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MINI REVIEW

Yeast as a Model to Study Apoptosis?

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Programmed cell death (PCD) serves as a major mechanism for the precise regulation of cell numbers, and as a defense mechanism to remove unwanted and potentially dangerous cells. Despite the striking heterogeneity of cell death induction pathways, the execution of the death program is often associated with characteristic morphological and biochemical changes termed apoptosis. Although for a long time the absence of mitochondrial changes was considered as a hallmark of apoptosis, mitochondria appear today as the central executioner of programmed cell death. This crucial position of mitochondria in programmed cell death control is not due to a simple loss of function (deficit in energy supplying), but rather to an active process in the regulation of effector mechanisms.

The large diversity of regulators of apoptosis in mammals and their numerous interactions complicate the analysis of their individual functions. Yeast, eukaryotic but unicellular organism, lack the main regulators of apoptosis (caspases, Bcl-2 family members, ...) found in mammals. This absence render them a powerful tool for heterologous expression, functional studies, and even cloning of new regulators of apoptosis.

Great advances have thus been made in our understanding of the molecular mechanisms of Bcl-2 family members interactions with themselves and other cellular proteins, specially thanks to the two hybrid system and the easy manipulation of yeast (molecular biology and genetics).

This review will focus on the use of yeast as a tool to identify new regulators and study function of mammalian apoptosis regulators.

KEY WORDS: Apoptosis; Bcl-2 family; caspases; yeast; two-hybrid system.

APOPTOSIS

The maintenance of pluricellular organisms implies that cell death is an important part of life. The central components of the intrinsic apoptotic pathway involve specific proteases (i.e. caspases), proteins of Bcl-2 family and mitochondria [1, 4]. First observed during amphibian metamorphosis, normal cell death was soon found to occur in many developing tissues in both invertebrates and vertebrates [3]. The term programmed cell death (PCD) was used to describe the cell deaths that occur in predictable places and at predictable times during development, to emphasize that the deaths are somewhat programmed into the development plan of the organism.

Apoptosis is a process whereby cells activate an intrinsic cell suicide program that is one of the potential cellular responses, such as differentiation and proliferation. It has been defined in 1972 by Kerr *et al.* in contrast to necrosis, which is a

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cell death generally due to aggressions from the external medium [4]. The apoptotic process is associated with characteristic morphological and biochemical changes, such as membrane blebbing, cell shrinkage, chromatin condensation, DNA cleavage and fragmentation of the cell into membrane-bound apoptotic bodies whose surface expresses potent triggers for phagocytosis. However, it must be kept in mind that although apoptosis is the most common form of PCD, dying cells may follow other morphological types [5–7]. Moreover, apoptotic cells do not always harbor all cardinal features of their cell death type, in particular DNA cleavage does not appear to be an absolute requirement [8]. Nevertheless, the observation that most cells undergoing PCD change similarly has suggested that apoptosis reflects the operation of an intracellular death program that can be activated or inhibited by a variety of physiological or pathological environmental stimuli.

Apoptosis is then a major mechanism for the precise regulation of cell numbers, and unwanted and potentially dangerous cells removal [9–11]. In addition to the beneficial effects of apoptosis in development and tissue homeostasis, the inappropriate activation of cell death may cause or contribute to a variety of diseases [12], including acquired immunodeficiency syndrome (AIDS) [13], neurodegenerative disorders, and ischemic strokes [14, 15]. Conversely, a defect in apoptosis activation could be responsible for some autoimmune diseases [16] and is also involved in oncogenesis [17].

Cascades of events leading to apoptosis can be divided into several phases (for a review see [18-20]). During the activation phase, multiple signaling pathways lead from the various death-triggering signals (lineage information, extracellular survival factors or signals, cell interactions, hormones, cell contacts, genotoxic and physical trauma, anoxia, oncogene expression, immune killing...), to the central control of the cell death machinery and activate it. This is followed by the execution stage, in which the activated machinery acts on multiple cellular targets, and, finally, the destruction phase in which the dead or dying cell is broken down.

INTRACELLULAR CONTROL OF THE CELL DEATH PROGRAM

A most important clue to the molecular nature of the death program came initially from genetic studies in *C. elegans* [21, 22] that led to the identification of a dozen cell death genes (*ced*) that are responsible for one aspect or another of cell death processes [23]. *Ced-3* and *ced-4* are essential for cell death, whereas *ced-9* antagonizes the activities of *ced-3* and *ced-4*, and thereby protects cells that should survive from any accidental activation of the death program. Biochemical approaches have provided insight into the mechanism by which programmed cell death is regulated in the nematode. CED-4 interacts directly and simultaneously with both CED-3 and CED-9 forming a multimeric protein complex [24, 25]. Furthermore, oligomerized CED-4, acting as a context dependent ATPase, promotes CED-3's activation and its ability to induce apoptosis [24]. CED-9 binding to CED-4, mutually exclusive with CED-4 oligomerization, prevents it from activating CED-3, thereby blocking cell death [26]. Remarkably, all three of these *C. elegans* cell

	C. elegans	Mammals	D. melonogaster
Caspases	CED-3	caspases 1 to 14	drICE, DCP1
Adaptors	CED-4	Apaf-1	D-Apaf-1
	CED-9	Bcl-2, Bcl-x _L	$\overline{?}$
Bcl-2 family members	?	Bax, Bok	Debcl/Drobl
	EGL-1	Bik, Bad	?

 Table 1. Programmed Cell Death in Nematodes, Drosophila and Mammals is Controlled by Homologous Proteins

Mammalian caspases act either during the activation or the execution phase of PCD [30]. A CED-4 homologue has been identified in human cells [83, 225] and Drosophila [226]. In mammals some members of the Bcl-2 related proteins are death antagonists while others are death agonists, whereas up to date, an only proapoptotic member is found in Drosophila [227]. For a recent review on the structure–function relations of Bcl-2 related proteins see [33].

death genes have mammalian and Drosophilia counterparts that are likely to play similar, albeit more complex, roles in cell death (Table 1).

These considerations have led to a different meaning of the term apoptosis: it now refers to any cell death that results from an intracellular death program, no matter what activates it and whatever the changes associated with the destruction of the cell. In this way PCD appears as a critical element in the repertoire of potential cellular responses, as are cell differentiation, quiescence or proliferation. Moreover, these studies demonstrated the very early occurrence of PCD in the course of metazoan evolution and the substantial conservation of its basal machinery from nematodes to humans.

The Caspases Family

CED-3 and its homologues form a family of proteases termed caspases (cysteinyl <u>aspartases</u> [27] (see Table 1). The mammalian and Drosophilia caspase families now comprises respectively at least fourteen and seven known members, most of which have been definitively implicated in PCD (for review see [28, 29]). All cleave their substrates after specific aspartic acids and are themselves activated by cleavage at specific aspartic acids [30]. Caspases mediate apoptosis by cleaving selected intracellular proteins, including proteins of nucleus, nuclear lamina, cytoskeleton, endoplasmic reticulum, and cytosol.

The Bcl-2 Family

CED-9 protein is homologous to a family of many members termed the Bcl-2 family (Bcl-2s) in reference to the first discovered mammalian cell death regulator (for review see [31]). The Bcl-2 family proteins are involved in positive (proapoptotic members) and negative regulations (antiapoptotic members) of apoptotic cell death (review in [32, 33]; see Table 1). They are characterized by the presence of conserved motifs, known as the Bcl-2 homology (BH) domain, termed BH1, BH2, BH3, and BH4. The *N*-terminal BH4 domain is only found in antiapoptotic members, whereas

BH1, BH2, and BH3 can generally be found among pro- and antiapoptotic members. These BH domains play a role in interactions between members of the family and BH3 mediates the proapoptotic function (some proapoptotic members only bear the BH3 domain) [34].

Among the antiapoptotic members, Bcl-2 and Bcl- x_L are negative regulators of cell death, able to prevent cells from undergoing apoptosis induced by various stimuli in a wide variety of cell types [35, 36], whereas others, such as Bax, Bid and Egl-1 promote or accelerate cell death. CED-9, Bcl-2 [37–43], Bcl- x_L [44], Mcl-1 [45, 46], the BHRF1 Epstein–Barr virus protein [47] and probably other members of the Bcl-2 family are localized to the cytoplasmic surfaces of the nuclear envelope, the endoplasmic reticulum and the outer mitochondrial membrane. It must be underlined that only a fraction of Bcl- x_L resides on membranes, even though it bears a *C*-terminal membrane anchor involved in the association of Bax with the membrane [48, 49].

However, the membrane association of Bcl-2 is of functional significance as mutant Bcl-2 molecules lacking this membrane anchorage capacity are less effective in preventing apoptosis in some systems [50–52]. Indeed, it has been reported that, in inhibiting apoptosis of MDCK cells, a mutant Bcl-2 molecule whose anchorage is targeted specifically to the mitochondria is as effective as the wild type protein, whereas mutant Bcl-2 targeted to the endoplasmic reticulum loses this capacity [52]. In contrast, Bcl-2 targeted to the endoplasmic reticulum in the Rat-1/myc fibroblasts proved to be more active than when targeted to mitochondria. Thus, Bcl-2 mutants with restricted subcellular location reveal distinct pathways for apoptosis depending on cell type. When associated to the endoplasmic reticulum membrane, Bcl-2 could be involved in maintenance of the calcium homoeostasis [53–55], while it could modulate protein subcellular trafficking through nuclear pores [56].

Possible Mechanisms of Action for Bcl-2s Proteins

The mechanism(s) by which proteins of the Bcl-2 family modulate apoptosis is not yet well known and several conflicting theories have been proposed. In nonstimulated cells, monomeric Bax is located in the cytosol and in peripheral association with intracellular membranes including mitochondria, but inserts into mitochondrial membranes after a death signal [57, 58]. A widely accepted model postulates that homodimers of Bax promote apoptosis [32, 58–61], and that the functional effect of Bcl-2 related proteins is to form competing heterodimers with Bax that cannot promote apoptosis [62, 63]. However, in some systems, Bax binding by Bcl-2 was not sufficient to prevent apoptosis and the overexpression of Bcl-2 or Bcl x_L can repress apoptosis in the absence of Bax [64, 65]. Moreover, mechanisms other than the simple dimerization among members of the Bcl-2 family may be required for the regulation of apoptosis [66]. Thus, while an *in vivo* competition exists between Bax and Bcl-2, each is able to regulate apoptosis independently.

In both worm and mammalian cells, the antiapoptotic members of the Bcl-2 family act upstream of the "execution caspases", somehow preventing their proteolytic processing into active killers [67–69]. Some proteins, such as Bap31 in the endoplasmic reticulum, may bind caspases and Bcl-2s family members, coordinating thus their activities [70]. Furthermore, two main mechanisms of action have been proposed to connect Bcl-2s to caspases. In the first one, Bcl-2 would act by regulating the release from mitochondria of apoptogenic factors, such as the Apoptosis-Inducing-Factor (AIF), some caspases activators (e.g., cytochrome c) and pro-caspases from mitochondria to cytosol [71–73]. Indeed, it was shown that (1) some Bcl-2s proteins have pore-forming properties with distinct ion-conducting properties, raising the possibility that these products can regulate the permeability of the intracellular membranes [74–79]; (2) activation of "execution caspases" required the preliminary shift to the cytosol of regulatory components, such as cytochrome c or procaspases, previously sequestered in mitochondria [71–73, 80, 81].

An alternative model proposes that the prosurvival proteins may function downstream the release of apoptogenic factors, by directly inhibiting the ability of CED-4 like proteins to activate caspases. This model arose from the elucidation of the role of the somewhat mysterious CED-4 protein in the nematode [28]. Indeed, it was first shown that CED-9 interacts with CED-3 via the bridging protein CED-4 that binds simultaneously to CED-9 and CED-3. CED-9 prevents CED-4 from inducing proteolytic processing and activation of CED-3. An equivalent ternary complex was found to be present in mammalian cells involving the mammalian equivalent of CED-4, Apaf-1 (apoptosis protease-activating factor 1, [83]), caspase-9 and Bcl- x_L , in which Bcl- x_L inhibits Apaf-l-mediated maturation of caspase-9 [84]. In its paradigm, proapoptotic relatives like Bik may free CED-4/Apaf-1 from the death inhibitor. Nevertheless, the prosurvival Bcl-2 homologues could not act by sequestering Apaf-1, but constrain its activity indirectly [85]. Additionally, there is some evidence that binding of prosurvival proteins to the apoptosome complex alter its location in cells, pulling it from the cytosol to the intracellular membranes where Bcl-2s often reside [24, 86].

These distinct and probably independent links between Bcl-2 and caspases match with the apparent redundancy of caspases and the existence of several alternative activation pathways that seem to be the rule for mammalian cell death. The multiplicity of caspases illustrates this complexity. Although caspases appear as the key components of the execution machinery of the cell death program, they also intervene in the death signal transduction cascade in some PCD, such as those triggered by surface receptor activation [87]. These different possible pathways of caspase activation would be consistent with the previously described possibility for some caspases to be activated by autoprocessing and for others to require transcleavage (the proteolytic cascade). Given the complexity and diversity of caspase activation pathways, these experiments point to the mitochondrion as a unifying element of apoptosis. An irony in the story of CPD studies, for a long time the absence of mitochondrial changes was considered as a hallmark of apoptosis [88] while, conversely, mitochondria appear today as the central executioner of programmed cell death [20, 32, 89–92].

YEASTS

For a long time, evidence for occurrence of endogenous PCD in unicellular organisms was not well documented. However, Skulachev termed phenoptosis a programmed cell death of an organism, as a sacrifice of individuals (unicellular or multicellular organisms) for the community [89, 93]. These types of cell suicide responses,

with mechanisms different from those of typical apoptosis, are found in prokaryotes (see [94]), pluricellular lower eukaryotes (such as dinoflagellates [95]), and unicellular eukaryotes [96–98].

An exciting question to resolve is the occurrence of an apoptosis-like phenotype associated with a specific mutation in *Saccharomyces cerevisiae* [99]. Indeed, the cdc48^{S565G} mutant show typical markers of apoptosis, including exposure of phosphatidylserine, chromatin condensation and fragmentation, DNA fragmentation, and formation of minicells, which look like apoptotic bodies. Some basic hallmarks of apoptosis were also recently detected during yeast cell death induced by acetic acid [100].

Do Yeasts Possess Homologues of Mammalian Regulators of Apoptosis?

Saccharomyces cerevisiae and Schizosaccharomyces pombe yeast species do not contain endogenous caspases or Bcl-2s gene [101–103] and their cytochrome c has been shown to be ineffective to induce nuclear apoptosis in a cellular assay [80]. However, Rad9, the human homologue of S. pombe (spRad9), is involved in the control of cell-cycle checkpoints. It contains a BH3 domain, and promotes apoptosis by binding to and inhibiting Bcl-2 and Bcl-x₁ in cells [104]. SpRad9, a protein essential for the resistance of S. pombe to gamma-rays and UV light which contains a BH3-like domain, physically interacts with Bcl-2, and equally induces apoptosis in mammalian cells [105]. Moreover, low concentration of H_2O_2 treatment or glutathione depletion could induce the pseudo-apoptosis in wild type yeast [106]. These data in yeast suggest the existence of yet unknown PCD pathways, independent of Bcl-2s and caspases families, in which the place and the role of mitochondria remains to be determined. It is tempting to speculate that a mitochondrial ROS production is involved in the cell death process. In agreement with this hypothesis, the apoptotic-like features observed in yeast is accompanied with ROS accumulation in the cells similarly to those observed in H₂O₂ treated cultures. Moreover, use of ROS scavengers attenuates the lethality in these models, and yeast mutants lacking superoxide dismutase were partially rescued by expression of the antioxidant Bcl-2 [107-1091.

The occurrence of pseudo-apoptosis in yeast is compatible with the lack of caspase. Indeed, if caspases are important in the mammalian apoptotic process, some models reveal a caspase-independent pathway. For example, Bax or Bak expression, and cytosolic AIF induce apoptosis in presence of broad caspase inhibitors [61, 110–112]. All these data argue for the use of yeast to study the regulators of higher eukaryotes PCD, in accordance with numerous publications praising this model [113–116]. The ability to manipulate the genome of yeast makes it an ideal organism in which to identify these evolutionary conserved pathways.

The Yeast Two-Hybrid System

Yeasts are genetically well-characterized organisms and provide useful tools for researchers. One of them, the two-hybrid system, was initially developed by Stanley Fields in *S. cerevisae* [117] to demonstrate physical interaction of proteins.

The yeast two-hybrid system is a powerful genetic method to identify protein/ protein interactions: A first plasmic generates a fusion of the GAL4 transcriptional activation domain (AD) and a protein of interest (X). A second plasmid generates a fusion of the GAL4 DNA binding domain (BD) and another protein of interest (Y). The hybrid proteins are expressed at high levels in yeast host cells from a constitutive promoter, and targeted to the yeast nucleus by the nuclear localization sequence. If a physical interaction between X and Y exists, AD and DB domains are brought together and reconstitute an active GAL4 transcription factor, which is able to activate transcription of a particular reporter gene (visual or prototrophic marker), whereas without any interaction, no transcription occurs.

This system is widely used and generally gives outstanding results. However, interpretations have to be carefully done to a certain extent, because of several limits: (i) the test is performed with fusion proteins, instead of native molecules; (ii) the expressed proteins are targeted to the nucleus, regardless of their real location in the cell; (iii) global structure and/or postraductional modifications are not inevitably the same in yeast and mammalian cells and may have an important role for interaction; (iv) levels of tested proteins are generally much higher than in physiological conditions, (v) other partners may be needed, even for a bipartite interaction. Other derived and improved systems were developed for specific uses (One-Hybrid, Three-Hybrid, cytosolic Two-Hybrid...), or to ascertain the observed interactions.

Studies of Interactions Between Known Bcl-2 Family Members

As described above, the different members of Bcl-2 family proteins can dimerize with one another, with one monomer antagonizing or enhancing the function of the other. In this way, it is assumed that the ratio of activators to inhibitors in a cell could determine the propensity of the cell to undergo apoptosis [118]. Nevertheless, it has been suggested that all the heterodimers are not formed in the cells [66]. The yeast two hybrid system has been extensively used to evidence global interactions between known Bcl-2 family proteins and/or to clone new members (see next paragraph and Tables 2 and 3) [62, 63, 119–132].

It was also used to precisely map the domains involved in formation of homoand/or heterodimers in the different Bcl-2s [102, 120, 133–139]. In a very simplified manner, evidence for importance of conserved BH domains in binding were in accordance with different assuagements according to the nature of the member (proor antiapoptotic). Table 2 summarizes the main interactions detected with this system between Bcl-2 family members. Bcl-2s not only interact together, but also with other cellular proteins, such as lamin [140, 141], Bag-1 [127], R-ras [142], Bap31/p28 [70], ced4/Apaf1 like proteins [83, 84, 143–146], ADP/ATP carrier [147], transcription factors [148] or the Raf-1 protein kinase [149].

Many laboratories took hold of this property in order to identify and clone by the two-hybrid test new members of Bcl-2 family and/or unrelated partners (see below). The relevance of these numerous interactions of Bcl-2s is not so evident every time and the functional significance has to be cautiously analyzed, especially when the supposedly partners have different cellular localization. However, the

	Al	Bad	Bfl1	Bak	Bax	Bcl-2	$Bcl-x_L$	Bcl-x _s	Bid	Ced-9	Mcl1
Bax	+	_		+	+	+	+	_		_	+
Bak	+	-		+	+	+	+	-		_	+
Bcl-2	(+)	(+)		-	+	+	+	+			(+)
Bcl-x _L					+		+				(+)
$Bcl-x_{S>}$	+			-	-	+	+				
Bad	+										-
(*)	_	-		-	-	+	+	-		+	+
CED9	+			_	_	+	+			+	
Mc11		+	(+)	+	+	(+)	(+)			(+)	
MAP1			, í		+	+	+		_	Ì,	
Bfl-1			-		+	-	-				

Table 2. Interactions Between Bcl-2 Family Proteins Observed in Two-Hybrid Tests

Data were obtained from: (62, 63, 102, 119–139).

+: strong interaction by a two-hybrid test; (+): weak interaction; -: no interaction.

(*) Bad interacts with a limited number of antiapoptotic Bcl-2s (but all when underphosphorylated) and negligibly with proapoptotic Bcl-2s.

growing number of data suggest that this family of proteins is involved in multiple signaling pathways that regulate apoptosis.

Identification of New Proteins Isolated for Their Ability to Interact with a Bcl-2 Family Member in a Yeast Two-Hybrid Screening

Many laboratories used the two-hybrid system to clone new Bcl-2 family or genes of which products interact with Bcl-2 members (Table 3). To perform such clonings, one of the partners (X or Y) in two-hybrid test can be a known protein, which serves as bait to screen libraries, whereas the second partner is replaced by cDNA in a fusion library: the clones positive for reporter gene transcription will correspond to those which contain a plasmid with a cDNA encoding a protein which interacts with the bait. Thus, the two-hybrid system can be a powerful genetic method to clone new genes, according to the ability of their products to physically interact with a particular protein. This strategy is advantageous to isolate new genes, which have a low degree of similitude at the DNA level with known genes, but yet interact at protein level. However, such a screen sometimes generates a large amount of positive clones, including some without biological relevance and false positive ones. Complementary assays have to be performed to confirm the interaction (coimmunoprecipitation, colocalization in the cell; . . .) [150].

The efficiency of the yeast two-hybrid system has permitted new Bcl-2s interacting proteins—and potentially regulators of apoptosis—to be regularly discovered, increasing in the same way the complexity of identified interactions and possible mechanisms. Independent discoveries of the same partner for a given Bcl-2 member by different screenings validate assessment of the corresponding interaction, even if other functional tests are needed (in vitro and in higher eukaryote cell). This interesting situation was encountered with Bak, a proapoptotic Bcl-2 family related protein identified in three different laboratories [129, 151, 152].

Identified				
interacting		Library		
protein	Bait	(prey)	Role	Ref.
Bad	Bcl-2	Mouse embryonic cDNA	BH3-only (proapoptotic) interacts only with Bcl-2 and Bcl-XL	[119]
Bak	E1B 19K	cDNA	Contains BH1, BH2, and BH3 (proapoptotic) interacts with Bcl-XL	[129]
Bax	E1B 19K	cDNA	Contains BH1, BH2, and BH3 (proapoptotic) interacts with diverse anti- and pro-	[228]
Bik	Bcl-2	Human B-cell cDNA	BH3-only (proapoptotic) Bik induces apoptosis upon overexpression	[156]
Bod	Mcl-1	Ovarian cDNA	BH3-only (proapoptotic) interacts with diverse antiapoptotic Bcl-2 not with proapoptotic ones	[124]
Bok	Mcl-1	Ovarian cDNA	Contains BH1, BH2, and BH3 Bok overexpression induces apoptosis	[125]
Hrk	Bcl-2	Hela cDNA	BH3-only (proapoptotic) induces apoptosis upon overexpression	[157]
MAP1	Bax	Human brain cDNA	Contains a BH3-like (proapoptotic) interacts with Bax, Bcl-2, and Bcl-XL	[123]
Bbp/53BP2	Bcl-2 P53	B-cell progenitor cell line	Interacts with Bcl-2 and p53	[159] [160]
NIP-1, -2; -3; -4	E1B 19K	cDNA	BH3-only (proapoptotic) proteins interact with E1B-19K, BCL-2, BCL-xL	[158]
Mcl1	Bad	Ovarian cDNA	Antiapoptotic interacts with proapoptotic	[131]
Bif-1	Bax	Human fetal brain cDNA	SH3 domain-containing protein	[166]
PrP	Bcl-2	Neurons	Cellular prion protein (PrP) interacts with Bcl-2, but not Bax	[164]
Aven	Bcl-XL	cDNA	Interacts with BCL-XL and Apaf-1 inhibitor of caspase activation	[165]

Table 3. Isolation of Bcl-2s Interacting Clones Using Two-Hybrid System.

This table is an inventory of the cloned genes upon the basis of interaction of their product (first column) with a member of Bcl-2 (Bait-second column) in a two-hybrid screening of particular library (third column).

In addition, use of a tissue specific cDNA library as a prey for a given bait allowed, for example, to study specific regulation of apoptosis in this tissue. As an example, two-hybrid tests with an ovarian cDNA library (prey) and the antiapoptotic Mcl-1 (myeloid cell leukemia protein 1) as a bait, identified the partners of the main ovarian Bcl-2s partners: the proapoptotic Bok (Bcl-2-related ovarian killer) and Bad, as well as Bod (Bcl-2-related ovarian death agonist) [124, 125, 153, 154] or fortilin [155]. Additive two-hybrid tests showed that Mcl-1 interacted preferentially with different proapoptotic (Bax, Bak, Bok, Bik, and BOD), as compared with antiapoptotic Bcl-2 family members (Bcl-2, Bcl- x_L , Bcl-w, Bfl-1, CED-9, and BHRF-1) [131].

In most of the cases, the discovered gene was shown to have a role in apoptosis in a mammalian model, and generally the encoded product contained BH-like domains (see Table 2). A new class of Bcl-2 related proteins progressively emerged with these two hybrid tests. Indeed, proteins such as Bad, Bid, Bik, Hrk, EGL1, Bnip, Map1,..., only contain a BH3 domain (death domain) and exhibited proapoptotic activity, notably by interaction with the antiapoptotic Bcl-2s [119, 123, 124, 156–158].

In addition, Bcl-2s do not only bind between themselves, but also with proteins unrelated to the family. The following proteins were also discovered as Bcl-2s' interacting proteins:

- Bhp/53BP2 a protein that interacts with both Bcl-2s and oncosuppressive p53 [159, 160];
- Fortilin, a nuclear antiapoptotic protein [155], identical to the human translationally controlled tumor protein (TCTP);
- Raf-1, a protein kinase associated to Bcl-2 for mitochondrial targeting [161] in order to phosphorylate and inactivate the proapoptotic protein Bad [149, 162];
- Bag-1, a protein able to associate with both Bcl-2 and Raf-1 [163];
- the cellular prion protein (PrP) [164];
- Aven has been characterized as an inhibitor of caspase activation and apoptosis, which binds both Bcl-x_L and the adaptor Apaf-1 [165];
- Btf is a proapoptotic transcriptional repressor [148];
- Bif-1, a cytoplasmic protein, containing a Src homology 3 (SH3) domain, which binds to proline-rich sequences in many intracellular proteins, and plays an important role in signal transduction pathways and is involved in cell-cell communication [166]
- the familial Alzheimer's disease gene products, presenilin-1 and presenilin-2, implicated in apoptosis by interaction with Bcl-X(L) and modulation of the antiapoptotic effects [167, 168];
- the mitochondrial membrane protein carnitine palmitoyltransferase CTP-I, suggesting that physical interaction could bridge fatty acid metabolism with apoptosis [169];
- the anti apoptotic activity of SMN gene product, which deficiency is implicated in spinal muscular atrophy disease [170, 171].

Heterologous Expression of Proapoptotic Bcl-2 Related Proteins

Proapoptotic Effect Upon Yeast Viability and Apoptosis Markers

The heterologous expression of mammalian proapoptotic genes (ced-4, bax, bak) provokes mitochondrial dysfunction and the death of yeast cells through a caspase-independent mechanism [101, 103, 122, 134, 172–175]. Several authors reported that this induced-cytotoxicity was characterized by typical phenotypic markers of apoptosis [103, 172, 173], identical to those observed in the pseudo-apoptotic mutant cdc48^{S565G} [99], and other features, such as release of cytochrome c [176], a decrease of the intracellular levels of total glutathione, and a substantial reduction of total

cellular phospholipids [177], diminishes the mitochondrial membrane potential, and alters the intracellular redox potential [106, 177]. The cytotoxicity is not due to non-specific disorders induced by a "foreign" protein, as punctual mutations of Bax, that rendered it inefficient in mammals, also abrogated negative effects upon yeast viability.

The last C-terminal domain, that contains the anchor sequence for membrane association in Bcl-2s, is not necessary for deleterious activity of Bax in veast [178, 179]. However, this domain could play a role in se, independently of membrane association, as its replacement by Bcl-x_L's one suppressed proapoptotic properties of this mitochondrial outer membrane-addressed Bax [180]. Contradictory results are often found in literature, concerning the phenotype of Bax-expressing yeast, partly due to different genetic backgrounds, and medium-dependent variations. Moreover, different molecular constructions were used for the Bax-expressing cassette-integration in the yeast genome, episomic plasmids at low or high number of copies, modified Bax protein sequence (deletion of a C-terminal domain for a better stability, fusion with more or less large tags, etc.) regardless Bax structure, although this aspect is determinant for Bcl-2 family members activity. Most of studies were done with the galactose-induced expression system as conditional heterologous Bax expression (GAL promoter). In this system, high expression level is obtained in presence of galactose as sugar source in the medium, whereas strong repression of transcription occurs with glucose. The nature of the sugar influences yeast metabolism, and especially mitochondrial one. More reliable results were obtained with inducible expression under control of a tetracyclin-driven promoter, where the Bax effects may be analyzed under either pure fermentative, or strict respiratory or fermentative + respiratory conditions [181].

Coexpression of Antiapoptotic Members

Moreover, as observed in mammals, coexpression of Bcl-2, Bcl- x_L , Al or Mcll with Bax or Bak abolishes the pseudo-apoptotic phenotype and the cell death [74, 121, 135, 172, 175, 176, 182, 183]. BFL-1 was able to suppress Bax lethality, but the rescue effect was weaker than that of Bcl- x_L [132]. Surprisingly, Ced-9 was unable to overcome Bax effects, and the proapoptotic Bad or Bcl-Xs potentiate the suppressing activity of the antiapoptotic Bcl-2 and Bcl- x_L [121, 184].

The exact mechanism for Bax inhibition are not fully understood, but it appears that formation of heterodimers Bax/Bcl-2 or Bax/Bcl- x_L allow to abrogate lethality. However, another mechanism should exist, as Bcl- x_L or Bcl-2 does not require Bax binding to inhibit its cytotoxicity [74, 121, 137]. The *C*-terminal anchor sequence for membrane association of Bcl- x_L and its effective mitochondrial targeting are required to efficiently suppress Bax lethality [137, 178, 179]. This could be linked to the ability for Bcl- x_L to form a sustained ion channel in membranes [75, 77, 185].

MITOCHONDRIAL ASPECTS

Bax induces specific mitochondrial changes in yeast, as mitochondrial matrix alkalinization (and cytosol acidification) [186], decrease in amount of cytochrome c

oxidase and cytochrome c release to the cytosol [176] and accumulation of ROS [106, 172, 175]. Release of cytochrome c to the cytosol was observed by several groups. However, this event is not indispensable for Bax toxicity, as Bax is still active and Bcl-x_L-inhibitable in a yeast strain deleted for both cytochrome c isoforms [175, 181]. Therefore, contrary to mammalian apoptosis models, cytosolic cytochrome c release does not activate the cell death process, specially because of the lack of adaptor, such as Ced4 or Apaf-1, or apoptosome formation and caspase activation. This is in accordance with inefficacy of yeast cytochrome c to induce nuclear apoptosis in a cellular assay [80].

As in mammals Bcl-2s act upon the regulation of opening and closure of Mitochondrial Permeability Transition Pore (MPTP), several laboratories interested in the yeast counterparts of its mitochondrial components (i.e. VDAC—Voltage Dependent Anion Carrier, AAC—ADP/ATP Carrier, CyclophilinD, ...) for baxinduced cytochrome c release. None of these proteins seemed indispensable for Baxinduced cell growth arrest or cytochrome c release [175, 181, 187, 188], even if individual reports suggest a crucial role of VDAC or AAC [147, 189, 190]. Thus, cytochrome c release is due to an increased mitochondrial outer membrane permeability for macromolecules, without alteration of the inner membrane or opening of MPTP [176, 188] (see Fig. 1).

Although some contradictory reports, Bax-induced cytotoxicty is not dependent on an efficient respiratory chain working and fully active mitochondria. However, Gross *et al.*, recently suggested that absence of some mitochondrial components, such as specific FoF1–ATPase subunits and mtDNA, acted differently upon Bax cytotoxicty, according to the studied parameter (growth arrest or cell killing) [175]. Inactive mitochondria were shown only to alter the kinetics of lethality, but not lethality itself [181]. This is in agreement with ATP requirements in yeast and *in vitro* for Bax activity on mitochondria, an active process rather than non-specific effects for cell death [188].

Genetic Screening of Bax Suppressors in Yeast

Search for yeast mutants able to resist to Bax-induced lethality led to identification of mitochondrial F0F1–ATPase subunits mutations [191], suggesting that, as in mammals, an intact F0F1–ATPase in the inner membrane of mitochondria was necessary for optimal function of Bax in yeast. However, these results were not fully corroborated by other laboratories [175] (see previous paragraph).

Another strategy was used in order to clone new mammalian PCD regulators by a functional genetic yeast-based assay. Considering that, on the one hand induction of Bax expression in yeast led to growth arrest and cell death, and on the other hand coexpression of an antiapoptotic abrogated this cytotoxicity, several groups transformed a Bax-inducible *S. cerevisiae* strain with higher eukaryotes cDNAexpressing libraries. The resultant transformants were then screened for their ability to resist to Bax cytotoxicity in a simple functional test. This classical genetic approach yielded to characterization of new more or less potent Bax suppressors in yeast [177, 192–197]. Interestingly, these suppressors retained their ability to interfere with cell death in higher eukaryotes models of apoptosis, confirming their role



Fig. 1. Specific alteration of the mitochondrial outer membrane. Pictures of green fluorescence (printed in black and white) emitted in yeast strains expressing BAX (lower panels) or not (upper panels) by a Green Fluorescent Protein constitutively targeted either to mitochondrial matrix–GFP(M); (left), or in the mitochondrial intermembrane compartment—GFP(IM); (right) [Christophe Fleury, Bernard Guiard, unpublished data]. Bax expression clearly tends to relocalize GFP(IM) from the intermembrane space to the cytosol (altered outer membrane), whereas initially matricial GFP(M) remains in mitochondria (intact inner membrane). This document illustrates a specific Bax-induced permeabilization of the outer mitochondrial membrane. White arrows indicate the mitochondrial network.

of apoptosis regulators. Human Bax suppressors were termed BI-1 (<u>Bax Inhibitor-1</u> [192], BAR (Bifunctional apoptosis regulator) [195], and BASS1 and BASS2 (<u>Bax Antagonist Selected in Saccharomyces [194]</u>. The two latter are novel proteins without any conserved motif, and their physiological role(s) remain(s) to be elucidated. BI-1 is an integral membrane protein, which physically interacts with Bcl-2/Bcl-x_L. Homologues exist in plants, with a converted ability to overcome mammalian Bax lethality in yeast and in plants [193, 196].

Bar is a particularly interesting new regulator of apoptosis, as this protein is able to bind Bcl-2 and caspases. A second mammalian protein, MRIT, was also shown to interact independently or simultaneously with both caspases and a Bcl-2 family member in two-hybrid tests [198]. Bar and Mrit thus form a new group of proteins, at the junction of the two main families of apoptosis regulators (i.e., Bcl-2s and caspases), that are able to activate caspase-dependent cell death, via physical interactions.

The last isolated Bax inhibitors with this functional assay are new proteins from plant. One of them exhibits glutathione S-transferase and weak glutathione peroxidase activities and restores mitochondrial functions [177]. The other is a vesicle associated membrane protein from Arabidopsis thaliana (AtVAMP), which also suppresses the sugar induced cell death and is involved in vesicules traffic and membrane recycling [197].

These results strengthen the link between oxidative stress and induced cell death in yeast. Conserved functions and mechanisms in eukaryotes render possible a functional screen to isolate new regulators of apoptosis. Surprisingly, no antiapoptotic member of the Bcl-2 family was identified with this genetic strategy, suggesting that the system did not reach saturation and will provide other new regulators of apoptosis in the future.

Heterologous Expression of Caspases

Yeasts do not contain any caspase gene, neither any caspase activity, but proteins containing baculovirus inhibitor of apoptosis repeats (BIRs domains), which are normally found in IAP (inhibitor of apoptosis protein) thought to inhibit caspase activation and apoptosis [199–201], have been identified in *S. cerevisiae* and *S. pombe* [202]. Rather than inhibiting caspase-mediated cell death, these yeast BIRPs, that partly resemble the human survivin [203, 204], have roles in cell division [205], in a similar way to the IAPs from *C. elegans* and the mammalian IAP Survivin [206]. Pbh1p (in *S. pombe*) and BIR1p (in *S. cerevisae*) are involved in chromosome segregation and in the pleiotropic control of cell division [207–209]. These results illustrate the fact that the role of IAPs is not restricted to apoptosis regulation, but also assumes other conserved cellular functions.

Heterologous expression of various mammalian procaspases and caspases were performed in yeast. Procaspases-8 or -10 (full length zymogens) are correctly processed in active proteases when expressed in *S. cerevisiae*, whereas procaspase-3 did not in *S. cerevisiae* but did in *S. pombe* [210–212]. An active caspase-3 was however reconstituted in *S. cerevisae* after coexpression of both subunits of the mature protein. Presence of an active caspase-3 or caspase-8 causes cell growth arrest or cytotoxicity, suppressed by the synthetic caspase inhibitor ZVAD-fmk, by coexpression of baculovirus p35 (a large broad caspase inhibitor) [210, 211, 213] or a mammalian Inhibitor of Apoptosis Proteins (XIAP, survivin). In the latter case, the suppressive strength varies according to the selected member of IAPs family [212].

These results show that yeast can be used as a model system to examine the regulation of caspase cascades and the nature of their cellular substrates. Functional assays were improved to identify targets of caspases and/or IAPs, and to supply for information on activity, structure–function relationship and specificity of caspases

and IAP [115, 214, 215]. Recently, Hawkins *et al.* described elegant methods to analyze inhibition of caspase activity by IAP in a reconstituted Apaf-1 activated mechanism [216], and to clone and identify caspases and their regulators and substrates in yeast [115, 217].

The latter method consists in a reporter system for caspase activity, generated by expression in yeast of a transcription factor fused to the intracellular domain of a transmembrane protein. The presence of an active caspase allows the release of the transcription factor part, due to a specific cleavage at junction (protease cleavage sites) and thus activates reporter transcription. Presence of a caspase activator increases activation of the reporter, whereas caspase inhibitor suppress reporter activity. Hawkins *et al.* applied this method and identified a new Drosophila IAP (DIAP1) that blocks the Drosophila DCP-1 caspase activity.

Identification of Caspase-Interacting Proteins

Recent use of caspase-2 as a bait allowed to study interactions between the different caspase-2 isoforms [218], and to identify ISBP, a calcium and integrinbinding protein that inhibit caspase-2 processing [219], and a novel proapoptotic caspase adaptor protein: proapoptotic caspase adaptor protein (PACAP) which binds caspase-2, caspase-9, cytochrome c and ATP [220]. Some caspases targets were also provided by a such screen: human gelsolin was, for example, identified as a substrate for caspase-3 [221].

CONCLUSION

Yeasts are easily manipulated model systems, that are widely used for heterologous expression of mammalian genes and structure-functions studies. Although they are devoid of homologues of all the main apoptosis regulators, some forms of pseudo-apoptosis were surprisingly observed in yeasts, under "physiological" conditions (mutants, acetic acid treated cultures, ...) or in case of ectopic expression of a mammalian proapoptotic gene. Indeed, some steps of apoptosis may be mimicked in yeasts, but the molecular mechanisms involved remain unclear. The only mutual event in the different models of pseudo-apoptosis in yeast is an increased ROS production and/or accumulation. As ROS are important signaling molecules in higher eukaryotes cell death (for a review, see [222, 223]), it is tempting to speculate the existence of an ancestral redox-sensitive death signaling pathway independent of Bcl-2s and caspases. Occurrence of some markers of oxidative stress and apoptosis in aged mother cells of *S. cerevisiae* [224] argues for their physiological role of cell death mediators. Moreover, antioxidant properties allow Bcl-2 to increase viability of Bax-expressing or superoxide dismutase-deificent yeast strains [107–109].

However, abrogation of yeast pseudo-apoptosis is not only obtained by molecules that are directly involved in ROS detoxification, such as plant peroxidases. Thus, overexpression of AtVAMP, which operates in vesicule trafficking and in an increased membrane recycling [197], counteracts Bax cytotoxicty in yeast downstream of oxidative burst. Moreover, the CDC48 gene, that is mutated in the pseudoapoptotic yeast mutant [99], controls the homotypic fusion of endoplasmous reticulum-derived vesicles. These data suggest a connection between intracellular vesicule trafficking and programmed cell deaths in yeast.

Application of several attractive yeast-based functional assays took yet an important place in the characterization of numerous mammalian apoptosis regulators. The yeast-based genetic approach will remain in the future a powerful tool for the isolation of novel antioxidant and antiapoptotic genes.

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