medium, in an orbital shaker, at 30 °C, 200 rpm.

3.2. Reagents

Zymolyase-20T was purchased from ImmunO™. *N*-(3-triethylammoniumpropyl)-4-(4-(dibutylamino) styryl) pyridinium dibromide (FM1-43), Fluo-4 AM, Acridine Orange and 9-amino-6-chloro-2-methoxyacridine (ACMA) were purchased from Invitrogen-Molecular Probes (Eugene, OR, USA). Yeast nitrogen base, peptone and yeast extract were purchased from Difco. Calcimycin, concanamycin A, amino acids and Ficoll PM400 were purchased from Sigma-Aldrich.

3.3. Vacuole isolation and purification

The protocol of Law *et al.* (1995) was combined with the procedure of Uchida *et al.* (1985) to isolate and purify the vacuole fraction from exponentially growing cells (OD_{640} = 0.5-0.8) of *S. cerevisiae*. The amount of cells was calculated considering that 1 OD_{550} corresponds to 1 g of dry weight per L of culture. Cells were collected by centrifugation in 250 mL bottles at 5000 rpm (Sigma 4K10) for 5 min and washed twice with deionised water. Cells were then incubated in 10 mL of pre-incubation buffer (0.5 M β-mercaptoethanol; 0.1 M Tris pH 7) per g of dry weight, for 15 min at 32 °C, centrifuged for 5 min at 5000 rpm, and washed twice with Tris/KCl buffer (10 mM Tris pH 7; 0.5 M KCl). Cell wall digestion occurred in 10 mL of digestion buffer (1.35 M sorbitol; 10 mM citric acid; 30 mM Na_2HPO_4; 1 mM EGTA) containing 10 mg of zymolyase 20T (previously dissolved in digestion buffer, at 32 °C) per 10 mL, at 32 °C under agitation. Digestion was monitored by phase contrast microscopy after 30 min of incubation and every 10 min afterwards. Cells with digested cell wall lose refringence comparatively to cells with non-digested cell wall. After most of the cell walls were digested, the reaction was stopped by centrifugation at 7700 *g* for 5 min, followed by a wash with digestion buffer without zymolyase. Supernatant was discarded, and the pellet was resuspended in 5 volumes of 12% Ficoll buffer (10 mM MES/Tris pH 6.9; 0.1 mM $MgC1_2$; 12% Ficoll), homogenised on the vortex and with a pipette, and centrifuged at 4500 *g* for 10 min. Supernatant (10 mL) was transferred to a centrifuge tube and overlaid with 5 mL of 12% Ficoll buffer. Centrifugation was performed in a swing-out bucket rotor (Beckman, rotor type SW28) at 26600 *g* for 30 min at 4 °C. The fraction floating on the top of the tube containing the crude vacuoles was collected and resuspended by vortexing in 5 mL of 12% Ficoll buffer. The homogenized crude vacuoles were overlaid in a centrifuge tube with a layer of 5 mL of 8% Ficoll buffer (10 mM MES/Tris pH 6.9; 0.1 mM $MgC1_2$; 8% Ficoll) and re-centrifuged in the same conditions. Purified vacuoles were collected from the top of the tube and resuspended in 8% Ficoll buffer. Protein concentration was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as the protein standard.

3.4. Fluorochrome solutions and staining protocols

The analysis of purified vacuole suspensions was performed by epifluorescence microscopy and by flow cytometry combined with different fluorescent probes. The preparation of the fluorochrome solutions used and the respective staining mechanisms are described below.

FM1-43 and similar styryl fluorescent dyes are amphipathic molecules that have been used to monitor membrane trafficking (Cochilla *et al.*, 1999). FM1-43 was used herein to stain the vacuolar membrane and monitor its integrity as described earlier (Emans *et al*., 2002; Conde *et al*., 2007b). This fluorochrome is readily soluble in water but essentially nonfluorescent until bound to membranes. A concentrated stock solution of FM1-43 (1 mM) was prepared in water, and stored at -20ºC. To analyse the membrane of isolated vacuoles 500 µL (13.4 mg protein /ml) of purified vacuole suspension was incubated with 0.5 µM of FM1-43, for 15 min at room temperature in the dark.

The calcium probe Fluo-4 AM (Molecular Probes, Eugene, OR, USA) was used to study the vacuole ability to accumulate calcium. This probe is commonly used as the non-fluorescent acetoxymethyl ester (Fluo-4 AM) which is cleaved inside the cell to give the free green fluorescent Fluo-4. Fluo-4 exhibits large fluorescence intensity increases on binding Ca^{2+}. A stock solution (455 µM) was prepared in DMSO and stored at -20ºC. To assess Ca^{2+} accumulation in the vacuole 500 µL (13.4 mg protein /ml) of purified vacuole suspension was incubated with 0.5 µM of Fluo-4 AM, for 60 min at room temperature in the dark. When used, the calcium ionophore calcimycin was added to the vacuolar suspension at a final concentration of 300 µM.

The fluorescent probe Acridine Orange (Gluck *et al.*, 1982) was used to monitor vacuolar pH. Acridine Orange (AO) is a weak base which accumulates in a pH dependent way in acidic compartments of cells by an ion-trap mechanism (Oparka, 1991). AO as a cation also interacts with nucleic acids by intercalation and electrostatic attractions. It has green fluorescence with an emission maximum at 525 nm (green fluorescence) when bound to double stranded nucleic acids (DNA), while upon association with single-stranded nucleic acids (RNA) its emission is shifted to ~650 nm (red fluorescence). This implies that caution is required in the interpretation of the

fluorescence pattern it generates (Steinberg and Swanson, 1994). For this study, a concentrated stock solution of AO (1 mM) was prepared in water, and stored at -20ºC.

To assess the effect of acetic acid on vacuolar pH, 500 µL (13.4 mg protein /ml) of purified vacuole suspension was incubated with 1 µM AO for 10 min in the dark at room temperature.

3.5. Analysis by epifluorescence microscopy

Whole cells and intact vacuoles were observed under a Leica Microsystems DM-5000B epifluorescence microscope with appropriate filter settings. Images were acquired with a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software. Membrane integrity was assessed by FM1-43, and Ca^{2+} sequestration into the vacuole was monitored with Fluo-4 AM.

3.6. Analysis by flow cytometry

Flow cytometry was exploited to characterize homogeneity and functionality of the purified vacuole samples, and to study the effect of acetic acid on vacuole membrane permeabilization and on the selective release of Pep4p. Sample analysis by flow cytometry was performed in an Epics® XL™ (Beckman Coulter) flow cytometer, equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. Monoparametric detection of red fluorescence from Acridine Orange was performed using FL-3 (488/620 nm) and detection of green fluorescence from Acridine Orange, FM1-43, Fluo-4, and EGFP was performed using FL-1 (488/525 nm). For each sample, at least 20000 events were analysed at low flow rate. An acquisition protocol was defined to measure forward scatter (FS), side scatter (SS) and green fluorescence (FL1) on four decade logarithmic scales.

3.7. Determination of the proton pumping activity of V-H^+-ATPase

For proton-pumping measurements the pH-sensitive fluorescent probe 9-amino-6-chloro-2-methoxyacridine (ACMA) was used (Fontes *et al.*, 2010; Queirós *et al.*, 2009, Silva *et al.*, 2010). The reaction medium (2 mL) contained 100 mM KCl, 2 mM $MgCl_2$, 0.1% BSA (w/v), 10 mM MOPS-Tris (pH 7.2), 2 μM ACMA and 100 μL intact vacuoles (1.34 mg protein). Reaction cuvettes of quartz were placed in a dark chamber of the spectrophotometer and were equilibrated with stirring for 5 min before fluorescence readings. Fluorescence quenching was monitored at 25 °C in a Perkin Elmer LS-5B fluorescence spectrophotometer at excitation and emission wavelengths of 415 nm and 485 nm, respectively. The reaction was initiated by the addition of ATP (0.22 - 3 mM, final concentrations) and quenching of fluorescence was allowed to proceed until a steady-state had been achieved. The initial rates of ACMA fluorescence quenching in the presence of ATP plus 500 nM concanamycin A, a specific inhibitor of V-H^+-ATPase, were regarded as the initial rates of H^+-transport activity (Δ%F min^{-1} mg^{-1} protein).

4. Results

4.1. Characterization of isolated vacuoles from *S. cerevisiae*

Spheroplasts were prepared by enzymatic digestion of yeast cells with Zymolyase-20T (Figure 3A), and vacuoles were released and purified by two steps of Ficoll gradient centrifugation (Figure 3 B). Each fractionation procedure yielded an average of 0.17 mg of total vacuolar protein per mg of cell dry weight.

To observe the vacuoles under the fluorescence microscope, the styryl dye FM 1-43 was used as described earlier (Fontes *et al.*, 2010). This fluorescent probe exhibits weak fluorescence in aqueous medium, but shines brightly when inserted into membranes (Betz *et al.*, 1996). Incubation with FM1-43 resulted in a strong staining of the vacuolar membrane (not shown), demonstrating the intactness of these extremely fragile organelles.

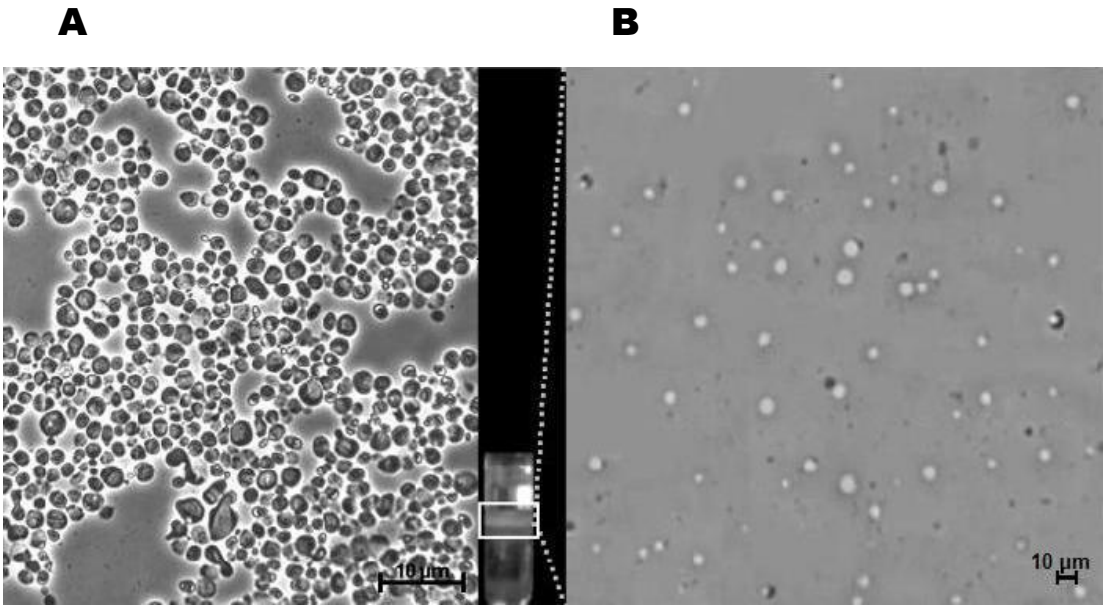

Fig. 3. Isolation and purification of intact vacuoles from the *S. cerevisiae* strain W303-1A. Spheroplast sample observed under the light microscope (A) and vacuole sample after spheroplast lysis followed by vacuole purification by a two-step density gradient centrifugation (B). Inset: Density gradient composed by an upper layer of 8% Ficoll over a layer of 12% Ficoll.

Vacuole samples were also labelled with the calcium probe Fluo-4 AM, prior to observation under the fluorescent microscope (Figure 4). The green fluorescence observed with Fluo-4 was substantially decreased after the addition of the calcium ionophore calcimycin (Figure 7). The capacity of the vacuoles to accumulate Ca^{2+} strongly suggests they retain both their structural and physiological integrity.

To measure the rate of transmembrane pH gradient formation through the activity of V-H^+-ATPase, the fluorescence quenching of ACMA was monitored after ATP addition. Figure 5A shows the fluorescence quenching signals obtained after addition of 0.22 to 3 mM ATP, which suggested the involvement of the V-H^+-ATPase on proton transport. To further demonstrate the involvement of V-H^+-ATPase, ATP-dependent ACMA fluorescence quenching was assayed in the presence of 100 - 500 nM concanamycin A, a specific inhibitor of this proton pump (Dröse *et al.*, 1993). This drug acts by inhibiting the subunit c which forms the major part of the proton translocating V_0 complex of V-H^+-ATPases (Huss *et al.*, 2002).

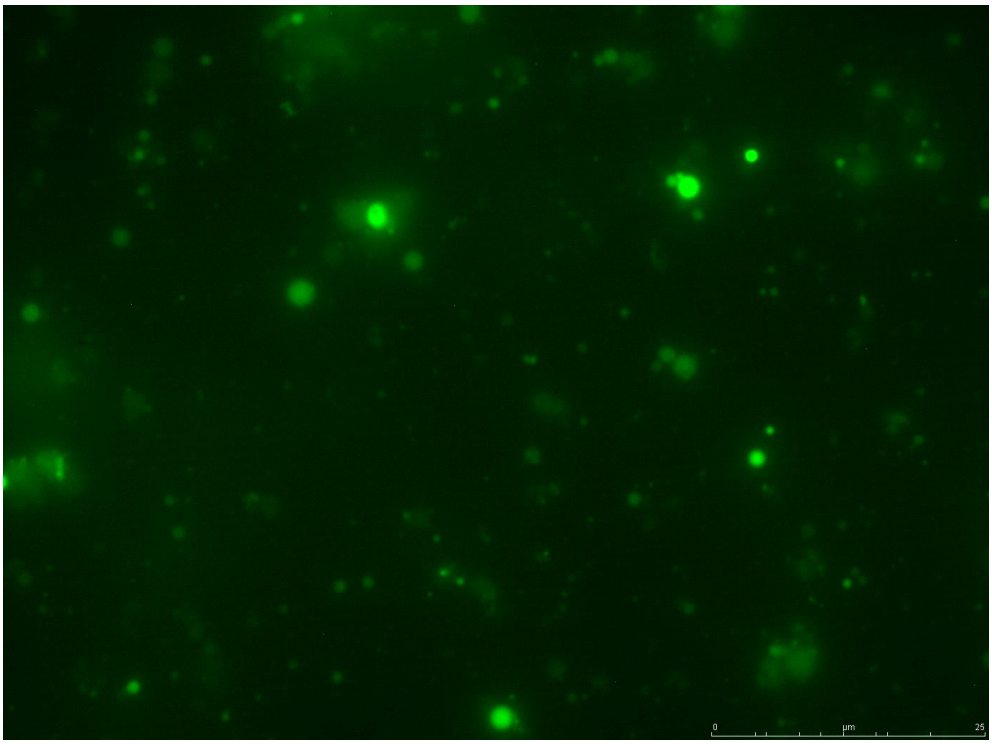

Fig. 4. Intact vacuoles from the *S. cerevisiae* strain W303-1A observed under the fluorescence microscope after 15 min incubation with the fluorescent membrane marker Fluo-4 AM.

Results showed that concanamycin A strongly inhibits proton transport in a dose-dependent manner (Figure 5B). The capacity of the vacuoles to sequester protons through the activity of V-H^+-ATPase supports the observations referred above made with the epifluorescence microscope that these organelles are intact and functional.

4.2. Flow cytometric analysis of vacuole populations

The purified vacuole preparations were further characterised by flow cytometry regarding their homogeneity and functionality. The analysis of the biparametric histograms of log SS against log FS, revealed a well-defined population corresponding to a homogeneous population of intact vacuoles (Figure 6). After a period of 15 min of incubation at room temperature with FM1-43 (Figure 6A) and the calcium probe Fluo-4 AM (Figure 6B), the fluorescence increased in both experimental conditions. The green fluorescence from Fluo-4 AM was substantially decreased after the addition of the calcium ionophore calcimycin (Figure 7).

These results confirm the previous observations made with the epifluorescence microscope that the isolated vacuoles preserved their integrity.

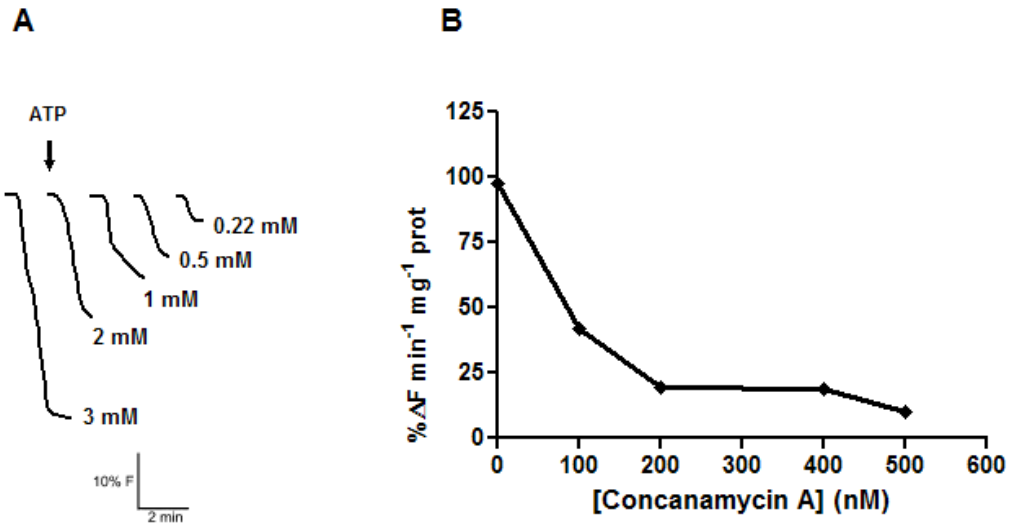

Fig. 5. Proton pumping activity in intact vacuoles purified from the *S. cerevisiae* strain W303-1A as assessed by the fluorescence quenching of ACMA. Typical fluorescence signals after addition of 0.22 to 3 mM ATP (A) and effect of concanamycin A on the initial velocities ACMA fluorescence quenching after addition of 1 mM ATP (B).

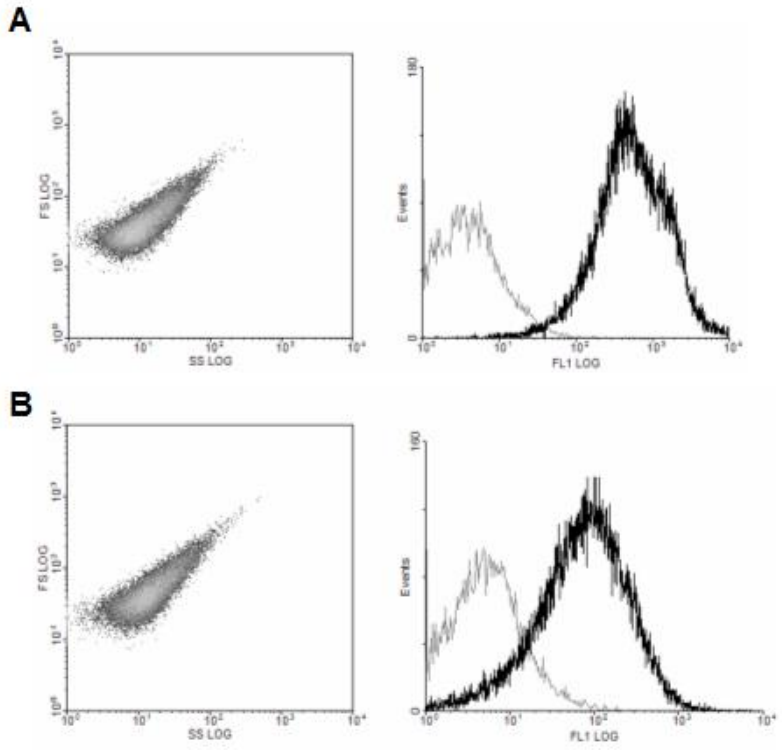

Fig. 6. Flow cytometric analysis of a vacuole population purified from the *S. cerevisiae* strain W303-1A. Scattergram (density plot) of a vacuole suspension after FM1-43 staining (A) and after Fluo-4 AM staining (B), and overlay of green fluorescence (FL1 LOG) histograms of stained and autofluorescence of the same vacuole suspension (right).

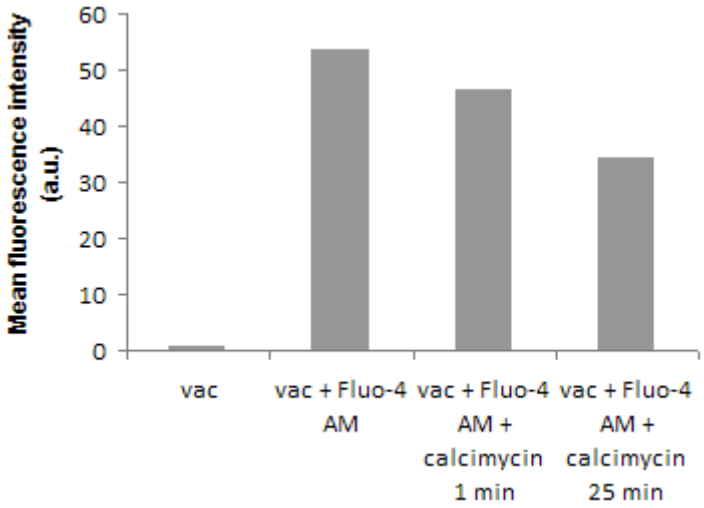

Fig. 7. Effect of calcimycin on isolated vacuoles from the *S. cerevisiae* strain W303-1A, incubated with the calcium fluorescent probe Fluo-4 AM. The calcium ionophore (300 μM) was added to the vacuole suspension after 60 min incubation with Fluo-4 AM. The flow cytometric analysis was performed right after calcimycin addition and 25 min after incubation at room temperature (vac, vacuolar suspension).

Vacuoles isolated from cells expressing a Pep4p-GFP analysed by flow cytometry displayed forward and side scatter signals lower than those corresponding to whole cells indicating that the two populations display different relative size and complexity. Comparison of the green fluorescence of both populations showed different values (Figure 8). Moreover the comparison of green fluorescence signals of vacuoles purified from cells non-transformed and transformed with Pep4p-EGFP showed that the purified vacuoles preserved the ability to retain Pep4p-EGFP (Figure 9).

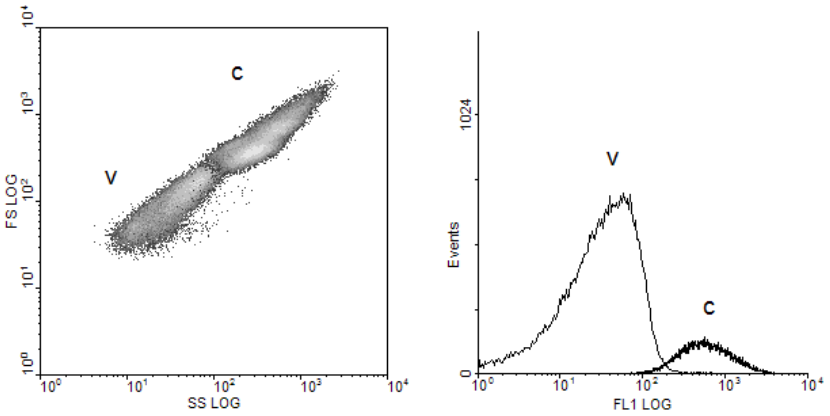

Fig. 8. Flow cytometric analysis of a vacuole and a cell population purified from the *S. cerevisiae* strain expressing a PEP4–EGFP fusion protein. Scattergram (A) of a vacuole suspension (v) and a cellular suspension (c), and overlay of green fluorescence (FL1 LOG) histograms of vacuolar and cellular suspension (B).

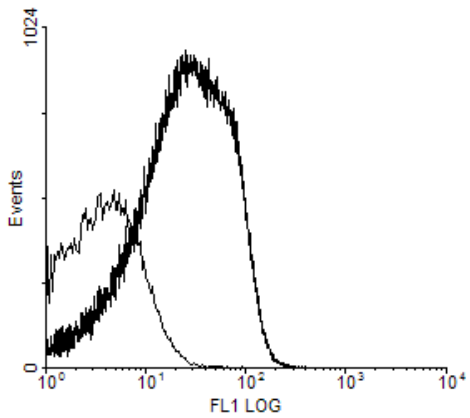

Fig. 9. Flow cytometric analysis of two vacuole population purified from the *S. cerevisiae* W303-1A strain and from a strain expressing a PEP4–EGFP fusion protein. Overlay of fluorescence (FL1 LOG) histograms of a vacuole suspension from PEP4–EGFP strain and autofluorescence of the wild type strain W303-1A.

4.3. Study of the effect of acetic acid on vacuole functionality and membrane permeabilization by flow cytometry

4.3.1. Effect of acetic acid on vacuolar pH

As referred to in the Introduction, acetic acid is able to induce apoptosis in yeast cells. Vacuoles isolated from the *S. cerevisiae* strain W303-1A readily stained with Acridine Orange, confirming the acidity of these organelles (Figure 10). When intact vacuoles were treated with 25 or 50 mM acetic acid, the ratio of red fluorescence/green fluorescence decreased after 30 min and then increased slightly or until reaching the control ratio value measured before the addition of the acid. After treatment for 30 min with 75 mM acetic acid the fluorescence ratio value increased but recovered the initial ratio value 60 min after incubation. These observations support that acetic acid is able to modify vacuolar pH.

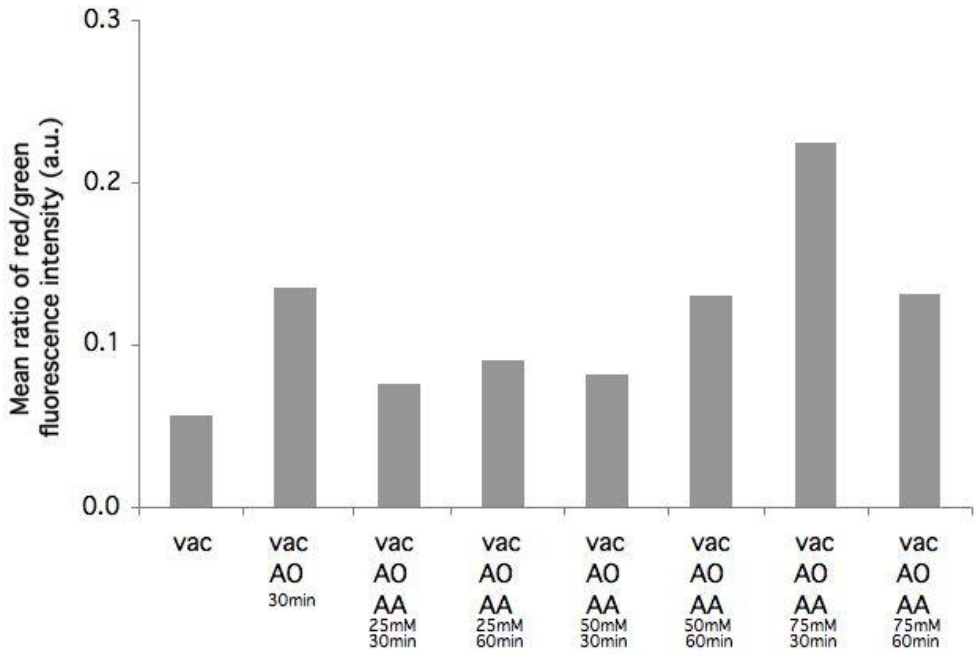

Fig. 10. Flow cytometric analysis of the effect of acetic acid on the pH of the yeast vacuole lumen. Vacuole populations were purified from the *S. cerevisiae* strain W303-1A, and incubated for 10 min with 10 µM Acridine Orange (AO) before acetic acid (AA) addition. (vac, vacuolar suspension).

4.3.2. Effect of acetic acid in vacuolar Ca^{2+} homeostasis

Vacuole is the main intracellular Ca^{2+} store in yeasts and fluctuations in the homeostasis of this divalent cation were recently associated to the onset of apoptosis (Mirnikjoo *et al.*, 2009). To assess the effect of acetic acid in the levels of vacuolar Ca^{2+}, vacuoles were labelled with Fluo-4 AM and analysed by flow cytometry. Results showed that after a treatment of 15 min with 50 mM acetic acid, the green fluorescence decreased to levels identical to green autofluorescence (Figure 11). This indicates that acetic acid induced a release of Ca^{2+} from the vacuole and that the Fluo-4 unspecific staining for the probe concentration used is insignificant.

4.3.3. Effect of acetic acid and H_2O_2 on Pep4p release from the vacuole

As referred before, Pep4p is a pepsin-like aspartic protease localized in the yeast vacuole homologous to human cathepsin D, and in whole yeast cells it has been shown that it translocates from the vacuole to cytosol during acetic acid-induced apoptosis (Pereira *et al.*, 2010). In the present study the effect of acetic acid and H_2O_2 on Pep4p release was evaluated by flow cytometry both in intact cells expressing Pep4p-EGFP and in intact vacuoles purified from these cells.

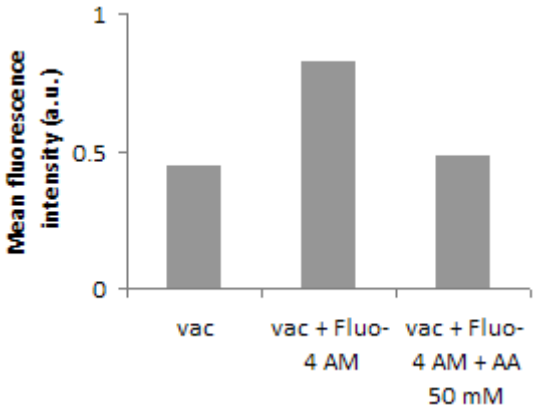

Fig. 11. Flow cytometric analysis of the effect of acetic acid on vacuolar Ca^{2+}. Vacuoles were isolated from the *S. cerevisiae* strain W303-1A, and incubated with the probe Fluo-4 AM for 60 min before treatment with acetic acid.

When transformed cells were observed under the epifluorescence microscope a strong green fluorescence co-localized to the vacuole (not shown) and intact vacuoles purified from these cells also showed a strong green fluorescence (not shown), confirming that the fractionation method yields intact and functional vacuoles.

Flow cytometric analysis showed a clear fluorescence decrease when intact vacuoles from transformed cells were treated with acetic acid, 21% when treated with 25 mM and 37.9% with 75 mM acetic acid (Figure 12). The effect was quite fast and identical for both acid concentrations. These results strongly suggest that acetic acid induces vacuolar release of Pep4p in isolated vacuoles and were corroborated when intact cells and intact vacuoles were observed under the fluorescent microscope before and after acetic acid treatment.

When intact cells were analysed by the flow cytometer 1 to 30 min after a pulse of 20 mM H_2O_2, a fluorescence decrease of 25.7% was observed (not shown), suggesting that H_2O_2 also acts on vacuolar membrane permeability. The study of the effect of H_2O_2 in intact vacuoles derived from transformant cells is in progress.

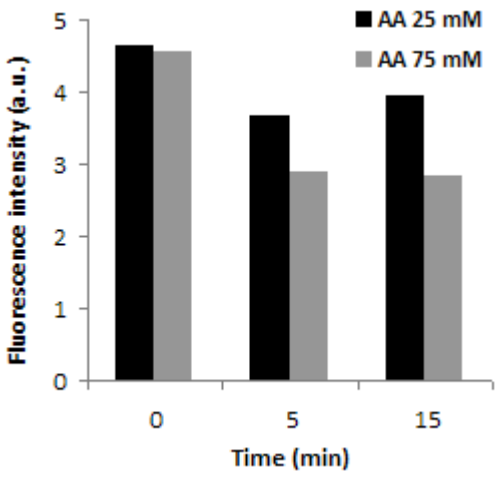

Fig. 12. Flow cytometric analysis of Pep4p release from intact yeast vacuoles 5 and 15 min after treatment with acetic acid. Vacuoles were isolated from the *S. cerevisiae* strain expressing a PEP4–EGFP fusion protein.

4.4. Effect of ATP on vacuole dynamics

As referred to in Introduction, the presence of the V_0 membrane complex of V-H^+-ATPase is required for fusion in yeast but not its pumping activity, while proton translocation activity is required for fission (Baars *et al.*, 2007). In present study the addition of 2 - 200 mM ATP to a vacuolar suspension promoted a split of the initial vacuole population and the emergence of a sub-population with lower relative size and complexity (Figure 13). These results, although preliminary, clearly suggest that the hydrolytic activity of V-H^+-ATPase is involved in the modification of the vacuole dynamics.

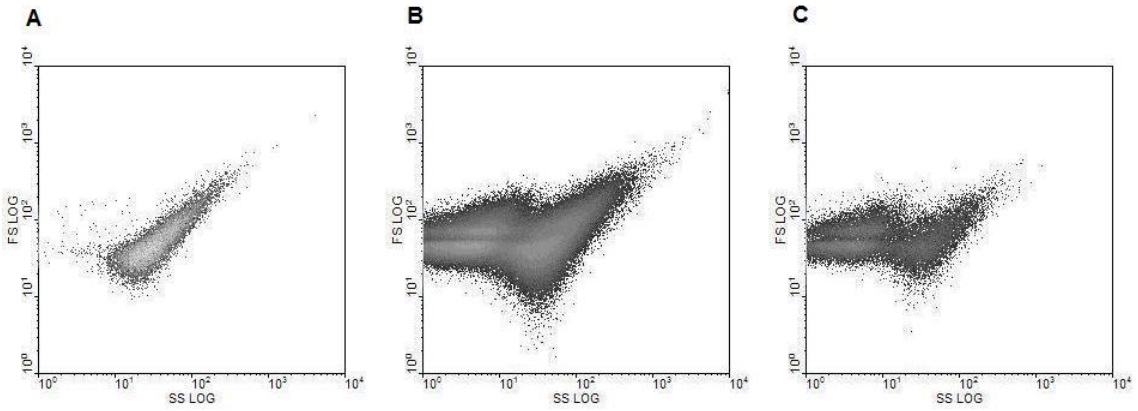

Fig. 13. Flow cytometric analysis of the effect of ATP on a vacuole population purified from the *S. cerevisiae* strain W303-1A. Scattergram (density plot) of a vacuole suspension (A), after addition of 2 mM ATP (B), and 200 mM (C).

5. Discussion

Previous studies developed in our laboratory have shown the involvement of the vacuole in the mitochondrial dependent apoptotic pathway induced by acetic acid (Pereira *et al.*, 2010). In the present study isolated and purified vacuoles were used as experimental models, which in combination with a flow cytometric approach will enable a further characterization of the role of this organelle in yeast apoptosis. The main bottleneck of this approach is to obtain highly purified vacuoles with a good yield. To the best of our knowledge, such approach based on the use of intact vacuoles to study yeast apoptosis by flow cytometry is reported for the first time in the present work.

5.1. Isolated vacuoles from *S. cerevisiae* are intact and functional

The results obtained supported that a fast and efficient method to isolate pure and functional vacuoles from yeast cells suspensions has been achieved. Also, the characterization of purified vacuole suspensions through complementary approaches, including epifluorescence microscopy, spectrofluorimetry and flow cytometry, showed that the isolated organelles are intact and functional. In particular, epifluorescence microscopy observation of isolated vacuoles labelled with FM1-43 or Fluo-4 AM allowed to visualize bright green fluorescent bodies, indicating the structural and physiological integrity of these organelles. Flow cytometric analysis of isolated vacuoles labelled with FM1-43 or Fluo-4 AM originated two distinct green fluorescent populations that were distinct from cell's green autofluorescence. These results confirmed the preservation of the vacuolar membrane integrity and of the organelle capacity to accumulate Ca^{2+}. The vacuoles ability to accumulated Ca^{2+} was further confirmed by the decrease of green fluorescence upon addition of the ionophore calcimycin.

Additionally, isolated vacuoles were able to sequester protons and to maintain a pH gradient across the vacuolar membrane through the activity of V-H^{+}-ATPase measured by spectrofluorimetry with the pH sensitive probe ACMA. This proton pump was strongly inhibited by concanamycin A in a dose-dependent manner. Concordantly,

flow cytometric analysis of vacuoles stained with acridine orange (AO) exhibited increased levels of red fluorescence as a consequence of the dye accumulation in an acidic compartment.

A clear-cut demonstration that the purification procedure yielded intact vacuoles was obtained by analysis of vacuoles isolated from cells expressing a Pep4p-EGFP fusion protein by flow cytometry. This approach allowed identifying a vacuole population able to retain this vacuolar protease clearly distinct from whole cells expressing Pep4p-EGFP both regarding relative size and complexity as well as fluorescence levels.

Altogether these experimental evidences suggested that purified vacuoles could be a good model system to study the mechanisms underlying vacuolar membrane permeabilization in response to an apoptotic stimulus and to study the role of this organelle in yeast apoptosis.

5.2. V-H^+-ATPase regulates vacuole dynamics

Flow cytometric analysis of isolated vacuoles clearly evidenced vacuolar morphological changes after addition of ATP. Addition of 2 - 200 mM ATP to a suspension of isolated vacuoles promoted a split of the initial vacuole population and the emergence of a sub-population with lower relative size and complexity. These results clearly suggest that the hydrolytic activity of V-H^+-ATPase is involved in the modification of the vacuole dynamics. As referred to before, the V_0 membrane complex of V-H^+-ATPase, but not its pumping activity, is required for vacuole fusion in yeast, while hydrolytic activity is required for fission (Baars *et al.*, 2007). This could be the case in this study, since vacuoles decrease in size and complexity with addition of ATP to the vacuolar suspension, suggesting the occurrence of vacuole fragmentation. Therefore, through our flow cytometric analysis of isolated vacuoles we also confirmed the involvement of V-H^+-ATPase activity in the modification of vacuolar morphology, previously studied in whole cells (Baars *et al.*, 2007). These results offer good perspectives for future studies to dissect the molecular/regulatory mechanisms involved in the regulation of vacuole morphology.

5.3. Acetic acid causes partial vacuole membrane permeabilization

Acetic acid directly induced a partial membrane permeabilization of isolated vacuoles from two strains of *S. cerevisiae*. Exposure to acetic acid caused a transient change in the ratio of the red/green fluorescence of AO indicating a transient change in the vacuolar pH. The observed transient increase of the vacuolar pH induced by 25 and 50 mM acetic acid 30 min after incubation is consistent with previous reports that H^+ can escape from lysosomes into the cytosol at the onset of apoptosis (Nilsson *et al.*, 2006). However, further experiments are needed to deepen our understanding of the mechanisms underlying the effect of acetic acid on vacuolar pH homeostasis. As a cation, AO also interacts with nucleic acids by intercalation and electrostatic attractions. Therefore some caution is needed in the interpretation of the fluorescence pattern it generates, although the vacuolar sample is probably impoverished, or even depleted, in nucleic acids. In addition, the possibility that AO binds to membrane lipids cannot be discarded (Cools and Janssen 1986). To confirm that acetic acid is able to induce a transient vacuolar acidification the utilization of other pH sensitive fluorescent probes is in progress.

The vacuole is involved in the regulation of Ca^{2+} levels in yeast and serves as the major intracellular calcium store, as in plants (Halachmi and Eilam, 1989; Dunn *et al.*, 1994). In the present study, exposure to 50 mM acetic acid caused a decrease of the green fluorescence of Fluo-4 AM labelled vacuoles similar to that observed after addition of calcimycin. This observation was interpreted as a Ca^{2+} release from the vacuole and is consistent with the identification of the yeast vacuole as a dynamic intracellular Ca^{2+} store. Moreover, these results are in line with the recognition that intracellular Ca^{2+} is an important signalling molecule for numerous cell responses, including cell death. In mammalian cells, most of intracellular Ca^{2+} is stored in the ER, and when released into the cytosol cell death can be initiated due to the activation of calpains, a family of Ca^{2+}-dependent cysteine proteases (Suzuki *et al.*, 2004), and/or mitochondrial Ca^{2+} overload. The prolonged high concentration of cytosolic Ca^{2+} and mitochondrial Ca^{2+} overload leads to PT and to the activation of calpains, which in turn can induce cell death by several mechanisms, e.g. activation of pro-apoptotic members of the Bcl-2 family (Chen *et al.*, 2001; Cao *et al.*, 2003), release of AIF from mitochondria (Polster *et al.*, 2005), and cleavage of essential components of the

cytoskeleton (Artal-Sanz and Tavernarakis, 2005). Calpains may also activate cathepsins and induce LMP, leading to release of lysosomal enzymes (Yamashima, 2004). Like many other insults, increased cytosolic Ca^{2+} can initiate either apoptosis or necrosis, depending on the concentration of cytoplasmatic Ca^{2+}. Low to moderate concentrations of cytosolic Ca^{2+} (0.2-0.4 μm) triggers apoptosis, whereas higher concentrations (>1 μm) are associated with necrosis (McConkey and Orrenius, 1996).

Though flow cytometric analysis of isolated vacuoles stained with Fluo-4 AM indicate that Ca^{2+} is discharged after addition of acetic acid, some cautious has to be taken on relying on Fluo-4 AM as a Ca^{2+} sensitive probe. Indeed, the interpretation of Fluo-4 fluorescence in whole cells is complicated by the following aspects (Rohrbach *et al.*, 2005): (i) Fluo-4 does not allow for ratiometric measurements, i.e. the signal depends on dye loading and/or partitioning and cannot be readily correlated with Ca^{2+} concentrations; (ii) Fluo-4 fluorescence is potentially pH-dependent, as has been shown for the related fluorochrome fluorescein (Haugland, 2002) and iii) Ca^{2+} measurements with Fluo-4 are impaired by problems arising from phototoxicity and photobleaching. While in the analysis of isolated vacuoles there is no need to account for different pH dependent Fluo-4 fluorescence signals generating from subcellular compartments with significant differences in pH values, it must be ascertained whether the change in fluoresce intensity is attributed to a pH change rather than to Ca^{2+} concentration alteration. Since ratiometric Ca^{2+} indicator Fura-Red provides Ca^{2+} reliable determinations measurements further assays with this probe should be performed.

Photo-induced lipid peroxidation of the *Plasmodium falciparum* vacuolar membrane has been reported by confocal microscopy (Wissing *et al.*, 2002). Though it is not known whether the yeast vacuole membrane is likely sensitive to photo-damage even in the case it is confirmed the analysis by flow cytometry of yeast vacuoles would overcome such major drawback due to the shorter exposure of each particle to the laser beam.

A recent study demonstrated that the vacuole communicates with mitochondria in yeast cells undergoing apoptosis (Pereira *et al.*, 2010). A partial release of Pep4p induced by acetic acid seems to be involved in the crosstalk between mitochondria and the vacuole, engaged in the proteolytic degradation of mitochondria, rather than an extensive vacuolar permeabilization. Although cathepsin D was shown to have a role in mammalian cell death by inducing mitochondrial dysfunction and subsequent release of

pro-apoptotic factors, the process of mitochondrial degradation by Pep4p appears to have a protective role in acetic acid-treated yeast cells (Pereira *et al.*, 2010). H_2O_2 also triggers the translocation of Pep4p from the vacuole into the cytosol in whole yeast cells Mason *et al.*, 2005). In the present study the addition of acetic acid to vacuoles isolated from a transformant strain expressing a PEP4-EGFP fusion protein acetic induced a decrease of vacuolar fluorescence as observed by flow cytometry, indicating the release of vacuolar Pep4p. So far it was unclear whether acetic acid acted directly on vacuoles, or activated pathways upstream to the vacuole, or even acted through other organelles, such a mitochondria. The observations made in this study support the first hypothesis, and suggest that similar mechanisms to LMP in mammalian cells may happen in yeast. As a result, acetic acid, as well as H_2O_2, induces vacuolar membrane destabilization, leading to the release of vacuolar content into the cytosol, which could explain the translocation of Pep4p from the vacuole to the cytosol and the probable release of Ca^{2+}. Whether the release of these molecules involves a non-specific mechanism rather than a selective transport mechanism across the membrane still remains to be elucidated. Moreover, the release of Ca^{2+}, Pep4p, H^+ and/or lysosomotropic dyes might not necessarily reflect a change in membrane integrity, but could be due to a change in ion pump activity (Johansson *et al.*, 2010). These novel results with isolated vacuoles allow us to conclude that acetic acid is able to directly induce permeabilization of the yeast vacuolar membrane, without the involvement of other organelles such as mitochondria or triggering upstream pathways.

5.4. Future prospects

One of the major challenges for further investigation is the elucidation of the molecular basis of LMP as well as of signalling pathways upstream and downstream of the lysosomes, and how they contribute to cell death. Therefore the developed methodologies based on flow cytometric analysis of isolated vacuoles as well as the aforementioned results open new perspectives for future work concerning the role of vacuolar pH and calcium, and on the study of other molecular components localized into the vacuole involved in cell death, besides the proteinase Pep4p. While relevant differences in cellular processes exist between yeast and mammalian cells, we anticipate

that the well established acetic acid-induced yeast apoptotic model will provide a better understanding of the role of vacuole in yeast apoptosis and may advance new insights on the function of lysosomes in mammalian cell death.

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