Molecular mechanisms linking the phosphorylation of the protein Bax to its activation during apoptosis
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Molecular mechanisms linking the phosphorylation of the protein Bax to its activation during apoptosis

Abstract

Programmed cell death is an important process to the normal development of an organism. Apoptosis is a type of programmed cell death process that can occur by two pathways the extrinsic or intrinsic. The intrinsic pathway is unleashed by an internal signal that actives the pro-apoptotic members of the Bcl-2 protein family. This protein family has an important role in the regulation of outer mitochondrial membrane permeabilization that may lead to the release of cytochrome c, and trigger the apoptotic cascade. These proteins differ in their structure and therefore in the function they perform.

Bax is a pro-apoptotic protein and its activation can be regulated in different ways. The activation can occur by interaction with other members of Bcl-2 protein family, by post-translation modification by phosphorylation of specific residues, and by interaction with lipids. Bax activation and its regulators can have an important role in diseases involving apoptotic dysfunctions.

Our working hypothesis was that the protein kinase Akt regulates the activation of Bax and its insertion in mitochondria directly (by phosphorylation of the residue Serine 184) or indirectly (by interaction with other proteins of the family Bcl-2). For this reason the objective of this work was to characterize the contribution of the presence of Akt in Bax activation, in yeast, using the mutant S184V (mimics the phosphorylation of the residue Serine 184).

Our results demonstrate that Akt presents an important role in activation of Bax, its effect may be directly connected with the phosphorylation of the residue Serine 184 but other effect might also be involved.

In conclusion, our results suggest that Akt contributes to the activation of Bax and the consequent cell death, but the exact mechanism of the interaction of this proteins is still to undercover.
Mecanismos moleculares vinculados à fosforilação da Proteína Bax e à sua ativação durante a apoptose

Resumo

Morte celular programada é um processo importante no desenvolvimento normal de um organismo. Apoptose é um tipo de morte celular programada que pode ocorrer por duas vias, a intrínseca ou a extrínseca. A via intrínseca é desencadeada por um sinal interno que ativa os membros pró-apoptóticos da família de proteínas Bcl-2. Esta família de proteínas tem um papel importante na regulação da permeabilização da membrana mitocondrial externa, que pode levar à libertação de citocromo c, e desencadear a cascata apoptótica. Estas proteínas diferem na sua estrutura e por consequente na sua função.

Bax é uma proteína pro-apoptótica e a sua ativação é regulada de diferentes formas. A ativação pode ocorrer por interação com outros membros da família proteica Bcl-2, por modificações pós-transicionais por fosforilação de resíduos específicos e pela interação com lípidos. A sua ativação e os reguladores podem ter um importante papel no desenvolvimento de doenças que envolvam disfunções apoptóticas.

A hipótese de trabalho deste projeto consistiu na suposição de que a proteína cinase Akt regula a ativação de Bax e a sua inserção na mitocôndria diretamente (fosforilação do resíduo Serina 184) ou indiretamente (interação com outras proteínas da família Bcl-2). Por essa razão foi nosso objetivo caracterizar a contribuição da presença de Akt na ativação de Bax, em levedura, usando o mutante S184V (mímica a fosforilação do resíduo Serina 184).

Os resultados demostraram que Akt apresenta um papel na ativação de Bax, podendo ser diretamente ligada pela fosforilação do resíduo Serina 184 ou outro mecanismo.

Em conclusão, os resultados sugerem que Akt contribui para a ativação de Bax e posteriormente para a morte celular sendo que o exato mecanismo desta interação ainda está por descobrir.
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Abbreviations

AIF - apoptosis inducing factor
Akt/PKB - Protein kinase A
APAF-1 - Apoptotic protease-activating factor 1
Apoptosis Protein (IAP)-Binding Protein With
ATP - Adenosine Triphosphate
BSA - Bovine Serum Albumin
c-FLIP - Cellular-FLICE (FADD-like IL-1β-converting enzyme)-inhibitory Protein
Co-IP - Co-immunoprecipitation
C-terminal - Carboxyl-terminal
DISC - Death Inducing Signaling Complex
DNA - Deoxyribonucleic acid
EDTA - Ethylenediaminetetraacetic acid
Endo G - Endonuclease G
ER - Endoplasmatic Reticulum
FADD - Fas-Associated Death Domain
GFP - Green Fluorescent Protein
GSK3 β - Glycogen Synthase Kinase
H2O2 – Hydrogen Peroxide
HtrA2/Omi - High Temperature Requirement
IAPs - Inhibitors of Apoptosis Proteins
JNK - Jun N-terminal kinase
MAC - Mitochondrial Apoptosis-induced Channel
MAPK - Mitogen-activated Protein Kinases
MOMP - Mitochondrial Outer Membrane
NADH - Nicotinamide Adenine Dinucleotide
NADPH - Nicotinamide Adenine Dinucleotide
N-terminal - Amino-terminal
OMM - Outer mitochondrial membrane
PKA - Protein kinase A
PKC - Protein kinase C
PTP - Permeability Transient Pore
ROS - Reactive oxygen species
SDS - Sodium Dodecyl Sulfate
Smac/Diablo - Second mitochondria-derived activator of caspases/IAP-binding mitochondrial protein
TM - Transmembrane
TNF - Tumor necrosis factor
TNF-R - Tumor-Necrosis Factor Receptor
TOM - Translocase of the outer membrane complex
TRADD - TNF-R-Associated Death Domain
TRAIL-R - TNF-related Apoptosis-Inducing
UV – Ultraviolet
VDAC - Voltage-dependent anion channel
1. Introduction
1.1. Bcl-2 protein family – crucial regulators of apoptotic cell death

Cell death is a crucial biological process for tissue homeostasis and the development of a multicellular organism, and has been studied for the last decades (Galluzzi et al., 2012, for review). Programmed cell death was first mentioned in 1965 when Lockshin and Williams used this term to differentiate this type of cell death from the accidental cell death. Posterior apoptosis that was known to be a type of cell death genetically controlled was considered the equivalent of programmed cell death, being necrosis an accidental cell death. This nomenclature has been altered when it was proved that necrosis can also be regulated (Kerr et al., 1972; Galluzzi et al., 2012).

The various processes that lead to cell death can be classified depending on morphological appearance, enzymatic aspects such as involvement of caspases, the functional aspects (if is programmed or accidental) or the immunological characteristics (Kroemer et al., 2009).

Apoptotic cell death does not affect the surrounding cells and is an essential process in embryonic and post-embryonic development, regulation of tissue homeostasis and elimination of damaged cells. This process occurs when a cell activates a suicide program as a result of an external stress signal or intracellular stress signal (Bras et al., 2005; Galluzzi et al., 2012). Cell suicide can be set off by changes in the cell equilibrium such as DNA damage or cell cycle aberrations and by other cells through signals and receptors that regulate gene expression (Hengartner, 2000; Fulda et al., 2010). This process is characterized by cellular shrinkage, degradation of chromosomal DNA, chromatin condensation and cell fragmentation into membrane-enclosed apoptotic bodies that are phagocyted by neighboring cells, not affecting the surrounding cells (Bursch et al., 2000; Kroemer et al., 2009). Its malfunction can lead to a variety of pathologies, including cancer and degenerative diseases (Adams and Cory, 2001; Green and Even, 2002).
1.1.1. The caspases

Apoptosis cell death can be triggered by an extrinsic pathway or an intrinsic pathway, depending on the type of stimulus received. Both pathways ultimately lead to the activation of a particular group of proteases, called caspases. Caspases are present in cells in an inactive form, after receiving an apoptotic stimulus, these caspases become activated through a sequence of proteolytic events that lead to structural changes, oligomerization, and further activation (Hengartner, 2000). These proteases contain a cysteine residue as the catalytic nucleophile and cleave substrates after an aspartic acid residue (hence their name that means Cysteine Aspartate Proteases). Caspases can be put in three types depending on the amino acid sequence that they target that is related to their cellular function. Initiator capases 8, 10, 9 and 2 participate to the signaling of apoptosis. Effector caspases 3, 6 and 7 participate to the degradation of specific substrates during apoptosis. Inflammatory caspases 1, 4 and 5 participate to the inflammation response and to necrosis but do not participate to apoptosis (Alnemri et al., 1996; Frejlich et al., 2013). Caspases are synthesized as a proenzyme (pro-caspase) with a pro-domain that is cleaved during their activation. The remaining protein is cleaved in two subunits, long and short, that form a heterotetramer formed by two long
subunits and two short subunits (Nicholson et al., 1995; Alnemri et al., 1996; Ho and Hawkins, 2005). Caspases 8 and 10 contain a dead effector domain and are involved in the extrinsic apoptotic pathway, whereas caspases 2 and 9 contain a caspase activation and a recruitment domain (CARD) and are involved in the intrinsic apoptotic pathway.

1.1.2. The extrinsic apoptotic pathway (or death-receptors pathway)

The extrinsic pathway is triggered by extracellular stimuli such as response to lack of nutrients, to an impact of chemical substances, to deficiency of physical factors or to a response to hormones and cytokines (Frejlich et al., 2013). Stimuli are sensed and propagated by death receptors present on the plasma membrane. These receptors have a cytoplasmic domain, also referred as death domain. Extracellular messengers, such as FAS/CD95 ligand, tumor necrosis factor α (TNFα) and TNF-related apoptosis inducing ligand (TRAIL), bind to their respective death receptors FAS/CD95, TNFα receptor 1(TNFR1) and TRAIL receptor (TRAILR). Upon ligand binding, the receptor suffers a conformational change that leads to the oligomerization of the death domains allowing the attachment of adaptor proteins, such as FADD and TRADD that possess a domain homologous to the death domain (Kischkel et al., 1995; Galluzzi et al., 2012).

Upon the formation of the Death Inducing Signaling Complex (DISC), it will recruit procaspases 8 or 10, provoking their activation. After their activation the caspases 8 or 10 (initiation caspases) trigger the activation of effector caspases 3, 6 and 7 that will cleave different substrates, resulting in cell death (Kischkel et al., 1995; Hengartner, 2000; Ho and Hawkins, 2005).

The extrinsic apoptotic pathway can be down regulated by the action of viral proteins FADD-like ICE inhibitory proteins (vFLIPs). These proteins prevent the recruitment of procaspases by the DISC; other viral proteins such as CrmA, directly inhibit the proteolytic activation of the initiator proscapases. Apoptosis can also be regulated by the expression of decoy receptors for TRAIL that sequesters TRAIL ligand preventing its binding to the extracellular messengers (reviewed by Budihardjo et al., 1999).

Extrinsic apoptosis can also occur through the action of dependence receptors such as netrin receptors UNC5A-D and DDC (deleted in colorectal carcinoma), leading to the caspase-9 and caspase-3 activation and cell death (Mehlen and Bredesen; 2011, Galluzzi et al., 2012).
1.1.3. The intrinsic apoptotic pathway (or mitochondrial pathway)

The intrinsic pathway, also known as the mitochondrial pathway, is triggered by intracellular signals such as DNA damage, oxidative stress, cytosolic Ca²⁺ overload, endoplasmic reticulum (ER) stress, and accumulation of incorrectly folded proteins, cellular deprivation of growth factors, and excessive production of reactive oxygen species (ROS) and the presence of oncogenes. These conditions generate pro-apoptotic signals that after being received by the mitochondria, leading to mitochondrial outer membrane permeabilization (MOMP). At the same time as the pro-apoptotic signal is propagated, anti-apoptotic signals are also emanating from different intracellular compartments, to allow cells to cope with the stress, these signals converge and the signal that prevails is integrated and decides the cell’s fate (Kroemer et al., 2007; Galluzzi et al., 2012).

Mitochondria plays a crucial role in apoptosis through the release of apoptogenic factors from the intermembrane space to the cytosol, upon permeabilization of the mitochondrial outer membrane. These are cytochrome c, apoptosis inducing factor (AIF), Endonuclease G (Endo G), Second Mitochondria-derived Activator of Caspases/Direct Inhibitor of Apoptosis Protein (IAP)-Binding Protein With Low Pi (Smac/Diablo) and High Temperature Requirement protein A2 (HtrA2/Omi). When released in the cytosol, these proteins participate to the activation of caspases.

When cytochrome c is released, it binds to apoptotic protease-activating factor 1 (APAF-1) and deoxyadenosine triphosphate (dATP) forming a large complex, the apoptosome, that will recruit pro-caspase 9, leading to activation of caspase 9 and caspase 3 provoking an apoptotic phenotype (Adams and Cory, 2007; Kroemer et al., 2007).

Smac/DIABLO and HtrA2/Omi also favor intrinsic apoptosis dependent of caspases by sequestering and degrading IAPs (Inhibitors of Apoptosis Proteins), that are endogenous inhibitors of caspases, thereby indirectly activate caspases. On note, the protein HtrA2/Omi also promotes caspase independent apoptosis by the action of its serine protease activity that cleaves cellular substrates such as cytoskeletal proteins. Like HtrA2/Omi, the proteins AIF and Endo G participate in a caspase-independent apoptosis. After relocating to the nucleus, these proteins mediate chromatin
condensation and large-scale DNA fragmentation (Kroemer et al., 2007; Galluzzi et al., 2012) (Figure 2).

A dissipation of the mitochondrial transmembrane potential can also take place with interruption of ATP synthesis and transport activities, that are both dependent on it. Also, the loss of mitochondrial cytochrome c inhibits the respiratory chain promoting ROS overproduction that will lead to an amplification of the apoptotic signal (Desagher and Martinou, 2000; Loeffler et al., 2001; Kroemer et al., 2007; Galluzzi et al., 2012).

The extrinsic and intrinsic pathways can be connected: indeed, caspase-8 can cleave the BH3-only protein Bid, generating a truncated fragment (truncated BID, tBID) that provokes the permeabilization of the outer mitochondrial membrane and consequentially the activation of caspase 3 by proteolytic cascade of caspase 9 that usually occurs in the intrinsic pathway (Antonsson, 2001; Favaloro et al., 2012).
1.1.4. The Bcl-2 family

The permeabilization of the mitochondrial membrane is due to the formation or the activation of a pore (termed permeability transition pore complex (PTPC)) in the mitochondrial outer membrane (Galluzzi et al., 2012). The formation and the regulation of this pores is controlled by a family of proteins, called the Bcl-2 family.

Bcl-2 family members play a crucial role in the regulation of the apoptotic pathway. They are able to activate cell death via the apoptotic program integrating diverse survival and death signals generated outside or inside the cell and directly regulate the permeability of the outer mitochondrial membrane (Vaux and Korsmeyer, 1999; Cory and Adams, 2002).

In 1985, the Bcl-2 (B-cell leukemia/lymphoma-2) protein was identified as an anti-apoptotic protein (Tsujimoto et al., 1985). Other proteins of the family were next identified based on the presence of Bcl-2 homology domains (BH1, BH2, BH3, and BH4). The proteins of this family are classified by the number of domains they contain, every member containing at least one of these domains. This structural classification reflects different functions (see below). It should be noted that several proteins that have a BH3 domain are not members of the Bcl-2 family, such as the autophagy regulator Beclin-1.

This family is divided in two functional groups, anti-apoptotic proteins and pro-apoptotic proteins. Pro-apoptotic proteins are subclassified as multidomain proteins and BH3-only proteins (Aouacheria et al., 2005; Er et al., 2006).

The anti-apoptotic proteins Bcl-2, Bcl-xL, Bcl-W, Bcl-B, Mcl-1 and A1 are characterized by the presence of a transmembrane domain (TM) localized at the C-terminal end of the proteins. Bcl-2, Bcl-xL, Bcl-W, Bcl-B present 4 BH domains (BH1, BH2, BH3 and BH4) whereas Mcl-1 and A1 do not have a BH4 domain (Er et al., 2006). The domains BH1, BH3 and BH4 present in Bcl-2 and Bcl-xL are required for the anti-apoptotic function of the proteins (Huang et al., 1998; Shimizu et al., 2000).

The pro-apoptotic multidomain proteins include the members Bax, Bak and Bok/Mtd and have a similarity in the sequence of the three homology domains BH1, BH2 and BH3. The presence of the BH3 domain in these proteins has been shown essential to their killing activity. This domain binds with the hydrophobic pocket of other proteins (Suzuki et al., 2000; Er et al., 2006). These proteins also have a C-
terminal hydrophobic helix, but it is still unclear if this helix is a genuine transmembrane domain.

The BH3 only proteins Bid, Bad, Puma, Bmf, Noxa Bim, Bik, Blk, Hrk/DP5, Bnip3 and Nix share only the BH3 domain. In this group, Bid is the only protein that does not present a transmembrane domain. The BH3 only proteins can promote apoptosis by activating pro-apoptotic Bcl-2 members or inhibiting anti-apoptotic Bcl-2 members, depending on the proteins involved. This behavior suggests that these proteins act like sensors and that the multidomain Bcl-2 pro-apoptotic proteins (Bax and Bak) are the executioners of those cell death signals. When BH3 only proteins are absent or inhibited in a cell, the anti-apoptotic Bcl-2 proteins block the action of the pro-apoptotic members (Antonsson, 2001; Er et al., 2006; Gallenne et al., 2009) (Figure 3).

Figure 3. The Protein Bcl-2 family: The three groups of Bcl-2 protein family are defined by the homologous structures, functions and the presence of BH domains. In this figure are represented the transmembrane domain (TM) and the helix (α), that constitute these proteins (Renault and Manon, 2011).

Anti-apoptotic proteins prevent the release of apoptogenic molecules from mitochondria like cytochrome c, hindering caspase activation, while pro-apoptotic Bcl-2 proteins favor this release. Therefore the relative protein concentrations of Bcl-2 family members can determine the cell response to the apoptotic stimuli (Antonsson, 2001; Schlesinger et al., 1997). These proteins can be detected in various locations, besides the outer mitochondrial membrane (OMM), such as the endoplasmic reticulum, the cytosol and microtubules. The regulation of this localization in different subcellular compartments can be accomplished, namely, by the formation of heterodimers, phosphorylation, and proteolysis (Sharpe et al., 2003).
The proteins of this family can regulate the permeability of the OMM, which is considered to be a major checkpoint of apoptosis. The molecular mechanism is still unclear, but it is generally considered that Bax and Bak suffer a conformational alteration that will lead to their membrane insertion, oligomerization and formation of a large channel permeable to small proteins, like cytochrome c. The control of OMM permeability by the Bcl-2 family members could also be related to the interaction of these proteins with pre-existing mitochondrial membrane pores or to the alteration of the membrane lipid order and the formation of lipidic pores, leading to the subsequent release of larger proteins, such as AIF. The presence of cytosolic Ca\(^{2+}\) can accelerate OMM permeabilization (Aouacheria et al., 2005; Er et al., 2006; Gallenne et al., 2009; Silva et al., 2011;). The phosphorylation of Bcl-2 family members can lead to a loss of function decreasing their binding to BH3 only protein members (Basu et al., 2006).

1.2. The pro-apoptotic Bax (Bcl-2 associated X) protein

Bax promotes cell death, and exists predominantly in the cytosol, as a monomer, before the induction of apoptosis by a stimulus. When cell death is induced, conformational changes lead to its translocation to mitochondria, and insertion in the OMM (Hsu et al., 1997). The insertion of Bax in mitochondria is associated to its activation and the formation of pores in the OMM conveying in apoptosis (Hsu et al., 1997; Goping et al., 1998; reviewed by Renault and Manon, 2011)

1.2.1. Bax structure and function

The structure of soluble Bax (reflecting its cytosolic conformation) has been determined by NMR. It is constituted by nine \(\alpha\) helices connected by loops; six hydrophilic \(\alpha\) helices and the hydrophobic \(\alpha9\), are clustered around two amphipathic \(\alpha\) helices in a central position (\(\alpha5\) and \(\alpha6\)). The BH3 domain is enveloped by helix \(\alpha2\), and the loop between the \(\alpha2\) and \(\alpha3\) helices presents rigidity. Bax has three flexible regions the N-terminal tail (15 residues), the loop between \(\alpha1\) and \(\alpha2\) helices (18 residues) and the loop between helices \(\alpha8\) and \(\alpha9\) (5 residues) (Suzuki et al., 2000; Petros et al., 2004).

Helices \(\alpha2\), \(\alpha3\) and \(\alpha4\) form a hydrophobic groove. The helices \(\alpha7\) and \(\alpha8\) form the BH2 domain, and the gap between helices \(\alpha4\) and \(\alpha5\) is filled by BH1 domain. The C-
terminal α helix is mostly hydrophobic. Hydrophilic residues Thr\textsuperscript{172}, Thr\textsuperscript{174}, Thr\textsuperscript{182} and Thr\textsuperscript{186}, are exposed to the solvent, but Ser\textsuperscript{184}, is oriented towards the hydrophobic pocket (Suzuki et al., 2000; Petros et al., 2004) (Figure 4).

![Figure 4](image)

**Figure 4** – Structure of Bax monomer determined by RMN, where the helices constituents of bax are observed, being the helix α9 represented at light green (Suzuki et al., 2000).

The structure of Bax presents a strong similarity with Bcl-x\textsubscript{L} in contrast with the relatively low degree of sequence similarity (Petros et al., 2004). The Bax C-Terminal α helix corresponds to the carboxy-terminal transmembrane domain of Bcl-x\textsubscript{L} and Bcl-2 (Petros et al., 2004). While this α-helix of Bcl-x\textsubscript{L} and Bcl-2 acts as a membrane anchor and a membrane-targeting signal (Janiak et al., 1994; Priault et al., 2003b), this role is less clear for the C-terminal α-helix of Bax: indeed, Bax can be located to the OMM even when the C-terminal α-helix is absent or replaced by a hydrophilic sequence (Priault et al., 2003b). However, the C-Terminal domain of Bax could participate to the formation of pores on the OMM, since it is able, by itself, to disrupt the barrier properties of membranes (Martinez-Senac et al., 2001). This helix may also play an important role in the localization of Bax, by defining the orientation and packing of the helices α2, α3 and α4 (Suzuki et al., 2000).
The binding of the C-terminus to the hydrophobic pocket containing the BH3 domain prevents the exposure of the BH3 prior apoptosis, and consequently prevents the dimerization and oligomerization events leading to the formation of a pore. The interaction of Bax BH3 domain with other Bcl-2 family proteins is very unlikely to happen, because of the binding existing between with C-terminal helix and its BH3 pocket, that prevents the exposure of the BH3 domain prior apoptosis (Suzuki et al., 2000).

The N-terminal of Bax, sometimes termed ART (Apoptotic Regulation of Targeting) is constituted by 20 mobile residues of which the deletion activates Bax. The connection between ART and the α1 helix support major conformational changes during Bax activation, since an antibody directed at this domain (6A7), is able to discriminate between the inactive and active conformation of Bax (Hsu and Youle, 1997).

The α1 helix, that presents some similarity to mitochondrial targeting sequences, might have an important regulatory function by allowing the interaction of Bax with mitochondrial receptors, such as Tom22 (Priault et al., 2003b, Bellot et al., 2007, Renault et al., 2012).

1.2.2. Bax signalling pathways

Bax activation is essential to its action on the permeabilization of OMM. After activation, and its binding to the OMM, the protein forms oligomers (Zhang et al., 2010). This oligomerization leads to the permeabilization of the OMM (Antonsson et al., 2001). Bax activation promotes apoptosis following exposure of the N-terminal, mitochondrial translocation, oligomerization, and focal clustering on the mitochondrial membrane (Sharpe et al., 2003).

Apoptotic stimuli can induce several conformational changes in Bax protein, which include interaction with BH3-only proteins such as Bim and Bid, exposure to chemical agents such as H2O2, low pH, mild heat treatment, proteolytic cleavage, and post-translational modification such as phosphorylation (Khaled et al., 1999; Westphal et al., 2011). It has been shown that conformational changes in the N- and C-terminal, BH3 domain and hydrophobic groove of Bax protein are related to its insertion on OMM (Suzuki et al., 2000; Priault et al., 2003b). Independently of the stimuli, the inactive conformation of Bax changes, exposing its inner hydrophobic surfaces and causing Bax activation (Gavathiotis et al., 2010).
When Bax activation occurs by the binding of BH3-only proteins to the α1/α6 trigger site (rear pocket) or to the hydrophobic groove, the binding leads to the opening of the α1-α2 loop, and its further displacement, that is followed by allosteric changes of the Bax BH3 domain and the helix α9. These conformational changes expose the N-terminal epitope (6A7), the BH3 domain (α2) and the helix α9. Following these conformational changes, Bax will be translocated to the OMM and will oligomerize, leading to OMM permeabilization, and subsequent release of cytochrome c (Hsu et al., 1997; Hsu and Youle, 1997; Lucken-Ardjomande and Martinou, 2005; Gavathiotis et al., 2008; Westphal et al., 2011). It is however still unclear in which order these changes take place (Westphal et al., 2011) (Figure 5).

The exposure of BH3 domain is essential to Bax oligomerization, since the exposure of the helix α9 facilitates Bax translocation and insertion in the OMM under an active conformation. Bax activation can also occur by auto-activating interactions between its own BH3 domain and trigger site (Gavathiotis et al., 2010; Westphal et al., 2011).

Bax oligomerization can occur by two different interfaces, one by the interface between front surfaces of neighboring molecules, such as interaction between α6 helices, or by interaction of the rear surfaces. The formations of these interfaces are dependent of one another. The oligomers can create pores with an undefined size by disrupting the lipid bilayer (Zhang et al., 2010) (Figure 6). Based on its conductance, it has been estimated that 10 Bax monomers are needed to form the pore (Martinez-Caballero et al., 2009).

Figure 5– Model of Bax activation by the family member BH3-only Bid, Bax in Blue is away from mitochondria by anti-apoptotic proteins Bcl-2 and Bcl-xL (red), after the apoptotic stimuli BH3-only proteins trap anti-apoptotic proteins, and the protein Bid helps the addressing and insertion of Bax to mitochondria membrane and its posterior oligmerization (adapted from Renault and Manon, 2011).
The outer mitochondrial membrane translocator (TOM) complex may play an important role in the Bax insertion into the mitochondria, acting as a receptor (Renault and Manon, 2011). However, mitochondrial Bax can also act as a receptor for cytosolic Bax (Bellot et al., 2007).

The interaction of other members of the Bcl-2 family, like Bcl-xL, with Bax can prevent the Bax conformational rearrangements, and therefore prevent its activation (Lucken-Ardjomande and Martinou, 2005; Jourdain and Martinou, 2009).

1.3. Mechanisms involved on Bax regulation

1.3.1. Bax regulation by interaction with Bcl-2 family members

Bax activity can be regulated by its interaction with other proteins from the Bcl-2 family. The anti-apoptotic proteins like Bcl-2 and Bcl-xL are well known as Bax inhibitors. The direct interaction of Bcl-2 and Bcl-xL leads to the inhibition of Bax activity. In another hand, the heterodimers formed by this interaction (Bax/Bcl-2 or Bax/Bcl-xL) can be destabilized by BH3-only proteins (Bad, tBid), by competing with Bax for the binding with these proteins (Billen et al., 2008; Renault and Manon, 2011; Gautier et al., 2011). In 2011, Gautier and colleagues suggested that the inhibition of the direct interaction between Bcl-xL and Bax can lead to apoptosis dependent on Bax, without the presence of efficient BH3 activators, suggesting that the release of Bax from its interaction with Bcl-xL is sufficient to promote Bax activation. The presence of molecules such ABT-737 or Terphenyl-K, that mimic BH3-only proteins, can induce apoptosis in these cases.
Bax interaction with BH3-only proteins like tBid, Bim and PUMA can lead to its activation, and therefore promote apoptosis. Bid is a cytosolic protein, that is activated by the cleavage of its amino portion by active caspase 8 (Lou et al., 1998). Activated Bid (tBid), interacts with Bax, inducing Bax N-terminal exposure and its oligomerization, mitochondrial permeability and subsequent cell death (Desagher et al., 1999; Moreau et al., 2003). This activation can require the direct interaction with BH3-only protein or not. Bax activation can be achieved by three paths: direct activation, indirect activation and priming–capture–displacement (Zhang et al., 2010; Strasser et al., 2011).

In the model of direct activation, BH3-only proteins bind directly to the BH3 domain of Bax, leading to its recruitment into the OMM.

In the indirect model, BH3-only proteins bind to the pro-survival proteins, preventing the already activated Bax to be neutralized by pro-survival proteins. This permits its oligomerization, so apoptosis can proceed when all pro-survival proteins are neutralized by the BH3-only proteins.

In the priming–capture–displacement model, Bax, that becomes active by direct activation by BH3-only proteins binding or by other signals, is captured by pro-survival relatives, these proteins remain bound to Bax until they are displaced by BH3-only proteins as in the indirect activation model (Adams and Cory, 2007; Strasser et al., 2011) (Figure 7).
Figure 7 – Models for Bax activation triggered by interaction with BH3-only protein, A - representation of the direct activation; B - indirect activation C- priming-capture-displacement, in which the red triangle represents the BH3 domain of Bax and the BH3-only proteins (Strasser et al., 2011).

The propagation of the death signal can be also executed by the auto-activation of Bax, by the interaction between its own BH3 domain and trigger site as discussed above (Gavathiotis et al., 2010). tBid can also indirectly help the formation of Bax-dependent lipidic pores by rearranging lipids, favoring Bax insertion into the OMM (Kuwana et al., 2002).

BH3-only proteins PUMA and Bim can regulate the activation of Bax by inducing structural changes in the α1/α2 loop of Bax (Gallenne et al., 2009; Gavathiotis et al., 2010; Gautier et al., 2011). BH3-only proteins like Bad, Bik and Noxa lack the capability to activate Bax protein and only bind to anti-apoptotic proteins (Moreau et al., 2003).

The exact mechanism underlying Bax inhibition by Bcl-xL and Bcl-2 is still debated. These studies came to raise the question if the interaction between the heterodimers Bax/Bcl-2 and Bax/Bcl-xL would prevent the formation of homodimers of Bax. Recent studies, such as Edlich et al. (2011), introduced the concept of retro-
translocation of Bax mediated by Bcl-xL. It is proposed that the protein Bcl-xL plays a role in the retro-translocation of Bax from mitochondria to the cytosol. Conversely, it was also observed that the presence of Bcl-xL increases the localization of Bax to mitochondria (Renault, PhD thesis, 2010). This suggests that Bcl-xL would regulate the "cycling" of Bax between mitochondria and the cytosol.

In 2013 Schellenberg and colleagues proposed that the interaction between Bax and Bcl-xL occurs in the helix α9 (C-terminal) of Bax and that this interaction promotes the translocation of Bax to the outer mitochondrial membrane (Figure 8).

1.3.2. Bax regulation by phosphorylation

The functions of Bcl-2 family proteins can also be regulated by post-translational modifications like phosphorylation. Bax activity can be regulated by the phosphorylation of some of its residues, with an impact on its translocation and activation (Er et al., 2006; Arokium et al., 2007). Ser184 phosphorylation prevents Bax translocation to mitochondria. This serine is present in the middle of α9 helix, oriented towards the core of the protein. The substitution of Ser184 by Asp, adds a negative charge, that prevents its insertion into the OMM. This might also be favorable to the heterodimerization with anti-apoptotic Bcl-2 proteins (Gardai et al., 2004).
Phosphorylation of Ser163 can regulate Bax activity, and promotes Bax dependent apoptosis (Linseman et al., 2004). Substitution of Ser163 to Asp did not increase, by itself, Bax activity. But when this mutation was introduced in the protein already carrying the substitutions Pro168 to Ala and Ser60 to Ala, the resulting triple mutant is highly active. This suggests that the addition of a negative charge on Ser163 can help the movement of Bax N-terminal, in the presence of other conformational changes (Linseman et al., 2004; Arokium et al., 2007) (Figure 9).

In vivo, these phosphorylations can be performed by the Glycogen Synthase Kinase (GSK3 β), Protein kinases PKA, Akt/PKB, PKC and Jun N-terminal kinase (JNK) and p38 kinase. GSK3 β can phosphorylate Ser163 inducing Bax activation. GSK3 β can be phosphorylated and inactivated by Akt/PKB (Linseman et al., 2004). Akt/PKB can inhibit Bax activation by phosphorylating Ser184, which prevents Bax translocation. Akt also can inhibit Bax activation by indirectly phosphorylating GSK3β thus preventing phosphorylation on Ser163 (Linseman et al., 2004; Gardai et al., 2004). PKC isoforms can also regulate Bax activity by increasing Bax content and stability (Silva et al., 2010). However, PKCζ was shown to phosphorylate Ser184, like Akt.

Other residues, like Ser60, might also affect Bax activity when phosphorylated. Although it has not directly demonstrated, Ser60 is a target for PKA. This residue is closed to a salt bridge formed by Asp33 and Lys64, which is involved in the

![Figure 9. Primary structures of human Bax. Constituted by 192 aminoacids, organized in nine helices Where the potentially phosphorylatable serines are represented (Renault and Manon, 2011).](image)
The negative charge brought by Ser60 phosphorylation might destabilize this bridge. Indeed, the substitution of Ser60 by Asp triggered Bax activation. It has been hypothesized that the phosphorylation of Ser60 by PKA might require the dephosphorylation of Ser184 and the phosphorylation of Ser163, that induce conformational changes that allow the accessibility of Ser 60 to PKA (Arokium et al., 2007) (Figure 10).

Xin and Deng, in 2006, proposed that nicotine can inactivate Bax through Ser184 phosphorylation and that Protein Phosphatase 2A can directly dephosphorylate and activate Bax. This dephosphorylation results in the exposure of the 6A7 epitope in Bax, facilitating its translocation to mitochondria, an disrupts Bcl-2/Bax binding, preventing Bcl-2 to inhibit Bax activation.

JNK and p38 kinase can phosphorylate Bax when activated by exposure to apoptosis-inducing agents such as staurosporine, H₂O₂, etoposide, and UV. These proline-directed Ser/Thr protein kinases phosphorylate Thr167, leading to Bax activation by the exposure of its N-terminus and possibly its C-terminal transmembrane domain (Kim et al., 2006). It should be noted that the phosphorylation of Ser163 by GSK3β may require the previous phosphorylation of Thr167.

Phosphorylation of other Bcl-2 family members can indirectly affect Bax activation, like Bcl-xl phosphorylation by PKC isoforms (Silva et al., 2012).
1.3.3. Bax regulation by sphingolipids

Bax interaction with lipids can induce the reorganization of the bilayer membrane structure and lead to the formation of lipidic pores (Basanez et al., 1999; Sharpe et al., 2003). This pore formation reveals to be more efficient in lipids with large head groups that induce curvature to membranes (Basanez et al., 2002; Billen et al., 2008).

Ceramide can form channels in the OMM that are permeable to proteins. This sphingolipid is found in membranes as is composed by an N-acylated sphingosine. Mitochondria contain enzymes responsible for ceramide synthesis and hydrolysis, such as ceramide synthase and ceramidase (Siskind, 2005). Both outer and inner mitochondrial membranes present acylated exogenous sphingosine, therefore are capable to generate ceramide. Mitochondria-associated membranes also contain ceramide synthases (Bionda et al., 2004). The levels of ceramide in mitochondria increase prior OMM permeabilization, thereby this permeabilization can be regulated by mitochondrial ceramide levels (Siskind, 2005). Apoptosis induced by CD95, TNFα (Tumor necrosis factor α), and UV radiation have been shown to occur via an increase in mitochondrial ceramide levels (Siskind, 2005).

The ceramide channels formed in the OMM are eliminated when ceramide is removed. These channels are not specific to cytochrome c, they also allow the release of low molecular weight proteins from mitochondria, such other pro-apoptotic proteins (Siskind, 2005).

Sphingolipids metabolism can regulate Bax activation. Ceramide can lead to Bax activation indirectly via PP2A-mediated Ser184 dephosphorylation (Xin and Deng, 2006) or, with Ser184 phosphorylated, by outer mitochondrial membrane reorganization. Ceramide forms a platform that allows Bax insertion, leading to its oligomerization (Lee et al., 2011; Chipuk et al., 2012).

Cardiolipin is essentially to the formation of Bax membrane pores, but its regulation is performed indirectly. Cardiolipin participates in the activation of Bid, that subsequently activates Bax. If cardiolipin is present in the OMM it will increase outer membrane sensitivity to Bax (Kuwana et al., 2002).
1.4. Yeast as a model system

Yeast is a unicellular eukaryotic organism that can be used as a model system to study the molecular mechanisms underlying mitochondria-dependent apoptosis. The actions of mammalian Bcl-2 family members on mitochondria are conserved when these proteins are expressed in yeast. Yeast model is therefore an important tool to study Bax regulation and find new modulators of its bioactivity (Pereira et al., 2008; Silva et al., 2011).

Yeast can be easily manipulated and can be used to study mitochondrial respiration, and allows cell cycle control and genetic tractability. In yeast, in contrast with mammalian cells, most apoptotic regulators are encoded by single copy genes (Clapp et al., 2012). Yeast presents apoptotic regulators that are homologs to the mammalian caspases (YCA1), a BH3-containing protein (yBH3p), an apoptosis inducing factor (AIF), an OMI serine protease (NMA111), and an Endonuclease G (NUC1) (Clapp et al, 2012). However yeast does not have homologs of mammalian Bcl-2 family members (Silva et al., 2011).

Yeast will trigger apoptosis in response osmolality alteration, pH variation, the over expression of caspases, oxidative stress and acid acetic (Clapp et al., 2012). During apoptosis the mitochondrial structure can suffer alterations like hyperpolarization and depolarization, production of reactive oxygen species (ROS), release of cytochrome c, yeast apoptosis inducing factor (Aif1p), and yeast endonuclease G (Nuc1p). Mitochondrial swelling, fragmentation and degradation can also be observed (Pereira et al., 2008).

To study apoptosis it is possible to use "humanized" yeast cells in which mammalian apoptotic regulators, like Bcl-2 family proteins, are expressed. When these proteins are expressed in yeast they maintain many molecular and biochemical functions, conserving the effects that these proteins have in apoptosis. These yeast cells are very important in the pursuit of knowledge concerning apoptosis mammalian regulators (Manon et al., 1997; Silva et al., 2011).

Like in mammalian cells, the heterologous expression of full-length Bax in yeast does not lead to its spontaneous interaction with mitochondria. Further Bax activation, through conformational changes, is required to its transition to the OMM. The deletion of either N- and C-terminal, increases Bax translocation and causes a stronger interaction with mitochondria (Cartron et al., 2003; Arokium et al., 2004). Also,
substitutions of residues located in critical domains of the protein, such as Pro13 (ART), Asp33 (helix α1), Glu69 (BH3 domain), Pro168 (α8-α9 loop) or Thr174 (α9 helix) promote Bax translocation and activation (Arokium et al, 2004, 2007).

The formation of a mitochondrial apoptosis-induced channel (MAC), similar to that observed in mammalian cells, is involved in OMM permeabilization after Bax is activated in yeast. This channel allows proteins like cytochrome c to diffuse to the OMM. VDAC or TOM do not have any role in the formation of MAC, this channel being formed only by Bax (Pavlov et al., 2001).

It was shown that tBid can stimulate cytochrome c release, by facilitating Bax oligomerization. Bax can also activate another cell death pathway, that involves mitochondrial protein Uth1p (Priault et al., 2003b; Pereira et al., 2008).

Some results show that the lack of cytochrome c release does not affect yeast cell death once Bax is activated, indicating that release of cytochrome c from mitochondria is not essential for Bax mediated cell death in yeast, suggesting that, once OMM permeabilization has occurred, the following death is not apoptotic (Manon et al., 1997; Roucou et al., 2000; Priault et al., 2003b) (Figure 11).

![Figure 11 - Bax inducted outer mitochondrial membrane permeabilization. Formation of MAC, after Bax activation and insertion in the outer membrane (Pereira et al., 2008).](image)
Bax activity in yeast can be regulated by PKC, the expression of PKCα (PKC isoform α) can increase the translocation and the OMM insertion of an active variant of Bax. PKCα enhances its cytotoxic effects like mitochondrial fragmentation, loss of viability, cytochrome c release and ROS production, but does not phosphorylate Bax (Silva et al., 2010). The authors propose that other subfamilies of PKC might regulate Bax in different ways (Saraiva et al., 2006).

Substitutions of phosphorylatable residues of Bax Ser184, Ser163 and Ser60 have been done in yeast (see above). Like in mammalian cells, the phosphorylation of Ser184 was found to prevent Bax translocation, while the phosphorylation of Ser60 and Ser163 was found to favor Bax translocation (Arokium et al., 2007; Renault, PhD thesis, 2010).

The co-expression of Bax with other Bcl-2 family members has also been done in yeast. Both Bcl-2 and Bcl-xL prevent the activation of Bax and subsequent death (Manon et al., 1997; Priault et al., 1999).

Priault et al. (2003), demonstrated that in yeast Bid and tc-Bid (truncated form of Bid) were unable to induce the conformational change of Bax that leads to its activation. But when Bax was already activated tc-Bid increases its effect, suggesting that tc-Bid plays a part in the oligomerization process of Bax (Gonzalvez et al., 2005).

The yeast model also provided new information about BH3-only protein Puma. Indeed, Puma is able to activate Bax when both proteins are co-expressed in yeast (Gallenne et al., 2009).
2. Objectives
This work aimed to understand the molecular mechanisms underlying Bax regulation by phosphorylation and its consequences in the different steps of Bax activation. A second objective is to assess the interaction of Bax protein and the anti-apoptotic protein Bcl-xL and the outcome that Bax phosphorylation can provoke on this interaction, affecting the process of apoptosis.

In addition to its localization to mitochondria, and its ability to be inserted in the OMM, Bax activation will be monitored by its capacity to promote the release of cytochrome c.

In this study, we will use humanized yeast cells, expressing human Bcl-2 family proteins co-expressed with protein kinases of interest.

The kinase AKT is already known to target Bax. The plasmids expressing AKT under the control of the regulatable promoter tet-off are already available. Wild-type, constitutively active, and constitutively inactive versions of this kinases will be co-expressed in yeast with wild-type Bax, and with different Bax mutants representing different steps of Bax activation (movement of the N-terminal end, of helix α1, of helix α9), to test if these kinases are able to modulate these conformational changes.
3. Material and methods
3.1. Yeast strains, plasmids and growth conditions

The wild-type haploid Sacharomyces cerevisiae strain W303-1B (mat a, ade1, his3, leu2, trpl, ura3) was used in this study. The cDNA encoding human Bax was cloned in the plasmid pYES3/CT and the cDNA encoding Bcl-xL was cloned in the plasmid pLM. Both genes are placed under the control of a GAL1 promoter that allows the inducible expression of the proteins following the addition of galactose. The cDNA encoding the kinase AKT was cloned in the plasmid pCM189, under the control of the tet-off promoter that is repressed by doxycycline. The plasmids carry yeast selectable markers, namely TRP1 for pYES3/CT, LEU2 for pLM, and URA3 for pCM189.

For the transformation of the W303-1B cells, the yeast cells were maintained in 10 mL of a YPD complete medium at a OD_{550} of 0.4 during 2 hours of incubation at 28°C on a mechanical shaker. The cells were collected by centrifugation, washed with water, and resuspended and washed with LiAc-TE buffer (Lithium acetate 0.1 M, Tris-HCL 10 mM, EDTA (Ethylenediaminetetraacetic acid) 1 mM, pH 8.0), in a final volume of 250 µl. 10 µl of DNA carrier (Salmon sperm 10 mg/mL, Invitrogen) and 100 ng of the plasmid of interest were added to 200µl of the cells and incubated at 4°C for 10 minutes. 600 µl of a solution of LiAc-TE/PEG (Lithium acetate 0.1 M, Tris-HCL 10mM, EDTA (Ethylenediaminetetraacetic acid) 1 mM, PEG (Polyethylene glycol) 50% w/v, pH 8.0), were then added and the suspension was incubated for 30 minutes at 28°C, then 15 minutes at 42°C and finally 10 minutes at 4°C. The cells were collected by centrifugation and resuspended in 100 µl of sterile water. After the cells were plated on selective YNB medium with the appropriate antibiotic (doxycyclin is added to repress the expression of AKT) and lacking the appropriate selective markers for selection, and grown at 28°C for 3-4 days. The clones were chosen and put in culture in a liquid glucose YNB (Yeast Nitrogen Base) medium with the appropriate markers and antibiotics.

The yeast strains were stored at -80 °C in their culture medium containing 50% glycerol.

Strains transformed with plasmid were grown in a YNB medium at pH 5.5 constituted by Yeast Nitrogen Base without aminoacids and ammonium sulfate 0.175% (1.75 g/L) (Becton-Dickinson), ammonium sulfate \((\text{NH}_4)_2\text{SO}_4\) 0.5% (5 g/L), Potassium dihydrogen phosphate \((\text{KH}_2\text{PO}_4)\) 0.1% (1 g/L), Drop Mix (a mixture of the
following amino acids A, R, N, D, C, E, Q, G, I, M, F, P, S, T, Y, V) 0.2% (2 g/L), glucose or lactate 2% (20 g/L), and the auxotrophic markers (Adenine, Uracile, H, L, K, W) 100 mg/L with the exception of the selectable markers already carried by the plasmids. To solid mediums is added agar 2.5% (25g/L).

The growth of the cells is accomplished in Erlenmeyer flasks containing a 5:1 ratio of air to liquid phase, and incubation on a mechanical shaker at 28°C. The optical density is measured at 550 nm.

Yeast cells were first grown in an YNB medium with glucose as a source of carbon, inducing a rapid growth of the cells, in the presence of doxycycline 5 µg/ml that represses the expression of the kinase AKT. The cells were then transferred to a medium with lactate as the carbon source to allow the respiratory growth and an optimal differentiation of mitochondria. When needed, the expression of AKT was induced by washing cells 3 times and resuspended them in the same medium without doxycyclin. The expression of proteins controlled by the promoter GAL1 is done by adding 0.8% galactose to the medium. The cells were cultured for 6 to 14 hours at 28°C.

3.2. Preparation of protein extracts

Mitochondria isolation

Cells were grown in 1600 ml of lactate medium, up to an OD at 550 nm between 3 and 6. The first steps aimed at converting cells to spheroplasts. After being washed with water, cells were treated (0.5 M β Mercaptoethanol, 0.1 M Tris, pH 9.3) to reduce the disulfide bonds of the cell wall. The pellet was incubated for 20 minutes with 10 ml of buffer per gram of dry weight (0.5 mg dry weight is the amount in one liter of culture 1 OD$_{550nm}$). Cells were then washed twice with Tris/KCl buffer (10 mM Tris, 0.5 M KCl, pH 7). To digest the cell wall, the cells were subjected to a 30 minutes incubation in 10 ml of digestion buffer (Sorbitol 1.35 M, Citric Acid 25 mM, Na$_2$HPO$_4$ 25 mM, EGTA 1 mM, pH 5.8) for mg of dry weight containing 1 mg/ml of Zymolyaze 20T (from Arhrobacter luteus). After 30 minutes, the cells were observed under a microscope to verify if the digestion took place. If the cells were still not completely digested, 10 to 15 minutes of incubation were added.
Spheroplasts are collected by centrifugation (10 minutes, 5000 x g) and washed twice (0.75 M Sorbitol, 0.4 M Mannitol, 10 mM Tris-maleate, BSA 0.1% w/v, pH 6.8) to remove zymolyase.

Spheroplasts were resuspended in a buffer that is hypo-osmotic for cells and iso-osmotic for mitochondria (0.6 M Mannitol, 0.2% BSA w/v, 10 mM Tris-maleate, 2mM EGTA, pH 6.8). Spheroplasts were homogeneized with a Waring Blendor (3 times, 5 seconds). The homogenate is centrifuged at 900 x g for 10 min to remove cellular fragments, nuclei, etc... The supernatant was centrifuged at 17000 x g for 10 min to collect the mitochondria-enriched fraction. Mitochondrial fraction was resuspended in the same buffer without BSA (recuperation buffer), gently homogeneized with a Teflon Potter, and the previous centrifugation steps were repeated to increase its purity.

Mitochondria were resuspended in recuperation buffer and preserved at -80 ºC. The concentration of proteins was determined by the biuret method.

**The Biuret method**

The concentration of protein was determined by the comparison of absorbance obtained at 540 nm from the samples to evaluate with known BSA (Bovine Serum Albumin) concentrations (BSA content in the samples used as standard from 0 to 0.5 mg). 25 µl of sample or the necessary quantity of a pre-prepared BSA solution with known concentration were added to water completing the volume of 100 µl, then 200 µl of Cholate 4% were added to the samples. After 10 minutes of reaction is added 2.7 ml of NaOH 10% and 0.3 ml of CuSO₄ 1%. After 20 minutes of reaction the absorbance was read at 540 nm.

**Total extracts preparation**

0.5 ml of culture were added with 50 µL of a lysis solution (NaOH 2 M, β-Mercaptoethanol 3.5%) and were incubated for at least 15 minutes at 4 ºC. Proteins were then precipitated with 50 µL of TCA 3 M. The pellet of proteins was washed twice with acetone, dried and resuspended in a Laemmli buffer for further electrophoresis.
Preparation of mitochondrial proteins for SDS-PAGE

1 mg of mitochondrial or cellular proteins were suspended in 0.5 mL of recuperation buffer, were precipitated through a 15 minutes-incubation at 4ºC after the addiction of 50 µl of 3 M Trichloroacetic acid (TCA). After a 5-minutes 10000 x g centrifugation, the pellet was washed 2 times with acetone. The proteins were solubilized in 30 µl of a SDS 2%, NaOH 0.1 N mixture, batch-sonicated, and added with 15 µl of Laemmli buffer 3X (150 mM Tris/HCl, pH 8.6, 6% SDS, 6% β-mercaptoethanol, 60% glycerol and 0.002% bromophenol blue).

Mitochondrial insertion of proteins

1 mg of mitochondria was suspended in 1 mL of recuperation buffer (Mannitol 0.6 M, EGTA 2 mM, Tris/Maleate 10 mM, pH 6.8) and subjected to three treatments: one of the samples is added with 100 µl of recuperation buffer as a control, to other sample 100 µl of 1M Na₂CO₃ pH 10 to remove non-inserted protein, and to the last sample 100 µl of 1% Triton Tx100 to remove inserted proteins. The samples were incubated for 10 minutes in ice, and centrifuged for 15 minutes at 100,000 x g at 4ºC. The pellet was resuspended with 100 µl of water and precipitated with TCA, washed with acetone and solubilized in Laemmli buffer.

Co-immunoprecipitation

Co-immunoprecipitation (CoIP) is a technique used to identify protein-protein interactions. This technique is based on the immunoprecipitation of a protein with specific antibodies, and the detection of proteins bound to the target protein.

20 mL of culture at a OD of 3 to 5 (at 550 mn), were collected by centrifugation and cells were resuspended in 1,75 mL of Recuperation buffer with Anti-proteases (Complete Protease inhibitor cocktail tablets, EDTA free, 1 tablet for 50 mL of recuperation buffer). The cells were then broken by vortexing with glass beads for 3 minutes. The lysate was then centrifuged at 900 x g for 5 minutes to remove the remaining beads cell debris. To the supernatant was then added 175 µl of 10x IP Buffer
(Sigma Protein G Immunoprecipitation Kit), and was incubated in the cold room for 45 minutes under gentle mixing.

2 µl (~4.4 µg) of Bax 2D2 antibody are added and the immunoprecipitation is done at 4º C over night under gentle mixing. The complex formed by the antibody and the proteins was then precipitated by the addition of 50 µl per sample of agarose beads coupled to protein G, for 4 hours at 4º C, to form a complex with beads/antibody/proteins. The samples with the beads were then transferred to a column with a filter and washed 5 times with IP Lysis buffer 1x. After washing the Laemmli buffer 1x (glycerol 20%, SDS 2%, Tris-HCl 50 mM, β Mercaptoethanol 3%, Blue bromophenol 0.04%, pH 6.8) is added to the samples and incubated at 75ºC for 30 minutes to elute the proteins, by breaking the interaction between protein G, antibody and the proteins of interest. After the incubation, the samples were centrifuged at 10 000 x g for 1 minute, to recover the solublized proteins through the column filter, the beads were then washed again with 5 µl of Laemmli buffer 1x and centrifuged at 10 000 rpm for 1 minute.

The obtained samples are ready to be separated on SDS-PAGE.

3.3. Cytochrome Measurements

Assessment of cytochrome c content was measured by redox spectra of isolated mitochondria. Differential spectra of the reduced (sodium dithionite) minus oxidized (potassium ferricyanide) extracts were recorded in a double-beam spectrophotometer (Varian Cary 4000). The difference spectra were acquired between 500 and 650 nm. Cytochromes content was measured from the differences of absorbance at the peak (550nm, 561nm and 603nm for cytochromes c+c1, b and a+a3, respectively) minus the absorbance at the isobestic points (540nm, 575nm and 630nm, respectively). Cytochrome b is a membrane protein that is not released and served as an internal standard, to normalize the protein content in different samples (Figure 12).

Due to the drift caused by the high optical density of the sample, isobestic points do not always gave the same values and a corrective factor was introduced.

\[ \delta = A_{575} - A_{540} \]
[Acytochrome $c$]corr. = (A550 −A540) − 2/7x $\delta$

[Acytochrome $b$]corr. = (A561 −A575) + 2/5x $\delta$

These values of absorbance can be converted to absolute cytochrome contents, with a molar extinction coefficient of 18,000M$^{-1}$ for both cytochrome $c$ and cytochrome $b$.

Figure 12. Spectrum redox of mitochondrial cytochrome. Representing the absorbance peaks and isobestic points used to calculate the release of cytochrome $c$ (adapted from Renault, PhD thesis, 2010).

3.4. SDS polyacrylamide gel electrophoresis/Western blot

The desaturating gel electrophoresis permits the separation of proteins as a function of their molecular mass. The samples were separated on 12.5% SDS-PAGE gels and transferred to PVDF membranes. The membranes were blocked with 3% milk or 1% BSA in PBS-Tween (PBS with 1% (v/v) Tween-20) or TBS-Tween (TBS with 1% (v/v) Tween-20) for 30 minutes at room temperature. Membranes were then incubated overnight at 4 °C with primary antibodies directed against human Bax (mouse monoclonal 2D2, 1:2000; Santa Cruz Biotechnology), yeast porin (mouse monoclonal, 1:50000; Invitrogen), human Bcl-xL (rabbit polyclonal, 1:2000; BD Transduction), human AKT (rabbit polyclonal, 1:2000; Cell Signaling) Membranes were incubated for 45 minutes at room temperature with peroxidase-coupled secondary antibodies directed to the specified IgG of the primary antibody.
After washing the membranes 7 times with PBS-tween or TBS-tween, the membranes were revealed with a solution of ECL reactive (Enhanced ChemoLuminescence, PerkiElmer), that permits the detection of the peroxidase activity coupled to the secondary antibodies. The light emitted is recorded in a camera GeneSys system.
4. Results
4.1. Co-expression of Bax with protein Kinase AKT in Saccharomyces cerevisiae

The importance of the conformational changes of the protein Bax to its activation and further action on intrinsic apoptosis is well-documented. The implication of the kinase AKT in this process was suggested by previous studies. This kinase is involved in the direct phosphorylation of Bax residue Ser184, which, as a result, inhibits the translocation of Bax to mitochondria. The addition of a negative charge, upon the phosphorylation of the Ser\textsuperscript{184} may prevent the insertion of the protein in mitochondria, and favor the heterodimerization of Bax with anti-apoptotic Bcl-2 proteins (Gardai et al., 2004).

The use of the yeast Saccharomyces cerevisiae to study the role of AKT in the expression of Bax, simplifies the model and permits the identification of its molecular characteristics.

Previous studies in the laboratory showed that when Bax was co-expressed with AKT, the translocation of Bax to the mitochondria and the subsequent release of cytochrome \( c \) were stimulated. To study the importance of the Serine residue that is targeted by AKT (Ser184), two mutants were used. A mutant that mimics the negative charge associated to the phosphorylation, by substituting the serine by an aspartatic acid, , was observed to increase the release of cytochrome \( c \) compared to wild type Bax, but did not have a significant effect on the translocation of Bax to mitochondria. Another mutant, where the serine is substituted by an alanine, thus eliminating the possibility of any sort of phosphorylation, displayed a significant translocation of Bax to mitochondria, but not a better capacity to release cytochrome \( c \) compared to the native protein.

These observations raise the question of a direct role of AKT in the behavior of Bax when co-expressed in yeast.

To study more precisely the role of AKT in Bax activation and phosphorylation, we used a wild type Bax or a mutant S184V, co-expressed with AKT. This mutant presents a substitution of a hydrophilic serine for a hydrophobic valine, that stabilizes helix $\alpha9$ of Bax in the lipidic membrane, and displays a constitutive mitochondrial localization.
The expression of wild type Bax and the mutant BaxS184V is done with a plasmid pYES3/CT, and is controlled by the regulated promoter \(pGAL1\), induced by galactose. A constitutively active mutant of AKT is expressed with a plasmid pCM189 and is controlled by the promoter tet-off that is repressed by doxycyclin. For optimized growth, cells were pre-grown in the presence of doxycycline, that represses AKT expression, that was observed to slow down cell growth. To induce the expression of AKT, the cultures were washed and the cells were cultivated overnight without doxycycline. The expression of Bax is induced by the addition of galactose and further incubation during 6 hours.

The level of expression of proteins is determined by immunodetection in cellular protein extracts. Antibodies against Pgk1 and Atp2 or Por1 are used as markers of cytosolic and mitochondrial compartments, respectively.

The mitochondrial localization of proteins Bax and AKT are evaluated by Western Blots on isolated mitochondria. The quantification of the Western blots was done using ImageJ, and analyzed statistically with the program GraphPad Prism. Bax content was quantified relatively to the expression of marker Atp2. Mitochondrial Bax content in the strains expressing the mutant S184V or wild-type Bax co-expressed with AKT, were compared to the content in strains expressing wild-type Bax alone.

![Figure 13](image-url)  
**Figure 13** – Mitochondrial Bax content, strains Wild type and S184V co-expressed with Akt. Bax content in isolated mitochondria estimated by analyze of western blots in the strains wild type and S184V when co-expressed with AKT. Relative to the expression of Bax WT. The western blots were quantified by ImageJ. Values are mean + SM of at least three independent experiments. Without significantly different values.
The co-expression of AKT with BaxWT slightly increased mitochondrial Bax content. Bax S184V had a spontaneously higher mitochondrial localization than Bax WT, but its localization was not further stimulated by AKT. These results did not present significantly different values (Figure 13).

To observe if the co-expression of Bax with AKT affects the insertion of Bax in mitochondria, isolated mitochondria was subjected to a carbonate treatment that removes the membrane-associated proteins but not membrane-inserted proteins (Figure 14). After the treatment with carbonate Bax is still present in mitochondria, suggesting that when Bax is co-express with AKT, Bax maintains its presence in the mitochondrial membrane.

![Figure 14](bax-content-after-carbonate-treatment.png)

**Figure 14** – Bax content in isolated mitochondria after subjected to a carbonate treatment. For the four different strains subjected to treatment with carbonate and triton for 10 minutes. Immunodetection of Bax protein. A representative experiment of at least three independent experiments with similar results is shown.

The mitochondria suspensions were also subject to cytochrome measurements where the cytochromes content was measured from the differences of absorbance at 550 minus 540nm and 561 minus 575nm, for cytochromes c and b, respectively. Cytochrome b is a membrane protein that is not released and served as an internal standard. The results were then expressed as cytochrome c/cytochrome b ratios, (Figure 15). We observed a significant increase of cytochrome c release from the mitochondria when Bax WT is expressed with Akt, compared to Bax WT alone. This suggests that
Akt stimulates the activity of Bax. On the opposite, the presence of Akt does not affect the release of cytochrome c induced by the mutant S184V.

![Figure 15. Release of cytochrome c, in strains Bax wild type and S184V co-expressed with Akt. The results are expressed as cytochrome c/cytochrome b concentration ratios, for the four different strains. The lines at 0.6 and 2 represent the maximum and minimum release of cytochrome c, respectively. Values are mean ± SM of at least four independent experiments. Values significantly different from Bax WT: *** P<0.001. One-way ANOVA and Turkey Test.](image)

The interaction between these proteins was verified using the method of immunoprecipitation where is possible to observe if the proteins are interacting, and to characterize the phosphorylation of Bax by the kinase Akt in yeast. Using an antibody Bax 2D2 to immunoprecipitate Bax protein, recognizing an antigenic domain located between residues 3 and 16 regardless of Bax conformation. The obtained samples from Bax wild type and the mutant S184V co-expressed with Akt were then run in SDS electrophoresis gel. The content of these proteins is determined by immunodetection in cellular protein extracts and the immunoprecipitated samples, with the antibodies Akt and Bax.

When the samples were revealed with Akt antibody, it was observed the immunodetection of Akt, this observation indicates that Bax interacts with Akt in this conditions, the detection of Bax with the molecular weight of 21 indicates that this Bax is active, and interacts with Akt to its activation.

Two protein bands having molecular weights apparent about 28 and 55 kDa were detected: they correspond to the molecular weight apparent light and heavy immunoglobulin chains. Its also possible to observe an unspecific binding of antibody
or proto-lysis of Akt when the western blot is revealed with Akt at the molecular weight of 21 kDa (Figure 16).

<table>
<thead>
<tr>
<th>IP: Bax</th>
<th>Total cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax WT</td>
<td>Bax S184V</td>
</tr>
<tr>
<td>+Akt</td>
<td>+Akt</td>
</tr>
</tbody>
</table>

Figure 16. Immunoprecipitation of Bax co-expressed with Akt, and with western Blot directed to Bax and Akt. The immunoprecipitation was realized from cultures of Bax wid type and Bax S184V co-expressed with Akt using the antibody Bax 2D2. The immunodetection in western blots was effectuated with the antibody Bax 2D2 and ant- Akt. A representative experiment of at least three independent experiments with similar results is shown.

To ascertain the role of the kinase activity of AKT in Bax activation we compared the effect of constitutively active AKT to a dominant negative mutant (AKTDN). Cells were subjected to the same conditions of growth and treatment. These results showned that when Bax is co-expressed with a dominant negative AKT (AKT DN), the release of cytochrome c decreases substantially (Figure 16). The results observed by analysis of the western blot, demonstrated that when Akt is inactive Bax does not translocate to mitochondria (Figure 17).
Figure 16. Release of cytochrome c, strains Bax wild type, S184V co-expressed with Akt or AktDN. The results are expressed as cytochrome c/cytochrome b concentration ratios, for the five different strains. Values are mean ± SM of at least two independent experiments. The lines at 0.6 and 2 represent the maximum and minimum release of cytochrome c, respectively. Values significantly different from Bax WT: *** P<0.001, One-way ANOVA and Turkey Test.

<table>
<thead>
<tr>
<th></th>
<th>Mitochondria</th>
<th>Cells</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>S184V</td>
</tr>
<tr>
<td>Akt</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Akt DN</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Porin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bax</td>
<td>+</td>
<td>-</td>
</tr>
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</table>

Figure 17 – Bax content in mitochondria and cells, strains Bax wild type and S184V co-expressed with Akt DN. Immunodetection of Bax protein and Porin. A representative experiment of at least two independent experiments with similar results is shown.
4.2. Co-expression of Bax with protein Kinase Akt and Bcl-xL in Saccharomyces cerevisiae

The protein Bcl-xL is mostly known as an anti-apoptotic protein that prevents the insertion of Bax in mitochondria. Recently Edlich et al. (2011) introduced the concept of retro-translocation of Bax mediated by Bcl-xL. The interaction between Bax and Bcl-xL can lead both to Bax translocation to the outer mitochondrial membrane and to its retro translocation to the cytosol, thus stimulating the dynamics of Bax localization.

Previous studies in this laboratory showed that when Bax is co-expressed with Bcl-xL the translocation of Bax to mitochondria is increased (Renault, PhD thesis, 2010). Schellenberg et al., 2013 publish a study where GFP-Bax retro-translocation by Bcl-xL was dependent on helix α9 (C-terminal) particularly on Serine 184. Since AKT has been shown to phosphorylate Bax on this residue, these observations raise the question of how the presence of AKT, and its possible phosphorylation of Bax, will affect the interaction of Bcl-xL with Bax and its capacity to stimulate the translocation /retrotranslocation cycling of Bax.

To study further the impact that AKT may have in the interaction between Bax and Bcl-xL and how this may affect the retro-translocation of Bax, we compared the effect of the co-expression of AKT and Bcl-xL on Bax WT and a mutant Bax-S184V.

Strains co-expressing Bax WT or BaxS184V with Bcl-xL and AKT were grown as above. The expression of AKT was induced overnight and the expression of Bax and Bcl-xL (that is controlled by the same promoter GAL1) was done for 6 or 14 hours.

The expression of the proteins is determined by immunodetection in whole extracts and the localization is determined in isolated mitochondria. Being detected by the antibodies Bax 2D2 and anti-AKT and anti-Bcl-xL and the antibody anti-porin is used as a marker to the mitochondrial compartment of the cell.

The amounts of proteins Bax, AKT and Bcl-xL are evaluated by Western Blots. The analysis of the Western blots was done using ImageJ and analyzed statistically with the program GraphPad Prism. The mitochondrial localization of Bax was compared to the amount of the mitochondrial marker Porin. The amount of Bax in mitochondria from strains co-expressing AKT and Bcl-xL, was compared to the amount of Bax in mitochondria from strains co-expressing only AKT (Figure 18).
The results obtained suggest that, in the presence of AKT, Bcl-xL decreased the mitochondrial localization of Bax WT. A similar decrease (although not statistically significant) was observed when the amount of mitochondrial Bax was compared to the total amount of Bax in the cells, instead of being compared to the amount of Porin (Figure 19/20).

This effect of Bcl-xL did not appear when the experiments was done on the mutant Bax-S184V instead of Bax WT, thus confirming that all the effect linked to the presence of AKT disappear on the mutant Bax-S184V.

**Figure 18.** Bax content in isolated mitochondria, from Bax Wild type or S184V co-expressed with Akt and Bcl-xL. Percentage of Bax content in isolated mitochondria by analyze of western blots in the strains Bax wild type and S184V when co-expressed with AKT and Bcl-xL, relative to the expression of BaxAkt WT. The western blots are quantified by ImageJ. Values are mean + SM of at least four independent experiments. Values significantly different from Bax WT Akt: ** P<0.01, One-way ANOVA and Turkey Test.
The cytochromes content of the different mitochondria preparations was measured from the differences of absorbance for cytochrome c and b, as above. The results were then expressed as cytochrome c/cytochrome b concentration ratios, and evaluated by comparing the residual cytochrome c content of mitochondria from the strain co-expressing BaxWT and AKT (Figure 21). It was observed that, as expected from its
anti-apoptotic function, Bcl-xL decreased the capacity of BaxWT+AKT to promote the release of cytochrome c. Since the co-expression of Bax-S184V and AKT did induce only a slight release of cytochrome c, no effect of Bcl-xL could be observed. These results did not present significantly different values.

![Figure 21](image)

**Figure 21.** Release of cytochrome c, strains Bax wild type and S184V co-expressed with Akt and Bcl-xL. The results are expressed as cytochrome c/cytochrome b concentration ratios, for the four different strains. The lines at 0.6 and 2 represent the maximum and minimum release of cytochrome c, respectively. Values are mean ± SM of at least three independent experiments. Without significantly different values.

The interaction between these proteins was verified using the method of immunoprecipitation where it is possible to observe if the proteins are interacting, and to characterize the phosphorylation of Bax by the kinase Akt in yeast. Using an antibody Bax 2D2 to immunoprecipitate Bax protein, recognizing an antigenic domain located between residues 3 and 16 regardless of Bax conformation. The obtained samples from Bax wild type and the mutant S184V co-expressed with Akt and Bcl-xL were then run in SDS electrophoresis gel. The content of these proteins is determined by immunodetection in cellular protein extracts and the immunoprecipitated samples, with the antibodies Akt, Bax and Bcl-xL.
When the samples were revealed with Akt antibody, Akt was detected indicating that Bax interacts with Akt in this conditions. The same results were observed with Bcl-x\textsubscript{L}, indicating that even in the presence of Bcl-x\textsubscript{L} Bax interacts with more with Akt.

Two protein bands having molecular weights apparent about 28 and 55 kDa were detected when the western blot was revealed with Bax 2D2 antibody, which correspond to the molecular weight apparent light and heavy immunoglobulin chains (Figure 22).

**Figure 22.** Immunoprecipitation of Bax co-expressed with Akt and Bcl-x\textsubscript{L} and with western Blot directed to Bax, Akt and Bcl-x\textsubscript{L}. The immunoprecipitation was realized from cultures of Bax wild type and Bax S184V co-expressed with Akt and Bcl-x\textsubscript{L} using the antibody Bax 2D2. The immunodetection in western blots was performed with the antibody Bax 2D2 and ant- Akt, and anti- Bcl-x\textsubscript{L}. A representative experiment of at least three independent experiments with similar results is shown.
4.3. Evaluation of the action of Akt and Bcl-xL on Bax stability in *Saccharomyces cerevisiae*

Post-translational modifications, including phosphorylation, may be a factor governing the stability of proteins. Previous studies in this laboratory showed that when Bax is expressed alone in yeast, its amount is slightly decreased after three hours of blockade of protein synthesis by cycloheximide. A Bax mutant with a substitution of the residue Serine 184 to an aspartic acid (S184D), that introduces a negative charge mimicking that of the phosphorylated residue, was more rapidly degraded since the protein completely disappeared after 3 hours of blockade of protein synthesis. However, the co-expression of Bcl-xL protected the Bax-S184D mutant against degradation. A Bcl-xL mutant that does not interact with Bax (Bcl-xL-G138A) did not have this protective effect. These results suggest that the interaction of Bcl-xL with Bax prevents the proteolysis of Bax (David Garrenna, Master Thesis, 2013).

We then investigated if Akt could have a similar effect as the mutation S184D on the stability of Bax, and if this is modulated by Bcl-xL.

Cells were grown under the same conditions as above in 10mL of medium. Cells were washed to remove doxycyclin and induce the expression of AKT overnight. Then galactose was added to induce the expression of Bax and/or Bcl-xL for 6 hours.

100 µl of cycloheximide from a solution of 60 mg/ml were added to stop protein synthesis (and the growth of the cells). After the addition of cycloheximide, 500µl of each sample were collected at time 0, 30 minutes, 60 minutes, 90 minutes, 120 minutes, 180 minutes and 240 minutes. These samples were treated as explained in the section of Material and methods, for total extract preparation.

The protein content is determined by immunodetection in protein extracts cellular. Bax was detected with the 2D2 antibody and an antibody against PGK was used as a control (this protein is abundant and stable during the time of the experiment).

The ratio Bax/PGK was calculated at each time and conditions, and expressed as the % of this ratio at time 0.
Figure 23. Cellular Bax content in the strain Bax wild type. In the several samples obtained in the different times after addiction of cycloheximide, compared by percentage to the expression in time zero. By analyze of western blots, the western blots are quantified by ImageJ. Values are mean + SM of at least five independent experiments.

Figure 24. Cellular Bax content in the strain Bax wild type co-expressed with the active Akt. In the several samples obtained in the different times after addiction of cycloheximide, compared by percentage to the expression in time zero. By analyze of western blots, the western blots are quantified by ImageJ. Values are mean + SM of at least five independent experiments.
Like it had been shown previously, Bax expressed alone in cells remains stable during the 4 hours of experiment. When Bax is co-expressed with Akt, a slight decrease of Bax content was observed, although less marked than the decrease that has been observed with Bax-S184D. When Bax is expressed with a kinase-inactive mutant of AKT, the slight decrease of Bax content was also observed, even less marked than with active AKT (~37% v/s ~73%) (Figure 23/24/25).

**Figure 25.** Cellular Bax content in the strain Bax wild type co-expressed with the dominant negative Akt (AktDN). In the several samples obtained in the different times after addiction of cycloheximide, compared by percentage to the expression in time zero. By analyze of western blots, the western blots are quantified by ImageJ. Values are mean ± SM of at least five independent experiments.

**Figure 26.** Cellular Bax content in the strain Bax wild type co-expressed with Bcl-xL. In the several samples obtained in the different times after addiction of cycloheximide, compared by percentage to the expression in time zero. By analyze of western blots, the western blots are quantified by ImageJ. Values are mean ± SM of at least five independent experiments.
When Bax is expressed with the anti-apoptotic protein Bcl-x<sub>L</sub>, it has the same stability as when it is expressed alone (Figure 26). Interestingly, the co-expression of Bcl-x<sub>L</sub> seems to slightly prevent the effect of AKT on the stability of Bax, since a decrease of Bax content by 31% was observed (Figure 27).

Figure 27. Cellular Bax content in the strain Bax wild type co-expressed with Bcl-x<sub>L</sub> and Akt. In the several samples obtained in the different times after addiction of cycloheximide, compared by percentage to the expression in time zero. By analyze of western blots, the western blots are quantified by ImageJ. Values are mean + SM of at least five independent experiments.

Figure 28. Cellular Bax content in the strain Bax S184V. In the several samples obtained in the different times after addiction of cycloheximide, compared by percentage to the expression in time zero. By analyze of western blots, the western blots are quantified by ImageJ. Values are mean + SM of at least five independent experiments.
Like wild-type Bax, the mutant Bax-S184V remains relatively stable when it is expressed alone. But, rather unexpectedly, its stability seems to be decreased by AKT, alone or in combination (Figure 28/29). The effect of AKT is more marked on Bax-S184V than on BaxWT.

Figure 29. Cellular Bax content in the strain Bax S184V co-expressed with active Akt. In the several samples obtained in the different times after addiction of cycloheximide, compared by percentage to the expression in time zero. By analyze of western blots, the western blots are quantified by ImageJ. Values are mean + SM of at least five independent experiments.

Figure 30. Cellular Bax content in the strain Bax S184V co-expressed with Bcl-xL. In the several samples obtained in the different times after addiction of cycloheximide, compared by percentage to the expression in time zero. By analyze of western blots, the western blots are quantified by ImageJ. Values are mean + SM of at least five independent experiments.
Surprisingly, the co-expression of Bcl-xL seems to decrease the stability of Bax-S184V, in the absence or in the presence of AKT (Figure 30/31). However, the effect of Bcl-xL was slightly less marked when AKT was present.

**Figure 31.** Cellular Bax content in the strain Bax S184V co-expressed with Bcl-xL and Akt. In the several samples obtained in the different times after addiction of cycloheximide, compared by percentage to the expression in time zero. By analyze of western blots, the western blots are quantified by ImageJ. Values are mean ± SM of at least five independent experiments.
5. Discussion and Future perspectives
The importance of Bax in the intrinsic pathway and its activation and insertion in mitochondria are largely recognized, but the exact mechanisms involved in this process are still to be described precisely. It has been hypothesized that the translocation of Bax to mitochondria might be a shift in the equilibrium between the cytosol and mitochondria (Schellenberg et al., 2013). According to this hypothesis, the activation of Bax as a result of the interaction of Bax with BH3 only proteins would be questioned, since Bax translocation and association to the mitochondrial membrane can occur independently of the activation of Bax by these proteins.

The activation of Bax by phosphorylation might be a crucial aspect of the mechanism leading to Bax activation. In mammalian cells, the phosphorylation of Bax by the kinase AKT prevents Bax translocation (Gardai et al., 2004). In our study, we observed that, when expressed alone in yeast, independently from the apoptotic network, AKT may, on the contrary, favor the mitochondrial translocation of Bax. It is important to note that this effect does not appear when a mutant Bax-S184V (supposedly not phosphorylated by AKT) was used. This result was, however, different from previous results obtained with a Bax-S184D mutant, that mimics the negative charge of phosphorylation: in yeast, like in mammalian cells, this mutant does not stimulate Bax translocation to mitochondria (Arokium et al., 2007). This suggest that the substitution S184D and the co-expression of AKT do not have identical consequences.

Different hypotheses can explain these results:

(i) The kinase activity might not be involved in the stimulation of Bax translocation by AKT. In this case, the effect of AKT would result from a non-specific interaction with Bax. However, we observed that a dominant-negative mutant of AKT was not able to stimulate Bax-induced release of cytochrome c (Figure 16).

(ii) In addition to Ser184, AKT may have another target residue in Bax. The phosphorylation of this residue X would have an opposite effect from the phosphorylation on Ser184, leading to a stimulation of Bax activation.

(iii) AKT may also phosphorylate another protein, different from Bax, that participates to Bax activation.
The dominant negative AKT did not stimulate the capacity of Bax to release cytochrome c, showing that the full effect of AKT on Bax activation requires the kinase activity. This suggests that AKT may have at least two distinct roles on the process leading to Bax activation.

The role of Bcl-xL as an anti-apoptotic protein is well known in mammalian cells, and is reproducible in yeast cells. The ability of Bcl-xL to translocate and retro-translocate Bax is a recent concept. The interaction between Bax and Bcl-xL can stimulate both Bax translocation to the outer mitochondrial membrane or retro-translocation to the cytosol (Edlich et al., 2011), this maintaining a dynamic equilibrium of Bax between both compartments (Schellenberg et al., 2013).

The helix α9 of Bax is involved in the interaction between Bax and Bcl-xL. The possibility that the phosphorylation of Ser184 (in helix α9) would participate to the regulation of the interaction between Bax and Bcl-xL and, consequently, to Bax retrotranslocation, is an interesting hypothesis. This makes the study of interaction between these Bax and Bcl-xL in the presence of AKT, important to comprehend the mechanism involved in the interaction between these two Bcl-2 family proteins (Schellenberg et al., 2013). We observed that the co-expression of these proteins down-regulated the presence of Bax in mitochondria, while it had been demonstrated before that Bcl-xL alone stimulated the mitochondrial localization of Bax. This may indicate that AKT may shift the dynamic equilibrium of Bcl-xL-dependent Bax localization towards a more cytosolic localization, that would indeed correspond to the fact that, in living cells, AKT-dependent Bax-phosphorylation prevents Bax translocation (Gardai et al, 2004).

Immunoprecipitation assays indicate that when Bax is expressed with Akt and Bcl-xL, the interaction between Akt and Bax increases. This results suggest that the interaction of Bcl-xL with Bax that induce conformational changes of Bax enabling its translocation to the outer mitochondrial membrane, might also induce an increase of the interaction of Bax with the Akt. Other hypothesis is that, as observed when Bcl-xL is expressed with Bax in the presence of Akt, the presence of Bax in mitochondria decreases in relation to the amount of Bax translocated to mitochondria when this protein is only expressed with Akt. These results suggest that the conformational alterations that Bcl-xL provokes on Bax may increase the interaction of Bax with Akt but prevent the conformational changes required for the translocation of Bax to mitochondria.
These results also show that Bax co-expressed with Akt in the presence of Bcl-x_L shows a decrease of activity, suggesting that Bcl-x_L may interact with Bax by its helix α9 preventing the phosphorylation of the residues of C-Terminal and the further activation of Bax. This suggests that the action of Akt on Bax translocation and activation is inhibited by Bcl-x_L.

We finally assessed if the ability of the Akt to increase the activity of Bax was linked to changes in the stability of the protein. We observed that the AKT slightly decrease the stability of Bax. However, the effect is very small and poorly reproducible, when compared to the loss of stability that had been previously observed for the mutant S184D.

Previous results have shown that the co-expression of anti-apoptotic protein Bcl-x_L made Bax more resistant to degradation. Our results support the same conclusion, since the slight loss of stability induced by Akt partly disappeared in the presence of Bcl-x_L. However, more experiments are required to ascertain these conclusions, since all the effects observed here lack reproducibility.

The use of the mutant S184V, that cannot be phosphorylated by AKT on residues S184, show that the effects of AKT on Bax stability might on the kinase activity or depend on the phosphorylation on another residues.

In conclusion our results support that the kinase Akt may have some role in the activation of Bax and its insertion in the outer mitochondrial membrane. Its effect may depend in part on the phosphorylation on Ser184, but other effect might also be involved. It is still to uncover the exact process of its interaction and what is its exact place in Bax conformational changes.

The capability of Akt to regulate indirectly the function of Bax by modulating its interactions with other Bcl-2 members, is also suggested by the fact that AKT changed the effect of Bcl-x_L on Bax mitochondrial localization. This may suggest that, depending on the activity of AKT, which is a kinase activated by survival signalling pathways, the response of Bax to the presence of Bcl-x_L might be different: Bcl-x_L would prevent Bax mitochondrial localization when AKT is active, but favors Bax mitochondrial localization when AKT is inactive. This might be an important aspect for the regulation of cells in tumors where AKT is over activated.

In order to investigate more precisely the role of the human kinases such as kinases such as Glycogen Synthase Kinase (GSK3 β), Akt/PKB, PKC p38MapK and JNK on the regulation of the different steps leading to Bax activation, these kinases
should be co-expressed with different Bax mutants. To investigate role of Bax phosphorylation in the regulation of the functional interactions between Bax and its partners of the Bcl-2 family, different Bcl-2 family members, antiapoptotic (Bcl-xL, Bcl-2, Mcl-1) or BH3-only (tBid, Bad, Bim, Puma). Different associations between Bax (wild-type or mutants) and Bcl-2 family members should be tested with our two experimental systems.

The plasmids expressing these kinases under the control of the regulatable promoter tet-off are already available. Wild-type, constitutively active, and constitutively inactive versions of these kinases should be co-expressed with wild-type Bax, and with different Bax mutants representing different steps of Bax activation (movement of the N-terminal end, of helix α1, of helix α9), to test if these kinases are able to modulate these conformational changes. Then the experiments should be repeated with Bax carrying both an activating mutation and a substitution of the potential residue targeted by the kinase.

The role of AKT-dependent phosphorylation of Ser184 on the movement of helix α9 is studied by co-expressing AKT with the mutants S184V and S60A.

The strains co-expressing wild-type or mutant Bax and the different Bcl-2 family members and human kinases and phosphatases are used to test the capacity of Bax to respond to the inhibiting (Bcl-2, Bcl-xL, Mcl-1) or activating (tBid, Bim, Puma) effect Bax phosphorylation can provoke on this interaction, affecting the process of apoptosis. Additional analyses should be performed, monitoring loss the degradation of Bax in the conditions previously mentioned, to access its impact on Bax integrity.

To evaluate the nature and extent of conformational changes of the mutants in which phosphorylatable residues are replaced by phosphomimetic ones to perform biophysical studies and in vitro reconstituted assays. For this investigation different Bax mutants with high yields and with a high degree of purity are produced obtaining pure protein. The expression system is a bacterial lysate supplemented namely, by different enzymatic systems to regenerate ATP and GTP. The reactive medium is separated from the reservoir containing metabolites and amino acids by a semi-permeable membrane.

Wild-type Bax and the constitutively active mutant Bax-P168A, Bax-S60A, Bax-S184V can be produced in the absence of detergents.

On the basis of this study, other Bax mutants, carrying substitutions on phosphorylatable residues (Ser/Thr to Ala or Asp) should be produced and analyzed through the same methods.
The results achieved would gather new information about the complex systems involved in apoptosis, such as Bax activation in mammalian cells, with the aim of identifying new therapeutics.
6. References


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