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Yeast as a model system for the study of Bax regulation by protein kinase C isoforms

Thesis for Doctoral degree in Sciences

Elaborated under the supervision of
Professor Manuela Côrte-Real,
Professor Lucília Saraiva
and
Professor Jorge Gonçalves

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Yeast as a model system for the study of Bax regulation by protein kinase C isoforms

Abstract

Programmed cell death (PCD), of which apoptosis is the most common morphological expression, is an orchestrated collapse of the cell resulting from the activation of a widely described cascade of molecular events. Due to the importance of this process in tissue homeostasis and development of multicellular organisms, its deregulation is associated with several disorders, including cancer and neurodegenerative diseases. A crucial event in mammalian apoptosis is the permeabilization of mitochondrial outer membrane and the release of several apoptogenic factors responsible for activation of different proteases involved in the dismantling of the apoptotic cell. The Bcl-2 protein family plays a central role in this permeabilization. A better knowledge of this process and its regulation will probably lead to the development of novel therapeutic strategies for treatment of apoptosis-related diseases. However, the mode of action of Bcl-2 protein family and its regulation are not completely understood. Protein kinase C (PKC) is a family of serine/threonine kinases that is involved in the transduction of signals that control different cellular processes including cell death and proliferation. In the last decade the role of PKC in apoptosis regulation has been highlighted. This family comprises at least 12 isoforms that regulate apoptosis in an isoform-specific manner. However, the co-existence of several PKC isoforms in the same cell and the different expression patterns observed in different cell types often lead to contradictory reports about the role of each individual isoform in apoptosis regulation. Yeast has proved to be a powerful tool to investigate the molecular aspects of several biological processes, including the steps of the apoptotic cascade involving mitochondria. Yeast does not have obvious homologues of the mammalian Bcl-2 family proteins and, though yeast has a PKC orthologue, the mammalian PKCs do not functionally complement this kinase. However, when these mammalian proteins are expressed in yeast, they conserve some of their molecular and biochemical functions. This favours the use of this simpler model system to unravel some of the functions of this family. Recently it has been

shown that distinct PKC isoforms can differently modulate Bcl-xL anti-apoptotic effect in yeast illustrating the possibility of using the yeast cell model to study regulation of Bcl-2 family proteins by PKC isoforms. Following these results we set out to exploit this model system to study the regulation of Bax by PKCα, δ, ε and ζ. In this thesis, it is shown that the classical PKCα increases the translocation and insertion of Bax *c*-myc (active and tagged human Bax with mitochondrial localization) into the outer membrane of yeast mitochondria. This is associated with an increase in cytochrome *c* (cyt *c*) release, reactive oxygen species production, mitochondrial network fragmentation and cell death. This cell death process is regulated, since it correlates with an increase in autophagy but not with plasma membrane permeabilization. Moreover, it was shown that Bax *c*-myc is not phosphorylated in yeast and that the observed increase in translocation and insertion of Bax into mitochondria in the presence of PKCα is not due to Bax *c*-myc phosphorylation. However, PKCα leads to dephosphorylation of Bax α (inactive human Bax with cytosolic localization) but does not interfere with cell viability or change Bax α translocation and insertion into mitochondria and cyt *c* release. Furthermore, it was demonstrated that the enhancement of Bax *c*-myc-induced cell death by PKCα is independent of the PKCα kinase activity. Nevertheless, PKCα inhibits cell death induced by Bax^{P168A} (human Bax with a single point mutation that increase its activity), an effect that is abolished when the PKCα kinase activity is abrogated. It was also demonstrated that the novel PKCε leads to Bax α dephosphorylation an effect that is associated to inhibition of Bax α translocation into mitochondria. The novel PKCδ and the atypical PKCζ had no detectable effect in Bax α phosphorylation and translocation into mitochondria. Altogether, these results give a mechanistic insight on apoptosis regulation by PKC isoforms showing that they distinctly regulate Bax activity. Moreover, these studies provide a proof of principle of yeast as an important tool to elucidate some the mechanisms by which PKC regulates the apoptotic process.

A levedura como modelo para o estudo da regulação de Bax por isoformas da proteína cinase C

Resumo

A morte celular programada (MCP), da qual a apoptose é a expressão morfológica mais comum, é o colapso orquestrado da célula resultante da activação de uma cascata de eventos moleculares amplamente descrita. Devido à importância deste processo na homeostasia e desenvolvimento de organismos pluricelulares, a sua desregulação está associada a diversas patologias, incluindo cancro e doenças neurodegenerativas. A permeabilização da membrana mitocondrial externa, e a libertação de vários factores apoptogénicos responsáveis pela activação das diferentes proteases envolvidas no desmantelamento da célula apoptótica é um evento crucial da apoptose em mamíferos. A família das proteínas Bcl-2 desempenha um papel decisivo na permeabilização. Um melhor conhecimento deste processo e da sua regulação irá provavelmente conduzir ao desenvolvimento de novas estratégias terapêuticas para o tratamento de doenças relacionadas com a apoptose. No entanto, o modo de acção das proteínas da família Bcl-2 e a sua regulação não está completamente esclarecido. As proteínas cinase C (PKC) são uma família de cinases serina/treonina envolvida na transdução de sinais que controlam diversos processos celulares, incluindo morte celular e proliferação. Na última década o papel da PKC na regulação da apoptose foi destacado. Esta família contém pelo menos 12 isoformas que regulam a apoptose de uma forma específica. No entanto, a coexistência de várias isoformas da PKC na mesma célula e os diferentes padrões de expressão observados em diferentes tipos celulares conduzem com frequência a resultados contraditórios sobre o papel de cada isoforma na regulação da apoptose. A levedura já provou ser uma ferramenta importante na investigação de vários processos biológicos, incluindo os passos da cascata apoptótica que envolvem a mitocôndria. A levedura não tem homólogos das proteínas da família Bcl-2 de mamíferos e, embora possua um homólogo da PKC, as PKCs de mamífero não complementam funcionalmente esta cinase. No entanto, quando estas proteínas de mamífero são expressas em levedura, elas conservam algumas das suas funções

moleculares e bioquímicas. Isto favorece o uso deste modelo mais simples para compreender algumas das funções desta família. Recentemente verificou-se que diferentes isoformas da PKC modulam distintamente o efeito anti-apoptótico de Bcl-xL em levedura demonstrando a possibilidade do uso da levedura como modelo para o estudo da regulação das proteínas da família Bcl-2 pelas isoformas da PKC. Após estes resultados decidimos explorar o modelo da levedura para estudar a regulação de Bax por PKCα, δ, ε e ζ. Nesta tese, mostra-se que a PKCα aumenta a translocação e inserção de Bax *c*-myc (Bax humano activo com localização mitocondrial) para a membrana mitocondrial externa da mitocondria de levedura. Este evento está associado a um aumento da libertação de citocromo c (cit *c*), produção de espécies reactivas de oxigénio, fragmentação da rede mitocondrial e morte celular. Este processo de morte celular é regulado, pois correlaciona-se com um aumento de autofagia mas não com a permeabilização da membrana plasmática. Também foi demostrado que Bax *c*-myc não é fosforilado em levedura e que o aumento da translocação e inserção de Bax na mitocôndria na presença de PKCα não se deve à fosforilação de Bax *c*-myc. No entanto, PKCα leva à desfosforilação de Bax α (Bax humano inactivo com localização citosólica) mas não interfere com a viabilidade celular ou altera a translocação e inserção de Bax α na mitocôndria e a libertação de cit *c*. Foi também demonstrado que o aumento da morte celular induzida por Bax *c*-myc pela PKCα é independente da actividade catalítica de PKCα. Contudo, PKCα inibe a morte celular induzida por Bax^{P168A} (Bax humano com uma mutação num aminoácido que aumenta a sua actividade), um efeito que desaparece quando a actividade catalítica de PKCα é eliminada. Foi também comprovado que PKCε induz desfosforilação de Bax α, um efeito que está associado à inibição da translocação de Bax α para a mitocôndria. As isoformas PKCδ e ζ não têm nenhum efeito detectável na fosforilação e translocação de Bax α para a mitocôndria. Em conjunto, os resultados aqui descritos contribuem para uma melhor compreensão da regulação da apoptose por isoformas de PKC, evidenciando uma diferente regulação da actividade de Bax por cada isofoma. Além disso, estes estudos demonstram a importância do uso da levedura como ferramenta para a elucidação dos mecanismos pelos quais as isoformas de PKC regulam o processo apoptótico.

Table of Contents

List of Abbreviations

λ-PPase	λ-protein phosphatase
AIF	apoptosis inducing factor
Akt/PKB	protein kinase B
ANT	adenine nucleotide translocator
Apaf-1	apoptotic protease activating factor 1
Bar	bifunctional apoptosis regulator
BI-1	Bax inhibitor-I
c.f.u.	colony forming units
Coleon U	6,11,12,14-tetrahydroxy-abieta-5,8,11,13-tetraene-7-one
Cyt *c*	cytochrome *c*
DAG	diacylglycerol
DNA-PK	DNA-dependent protein kinase catalytic subunit
DOPPA	12-deoxyphorbol 13-phenylacetate 20-acetate
Endo G	endonuclease G
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
GSK3β	glycogen synthase kinase 3β
H_2DCFDA	dichlorodihydrofluorescein diacetate
JNK	Jun N-terminal kinase

MAC	mitochondrial apoptosis-induced channel
MOMP	mitochondrial outer membrane permeabilization
MPMA	phorbol-12-myristate-13-acetate-4-O-methyl-ether
Par-4	prostate androgen responsive-4
PDK-1	phosphoinositide-dependent kinase
PI	propidium iodide
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol-12-myristate-13-acetate
PP2A	protein phosphatase 2A
PS	phosphotidylserine
PTP	permeability transition pore
RACKs	receptors for activated C kinases
ROS	reactive oxygen species
VDAC	voltage dependent anion channel

List of Figures

Chapter 1

General Introduction

This chapter comprises parts from the following publications:

Silva RD, Manon S, Gonçalves J, Saraiva L and Côrte-Real M (2011) The importance of humanized yeast to better understand the role of Bcl-2 family in apoptosis: finding of novel therapeutic opportunities. *Curr Pharm Des*. 17:246-255

Silva RD, Coutinho I, Gonçalves J, Saraiva L and Côrte-Real M. (2011) Yeast as model system to study the regulation of apoptosis by PKC isoforms. *Manuscript in preparation*

1.1. Introduction

1.2. Contributions of the yeast model system towards the understanding of the role of Bcl-2 family proteins in apoptosis

1.2.1. Regulation of yeast cell death by Bcl-2 family proteins

1.2.1.1. Bax-induced cell death in yeast
1.2.1.2. Anti-apoptotic activity of Bcl-2 and Bcl-xL in yeast

1.2.2. Regulation of yeast mitochondrial outer membrane permeabilization by Bcl-2 family proteins

1.2.2.1. The role of PTP
1.2.2.2. The role of MAC
1.2.2.3. The two-stage model
1.2.2.4 Therapeutic modulation of MOMP

1.2.3. Bax activation and translocation to yeast mitochondria

1.2.4. Regulation of Bcl-2 family proteins in yeast

1.2.4.1. Interaction mechanisms involved in Bcl-2 family regulation
1.2.4.2. Bcl-2 family regulation by post-translational modifications

1.3. Yeast as model system to study the regulation of apoptosis by PKC isoforms

1.3.1. PKC structural features and activation

1.3.2. Regulation of apoptosis by PKC isoforms: contribution of yeast studies

1.3.2.1. cPKCs in apoptosis
1.3.2.2. nPKCs in apoptosis
1.3.2.3 aPKCs in apoptosis

1.3.3. Yeast as a model system for the identification and characterization of isoform-selective PKC modulators

1.4. Scope of this thesis

1.5. References

1.1. Introduction

Apoptosis is the best-characterized form of programmed cell death. It was originally defined by Kerr *et al.,* (1972), based only in morphological features found in dying mammalian cells like nuclear condensation, nuclear fragmentation, membrane blebbing, cellular fragmentation, phagocytosis of the dying cell, and lack of an ensuing inflammatory response. However, only recently the enormous impact of the apoptotic process on human health was recognized. In adult humans, 50 to 70 billion cells are eradicated by this process every day (Matsuyama *et al.*, 1999), therefore it is not surprising that deregulation of this process can contribute to several diseases including cancer and neurodegenerative disorders.

Apoptosis can be initiated by activation of the plasma membrane death receptor or through permeabilization of the mitocondrial membrane and the release of several apoptogenic factors like cytochrome *c* (cyt *c*), apoptosis inducing factor (AIF), Endonuclease G (Endo G), HtrA2/OMI and Smac/DIABLO. Initiation of apoptosis leads to the activation of caspases (cysteine-dependent aspartate-specific proteases) the main proteases involved in the dismantling of the apoptotic cell. These proteases are responsible for morphological and biochemical alterations typical of apoptosis and for the rapid clearance of the dying cell (Hengartner, 2000). Due to the importance of apoptosis to tissue homeostasis and development of multicellular organisms it is not surprising that is process is tightly regulated. In fact, during the last two decades a multitude of apoptotic regulators have been described. However, this high variety of apoptotic regulators, their numerous interactions and its dependence of the cell type and apoptotic stimuli complicates the analysis of their interactions and roles in the regulation of apoptosis.

The discovery in the late 1990s early 2000s that yeast can undergo cell death with typical markers of mammalian apoptosis in response to different stimuli like H_2O_2 (Madeo *et al.*, 1999), acetic acid (Ludovico *et al.*, 2001), UV radiation (Del Carratore *et al.*, 2002), osmotic stress (Silva *et al.*, 2005) and amiodarone (Pozniakovsky *et al.*, 2005) made yeast an attractive cell model to study the apoptotic cell death. Moreover, it was recognized in yeast a mitochondria-mediated apoptotic pathway similar to the mammalian intrinsic apoptotic pathway (Ludovico *et al.*, 2002; Pereira *et al.*, 2008).

Therefore it was reasonable to consider that our understanding of apoptosis could be greatly improved by studying this process in yeast. Additionally, the genetic tractability of yeast and easy manipulation of its mitochondria led to an increase interest in using this cell model to unveil unknown features of the mammalian intrinsic apoptotic pathway. The existence of orthologues of some mammalian apoptotic regulators like cyt *c*, AIF (Wissing *et al.*, 2004), HtrA/OMI (Fahrenkrog *et al.*, 2004) and endonuclease G (Buttner *et al.*, 2007) in yeast support the existence of a primordial apoptotic machinery similar to that present in mammalian cells. Moreover, although orthologues of other key mammalian apoptotic regulators like Bcl-2 family members (Madeo *et al.*, 2002) and p53 (Nigro *et al.*, 1992) are absent, when some of these proteins are expressed in yeast, they conserve their functional and molecular roles at several cellular levels, namely at the mitochondria. In this context, the use of yeast cells expressing mammalian apoptotic regulators is even more appealing as it can serve to better understand their mode of action, screen for new genetic and pharmacological modulators of these proteins, find novel interactions and uncover the role of new proteins.

In this chapter, the use of the yeast cellular model to study the structural, functional and mechanistic properties of members of the Bcl-2 family is examined. It also includes an overview of the actual knowledge on the role of mammalian protein kinase C (PKC) isoforms in apoptosis and possible use of the yeast model system for the elucidation of the mechanisms of apoptosis regulation by these members of the signalling cascades. Moreover the use of yeast cells for the screening and characterization of new modulators of Bcl-2 family proteins or isoforms of the PKC family is also discussed.

1.2. Contributions of the yeast model system towards the understanding of the role of Bcl-2 family proteins in apoptosis

Bcl-2 family members are critical for the regulation of the mitochondrial pathway of apoptosis in vertebrates (Roset *et al.*, 2007). Complex interactions between members of this family control the integrity of the mitochondrial outer membrane (Green and Evan,

2002). The pro-apoptotic members of this family (Bax and Bak) are essential for this integrity since deletion of both proteins completely impairs mitochondrial membrane permeabilization (Wei *et al.*, 2001). Despite the importance of these proteins, their mechanisms of regulation are not fully understood. An aspect that has been hampering the study of the role of these proteins is the co-existence of a large number of members in the same cell that complicates the analysis of their individual function in the apoptotic process.

Due to the advantages of the yeast model system described above, numerous studies regarding the role of the Bcl-2 family in apoptosis have been performed using the yeast model. In this section we will discuss the advances made in the understanding of role of the Bcl-2 family proteins using the yeast model system and how this model system can help in the identification of new apoptotic modulators that interfere with the mechanism of action of this protein family.

1.2.1. Regulation of yeast cell death by Bcl-2 family proteins

Mammalian mitochondrial apoptosis is mainly regulated by Bcl-2 family proteins (Roset *et al.,* 2007). A variety of Bcl-2 family members have been identified and classified accordingly to their structure and function. At first, this family was usually divided in anti- and pro-apoptotic members. Currently, with new results obtained for a sub-group of this family, the BH3 only proteins, they are divided into four categories. The anti-apoptotic Bcl-2 proteins (A1, Bcl-2, Bcl-w, Bcl-xL and Mcl-1), Bcl-2 effector proteins, (Bak and Bax), direct activators BH3 only proteins (Bid, Bim and Puma) and sensitizers/de-repressors proteins (Bad, Bik, Bmf, Hrk and Noxa) (Chipuk *et al.*, 2010). Some of these proteins have been extensively expressed in yeast in order to address basic questions about their mechanism of action. The idea of using yeast cells as an alternative system to study the molecular aspects of the function of these proteins arose accidentally. When the yeast two-hybrid system was used to test possible interactions between Bcl-2 family members, namely between the anti-apoptotic Bcl-2 and Bcl-xL with the effector Bax, it was found that the chimeric protein LexA-Bax was able to kill yeast. Additionally, it was shown the prevention of this Bax-induced cell death by the native and chimeric proteins derived from Bcl-2 or Bcl-xL (Sato *et al.*, 1994). This was the first report suggesting that Bcl-2 family proteins are able to conserve at least part of

their function when expressed in a heterologous system without endogenous homologue proteins. Later, several reports demonstrated that some proteins of this family act on conserved components of the yeast mitochondria, homologues of mammalian proteins involved in the intrinsic pathway of mammalian apoptosis, generating similar biochemical and physiological responses (Guscetti *et al.*, 2005; Polcic and Forte, 2003; Priault *et al.*, 2003b). Moreover, yeast has allowed the identification of new mammalian apoptotic regulators, such as Bax inhibitor-I (BI-1) (Xu and Reed, 1998), bifunctional apoptosis regulator (Bar) (Zhang *et al.*, 2000) and the Calnexin ortologue Cnx1 (Torgler *et al.*, 1997). Altogether, these studies led to the emergence of a new research field where data obtained from the heterologous expression of these mammalian apoptotic regulators in yeast contribute to a better understanding of the function and regulation of Bcl-2 family proteins.

Various malignancies are associated to overexpression of several anti-apoptotic members of the Bcl-2 family proteins highlighting them as potential targets for therapeutic modulation. Currently, some drugs that modulate the activity of these proteins are in clinical trials. However, none of them have reached the market yet (Reed, 2006). Though pro-apoptotic proteins of this family can also be important targets for therapeutic modulation of apoptosis, they have not been extensively explored. All the aforementioned studies support the notion that the yeast model can be an important tool to find new modulators of the Bcl-2 family proteins, either by increasing our understanding of the biochemical actions of these proteins that will eventually be translated into clinical benefits, or by using humanized yeasts to screen for new drugs that modulate these proteins.

1.2.1.1. Bax-induced cell death in yeast

Bax is the best-studied member of the Bcl-2 protein family. It is a cytosolic multidomain protein (Fig **1.1**) with important functions in mitochondrial morphogenesis and cell death (Karbowski *et al.*, 2006). Expression of Bax in different yeast species induces cell death. It was shown that the effector Bax causes growth arrest and cell death in *Saccharomyces cerevisiae* (Greenhalf *et al.*, 1996; Kissova *et al.*, 2006; Priault *et al.*, 1999b), *Schizosaccharomyces pombe* (Ink *et al.*, 1997; Jurgensmeier *et al.*, 1997),

Pichia pastoris (Martinet *et al.*, 1999), *Candida albicans* (De Smet *et al.*, 2004) and *Kluyveromyces lactis* (Poliakova *et al.*, 2002).

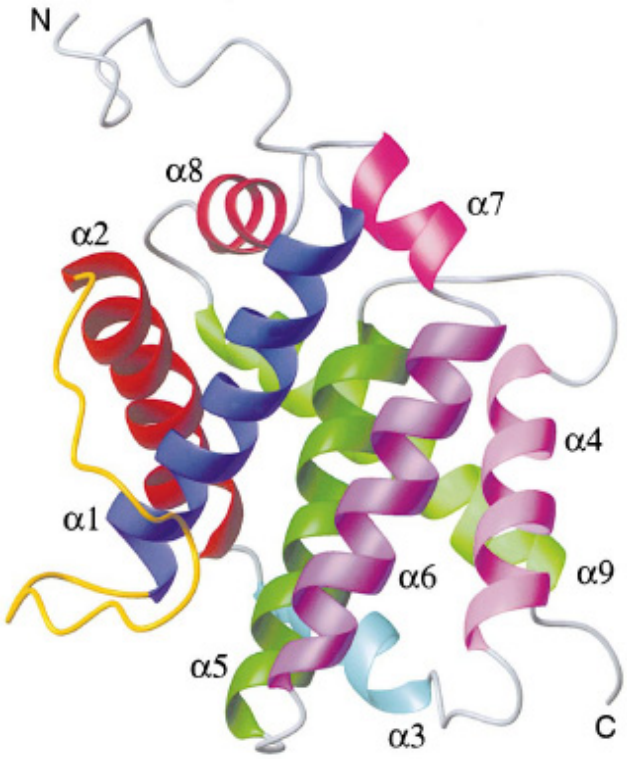

Figure 1.1 – Structure of Bax α. Ribbon representation of an averaged minimized NMR structure for Bax α. Helices are distinguished by different colors. From Suzuki *et al.*, 2000.

Several studies highlighted the role of mitochondria in Bax-induced cell death. Immunofluorescence studies showed that active Bax is predominantly associated with mitochondria in yeast cells. Furthermore, they showed that abolishing Bax mitochondrial targeting completely abolished its cytotoxic functions (Zha *et al.*, 1996). In addition, Bax-induced cell death is delayed in cells lacking mitochondrial DNA and in strictly fermentative conditions (Greenhalf *et al.*, 1996; Priault *et al.*, 1999b). Moreover, strains lacking the ability to perform oxidative phosphorylation are more resistant to Bax-induced growth arrest (Harris *et al.*, 2000). Also, Bax expression induces hyperpolarization of mitochondria (Gross *et al.*, 2000), reactive oxygen species (ROS) production (Gross *et al.*, 2000; Madeo *et al.*, 2002), cyt *c* release (Manon *et al.*, 1997) and mitochondrial network fragmentation (De Smet *et al.*, 2004). The dependence of Bax-induced cell death on mitochondrial lipid oxidation (Priault *et al.*, 2002) reinforces the importance of mitochondria in Bax killing effect.

There still is some controversy regarding the type of cell death induced by Bax in yeast. Ligr *et al.* (1998) reported that overexpression of Bax in *S. cerevisiae* induced cell death accompanied by morphological changes similar to those of apoptotic metazoan cells, such as phosphatidylserine exposure, plasma membrane blebbing, chromatin condensation and margination, and DNA fragmentation. These changes were prevented by co-expression with Bcl-xL. Bak, was also shown to induce cell death in yeast accompanied by condensation and fragmentation of chromatin and specific cleavage of chromosomal DNA (Ink *et al.*, 1997). The detection in yeast cells expressing Bax of cyt *c* release from the mitochondrial intermembrane space, mimicking that observed in the early steps of mammalian apoptosis, could support the hypothesis that Bax induced an apoptotic like cell death in yeast (Manon *et al.*, 1997). However, since yeast cells lack orthologues of the mammalian apoptotic protease activating factor 1 (Apaf-1) that forms the apoptosome together with cyt *c* and ATP, the relevance of cyt *c* release in yeast expressing Bax was questioned. In fact, the finding that yeast cells lacking cyt *c* still die after Bax expression, although at a slower rate, indicate that cyt *c* release is not essential for Bax-induced cell death (Priault *et al.*, 1999b). This was further supported by the demonstration that a strain with a cyt *c*-GFP fusion that is not released to the cytosol after Bax expression died at the same rate of a strain with a releasable cyt *c* (Roucou *et al.*, 2000). These results discarded the hypothesis of cyt *c* involvement in Bax-induced cell death. Apoptotic cell death independent of cyt *c* was also observed in yeast in response to acetic acid or H_2O_2 (Pereira *et al.*, 2007). Though the role of cyt *c* release in yeast apoptosis is uncertain it appears to be a dispensable event in cells expressing Bax or committed to death in response to apoptotic stimuli. In contrast to the former studies by Ligr *et al.* (1998), others showed the absence of typical apoptotic markers like caspase activation, phosphatidylserine exposure and DNA fragmentation in Bax-induced yeast cell death (Guscetti *et al.*, 2005; Kissova *et al.*, 2006), ruling out a role for apoptosis in Bax-induced cell death. Instead they found that autophagy is activated in yeast cells after Bax expression. Indeed, heavy vacuolization of the cytoplasm have been reported in *S. cerevisiae* and *P. pastoris* cells expressing Bax (Kissova *et al.*, 2006; Martinet *et al.*, 1999). Other autophagic features have also been observed in yeast cells expressing Bax, including increased accumulation of Atg8p and activation of the targeting-deficient mutant of the vacuolar alkaline phosphatase. Inactivation of autophagy slightly accelerated Bax-induced cell death showing a protective role for this process (Kissova *et al.*, 2006). Although an increased number of autophagosomes has

been associated with forms of non-apoptotic cell death in metazoan cells autophagy has been mainly demonstrated as a cytoprotective and anti-apoptotic process (Levine and Yuan, 2005; Mizushima *et al.*, 2008). Curiously, mitophagy (a selective autophagic process of mitochondrial degradation) is also involved in Bax-induced cell death, since hampering this process by deletion of *Uth1* delays Bax-induced cell death (Camougrand *et al.*, 2003). However the slow loss of plating efficiency in *uth1*Δ cells expressing Bax was correlated with loss of plasma membrane integrity suggesting that selective degradation of altered mitochondria through mitophagy is required for a regulated loss of growth capacity (Kissova *et al.*, 2006). This suggests that mitophagy is able to convert a necrotic form of death into a regulated form of death. The apparent contradictory results regarding the mode of cell death induced by Bax expression in yeast may reflect the use of different expression systems, tags and strains.

A role for Bcl-2 family proteins in the regulation of the crosstalk between autophagy, a process primarily involved in cell survival, and apoptosis, a pathway that invariably leads to death has also been ascertained in mammalian cells (Levine *et al.*, 2008; Luo and Rubinsztein, 2009). Therefore the yeast model system may also provide new data that will improve our understanding about the complex crosstalk between these two processes in mammalian cells.

Early studies reported the collapse of mitochondrial network during apoptosis into short punctuate fragments in the perinuclear region of mammalian cells. This morphological change was frequently perceived as a passive post-mortem event, and its role was not carefully investigated (Brooks and Dong, 2007). However, the finding that fragmentation of the mitochondrial network was an early event in apoptosis and that inhibition of mitochondrial fragmentation by the dominant-negative Drp1 suppresses cyt *c* release and blocks apoptosis suggests a role for this process in apoptosis (Frank *et al.*, 2001). Several studies highlighted a role for Bcl-2 family proteins in this process (Brooks and Dong, 2007). Mitochondrial network fragmentation after Bax expression in yeast was shown for the species *C. albicans* (De Smet *et al.*, 2004), *S. pombe* (Pevala *et al.*, 2007) and *S. cerevisiae* (Kissova *et al.*, 2006). However, the role of mitochondrial network fragmentation in Bax-induced cell death in yeast is not completely clarified.

1.2.1.2. Anti-apoptotic activity of Bcl-2 and Bcl-xL in yeast

Bcl-2 and Bcl-xL are able to antagonize Bax effects in yeast (Greenhalf *et al.*, 1996; Gross *et al.*, 1998; Ink *et al.*, 1997; Jurgensmeier *et al.*, 1997; Ligr *et al.*, 1998; Manon *et al.*, 1997; Matsuyama *et al.*, 1998; Priault *et al.*, 1999c; Sato *et al.*, 1994; Tao *et al.*, 1997; Zha *et al.*, 1996), imitating the anti-death effects observed in mammalian cells. Similar to mammalian cells undergoing apoptosis, Bax-induced mitochondrial changes in yeast are inhibited by Bcl-xL through heterodimerization-dependent and -independent mechanisms (Minn *et al.*, 1999). This indicates the conservation of both anti-apoptotic mechanisms of Bcl-xL in yeast. Polcic and Forte (2003) showed that Bcl-xL inhibits the stable integration of Bax into mitochondrial membranes hindering Bax activity. Moreover, Bcl-xL and Bcl-2 are able to protect yeast from apoptotic cell death induced by several stimuli. This makes yeast a good cell model to study the heterodimerization-independent role of Bcl-2 and Bcl-xL. In some cases, the anti-apoptotic mechanism of Bcl-2 in yeast seems to be related with their antioxidant properties. Expression of Ced-9, Bcl-2, or Bcl-xL in yeast makes the cells more resistant to H_2O_2-, menadione- and heat shock-induced cell death (Chen *et al.*, 2003) allowing yeast cells to remain viable under conditions that were lethal to wild-type yeast. The protective effect of Bcl-xL in acetic acid-induced apoptosis in yeast is also accompanied by a reduction in mitochondrial ROS production (Saraiva *et al.*, 2006). It has also been reported that Bcl-2 protein improves survival deficiency of a strain defective in antioxidant protection (Longo *et al.*, 1997). However, in chronologically aged cells the protective effect of Bcl-xL against death seems to involve a mitochondrial mechanism which is distinct from the antioxidant activity of Bcl-xL (Trancikova *et al.*, 2004). In yeast, the anti-apoptotic Bcl-2 and Bcl-xL proteins, not only inhibit cell death triggered by several stimuli, but also mitochondria network fragmentation (Fannjiang *et al.*, 2004). However, the mechanisms by which these proteins prevent mitochondrial network fragmentation are still unclear.

1.2.2. Regulation of yeast mitochondrial outer membrane permeabilization by Bcl-2 family proteins

In mammalian cells, mitochondria's principal responsibility in early apoptosis is to release several lethal factors like cyt *c*, AIF and EndoG from the mitochondrial

intermembrane space into the cytosol. This release occurs after mitochondrial outer membrane permeabilization (MOMP) and allows these proteins to play a crucial role in later steps of apoptosis. Additionally, MOMP may also lead to cell death by lethal ROS production or collapse of mitochondrial functions. The mechanism by which MOMP occurs is still under study but it has been attributed to: i) the formation of the permeability transition pore (PTP), an inner membrane unselective channel formed at the contact points between the inner and outer mitochondrial membrane and that, in the open state, leads to mitochondrial swelling with rupture of the outer mitochondrial membrane; ii) the formation of pores in the outer mitochondrial membrane by Bax and Bak; iii) interactions between both models (Rasola and Bernardi, 2007; Schwarz *et al.*, 2007; Tsujimoto and Shimizu, 2007). More recently, formation of ceramide channels has been proposed as another mechanism mediating the release of pro-apoptotic proteins from mitochondria during the induction phase of apoptosis. Both anti- and pro-apoptotic members of the Bcl-2 family (Ganesan *et al.*, 2010; Siskind *et al.*, 2008) were shown to interact with ceramide channels and modulate MOMP. These different mechanisms are discussed below.

1.2.2.1. The role of PTP

Although yeast does not possess obvious homologues of members of the Bcl-2 family proteins, they have homologues of the main putative components of the mammalian PTP, namely the adenine nucleotide translocator (ANT), the voltage dependent anion channel (VDAC) and cyclophilin D. The discovery that Bax also leads to MOMP and cyt *c* release in yeast pointed out this organism as an alternative model system to clarify how MOMP occurs during apoptosis.

The role of PTP in Bax-induced MOMP and cyt *c* release in yeast was studied by examining the involvement of homologues of the major putative components of this pore, namely Por1p (homologous to VDAC), AAC (homologous to ANT) and cyclophilin. The results obtained were quite contradictory indicating either a role for Por1p and AAC in Bax effects (Marzo *et al.*, 1998; Shimizu *et al.*, 2000) or showing that Bax is able to induce cyt c release and/or cell death independently of the putative PTP components, Por1p, AAC and cyclophilin (Gross *et al.*, 2000; Kissova *et al.*, 2000; Priault *et al.*, 1999b; Priault *et al.*, 1999c). These distinct results obtained have been

attributed to differences in expression levels, different epitope tags fused to the native Bax protein, metabolic differences caused by growth on different carbon sources, and to the different genetic background of the strains used in those studies. However, some findings clearly indicate that cyt *c* release after Bax expression is independent of the PTP. For instance, it was shown that a cyt *c*-histidine x6 tag of about 1kDa does not affect the release of cyt *c*, but a fusion with the F1-ATPase subunit epsilon of about 6 kDa prevented it. This pointed to a dependence on the size of the protein, and therefore to a channel-mediated cyt *c* release. Also, Priault *et al.* (1999c) demonstrated the conservation of the mitochondrial inner membrane integrity in mitochondria isolated from Bax *c*-myc expressing yeast cells. Indeed in this case, no swelling is detected and the osmotic properties of mitochondria are not altered. These data indicate that the mechanisms underlying MOMP and cyt *c* release induced by Bax expression in yeast are independent of PTP. Recent studies with animal cells show that sustained PTP opening is actually a consequence of apoptosis (Kinnally and Antonsson, 2007). Other studies demonstrated that PTP is predominantly involved in necrosis and ischemia-reperfusion injury (Baines *et al.*, 2005; Basso *et al.*, 2005; Nakagawa *et al.*, 2005).

1.2.2.2. The role of MAC

Bcl-2 family proteins have structural similarities with pore forming domains of bacterial toxins, such as colicins and diphtheria toxins (Muchmore *et al.*, 1996). This led to the suggestion that the members of this family are able to form pores in membranes. In fact, these proteins are able to form channels in synthetic membranes (Antonsson *et al.*, 1997; Schlesinger *et al.*, 1997). Furthermore, Bax can induce transport of cyt *c* in liposomes (Saito *et al.*, 2000). Electrophysiological studies of the mitochondrial outer membrane purified from a Por1p-less yeast strain expressing Bax showed a novel channel activity that is absent in mitochondrial outer membranes of the same yeast strain not expressing Bax (Pavlov *et al.*, 2001). This channel was named mitochondrial apoptosis-induced channel (MAC) (Fig. **1.2**, step 3). It was calculated that the size of this channel is about 4 nm and is capable to discriminate between positively, neutral or negatively charged proteins, exhibiting higher selectivity for small, positively charged proteins (Priault *et al.*, 2003a). Cyt *c* is a protein with a diameter of about 3 nm and is positively charged, therefore MAC is the probable candidate involved in MOMP and cyt *c* release after Bax expression in yeast. A channel with similar properties has been

found in mammalian cells (Pavlov *et al.*, 2001) and immunodepletion of MAC activity from lysates of apoptotic HeLa cells using anti-Bax antibodies provided a direct proof that oligomeric Bax is a component of MAC (Dejean *et al.*, 2005). This last study also showed that Bax and Bak are functionally redundant with respect to this channel.

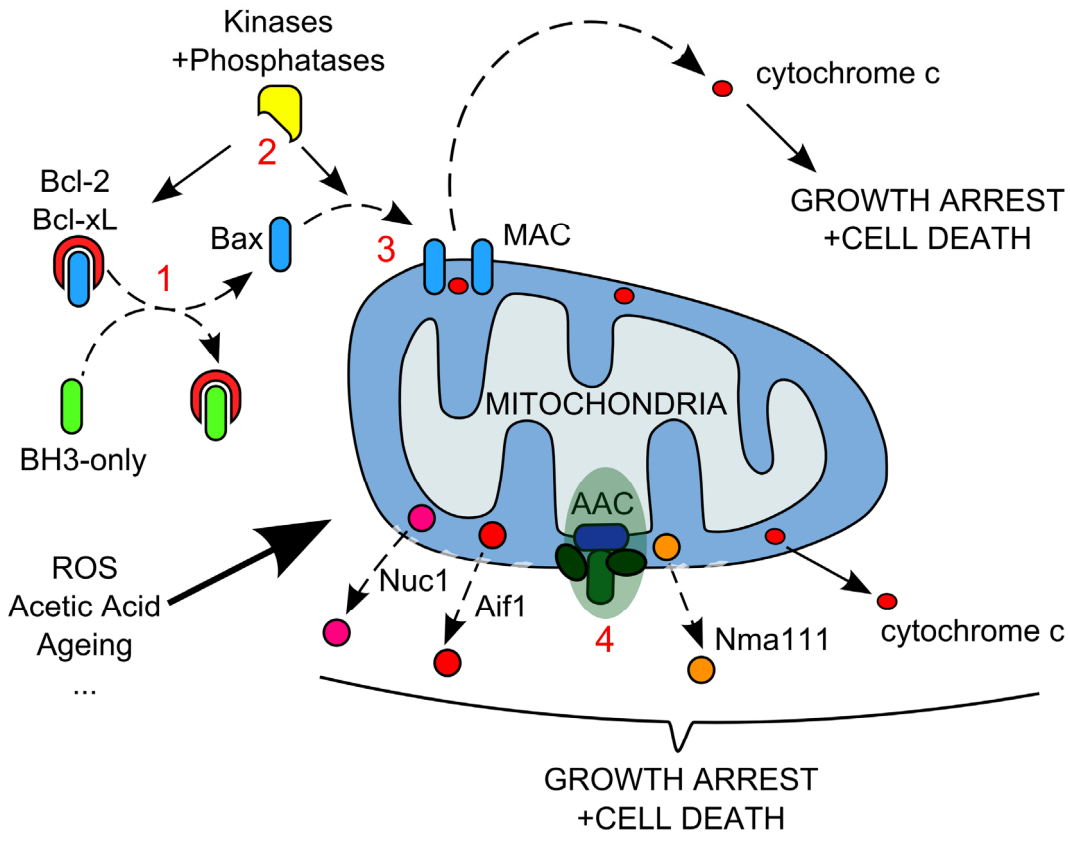

Figure 1.2 – Bax regulation and mode of action in yeast. Native untagged human Bax expressed alone in yeast remains inactive, either cytosolic or weakly bound to the outer mitochondrial membrane. The network of interaction with anti-apoptotic proteins Bcl-2 and Bcl-xL, and with BH3-only proteins (Bad, tBid, PUMA,…) can be reconstituted by co-expressing these proteins with Bax (1). The network of interactions is regulated by kinases and phosphatases, which phosphorylate and dephosphorylate Bax, anti-apoptotic proteins and BH3-only proteins. Mammalian kinases and phosphatases can be co-expressed with proteins of the Bcl-2 family to study this regulation (2). Once Bax is activated, it is translocated and inserted into the outer mitochondrial membrane, and its oligomerization leads to the formation of the MAC channel that is able to release cyt *c* (3). Since the release of cyt *c* leads to growth arrest and eventually cell death, a screening of molecules based on the capacity of yeast expressing adequate combination of Bcl-2 family members and kinases/phosphatases will help to identify molecules acting on these different steps. Alternatively, apoptotic cell death can be triggered in yeast by different conditions (H_2O_2, acetic acid, ageing,…) and involve the release of different mitochondrial proteins, homologous to mammalian apoptogenic factors (4). This release involves the yeast ANT homologue AAC and alteration of the permeability of the outer mitochondrial membrane, in a similar way as mammalian PTP. Note that Bax-expression does not stimulate this second mitochondrial death pathway. This figure was kindly provided by Dr. Stépen Manon.

Consistently, patch clamp studies of mitochondria isolated from cells deficient in Bax and Bak show that at least one of these proteins must be present for MAC formation and consequent cyt *c* release. These studies also show that MACs containing exclusively Bax or Bak function in a similar way (Pavlov *et al.*, 2001). Moreover, truncated Bid catalyzes MAC formation in isolated mitochondria containing Bax and/or Bak and does not require VDAC1 or VDAC3.

Several stimuli lead to apoptosis, MOMP and cyt *c*, Aif1p and Nuc1p (the yeast EndoG) release, in yeast cells (Pereira *et al.*, 2008). However, since yeast does not possess homologues of Bcl-2 family proteins, it is apparent that MOMP occurs during yeast apoptosis through a process distinct of MAC formation (Fig. **1.2**, step 4). Therefore, yeast, besides being a unique model to study MOMP independent of MAC, also seems adequate to elucidate the molecular mechanisms underlying MAC-mediated MOMP that occurs in mammalian cells. It could also help in identifying other possible components of MAC and to assess whether formation of this channel is sufficient to induce cyt *c* release. The synergistic permeabilization of the mitochondrial outer membrane by activated Bax and ceramide (Ganesan *et al.*, 2010) can also be uncovered using the yeast model.

1.2.2.3. The two-stage model

Although MAC is probably the channel responsible for the cyt *c* release observed during apoptosis in mammalian cells, it is unlikely to transport other larger proteins that are translocated from mitochondria during apoptosis such as Smac/DIABLO, AIF and EndoG. For this reason, it has been proposed a two-stage model for MOMP in mammalian cells. In the first step, Bax oligomerizes in the mitochondrial outer membrane and leads to the formation of MAC, facilitating the release of cyt *c*. Activation of caspases by cytosolic cyt *c* should lead to further permeabilization of outer mitochondrial membrane by an unknown mechanism, but probably involving PTP. This model is further corroborated by recent evidence showing that the yeast ANT homologue and one particular ANT isoform in *Caenorhabditis elegans,* is required for MOMP (Pereira *et al.*, 2007; Shen *et al.*, 2009). Together these studies reinforce the existence of an intricate, phylogenetically conserved crosstalk between Bcl-2 family proteins and components of the PTP.

1.2.2.4 Therapeutic modulation of MOMP

MOMP is considered a "point of no return" in the apoptotic cascade. Therefore pharmacological inhibition of this event can be of extreme importance in controlling disease caused by excessive apoptosis. An approach for the control of MOMP is to control MAC, the first stage of MOMP. Although the pharmacological profile of MAC activity is still limited, some compounds have already been identified as MAC inhibitors. The first potent small molecule inhibitors of cyt *c* release were several derivatives of 2-propanol, identified in an *in vitro* assay using Bid-induced Bax activation in isolated mitochondria (Bombrun *et al.*, 2003). Dibucaine, propranolol and trifluoperazine have also been identified as dose-dependent MAC inhibitors in patch-clamp experiments, while lidocaine, a structural homologue of dibucaine, has a small effect (Martinez-Caballero *et al.*, 2004). Curiously, cyclosporine A, a potent PTP blocker has no effect on MAC activity (Martinez-Caballero *et al.*, 2004). More recently, Hetz *et al.* (2005) identified two small molecule inhibitors of MAC, which blocked the Bax channel activity, cyt *c* release, inhibited mitochondrial membrane depolarisation and apoptosis. Although yeast has not yet contributed to MAC pharmacological profiling, yeast expressing activated Bax can be used to screen for potent MAC modulators and help in the generation of novel therapeutic regimes for apoptotic diseases.

1.2.3. Bax activation and translocation to yeast mitochondria

The 3D structure of soluble Bax has been determined by nuclear magnetic resonance spectroscopy (Suzuki *et al.*, 2000). Like other members of the Bcl-2 family, Bax is formed by alpha helices connected by loops. But, unlike the other multidomain members of this family, Bax cellular localization is not always in the mitochondria. Bax is able to adopt at least two stable conformational states, inactive Bax with cytosolic localization and fully activated Bax, inserted in mitochondria and inducing cyt *c* release. It is possible that an intermediate state exists that allows Bax to be inserted in the mitochondrial membrane but does not lead to MAC formation and cyt *c* release. In fact, unlike other pore-forming proteins, oligomerization of Bax occurs mainly in the mitochondrial membrane (Annis *et al.*, 2005; Cartron *et al.*, 2008). Dimers of Bax can

also be detected in the cytosol, however they are not efficiently inserted in the membrane (Cartron *et al.*, 2008; D'Alessio *et al.*, 2005).

In aqueous solutions, Bax is composed by 9 alpha helices and the two central helices (Hα5 and Hα6) are the mostly hydrophobic. These two helices are embedded within the other 7 helices, which have amphipathic properties and keep their hydrophilic residues exposed to the exterior. This type of organization enables this protein to remain soluble in its native cytosolic conformation (Lalier *et al.*, 2007). By analogy to the C-terminal transmembrane domain of Bcl-2 and Bcl-xL, it was proposed that the hydrophobic helix 9 is responsible for Bax anchoring to the mitochondrial outer membrane. In fact, C-terminal truncated versions of Bcl-2 and Bcl-xL lose their ability to insert into membranes and prevent apoptosis in mammalian cells (Janiak *et al.*, 1994; Nguyen *et al.*, 1993). This shows the importance of this domain to Bcl-2 and Bcl-xL function. In yeast cells these truncated versions are unable to prevent Bax cytotoxic effects (Clow *et al.*, 1998; Greenhalf *et al.*, 1996; Priault *et al.*, 1999a). However, in the 3D structure of Bax it is clear that, in the native conformation, helix 9 is tightly sequestered in Bax hydrophobic pocket and cannot insert into membranes in this conformation.

In order to be inserted into the mitochondrial membrane and to oligomerize, Bax must undergo profound conformational changes. Several relevant aspects about this conformational changes and their role in mitochondrial insertion have been obtained using yeast. Due to its homology to the C-terminal transmembrane domain of Bcl-2 and Bcl-xL, the Hα9 has deserved a considerable attention and its role has been extensively studied using the yeast system. Expression of Bax in its native conformation does not induce cell death in yeast. This shows that this protein does not spontaneously interact with mitochondria. However, single point substitutions on this helix, its deletion or replacement strongly enhance Bax capacity to translocate to mitochondria to release cyt *c* and induce cell death (Arokium *et al.*, 2004; Clow *et al.*, 1998; Priault *et al.*, 2003b). Moreover, these single point substitutions or deletions impair the inhibitory effect of Bcl-2 and Bcl-xL. This suggests an important role of Hα9 in Bax/Bcl-2 and Bax/Bcl-xL interaction (Arokium *et al.*, 2004; Clow *et al.*, 1998). These results indicate that this helix is able to regulate negatively Bax activation and translocation and helps stabilizing Bax in an inactive cytosolic conformation. In order to induce apoptosis, Bax C-terminal has to undergo some conformational changes allowing the insertion of Bax into the mitochondria.

Bax N-terminal residues also control its translocation. Deletion of the first 20 aminoacids of Bax α structurally corresponds to a variant called Bax ψ, which is encoded by a transcript distinct from that of Bax α (Cartron *et al.*, 2002b). To better explore the role of these 20 aminoacids, a deletion construct of Bax lacking the first 20 aminoacids (BaxΔN) was created and studied using the yeast system. This deletion enhances the association of Bax with mitochondria and increases cyt *c* release and cell death (Priault *et al.*, 2003b). This finding was also confirmed using a cell-free system and mammalian cells (Cartron *et al.*, 2002a; Priault *et al.*, 2003b). These results indicate that this part of the protein has also an inhibitory effect on Bax activity. The role of Hα1 (aminoacids 24-37) was subsequently studied by investigating the effect of mutations in the Hα1 in Bax mitochondrial insertion and induction of cyt *c* release. Substitution of ala24 for Arg (A24R) inhibits the binding of BaxΔN to mitochondria and cyt *c* release. The double substitution on this helix of leu26 for gly (L26G) and leu27 for val (L27V) also have the same inhibitory effect (Cartron *et al.*, 2003). These effects were also assessed in mammalian cells with similar results (Cartron *et al.*, 2003). Additionally, a construct lacking the first 37 aminoacids (N-terminal and Hα1) does not to bind mitochondria in a cell-free system (Cartron *et al.*, 2003). These results suggest an important role for Hα1 in Bax addressing to mitochondria.

More recently, Arokium *et al.* (2007) tried to uncover the role of some putative ionic interactions in the stabilization of Bax in the cytosolic conformation. A possible weak interaction between the positive charged residue arg9 in the N-terminal region and the negatively charged residue asp154 in Hα7 was studied by single substitution of glu for arg9 or lys for asp154. Both of these substitutions triggered a massive addressing of Bax to mitochondria and release of cyt *c* to the cytosol. A second ionic interaction between as33 in the Hα1 and lys64 in the Hα2 has also been described using mammalians cells and cell-free systems (Cartron *et al.*, 2004). These results were reproduced using the yeast system (Arokium *et al.*, 2007).

The above mentioned results allow us to conclude that, in native Bax, the position of its C-terminal and N-terminal allows Bax to keep a cytosolic stable conformation. This conformation is stabilized by ionic interactions between some residues of the protein and its disruption can lead to Bax activation. The Hα1 is necessary for Bax translocation to mitochondria and contains an addressing signal to the mitochondria.

Bax needs to suffer dramatic conformational alterations in order to insert in the mitochondria (Peyerl *et al.*, 2007) that can be mimicked by deletions of portions of the protein, single substitutions or addition of an epitope.

Major conformational changes are not the only event required for Bax insertion into the mitochondria. The existence of a mitochondrial receptor is also important for Bax translocation and insertion. The translocase of outer mitochondrial membrane (TOM) complex has been implicated as a receptor of Bax. However there are some contradictory results about the component of the TOM complex that is required for Bax insertion. Bellot *et al.* (2007) identified TOM22 as the mitochondrial receptor of Bax using the yeast and mammalian cells systems. In this study, blocking expression of TOM22 inhibited association of Bax with mitochondria and prevented Bax-dependent apoptosis in mammalian cells. Treatment of mammalian mitochondria with antibodies against TOM22 or with trypsin to degrade outer mitochondrial membrane proteins with cytosolic exposed domains, such as TOM22, also inhibited association of Bax with mitochondria. A yeast strain with decreased expression of Tom22p also exhibited decreased association of Bax with mitochondria.

In a different study, using isolated yeast mitochondria, the Tom40p subunit of the TOM complex was identified as the mitochondrial Bax receptor (Ott *et al.*, 2007). They showed that yeast mitochondria defective in Tom40p have reduced Bax insertion. This work also presented some contradictory results regarding the role of TOM22 in Bax mitochondrial insertion. While digestion with proteinase K of outer mitochondrial membrane proteins with cytosolic exposed domains did not influenced Bax-induced cyt *c* release, treatment of isolated mitochondria with antibodies against TOM22 prevented Bax-induced cyt *c* release. In a latter work, using mitochondria isolated from rat liver it was showed that actually both TOM22 and TOM40 are required for integration of Bax monomers (Cartron *et al.*, 2008).

1.2.4. Regulation of Bcl-2 family proteins in yeast

The pro-apoptotic function of Bax depends on its ability to translocate, oligomerize and insert into the mitochondrial membrane following stress. Therefore, in order to control the apoptotic process these events are tightly regulated. In mammalian cells, these events are regulated at transcriptional and post-translational levels. Since some of the

Bax effects are conserved in yeast, this organism is a useful tool to study the regulation of Bax at the post-translational level. In this sub-section we will describe some results obtained with the use of humanized yeasts that have improved our knowledge about the mechanisms of post-translational regulation of Bax.

1.2.4.1. Interaction mechanisms involved in Bcl-2 family regulation

Regulated protein-protein interactions are a key event in the regulation of the Bcl-2 family. These interactions can occur not only between proteins of this family but also with other cytosolic or mitochondrial proteins. Several interactions between Bax and other cytosolic or mitochondrial proteins, namely adenine nucleotide translocator [66], voltage-dependent anion channel protein (Shimizu *et al.*, 2001), humanin (Guo *et al.*, 2003), 14-3-3 (Nomura *et al.*, 2003), heat shock protein Hsp60 (Kirchhoff *et al.*, 2002), PKCε (McJilton *et al.*, 2003), and Asc (Ohtsuka *et al.*, 2004) have been described in mammalian cells. In fact, so far none of these Bax-interacting proteins have been described using the yeast model system.

Bcl-2 anti-apoptotic proteins and the pro-apoptotic member Bak have a mitochondrial localization, while Bax localizes in the cytosol when in an inactive conformation. However, members of this family can interact with each other forming heterodimers and, by this way, block each other's activity. The yeast cells have provided some important information regarding mechanisms of apoptosis inhibition involving interaction between Bcl-2 and pro-apoptotic members. Functional analysis of deletion mutants of human Bcl-2 in yeast demonstrated the presence of at least four conserved domains that are required to suppress Bax-mediated cell-killing (Hanada *et al.*, 1995). This suppression is not dependent on its ability to heterodimerize with Bax (as already discussed above), since some of these mutants suppress Bax activity and are not able to heterodimerize. Moreover, it was shown that the N-terminal region of Bcl-2 (amino acids 1-82) is necessary for Bcl-2 ability to homodimerize. Greenhalf *et al.* (1996) also showed that the C-terminal membrane anchor of Bcl-2 is necessary to rescue Bax-mediated cell killing by Bcl-2.

Yeast has also been used to study the function of other Bcl-2 family proteins. The cytosolic Bid, a direct activator BH3-only protein, is cleaved by caspase 8 generating a 15 kDa fragment that corresponds to the C-terminal of Bid, termed truncated Bid (tBid).

When this fragment is co-expressed with an active form of Bax, it enhances cell death and cyt *c* release, while co-expression with Bid has no effect (Gonzalvez *et al.*, 2005). Interestingly, while Bid has a cytosolic localization in yeast, tBid is found in mitochondria and its concentration in this organelle is higher when co-expressed with active Bax. On the opposite, the localization of active Bax is not altered by co-expression with tBid (Gonzalvez *et al.*, 2005). This effect of tBid in Bax-induced cyt *c* release has been attributed to alterations of mitochondrial bioenergetics after tBid binding to mitochondria (Gonzalvez *et al.*, 2005).

In a recent study, the yeast model also provided new insights about the controversial issue of whether the Bcl-2 protein Puma, acts as either an activator or a sensitizer in Bax activation (Gallenne *et al.*, 2009). As mentioned above, full-length untagged human Bax remains cytosolic and does not induce cyt *c* release or cell death when expressed in yeast. In this study, Puma was co-expressed with untagged human Bax which led to increased cyt *c* release and cell death. Physical interaction between both proteins was detected by co-immunoprecipitation. Bcl-xL expression inhibits this interaction, cell death and cyt *c* release. Moreover, Puma does not enhance the killing effect of the active and lethal BaxD33A mutant. These results point to the specificity of the observed effects. Puma is a direct activator of Bax and not a sensitizer/de-repressor since Bcl-xL inhibits Bax/Puma effects in yeast (Fig. **1.2**, step1).

Inhibiting protein interactions might be a method for pharmacological intervention. A class of compounds, peptide and non-peptide mimetics of the BH3 domain are able to interfere with Bax/Bcl-2 interaction. These BH3 mimetics are small molecule antagonists of the anti-apoptotic Bcl-2 members that function as competitive inhibitors of the pro-apoptotic proteins through binding to the hydrophobic cleft of the anti-apoptotic proteins (for a review see Chonghaile and Letai, 2008). Antagonising anti-apoptotic Bcl-2 family proteins through BH3-mimetics can unleash pro-death molecules and trigger cell death. Because anti-apoptotic Bcl-2 family proteins are overexpressed in cancer cells these compounds form a new class of cancer drugs that specifically target a mechanism of cancer cell survival to selectively kill cancer cells. A number of cell permeable natural compounds mimicking the BH3 domain have already been identified using a library screening process, including Tetrocarcin A, Antimycin and gossypol (Kitada *et al.*, 2003; Nakashima *et al.*, 2000; Tzung *et al.*, 2001). By means of nuclear magnetic resonance-based screening, parallel synthesis and structure-based design

Oltersdorf *et al.* (2005) discovered a small molecule inhibitor of the anti-apoptotic proteins Bcl-2, Bcl-xL and Bcl-w, that was designated as ABT-737. Treatment with ABT-737 does not directly initiate the apoptotic process, but enhances the effects of death signals indicating that it could be used in conjugation with other treatments. The effect of ABT-737 in Bax-induced cell death in yeast has been recently studied. Treatment of yeast cells expressing Bax and Bcl-xL with ABT-737 significantly affected the viability, while treatment of yeast cell expressing only Bax or Bcl-xL had no effect (Gautier *et al.*, 2011). This indicated that disruption of Bax/Bcl-xL interaction by ABT-737 suffices to initiate cell death. The same work also studied the role of Terphenyl 14 (a cell permeant Terphenyl-based peptidomimetic designed to mimic a α-helical BH3 domain (Kutzki *et al.*, 2002). Using cell free-assays, yeast and mammalian systems they showed that this compound displaces Bax from Bcl-xL and that, like with ABT-737, this is sufficient to activate Bax.

1.2.4.2. Bcl-2 family regulation by post-translational modifications

In the last years, a big emphasis has been given to the regulation of Bcl-2 family proteins by post-translational modifications. Among several post-translational modifications, phosphorylation has been a subject of increased attention. Regarding Bax, it has already been reported that phosphorylation of different Bax residues modulates its activity. Using animal cells it was discovered that phosphorylation of ser184 by protein kinase B (Akt/PKB) and PKCζ (Gardai *et al.*, 2004; Xin *et al.*, 2007; Yamaguchi and Wang, 2001) promotes cell survival that is prevented by dephosphorylation by the protein phosphatase 2A (Xin and Deng, 2006). Phosphorylation of ser163 by glycogen synthase kinase 3β (GSK3β) (Linseman *et al.*, 2004) and of thr167 by Jun N-terminal kinase (JNK) and p38 kinase (Kim *et al.*, 2006) lead to Bax activation and cell death. This showed the importance of phosphorylation that can induce slight movements in strategic positions that are able to initiate profound conformational changes in Bax structure and allow the shift from inactive/soluble to active/membrane-inserted Bax. Other members of the Bcl-2 family proteins can also be regulated by phosphorylation events. For example, Akt/PKB also phosphorylates Bad increasing cell survival (Datta *et al.*, 1997; del Peso *et al.*, 1997), Bcl-2 phosphorylation is required for its anti-apoptotic function (Ito *et al.*, 1997) and Bcl-xL is phosphorylated and inactivated by the JNK (Basu and Haldar, 2003; Fan *et al.*, 2000).

As already showed above, yeast is a potent tool to study the events that lead to destabilization of Bax soluble conformation. Arokium *et al.*, (2007) used the yeast model system to study how substitutions of potentially phosphorylatable serine residues of Bax regulate its interaction with mitochondria. By substitution of key serine residues with residues that introduces a negative charge and, therefore mimicking a phosphorylation event, they were able to modulate Bax translocation and cyt *c* release activity. Moreover, they identified a new putative phosphorylation site ser60, which is located in a consensus target sequence for PKA. These results show that yeast allows the study of the complex phoshorylation/dephosphorylation events that regulates Bcl-2 protein family activity by heterologous expression of different kinases and phosphatases (Fig. 1.2, step 2). Additionally, the yeast system was also used to study the regulation of Bax and Bcl-xL by different mammalian PKC isoforms (Saraiva *et al.*, 2006; Silva *et al.*, 2011). These results will be discussed on section 1.3.

1.3. Yeast as model system to study the regulation of apoptosis by PKC isoforms

PKC is a family of 12 intracellular serine/threonine kinases with distinct, and in some cases opposing, roles in the regulation of several mechanisms, namely cell cycle, apoptosis, differentiation, angiogenesis, multidrug resistance, invasiveness and senescence (Ali *et al.*, 2009; Battaini and Mochly-Rosen, 2007; Gutcher *et al.*, 2003; Reyland, 2009). Due to the variety of mechanisms regulated by PKC isoforms it is not surprising that deregulation of expression or activity of PKC isoforms is therefore involved in several malignancies, such as cancer (reviewed in Ali *et al.*, 2009), cardiac (Churchill *et al.*, 2008) and neurodegenerative disorders (reviewed in Cuny, 2009). The role attributed to each PKC isoform in mammalian cellular processes is somewhat contradictory. Depending on the biological context, PKC isoforms can display distinct and even opposing functions, and overlapping functions of PKC isoforms in both

promoting and inhibiting cell proliferation and/or survival have been described (reviewed in (Ali *et al.*, 2009; Battaini and Mochly-Rosen, 2007; Gutcher *et al.*, 2003; Reyland, 2009). The coexistence of several PKC isoforms in a same cellular environment, and the different expression patterns observed in different cell types and tissues may explain such contradicting results (Hofmann, 2004). Additionally, it was reported that, in a same cellular environment, the function of a PKC isoform depends on its sub-cellular localisation (Shirai and Saito, 2002). These features have been hampering the development of new therapeutic strategies involving modulation of PKC activity with many PKC modulators being discarded in early phases of clinical trials (Mackay and Twelves, 2007). Potent and selective modulators of individual PKC isoforms are urgently required in the PKC research field. To circumvent these limitations, an individual analysis of each PKC isoform should be carried out. Indeed, studies with mammalian cells lacking some isoforms of the PKC signalling cascade have contributed substantially to our understanding about the pharmacology and molecular biology of PKC isoforms. However, it would be almost impossible to use cells expressing only a PKC isoform by silencing or knocking out the genes coding for the remaining PKC isoforms.

The remarkable high degree of conservation of many pathways and cellular processes between yeast and mammalian cells has allowed the use of yeast as valuable cell models for genetic and molecular analysis of a variety of complex pathways and processes, such as cell division, transcription, receptor-mediated signal transduction secretion, cell proliferation and cell death. As refered in section 1.1 of this thesis, the genetic tractability of these organisms makes them ideal for applying genetic approaches to many biological questions. Since the yeast PKC (Pkc1p in *Saccharomyces cerevisiae*) is a structural but not a functional homologue of mammalian PKC isoforms (Iwai *et al.*, 1992; Perez and Calonge, 2002), yeast has been considered a well-suited model system to study individual mammalian PKC isoforms (Parissenti and Riedel, 2003; Parissenti *et al.*, 1993; Riedel *et al.*, 1993a; Riedel *et al.*, 1993b; Riedel *et al.*, 1993c). More recently, yeast expressing individual PKC isoforms, has been extensively used as an "*in vivo*" system for pharmacological, functional and molecular studies of relevant mammalian PKC isoforms representative of the three sub-families (Coutinho *et al.*, 2011; Coutinho *et al.*, 2009b; Saraiva *et al.*, 2004; Saraiva *et al.*, 2006; Silva *et al.*, 2011). In the yeast studies, a particular focus was given to the screening of selective PKC modulators and

the understanding of the regulation of the Bcl-2 family proteins and p53 by PKC isoforms.

The present section includes a current overview on the more recent data about the role of PKC isoforms in apoptosis regulation and how the yeast model system contributed to our knowledge in this research field. Moreover, the use of this cell system in the identification and characterization of isoform-selective PKC modulators will also be discussed.

1.3.1. PKC structural features and activation

The PKC family comprises at least 12 related serine/threonine kinases that are coded by nine different genes. Currently, these isoforms are classified into three different subfamilies based on their structure and co-factors required for activation: classical or conventional PKCs (cPKCs; α, βI, βII and γ) that require calcium, phosphotidylserine (PS) and diacylglycerol (DAG) for activation; novel PKCs (nPKCs; δ, ε, η and θ) that require PS, DAG, but not calcium; and atypical PKCs (aPKCs; ζ and λ/ι) that only require PS for activation (Newton, 1995; Reyland, 2009). The differential dependence on cofactors for optimal activation can be explained by differences in the protein structure between the subfamilies (Fig. 1.3). The overall PKC isoform structure comprises four conserved (C1-C4) and five variable (V1-V5) domains. The regulatory domain includes the C1 and C2 regions as well as a pseudo-substrate region that interacts with the catalytic domain, thereby maintaining the enzyme in an inactive state within the cytosol (Newton, 1995; Soderling, 1990). The C1 domain contains two cysteine-rich zinc fingers motifs that constitute the binding site for DAG. aPKCs have only one zinc finger, thus explaining their inability to be activated by DAG or phorbol esters. The C2 domain binds calcium and is absent in nPKCs and aPKCs. The regulatory domain is also responsible for the binding of receptors for activated C kinases (RACKs), anchoring proteins that ultimately determines the sub-cellular locations of PKCs after activation (Schechtman and Mochly-Rosen, 2001). The catalytic segment contains the C3 and C4 domains, which function as the ATP binding site and the kinase catalytic centre, respectively. The variable domain, V3, is the hinge region where the PKC isoforms can be cleaved by caspases separating the catalytic and regulatory domains.

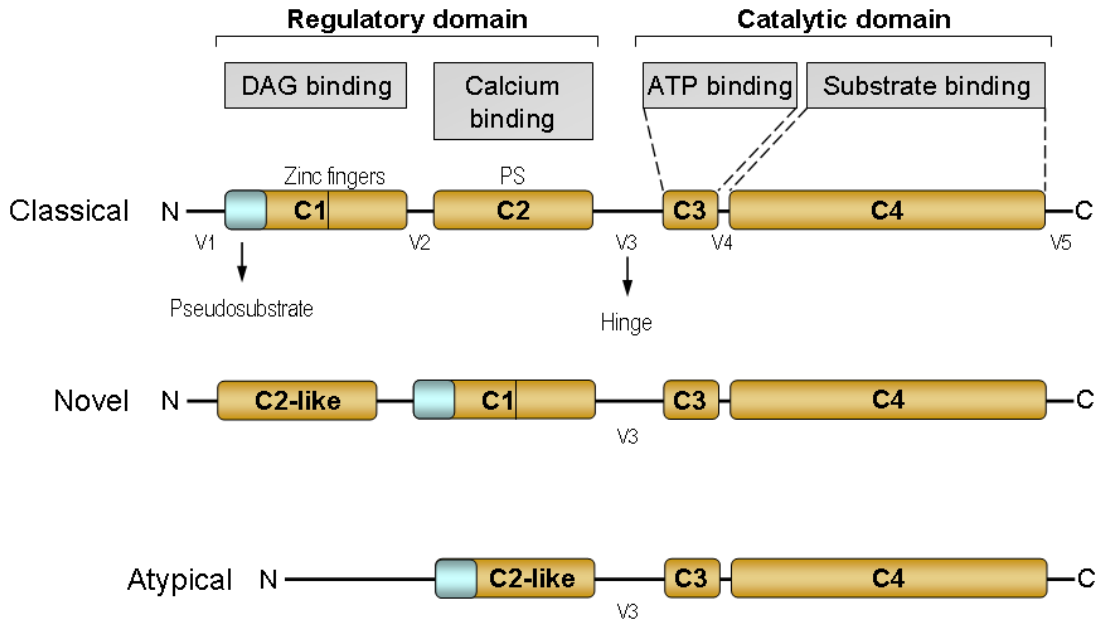

Figure 1.3 – Structural representation of the PKC family. PKC has four conserved domains (C1-C4) and five variable (V1-V5) domains. The catalytic domains comprise the C3 and C4 domains responsible for ATP and substrate binding, respectively. The domains are well conserved between the different sub-families. The differences between the different subfamilies reside mainly in the regulatory domain. The C1 domain that comprises 2 cysteine-rich motifs forming the DAG binding site has only 1 cysteine-rich in aPKCs and is not able bind DAG. The C2 domain that binds calcium and PS in cPKCs is changed in nPKCs and aPKCs which lost the ability to bind calcium.

PKC is synthesized as an inactive dephosphorylated form. Mass spectrometry studies revealed that classical and novel PKC isozymes are phosphorylated in three conserved sites in the catalytic domain. Phosphorylation at all these three sites is required to generate a mature form of the kinase, but does not activate PKC, it only prime PKC for activation by second messengers. The first phosphorylation event is executed by the 3-phosphoinositide-dependent kinase (PDK-1) and occurs at a conserved threonine in the activation loop of the carboxy terminus (Parekh *et al.*, 2000). Phosphorylation of the other two additional C-terminal sites contributes to stability of the kinase. These include an autophosphorylation site and another site in a hydrophobic region (Dempsey *et al.*, 2000; Newton, 1995; Newton, 2001).

Most cellular PKCs are fully phosphorylated and in an auto-inhibited conformation in the cytoplasm. Several stimuli can lead to activation of phospholipase C (PLC) that hydrolyses inositol-containing phospholipids producing DAG and, therefore, activation of classical and novel PKCs. First, binding of DAG to the C1 domain increases the

affinity of PKCs for cell membranes. In the case of classical PKC isoforms Ca^{2+} is thought to increase the affinity of DAG to the C1 domain. A second interaction with acidic membrane lipids provides the necessary energy to the release of the pseudo-substrate from the substrate binding pocket and activation of the kinase (Newton, 2001). While lipid-dependent isoforms are largely activated by this mechanism, little is know about the mechanism of aPKCs activation. Recent studies suggest that the activity of these isoforms may be regulated through protein interactions involving the Phox Bem 1 (PB1) domain within the regulatory region of aPKCs (Etienne-Manneville and Hall, 2003).

Activated PKCs then activate several signalling pathways such as the extracellular signal-regulated kinase (ERK) pathway, the JNK and the Akt/PKB pathway (Balendran *et al.*, 2000; Cai *et al.*, 1997; Ueda *et al.*, 1996).

1.3.2. Regulation of apoptosis by PKC isoforms: contribution of yeast studies

The initial results that aimed to establish a role for PKC in apoptosis were quite contradictory. The studies either indicated a pro-apoptotic or an anti-apoptotic role for PKC depending on the cell type (Azuma *et al.*, 1993; McConkey *et al.*, 1989). The contradictory nature of these results was in part due to the use of phorbol esters as PKC activators. Phorbol esters can stimulate classical and novel PKCs, but prolonged treatments with these compounds lead to PKC down-regulation and cellular depletion (Gutcher *et al.*, 2003). In addition, different cellular types can vary extensively in PKC isoforms expression profiles puzzling the interpretation of the results obtained. It is now clear that PKC isoforms have specific functions in the regulation of apoptosis. Therefore, apoptosis regulation by PKC is dependent of the function of the isoform that is activated and of the PKC isoforms expression profile of the cell type under study. Moreover, in a same cellular environment, the function of a PKC isoform depends on the cellular compartment where it is relocalised after activation (Shirai and Saito, 2002).

The development of activators and inhibitors with higher PKC isoform-selectivity contributed to the study of the role of PKC isoforms in the regulation of apoptosis (Dempsey *et al.*, 2000). In general, PKCα, ε, ζ and λ/ι are considered predominantly anti-apoptotic, promoting survival and proliferation, while PKCδ is characterized as pro-apoptotic, having a tumour suppressor role (Reyland, 2009). However, recent

results show that such a general classification is not completely accurate. Therefore, the establishment of the relative contribution of individual PKC isoforms in apoptosis is still challenging.

1.3.2.1. cPKCs in apoptosis

Several reports indicating that PKCα activation can prevent apoptosis and that its inhibition is associated with apoptosis have been published. Down-regulation of PKCα using antisense nucleotides or phorbol esters treatment leads to apoptosis in different cell types (Ahmad *et al.*, 1994; Dooley *et al.*, 1998; Haimovitz-Friedman *et al.*, 1994). Induction of apoptosis is also observed after expression of a dominant negative form of PKCα (Whelan and Parker, 1998). The mechanism for apoptosis inhibition by PKCα is still poorly understood. One target already identified is the anti-apoptotic protein Bcl-2. It was demonstrated that suppression of apoptosis in murine growth factor dependent cell lines involves Bcl-2 phosphorylation at serine 70 by PKCα. Phosphorylation of this site leads to Bcl-2 stabilization and enhanced anti-apoptotic activity (Ito *et al.*, 1997).

However, some contradictory results suggesting a pro-apoptotic role for PKCα have also been reported. PKCα was shown to mediate activation of caspase-3 downstream cyt *c* release in renal proximal tubule cells treated with the chemotherapeutic drug cisplatin (Nowak, 2002) and activation of DNA fragmentation and nuclear apoptosis through Lamin B phosphorylation in HL60 cells (Shimizu *et al.*, 1998). In a human prostate cancer cell line, the presence of PKCα in non-nuclear membranes was associated with apoptosis, while its absence resulted in resistance to apoptosis (Powell *et al.*, 1996). In the same cell line, Tanaka and colleagues showed that p38 MAPK is activated by PKCα, mediating PKCα-induced apoptosis (Tanaka *et al.*, 2003). Moreover, they demonstrated that PKCα leads to protein phosphatase 2A-mediated dephosphorylation and inactivation of the survival kinase Akt/PKB, giving some insight about a possible pro-apoptotic mechanism of PKCα (Tanaka *et al.*, 2003).

Recently studies using the yeast model system gave some important insights regarding the mechanisms of apoptosis regulation by several PKC isoforms, including PKCα. When expressed in yeast, this isoform stimulated acetic acid-induced apoptosis exhibiting pro-apoptotic functions under this stress conditions (Saraiva *et al.*, 2006).

However, it did not stimulate H_2O_2-induced apoptosis (Coutinho *et al.*, 2011). Similarly to that reported for mammalian cells (Shirai and Saito, 2002; Wang *et al.*, 2004), these results obtained with yeast corroborate that a specific PKC isoform can induce, in a same cellular background, different cellular responses depending on the stimulus applied. This can be explained by the ability of different stimuli to selectively translocate a PKC isoform to distinct subcellular compartments (Shirai and Saito, 2002; Wang *et al.*, 2004). Though this possible explanation was not confirmed in yeast for acetic acid and H_2O_2 stimuli, distinct translocations associated with distinct cellular responses were observed in yeast cells expressing PKCδ or PKCε when expose to phorbol-12-myristate-13-acetate (PMA) and 6,11,12,14-tetrahydroxy-abieta-5,8,11,13-tetraene-7-one (coleon U) (Coutinho *et al.*, 2011). Together, the data obtained from yeast cells highlight that the function of a PKC isoform, such as PKCα, in apoptosis is also highly dependent of the stimulus applied.

Using the yeast cell model, it was also showed that PKCα is a regulator of several members of the Bcl-2 family, namely Bax and Bcl-xL, promoting cell death (Saraiva *et al.*, 2006; Silva *et al.*, 2011). In fact, with yeast cells co-expressing murine Bcl-xL and bovine PKCα it was shown that PKCα abolished the protective effect of Bcl-xL on acetic acid-induced yeast cell death. This inhibition of the anti-apoptotic effects of Bcl-xL was associated to an increase in the levels of phosphorylated Bcl-xL, the inactive form of Bcl-xL (Saraiva *et al.*, 2006). PKCα was also able to regulate Bax function in yeast. Using yeast cells co-expressing an active form of human Bax and bovine PKCα, it was shown that PKCα increases the translocation and insertion of Bax into the outer mitochondrial membrane, with an increase in cyt *c* release, ROS production, mitochondrial network fragmentation and cell death. The cell death process observed is regulated, since it correlates with an increase in autophagy but not with plasma membrane permeabilization. However, in this case, the observed increase in Bax translocation and insertion by PKCα was not due to Bax phosphorylation. Additionally, it revealed to be independent of the PKCα kinase activity. This observation obtained with yeast supports the notion that PKCα can promote apoptosis by a kinase-independent way. In fact, non traditional PKC activation mechanisms, such as kinase-independent activity of the PKC regulatory domain, are frequently reported for PKCs (Steinberg, 2008). For example, a PKC pro-apoptotic function independent of its kinase activity was reported for PKCδ in the vascular smooth muscle cell line A7r5 (Goerke *et*

al., 2002). Additionally, PKCε, via its regulatory domain and independently of its catalytic domain, induced neurite-like processes in neuroblastoma cells (Zeidman *et al.*, 1999). Therefore some caution must be taken in the interpretation of results obtained with PKC inhibitors (as Ro 32-0432) which act through interaction with the PKC catalytic region (namely the ATP binding site), inhibiting the protein kinase activity by inhibition of the PKC capability to phosphorylate its substrates. Recently, the regulation of apoptosis through modulation of the tumour suppression protein p53 was also studied using H_20_2-treated yeast cells co-expressing bovine PKCα and human p53 (Coutinho *et al.*, 2011). However, in opposition to that observed with other PKC isoforms, PKCα did not interfere with the p53 activity. This result suggests that p53 is not a substrate of this PKC isoform.

The role of PKCβ in apoptosis is also somewhat contradictory. The gene encoding for this protein generates two isoforms by alternative splicing PKCβI and PKCβII and some of the studies reported failed to distinguish the contribution of each of these variants in apoptosis. Results favouring an anti-apoptotic role for PKCβ show that expression of the oncogene v-*abl* induces translocation of PKCβII to the nucleus and prevention of apoptosis, implying an anti-apoptotic role for this isoform (Evans *et al.*, 1995). Moreover, it was reported that PKCβII leads to an increase of Bcl-2 levels in the mitochondrial membrane and to the inhibition of apoptosis in HL60 cells treated with arabinofuranosylcytosine (Whitman *et al.*, 1997). Studies with PKCβ transgenic mice demonstrated that this isoform is specially required for NFkB activation and NFkB-mediated cell survival in B lymphocytes (Su *et al.*, 2002). A pro-apoptotic role for PKCβ has also been described. Activation of PKCβI by 12-deoxyphorbol 13-phenylacetate 20-acetate (DOPPA) induces apoptosis in HL60 cells (Macfarlane and Manzel, 1994). PKCβ targets the JNK/SAPK to the mitochondria. JNK/SAPK phosphorylates Bcl-xL in the mitochondria inactivating it and thereby promoting cyt *c* release and apoptosis (Ito *et al.*, 2001).

1.3.2.2 nPKCs in apoptosis

Among the several isoforms of this subfamily, PKCδ and ε are the most extensively studied. Although PKCδ and ε display a high degree of homology and similar substrate specificity, suggesting similar targets in signal transduction pathways for both nPKCs,

they are frequently described as mediating quite contrasting physiological effects (Gutcher *et al.*, 2003; Hofmann, 2004; Reyland, 2009). While PKCδ is often reported as pro-apoptotic, PKCε is widely considered an oncogene with anti-apoptotic functions (Basu and Pal, 2010; Basu and Sivaprasad, 2007; Gorin and Pan, 2009; Reyland, 2009).

Activation of PKCδ occurs in response to several apoptotic stimuli including genotoxic stress (Yoshida *et al.*, 2006), oxidative stress (Majumder *et al.*, 2001), UV (Denning *et al.*, 1998), etoposide (Reyland *et al.*, 1999) and death receptors (Khwaja and Tatton, 1999). In most of these cases, inhibition of PKCδ activity leads to inhibition of apoptosis. A regulator of PKCδ activation is caspase-3 and it was shown that during apoptosis, caspase-3 proteolytically cleaves PKCδ at a site within the V3 domain leading to the release of the catalytically active 40kDa fragment from the regulatory domain, thereby causing activation of PKCδ in the absence of any cofactors (Emoto *et al.*, 1995). The identification of PKCδ as a substrate of caspase-3 led to the publication of an overwhelming amount of literature linking PKCδ to apoptosis induction. However, it is important to note that the PKCδ cleavage by caspase-3 seems to depend on the cell type (D'Costa and Denning, 2005; Sun *et al.*, 2008).

While most of the reports associate a pro-apoptotic function to PKCδ, this isoform can also have anti-apoptotic functions during receptor-mediated cell death. Depletion of PKCδ by siRNA, overexpression of the kinase-dead mutant of PKCδ, or inhibition of PKCδ with rottlerin enhanced TRAIL-induced apoptosis. Interestingly, proteolytic cleavage of PKCδ and phosphorylation of several key residues were necessary for the anti-apoptotic effect of PKCδ during TRAIL-induced apoptosis (Basu and Miura, 2002; Lu *et al.*, 2007). PKCδ has been shown to promote survival of non-small cell lung cancer, breast cancer, pancreatic cancer, liver cancer, and chronic lymphocytic leukemia cells (reviewed in Basu and Pal, 2010). But, the pro- and anti-apoptotic function of PKCδ does not seem to depend only on the cell type. PKCδ apoptotic activity is highly dependent on the cellular stimulus that, as referred above, interferes with the cellular localization of PKC isoforms. In fact, the intracellular localization of PKCδ has a pronounced impact on its pro-apoptotic activity. Constitutively active PKCδ targeted to the cytosol, mitochondria, or nucleus often behaved as pro-apoptotic, whereas PKCδ targeted to the endoplasmic reticulum (ER) protected against tumor necrosis factor-related apoptosis, ligand-induced apoptosis and etoposide-induced apoptosis (reviewed

in Basu and Pal, 2010). PKCδ targeted to the cytosol and mitochondria, but not to the nucleus or ER, undergoes proteolytic cleavage by caspase 3 (Gomel *et al.*, 2007). Recent reports showed that upon exposure to a genotoxic stress, such as etoposide, PKCδ accumulates in the nucleus. Whereas retention of PKCδ in the cytoplasm is compatible with cell survival, its nuclear retention is required for commitment to apoptosis, showing that cellular localisation of PKCδ regulates the survival/death pathway (DeVries-Seimon *et al.*, 2007; Reyland, 2007; Yoshida, 2008). Indeed, nuclear targeting of kinases such as PKCδ is considered a new and essential regulatory mechanism that directly influences the induction of apoptosis (Yoshida, 2008). Interestingly, in yeast cells expressing rat PKCδ or ε, it was observed that while PMA induced PKCδ/ε translocation from the cytosol to the plasma membrane, coleon U induced PKCδ/ε translocation from the cytosol to the nucleus of yeast cells. These different translocations were associated with different cellular responses. In fact, while PMA caused a G2/M cell cycle arrest, coleon U induced an apoptotic cell death in yeast expressing PKCδ/ε. The results obtained in yeast corroborated the data from mammalian cells showing not only that the correct cellular localisation is critical to the function of these two nPKCs, but also that the induction of apoptosis by these kinases can be driven by their nuclear retention. In fact, many PKCδ substrates in apoptotic cells are nuclear proteins (Reyland, 2009). One example is the DNA-dependent protein kinase catalytic subunit (DNA-PK). It was shown that the functional interactions between PKCδ and DNA-PK contribute to DNA damage-induced apoptosis (Bharti *et al.*, 1998).

Besides ser/thr phosphorylation, unlike other PKC isoforms, PKCδ is also phosphorylated in several tyrosine residues in response to various stimuli. Depending on the site of phosphorylation and the stimulus, tyrosine phosphorylation of PKCδ can either lead to its activation or inhibition. Tyrosine phosphorylation of PKCδ has been shown to regulate both nuclear localization and proteolytic cleavage of PKCδ and, therefore, its apoptotic function (Reyland, 2009; Steinberg, 2004). After activation and translocation of PKCδ to different intracellular compartments PKCδ regulates several signalling pathways. PKCδ interacts with several members of the mitogen-activated protein kinase (MAPK) family, including p38 (Gomel *et al.*, 2007), ERK (Basu and Tu,

2005; Lomonaco *et al.*, 2008) and JNK (Gomel *et al.*, 2007; Humphries *et al.*, 2006). It is also able to phosphorylate the Bcl-2 family member, Mcl-1 (Sitailo *et al.*, 2006).

The effect of PKCδ on Bcl-xL anti-apoptotic function was also analysed in yeast. However, unlike other PKC isoforms, in these cells PKCδ did not regulate Bcl-xL phosphorylation state or its anti-apoptotic role (Saraiva *et al.*, 2006). PKCδ is also described as a major regulator of p53 activity at transcription and post-translational levels (Abbas *et al.*, 2004; Yoshida *et al.*, 2006). In fact, studies performed in yeast also indicate that PKCδ is a positive regulator of p53 activity both in cell proliferation and death processes. It was observed that PKCδ enhanced p53-induced yeast growth inhibition/S-phase cell cycle arrest and p53-mediated apoptosis through p53 phosphorylation at ser376-378 (Coutinho *et al.*, 2009a; Coutinho *et al.*, 2009b). Though several works have already reported the p53 phosphorylation and the activation of a transcription-dependent p53 apoptotic mechanism by PKCδ, the studies performed in yeast revealed crucial aspects about the regulation of p53 apoptotic activity, namely in its transcription-independent mechanisms, by PKCδ. In fact, using yeast cells, it was shown, for the first time, the activation of mitochondrial p53 translocation by PKCδ. This study provided new insights about an unclear issue concerning the regulation of p53 translocation to mitochondria (Coutinho *et al.*, 2011).

As referred above, PKCε is often regarded as having anti-apoptotic properties, the mechanisms responsible for its anti-apoptotic function are unclear, and little is still known about the physiologically relevant substrates of this isoform. An important target of this isoform is the Akt/PKB pathway (Lu *et al.*, 2006; Wu *et al.*, 2004; Zhou *et al.*, 2002). Several evidences reported physical interaction between PKCε and Akt and decreased phosphorylation of Akt when a kinase-dead mutant of PKCε is expressed in apoptotic cells (Matsumoto *et al.*, 2001). But, the anti-apoptotic properties of PKCε seem to also involve the regulation of several members of the Bcl-2 family. PKCε enhances the expression of anti-apoptotic members of this family and inhibits pro-apoptotic Bcl-2 family members, such as Bax and Bad (Gubina *et al.*, 1998; Sivaprasad *et al.*, 2007). In fact, the physical interaction of PKCε with Bax decreases Bax translocation to mitochondria (McJilton *et al.*, 2003). Additionally, it was also shown the inactivation of BAD by PKCε through an rsk-dependent pathway (Bertolotto *et al.*, 2000). Using yeast cells co-expressing a mouse PKCε and the human anti-apoptotic

protein Bcl-xL, we showed that PKCε markedly enhanced the Bcl-xL anti-apoptotic activity, which was accompanied by a pronounced decrease of the Bcl-xL phosphorylated form (Saraiva *et al.*, 2006).

Although several studies suggest that PKCε favours life over death, some reports also showed that PKCε activation can contribute to apoptosis. For example, it was demonstrated that ethanol-induced apoptosis in hepatocytes via activation of the nPKCs, PKCδ and ε (Zhang *et al.*, 2007). In fact, in yeast, PKCε stimulated acetic acid- and H_2O_2-induced cell death exhibiting therefore a pro-apoptotic activity in these cells (Coutinho *et al.*, 2011; Saraiva *et al.*, 2006). Furthermore, activation of PKCε by coleon U induced apoptosis in yeast (Coutinho *et al.*, 2009b). Additionally, similarly to that reported for PKCδ, in yeast, PKCε stimulated the p53 activity through phosphorylation at ser376-378 (Coutinho *et al.*, 2011; Coutinho *et al.*, 2009a). It was also shown that PKCε stimulated a p53-mediated apoptosis in yeast through activation of transcription-dependent and -independent p53 apoptotic mechanisms (Coutinho *et al.*, 2011).

1.3.2.3 aPKCs in apoptosis

The atypical PKC isoforms are considered anti-apoptotic proteins with a critical role in the cell survival signalling (Reyland, 2009). However, very little is known about their mechanisms of action and physiological targets. It has been described that PKCλ/ι promotes inhibition of apoptosis in the absence of the correct extracellular matrix (Pongracz *et al.*, 1995). Moreover, PKCλ/ι is genomically amplified and overexpressed in serous epithelial ovarian cancers and contributes to poor prognosis (Eder *et al.*, 2005). The proliferative and anti-apoptotic role of PKCζ involves the ERK and the NF-κB/IκB pathways. It was shown that loss of PKCζ results in inhibition of cell proliferation and survival, as well as defects in the activation of ERK and the transcription of NF-κB-dependent genes (Martin *et al.*, 2002). Moreover, it was shown that PKCζ induces phosphorylation and inactivation of IκB-α in vitro (Diaz-Meco *et al.*, 1994). Another evidence for the involvement of the NF-κB pathway in the PKCζ anti-apoptotic activity was the identification, by yeast two-hybrid, of the prostate androgen responsive-4 (Par-4), a pro-apoptotic protein that induces apoptosis trough the NF-κB pathway, as a binding protein partner of aPKCs (Diaz-Meco *et al.*, 1999; Diaz-Meco *et al.*, 1996). This interaction inhibits Par-4 enzymatic activity and consequently

apoptosis. Recently, Xin *et al.* (2007) showed that PKCζ also modulates Bax through phosphorylation. They demonstrated that the treatment of cells with nicotine leads to enhanced PKCζ activity and phosphorylation of Bax at ser184, inhibiting Bax pro-apoptotic activity.

As described above for PKCα, distinct responses were also obtain with PKCζ in yeast under distinct apoptotic stimuli. In fact, though PKCζ did not interfere with H_2O_2-induced cell death, it stimulated the acetic acid-induced apoptosis in yeast. Therefore, in opposition to the anti-apoptotic activity attributed to PKCζ in mammalian cells, a pro-apoptotic activity was exhibited by this isoform in yeast. However, when this isoform was co-expressed with Bcl-xL, it markedly enhanced the Bcl-xL anti-apoptotic activity through a pronounced decrease of the Bcl-xL phosphorylated form (Saraiva *et al.*, 2006). In this work, a direct correlation between the anti-apoptotic activity of PKCζ and the regulation of Bcl-xL activity was established. Indeed, in the absence of Bcl-xL, the anti-apoptotic activity of PKCζ is completely lost. Using the yeast cell model, we also showed that, in opposition to Bcl-xL, p53 was not a cellular substrate of PKCζ. In fact, when co-expressed with p53 in yeast, PKCζ did not interfere with the p53 activity and phosphorylation state (Coutinho *et al.*, 2011).

1.3.3. Yeast as a model system for the identification and characterization of isoform-selective PKC modulators

Yeast has been considered an important tool to uncover the different cellular targets of several drugs (Sturgeon *et al.*, 2006). Moreover, the preservation in yeast of the functional characteristics of mammalian PKCs (Parissenti and Riedel, 2003; Sprowl *et al.*, 2007) allowed to identify and characterize the mode of action of several PKC modulators in yeast cells expressing individual PKC isforms.

An *in vivo* assay, based on growth stimulation of yeast expressing an individual mammalian PKC isoform (proportional to the degree of PKC inhibition), revealed that calphostin C inhibits PKCδ more efficiently than PKCγ and that chelerythrine is a more efficient inhibitor of PKCγ (Keenan *et al.*, 1997). It was also demostrated that xanthonolignoids inhibit PKC isoforms but with differences in their potency towards the distinct isoforms tested (PKCα, PKCβI, PKCδ, PKCη and PKCζ), with higher potency

towards PKCζ (Saraiva *et al.*, 2003a). This form of yeast phenotypic assay was also used to characterize the activities of the PKC activators PMA and its analogues on different PKC isoforms, namely on the classical (α and βI), novel (δ and η) and atypical (ζ). Saraiva and co-workers demonstrated that the different analogues of PMA differ on their potency to activate a given PKC isoform, presenting isoform-selectivity. Furthermore, they showed that two of the analogues tested, phorbol-12-myristate-13-acetate-4-O-methyl-ether (MPMA) and 4αPMA, caused effects similar to those expected from PKC inhibition (Saraiva *et al.*, 2004).

Yeast cells expressing individual PKC isoforms were used to elucidate the molecular mechanism of action of coleon U, a diterpene compound isolated from *Plectranthus grandidentatus*. Coleon U showed to inhibit the growth of several human cancer cell lines, such as MCF-7 (breast), NCI-H460 (lung), SF-268 (CNS), TK-10 (renal) and UACC-62 (melanoma), in a dose dependent manner (Marques *et al.*, 2002). In a later study, a dose-dependent anti-proliferative effect of coleon U on T- and B-lymphocyte cells was also reported (Cerqueira *et al.*, 2004) and correlated with its capacity to induce apoptosis in these cells. However, the molecular mechanisms of action associated with coleon U-induced apoptosis in human cell lines were not elucidated. Since several diterpene compounds, such as phorbol esters, were identified as potent PKC activators, the yeast PKC expression system was exploited to study the modulatory activity of coleon U on individual PKC isoforms of the three PKC subfamilies. In agreement with that reported for human cells, coleon U also induced yeast growth inhibition but only in cells expressing the nPKCδ or ε. Together, these results led to discover of coleon U as a potent and selective activator of nPKCδ and ε, as later confirmed in mammalian cells (Maghzal *et al.*, 2010).

Together these results show that the yeast model system can be used, not only for a high-throughput screening of PKC modulators but also to elucidate the molecular mechanisms of action by which these compounds modulate the different cellular processes, namely apoptosis.

1.4. Scope of this thesis

As illustrated in previous sections the yeast model system has provided important information regarding the mechanism of apoptosis regulation by Bcl-2 family proteins and PKC isoforms. The scope of this thesis is to analyse the mechanisms of Bax regulation by four different PKC isoforms in yeast (PKCα, δ, ε and ζ) and discuss the relevance of these mechanism in mammalian apoptosis. In chapter 2, several experimental evidences were included showing a regulation of Bax *c*-myc (an active and tagged human Bax with mitochondrial localization) activity by PKCα (section 2.3). A distinct regulation of Bax α (an inactive human Bax with cytosolic localization) by mammalian PKC isoforms in yeast is also demonstrated in this chapter (section 2.4). Chapter 3 is dedicated to a general discussion focusing on the main contributions of the present work to the understanding of apoptosis regulation by PKC isoforms and the use of humanized yeast as a powerful tool for these studies.

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Chapter 2

Modulation of Bax by mammalian protein kinase C isoforms in yeast

This chapter comprises parts from the following publications:

Silva RD, Manon S, Gonçalves J, Saraiva L and Côrte-Real M (2011) Modulation of Bax mitochondrial insertion and induced cell death in yeast by mammalian protein kinase Calpha. *Exp Cell Res*. 317:781-790.

Silva RD, Manon S, Fernandes S, Gonçalves J, Saraiva L and Côrte-Real M (2011) Mammalian PKC isoforms distinctly regulate Bax in yeast. *Manuscript in preparation*.

Modulation of Bax by mammalian PKC isoforms in yeast

2.1. Introduction

Apoptosis is a highly regulated genetic program that culminates in cell death. It is fundamental in development and tissue homeostasis and its deregulation leads to several diseases, including cancer and neurodegenerative disorders. Due to its significance, this process is under tight regulation by a large variety of apoptotic proteins and signalling pathways. A crucial feature of mammalian apoptosis is the permeabilization of membrane organelles, namely mitochondria (see section 1.2). This permeabilization leads to the release of several apoptogenic factors and irreversible activation of the apoptotic cascade. Mitochondrial outer membrane permeabilization (MOMP) is regulated by complex interactions between members of the Bcl-2 family proteins (Green and Evan, 2002). Despite the importance of these proteins, the mechanisms by which they are regulated are not fully understood. Moreover, the existence of a large number Bcl-2 family members, their dependence of the cell type and stimulus complicate the analysis of their individual role.

The Bcl-2 family comprises several members divided into four sub-families. Among the different subfamilies, the Bcl-2 effector proteins sub-family (Bak and Bax) is essential for the completion of MOMP and apoptosis through the intrinsic pathway, since deletion of both of its members completely impairs the mitochondrial apoptotic process (Wei *et al.*, 2001). Bax is the most well studied member of this subfamily. It is a cytosolic multidomain protein and its pro-apoptotic function depends on its ability to translocate, oligomerize and insert into the mitochondrial membrane following stress where it oligomerizes and induces MOMP (Gross *et al.*, 1998; Mikhailov *et al.*, 2001; Nechushtan *et al.*, 2001). In the last years a bulk of evidences showed that Bax activity can be regulated by phosphorylation events and interaction with other proteins (Gardai *et al.*, 2004; Guo *et al.*, 2003; Kim *et al.*, 2006; Kirchhoff *et al.*, 2002; Linseman *et al.*, 2004; Marzo *et al.*, 1998; McJilton *et al.*, 2003; Nomura *et al.*, 2003; Ohtsuka *et al.*,

2004; Sato *et al.*, 1994; Shimizu *et al.*, 2001; Xin and Deng, 2006; Xin *et al.*, 2007; Yamaguchi and Wang, 2001; Yin *et al.*, 1994).

Regulation of apoptosis by signalling pathways involving different protein kinase C (PKC) isoforms has been subject of increased attention in the last decade. PKC is a family of serine/threonine kinases comprising at least twelve isoforms that are classified into three different subfamilies based on their structure and activation requirements (see chapter 1, section 1.3). PKC isozymes are ubiquitously expressed, and PKCα, β, and δ are the most abundant isozymes in various tissues (Wetsel *et al.*, 1992). Although, it has been clearly demonstrated that PKC regulates apoptosis in an isoform-specific manner, the role of each isoform in apoptosis regulation is still controversial (Gutcher *et al.*, 2003). For most of the PKC isoforms there are reports either indicating a role in apoptosis promotion (Su *et al.*, 2002; Whelan and Parker, 1998) or in apoptosis inhibition (Basu and Pal, 2010; Ito *et al.*, 2001; Tanaka *et al.*, 2003). These contradictory results can be explained by the co-existence in the same cell of different PKC isoforms with opposing roles in apoptosis, different tissue distribution patterns and different sub-cellular localisations (chapter 1, section 1.3; Shirai and Saito, 2002). These features have been hampering the study of the role of PKC isoforms in apoptosis regulation. While studies of mammalian cell lines lacking specific components of the apoptotic machinery or isoforms of the PKC signalling cascade have contributed substantially to our understanding, it would be almost impossible to use cells with all the relevant genes silenced or knocked out.

The genetic tractability of yeast cells makes this organism ideal for applying genetic approaches to many biological questions, including apoptosis (chapter 1). It was recognized in yeast a mitochondria-mediated apoptotic pathway similar to the mammalian intrinsic apoptotic pathway (Pereira *et al.*, 2008; Silva *et al.*, 2011a). Moreover, the existence of orthologues of some mammalian apoptotic regulators in yeast, support the existence of a primordial apoptotic machinery similar to the one present in mammalian cells (Buttner *et al.*, 2007; Fahrenkrog *et al.*, 2004; Ludovico et al 2002; Madeo *et al.*, 2002; Wissing *et al.*, 2004). Although orthologues of key mammalian apoptotic regulators like Bcl-2 family proteins are absent, when some of these proteins are expressed in yeast they conserve their functional and molecular roles at several cellular levels, namely at the mitochondria. This has allowed the use of yeast cells to uncover several important features of apoptosis regulation by Bcl-2 family

proteins (see Silva *et al.*, 2011a). As discussed in section 1.3, yeast is also considered a good model system to study the role of individual mammalian PKC isoforms (Parissenti and Riedel, 2003) and has allowed study of isoform-selective PKC modulators (Saraiva *et al.*, 2004; Saraiva *et al.*, 2002; Saraiva *et al.*, 2003a; Saraiva *et al.*, 2003b). Recently, this system was used to study regulation of human apoptotic regulators such as Bcl-xL and p53 by different PKC isoforms (Coutinho *et al.*, 2011; Coutinho *et al.*, 2009; Saraiva *et al.*, 2006).

In the present chapter, we used the yeast model to investigate the role of mammalian PKCα, δ, ε and ζ in Bax regulation. We started to study the role of PKCα in the modulation of Bax *c*-myc (a human and active Bax with mitochondrial localization), in mitochondrial insertion and induced cell death in yeast (section 2.3). Our results demonstrate that PKCα increases the translocation and insertion of Bax *c*-myc into the yeast mitochondria by a mechanism independent of the PKCα kinase activity. Following these results and aiming to address if this effect was isoform-specific we extended our studies to PKCδ, ε and ζ (section 2.4) but, instead of studying their role in the regulation of the tagged form Bax *c*-myc, we evaluated their effect on regulation of Bax α, an inactive human Bax with cytosolic localization. We showed that these different PKC isoforms distinctly regulate Bax and therefore its apoptotic-inducing activity.

2.2. Material and methods

2.2.1. Yeast strains, plasmids and growth conditions

The wild-type haploid *Sacharomyces cerevisiae* strain CG379 (*mat* α *ade5 his7-2 leu2-112 tryp1-289α ura3-52* [*kil-O*], yeast genetic stock center, University of California, Berkeley, USA) was used in the studies described in section 2.3 and the strain W303-1A (MAT*a*, *ade2*, *his3*, *leu2*, *trp1*, *ura3*, *can1*) was used in the studies described in section 2.4. For PKCα expression, the bovine PKCα was cloned into the YEp51 yeast

expression plasmid (*LEU2*). For PKCδ, PKCε and PKCζ expression, the rat PKCδ, the mouse PKCε or PKCζ cDNA, respectively, were cloned into the YEplac181 yeast plasmid. Both, Yep51 and YEplac181 have the *LEU2* gene as a selective marker. All the PKC constructs are under control of the *GAL1/10* promoter. For Bax *c*-myc expression, the isoform α of the human *bax* gene was chemically synthesized with yeast codon bias and fused to the *c*-myc epitope cloned into the centromeric plasmid pCM184 (*TRP1*) under the control of a Tet-Off promoter (repressed by the addition of doxycycline) as described in (Priault *et al.*, 1999). Bax α and Bax^{P168A} were cloned in the pYES3/CT plasmid (Invitrogen) as described in (Arokium *et al.*, 2004; Arokium *et al.*, 2007). The pYES3/CT plasmid has the *TRP1* gene as a selective marker and a *GAL1/10* promoter. The GFP-Atg8p construction (as described in Shintani and Klionsky, 2004) is in the pRS416 plasmid under control of the endogenous Atg8p promoter.

Site directed mutagenesis of bovine PKCα was done using the QuickChange method (Stratagene) with the primers gagctgtacgccatccgtatcctgaagaaggacgtgg and ccacgtccttcttcaggatacggatggcgtacagctc. The mutant PKCα was sequenced to verify the introduction of the desired substitution. pCLbGFP (*URA3)*, encoding GFP fused to the mitochondrial presequence of citrate synthase (mt-GFP) under the control of the *GAL1*/*10* promoter was used to monitor mitochondrial morphology (Okamoto *et al.*, 1998).

Expression of PKCα and Bax *c*-myc was done sequentially. Yeast cells were first grown in synthetic compleate medium (SC; 0.17% yeast nitrogen base, 0.5% ammonium sulphate, 0.1% potassium phosphate and 80 μg/ml of all auxotrophic requirements except leucine and tryptophan, pH 5.5) with 2% glucose, 10 μg/ml of doxycycline to repress Bax *c*-myc expression. Cells were then transferred to SC medium with 2% galactose, 1% raffinose, 3% glycerol and 10 μg/ml doxycycline to induce PKCα expression and grown to an OD at 640 nm of 1.0. Finally, cells were transferred to SC medium with 2% galactose without doxycycline and diluted to an OD at 640 nm of 0.1 to induce both proteins. To induce expression of Bax α or Bax^{P168A} and PKC, yeast cells bearing the different plasmids were grown in SC medium with 2% glucose. Then cells were transferred to SC with 2% lactate and grown to an OD at 640 nm of 0.5. At this OD, galactose to a final concentration of 2% was added to the

medium to induce Bax α and PKC expression. Cells were collected at different times and further processed. All incubations were performed at 30 °C, 200 r.p.m.

2.2.2. Cell death assay and effect of PKCα inhibitors on cell death

For cell death assays, samples were harvested at the indicated times, the number of cells counted, and 100 cells plated in YPD plates. Cells harbouring the pCM184-Bax *c*-myc plasmid were plated in YPD plates containing 10 μg/ml of doxyxycline. Plates were incubated at 30 ºC and the number of colonies counted after 48 hours. For the results obtained with cells harbouring the plasmid pCM184-Bax *c*-myc, the data shown represent the number of c.f.u. at time *t* divided by the number of c.f.u. in the control (cells carrying the empty vector of PKCα and grown in the presence of doxycycline) for the same time. For the results obtained with cells harbouring the plasmid pYES3/CT-Bax or pYES3/CT-Bax^{P168A}, the data shown represents the number of c.f.u. at time t divided by the number of c.f.u. at time 0 h.

The PKCα inhibitors Gö 6976 (Sigma) and Ro 32-0432 (Sigma) were prepared in dimethyl sulfoxide (DMSO) at a final concentration of 1 mM. Cells were transferred to synthetic medium with 2% galactose without doxycycline and diluted to an OD at 640 nm of 0.1, to express both proteins, and DMSO, Gö 6976 or Ro 32-0432 were added to the culture at a final concentration of 0.1% and 1μM, respectively. Cell survival was measured by platting efficiency as described above.

2.2.3. PI staining and ROS production

Propidium iodide (PI) staining and ROS production were monitored by flow cytometry. Labelling with PI was performed by incubating 10^6 cells in culture medium containing 2 μg/ml of PI (Sigma) for 15 minutes. ROS production was monitored in cells preserving plasma membrane integrity by double staining with PI and dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes). Conversion of H_2DCFDA to DCF was analysed in PI negative cells. About 10^6 cells were incubated in culture medium containing 40 μg/ml H_2DCFDA for 45 minutes at 30ºC. 2 μg/ml of PI was added after 30 minutes of incubation. Flow cytometric analysis was performed in an Epics® XL™ (BeckmanCoulter) flow cytometer equipped with an argon-ion laser emitting a 488 nm

beam at 15 mW. Green fluorescence was collected through a 488 nm blocking filter, a 550 nm long-pass dichroic and a 525 nm band-pass filter. Red fluorescence was collected through a 560 nm short-pass dichroic, a 640 nm long-pass, and another 670 nm long-pass filter. 20,000 cells were analysed per sample at low flow rate. Data were analysed by WinMDI 2.8 software.

2.2.4. Mitochondrial network fragmentation

Cells expressing PKCα, Bax *c*-myc, PKCα and Bax *c*-myc or none of the proteins (control) were co-transformed with pCLbGFP. Cells were collected at different times and fragmentation of the mitochondrial network evaluated by epifluorescence microscopy. At least 150 cells per sample were classified. In this set of experiments uracil was also omitted from the growth medium.

2.2.5. Western blot analysis

Cells extracts were prepared as described in Camougrand *et al.* (2003). Protein lysates were separated on 12.5% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes (hybond-P; Amersham). The membranes were blocked with 5% non-fat milk in phosphate buffered saline containing 0.05% Tween 20, for 1 h at room temperature. Membranes were then incubated overnight at 4 °C with primary antibodies directed against human Bax (rabbit polyclonal, 1:10,000; Sigma), bovine PKCα (mouse monoclonal, 1:2000; Upstate), PKCδ (mouse monoclonal, 1:100; Santa Cruz Biotechnology), PKCε (rabbit polyclonal, 1:100; Santa Cruz Biotechnology), PKCζ (mouse monoclonal, 1:1,000; Santa Cruz Biotechnology), yeast Atg8p (rabbit polyclonal, 1:200; Santa Cruz Biotechnology), GFP (mouse monoclonal, 1:3000; Roche Applied Science), yeast cyt *c* (rabbit polyclonal, 1:2,000) yeast phosphoglycerate kinase (mouse monoclonal, 1:10,000; Molecular Probes) and yeast Por1p (mouse monoclonal, 1:5000; Molecular Probes). Membranes were incubated for 1 h at room temperature with peroxidase-coupled secondary antibodies from Jackson ImmunoResearch Laboratories (1:10,000). Peroxidase activity was revealed by chemioluminescence (Immobilon Western, Millipore).

2.2.6. Mitochondria preparation, carbonate treatment and cyt *c* content analysis

Mitochondria were isolated by differential centrifugation from zymolyase-treated cells, as described previously (Arokium *et al.*, 2004). For carbonate and Triton X-100 extraction, 1 mg of protein from isolated mitochondria was incubated in the presence of 0.1 M Na_2CO_3 (pH 10.0) or Triton X-100 for 15 minutes and centrifuged for 15 minutes at 105,000g. The presence of Bax *c*-myc in the pellet and the supernatant was verified by western blot.

Assessment of cyt *c* content was measured by redox spectra of isolated mitochondria essentially as described previously (Manon *et al.*, 1997). Differential spectra of the reduced (sodium dithionite) minus oxidized (potassium ferricyanide) extracts were recorded on a double-beam/double wavelength spectrophotometer (Aminco DW2000). The maxima absorption for cyt *b* and for cyt *c*+*c1* used were 561 and 550 nm, respectively.

2.2.7. Immunoprecipitation of Bax, detection of Bax phosphorylation and phosphatase assay

Imunoprecipitation was performed using protein G-coupled Dynabeads (Invitrogen). Briefly, cells were ressuspended in resuspension buffer (Tris–malate 10 mM, Mannitol 0.6 M, EGTA 1 mM, pH 6.7) supplemented with a mixture of protease home made protease inhibitors (0.4µl/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (PhosSTOP phosphatase inhibitor cocktail tablets; Roche Applied Science). Cells were broken mechanically by vortexing with glass beads, after which 100 µl of 10× IP buffer (1x IP buffer: 50mM Tris-HCl, pH7.5; 100mM NaCl; 2mM EDTA) was added to 1 ml of cell lysate and incubated at 4 °C during 1 h. Alternatively, the mitochondrial and supernatant fractions were separated before incubation with IP buffer, by centrifuging the cell lysate at 17000g for 10min. The supernatant corresponded to the cytosolic and microsomal fractions and the pellet, which corresponded to the mitochondrial fraction, was resuspended in resuspension buffer. After incubation with IP buffer, 2 µg of monoclonal anti-Bax antibody (2D2; Sigma) was added, and the lysate incubated overnight at 4 °C. Protein G-coupled Dynabeads (Invitrogen) were added and incubated for 6 h. Washing

was performed with 1x IP buffer and elution was performed with Laemmli sample buffer, or when non-denaturing conditions were required, with 50 mM Glycine pH 2.8.

For detection identical samples of purified Bax was loaded in parallel onto two SDS-PAGE gels and blotted. One was probed with a monoclonal anti-phosphoserine antibody (7F12; 1:5000; ALEXIS Biochemicals), and the other was probed with a polyclonal anti-Bax antibody.

Bax phosphorylation was also performed by monitoring the appearance slow migrating bands in the western blot with purified Bax. The λ-protein phosphatase (λ-PPase) assay was performed to confirm that these slow migrating bands corresponded to phosphorylated Bax. For the phosphatase assasy, 0.1-0.2 µl of phosphatase (New England Biolabs), were added to12 µl of eluate, 1.5 µl of 10x buffer and 1.5µl of $MnCl_2$. The mixture was incubated for 30 min at 37ºC and the reaction was stopped by addition of Laemmli sample buffer and incubation at 65ºC for 30 min.

2.2.8. [^{32}P] Phosphate labelling

For phosphate labelling, expression of PKCα and Bax *c*-myc were done in a low phosphate medium (Pinson *et al.*, 1996) as in (Arokium *et al.*, 2007). Briefly, ^{32}P phosphate (0.25mCi/ml) was added 6 hours after Bax *c*-myc induction, and cells collected after 2 hours. Bax *c*-myc was immunoprecipitated using the protocol described above, loaded onto two SDS-PAGE gels and blotted. One membrane was exposed to autoradiography film, and the other was probed with a polyclonal anti-Bax antibody.

2.3. Modulation of Bax mitochondrial insertion and induced cell death in yeast by mammalian protein kinase Cα

2.3.1. Abstract

Protein kinase Cα (PKCα) is a classical PKC isoform whose involvement in cell death is not completely understood. Bax, a major member of the Bcl-2 family, is required for apoptotic cell death and regulation of Bax translocation and insertion into the outer mitochondrial membrane is crucial for regulation of the apoptotic process (as referred in section 1.2). Here we show that PKCα increases the translocation and insertion of Bax *c*-myc (an active form of Bax) into the outer membrane of yeast mitochondria. This is associated with an increase in cytochrome *c* (cyt *c*) release, reactive oxygen species production (ROS), mitochondrial network fragmentation and cell death. This cell death process is regulated, since it correlates with an increase in autophagy but not with plasma membrane permeabilization. The observed increase in Bax *c*-myc translocation and insertion by PKCα is not due to Bax *c*-myc phosphorylation, and the higher cell death observed is independent of the PKCα kinase activity. PKCα may therefore have functions other than its kinase activity that aid in Bax *c*-myc translocation and insertion into mitochondria. Together, these results give a mechanistic insight on apoptosis regulation by PKCα through regulation of Bax insertion into mitochondria.

2.3.2. Results

2.3.2.1. Mammalian PKCα enhances Bax c-myc-induced cell death without disturbing plasma membrane integrity

Bax α needs to be activated in order to induce organelle membrane permeabilization, and thus trigger apoptosis. So, expression of native human Bax in yeast, a system that lacks several homologues of mammalian apoptotic regulators, has no effect on yeast viability (Gallenne *et al.*, 2009). Therefore, in order to study the effect of mammalian PKCα in the regulation of Bax using yeast, we expressed a form of Bax in the active

conformation that is cytotoxic for this organism (Bax *c*-myc) (Priault *et al.*, 1999). Our results show that cell death induced by expression of Bax *c*-myc in yeast is increased by co-expression with PKCα (Fig. 2.1A). This increase in cell death is not accompanied by loss of plasma membrane integrity, measured by PI staining (Fig. 2.1B). The maintenance of plasma membrane integrity suggests that, as already described for expression of Bax *c*-myc alone (Kissova *et al.*, 2006), the death process in cells co-expressing PKCα and Bax *c*-myc is a regulated event.

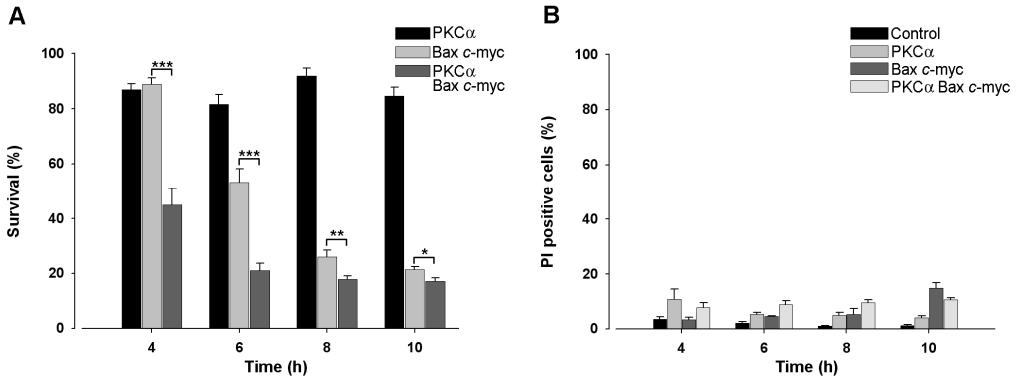

Figure 2.1 - PKCα enhances Bax *c*-myc induced cell death in yeast without disturbing plasma membrane integrity. (A) Percentage of cell survival evaluated by c.f.u. About one hundred cells expressing PKCα, Bax *c*-myc, PKCα and Bax *c*-myc or none of the proteins (control) were taken at different times, plated and the number of c.f.u. evaluated. 100% survival corresponds to the number of c.f.u. obtained with the control for each time point. Data are the mean ± s.e.m. of five independent experiments. (B) Percentage of cells displaying loss of plasma membrane integrity evaluated by PI staining. Cells expressing PKCα, Bax *c*-myc, PKCα and Bax *c*-myc or none of the proteins (control) were collected at different times and the percentage of PI-positive cells was evaluated by flow cytometry. Data are the mean ± s.e.m. of three independent experiments. Significant differences obtained between Bax *c*-myc expression and PKCα and Bax *c*-myc co-expression are indicated by **P*<0.05; **0.01>*P*>0.001; ****P*<0.001 (unpaired Student's *t*-test).

2.3.2.2. Expression of PKCα enhances Bax c-myc-induced ROS production, cyt c release and mitochondrial network fragmentation

Yeast cell death induced by Bax *c*-myc is usually accompanied by several functional and biochemical markers such as ROS production (Camougrand *et al.*, 2003; Madeo *et al.*, 1999), cyt *c* release (Manon *et al.*, 1997), and fragmentation of the mitochondrial network (Kissova *et al.*, 2006). The effect of PKCα in Bax *c*-myc ROS production, cyt *c* release, and fragmentation of the mitochondrial network was evaluated in cells co-expressing PKCα and Bax *c*-myc and compared to cells expressing Bax *c*-myc alone.

ROS production increases in cells co-expressing PKCα and Bax *c*-myc (Fig. 2.2A). In addition, cells co-expressing PKCα and Bax *c*-myc have a lower cyt *c* content (Fig. 2.2B) and increased mitochondrial network fragmentation (Fig. 2.2C,D). These results indicate that PKCα enhances the cytotoxic effects of Bax *c*-myc expression in yeast cells.

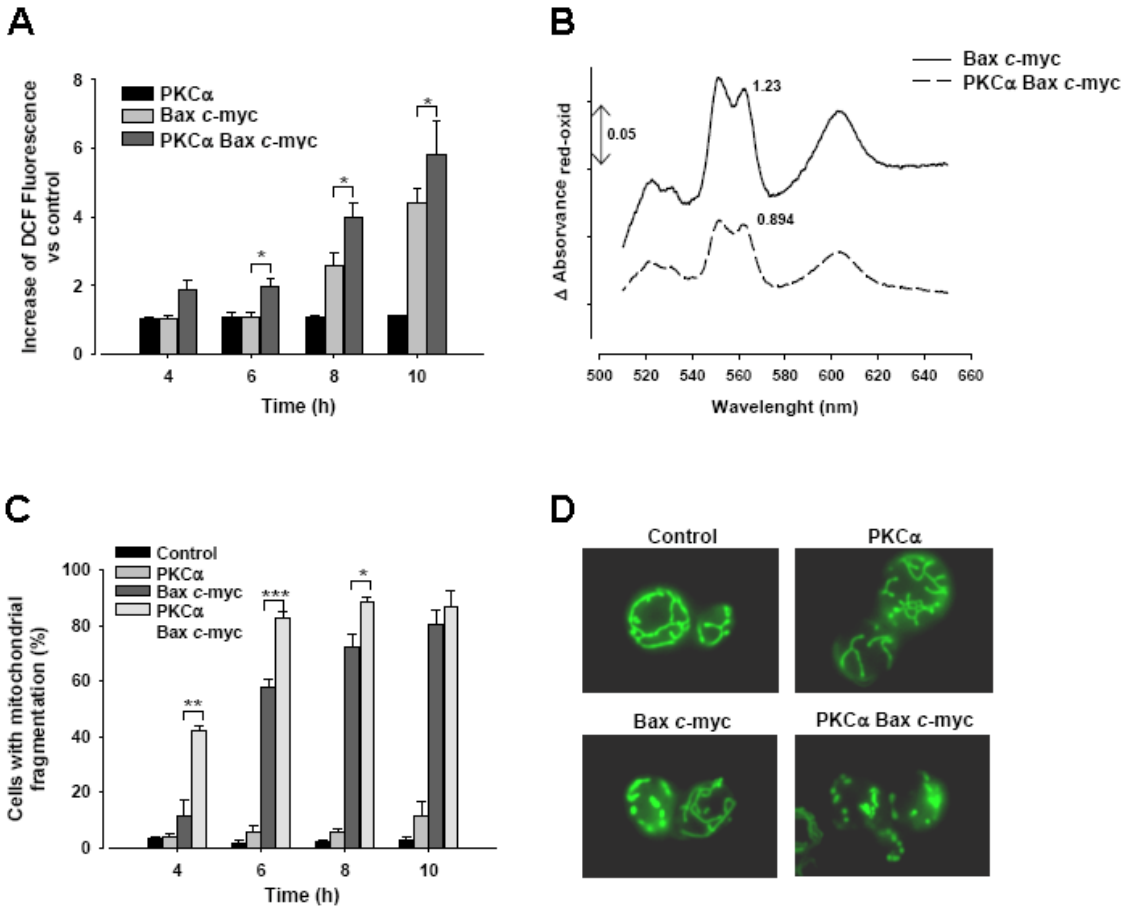

Figure 2.2 – Expression of PKCα enhances Bax *c*-myc-induced ROS production, cyt *c* release and mitochondrial network fragmentation in yeast. (A) ROS production was evaluated by monitoring the conversion of H_2DCFDA to DCF by flow cytometry. Results are expressed as ratio values estimated by dividing the mean fluorescence intensity of each sample by the mean fluorescence intensity of the control cells for the same time. Data are the mean ± s.e.m. of five independent experiments. (B) Cyt *c* content in mitochondria assessed by redox spectra analysis of isolated mitochondria from cells expressing Bax *c*-myc (full line) and cells co-expressing PKCα and Bax *c*-myc (dashed line). The values of the cyt *c*/cyt *b* ratios are indicated in the image. (C) Percentage of cells displaying fragmentation of the mitochondrial network. Cells expressing mt-GFP and expressing PKCα, Bax *c*-myc, PKCα and Bax *c*-myc or none of the proteins (control) were collected at different times and fragmentation of the mitochondrial network was evaluated by epifluorescence microscopy. Data are the mean ± s.e.m. of three independent experiments; means correspond to counts of at least 150 cells per sample. (D) Fluorescence microscopy images of cells expressing mt-GFP and expressing PKCα, Bax *c*-myc, PKCα and Bax *c*-myc or none of the proteins (control) after 6 hours. Significant differences between Bax expression and co-expression of PKCα and Bax *c*-myc are indicated by **P*<0.05; **0.01>*P*>0.001; ****P*<0.001 (unpaired Student's *t*-test).

2.3.2.3. Co-expression of PKCα and Bax c-myc stimulates autophagy

An increased amount of Atg8p has been observed in yeast following nitrogen starvation, rapamycin treatment or Bax *c*-myc expression. The increase in the amount of this autophagic protein is considered one of the typical markers of autophagy induction (Kirisako *et al.*, 1999; Kissova *et al.*, 2006). In order to determine whether PKCα also interferes with Bax *c*-myc-induced autophagy, Atg8p expression was evaluated by western blot in cells expressing PKCα, Bax *c*-myc, co-expressing PKCα and Bax *c*-myc, and in control cells. It has been previously shown that Bax *c*-myc stimulates Atg8p expression (Kissova *et al.*, 2006). Accordingly we were also able to detect a two-fold increase in Atg8p expression after Bax *c*-myc expression. However we did not detect any difference in Atg8p expression between control cells and PKCα expressing cells (Fig. 2.3A). In cells co-expressing both proteins there was a seven-fold increase in Atg8p expression, indicating that autophagy is increased.

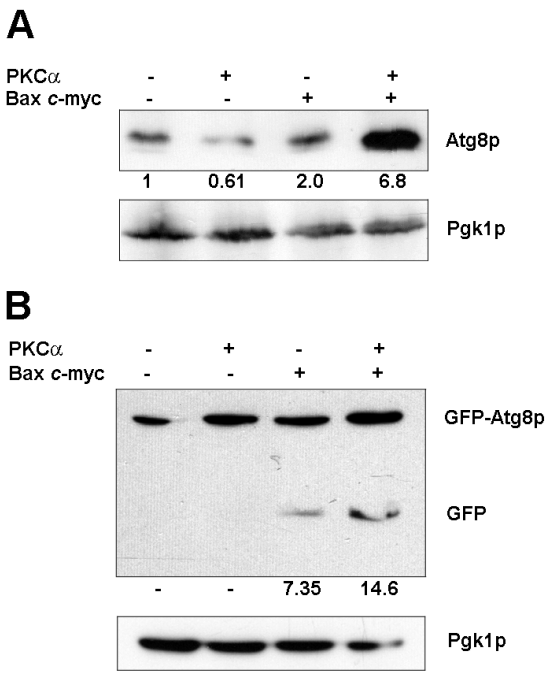

Figure 2.3 - Co-expression of PKCα and Bax *c*-myc increases the level of autophagy. (A) Detection of Atg8p expression in whole cell extracts of control cells and cells expressing PKCα, Bax *c*-myc and co-expressing PKC-α and Bax *c*-myc, after 10 hours. Pgk1p was used as loading control. The amount of Atg8p was quantified by densitometry analysis of non saturated immunoblots. All values were normalised to the loading control. (B) Detection of free GFP generated from the GFP-Atg8p fusion protein in whole cell extracts of cells expressing this fusion and expressing PKCα, Bax *c*-myc and co-expressing PKC-α and Bax *c*-myc, after 14 hours. Pgk1p was used as loading control. The amount of GFP was quantified by densitometry analysis of non saturated immunoblots and the values showed are the percentage of the GFP in the cells that is not fused to Atg8p.

In order to further confirm that the higher Atg8p expression detected was associated to autophagy induction we also monitored the level of Atg8p that is delivered into the vacuole. For this purpose a GFP-Atg8p fusion was also expressed in our transformed

cells. When this fusion is delivered into the vacuole the Atg8p is rapidly degraded by vacuolar hydrolases while free GFP is not degraded. So, accumulation of the GFP moiety reflects delivery of Atg8p into the vacuole and therefore the level of autophagy induction (Shintani and Klionsky, 2004). Cells expressing the GFP-Atg8p fusion displayed an accumulation of free GFP corresponding to 7% and 15% of the total GFP, when Bax *c*-myc is expressed, or PKCα and Bax *c*-myc are co-expressed, respectively. These observations indicate a higher delivery of Atg8p into the vacuole and confirmed a higher autophagy level when both proteins are co-expressed (Fig. 2.3B). In control cells and in cells expressing PKC no accumulation of free GFP was detected (Fig. 2.3B).

2.3.2.4 PKCα increases the insertion of Bax c-myc into the mitochondrial membrane

When expressed in yeast cells, Bax *c*-myc translocates to the mitochondria and inserts into the mitochondrial membrane, leading to several downstream events described above. The presence of PKCα and Bax *c*-myc in whole cell extracts and in the mitochondrial fraction was verified by western blot. Both proteins were expressed in yeast cells, and there was an accumulation of Bax *c*-myc (about 2.3 times) in cells co-expressing PKCα (Fig. 2.4A). The possibility that this increase could be due to interference by PKCα with the promoter of Bax *c*-myc was unlikely. However we did check this possibility by expressing PKCα with Bcl-xL, another protein with mitochondrial localization, under control of the same expression system (pCM184) used for Bax *c*-myc expression. We could confirm that there was no effect on the expression of Bcl-xL, thus ruling out the hypothesis of a non-specific effect of PKCα on the promoter of the plasmid used for Bax *c*-myc expression (Fig. 2.4B). Analysis of the mitochondrial fraction confirmed the translocation of Bax *c*-myc to the mitochondria as revealed by an increase in the amount of Bax *c*-myc in the mitochondrial fraction (about 4.6 times) when PKCα is co-expressed (Fig. 2.4A). This increase is much higher than that observed in whole cell extracts, indicating that the accumulation of Bax *c*-myc observed under co-expression conditions occurs preferably at mitochondria. In fact, the accumulation observed in whole cell extracts might be due to a higher translocation to mitochondria since Bax *c*-myc is more protected from degradation in the lipidic environment of the outer mitochondrial membrane.

PKCα could lead to an increase in the actual insertion of Bax *c*-myc into the mitochondrial membrane or only to an enhanced association. Isolated mitochondria from cells expressing Bax *c*-myc or co-expressing PKCα and Bax *c*-myc were therefore treated with Na_2CO_3 or Triton X-100 to remove loosely bound or inserted proteins, respectively. Bax *c*-myc was partially insensitive to carbonate treatment but sensitive to Triton X-100, showing that it is mainly inserted into the mitochondrial membrane (Fig. 2.4C). The maintenance of the ratio between associated and inserted Bax *c*-myc in yeast cells expressing Bax *c*-myc and co-expressing PKCα and Bax *c*-myc shows that the

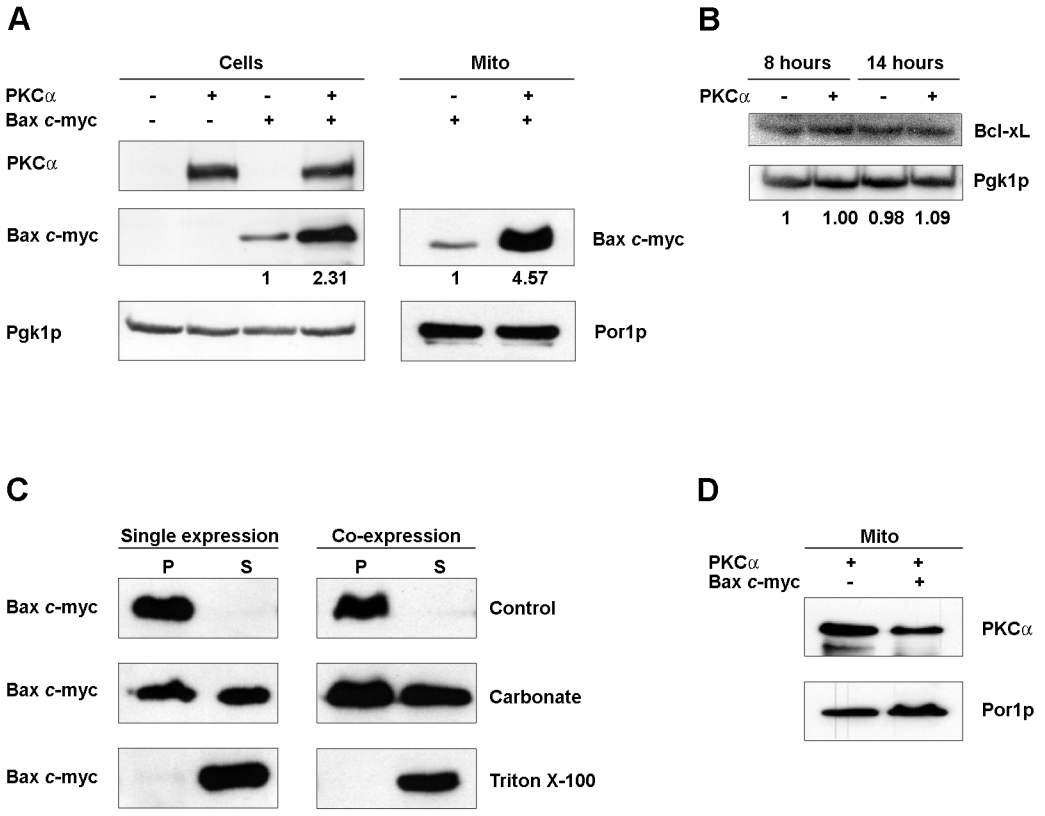

Figure 2.4 - PKCα increases the translocation and insertion of Bax *c*-myc into the mitochondria. (A) Detection of PKCα and Bax *c*-myc in whole cell extracts and in the mitochondrial fraction. Pgk1p and Por1p were used as loading controls for the whole cell extracts and mitochondrial fraction, respectively. The amount of Bax *c*-myc was quantified by densitometry analysis of non saturated immunoblots. All values were normalised to the loading control. (B) Detection of Bcl-xL in whole cell extracts. Por1p was used as loading control. The amount of Bax *c*-myc was quantified by densitometry analysis of non saturated immunoblots. (C) Mitochondria isolated from cells expressing Bax *c*-myc only and co-expressing PKCα and Bax *c*-myc were treated with Na_2CO_3 or Triton X-100 to remove loosely bound or inserted proteins, respectively. (D) Detection of PKCα in the mitochondrial fraction. Por1p was used as loading control.

higher translocation of this protein is associated with a higher insertion.

Analysis of the mitochondrial fraction also revealed the presence of PKCα in mitochondria independently of the co-expression with Bax *c*-myc (Fig. 2.4D).

2.3.2.5. PKCα does not alter Bax c-myc phosphorylation in yeast

Arokium *et al.* (2007) showed that human Bax α is phosphorylated in yeast cells and mutation of possible phosphorylation serine sites in the protein enhances the ability of Bax α to insert into the mitochondria and to induce cyt *c* release. Interestingly, we were not able to detect phosphorylation of Bax *c*-myc either in cells expressing Bax *c*-myc or

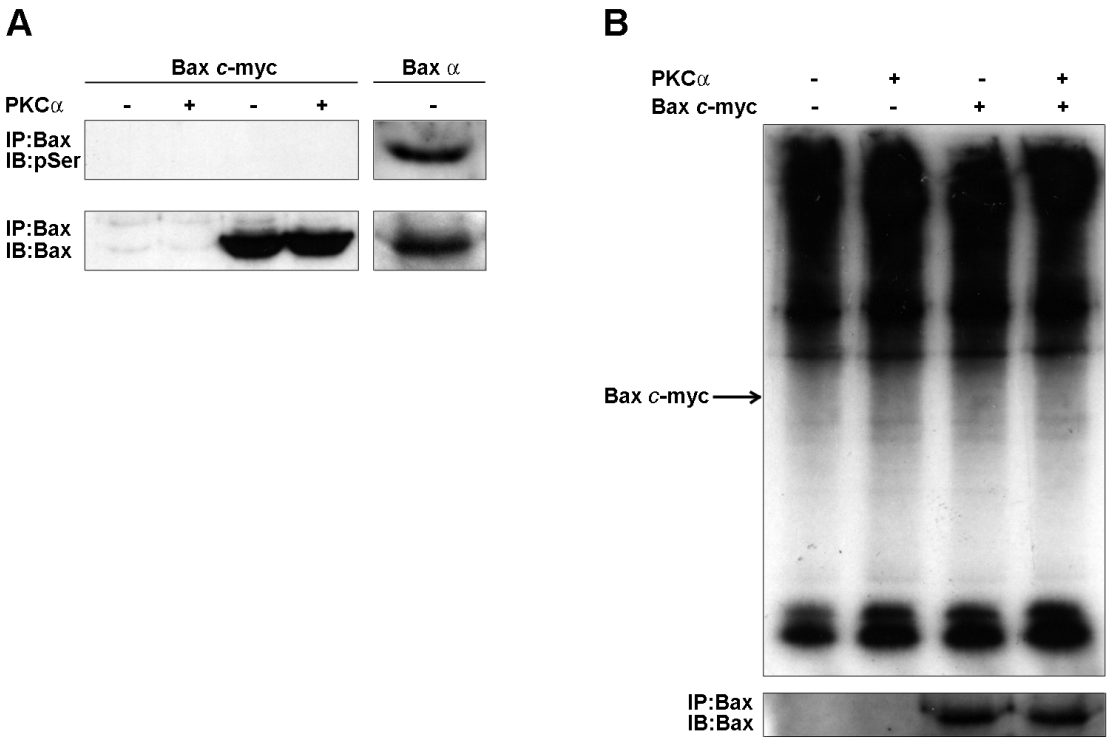

Figure 2.5 - Bax *c*-myc is not phosphorylated in yeast. Phosphorylation of Bax *c*-myc was evaluated by using an anti-phosphorylated serine antibody (A) and by [^{32}P] phosphate labelling (B). (A) The search for phosphorylated serine residues in Bax *c*-myc was performed after 14 hours of induction. Membranes were probed with a monoclonal anti-phosphoserine antibody (7F12) and with a polyclonal anti-Bax antibody. As a positive control, immunoprecipitated Bax α (a phosphorylatable form of Bax in yeast) from yeast cells expressing Bax α, was also probed with the anti-phosphoserine antibody. (B) [^{32}P] phosphate labelling of cells grown in a low phosphate medium. Expression of Bax *c*-myc was induced by removal of doxycycline, ^{32}P phosphate (0.25mCi/ml) was added 6 hours later and cells were further incubated for 2 hours. Membranes were revealed by autoradiography or with a polyclonal anti-Bax antibody.

co-expressing PKCα and Bax *c*-myc, using an antibody previously shown to detect Bax α with phosphorylated serines (Gardai *et al.*, 2004). As a positive control, Bax α immunoprecipitated from yeast cells was used (Fig. 2.5A). To confirm that Bax *c*-myc is not phosphorylated in yeast cells, in vivo radioactive labelling was performed. Phosphorylation of Bax *c*-myc was not detected, with or without expression of PKCα (Fig. 2.5B). These results indicate that the higher insertion of Bax *c*-myc in the presence of PKCα, and its associated effect described above is not related to an alteration of the Bax *c*-myc phosphorylation state.

2.3.2.6. PKCα kinase activity is not involved in enhancing the effect of Bax c-myc

To study the relation between PKCα kinase activity and the enhancement of the events induced by Bax *c*-myc, the viability of yeast cells expressing both proteins was assessed in the presence of two PKC inhibitors, Gö 6976 and Ro 32-0432. The concentration of

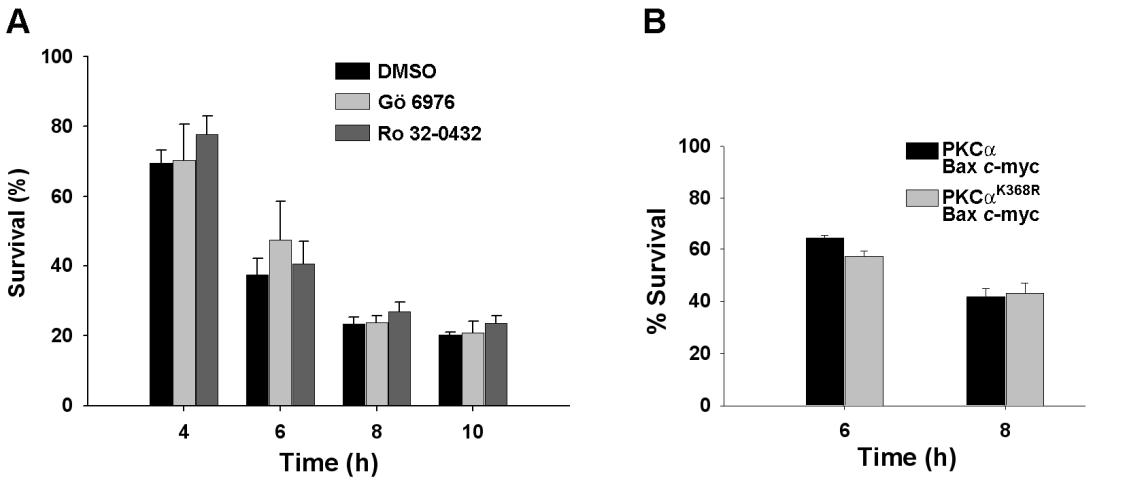

Figure 2.6 - The effect of PKCα is independent of its kinase activity. (A) Effect of PKC inhibitors Gö 6976 and Ro 32-0432 in the stimulation of Bax *c*-myc induced cell death by PKCα. Survival of cells co-expressing PKCα and Bax *c*-myc in the absence or presence of the inhibitor was evaluated by c.f.u.. 100% survival corresponds to the number of c.f.u. obtained for time 0 (hours). (B) Comparison of survival percentage between cells co-expressing PKCα and Bax *c*-myc or co-expressing PKCα^{K368R} and Bax *c*-myc after 6 and 8 hours. Survival was evaluated by c.f.u. and 100% survival corresponds to the number of c.f.u. obtained with the control (cells expressing none of the proteins) for each time. Data are the mean ± s.e.m. of at least three independent experiments. Two-way ANOVA analysis revealed no significant effect on cell death of both PKC inhibitors and of the single point mutation K368R in PKCα.

both inhibitors tested was selected using a yeast phenotypic assay as described in (Saraiva *et al.*, 2004). Curiously, the results obtained showed that these inhibitors have no effect on the viability of yeast cells expressing both proteins (Fig. 2.6A). A catalytically inactive mutant of PKCα (the PKCα^{K368R}) was also co-expressed with Bax *c*-myc and its effect on cell viability compared with that obtained with wild-type PKCα. In this mutant, a lysine residue in the ATP-binding site of the protein was replaced with an arginine, leading to the loss of phosphorylation activity (Baier-Bitterlich *et al.*, 1996). Co-expression of PKCα^{K368R} and Bax *c*-myc was confirmed by western blot (data not shown). Co-expression of PKCα^{K368R} or PKCα with Bax *c*-myc had similar effects in cell viability (Fig. 2.6B). These results indicate that the effect of PKCα on Bax *c*-myc expressing yeast cells does not depend on PKCα kinase activity.

2.3.3. Discussion

In previous works, we took advantage of yeast to study the role of mammalian PKC isoforms on the regulation of apoptosis and the Bcl-2 anti-apoptotic protein Bcl-xL (Saraiva *et al.*, 2006). In the present study, yeast was used to study the role of PKCα on the regulation of Bax, one of the most important proteins in the mitochondrial apoptotic cascade. We assessed whether PKCα, a member of the classical PKC subfamily, modulates Bax without the interference of other Bcl-2 family proteins and PKC isoforms by expressing these two proteins in yeast.

We found that PKCα regulates the effect of Bax *c*-myc, an active form of Bax α, by increasing its translocation and insertion into the outer mitocondrial membrane. This leads to an enhancement of other Bax *c*-myc induced-downstream events in yeast cells, such as loss of viability, ROS production, mitochondrial network fragmentation, cyt *c* release, and higher Atg8p expression and vacuolar delivery. In contrast, no increase in loss of plasma membrane integrity was detected. Several reports show that autophagy is activated following Bax *c*-myc expression (Camougrand *et al.*, 2003; Kissova *et al.*, 2006). These authors showed that autophagy was not responsible for the loss of plating efficiency but rather played a minor role in maintaining cell survival. However, they found that mitophagy is required for regulated loss of cell survival since absence of Uth1p (a protein required for mitophagy) led to a higher percentage of PI-positive cells. Here, the enhancement of Bax *c*-myc induced cell death by PKCα is unlikely related to

an inhibition of autophagy, since there is an accumulation of Atg8p, a higher delivery of this protein to the vacuole and no increase in the percentage of PI positive cells. The higher amount of Atg8p and the higher vacuolar delivery detected in cells co-expressing PKCα and Bax *c*-myc is likely due to the observed higher translocation of Bax *c*-myc to mitochondria, which in turn results in higher autophagy induction.

A great benefit of studies with animal tissue cultures is the possibility of determining the final cellular effect of a given modulator. However, it is difficult to study the specific effect of such modulator on a particular protein. The effect of PKCα on other Bcl-2 family proteins such as Bax is difficult to study in an environment where other PKCα-regulatable apoptosis modulators are present. By expressing PKCα and Bax *c*-myc in yeast, we were able to study the regulation of Bax *c*-myc by PKCα in the absence of all other Bcl-2 family proteins. We found a mitochondrial localization of PKCα, higher insertion in Bax *c*-myc on the outer mitochondrial membrane and higher cell death in cells co-expressing PKCα. Previous studies with mammalian cells have uncovered a mitochondrial localization of PKCα (Cerioni *et al.*, 2006; Wang *et al.*, 2007). However, it was linked with an increase of cell survival. Whether the presence of PKCα in the mitochondria is essential for enhancement of Bax *c*-myc induced cell death in yeast is unknown.

PKCα regulates several apoptotic proteins, as well as proteins upstream of the apoptotic cascade, through phosphorylation. Therefore, it would be reasonable to consider that PKCα regulates Bax *c*-myc through phosphorylation. It was surprising to find that the presence of PKCα does not alter the Bax *c*-myc phosphorylation state. In fact, phosphorylated Bax *c*-myc is not detected in yeast, in contrast with what was previously described for Bax α (Arokium *et al.*, 2007, this study). It is possible that the conformational changes induced by the *c*-myc epitope or the insertion of Bax *c*-myc in the outer mitochondrial membrane protect target residues from phosphorylation. Our data clearly demonstrate that the enhancing effect of PKCα on Bax *c*-myc is not mediated by phosphorylation. In fact, the kinase-dead PKCα^{K368R} mutant has the same effect on the increase of Bax *c*-myc induced cell death as the wild-type PKCα. Consistently, the PKC inhibitors used in this study had no effect on Bax *c*-myc induced cell death in cells co-expressing Bax *c*-myc and PKCα. This shows that the kinase activity of PKCα is not necessary for the enhancement of Bax *c*-myc induced cell death

and that a phosphorylation cascade is not involved in this process. It has previously been shown that PKCα enhances phosphorylation of Bcl-xL in yeast, abolishing its anti-apoptotic activity (Saraiva *et al.*, 2006). Here we show that PKCα also has a pro-apoptotic role in the modulation of Bax. However, this role is independent of its kinase activity. It was reported that PKCε interacts with Bax, sequestering it in the cytosol. It is possible that a similar interaction between Bax *c*-myc and PKCα exists in this compartment or even at mitochondria. However, we could not detect it by immunoprecipitation (data not shown). The present study only focused on the regulation of Bax *c*-myc by PKCα.

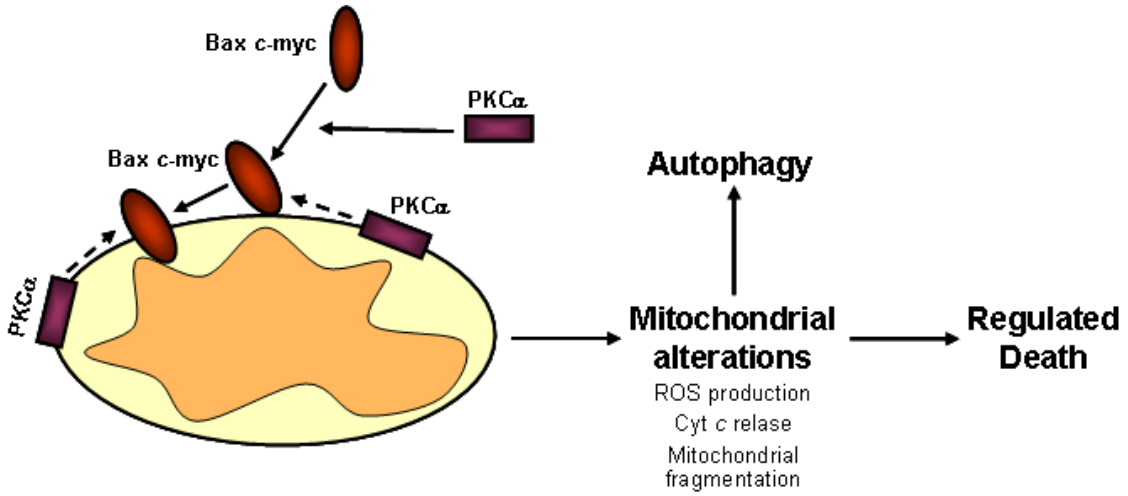

Figure 2.7 – Proposed model for PKCα regulation of Bax *c*-myc induced cell death. PKCα in the cytosol, mitochondria or in both compartments directly or indirectly increases translocation and/or insertion of Bax *c*-myc to mitochondria by an unknown mechanism leading to an increase in cyt *c* release, ROS production and mitochondrial network fragmentation. These alterations lead to an increase in autophagy that regulates the mode of cell death.

In conclusion, our findings show that PKCα has a pro-apoptotic effect on Bax *c*-myc, increasing Bax *c*-myc induced cell death, translocation and insertion of Bax *c*-myc into the outer mitochondrial membrane, and enhances several other cellular events associated with Bax *c*-myc-induced death. We therefore propose a model where PKCα aids in the translocation and/or the insertion of Bax *c*-myc into the outer mitochondrial membrane by a still unknown mechanism, subsequently leading to an increase in cyt *c* release, ROS production, mitochondrial network fragmentation and cell death. Furthermore, an increase in the autophagic process allows the maintenance of a

regulated form of cell death (Fig. 2.7). This work together with our previous data on specific modulation of apoptosis and Bcl-xL phosphorylation by distinct mammalian PKC isoforms (Saraiva *et al.*, 2006) further reinforces the yeast model to study the regulation of Bcl-2 family proteins by PKC isoforms. Finally, a mechanistic insight on apoptosis regulation by PKCα through regulation of Bax insertion into mitochondria is provided.

2.4. Mammalian protein kinase C isoforms distinctly regulate Bax in yeast

2.4.1. Abstract

Bax, a major pro-apoptotic member of the Bcl-2 family, is required in some types of apoptotic cell death and several reports have suggested that phosphorylation is implicated in the regulation of Bax activation and translocation. Protein kinase C (PKC) regulates apoptosis in an isoform-specific manner that has been implicated in the modulation of Bcl-2 family proteins. However, as refered in section 1.3., co-existence of several PKC isoforms in the same cell and the different expression patterns observed in different cell types lead to often contradictory results. Recently, we showed that distinct PKC isoforms can differently modulate Bcl-xL anti-apoptotic effect in yeast, and in section 2.3 we showed that PKCα enhances translocation and insertion of Bax *c*-myc (an active human Bax with mitochondrial localization) into yeast mitochondria. Following these results, we set out to exploit this model system to study the regulation of Bax α (an inactive human Bax with cytosolic localization) by PKCα, δ, ε and ζ and Bax^{P168A} (a human Bax with a single point mutation that increase its activity) by PKCα. We found that PKC isoforms differently regulate Bax α phosphorylation. PKCα and ε leads to Bax α dephosphorylation while PKCδ and ζ has no detectable effect in Bax α phosphorylation. Moreover, we showed that PKCα inhibits Bax^{P168A}-induced cell death, an effect that is abolished when the PKCα kinase activity is abrogated. It is also demonstrated that the Bax α dephosphorylation observed in the presence of PKCε is associated with the inhibition of Bax α translocation to mitochondria. Together, these

results show a distinct regulation of Bax α by different PKC isoforms and provide a proof of principle of yeast as an important tool for this study.

2.4.2 Results

2.4.2.1. PKCα leads to Bax α phosphorylation but does not increase its activity

In section 2.3 we showed that PKCα increases the translocation and insertion of Bax *c*-myc into the outer mitochondrial membrane, and consequently, higher cyt *c* relase and cell death (Silva *et al.*, 2011b). This effect did not depend on PKCα kinase activity and Bax *c*-myc phosphorylation state was not altered by co-expression with PKCα. Since Bax *c*-myc is an active form of Bax with mitochondrial localization, only allowing the study of the role of PKCα in the regulation of mitochondrial active Bax, we decided to study the effect of PKCα in human untagged Bax α. Our results show that accordingly to the observed by Arokium *et al.* (2007), and in opposite to what have been observed for Bax *c*-myc, Bax α is phosphorylated in yeast (Fig. 2.8A). Moreover, co-expression of PKCα with Bax α leads to Bax α dephosphorylation (Fig. 2.8A). However, this dephosphorylation does not seem to increase Bax α cell death activity, since co-expression of PKCα with Bax α does not significantly affect cell viability when we compare to single-expression of Bax α (Fig. 2.8B). Consistently with the absence of effect on cell viability, no significant difference is observed in the levels of Bax α in mitochondria isolated from cells expressing Bax α and from cells co-expressing PKCα and Bax α (Fig. 2.8C). It was also detected PKCα in isolated mitochondria showing that PKCα translocates to mitochondria in yeast and confirming our previous (Silva *et al.*, 2011b; section 2.3) (Fig. 2.8C). We also studied if PKCα could interfere with the levels of Bax α associated with mitochondria, but not inserted into the mitochondria outer membrane, by treatment with Na_2CO_3. No differences in the levels of inserted and associated Bax α is observed after PKCα expression (Fig. 2.8D). Cyt *c* content in mitochondria isolated from cells expressing Bax α and co-expressing PKCα and Bax α was assessed by redox spectra analysis. The values of the cyt *c*/cyt *b* ratios obtained indicate that the amount of cyt *c* in mitochondria is not affected by the co-expression of PKCα with Bax α (Fig 2.8E).

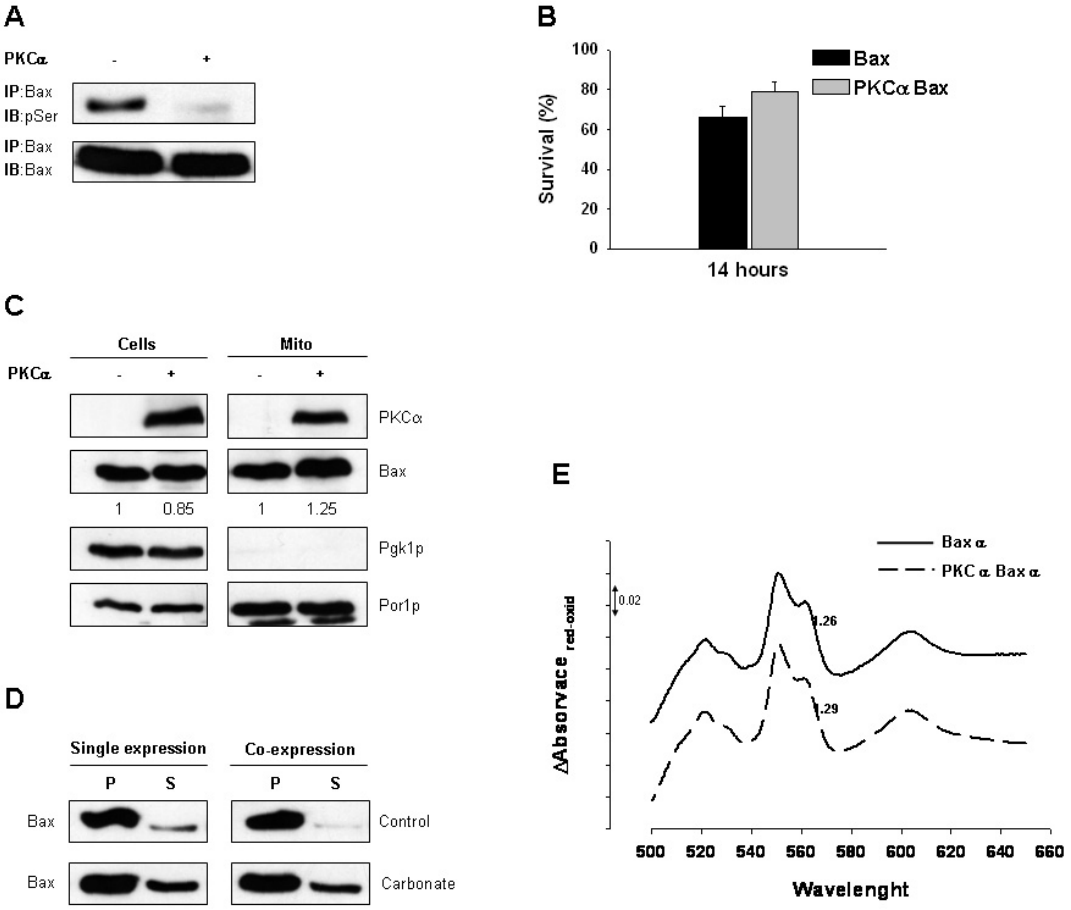

Figure 2.8 - PKCα does not affect Bax α activity. (A) Phosphorylation of Bax α evaluated with the monoclonal anti-phosphoserine antibody (7F12). Bax α from cells expressing Bax α and co-expressing PKCα and Bax α was immunoprecipitated after 14 h of expression. (B) Percentage of cell survival evaluated by c.f.u.. About 100 cells expressing Bax α and co-expressing PKCα and Bax α were taken at 14hours, plated and the number of c.f.u. evaluated. 100% survival corresponds to the number of c.f.u. obtained for time 0 h. Data are the mean±s.e.m. of three independent experiments. (C) Detection of PKCα and Bax α in whole cell extracts and in the mitochondrial fraction after 14 h of expression. Pgk1p and Por1p were used as loading controls for the whole cell extracts and mitochondrial fraction, respectively. The amount of Bax α was quantified by densitometry analysis of nonsaturated immunoblots. All values were normalised to the loading control. (D) Mitochondria isolated from cells expressing Bax α and co-expressing PKCα and Bax α for 14h were treated with Na_2CO_3 to remove loosely bound proteins. (E) Cyt *c* content in mitochondria assessed by redox spectra analysis of isolated mitochondria from cells expressing Bax α (full line) and cells co-expressing PKCα and Bax α (dashed line) for 14 h. The values of the cyt *c*/cyt *b* ratios are indicated in the image.

2.4.2.2. PKCα inhibits Bax^{P168A} induced cell death

The full-lenght unaltered human Bax α does not significantly induce cell death when expressed in yeast. Therefore, the results above described do not exclude a possible anti-apoptotic effect of PKCα in Bax α regulation. To study the existence of this putative anti-apoptotic effect, PKCα was co-expressed with Bax^{P168A} and the viability of yeast cells expressing those proteins was assessed. The expression of Bax^{P168A}, PKCα and PKCα^{K368R} was confirmed by western blot analysis (Fig. 2.9A). Bax^{P168A} is an active Bax α variant in yeast and mammalian cells due to the single substitution of pro168 by ala that increases the mobility of the α9 helix (Arokium *et al.*, 2004; Cartron *et al.*, 2005). The results obtained show that Bax^{P168A} expression leads to cell death in yeast, and co-expression of PKCα with Bax^{P168A} inhibits the death-promoting activity of Bax^{P168A} (Fig. 2.9B). Expression of a kinase death mutant of PKCα (PKCα^{K368R}) with Bax^{P168A} abolished the PKCα effect on Bax^{P168A}-induced cell death (Fig. 2.9B).This shows that the anti-apoptotic effect of PKCα depends on its kinase activity.

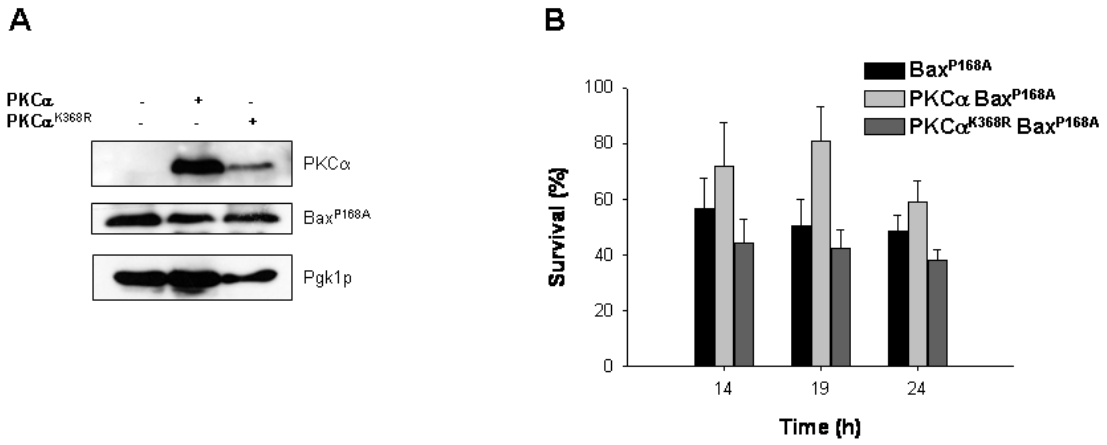

Figure 2.9 – Inhibition of Bax^{P168A}-induced cell death by PKCα is dependent of PKCα kinase activity. (A) Detection of PKCα and Bax^{P168A} in whole cell extracts after 14 h of expression. Pgk1p was used as loading control. (B) Percentage of cell survival evaluated by c.f.u.. About 100 cells expressing Bax^{P168A}, co-expressing PKCα and Bax^{P168A} and co-expressing PKCα^{K368R} and Bax^{P168A} were taken at the indicated times, plated and the number of c.f.u. evaluated. 100% survival corresponds to the number of c.f.u. obtained for time 0 h. Data are the mean±s.e.m. of three independent experiments. Survival between cells expressing Bax^{P168A} and co-expressing Bax^{P168A} and PKCα is statistically different (P<0.05; Two-way ANOVA).

2.4.2.3. PKCδ, ε and ζ distinctly regulate Bax α phosphorylation and translocation to mitochondria

The results obtained with PKCα in Bax α and Bax^{P168A} regulation supported our purpose to also study Bax α regulation by other PKC isoforms. PKCα is a member of the classical PKC subfamily, therefore it was interesting to study other PKCs. For this reason the role of members of novel PKCs (PKCδ and PKCε) and one member of the atypical PKCs (PKCζ) in Bax α regulation was studied. For that purpose, yeast cells co-expressing PKCδ and Bax α, PKCε and Bax α and PKCζ and Bax α were obtained and expression of these proteins confirmed by Western blot (Fig. **2.10**A). Using these cells, we studied the effect of PKCδ, ε and ζ in the regulation of Bax α phosphorylation state. To this purpose, Bax α from the mitochondrial fraction and the supernatant (corresponding to the cytosolic and microsomal fractions) of cells expressing Bax α, co-expressing PKCδ and Bax α, PKCε and Bax α and PKCζ and Bax α was immunoprecipitated and analysed by Western blot. This analysis show the existence of slow migrating bands recognized by the anti-Bax α antibody in the supernatant fraction of cells expressing Bax α, co-expressing PKCδ and Bax α and PKCζ and Bax α (Fig. **2.10**B). Treatment of Bax α, immunoprecipitated from the supernatant of cells expressing Bax α, with λ-PPase leads to the disappearance of these slow migrating bands showing that these bands corresponds to phosphorylated Bax α (Fig. **2.10**B). While PKCδ and ζ have no effect on Bax α phosphorylation, PKCε leads to the disappearance of these bands and therefore to Bax α dephosphorylation (Fig. **2.10**B). Curiously, these slow migrating bands are not found in Bax α immunoprecipitated from the mitochondrial fraction of all transformants, demonstrating that Bax α present in the mitochondria is not phosphorylated (Fig. **2.10**B). These results are in agreement with previous results (Renault and Manon, personal communication).

The effect of PKCδ, ε and ζ on Bax α translocation to mitochondria was also assessed. With that purpose, the levels of Bax α in mitochondria isolated from yeast cells expressing Bax α, co-expressing PKCδ and Bax α, PKCε and Bax α and PKCζ and Bax α were analysed by Western blot. The results obtained show that while PKCδ and ζ

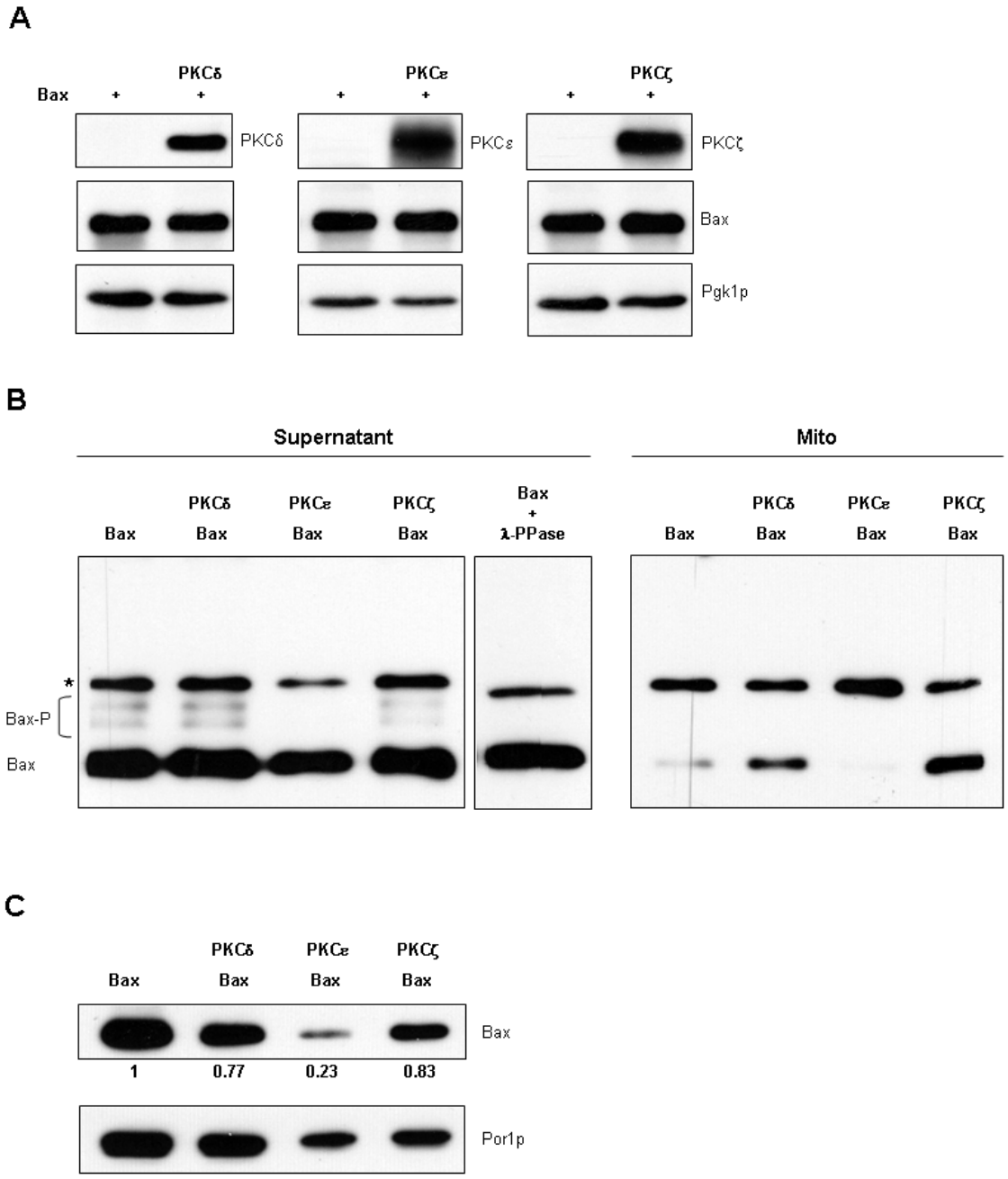

Figure 2.10 – PKCδ, ε and ζ distinctly modulates Bax α in yeast. (A) Detection of PKCδ, PKCε, PKCζ and Bax α in whole cell extracts after 14 h of expression. Pgk1p was used as loading control. (B) Phosphorylation of Bax α immunoprecipitated from the supernatant and the mitochondrial fractions of cells expressing Bax α, co-expressing PKCδ and Bax α, PKCε and Bax α and PKCζ and Bax α after 14 h of expression. Bax α phosphorylation was monitored by the appearance of slow migrating bands of Bax α. In vitro λ-PPase treatment abolished the appearance of these bands confirming that they corresponded to phosphorylated Bax α. (C) Detection of PKCδ, PKCε, PKCζ and Bax α in the mitochondrial fraction after 14 h of expression. Por1p was used as loading controls for the mitochondrial fraction. The amount of Bax α was quantified by densitometry analysis of nonsaturated immunoblots. All values were normalised to the loading control. * Unspecific band.

have no effect on Bax α translocation to the mitochondria, PKCε dramatically reduced Bax α translocation (Fig. 2.10C). Therefore PKCε does not only inhibit Bax α phosphorylation but also Bax α translocation to mitochondria.

2.4.3. Discussion

In the previous section we showedthat PKCα increases Bax α *c*-myc-induced cell death and translocation into mitochondria through a mechanism that is independent of PKCα kinase activity (Saraiva *et al.*, 2006; Silva *et al.*, 2011b; section 2.3). In this section we used the yeast cell model to study the regulation of a different form of Bax (Bax α), that is an inactive, untagged and with cytosolic localization, by PKC α, δ, ε and ζ.

Different reports using yeast and animal cells have suggested that phosphorylation of members of the Bcl-2 family proteins regulate their function (Arokium *et al.*, 2007; Datta *et al.*, 1997; Gardai *et al.*, 2004; Ito *et al.*, 1997; Saraiva *et al.*, 2006; Xin *et al.*, 2007). Previous reports have shown that Bax α is phosphorylated in yeast cells by unidentified yeast kinases (Arokium *et al.*, 2007). After co-expression of Bax α with PKCα, δ, ε and ζ we studied the role of these isoforms in the regulation of Bax α phosphorylation state in yeast. Our results show a distinct modulation of Bax α phosphorylation state by the PKC isoforms studied. While no effect on Bax α phosphorylation was detected a PKCδ and ζ, PKCα and ε and lead to Bax α dephosphorylation.

The effect of dephosphorylation in Bax α activity was studied. Dephosphorylation of Bax α by PKCα does not lead to Bax α activation, since no effect on cell death, Bax α translocation and insertion, and cyt *c* release was detected. However, this lack of effect does not exclude a possible anti-apoptotic effect of PKCα in Bax α regulation, since the human and untagged Bax α used has little or no effect in yeast. In fact, when PKCα was co-expressed with a more active form of human Bax α (Bax^{P168A}), an inhibition of Bax α-induced cell death, through a kinase activity-dependent mechanism, was observed. Dephosphorylation of Bax^{P168A} in yeast in the presence of PKCα was not confirmed, although the similarity of this form of Bax α with unaltered Bax α favours the existence of a dephosphorylation effect of PKCα on Bax^{P168A}. The mechanism involved in Bax α dephosphorylation by PKCα is unknown. These results are the contrary to what had

been described in section 2.3 for Bax *c*-myc, where a pro-apoptotic role of PKCα, independent on its kinase activity, was observed. It seems that PKCα has opposite roles in Bax α regulation, it increases the activity of full active mitochondrial Bax *c*-myc through a mechanism independent of its kinase activity, but inhibits activation of cytosolic Bax α through a kinase activity-dependent mechanism. It is interesting to find a putative dual role in Bax α modulation for this isoform. In fact, several contradictory results regarding the role of PKCα in apoptosis regulation have been observed with reports describing either a pro-apoptotic or an anti-apoptotic function in cell death (Ahmad *et al.*, 1994; Dooley *et al.*, 1998; Haimovitz-Friedman *et al.*, 1994; Nowak, 2002; Powell *et al.*, 1996; Shimizu *et al.*, 1998; Tanaka *et al.*, 2003; Whelan and Parker, 1998). Perhaps PKCα has the ability to regulate apoptosis at different levels with opposing roles. Regulation of PKCα function in apoptosis may occur through modulation of its kinase activity.

PKCε is considered an oncogene with anti-apoptotic functions (Basu and Pal, 2010; Basu and Sivaprasad, 2007; Gorin and Pan, 2009). A mechanism of apoptosis inhibition by PKCε is through interaction with Bax α and its retention in the cytosol (McJilton *et al.*, 2003). Here, we found that PKCε leads to Bax α dephosphorylation and inhibits Bax α translocation into mitochondria. Although we did not search for interaction between PKCε and Bax α in yeast in order to verify if this mechanism is conserved in yeast cells, the conservation of this interaction in yeast could explain the results obtained. An interaction between these two proteins in yeast would probably prevent Bax α phosphorylation by endogenous yeast kinases by blocking their access to Bax α and would also retain Bax α in the cytosol. The confirmation of conservation of this mechanism in yeast cells will further reinforce the yeast cell model as a powerful tool to study the regulation of Bax α by the oncogene PKCε. This model system can be used to better understand the regulation of this interaction and to find new modulators through drug screens that specifically regulate this interaction and therefore apoptosis.

In this study, no effect of PKCδ and ζ in Bax α phosphorylation and translocation into mitochondria was detected. During apoptosis, PKCδ is cleaved by caspase 3 leading to the release of the catalytically active fragment from the regulatory domain, thereby activating of PKCδ in the absence of any cofactors (Emoto *et al.*, 1995). After activation, PKCδ translocates to several different compartments, including mitochondria

(Gomel *et al.*, 2007). However, with exception of Mcl-1 (Gomel *et al.*, 2007), no other Bcl-2 family members was shown to be regulated by this PKC isoform (Sitailo *et al.*, 2006). Also, contrary to PKCα, ε and ζ, PKCδ did not regulate Bcl-xL phosphorylation state nor its anti-apoptotic role in a yeast cell model system (Saraiva *et al.*, 2006). Therefore, the lack of effect of PKCδ in the regulation of Bax α observed in this study may indicate a modest involvement of the Bcl-2 family proteins in the mechanism of apoptosis regulation by PKCδ.

It was been already shown that PKCζ modulates Bax α in mammalian cells through Bax α phosphorylation in ser184, inhibiting Bax pro-apoptotic activity (Xin *et al.*, 2007). In our model system no increase in Bax α phosphorylation was detected when Bax α was co-expressed with PKCζ. Since Bax α is already phosphorylated in yeast, a possible explanation could be the lack of sensitivity of the method used to detect small increases in the phosphorylation of Bax α. Another possible explanation could be the phosphorylation of Bax α in ser184 by endogenous yeast kinases masking the effect of PKCζ. In fact, the yeast kinase Sch9p is a homologue of the mammalian Akt/PKB (Geyskens *et al.*, 2000) that phosphorylates Bax α in the residue ser184. Therefore, phosphorylation of Bax α by the yeast Sch9p might mask an effect of PKCζ in Bax α phosphorylation.

In conclusion, this work shows a distinct modulation of Bax α by PKC α, δ, ε and ζ. Moreover, it highlights the role of phosphorylation events in the regulation of Bax α activity and reinforces the usefulness of the yeast model system in the study Bax α regulation by PKC isoforms.

2.5. Acknowledgements

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Chapter 3

General Discussion

In recent years, a number of important features and mechanisms implicated in Bax regulation have been clarified by expressing this protein in yeast allowing the identification of: i) mutations in Bax that affect its function; ii) yeast proteins that interact or are involved in its function; iii) members of the mammalian signalling pathways that are involved in its regulation (discussed in chapter 1). With the dissection of the mechanisms involved in Bax mode of action and regulation, a comprehensive picture is starting to emerge. Hopefully, this new knowledge will eventually be translated into new apoptosis-based therapies to be used in the treatment of several diseases. The studies described in this thesis aimed to analyse the role of mammalian PKC isoforms in Bax regulation. To accomplish this goal we exploited the use of the yeast model system as an alternative an advantageous approach. The main outputs of the work will be described here and its importance for a better therapeutic modulation of apoptosis will be discussed. Some research lines that should be pursued are also identified.

The crucial role of the Bcl-2 family proteins in the regulation of mitocondrial outer membrane permeabilization (MOMP), and therefore apoptosis, led to an increased concern for the study of their regulation. Recently, it has emerged that activity of Bcl-2 family proteins could be regulated by different protein kinases, such as protein kinase C (PKC). It has been shown that PKCα phosphorylates Bcl-2 (Ito *et al.*, 1997), that PKCε interacts with Bax (McJilton *et al.*, 2003), that PKCζ phosphorylates Bax (Xin *et al.*, 2007) and that PKCε and PKCθ also lead to Bad phosphorylation (Bertolotto *et al.*, 2000). Despite these results, several contradictory data regarding the role of the different PKC isoforms in cell death have been obtained with mammalian cell lines and it has been challenging to establish the relative contribution of the individual PKC isoforms in apoptosis. The co-existence of several PKC isoforms in the same cell and the different roles of PKC isoforms according to the cell type and cellular localization can explain this complexity (Hofmann, 2004; Shirai and Saito, 2002).

Among the several PKC isoforms, PKCα, δ, ε and ζ are the most abundant isozymes in various tissues and the major forms in carcinogenesis (Wetsel *et al.*, 1992; Gutcher *et al.*, 2003). Therefore, we have proposed with this work to study their roles in the regulation of Bax. The lack of obvious homologues of many key mammalian apoptotic

regulators, including of the Bcl-2 family, in yeast and the existence of a PKC homologue (Pkc1p) that is not functionally complemented by its mammalian counterparts led us to use the yeast model system in our study. With this thesis we show a distinct regulation of Bax activity by PKC isoforms in yeast. Moreover we demostrate an isoform-specific regulation of several features in Bax regulation.

3.1. Regulation of Bax by PKCα

The classical PKCα was the isoform most extensively studied in this thesis regarding its regulation of Bax. The role of PKCα in Bax regulation was studied in three variants of Bax, these variants being: i) the human untagged Bax α with cytosolic localization and reduced activity; ii) an untagged variant of Bax α with a single point mutation that increases its activity, Bax^{P168A}; iii) the human C-terminal tagged variant of Bax α, fully active and with mitochondrial localization (Bax *c*-myc). The results obtained (chapter 2) show a distinct modulation of these three variants of Bax by PKCα.

PKCα has no effect in the viability of cells expressing Bax α, in the translocation and insertion of Bax α into mitochondria and does not alter cytocrome *c* (cyt *c*) content of mitochondria of cells expressing Bax α. However, PKCα leads to Bax α dephosphorylation in yeast. When the effect of PKCα on Bax^{P168A}-induced cell death was studied, an inhibition of cell death induced by Bax^{P168A} was observed. Moreover, this anti-death activity of PKCα was abolished when the kinase activity of PKCα is eliminated. Whether the PKCα-induced dephosphorylation observed for Bax α also occurs with Bax^{P168A} is not known. However, the structure similarities between Bax α and Bax^{P168A} make us hypothesise that the PKCα-induced dephosphorylation observed for Bax α also occurs with Bax^{P168A}.

Regarding the role of PKCα in Bax *c*-myc regulation, a completely distinct result was observed. PKCα increases Bax *c*-myc-induced cell death and its translocation and insertion into mitochondria. It also increases Bax *c*-myc-induced cyt *c* release.

Moreover, it is shown that Bax *c*-myc is not phosphorylated in yeast in opposite to what has been previously observed for Bax α (Arokium *et al.*, 2007; section 2.4) and that PKCα does not alter its phosphorylation state. It is also shown that PKCα regulation of Bax *c*-myc is independent of PKCα kinase activity. Apparently, in yeast, PKCα has a dual role in Bax regulation. It not only increases the activity of full active mitochondrial Bax *c*-myc through a kinase-independent mechanism, but also inhibits the activation of cytosolic Bax α through a kinase-dependent mechanism.

The kinase activity of the different PKC isoforms was thought to be essential for the plethora of biological processes attributed to these kinases. However, apoptosis regulation independent of the catalytic activity has also been shown for PKCδ (Goerke *et al.*, 2002). Moreover, it was also shown that several yeast kinases modulate Bax through a pathway that is independent of their kinase activity (Renault and Manon, personal communication). Here we show that the function of PKCα in the modulation of an active form of Bax is also independent of its kinase activity. These results reveal PKCα as a multifunctional protein, whose biological role is not solely determined by its kinase activity. Since, PKCα function does not depend on its catalytic activity we assume that its role occurs through interaction with Bax or other yeast proteins yet to be determined. In mammalian cells, several opposing results regarding the role of PKCα in apoptosis have been obtained (Ahmad *et al.*, 1994; Dooley *et al.*, 1998; Haimovitz-Friedman *et al.*, 1994; Nowak, 2002; Powell *et al.*, 1996; Shimizu *et al.*, 1998; Tanaka *et al.*, 2003; Whelan and Parker, 1998). An apparently explanation for these contradictory results observed in mammalian cells might be the dual role of PKCα in Bax regulation as observed in yeast. It can be speculated that, depending on the death signal and cellular type, a PKCα kinase-dependent or -independent mechanism is activated, leading to different and opposing effects in mammalian cell death.

3.2. Regulation of Bax phosphorylation state by different PKC isoforms

In the last years a bulk of evidences supported the hypotesis that the function of Bcl-2 family members is regulated by phosphorylation. Different reports identified several kinases involved in the phosphorylation of Bcl-2 family members, including several PKC isoforms, as referred above (reviewed in chapter 1). The yeast model system has been used to study how these phosphorylation events regulate the function of members of the Bcl-2 family. Arokium *et al.* (2007) showed that substitutions of potentially phosphorylatable serine residues of Bax α regulate its interaction with mitochondria. Moreover, they showed that human Bax is phosphorylated by endogenous yeast kinases. Using the yeast model system it has been shown a differential regulation of Bcl-xL phosphorylation state that is associated to differential modulation of Bcl-xL anti-apoptotic effect in acetic acid-induced apoptosis (Saraiva *et al.*, 2006).

In this thesis, we show that PKC isoforms distinctly regulate Bax α phosphorylation state in yeast. But conversely to what we would expect from a kinase, we observe that PKCα and PKCε lead to Bax α dephosphorylation in yeast. Dephosphorylation of Bax α in yeast can occur by activation of phosphatases that dephosphorylate Bax α, or inhibition of Bax α phosphorylation either by inhibition of the activity of the endogenous yeast kinases responsible for Bax α phosphorylation, or by limiting the access of these kinases to Bax α.

PKCε is widely considered as having anti-apoptotic functions and it is considered to be an oncogene (Basu and Pal, 2010; Basu and Sivaprasad, 2007; Gorin and Pan, 2009). Although the mechanisms responsible for its anti-apoptotic function are unclear, several evidences showed that PKCε regulate members of the Bcl-2 protein family. It was shown a physical interaction between PKCε and Bax that decreases Bax α translocation to mitochondria (McJilton *et al.*, 2003) and also that PKCε leads to phosphorylation and inactivation of BAD trough a rsk-dependent pathway (Bertolotto *et al.*, 2000). Here we show that Bax α is dephosphorylated in yeast cells co-expressing PKCε. The mechanism that lead to Bax α dephosphorylation in yeast in the presence of PKCε was not elucidated. However, a possible conservation in yeast of the interaction between

Bax α and PKCε, identified in mammalian cells, would explain the detected dephosphorylation in yeast, since this interaction will probably block the access of endogenous yeast kinases to phosphorylatable Bax residues. The inhibition of Bax translocation to mitochondria by PKCε in yeast also supports the hypothesis of an interaction between PKCε and Bax, since this interaction should retain Bax in the cytosol.

In this study, PKCδ and PKCζ did not interfere with Bax phosphorylation state. Results obtained so far with mammalian cells show that, with exception of Mcl-1, no other Bcl-2 family member was shown to be regulated by PKCδ (Sitailo *et al.*, 2006). Moreover, it has been shown that, contrary to PKCα, ε and ζ, PKCδ did not regulate Bcl-xL phosphorylation state nor its anti-apoptotic role in yeast (Saraiva *et al.*, 2006). Due to the small involvement of Bcl-2 family proteins in the mechanism of apoptosis regulation by PKCδ identified so far, it is not surprising the absence of effect on Bax observed in this study. The absence of effect of PKCζ in Bax phosphorylation is more intriguing, since it has been shown that PKCζ phosphorylates Bax in mammalian cells (Xin *et al.*, 2007). However, the absence of effect of PKCζ can be explained by the lack of sensitivity of the method or due to phosphorylation of Bax by endogenous yeast kinases that may mask the effect of PKCζ (discussed in section 2.4). The absence of a detectable effect of PKCδ and PKCζ on Bax phosphorylation shows an isoform-specific regulation of Bax phosphorylation state, since not all isoforms studied regulate Bax phosphorylation in the same manner.

It was curious to find that either Bax or Bax *c*-myc immunoprecipitated from the mitochondrial fraction are not phosphorylated in yeast even in the presence of different PKC isoforms. This shows that the lipidic environment of the mitochondria membrane protects Bax from phosphorylation by yeast kinases. Since Bax *c*-myc is a mitochondrial-localized form of Bax, this probably explains why Bax *c*-myc is not phosphorylated in yeast, conversely to what was observed for Bax α.

3.3. Concluding remarks and future perspectives

PKC has been implicated in the regulation of a variety of cellular processes in an isoform-specific manner. The finding that PKC is involved in apoptosis regulation gives us the opportunity of targeting PKC during therapeutic intervention of diseases where the apoptotic process is deregulated, such as cancer and neurodegenerative disorders. However, the role of PKC isoforms in apoptosis is not well elucidated. Most of the research has been focused on a limited number of isoforms while the role of many others remains obscure. Moreover, the mechanisms by which distinct PKC isoforms regulate different signalling pathways, as well those underlying their translocation to different sub-cellular compartments are not completely clarified. In addition, the limited number of isoform-specific PKC regulators leads to the requirement of intelligent trial design in the screening for new isoform-specific PKC modulators. A lot of work has to be done, both in the clinic and in the laboratory, for a successful therapeutic modulation of PKC activity in several apoptotic diseases.

The finding in this thesis of a PKC isoform-specific regulation of Bax in yeast, both dependently and independently of their kinase activity, gives us the opportunity of using the yeast model system as a tool for discovering new forms of targeting the different PKC isoforms during therapeutic intervention of diseases, where the apoptotic process is deregulated such as cancer and neurodegenerative disorders.

Although this thesis has allowed some important improvements in our understanding of the mechanisms of Bax regulation by PKC isoforms, several relevant questions were also raised with this work that should be covered in the future, namely:

- Does PKCα-induced dephosphorylation observed for Bax α also occurs with Bax^{P168A}?

The conservation of this phosphorylation between Bax α and Bax^{P168A} would allow attributing an anti-apoptotic kinase-dependent role for the dephosphorylation of Bax α, observed in the presence of PKCα. Currently, we are trying to verify if this dephosphorylation also occurs in Bax^{P168A}.

- Which mechanism leads to Bax α dephosphorylation in the presence of PKCα?

Previous results obtained with mammalian cells showed that selective activation of PKCα in androgen-dependent prostate cancer (LNCaP) cells lead to activation of protein phosphatase 2A (PP2A) (Tanaka *et al.*, 2003). Moreover, it has been shown that PP2A dephosphorylates Bax in A549 cells (Xin and Deng, 2006). These results led us to hypothesise that the yeast PP2A homologue might be responsible for the observed dephosphorylation of Bax α in yeast. Currently, several studies are under way in our laboratory in order to verify the role of the yeast PP2A in Bax α dephosphorylation when PKCα is co-expressed. With this purpose Bax α will be co-expressed with PKCα in yeast cells lacking the catalytic subunit of the yeast PP2A.

- Which proteins interact with PKCα in yeast?

The yeast model system can further contribute for the elucidation of the mechanisms of apoptosis regulation by PKCα. Identification of PKCα interactome using a yeast two-hybrid screen or a pull-down assay, and identification of positive PKCα interactors in yeast by mass-spectrometry might allow the identification of novel interactions between yeast proteins and PKCα. This might provide some clues regarding the mechanism that leads to higher mitochondrial translocation and insertion of Bax *c*-myc in yeast in the presence of PKCα and, therefore a better understanding of the PKCα pro-apoptotic kinase-independent function. Identification of these interactors may lead to the identification of new mechanisms of Bax regulation in mammalian cells by PKCα.

- Does inhibition of Bcl-xL anti-apoptotic activity in acetic acid-induced yeast cell death by PKCα depends on PKCα catalytic activity?

It would be interesting to find a dual, opposite, role in the regulation of apoptosis by PKCα, where the kinase activity-dependent mechanism of apoptosis regulation is anti-apoptotic and the activity-independent mechanism is pro-apoptotic. Saraiva *et al.* (2006) showed that PKCα leads to dephosphorylation of Bcl-xL inhibiting its anti-apoptotic activity in acetic acid-induced yeast cell death. Studying the dependence of this effect on PKCα kinase activity would help to establish a connection between the dependence of PKCα kinase activity and its final outcome in cell death.

- Does isoforms of other PKC sub-families regulate Bax *c*-myc differentlty?

In the present thesis we only focused on the regulation of Bax *c*-myc by PKCα. Although, we expect a distinct regulation Bax *c*-myc by isoforms from other PKC sub-families, as reported for Bax α (section 2.3) and for Bcl-xL (Saraiva *et al.*, 2006), it would be interesting to study the role of other PKC isoforms in the regulation of Bax *c*-myc.

- Does PKCε and Bax interact in yeast?

Currently we are trying to verify by if PKCε and Bax α interact in yeast cells by co-immunoprecipitation, confirming results already obtained with mammalian cells (McJilton *et al.*, 2003). The conservation of this interaction in yeast will, not only explain our results obtained in section 2.4 showing Bax α dephosphorylation and inhibition of its translocation into mitochondria in the presence of PKCε, but will allow the identification of the PKCε residues required for interaction between these two proteins. This information will allow the development of new drugs that modulate this interaction through rational drug design or by conventional drug screenings.

The answer to those questions would greatly benefit our knowledge regarding Bax modulation by PKC isoforms and will open new opportunites for therapeutic modulation of apoptosis.

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