Minireview

# Mitochondria and programmed cell death: back to the future

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**Abstract Programmed cell death, or apoptosis, has in the past few years undoubtedly become one of the most intensively investigated biological processes. However, fundamental questions concerning the molecular and biochemical mechanisms remain to be elucidated. The central question concerns the biochemical steps shared by the numerous death induction pathways elicited by different stimuli. Heterogeneous death signals precede a common effector phase during which cells pass a threshold of 'no return' and are engaged in a degradation phase where they acquire the typical onset of late apoptosis. Alterations in mitochondrial permeability transition linked to membrane potential disruption precede nuclear and plasma membrane changes. In vitro induction of permeability transition in isolated mitochondria provokes the release of a protein factor capable of inducing nuclear chromatin condensation and fragmentation. This permeability transition is regulated by multiple endogenous effectors, including members of the bcl-2 gene family. Inhibition of these effects prevents apoptosis.**

*Key words:* Apoptosis; Bcl-2; Mitochondria; Permeability transition pore; Protease

## 1. Introduction

Apoptosis, or programmed cell death (PCD), is a naturally occurring process of cell 'suicide' that plays a crucial role in the development and maintenance of metazoans by eliminating superfluous or unwanted cells [1,2]. Disturbed apoptosis plays a major role in diseases such as cancer, acquired immune deficiency syndrome, autoimmune disease, and neurodegeneration. The biochemical basis for apoptotic cell death is constitutively present in virtually all mammalian cells and can be activated by a wide variety of extra- and intra-cellular signals [3]. The induction phase of PCD or apoptosis is characterized by an extreme heterogeneity of potential PCD-triggering signal transduction pathways.

Although apoptosis research has exponentially grown the last few years, fundamental questions concerning the molecular and biochemical mechanisms of apoptosis remain to be elucidated. A great deal of effort has been dedicated to biochemical studies of its initiation and regulation, in order to develop new methods of enhancement or inhibition of the process in disease, with considerable benefit for patients [4]. In contrast, relatively little is known about the mechanism involved in the execution of apoptosis [5]. The central question concerns the hypothetical biochemical steps shared by the numerous death induction pathways elicited by different stimuli. Structural and functional similarities between mammalian proteins that regulate PCD and those encoded by cell death genes in the nematode worm *Caenorhabditis elegans* indicate that the molecular mechanism of PCD has been evolutionarily conserved. Moreover, recent findings indicate that a similar process of socially advantageous regulation of cell survival also operates in single-cell eukaryotes [6].

The hypothesis of a central executioner of the PCD was reinforced by the discovery of the proto-oncogene *bcl-2* which is structurally and functionally related to the product of the *Caenorhabditis elegans* cell death-protecting gene *ced-9* and its protective action in a variety of cell death systems [7,8]. *Ced-9* is an element of a polycistronic locus that also contains the gene *cyt-1*, which encodes a protein similar to the cytochrome $b_{560}$ of complex II of the mitochondrial respiratory chain [8].

Bcl-2 is the product of the *bcl-2* apoptosis-inhibitory proto-oncogene. The human *bcl-2* gene can function in *C. elegans* to suppress PCD [2,8]. In a number of systems Bcl-2 fails to protect against cell death. Bcl-2 is only one, however, of a numerous family of recently discovered dimerizing proteins (Bcl-$X_L$, Mcl-1, Bfl-1, A1 etc.) which influence cell death [9]. Some, like Bax (or Bad, Bak) heterodimerize with Bcl-2 and others to abrogate its inhibition of apoptosis. Susceptibility to apoptosis may therefore be determined by multiple competing dimerizations in which Bax and its relatives may be common partners [10]. Some family members, like Bcl-2, suppress or delay PCD, while others promote death, although the mechanisms are unknown. The failure of Bcl-2 to protect against apoptosis can be explained by postulating that there are additional parallel death pathways that are related to, yet distinct from Bcl-2. It also is reasonable to suppose that the molecular targets of the Bcl-2 family members are part of a pathway leading to apoptotic death which has been conserved throughout evolution.

## 2. Reactive oxygen species (ROS), Bcl-2 and programmed cell death

Bcl-2 was initially thought to be located in the mitochondrial membrane [11], suggesting that it might directly affect primarily mitochondrial function. It has since been found by immunolocalization to be in the outer mitochondrial membranes but also in the membranes of the endoplasmic reticu-

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*Abbreviations:* AIF, apoptosis inducing factor; $\Delta\Psi_m$, mitochondrial membrane potential; ICE, interleukin-1β converting enzyme; PCD, programmed cell death; PT, permeability transition pore; ROS, reactive oxygen species; TNF, tumor necrosis factor


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Table 1
Models in which the $\Delta\Psi_m$ reduction precedes apoptosis

| Cells | Inducer | Reference |
|---|---|---|
| Neurons | Deprivation of nerve growth factor | [81] |
| Fibroblasts | p53 | [55] |
| Pre-B cells (WEHI-231) | Anti-IgM | [57] |
| T lymphocytes and T cell hybdridomas | Glucocorticoids | [57] |
| | Superantigen | [82] |
| | T cell receptor crosslinking | [69] |
| | Ceramide | |
| | HIV infection | |
| Thymocytes | Glucocorticoid (dexamethasone) | [56] |
| | Self-antigen (transgenic mice) | [58] |
| | Irradiation | [69] |
| | Etoposide | |
| Myelomonocytic cells (U937) | TNF-α | [57,83,84] |
| | Cycloheximide | |

lum and the nuclear envelope [12,13]. At the mitochondrial level, Bcl-2 has a patchy distribution linked to the contact sites between the outer and the inner membranes [14]. The role of mitochondria and the importance of the C-terminal membrane anchor of Bcl-2 in Bax-induced growth arrest and mortality has been recently demonstrated in *Saccharomyces cerevisiae* [15]. Importantly, Bax kills only yeast cells which have functional respiring mitochondria.

Recent models proposed that Bcl-2 has antioxidant properties and inhibits PCD by suppressing the formation or effects of ROS [16,17]. This fits well with the location of Bcl-2 in organelles known to participate in redox reactions and the formation of ROS [18]. Bcl-2 may thus function to protect cells against cytoplasmic oxidants involved in mediating apoptotic cell death and/or from ROS generated as normal by-products of the normal mitochondrial reactions during ATP synthesis.

Aerobic cells are endowed with extensive antioxidant defence mechanisms to counteract the damaging effects of ROS. Under a continual 'oxidative siege' their survival depends on the balance between ROS production and antioxidant activity (for review, see [19]). ROS, being highly reactive and generally non-specific, are unlikely to mediate the highly co-ordinated and controlled changes that occur in PCD. Nevertheless, ROS seemed to be involved in some PCD models [18]: (i) the addition of ROS or the depletion of endogenous antioxidants can induce PCD; (ii) PCD can sometimes be inhibited by endogenous or exogenous antioxidants [18]; (iii) PCD is associated with increases in cellular ROS levels [16]. Particularly in TNF-induced apoptosis associated with mitochondrial reactive oxygen intermediate production [20]. It is unknown whether the production of ROS is essential to the progression of PCD. In cells cultured in near anaerobic conditions, ROS are unlikely to be produced, although the cells can still undergo PCD [21,22]. Hypoxia itself can induce PCD and Bcl-2 and Bcl-$X_L$ can inhibit cell death [23]. These findings demonstrate that Bcl-2 and Bcl-$X_L$ can inhibit PCD in the absence of ROS and that whatever antioxidant properties Bcl-2 might have, they are not necessary for Bcl-2 to inhibit PCD.

Other evidence supports the fact the ROS are not required for the execution of the cell death program: (i) although some antioxidants can inhibit or delay PCD in some systems [24–27], they fail to do so in all systems, at least not in PCD induced by Fas/APO-1 [28,29] or staurosporine [28]; (ii) antioxidants and radical scavengers have other activities that might exert side effects on molecules required for PCD. Many antioxidants are reducing agents and, as such, might modify sulfhydryl groups of proteins and alter their functions. They also have indirect effects on cellular biochemistry, for example, the $Cu^+$ chelator 1,10-phenanthroline (OPT) can inhibit PCD in thymocytes, suggesting ROS involvement [25]. OPT can also inhibit ICE in vitro by preventing the metal-catalysed oxidation of an essential thiol suggesting that it might inhibit PCD by interaction with an ICE-family protease.

Although ROS do not seem to be required in the execution phase of PCD, they can be involved in the activation phase, as intracellular signaling molecules [30]. It is necessary to distinguish ROS molecules involved in such signaling pathways from those that mediate general cellular damage. It seems unlikely that hydroxyl radicals, which are reactive but lack of biological specificity, can be specific signalling intermediates. However, the hydroxyl radicals might participate to very localized processes, within cytoplasmic membranes, during the execution phase of PCD.

## 3. Proteases as mediators of apoptosis

In view of these results many investigators have purchased apoptotic effectors other than ROS. Cysteine proteases with 'asp-ase' activity such as the prototypical ICE/ced3 protease family, have attracted attention as candidates for common apoptotic effector molecules (for review, see [31–33]). The emergence of ICE-like proteases detracted from the early interest in ROS and mitochondria.

Intracellular proteases might play a critical role in the initiation of apoptosis: (i) specific and reproducible proteolytic cleavage has been identified in apoptosis [34–39]; (ii) certain protease inhibitors have been shown to inhibit apoptosis [35,40,41]; (iii) some viral proteins capable of inhibiting apoptosis are protease inhibitors [42,43]; (iv) gene knockout experiments have demonstrated an essential role for a specific protease in some physiologic models of apoptosis [44,45].

Inhibitors of ICE family proteases are capable of blocking PCD triggered by diverse signals, both in vivo and in vitro (for a review, see [32]). Among the numerous ICE-like protease inhibitors, Z.VAD is a broad-spectrum antagonist of numerous proteases that inhibits apoptosis in mammalian and insects cells [46,47]. These data suggest that the ICE pro-

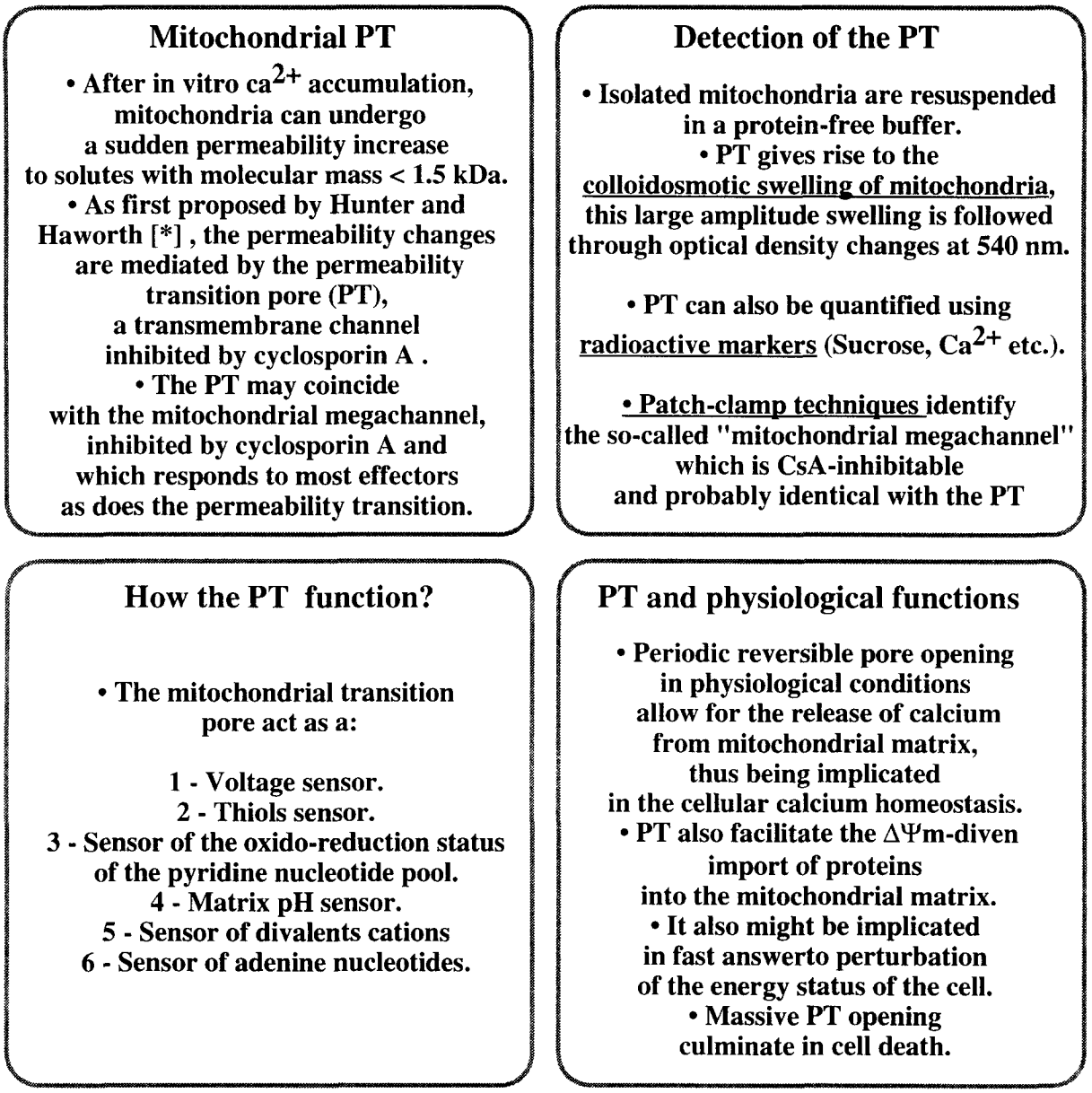


Fig. 1. Summary of the structure and function of the mitochondrial permeability transition. (*) Hunter and Haworth [79]. All information summarized from Bernardi et al. [68], Zoratti et al. [66] and Constantini et al. [80].

tease family plays a central shared functional role in apoptotic death pathways. Two examples of apoptotic cell death that are not blocked by inhibitors of ICE-like proteases (AcY-VAD-peptide) concern: (1) IL-2 withdrawal-induced cell death and DNA fragmentation in murine CTLL-2 cells even though these inhibitors blocked IL-1β production and (2) Fas-mediated apoptosis in other murine cells [48]. The significance of the failure of the inhibitors of the ICE-like proteases to block cell death is difficult to interpret given our incomplete knowledge of the specificity of the proteases. It may also well be that some cases of PCD are not mediated by ICE-family proteases.

### 4. Alterations of mitochondrial functions as early event of apoptosis

Until recently, mitochondria were thought to be morphologically normal during apoptotic cell death whereas they appear swollen in necrotic cells. However, some data indicate a breakdown of mitochondrial function during apoptosis. An inhibition of oxidative ATP production has been reported to be associated with glucocorticoid-induced lymphocyte apoptosis [49] and a decreased mitochondrial dehydrogenase ability to cleave tetrazolium salt (MTT) [50] has been reported in anti-CD3-induced apoptosis of T-cells [51]. In the case of TNFα-induced apoptosis [52], early disruption of mitochondrial function [53,54] has been described. More recently, an early drop of mitochondrial membrane potential ($\Delta\Psi_m$) which correlates with an uncoupling of electron transport from ATP production, a drop of the rate of mitochondrial translation and defect in mitochondrial protein cytoplasmic precursors maturation have been observed during the commitment to apoptosis of cell conditionally immortalized with SV40 [55]. A similar drop of $\Delta\Psi_m$ associated with cardiolipin alteration

Table 2
Inducers and inhibitors of the mitochondrial PT pore and/or apoptosis

| Substances | Interaction with | Effect on the PT | Effect on apoptosis | Reference |
|---|---|---|---|---|
| Calcium | Unknown | **Induction** | **Induction** | [a] |
| Pro-oxidants (*tert.*-butyl-hydroperoxide, $H_2O_2$) | Unknown | **Induction** | **Induction** | [a] |
| Diamide | Vicinal thiols (crosslinker) | Induction | **Induction** | [85] |
| Uncoupling agents (mClCCP) | Membranes (protonophore) | **Induction** | **Induction** | [62] |
| Protoporphyrin IX (PPIX) | Ligand of the peripheral (outer mitochondrial membrane) benzodiazepine receptor, a putative constituent of the multi-subunit PT pore | **Induction** | **Induction** | [63] |
| Atractyloside | Interaction with the external domains of the adenine nucleotide translocator, another putative constituent of the PT pore | **Induction** | **No effect** (not membrane-permeable) | [62] |
| Bongkrekic acid (BA) | Ligand of the adenine nucleotide translocator (matrix side) | **Inhibitor** of PT induced by atractyloside, oxidants, and uncoupling agents, not calcium or diamide | **Inhibitor of apoptosis** induction by oxidants, uncoupling agents, glucocorticoids, or DNA damage | [62] |
| Cyclosporin A (CsA) | Interaction with the cyclophilin isoenzyme of the mitochondrial matrix | **Short-term inhibitor** ($< 60$ min) | **No major effect,** synergistic inhibitory effects with BA | [62] |
| Ruthenium red | Inhibitor of the inner mitochondrial membrane calcium uniport | **Inhibitor** of PT induced by calcium, not pro-oxidants, uncoupling agents, or diamide | **No effect** | Unpublished |
| Monochlorobimane | Thiols (inhibits disulfide bridge formation between vicinal thiols) | **Inhibitor** of PT induced by diamide | **Inhibitor** of apoptosis induced by glucocorticoid and DNA damage | Unpublished |

[a]For $Ca^{2+}$ and pro-oxidants, for isolated mitochondria, cf. [66,68].

is also an early event of dexamethasone-induced thymocyte apoptosis [56–58] (Table 1).

The finding that the Bcl-2 proto-oncogene product can locate to the mitochondrial membrane [11] and that its ability to suppress apoptosis is reduced in constructs that lack the C-terminal transmembrane segment [16,59] has suggested that Bcl-2 acts at the mitochondrial level. Since it has been found that Bcl-2 blocks apoptosis in cells that do not contain a functional respiratory chain (cells lacking mtDNA) [60], it has been concluded that apoptosis itself and the antiapoptotic activity of Bcl-2 are not related to mitochondrial respiration. These experiments do not exclude that mitochondrial events might be involved in apoptosis. Cells devoid of mtDNA have been selected for their ability to grow in the presence of ethidium bromide (which inhibits mtDNA replication). The clones are selected for their ability to grow and survive in the absence of respiration. These cells generates ATP by glycolysis and maintain a normal $\Delta\Psi_m$, probably by using the ADP/ATP translocator to import glycolytic ATP in the mitochondria [61–63]. Thus it remains possible that a mitochondrial function, not directly linked to respiration but possibly to the $\Delta\Psi_m$, is involved in apoptosis. Second, a depletion of the mitochondrial electron transport abrogates the cytotoxic effect of TNFα [64]. In the same system, the overexpression of Bcl-2 has been shown to increase $\Delta\Psi_m$ and this effect could be linked to its anti-apoptotic activity since the ionophore nigericin, known to increase $\Delta\Psi_m$, has a similar effect [65]. A new interest emerges from recent work [57,58,62] and brings back the mitochondria under the apoptosis searchlights, as a crucial common step in PCD is the opening of the mitochondrial 'megachannel' or permeability transition pore (PT).

## 5. Modulation of mitochondrial permeability transition decides cell fate

A number of different substances regulate the probability of PT pore opening and closing [66]. The heterogeneous catalogue of PT inducers [67,68] comprises divalent cations, pro-oxidants, thiol-crosslinking agents, and more specifically ligands of proteins thought to be involved in the formation of PT pores (adenine nucleotide translocator, peripheral benzodiazepine receptor). Such substances, which can induce PT in isolated mitochondria (Fig. 1), are also highly efficient PT inducers in cells. They provoke one of the cardinal feature of PT, namely $\Delta\Psi_m$ disruption and by superoxide anion generation. In doing so, PT opening induces apoptosis in lymphoid cells and cell lines (Table 2). Inhibitors of PT such as bongkrekic acid or the monovalent thiol-reactive agent monochlorobimane can inhibit apoptosis induced by glucocorticoids, irradiation or topoisomerase inhibition (Table 2). $\Delta\Psi_m$ disruption and subsequent nuclear apoptosis are strictly co-regulated, i.e. whenever a cell loses its $\Delta\Psi_m$ it will die from apoptosis, and vice versa, whenever a cell is protected against apoptosis induction, it will maintain its $\Delta\Psi_m$. Inhibition of thymocyte apoptosis by inhibitors of mRNA synthesis, mRNA translation, protease activation, antioxidants or p53 null mutation prevents the $\Delta\Psi_m$ disruption that normally follows exposure to the pro-apoptotic stimulus [69]. Bcl-2 prevents the PT-mediated $\Delta\Psi_m$ collapse, both in cells and in isolated mitochondria [62]. Thus, a proto-oncogene product exerts a direct control on mitochondrial PT. In a cell-free system, in which purified nuclei are exposed to cytoplasmic compounds, mitochondria are necessary to reproduce the fea-

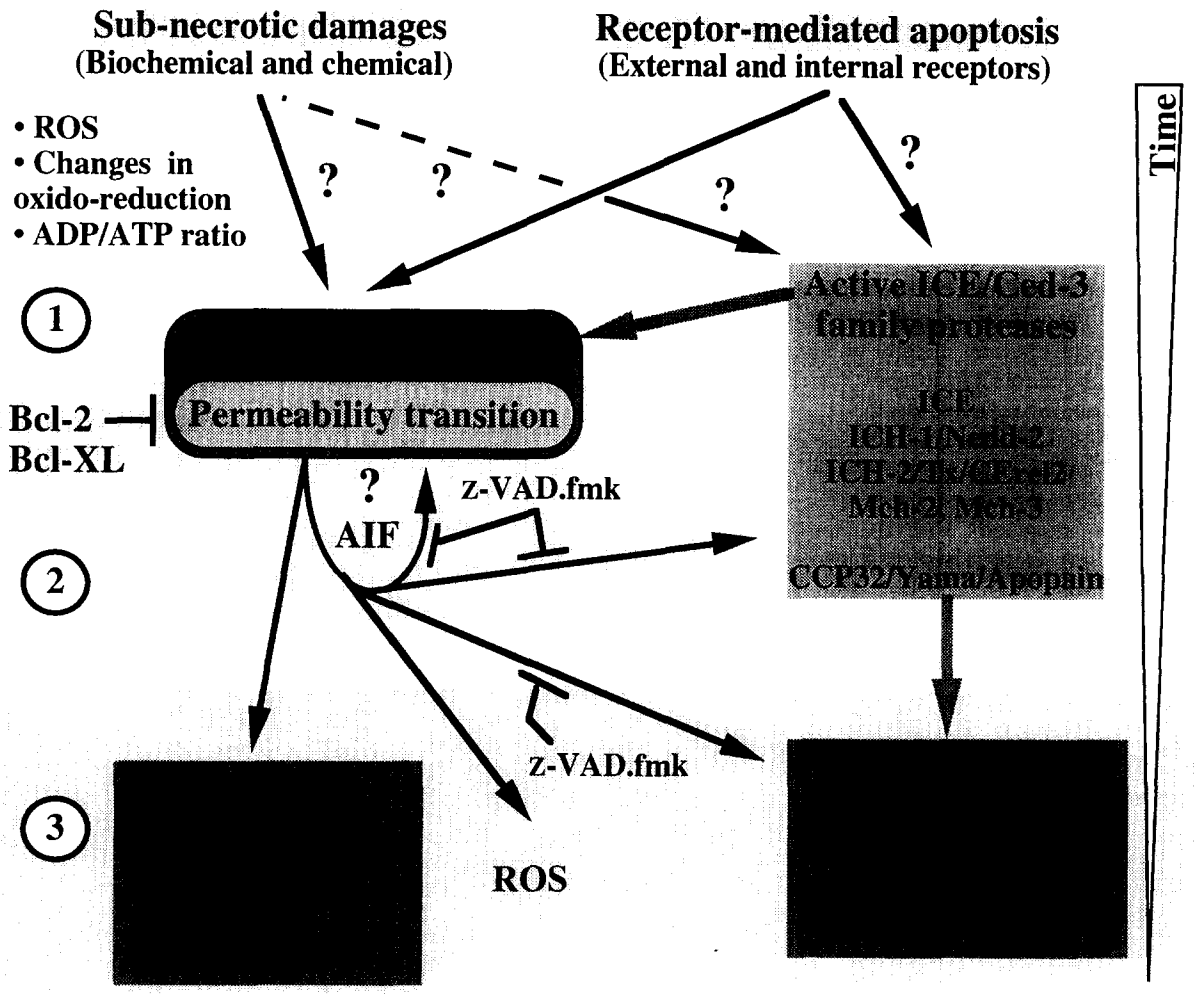


Fig. 2. Hypothetical model of apoptosis regulation. Apoptosis can be induced by biochemical or chemical signals mediated via internal or external receptors. Signal transduction pathways after apoptotic stimuli are numerous. Reactive oxygen species (ROS), alteration of the cellular redox potential and/or changes in ADP/ATP ratio involve some but not all of the pathways of apoptosis induction. ICE and ICE/Ced-3 proteases, as well serine proteases appear to intervene in the 'private pathway' of apoptosis. The different signal transduction pathways converge into a common apoptotic pathway to all cells apoptosis through the induction of mitochondrial permeability transition (MPT). The Bcl-2 proto-oncogene intervenes at the level of MPT induction in response to some but not all MPT inducers. As a consequence of the pore opening, mitochondria release a protein (apoptosis inducing factor, AIF) capable of causing nuclear apoptosis in cell-free systems. PT is related to the mitochondrial generation of ROS, as well as a rapid exposure of phosphatidyl serine residues at the outer plasma membrane surface. These changes form a part of the degradation phase of apoptosis. It is probable that the permeability transition is linked to an activation of specific proteases, although the exact mechanisms underlaying their action remain unknown. Enhanced ROS generation, depletion in reduced glutathione, increase in cellular calcium and nuclear apoptosis are likely to be independent events not linked in a strict cause-effect relationship but all belonging to the degradation phase of apoptosis. 1: induction phase; 2: effector phase; 3: degradation phase.

tures of nuclear apoptosis [70]. Only mitochondria that are undergoing PT are pro-apoptotic in this system [62], and inhibition of PT prevents mitochondria from apoptotic cells from inducing apoptosis in isolated nuclei.

Other recent data suggest that a pre-formed $> 10$ kDa protein is released from mitochondria upon $\Delta\Psi_m$ disruption which causes isolated nuclei to undergo chromatin condensation and nuclear fragmentation [62,71]. The effects of this apoptosis inducing factor (AIF) are blocked by *N*-benzyloxycarbonyl-Val-Ala-Asp.fluoromethylketone (z-VAD.fmk), an antagonist of interleukin-1β converting enzyme (ICE)-like proteases which is also an efficient inhibitor of apoptosis in cells. Bcl-2 hyperexpressed in the outer mitochondrial membrane also impedes the release of AIF from mitochondria in vitro without affecting its formation. Either the presence of Bcl-2 in the nuclear membrane nor the Bcl-2 hyperexpression protects cells against AIF [71]. Bcl-2 may thus also prevent apoptosis by favoring the retention of an apoptogenic mitochondrial protease. These new results have the advantage of giving a link between mitochondria and proteases without excluding the possibility that proteases act upstream of the PT pore opening in other cell death systems (Fig. 2).

### 6. Bcl-$X_L$ (Bcl-2) and pore regulation

The recent crystallization of Bcl-$X_L$ [72], a member of the Bcl-2 family [73], has revealed a central structure containing two α-helices surrounded by amphiphatic helices. Three functionally important Bcl-2 proteins homology regions (BH1, BH2 and BH3) [74–76] are in close spatial proximity and form an hydrophobic cleft which may be the binding site for other Bcl-2 family members.

The arrangement of the α-helices in a pore like structure is reminiscent of the membrane translocation domain of bacterial toxins, like diphtheria toxin and colicins [77]. By analogy to the diphtheria toxin translation domain [78], Bcl-2 proteins may form pores in the membranes where they localized [72]. The insertion of Bcl-2 proteins like the bacterial proteins, may be regulated by signals dependent on voltage and pH [72]. It has also been demonstrated that Bcl-2 inhibits the release of the mitochondrial Z.VAD inhibitable protein factor (AIF) which is released upon $\Delta\Psi_m$ disruption and causes nuclei to undergo chromatin condensation and internucleosomal fragmentation [71]. These data suggest that Bcl-2 proteins and related family members may directly or indirectly affect the permeability of the mitochondria and thus regulate or either destroy cellular homeostasis and death.

As Bcl-2 (Bcl-$X_L$) is located in the outer mitochondrial membrane, it is unlikely that the membrane insertion of Bcl-2 can directly lead to the uncoupling of electron transport and $\Delta\Psi_m$ disruption. Interaction with the mitochondrial mega-channel would have to be based on a two step mechanism, which may implicate Bcl-2 insertion into membrane, outer membrane pore formation and translocation capability into the intermembrane space or inner mitochondrial membrane of proteins known to form heterodimers with Bcl-2 (or Bcl-2 family members). But this is only speculation.

Together, these findings suggest the action of Bcl-2 and its homologues as direct regulators of the mitochondrial permeability transition, a hypothesis that would be in accord with genetic, functional and structural (crystallographic) data.

Future investigations will unravel whether apoptosis inducing Bcl-2 homologues function themselves as mitochondrial pores, whether they have to assemble with additional structure to form permeability transition pore and their control thereof.

These findings open new perspectives concerning the cell death machinery and its regulatory properties, in which mitochondria make their comeback!

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