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The importance of humanized yeast to better understand the role of Bcl-2 family in apoptosis: finding of novel therapeutic opportunities

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Keywords: apoptosis, Bcl-2 family proteins, Bax, Bcl-2, yeast, apoptosis modulation, MOMP

Abstract

The Bcl-2 protein family plays a central role in mitochondrial membrane permeabilization. This event and the ensuing release of cytochrome *c* are decisive in the apoptotic cascade. Therefore, a better knowledge of these processes and their 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. 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. The fact that yeast does not have obvious homologues of the mammalian Bcl-2 family proteins and that these proteins conserve some of their molecular and biochemical functions when expressed in yeast favours the use of this simpler model system to unravel some of the functions of this family. In this review we attempt to encompass the current knowledge regarding Bcl-2 family mode of action and regulation obtained using the yeast model system. Moreover, we discuss how this model system can be used in the future to gain new understanding about the intricate mechanisms of Bcl-2 family protein regulation, and highlight novel therapeutic targets revealed by this system. We believe that the studies here summarized also provide a proof of principle of yeast as an important tool to elucidate some of the complex mechanisms of apoptotic cell death in higher eukaryotes.

1. Introduction

Apoptosis is a highly regulated genetic program that culminates in cell death. This program is crucial for the survival of multicellular organisms. It allows the elimination of damaged or infected cells and, when inappropriately controlled, causes several pathologies. 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 caspase (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 (e.g., cellular blebbing and shrinkage, DNA fragmentation, and plasma membrane changes) and for the rapid clearance of the dying cell [1].

Beside caspases, members of the Bcl-2 family proteins are critical for the regulation of the mitochondrial pathway of apoptosis in vertebrates [2]. Complex interactions between members of this family control the integrity of the mitochondrial outer membrane [3]. 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 [4]. 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.

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 [5],

acetic acid [6], osmotic stress [7] and amiodarone [8] 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 [9, 10]. 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 [11], HtrA/OMI [12] and endonuclease G [13] 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 [14] and p53 [15] 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 interaction and uncover the role of new proteins.

In this review, we examine the effect of mammalian Bcl-2 proteins in yeast and how this model system can contribute to study their structural, functional and mechanistic properties. Moreover, the use of yeast to better understand how components of some of the mammalian signalling cascades regulate the function of these proteins as well to identify new apoptotic regulators is also discussed. Novel therapeutic opportunities revealed with the new data obtained from the yeast model system, and the use of yeast for drug screens will also be addressed.

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

Mammalian mitochondrial apoptosis is mainly regulated by Bcl-2 family proteins [2]. 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 BH-3 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) [16]. 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 [17]. 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 [18-20]. Moreover, yeast has allowed the identification of new mammalian apoptotic regulators, such as Bax inhibitor-I (BI-1) [21], bifunctional apoptosis

regulator (Bar) [22] and the Calnexin ortologue Cnx1 [23]. Altogether, these studies led to the emergence of a new research field where data obtained from the heteroulogous 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 [24]. Though pro-apoptotic proteins of this family can also be important targets for therapeutic modulation of apoptosis, they have not been so 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.

2.1. Bax-induced cell death in yeast

Bax is the best-studied member of the Bcl-2 protein family. It is a cytosolic protein with important functions in mitochondrial morphogenesis and cell death [25]. 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* [26-28], *Schizosaccharomyces pombe* [29, 30], *Pichia pastoris* [31], *Candida albicans* [32] and *Kluyveromyces lactis* [33].

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 [34]. In addition, Bax-induced cell death is delayed in cells lacking mitochondrial DNA and in strictly fermentative conditions [26, 28]. Moreover, strains lacking the ability to perform oxidative phosphorylation are more resistant to Bax-induced growth arrest [35]. Also, Bax expression induces hyperpolarization of mitochondria [36], reactive oxygen species (ROS) production [5, 36], cyt *c* release [37] and mitochondrial network fragmentation [32]. The dependence of Bax-induced cell death on mitochondrial lipid oxidation [38] 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) [39] 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 [30]. 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. [37]. 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 [28]. 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* [40]. 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 [41]. 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. [39], others showed the absence of typical apoptotic markers like caspase activation, phosphatidylserine exposure and DNA fragmentation in Bax-induced yeast cell death [18, 27], ruling out a role of 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 [27, 31]. 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 [27]. [39]. Although 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 [43, 44]. 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 [45]. However the slow loss of plating efficiency in *uth1*Δ cells expressing Bax was correlated with loss of plasma membrane integrity [27] suggesting that selective degradation of altered mitochondria though mitophagy is required for a regulated loss of growth capacity. 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 cross-talk between autophagy, a process primarily involved in cell survival, and apoptosis, a pathway that invariably leads to death [46, 47] has also been ascertained in mammalian cells. Therefore the yeast model system may also provide new data that will improve our understanding about the complex cross-talk 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 [48]. 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 [49]. Several studies highlighted a role for Bcl-2 family proteins in this process [48]. Mitochondrial network fragmentation after Bax expression in yeast was shown for the species *C. albicans* [32], *S. pombe* [50] and *S. cerevisiae* [27]. Though the role of this mechanism in Bax-induced cell death in yeast is not completely clarified.

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

Bcl-2 and Bcl-xL are able to antagonize Bax effects in yeast [17, 26, 29, 30, 34, 37, 39, 51-54], 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 [55]. This indicates the conservation of both anti-apoptotic mechanisms of Bcl-xL in yeast. Polčic and Forte (2003) [20] 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 [56] 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 [57]. It has also been reported that Bcl-2 protein improves survival deficiency of a strain defective in antioxidant protection [58]. 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 [59]. 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 [60]. However, the mechanisms by which these proteins prevent mitochondrial network fragmentation are still unclear.

3. 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 [61-63]. 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 [64, 65] were shown to interact with ceramide channels and modulate MOMP. These different mechanisms are discussed below.

3.1. 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 [66, 67] 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 [28, 36, 51, 68]. 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*. (1999) [51] 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 [69]. Other studies demonstrated that PTP is predominantly involved in necrosis and ischemia-reperfusion injury [70-72].

3.2. The role of MAC

Bcl-2 family proteins have structural similarities with pore forming domains of bacterial toxins, such as colicins and diphtheria toxins [73]. 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 [74, 75]. Furthermore, Bax can induce

transport of cyt *c* in liposomes [76]. 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 [77]. This channel was named mitochondrial apoptosis-induced channel (MAC). 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 [78]. 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 [77] 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 [79]. This last study also showed that Bax and Bak are functionally redundant with respect to this channel. 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 [77]. Moreover, truncated Bid catalyzes MAC formation in isolated mitochondria containing Bax and/or Bak and does not require VDAC1 or VDAC3.

Several stimuli leads to apoptosis, MOMP and cyt *c*, Aif1p and Nuc1p (the yeast EndoG) release, in yeast cells [9]. 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. 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 [65] can also be uncovered using the yeast model.

3.3. 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,* are required for MOMP [41, 80]. Together these studies reinforce the existence of an intricate, phylogenetically conserved crosstalk between Bcl-2 family proteins and components of the PTP.

3.4 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 [81]. 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 [82]. Curiously, cyclosporine A, a potent PTP blocker has no effect on MAC activity [82]. More recently, Hetz *et al.* (2005) [83] identified two small molecule inhibitors of MAC, that 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.

4. Bax activation and translocation to yeast mitochondria

The 3D structure of soluble Bax has been determined by nuclear magnetic resonance spectroscopy [84]. 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 exist 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 [85, 86].

Dimers of Bax can also be detected in the cytosol, however they are not efficiently inserted in the membrane [86, 87].

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 [88]. 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 [89, 90]. 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 [26, 91, 92]. 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 [19, 91, 93]. 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 [91, 93]. 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 [94]. 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 [19]. This finding was also confirmed using a cell-free system and mammalian cells [19, 95]. 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 Ala-24 for Arg (A24R) inhibits the binding of BaxΔN to mitochondria and cyt *c* release. The double substitution on this helix of Leu-26 for Gly (L26G) and Leu-27 for Val (L27V) also have the same inhibitory effect [96]. These effects were also assessed in mammalian cells with similar results [96]. Additionally, a construct lacking the first 37 aminoacids (N-terminal and Hα1) does not to bind mitochondria in a cell-free system [96]. These results suggest an important role for Hα1 in Bax addressing to mitochondria.

More recently, Arokium *et al.* (2007) [97] 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 Arg-9 in the N-terminal region and the negatively charged residue Asp-154 in Hα7 was studied by single substitution of Glu for Arg-9 or Lys for Asp-154. 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 Asp-33 in the Hα1 and Lys-64 in the Hα2 has also been described using mammalians cells and cell-free systems [98]. These results were reproduced using the yeast system [97].

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 [99] 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) [100] 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 [101]. 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 [86].

5. 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 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.

5.1. 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 [102], humanin [103], 14-3-3 [104], heat shock protein Hsp60 [105], PKCε [106], and Asc [107] 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 localize 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 [108]. 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) [26] 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 [109]. 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 [109]. This effect of tBid in Bax-induced cyt *c* release has been attributed to alterations of mitochondrial bioenergetics after tBid binding to mitochondria [109].

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 [110]. 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.

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 though binding to the hydrophobic cleft of the anti-apoptotic proteins (for a review see [111]). 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 [112-114]. By means of nuclear magnetic resonance-based screening, parallel synthesis and structure-based design Oltersdorf *et al.* (2005) [115] 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 [116]. 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 [117]). 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.

5.2. 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 protein kinase Cζ (PKCζ) [118-120] promotes cell survival that is prevented by dephosphorylation by the protein phosphatase 2A [121]. Phosphorylation of ser163 by glycogen synthase kinase 3β (GSK3β) [122] and of thr167 by Jun N-terminal kinase (JNK) and p38 kinase [123] 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 [124, 125], Bcl-2 phosphorylation is required for its anti-apoptotic function [126] and Bcl-xL is phosphorylated and inactivated by the JNK [127, 128].

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) [97] used the yeast model system to study how substitutions of potentially phosphorylatable serine residues of Bax regulates 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. Additionally, the yeast system was also used to study the regulation of Bcl-xL by different mammalian PKC isoforms [57]. Using yeast co-expressing an individual PKC isoform and the Bcl-xL protein, it was shown that PKC isoforms differently interfere with the cytoprotective effect of Bcl-xL in acetic acid-induced yeast cell death, by affecting the balance between the

phosphorylated (inactive) or dephosphorylated (active) forms of Bcl-xL. Co-expression of PKCα with Bcl-xL completely abolishes the Bcl-xL anti-apoptotic activity, whereas co-expression of PKCε or ζ with Bcl-xL causes a marked enhancement of the Bcl-xL anti-apoptotic activity. Abolishment of the Bcl-xL anti-apoptotic effect by PKCα co-expression is accompanied by a pronounced decrease of the Bcl-xL dephosphorylated form, and the remarkable increase in the Bcl-xL anti-apoptotic effect by PKCε or ζ co-expression is accompanied by a pronounced decrease of the Bcl-xL phosphorylated form. By contrast, PKCδ practically has no effect on Bcl-xL phosphorylated state. These results corroborate the interpretation that the different influences of mammalian PKC isoforms on apoptosis can also be due to distinct modulation of members of the Bcl-2 family by each isoform. Recently, it was also shown that PKCα regulates Bax *c*-myc translocation and cyt *c* release activity, but in this case, this regulation is not dependent on PKCα kinase activity [129] showing that kinases can also have a regulatory effect independent of its kinase activity.

6. Final remarks

Bcl-2 family proteins are decisive players in the control of the mammalian intrinsic apoptotic pathway. However, the high number of members of this family co-existing in the same cell and its intricate regulatory system complicates the study of the different individual members. Yeast lacks obvious homologues of mammalian Bcl-2 family members however, as shown above, when expressed in yeast these proteins retain many of their molecular and biochemical functions. This feature presents an important opportunity to study the function and regulation of individual family members in a less complex model system. Moreover, yeast can be an important tool to screen for drugs

that modulate specifically a member of this family or the mechanisms that regulate its function.

In this review we attempt to present an overview of the knowledge already provided by yeast cells regarding Bcl-2 family member's function and regulation, and emphasizes the novel therapeutic opportunities uncovered by this new data. Although considerable date is already available for the function of Bcl-2 family, its regulation is still not completely understood. For example, a lot is known about how these proteins function in the cytosol but little is known about their function in the lipidic environment of the membrane. Moreover, until now research has been focused on Bax, Bcl-2 and Bcl-xL but the function of other members of this family is still obscure. More recent data showed that Bcl-2 family function is beyond control of MOMP and a role in autophagy regulation has been found, revealing a function of these proteins in the crosstalk between apoptosis and autophagy. However the interplay between these two processes is not completely understood. Due to easy manipulation and genetic tractability yeast emerged as an important model system to answer some of these biological questions. Furthermore, this model system can allow us to find new drugs through drug screens, and new drug targets within the core apoptotic machinery helping us in translating our growing knowledge about these targets into new therapies.

Acknowledgements

R. D. Silva (SFRH/BD/23774/2005) have a fellowship from Fundação para a Ciência e Tecnologia, Portugal. This work was supported by a FCT funded project (PTDC/BIA-BCM/69448/2006).

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