Apoptosis 1996; 1: 231–242

The cell biology of apoptosis: Evidence for the implication of mitochondria

S. A. Susin, N. Zamzami and G. Kroemer

Centre National de la Recherche Scientifique-UPR420, 19 rue Guy Môquet, B.P.8, F-94801 Villejuif, France

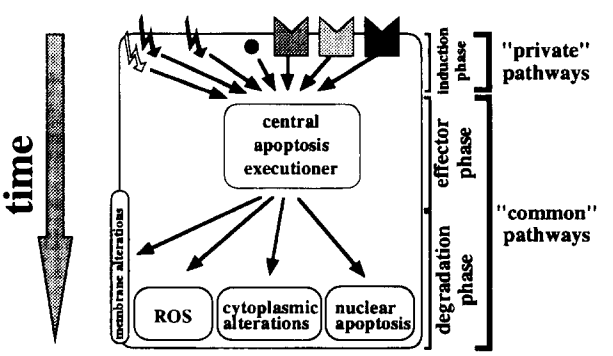
The apoptotic process can be subdivided into three phases: a death-stimulus-dependent heterogeneous induction phase, a common effector phase during which the central apoptotic executioner is activated, and a common degradation phase during which cells acquire the biochemical and morphological features of end-stage apoptosis. Recently, it has become clear that the central apoptosis executioner is dictated by cytoplasmic (non-nuclear) events and that nuclear changes that define apoptosis (chromatin condensation and oligonucleosomal DNA fragmentation) only become manifest beyond the point-of-no-return of apoptosis, during the late degradation phase. It appears that one obligatory event of the apoptotic cascade involves a characteristic change in mitochondrial function, namely the so-called mitochondrial permeability transition. Permeability transition leading to disruption of the mitochondrial transmembrane potential precedes nuclear and plasma membrane features of apoptosis. Induction of permeability transition in cells suffices to cause the full-blown picture of apoptosis. In vitro induction of permeability transition in isolated mitochondria provokes the release of a factor capable of inducing apoptotic changes in isolated nuclei. Permeability transition is subject to regulation by multiple endogenous effectors, including members of the *bcl-2* gene family. Its inhibition by pharmacological agents or hyperexpression of Bcl-2 prevents apoptosis, indicating that PT is a central coordinating event of the apoptotic effector stage.

Key words: Bcl-2; mitochondrial transmembrane potential; permeability transition; proteases.

Introduction: From nuclear to cytoplasmic control

It is currently assumed that the apoptotic process can be divided into at least three functionally distinct phases (Figure 1).[1,2] During the heterogeneous *initiation phase*, cells receive the death-inducing stimulus: lack of growth or survival factors, shortage of oxygen or metabolic supply, signalling via certain receptors such as Fas/APO-

Figure 1. Phases of the apoptotic process. Apoptosis can be induced by subnecrotic damage of the cell or via signals mediated via receptors. The particular apoptosis-inducing second messenger system depends on the stimulus and thus constitutes a 'private' pathway. Integration of various private pathways into a pathway that is common to all types of apoptosis, irrespective of the particular apoptosis inducer, is achieved during the effector phase of apoptosis via activation of the common central apoptosis executioner. Once the executioner has been activated and the point-of-no-return has been passed, the cell manifests alterations of the plasma membrane (exposure of phosphatiylserine residues) that renders the cell 'palatable', facilitating its recognition and heterophagic removal by adjacent cells. Moreover, the cell manifests strong alterations in redox regulation (depletion of glutathione, hyperproduction of reactive oxygen species, ROS), cytoplasmic alterations (vacuolization, reduction of cell size, increase in free cytosolic calcium, disorganization of the cytoskeleton *etc.*), and nuclear apoptosis (chromatin condensation, activation of endonucleases, degradation of specific nuclear proteins). These changes become manifest during the degradation phase of apoptosis and are coordinated by the central apoptosis executioner.



Supported by ARC, ANRS, CNRS, FRM, Fondation de France, INSERM, NATO, Ligue contre le Cancer. Ministère de la Recherche et de l'Industrie (France), and Sidaction (to GK). SAS receives a fellowship from the Spanish Government (Ministerio de Ciencia y Educación).

Correspondence to Dr G. Kroemer, 19 rue Guy Môquet, B.P. 8, F-94801 Villejuif, France. Tel: (+33) 1-49 58 35 13; Fax: (+33) 1-49 58 35 09.

1/CD95 or tumour necrosis factors (TNF) receptors, contradictory internal signals concerning cell cycle advancement, or subnecrotic physical and chemical damage. The biochemical events participating in the initiation phase constitute 'private' pathways in the sense that they depend on the lethal stimulus. It is only during the subsequent *effector phase* that these initiating events are translated into a regular common pattern of metabolic reactions. The common pathway can be subdivided into an initial effector phase, during which the 'decision to die' is still subject to regulatory mechanisms, and a later *degradation phase*, beyond the 'point-of-no-return', during which an increase in the overall entropy including activation of catabolic enzymes precludes further regulatory effects.

Investigations on the common mechanisms of apoptosis have yielded a number of wrong hypotheses which are still applied by some researchers. In the following paragraphs, we will enumerate a few of these incorrect assumptions:

Apoptosis as a result of the activation of 'killer genes'

In some special cases, inhibition of mRNA or protein synthesis can prevent cell death. This is true for instance for glucocorticoid-induced thymocyte death.[3] Based on this finding, it has been postulated that apoptosis would require activation of 'killer genes' and hundreds of research teams have engaged in differential screening procedures to identify such 'killer genes' in subtracted cDNA libraries. Nobody yet has come up with a universal 'killer gene' involved in all types of cell death, although expression of certain genes is involved in apoptosis induction in some special circumstances, probably as part of private apoptosis induction pathways.[4] By now, this failure to identify a common genetic 'death programme' appears plausible in view of the facts that: (i) any cell type can undergo apoptosis;[5] (ii) inhibition of mRNA or protein synthesis does not affect apoptosis induction in many systems (*e.g.* when apoptosis is induced via cross-linking of Fas/APO-1/CD95 or granzymes);[3,6,7] (iii) inhibition of macromolecule synthesis causes apoptosis in many (or perhaps all?) cells types 'by default'.[5,8,9] This latter finding implies that cells constitutively express all of the protein components required to execute the death programme.

Apoptosis as an 'abortive cell cycle' or 'mitotic catastrophe'

It has been speculated that apoptosis constitutes the result of an 'abortive cell cycle' or of a 'mitotic catastrophe'.[10] Although it is true that some apoptosis inducers kill cells during a determined phase of the cell cycle and that some oncogenes involved in cell cycle regulation (*e.g.* c-myc, p53) also participate in apoptosis regulation,[11,12] it is incorrect to assume that apoptosis is always linked to cell cycle regulation. Indeed, in response to certain inducers, apoptosis can occur in any phase of the cell cycle (including in terminally differentiated cells such as neurons or cardiomyocytes).[13] Thus, although in some systems PCD and cell cycle may be controlled in part by a common pathway, it is wrong to assume that any kind of PCD constitutes the outcome of a 'mitotic catastrophe'. It appears rather that an abnormal sequence of cell cycle-triggering events is just one among many different triggers of PCD (reviewed in Ref. 2).

Apoptosis as a result of oxidative stress

Oxidative stress is an inducer of apoptosis and, in several systems, the susceptibility of cells to undergo apoptosis can be reduced by antioxidants.[14] This finding, together with the observation that cells undergoing apoptosis are characterized by an enhanced generation and/or action of reactive oxygen species (ROS)[15,16] led to the hypothesis that ROS and/or changes in cellular redox potentials would participate in the effector phase of apoptosis. However, culture of cells in the absence of oxygen (which obviously prevents the generation of ROS) can induce apoptosis[17–19] and does not prevent apoptosis induction by some stimuli such as staurosporine or cross-linking of the Fas/APO-1/CD95 surface receptor.[20,21] Hence, enhanced generation of ROS is a common consequence rather than a common cause of apoptosis.[2,15,16]

Apoptosis due to the action of invariant second messengers

A few second messengers have been accused of participating in the induction of cell death. This applies in particular to cytosolic calcium[22,23] and second messengers generated by sphingomyelinase activation (ceramide, sphingosin).[24–26] However, it has become clear that such second messengers are not always involved in apoptosis and that, on the contrary, both calcium and ceramide can mediate activation signals, depending on the cell type and additional second messengers.

Apoptosis in cells lacking mitochondrial DNA

Cells lacking mitochondrial DNA (ρ^0 cells) undergo apoptosis normally. This finding has been widely (over)

interpreted to mean that mitochondria are not involved in the apoptotic process. However, cells lacking mitochondrial DNA possess morphologically normal mitochondria that normally fulfil several crucial mitochondrial functions including those involved in apoptosis (reviewed in Ref. 27).

Apoptosis as a nuclear event

In most cases, the process of apoptosis culminates in characteristic alterations of nuclear morphology (mainly chromatin condensation and pyknosis) and chromatin biochemistry (a step-wise degradation by DNAses culminating in the formation of mono- or oligomers of ~200 bp or DNA).[28] Based on this observation, many investigators assumed that activation of endonucleases was responsible for apoptosis and that inhibition of endonucleases by drugs such as aurintricarboxylic acid and zinc would prevent apoptosis. This idea is certainly wrong because (i) programmed cell death can be induced in cells lacking nuclei;[29–31] (ii) endonucleases are only involved in the late degradation phase of apoptosis ('cleaning up *after* death');[32] and (iii) supposed 'endonuclease inhibitors' such as zinc and aurintricarboxylic acid have pleiotropic effects on many different enzyme systems (reviewed in Ref. 6). In other words, although chromatin condensation and oligonucleosomal DNA fragmentation constitute hallmarks of advanced apoptosis, they do not reflect the decisive mechanism of apoptosis. Nuclear changes constitute useful epiphenomena[33] for the detection of apoptosis — at the stage of degradation — yet do not constitute the essence of the apoptotic effector phase.

The above findings do not answer one of the fundamental questions of apoptosis research: What is/are the biochemical event(s) that define the initiation of the common effector phase of apoptosis and simultaneously allow for the convergence of disparate initiation pathways into one, stereotypical sequence of apoptotic alterations? Today's consensus is that cytoplasmic (= non-nuclear) events determine the effector phase of apoptosis.[29–31] According to one widely accepted theory, the effector phase involves the activation of specific protease cascades (reviewed in Ref. 34–36). We[2,16,27,37–47] and others[48–54] have recently provided evidence indicating that mitochondria undergo profound perturbations early during apoptosis. Moreover, we[2,40–47] and others[49,53,55] have obtained compelling functional evidence indicating that mitochondria may play an important role in the process of apoptosis.

The present review will focus on three aspects of the putative mitochondrial implication in apoptosis regulation. First, data suggesting the involvement of mitochondrial permeability transition (PT) in the apoptosis effector phase will be discussed. Second, we will discuss the characterization of an apoptogenic mitochondrial protein that is released from mitochondria undergoing PT and that suffices to cause apoptotic changes in isolated nuclei *in vitro*. Third, the mode of action of the onco-protein Bcl-2, one of the major endogenous inhibitors of apoptosis, will be discussed. Data suggesting that Bcl-2 directly controls mitochondrial permeability transition will be presented.

Mitochondrial permeability transition: a critical co-ordinating event of the apoptotic cascade

The mitochondrial transmembrane potential ($\Delta\Psi_m$) results from the asymmetric distribution of protons on both sides of the inner mitochondrial membrane, giving rise to a chemical (pH) and electric gradient which is essential for mitochondrial function.[56] The inner side of the inner mitochondrial membrane is negatively charged. Consequently, cationic lipophilic fluorochromes such as rhodamine 123, 3,3'dihexyloxacarbocyanine iodide [DiOC$_6$(3)], chloromethyl-X-rosamine (CMXRos) or 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) distribute to the mitochondrial matrix as a function of the Nernst equation, correlating with the $\Delta\Psi_m$. Using a cytofluorometer, these dyes can be employed to measure variations in the $\Delta\Psi_m$ on a per-mitochondrion or per-cell basis. We[2,16,27,37–40,42–47] and others[48,50–52] have shown that cells induced to undergo apoptosis manifest an early reduction in the incorporation of $\Delta\Psi_m$-sensitive dyes, indicating a disruption of the $\Delta\Psi_m$. This $\Delta\Psi_m$ collapse can be detected in many different cell types, irrespective of the apoptosis-inducing stimulus. $\Delta\Psi_m$ disruption also precedes nuclear apoptosis in cells lacking mitochondrial DNA.[27,42] It becomes manifest before cells exhibit nuclear DNA fragmentation, hyperproduce ROS or aberrantly expose phosphatidylserine (PS) on the outer cell membrane leaflet.[16,37,38,45] Thus, the $\Delta\Psi_m$ collapse constitutes an early common event of the apoptotic cascade. Since an intact $\Delta\Psi_m$ is indispensable for normal mitochondrial function,[56] cells undergoing apoptosis manifest a cessation of mitochondrial biogenesis, both at the transcription and translation levels.[4,48] Moreover, during apoptosis mitochondrial electron transport ceases,[54] and mitochondrial intermembrane proteins such as cytochrome *c* leak out into the cytosol.[53]

To understand the mechanism by which cells undergoing apoptosis lose their $\Delta\Psi_m$, we performed a series of experiments in which cells were first labelled with $\Delta\Psi_m$-sensitive fluorochromes and then purified in a

fluorocytometer, based on their $\Delta\Psi_m$. In appropriate conditions, this procedure allows for the purification of cells with low $\Delta\Psi_m$ values and a still normal DNA content and morphology (= pre-apoptotic cells) or, alternatively, of cells with a still high $\Delta\Psi_m$ that will lose their $\Delta\Psi_m$ upon a short-term (30–120 min) culture period.[16,37,41] We have used this system to show that $\Delta\Psi_m^{low}$ (but not $\Delta\Psi_m^{high}$) cells will undergo oligonucleosomal DNA fragmentation upon short-term culture at 37°C. Moreover, we have found that some drugs inhibit the $\Delta\Psi_m$ loss of $\Delta\Psi_m^{high}$ cells, namely cyclosporin A (CsA) and bongkrekic acid (BA).[37,41,43] This data suggests that the so-called permeability transition (PT), which is inhibited by CsA and BA,[57,58] accounts for the $\Delta\Psi_m$ collapse observed during pre-apoptosis.

PT involves the formation of proteaceous pores ('PT pores' or 'megachannels'), probably by apposition of inner and outer mitochondrial membrane proteins (Figure 2), allowing for the diffusion of solutes < 1500 Da (and *ipso facto* dissipation of the $\Delta\Psi_m$). CsA is one of the best studied (and definitory) inhibitors of PT. Its PT-inhibitory effect is mediated via a conformational change in a mitochondrial CsA receptor, the matrix cyclophilin D.[59] In contrast, its immunosuppressive effect is mediated via an effect on calcineurin-dependent signalling. A CsA derivative that loses its immunosuppressive (calcineurin-mediated) properties, *N*-methyl-Val-4-CsA, still conserves its $\Delta\Psi_m$-stabilizing (cyclophilin-mediated) effect in apoptotic cells.[41] This observation is again compatible with the implication of PT in apoptotic $\Delta\Psi_m$ disruption.

To demonstrate that PT might indeed be important for the apoptotic process, we have used four different approaches:

First, we have shown that pharmacological induction of PT with agents specifically affecting mitochondria is sufficient to cause full-blown apoptosis.[44] Inducers of PT (Table 1) that also trigger signs of nuclear apoptosis include protoporphyrin IX (a ligand of the mitochondrial benzodiazepin receptor, one of the putative constituents of the PT pore, see Figure 2),[44] the protonophore carbomyl cyanide *m*-chlorophenylhydrazone (mClCCP, that causes dissipation of the $\Delta\Psi_m$)[46] and diamide (a divalent thiol-substituting agents causing the cross-linking of vicinal thiols in the mitochondrial matrix).[60] This indicates that triggering of PT is sufficient to cause apoptosis.

Second, we have used BA to evaluate the effect of PT inhibition in cells in long-term experiments (> 120 min). (In this system CsA is not useful because it is a transient inhibitor of PT). BA does not only prevent the mitochondrial manifestations of apoptosis,[41] but it also abolishes all changes of the apoptotic degradation phase concerning the nucleus (DNA condensation and fragmentation), the cytoplasma (vacuolization, glutathione depletion, ROS hypergeneration and NFκB translocation), and the plasma membrane (exposure of phosphatidylserine residues in the outer membrane leaflet).[43] Similarly, chloromethyl-X-rosamine (CMXRos), a substance that prevents oxidation of thiols located in the mitochondrial matrix, can be used as an inhibitor of PT and apoptosis.[60] BA and CMXRos prevent apoptosis induced via both p53-dependent and p53-independent

Figure 2. Hypothetical composition of the permeability transition (PT) pore, also called the mitochondrial megachannel. The adenine nucleotide translocator (ANT), localized in the inner mitochondrial membrane, interacts with proteins inserted in the outer membrane such as the peripheral benzodiazepin receptor (PBR) and the voltage dependent anion channel (VDAC, also called porin). The structural correlate of this interaction is the inner–outer membrane contact site. Additional yet-to-be characterized proteins may interact with either the ANT or ANT-associated outer membrane proteins. The ANT can adopt two different conformations, the *c*-state or the *m*-state that are compatible with pore opening or closing, respectively. Atractyloside, which interacts with an external site of the ANT, causes pore opening, whereas bongkrekic acid, which interacts with an internal site of the ANT, favours pore closing. Other agents that may act on the ANT are thiol-derivatizing substances such as diamide (which causes thiol cross-linking and pore opening) or monovalent thiol-reactive agents (which prevent disulfide bridge formation and inhibit pore opening). In addition, cyclosporin A (CsA) can prevent cyclophilin D from interacting with the inner mitochondrial membrane. The interaction of matrix cyclophilin D with the inner membrane is essential for pore opening. Some non-immunosuppressive CsA derivatives such *N*-methyl-Val-4-CsA, which conserve cyclophilin D binding, have the same effect as CsA on pore opening. At present, it is not known whether the PT-modulatory thiol residues are located within the ANT. Moreover, it remains speculative that cyclophilin D would interact directly with the ANT.

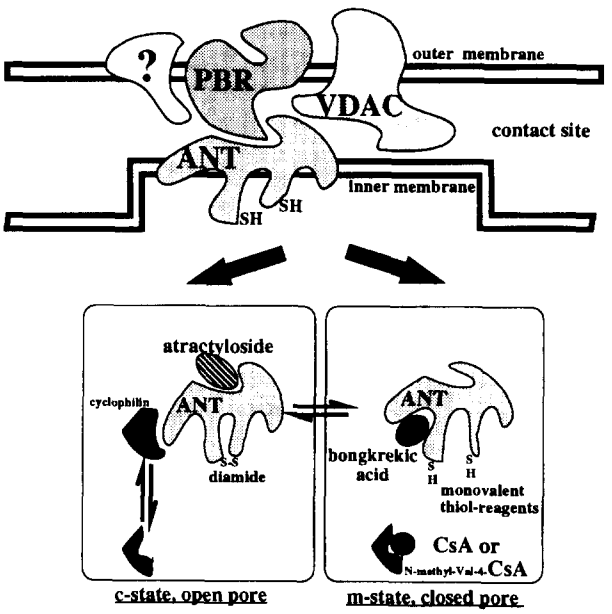


Table 1. Inducers and inhibitors of apoptosis acting on permeability transition pores

Inducers of permeability transition		Inhibitors of permeability transition	
Substance	**Target molecule**	**Substance**	**Target molecule**
Protoporphyrine IX	PBR	Bongkrekic acid	ANT
Atractyloside	ANT	Cyclosporin A	Cyclophilin D
Calcium	Unknown	Calcium chelators	Calcium
Oxidizing agents	Matrix thiols?	Monochlorobiman Monobromobiman	Matrix thiols?
Protonophores	Inner mitochondrial membrane	Chloromethyl-X-rosamin	Matrix thiols
Disulfide bridge formers	Matrix thiols	Calpain inhibitors	Mitochondrial calpain-like protease
Proteases	Unknown	Phosphotyrosine	Tyrosine kinases?
Protons	Reversible histidyl protonation		

Abbreviations: ANT = adenine nucleotide translocator; PBR = peripheral benzodiazepin receptor

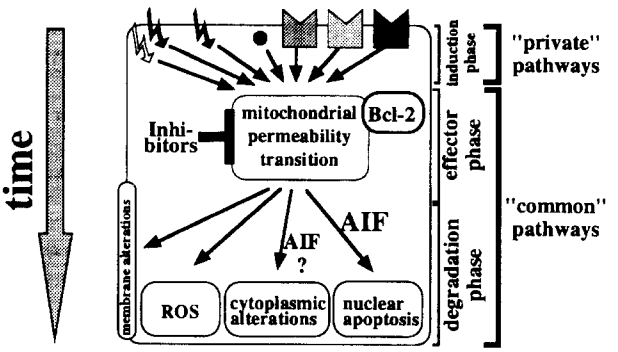
pathways.[43,60] The fact that pharmacological inhibition of PT can prevent all manifestations of apoptosis[40,43] suggests that PT constitutes a central co-ordinating event of the apoptotic process.

Third, we have constructed a cell-free system of apoptosis in which isolated mitochondria are cultured with purified nuclei *in vitro*. Using this approach, we have demonstrated that control mitochondria are not apoptogenic. However, mitochondria induced to undergo PT do acquire the capacity to induce nuclear apoptosis (chromatin condensation + DNA fragmentation) *in vitro*.[40] The molecular basis of this observation well be discussed later in this article.

Fourth, we have purified mitochondria from hepatocytes or lymphocytes undergoing apoptosis, showing that such mitochondria (but not those isolated from non-apoptotic controls) induce DNA fragmentation in healthy nuclei *in vitro*. This effect is partially antagonized by the PT inhibitor BA.[40] Thus, mitochondria undergoing PT can transfer apoptosis induction from one system to another.

Altogether, these data provide compelling evidence that PT is involved in the apoptotic cascade (Figure 3). In addition, a number of indirect arguments (Table 2) suggest that PT could fulfil a central role in the apoptotic effector phase.

Figure 3. Hypothetical model of apoptosis regulation. Mitochondrial permeability transition constitutes one of the critical steps of the apoptotic effector stage. Its inhibition by pharmacological agents specifically acting at the level of the mitochondrion, such as bongkrekic acid, prevents all manifestations of apoptosis, at the levels of the plasma membrane, of the redox state, of cytoplasmic changes and of the nucleus. Mitochondrial permeability transition is accompanied by the mitochondrial release of an apoptosis-inducing factor (AIF) that is sufficient to cause nuclear apoptosis. The impact of AIF on non-nuclear manifestations of apoptosis remains elusive. Bcl-2 functions as an endogenous inhibitor of permeability transition. Thus, Bcl-2 inhibits the mitochondrial release of AIF. In contrast, Bcl-2 does not affect the formation or action of AIF. For details and references consult main text.



A novel apoptogenic protein released from mitochondria undergoing permeability transition: 'apoptosis-inducing factor' (AIF)

Supernatants from mitochondria that have undergone PT but not those from control mitochondria induce signs of apoptosis such as nuclear chromatin condensation in a cell-free system.[40,46] Thus, mitochondria treated with the PT-inducers calcium, atractyloside [a specific ligand of the adenine nucleotide translocator (ANT); Figure 2], carbamoyl cyanide *m*-chlorophenylhydrazone (a protonophore), diamide (a thiol-cross-linking agent) or *ter*-butylhydroperoxide (a pro-oxidant) release an apoptogenic activity into the supernatant that we have baptized 'apoptosis-inducing factor' (AIF).[40] In addition to these PT-inducing treatments, destruction of mitochondrial membranes via sonication, osmotic shock, or digitonin treatment (which specifically lyses the outer but not the inner membrane, yielding the intermembrane fraction of proteins) also releases AIF, indicating that AIF is pre-formed.[46] Proteinase K treatment as well as mild heat treatment (70°C, 5 min) destroy AIF activity,

Table 2. Why is mitochondrial permeability transition an attractive candidate for the central apoptosis executioner?

Argument	Reference
PT induction is sufficient to induce apoptosis.	44,60
PT is necessary for apoptosis to occur, because its prevention abolishes apoptosis.	40,41,43,60
Opening of PT pores entails multiple potentially lethal alterations of mitochondrial physiology (loss of $\Delta\Psi_m$, uncoupling of respiratory chain, hypergeneration of superoxide anion, loss of mitochondrial glutathione and calcium) and provokes the mitochondrial release of an apoptogenic protease.	16,57,83
The PT pore functions as a sensor for multiple physiological effectors (divalent cations, ATP/ADP, NAD, $\Delta\Psi_m$, NAD, pH, thiols, *etc.*) and thus may allow for the convergence of different death pathways.	57
Opening of the PT pore has self-amplificatory properties in the sense that some consequences of PT themselves induce PT. As a consequence, PT must function in an all-or-nothing fashion, as may be expected from a molecular device deciding between life and death.	40,57
The PT pore is built up by components essential for normal metabolism, a fact that precludes apoptosis-inhibitory (oncogenic) mutations at this level of the apoptotic cascade.	57
Several of the PT pore constituents exist as isoenzymes with strict tissue specificity (ANT, hexokinase, *etc.*), indicating that the pore can be regulated in each cell type in slightly different fashions.	84
Opening of the PT pore is regulated by Bcl-2 (and probably other members of the Bcl-2 family).	46,47

indicating that AIF is a protein. Anion exchange chromatography, molecular sieve chromatography, and SDS-PAGE identify mouse hepatocyte AIF as a single ~50 kDa protein with an estimated isoelectric point of 5.5 (Table 3). AIF is low-abundant (< 0.1% of mitochondrial proteins) and labile at room temperature, requiring purification at 4°C.[46] AIF activity has been detected in mitochondria from several cell types (liver, heart, brain, myelomonocytic cells and lymphoid cells) and species (mouse, human). It appears phylogenetically conserved, since human AIF induces apoptosis in mouse nuclei and vice versa.[40] AIF is present in mitochondria from cells lacking mitochondrial DNA, indicating that it is encoded for by the nuclear rather than by the mitochondrial genome.[40,42]

Purified AIF suffices to induce hallmarks of nuclear apoptosis such as chromatin condensation and oligonucleosomal DNA fragmentation, in the absence of additional cytoplasmic components.[46] AIF thus differs from cytotochrome *c*, which requires additional factors to induce nuclear apoptosis *in vitro*.[53] Moreover, AIF induces nuclear apoptosis much more rapidly (< 15 min)[40] than ceramide, another mitochondrial product implicated in apoptosis, that even at high doses (> 100 μM) requires ≥ 3 h to induce nuclear apoptosis.[55] Purified AIF has no intrinsic DNAse activity, indicating that it probably induces DNA fragmentation via activating pre-existing nuclear DNAses.[46] Although AIF has a proteolytic activity on unidentified nuclear substrates (our unpublished data), it fails to cleave poly (ADP ribose) polymerase (PARP) or lamin in isolated nuclei.[46] As a consequence, the proteolytic spectrum of AIF differs from that of other protease previously implicated in apoptosis induction.[35]

To gain information on the mode of action of AIF, we determined the inhibitory profile of this factor (Table 4). The chromatin condensation-inducing activity of AIF is inhibited by the thiol reagents *p*-chloromercuryphenylsulfonic acid and *N*-phenylmaleimide[46] but not by specific inhibitors of different calcium, serine or cysteine proteases including specific inhibitors of interleukin-1β converting enzyme (ICE) and CPP32/YAMA/apopain (Table 3).[40] The only selective protease inhibitor which blocks AIF activity is *N*-benzyloxycarbonyl-Val-Ala-Asp.fluoromethylketone (Z-VAD.fmk),[46] an inhibitor of ICE-like proteases[9,61–66] Z-VAD.fmk prevents all manifestations of apoptosis induced by AIF: chromatin condensation, oligonucleosomal DNA fragmentation, and DNA loss from nuclei[46] (Table 3). By virtue of its tripeptidic structure, Z-VAD.fmk is thought to be a less specific protease inhibitor than conventional tetrapeptidic inhibitors of ICE or CPP32/YAMA/apopain.[9,61–66] Altogether these data suggest that AIF possesses a cysteine-dependent catalytic activity not identical with but distantly related to proteases from the ICE/CPP32/Ced-3 family. Accordingly, AIF's molecular mass, subcellular localization and proteolytic spectrum differ from those of known members of the ICE/CPP32/Ced-3 family.[36,67] Experiments performed in intact cells indicate that Z-VAD.fmk inhibits apoptosis in both mammalian[9,61–63,65,66] and insect cells,[64] in response to a wide array of apoptosis triggers including inducers of PT such as protoporphyrin IX and mClCCP.[44,46] Although this is not formal proof, this observation underscores the probable importance of AIF as a rate-limiting factor of the apoptotic process *in vivo*.

In conclusion, mitochondria undergoing PT release a Z-VAD.fmk-inhibitable protease, AIF, which is suffi-

Table 3. Properties of apoptosis-inducing factor (AIF)

Physicochemical characteristics of mouse hepatocyte AIF:
One instable protein, one chain
Apparent M.W ~50 kDa, estimated pI ~5.5
Localization:
Constitutively expressed in different cell types including cells lacking mitochondrial DNA
Associated with mitochondrial intermembrane space, not with membranes nor with matrix
Released upon induction of permeability transition
Enzymatic activities:
No DNAse activity
Selective protease activity?
Effects of AIF on isolated nuclei:
Activation of nuclear endonucleases causing oligo-nucleosomal DNA fragmentation
Induction of chromatin condensation and redistribution of nuclear matrix
Proteolysis of selected proteins (not PARP nor lamins)
Inhibitors of apoptogenic effects mediated by AIF:
p-mercuryphenylsulfonic acid (ID_{50} ~250 μM)
N-phenylmaleimide (ID_{50} ~50 μM)
N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (ID_{50} ~30 μM)
Inefficient inhibitors of AIF:
Acetyl-Asp-Glu-Val-Asp-aldehyde (ID_{25} > 250 μM)
Acetyl-Tyr-Val-Ala-Asp-aldehyde (ID_{25} > 250 μM)
Acetyl-Tyr-Val-Ala-Asp-chloromethylketone (ID_{25} > 250 μM)
N-acetyl-Leu-Leu-norleucinal (ID_{25} > 1 mM)
N-acetyl-Leu-Leu-methioninal (ID_{25} > 1 mM)
3-aminobenzamide (ID_{25} > 2.5 mM)
N-benzyloxycarbonyl-Asp-$CH_2OC(O)$-2-6,-dichlorbenzene (ID_{25} > 250 μM)
N-benzyloxycarbonyl-Phe-Ala-fluoromethylketone (ID_{25} > 250 μM)
β-butirolactone (ID_{25} > 1mM)
3,4 dichloroisocoumarin (ID_{25} > 100 μM)
Diisoproylfluorophosphate (ID_{25} > 100 μM)
N-ethylmaleimide (ID_{25} > 1 mM)
Iodoacetamide (ID_{25} > 3 mM)
m-iodobenzylguanidine (ID_{25} > 500 μM)
Leupeptin (ID_{25} > 50 μM)
Thymerosal (ID_{25} > 3 mM)
Trans-epoxysuccinyl-L-leucylamido (4-guanidino)butane (E64) (ID_{25} > 50 μM)
N-tosyl-L-lysyl chloromethylketone (ID_{25} > 1mM)
N-tosyl-L-phenyl chloromethylketone (ID_{25} > 1 mM)

cient and probably necessary to cause nuclear apoptosis. Altogether, our data suggest a two-step-model of apoptosis. At a first level, different apoptosis inducers cause PT, which marks the beginning of the effector stage. PT, in turn, is linked to the release of AIF, which then acts on the nucleus (and further non-nuclear structures?) to initiate the degradation phase of apoptosis.

Mechanism of the anti-apoptotic action of the proto-oncogene product Bcl-2

Bcl-2 belongs to a growing family of proteins which can either inhibit (Bcl-2, Bcl-X_L, Mcl-1, Bfl-1, A1, *etc.*) or favour (Bax, Bcl-X_S, Bad, Bak, Bik, *etc.*) apoptosis.[68–70] The Bcl-2 p26 protein possesses a transmembrane domain allowing for its incorporation into different intracellular membranes, including the outer mitochondrial membrane, the endoplasmatic reticulum and the nuclear envelope.[71–74] In hematopoietic cells, the mitochondrial localization of Bcl-2 is the quantiatively most important one. At least in certain systems, the specific expression of Bcl-2 in the mitochondrion is sufficient and necessary for its apoptosis action.[75–79]

Based on the findings discussed above, we have formulated three alternative hypotheses: (i) Bcl-2 could neutralize AIF or interfere with its apoptogenic action; (ii) Bcl-2 could interfere with AIF formation, *e.g.* by inhibiting the synthesis or the mitochondrial uptake of AIF; (iii) Bcl-2 might interfere with the PT-triggered AIF release from the mitochondrial intermembrane space. These three possibilities are discussed below.

Does Bcl-2 interfere with the action of AIF? Nuclei that are purified from cells transfected with the human *bcl-2* gene contain detectable amounts of Bcl-2 protein in their envelope. Such nuclei were incubated with purified AIF to determine a possible inhibitory effect on its apoptosis-inducing effect. Bcl-2 expressing nuclei undergo apoptotic changes in response to AIF, exactly as this is the case for control nuclei from vector-only-transfected

Table 4. Spectrum of inhibitors of mitochondrial permeability transition (PT) *in vitro* [a]

Inhibitor of PT	Inducer of PT					
	Atractyloside	*ter*-BHP	mClCCP	(500 μM) Ca^{2+}	Diamide	PPIX
Bongkrekate	+[b]	+	+	–	–	+
Phosphotyrosine	+	+	+	–	–	ND
Bcl-2 transfection	+	+	+	–	–	+
Zn^{2+}	+	–	–	–	+	ND
Monochlorobiman	+	–	–	–	+	ND
Cyclosporin A	+	–	–	–	–	+
Ruthenium red	–	–	–	+	–	ND

[a] Tested on purified mouse liver mitochondria during an interval of 60 min. Data are compiled from Refs. 40,44.
[b] Positive signs indicate inhibition ≥ 50% of permeability transition as assessed by large amplitude swelling.

cells. Moreover, both control and Bcl-2-overexpressing nuclei manifest the same pattern of chromatin condensation and DNA fragmentation (Figure 4).[46] This indicates that the nuclear expression of Bcl-2 does not affect the action of AIF. Since Bcl-2 exists also in localizations outside of the nuclear and mitochondrial membrane,[71–73] we evaluated the effect of Bcl-2 overexpression on intact cells exposed to AIF. Introduction of AIF into saponin-treated cells causes the same extent of DNA fragmentation in control and in Bcl-2-hyperexpressing cells.[46] Thus, Bcl-2 has no detectable effect on AIF action, both in cells and in isolated nuclei.

Does Bcl-2 interfere with the formation and/or mitochondrial uptake of AIF? On theoretical grounds, Bcl-2 could influence genetic programmes of gene expression, could interact with the AIF precursor synthesized in the endoplasmatic reticulum or could influence the export of AIF from the cytoplasma to the mitochondrial intermembrane space. We therefore determined whether isolated mitochondria from *bcl-2* transfected cells or vector-only-transfected controls would contain similar amounts of AIF. We found that lysis of control and Bcl-2-overexpressing mitochondria with detergents or osmotic shock releases equal amounts of AIF activity and AIF protein.[46] Therefore, Bcl-2 does not affect the formation and/or mitochondrial uptake of AIF.

Does Bcl-2 interfere with the PT-mediated AIF release? Transfection-enforced hyperexpression of Bcl-2 prevents the disruption of the mitochondrial transmembrane potential that normally precedes apoptosis induced by glucocorticoids or ceramide.[16] The Bcl-2-mediated protection against ceramide-induced $\Delta\Psi_m$ dissipation is observed both in intact cells and in anucleate cells (cytoplasts),[16,45] in which Bcl-2 conserves its death-inhibitory function,[29] indicating that its nuclear localization is dispensable for its function. More importantly, Bcl-2 overexpressed in the outer mitochondrial membrane inhibits the PT induced by a variety of PT inducers (*ter*-butylhydroperoxide, mClCCP, atractyloside, protoporphyrin IX) but not by others (calcium,

Figure 4. Effect of mitochondrial apoptosis inducing factor (AIF) on isolated nuclei *in vitro*. Nuclei were purified either from control vector-only-transfected (Neo) T cell hybridoma cells or from Bcl-2-transfected cells. Isolated nuclei were then incubated in the presence or absence of purified AIF (250 ng/ml; 90 min at 37°C), followed by electron microscopic analysis. Note the peripheral chromatin condensation induced by AIF. The effect of AIF is not influenced by the presence or absence of Bcl-2 in the nuclear envelope. The experiment was performed as described in Ref. 46.

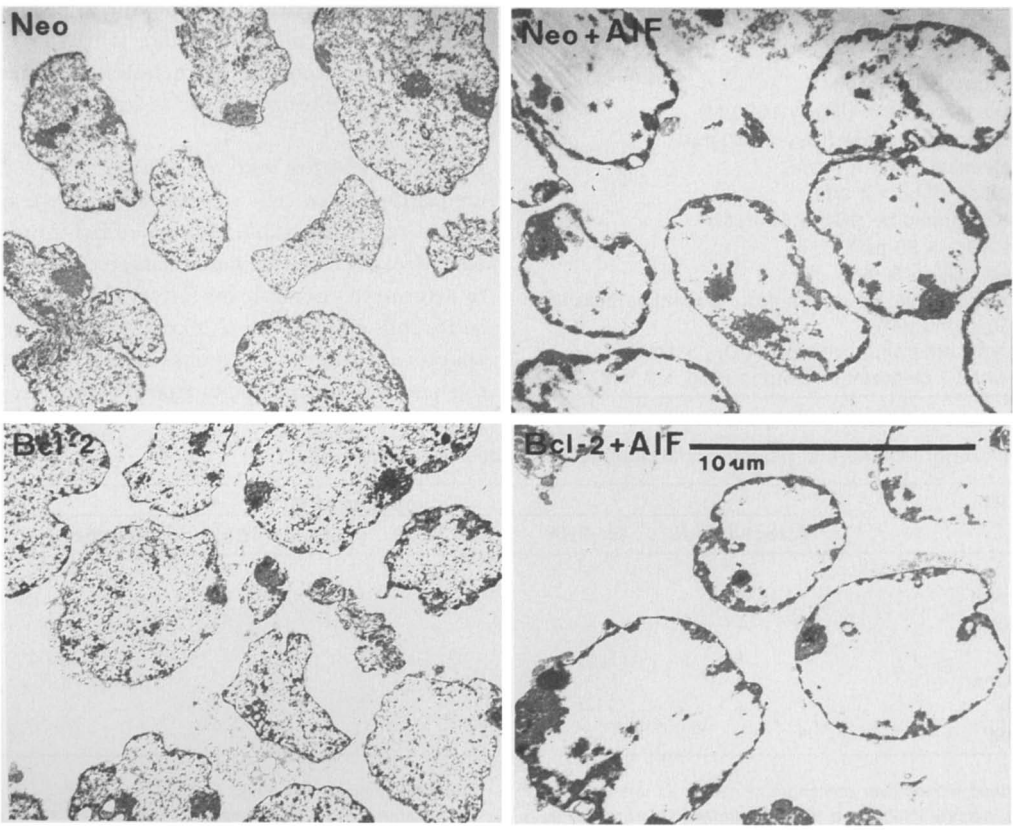


Table 5. Spectrum of inhibitors of mitochondrial permeability transition (PT) in cells[a]

Inhibitor of PT	Inducer of PT			
	DEX	DNA damage	Anti-Fas	Diamide
BA	+	+	–	–
CMXRos	+	+	–	+
Bcl-2	+	+	–	–

[a] Tested on mouse thymocytes or T cell hybridoma cells. Data are compiled from Refs. 43,44,60.
[b] Positive signs indicate significant inhibition of the apoptotic $\Delta\Psi m$ disruption and posterior nuclear apoptosis.

diamide).[41,44,46] This effect is observed in isolated mitochondria (Table 4), correlating with the anti-apoptotic spectrum of Bcl-2 effects in cells (Table 5). For example, the incapacity of Bcl-2 to interfere with diamide-induced PT correlates with its incapacity to inhibit the diamide-triggered $\Delta\Psi_m$ disruption and subsequent nuclear apoptosis in cells.[40] (Tables 4 and 5). Thus, Bcl-2 does exert direct PT-inhibitory effects on mitochondria, although with a limited inhibitory spectrum. The spectrum of activity of Bcl-2 resembles most closely that of BA, a ligand of the ANT (Tables 4 and 5). When inhibiting PT, Bcl-2 prevents the release of AIF from the intermembrane space.[40,46] Thus, AIF is present in the supernatants of control mitochondria treated with atractyloside, *ter*-butylhydroperoxide or mClCCP, yet is absent or greatly reduced in the supernatant of Bcl-2 hyperexpressing mitochondria treated with these reagents.[46]

In synthesis, Bcl-2 suppresses apoptotic PT and AIF release from mitochondria, yet does not interfere with the formation or action of AIF. This interpretation is in accord with previous genetic[75–78] and functional data,[29,49,78] suggesting that, at least in some experimental systems, the mitochondrial but not the nuclear localization of Bcl-2 would determine its anti-apoptotic capacity. In the mitochondrion, Bcl-2 demonstrates a patchy distribution to the contact sites between the outer and the inner mitochondrial membrane.[74] Bcl-2 expression correlates in a quasi-stoichiometric fashion with that of the peripheral benzodiazepine receptor (one of the putative constituents of the PT pore,[80] see Figure 2) suggesting that Bcl-2 might associate with the PBR or with a PBR-associated protein.[81] Thus, it is conceivable that Bcl-2 or its homologs might exert a direct regulatory effect on the PT pore complex. However, the mechanism via which Bcl-2 prevents PT remains to be clarified. This question is presently addressed in our laboratory.

Concluding remarks and perspectives

The available data are compatible with our current working hypothesis that mitochondrial PT is a central co-ordinating event of the apoptotic effector phase (Figure 3). This hypothesis predicts that various 'private' pathways of apoptosis converge at the level of PT, in line with the fact that PT can be induced by numerous physiological effectors (Table 1). Once PT has been triggered, a series of common pathways of apoptosis are initiated, each of which may be lethal. Thus, PT causes at least three common, yet dissociable hallmarks of the apoptotic degradation phase: (i) changes in the cytoplasmic redox state such as depletion of reduced glutathione and enhanced generation of reactive oxygen species (ROS);[16] (ii) exposure of phosphatidylserine residues on the cell surface;[45] and (iii) nuclear apoptosis[40] (Figure 3).

The central role of PT in the apoptotic cascade might allow for an operative reinterpretation of a number of hitherto controversial and apparently contradictory observations. First, it predicts a dual role of ROS in the apoptotic process, either as a facultative inducer of PT in determined private pathways of the apoptotic inducer phase or as a common by-product of PT. Second, it explains why Bcl-2 can both inhibit the effects of ROS (via inhibition of ROS-mediated PT) and the generation of ROS (which is a consequence of PT).[15,82] Third, it may explain other pleiotropic effects of Bcl-2. If Bcl-2 acts as an inhibitor of apoptosis at the level of PT, it must prevent all downstream events such as ROS generation, increases in cytosolic and nuclear calcium levels or activation of proteases and nucleases. Fourth, this model may provide clues to decipher the hierarchy between the 'checkpoints' controlled by Bcl-2 and specific proteases.[34] Whereas some proteases (such as ICE) would serve directly or indirectly as inducers of PT,[43,45] other proteases released from the mitochondrion and/or activated by mitochondrial products such as AIF might act downstream of PT.[44,46] Thus, it is possible that the regulation of PT is intertwined with that of specific proteases.

Future studies will have to explore numerous incognita: the nature of the PT-inducing trigger in different private pathways of apoptosis induction, the molecular partner(s) of Bcl-2 and its homologs at the level of the outer-inner membrane contact sites, the putative relationship between AIF and proteases of the

ICE/CPP32/Ced-3 family, the molecular biology of AIF formation and release and the essential molecular targets of AIF. Moreover, our hypothesis will require the performance of Popperian falsification experiments. Does neutralization of AIF using truly specific inhibitors or neutralizing antibodies retard or abolish apoptosis? Does genetic manipulation of AIF (knock-out, targeting to other subcellular compartments than mitochondria) influence apoptosis? Are the AIF substrates identified in cell-free experiments also cleaved in naturally occurring apoptosis *in vitro?* Only an affirmative answer to all these questions will allow us to maintain our working hypothesis without further modifications.

Acknowledgements

We are indebted to Drs Maria Castedo, Tamara Hirsch, Isabel Marzo, Antonio Macho and Patrice X. Petit (UPR420, CNRS) for stimulating discussions. Electron microscopic data were provided by Dr Maurice Geuskens (Université Libre de Bruxelles, Belgium).

References

1. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. *Science* 1995; **267**: 1456–1462.
2. Kroemer G, Petit PX, Zamzami N, Vayssière J-L, Mignotte B. The biochemistry of apoptosis. *FASEB J* 1995; **9**: 1277–1287.
3. Cohen IJ, Duke RC, Fadok VA, Sellins KS. Apoptosis and programmed cell death in immunity. *Ann Rev Immunol* 1992; **10**: 267–293.
4. Osborne BA, Smith SW, Liu Z-G, McLaughlin KA, Grimm L, Schwartz LM. Identification of genes induced during apoptosis in T cells. *Immunol Rev* 1994; **142**: 301–320.
5. Weil M, Jacobson MD, Coles HSR, *et al.* Constitutive expression of the machinery for programme cell death. *J Cell Biol* 1996; **133**: 1053–1059.
6. Kroemer G, Martínez-A C. Pharmacological inhibition of programmed lymphocyte cell death. *Immunol Today* 1994; **15**: 235–242.
7. Kroemer G. The pharmacology of T cell apoptosis. *Adv Immunol* 1995; **58**: 211–296.
8. Ishizaki Y, Cheng L, Mudge AW, Raff MC. Programmed cell death by default in embryonic cells, fibroblasts, and cancer cells. *Mol Biol Cell* 1995; **1995**: 1443–1458.
9. Jacobson MD, Weil M, Raff MC. Role of Ced-3/ICE-family proteases in staurosporine-induced programmed cell death. *J Cell Biol* 1996; **133**: 1041–1051.
10. Colombel M, Olsson CA, Ng P-Y. *Cancer Res* 1992; **52**: 4313–4319.
11. Evan GI, Wyllie AH, Gilbert CS, *et al.* Induction of apoptosis in fibroblasts by c-myc protein. *Cell* 1992; **69**: 119–128.
12. Yonish-Rouach E, Resnitzky D, Lotem J, Sachs L, Kimchi A, Oren M. Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. *Nature* 1991; **352**: 345–347.
13. Norbury C, MacFarlande M, Fearnhead H, Cohen GM. CDC2 activation is not required for thymocyte apoptosis. *Biochem Biophys Res Comm* 1994; **202**: 1400–1406.
14. Buttke TM, Sandstrom PA. Oxidative stress as a mediator of apoptosis. *Immunol Today* 1994; **15**: 7–10.
15. Hockenbery DM, Oltvai ZN, Yin X-M, Milliman CL, Korsmeyer SJ. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* 1993; **75**: 241–251.
16. Zamzami N, Marchetti P, Castedo M, *et al.* Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. *J Exp Med* 1995; **182**: 367–377.
17. Yao KS, Clayton M, O'Dwyer PJ. Apoptosis in human adenocarcinoma HT29 cells induced by exposure to hypoxia. *JNCI* 1995; **87**: 117–122.
18. Shimizu S, Eguchi Y, Kosaka H, Kamlike W, Matsuda H, Tsujimoto Y. Prevention of hypoxia-induced cell death by Bcl-2 and Bcl-xL. *Nature* 1995; **374**: 811–813.
19. Graeber TG, Osmanian C, Jacks T, *et al.* Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumors. *Nature* 1996; **379**: 88–91.
20. Hug H, Enari M, Nagata S. No requirement of reactive oxygen intermediates in Fas-mediated apoptosis. *FEBS Lett* 1994; **351**: 311–313.
21. Jacobson MD, Raff MC. Programmed cell death and Bcl-2 protection in very low oxygen. *Nature* 1995; **374**: 814–816.
22. McConkey DJ, Hartzell P, Nicotera P, Orrenius S. Calcium-activated DNA fragmentation kills immature thymocytes. *FASEB J* 1989; **3**: 1843–1849.
23. McConkey DJ, Nicotera P, Orrenius S. Signalling and chromatin fragmentation in thymocyte apoptosis. *Immunol Rev* 1994; **142**: 343–363.
24. Obeid LM, Linardic CM, Karolak LA, Hannun YA. Programmed cell death induced by ceramide. *Science* 1993; **259**: 1769–1771.
25. Haimovitz-Friedman A, Kan C-C, Ehleitner D, *et al.* Ionizing radiation acts on cellular membranes to generate ceramide and initiate apoptosis. *J Exp Med* 1994; **180**: 525–535.
26. Santana P, Peña LA, Haimovitz-Friedman A, *et al.* Acid sphingomyelinase-deficient human lymphoblasts and mice are deficient in radiation-induced apoptosis. *Cell* 1996; **86**: 189–199.
27. Marchetti P, Zamzami N, Susin SA, Patrice PX, Kroemer G. Apoptosis of cells lacking mitochondrial DNA. *Apoptosis* 1996; **1**: 119–125.
28. Willie AH. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* 1980; **284**: 555–556.
29. Jacobson MD, Burne JF, Raff MC. Programmed cell death and Bcl-2 protection in the absence of a nucleus. *EMBO J* 1994; **13**: 1899–1910.
30. Schulze-Osthoff K, Walczak H, Droge W, Krammer PH. Cell nucleus and DNA fragmentation are not required for apoptosis. *J Cell Biol* 1994; **127**: 15–20.
31. Nakajima H, Golstein P, Henkart PA. The target cell nucleus is not required for cell-mediated granzyme- or Fas-based cytotoxicity. *J Exp Med* 1995; **181**: 1905–1909.
32. Peitsch MC, Mannherz HG, Tschopp J. The apoptotic endonucleases: cleaning up after cell death? *Trends Cell Biol* 1994; **4**: 37–41.
33. Henkart PA. Apoptosis: O death, where is thy sting? *J Immunol* 1995; **154**: 4905–4908.
34. Martin SJ, Green DR. Protease activation during apoptosis: death by a thousand cuts? *Cell* 1995; **82**: 349–352.
35. Lazebnik YA, Takahashi A, Poirier GG, Kaufman SH, Earnshaw WC. Characterization of the execution phase of apoptosis in vitro using extracts from condemned-phase cells. *J Cell Sci*

1995; S19: 41–49.
36. Henkart PA. ICE family proteases: Mediators of all apoptotic cell death? *Immunity* 1996; 4: 195–201.
37. Zamzami N, Marchetti P, Castedo M, *et al.* Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. *J Exp Med* 1995; 181: 1661–1672.
38. Macho A, Castedo M, Marchetti P, *et al.* Mitochondrial dysfunctions in circulating T lymphocytes from human immunodeficiency virus-1 carriers. *Blood* 1995; 86: 2481–2487.
39. Castedo M, Macho A, Zamzami N, *et al.* Mitochondrial perturbations define lymphocytes undergoing apoptotic depletion in vivo. *Eur J Immunol* 1995; 25: 3277–3284.
40. Zamzami N, Susin SA, Marchetti P, *et al.* Mitochondrial control of nuclear apoptosis. *J Exp Med* 1996; 183: 1533–1544.
41. Zamzami N, Marchetti P, Castedo M, *et al.* Inhibitors of permeability transition interfere with the disruption of the mitochondrial transmembrane potential during apoptosis. *FEBS Lett* 1996; 384: 53–57.
42. Marchetti P, Susin SA, Decaudin D, *et al.* Apoptosis-associated derangement of mitochondrial function in cells lacking mitochondrial DNA. *Cancer Res* 1996; 56: 2033–2038.
43. Marchetti P, Castedo M, Susin SA, *et al.* Mitochondrial permeability transition is a central coordinating event of apoptosis. *J Exp Med* 1996; 184: 1155–1160.
44. Marchetti P, Hirsch T, Zamzami N, *et al.* Mitochondrial permeability transition triggers lymphocyte apoptosis. *J Immunol* 1996; 157: 4830–4836.
45. Castedo M, Hirsch T, Susin SA, *et al.* Sequential acquisition of mitochondrial and plasma membrane alterations during early lymphocyte apoptosis. *J Immunol* 1996; 157: 512–521.
46. Susin SA, Zamzami N, Castedo M, *et al.* Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. *J Exp Med* 1996; 184: 1331–1341.
47. Kroemer G, Zamzami N, Susin SA. Mitochondrial control of apoptosis. *Immunol Today* 1996; in press.
48. Vayssière J-L, Petit PX, Risler Y, Mignotte B. Commitment to apoptosis is associated with changes in mitochondrial biogenesis and activity in cell lines conditionally immortalized with simian virus 40. *Proc Natl Acad Sci USA* 1994; 91: 11752–11756.
49. Newmeyer DD, Farschon DM, Reed JC. Cell-free apoptosis in xenopus egg extracts: inhibition by Bcl-2 and requirement for an organelle fraction enriched in mitochondria. *Cell* 1994; 79: 353–364.
50. Petit PX, LeCoeur H, Zorn E, Dauguet C, Mignotte B, Gougeon ML. Alterations of mitochondrial structure and function are early events of dexamethasone-induced thymocyte apoptosis. *J Cell Biol* 1995; 130: 157–167.
51. Cossarizza A, Franceschi C, Monti D, *et al.* Protective effect of N-acetylcysteine in tumor necrosis factor-alpha-induced apoptosis in U937 cells: the role of mitochondria. *Exp Cell Res* 1995; 220: 232–240.
52. Polla BS, Kantengwa S, Francois D, *et al.* Mitochondria are selective targets for the protective effects of heat shock against oxidative injury. *Proc Natl Acad Sci USA* 1996; 93: 6458–6463.
53. Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptic program in cell-free extracts: requirement for dATP and cytochrome C. *Cell* 1996; 86: 147–157.
54. Krippner A, Matsuno-Yagi A, Gottlieb RA, Babior BM. Loss of function of cytochrome c in Jurkat cells undergoing Fas-mediated apoptosis. *J Biol Chem* 1996; 271: 21629–21636.
55. Martin SJ, Newmeyer DD, Mathisa S, *et al.* Cell-free reconstitution of Fas-, UV radiation- and ceramide-induced apoptosis. *EMBO J* 1995; 14: 5191–5200.
56. Attardi G, Schatz G. Biogenesis of mitochondria. *Ann Rev Cell Biol* 1988; 4: 289–333.
57. Zoratti M, Szabò, I. The mitochondrial permeability transition. *Biochem Biophys Acta Rev Biomembr* 1995; 1241: 139–176.
58. Bernardi P, Petronilli V. The permeability transition pore as a mitochondrial calcium release channel; a critical appraisal. *J Bioenerg Biomembr* 1996; 28: 129–136.
59. Nicolli A, Basso E, Petronilli V, Wenger RM, Bernardi P. Interactions of cyclophilin with mitochondrial inner membrane and regulation of the permeability transition pore, a cyclosporin A-sensitive channel. *J Biol Chem* 1996; 271: 2185–2192.
60. Marchetti P, Decaudin D, Macho A, *et al.* Redox regulation of apoptosis: impact of thiol redoxidation on mitochondrial function. *Eur J Immunol* 1997; in press.
61. Zhivotovsky B, Gahm A, Ankarcrona M, Nicotera P, Orrenius S. Multiple proteases are involved in thymocyte apoptosis. *Exp Cell Res* 1995; 221: 404–412.
62. Fearnhead HO, Dinsdale D, Cohen GM. An interleukin-1 beta-converting enzyme-like protease is a common mediator of apoptosis in thymocytes. *FEBS Lett* 1995; 375: 283–288.
63. Zhu HJ, Fearnhead HO, Cohen GM. An ICE-like protease is a common mediator of apoptosis induced by diverse stimuli in human monocytic THP.1 cells. *FEBS Lett* 1995; 374: 303–308.
64. Pronk GJ, Ramer K, Amiri P, Williams LT. Requirement of an ICE-like protease for induction of apoptosis and ceramide generation by REAPER. *Science* 1996; 271: 808–810.
65. Cain K, Inayathussain SH, Couet C, Cohen GM. A cleavage-site-directed inhibitor of interleukin 1 beta-converting enzyme-like proteases inhibits apoptosis in primary cultures of rat hepatocytes. *Biochem J* 1996; 314: 27–32.
66. Slee EA, Zhu HJ, Chow SC, Macfarlane M, Nicholson DW, Cohen GM. Benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD.fmk) inhibits apoptosis by blocking the processing of CPP32. *Biochem J* 1996; 315: 21–24.
67. Patel T, Gores GJ, Kaufman SH. The role of proteases during apoptosis. 1996; 10: 587–597.
68. Reed JC. Bcl-2 and the regulation of programmed cell death. *J Cell Biol* 1995; 124: 1–6.
69. Cory S. Regulation of lymphocyte survival by the Bcl-2 gene family. *Ann Rev Immunol* 1995; 13: 513–543.
70. Yang E, Korsmeyer SJ. Molecular Thanatopsis: A discourse on the Bcl-2 family and cell death. *Blood* 1996; 88: 386–401.
71. Krajewski S, Tanaka S, Takayama S, Schibler MJ, Fenton W, Reed JC. Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. *Cancer Res* 1993; 53: 4701–4714.
72. Lithgow T, Vandriel R, Bertram JF, Strasser A. The protein product of the oncogene Bcl-2 is a component of the nuclear envelope, the endoplasmic reticulum, and the outer mitochondrial membrane. *Cell Growth Differ* 1994; 5: 411–417.
73. Janiak F, Leber B, Andrews DW. Assembly of Bcl-2 into microsomal and outer mitochondrial membranes. *J Biol Chem* 1994; 269: 9842–9849.
74. Riparbelli MG, Callaini G, Tripodi SA, Cintorino M, Tosi P, Dallai R. Localization of the Bcl-2 protein to the outer mitochondrial membrane by electron microscopy. *Exp Cell Res* 1995; 221: 363–369.
75. Tanaka S, Saito K, Reed JC. Structure-function analysis of the Bcl-2 oncoprotein. Addition of a heterologous transmembrane domain to portions of the Bcl-2β protein restores function as a regulator of cell survival. *J Biol Chem* 1993; 268: 10920–10926.

76. Nguyen M, Branton PE, Walton PA, Oltvai ZN, Korsmeyer SJ, Shore GC. Role of membrane anchor domain of Bcl-2 in suppression of apoptosis caused by E1B-defective adenovirus. *J Biol Chem* 1994; **269**: 16521–16524.
77. Hanada M, Aimesempe C, Sato T, Reed JC. Structure-function analysis of Bcl-2 protein identification of conserved domains important for homodimerization with Bcl-2 and heterodimerization with Bax. *J Biol Chem* 1995; **270**: 11962–11969.
78. Greenhalf W, Stephan C, Chaudhuri B. Role of mitochondria and C-terminal membrane anchor of Bcl-2 in Bax induced growth arrest and mortality in Sacharomyces cerevisiae. *FEBS Lett* 1996; **380**: 169–175.
79. Zhu W, Cowie A, Wasfy GW, Penn LZ, Leber B, Andrews DW. Bcl-2 mutants with restricted subcellular localization reveal spatially distinct pathways for apoptosis in different cell types. *EMBO J* 1996; **15**: 4130–4141.
80. McEnery MW, Snowman AM, Trifiletti RR, Snyder SH. Isolation of the mitochondrial benzodiazepine receptor: Association with the voltage-dependent anion channel and the adenine nucleotide carrier. *Proc Natl Acad Sci USA* 1992; **89**: 3170–3174.
81. Carayon P, Portier M, Dussossoy D, *et al*. Involvement of peripheral benzodiazepine receptors in the protection of hematopoietic cells against oxygen radical species. *Blood* 1996; **87**: 3170–3178.
82. Kane DJ, Sarafian TA, Anton R, *et al*. Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. *Science* 1993; **262**: 1274–1277.
83. Reed DJ, Savage MK. Influence of metabolic inhibitors on mitochondrial permeability transition and glutathione status. *Biochem Biophys Acta* 1995; 1271: 43–50.
84. Walker JE, Runswick MJ. The mitochondrial transport protein superfamily. *J Bioenerg Biomembr* 1993; **25**: 435–446.