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REVIEW

Apoptosis in Unicellular Organisms: Mechanisms and Evolution

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Abstract—Data about the programmed death (apoptosis) in unicellular organisms, from bacteria to ciliates, are discussed. Firstly apoptosis appeared in lower eukaryotes, but its mechanisms in these organisms are different from the classical apoptosis. During evolution, the apoptotic process has been improving gradually, with reactive oxygen species and Ca^{2+} playing an essential role in triggering apoptosis. All eukaryotic organisms have apoptosis inhibitors, which might be introduced by viruses. In the course of evolution, caspases and apoptosis-inducing factor appeared before other apoptotic proteins, with so-called death receptors being the last among them. The functional analogs of eukaryotic apoptotic proteins take parts in the programmed death of bacteria.

Key words: apoptosis, bacteria, Ca^{2+}, caspases, death receptors, evolution, eukaryotes, reactive oxygen species

Apoptosis, or programmed cell death, is a regulated process of cell self-killing. It plays an important role in individual development and the maintenance of the tissue homeostasis in multicellular organisms. Disturbances of apoptosis regulation bring about the development of several kinds of illnesses [1].

In multicellular organisms, every cell is ready to die if it is necessary for the whole organism. But recent studies have indicated that programmed cell death could also take place in primitive single-celled eukaryotes [2, 3] and prokaryotes. What is the aim of this process in unicellular organisms? When did programmed cell death appear in phylogenesis? And how did cells manage to die when necessary? We discuss these questions in this paper. But let us first provide a brief review of the classical mammalian apoptosis.

WHAT IS APOPTOSIS?

Apoptosis principally differs from programmed necrosis [4] by several biochemical and structural characteristics. Apoptosis involves separation of cells from the matrix [5], chromatin condensation, cytoplasmic shrinkage, plasma membrane blebbing, and swelling of the outer mitochondrial membrane. This results in the formation of specific apoptosis bodies, membranous vesicles with cellular content consumed by macrophages and adjacent cells. Molecular changes during apoptosis include internucleosomal DNA cleavage and randomization of the distribution of phosphatidylserine between the inner and outer leaflets of the plasma membrane. Macrophages excrete specific glycoprotein MFG-E8 (milk fat globule-EGF-factor 8), which is subjected to specific binding with phosphatidylserine at the surface of apoptotic cells [6]. This is probably a marker for macrophages to discriminate dead cells. Such morphological and molecular changes are observed in cells from diverse tissue types and species [7]. In contrast, necrosis involves cell swelling, damage to organelles, disruption of membrane integrity, and cell lysis. Both apoptosis and necrosis are accompanied by rapid ATP depletion and dramatic changes in mitochondrial ultrastructure [8, 9].

Four principal components of apoptotic biochemical mechanisms are usually considered: 1) Cys-Asp-proteases or caspases; 2) specific "death receptors" at the cell surface; 3) mitochondria and cytochrome *c* released from them, and 4) specific pro- and anti-apoptosis proteins [10]. Such secondary messengers as Ca^{2+}, reactive oxygen species (ROS), and nitric oxide (NO) are also very important for apoptosis [11].

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MAIN COMPONENTS OF APOPTOSIS

Caspases (a family of cysteine aspartate-specific proteases) play the central role in triggering apoptosis [12]. The caspases family in mammals consists of 14 proteins, which are constantly synthesized almost in all cells in the form of proenzymes (zymogens) that are activated during apoptosis. Procaspases (32-56 kD) contain four domains: N-terminal domain, larger (17-21 kD) and smaller (10-13 kD) subunits, and a short linker region between the large and small subunits [12]. Caspase activation involves proteolytic processing of the proenzyme at specific aspartate residues between the domains, resulting in removal of the N-terminal domain as well as the linker region and formation of a heterodimer containing one large and one small subunit [13]. The active caspase is a tetramer composed of two such heterodimers [14].

Evidence for the sequential activation of caspases has lead to the concept of a caspase cascade [15]. This cascade begins with autocatalytic activation of initiator caspases that, in turn, transmit the signal by cleaving and thereby activating the downstream effector caspases [16]. There are initiator caspases (procaspase-2, -8, -9, -10, -12) and effector caspases (procaspase-3, -6, -7). Cleavages by effector caspases result in diminished integrity of the actin and intermediate filament networks within the cell, inhibition of protein synthesis, and activation of DNase [17]. Other caspases (procaspase-1, -4, -5, -11, -13) are not involved in apoptosis [12]. They are involved in inflammatory processes and, along with the effector caspases, in the proliferation of T-lymphocytes and terminal differentiation of epithelial cells of the lens and keratinocytes [14, 18].

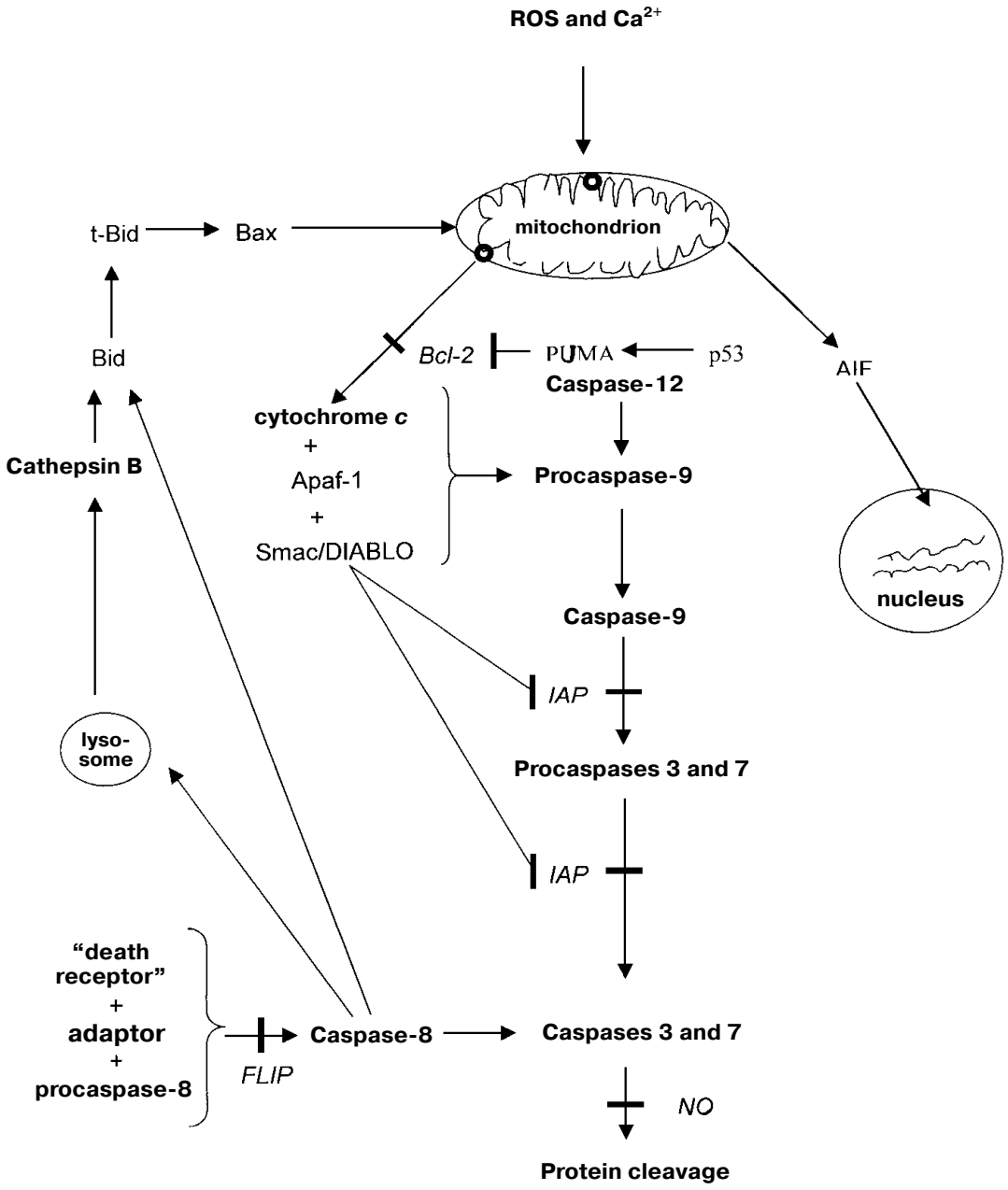
Cell-surface "death receptors". Apoptosis is rapidly induced in mammals by activation of "death-inducing signaling complexes" at the plasma membrane. They are formed when extracellular ligands, such as Fas and TNF (tumor necrosis factor), interact with cell surface receptors belonging to the cysteine-rich tumor necrosis factor receptor (TNFR) gene superfamily [10], called the "death receptors". The death receptors Fas (also called CD95 or Apo1) and TNFR1 (also called p55 or CD120a) are two of the best characterized. They trigger activation of caspase-8 through the formation of a death-inducing signaling complex containing a death receptor, adapters such as TRADD (tumor necrosis factor receptor-1-associated protein with death domain) and FADD (Fas-associated protein with death domain)/MORT1, and the procaspase-8 [16]. Activated caspase-8 is released into the cytoplasm and initiates a protease cascade that activates downstream effector caspases, including caspase-3 [19]. Caspase-3 is the convergence point of the death receptor and **mitochondrial pathways** of caspase activation [12] (figure).

Mitochondria have emerged as the central processing organelles in the majority of apoptotic pathways in mammals. Signals from cell surface death receptors or from damaged sites converge on mitochondria, leading to permeabilization of both mitochondrial membranes, dissipation of the inner membrane transmembrane potential ($\Delta\Psi_m$), and release of apoptosis-related proteins such as apoptosis-inducing factor (AIF) [20], SMAC (second mitochondria-derived activator of caspases), and certain procaspases from the intermembrane space.

The release of these proteins results from the opening of a large conductance channel known as the permeability transition pore complex (PTPC), which is formed at contact sites of the outer and inner mitochondrial membranes. The PTPC is thought to be comprised of at least three transmembrane proteins: the 30-kD inner membrane adenine nucleotide translocator; the 32-kD outer membrane voltage-dependent anion channel or mitochondrial porin, and the 18-kD outer membrane peripheral benzodiazepine receptor [21-23]. There is also evidence for the association of other proteins with the PTPC: members of the Bax/Bcl-2 family, cyclophilin D, and enzymes of energy metabolism, such as hexokinase and mitochondrial creatine kinase. Binding of Ca^{2+} is accompanied by the formation of the permeability transition pore with diameter 2.6-2.9 nm, which could transmit low-molecular-weight substances with $M_r \leq 1500$, bringing about rapid release of Ca^{2+}, reduction of $\Delta\Psi_m$, and high-amplitude mitochondrial swelling [21-24]. Opening of the permeability transition pore is stimulated by several factors, such as inorganic phosphate, prooxidants, SH-reagents, and depletion of the mitochondrial ATP pool. On the contrary, Mg^{2+} [25], adenine nucleotides [21, 24], antioxidants, spermine [26], and cyclosporin A [27] facilitate the closure of the pore. The opening of the Ca^{2+}-dependent permeability transition pore is considered as a way of efficient removal of the cations from mammalian mitochondria when the concentration of free Ca^{2+} ($[Ca^{2+}]_{in}$) reaches high values and as a key event in triggering apoptosis [1, 21, 24, 28] and necrosis [29, 30] of animal cells.

Cytochrome *c*, released from the intermembrane space of mitochondria into the cytosol together with specific apoptotic proteins, binds to Apaf-1 (apoptotic protease activating factor-1) to form the apoptosome complex and initiate activation of the cascade of caspases. With the aid of Smac/DIABLO and Omi/HtrA2 (Omi stress-regulated endopeptidase/high temperature requirement protein A2), cytochrome *c* triggers Apaf-1-dependent activation of caspase-9 [31]. Caspase-9 cleaves and activates procaspase 3 and 7 (figure); these effector caspases are responsible for the cleavage of various proteins leading to biochemical and morphological features characteristic of apoptosis [32].

Cytochrome *c* is bound to the inner membrane by anionic phospholipids, primarily cardiolipin. This binding involves at least two conformations: i) a loosely bound conformation provided by electrostatic (i.e., ion–ion



A common scheme of classical mammalian apoptosis (based on the scheme from [11]). ROS and Ca^{2+} activate the opening of the mitochondrial permeability transition pore, which causes swelling, rupture of the outer membrane, and the release into the cytoplasm of cytochrome *c* and AIF. Cytochrome *c* forms a complex with cytosolic Apaf-1, Smac/DIABLO, and procaspase-9, producing active caspase-9. This in turn activates procaspase 3 and 7. Caspases activation processes are blocked by inhibitors of apoptosis proteins (IAP), which, in turn, are inhibited by Smac/DIABLO. Caspase-12 could activate caspase-9 without participation of cytochrome *c* [39]. Procaspases 3 and 7 are also activated by caspase-8, which, in turn, are activated by so-called "death inducing signal complexes" in plasmalemma. They consist of the "death receptor" and specific adaptor, interacting with procaspase-8. Interacting with them FLIP protein blocks activation of caspase-8 [18]. Caspase-8 releases activated cathepsin B from lysosomes; further one of them (cathepsin B or caspase-8) activates by partial cleaving of cytosolic protein Bid. Then activated protein tBid activates another proapoptotic protein Bax. Interacting with mitochondrial protein porin, it forms channels for cytochrome *c* and AIF in the outer mitochondrial membrane [41]. AIF directs immediately to the nucleus and causes DNA degradation. Bcl-2 prevents the release of cytochrome *c* from mitochondria. (In contrast, Bax stimulates it via channel formation.) P53 activates PUMA. Then PUMA stimulates cytochrome *c* release via Bcl-2-binding [44]. NO blocks apoptosis via selective nitrosylation of effector caspases [11]

interaction with positively charged lysine residues of cytochrome *c* and negatively charged phosphate groups of cardiolipin [33]), and ii) a tightly bound conformation wherein hydrophobic interactions accompany a loosening of the tertiary structure, resulting in partial embedding of the protein into the membrane [34].

In either case, because of its association with cardiolipin, it seems that permeabilization of the outer membrane alone would be insufficient to stimulate the release of cytochrome *c*. Electrostatically bound cytochrome *c* is mobilized by changes in ionic strength, surface-charge density, or pH [35], and hydrophobically bound when mitochondrial lipids are subjected to oxidative modification [36].

Thus, mitochondria are not only the powerhouse of energy generation within the eukaryotic cell but they also play a major role in inducing apoptotic cell death through the release of redox proteins such as cytochrome *c* and AIF. AIF homologs are present in animals and plants, eubacteria, and archaebacteria [37]. Recent evidence indicates that some present day prokaryotes release redox proteins that induce apoptosis in mammalian cells through stabilization of the tumor suppressor protein p53. This is very interesting within the context of the symbiotic theory of the origin of mitochondria [38].

Nonetheless, cytochrome *c* is not always necessary for the apoptosis chain [9]. For example, apoptosis of cardiac tissue is not accompanied its release from the intermembrane space [9]. Caspase-12 could activate caspase-9 even without its participation [39] (figure).

It was shown on isolated liver mitochondria that cytochrome *c* release occurs by distinct mechanisms that are either Ca^{2+}-dependent or Ca^{2+}-independent [40]. In the first case, mitochondrial Ca^{2+} overload promotes the opening of the permeability transition pore. This increased permeability of the inner mitochondrial membrane leads to matrix swelling, rupture of the outer mitochondrial membrane, and the release of cytochrome *c*. Ca^{2+}-independent cytochrome *c* release seems to be governed by different members of the Bcl-2 family of proteins (B-cell leukemia-2).

Bcl-2 family proteins. Bcl-2 family members regulate apoptosis, induced by many apoptotic stimuli, primarily at the level of the mitochondria. Anti-apoptotic members of this large protein family include Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Al, and Boo; whereas pro-apoptotic members include Bax, Bad, Bok, Bcl-xS, Bak, Bid, Bik, Bim, Krk, and Mtd.

Cytosolic Bid (p22) is cleaved by caspase-8 activated by the death receptors and lysosomal cathepsin proteases. The resulting protein, truncated Bid (t-Bid), then changes the conformation of another pro-apoptosis protein, Bax, which integrates into the membrane and forms a complex with porin. They form ion channels, so that cytochrome *c* and other proteins are released from the intermembrane space [41] (figure).

Proteins of this family are usually linked with the cytoskeleton and after the inductions of apoptosis are transmitted to mitochondria where they deactivate anti-apoptosis proteins integrated in their membranes. Bcl-xL and Bax can form ion channels in artificial membranes, suggesting regulation of apoptosis *via* the formation of pores. Bcl-2 acts as an antioxidant and blocks the release of cytochrome *c* (figure). It is hypothesized that the ratio between pro- and anti-apoptosis members of the family determines whether the cell will live or die [9].

Cathepsin B (cat B), a lysosomal cysteine protease, has recently been implicated in mammalian apoptosis. Caspase-8 caused release of active cat B from purified lysosomes and that cat B, in turn, increased cytosol-induced release of cytochrome *c* from mitochondria [42] (figure).

P53 is involved in the induction of apoptosis caused by DNA damage, activation of oncogenes, and hypoxia [43] interacting with Bax, stimulating the death receptors and activating apoptosis genes. p53 activates so-called PUMA (p53 upregulated modulator of apoptosis), which then binds Bcl-2, thereby stimulating the release of cytochrome *c* from mitochondria [44] (figure).

Several Ca^{2+}-binding proteins, for example apoptosis-linked gene-2 (**ALG-2**), take parts in apoptosis too. ALG-interacting protein X (**Alix**), also known as AIP1, may regulate apoptosis since it binds ALG-2 [45]. The molecule ALG-2 includes sorcin, peflin, grancalcin, and calpain [46].

Unlike caspases and cathepsins, **calpains** specifically cleave proteins containing a region rich in proline, aspartate, serine, and threonine residues called PAST motifs [47]. They include DNA ligase III [48] containing such a motif in the N-terminal portion. Additionally, calpains release caspases from bound inhibitors of apoptosis proteins [49].

Inhibitors of apoptosis proteins. Because of the potentially dire consequences of inadvertent caspase activation, caspase activity within cells is tightly regulated. A family of polypeptides known as IAPs (inhibitors of apoptosis proteins) has been implicated in this process. First identified in baculoviruses [50], IAPs homologs have been found in eukaryotic species from yeast to mammals [51]. In mammals, there are currently eight known IAP family members, all of which contain from one to three ~70-amino-acid BIR (baculoviruses inhibitor of apoptosis repeat) domains [52]. X-Chromosome linked IAP (XIAP) [53] and cellular inhibitors of apoptosis protein (cIAP1,2) each having three N-terminal BIR domains followed by a C-terminal RING (really interesting new gene) finger that has ubiquitin-ligase activity [54]. X-Chromosome linked IAP and cellular inhibitors of apoptosis protein (cIAP1,2) have been shown to inhibit caspases 3, 7, and 9 [55]. Zinc finger-like BIR domains directly bind to active caspases, allowing BIR linker sequences to block the caspase-active sites [56]. The

RING domain mediates caspase ubiquitination and degradation [57]. The 76-amino-acid-residue protein ubiquitin represents a kind of "black mark", because the proteins bound it are subjected to proteolysis [58].

As in the case with other protease inhibitors, XIAP, cIAP1, cIAP2 are themselves subject to negative regulation. In particular, the 25-kD mitochondrial polypeptide Smac/DIABLO (direct IAP-binding protein with low p*I*) has been shown to bind these IAPs and disrupt their binding to caspases [59]. During apoptosis, Smac/DIABLO is released from mitochondria into the cytosol, where it binds to IAPs and facilitates caspase activation [60]. Omi/HtrA2 binds IAP proteins and also suppresses their ability to inhibit caspases [61]. At high molar excess, caspase-3 and -7 can also overcome XIAP inhibition and cleave XIAP [62].

Recent data suggest that XIAP and cIAP1 and -2 could resist the inhibitor effect of Smac/DIABLO by binding ubiquitin [63, 64]. One isoform of Smac/DIABLO, Smac3, in turn, stimulates ubiquitin binding to XIAP [65]. Another endogenous caspases inhibitor, FLIP (FLICE-inhibitory protein), bounds with the death inducing signal complexes, blocking the activation of caspase-8 and the transmission of pro-apoptotic signal from the TNFR family death receptors [18].

ROS, Ca^{2+}, and nitric oxide in triggering of apoptosis. The production of ROS is associated with many forms of apoptosis [66]. In multicellular organisms, ROS could be in the beginning or at the end of the programmed death process. In apoptosis caused by ischemia, ROS acts before Bax and caspases [67]. In such cases, ROS scavenging prevents the activation of caspases, indicating that ROS not only cause the damage, but also act as signaling molecules [68]. ROS could become involved in apoptosis at its later stages. The absence of K^+, leading to hyperpolarization of plasmalemma, increasing $[Ca^{2+}]_{in}$ and causing cell swelling, induced apoptosis in cerebellum neurons, bringing about ROS generation. Actinomycin D, cycloheximide, and caspases inhibitors prevented ROS production, suggesting that it is the result of transcription, translation, and posttranslational modification of procaspases [69, 70].

Mitochondrial Ca^{2+} uptake is an early and necessary event in both apoptosis and necrosis [8]. It has been shown by many authors that Ca^{2+} and ROS levels are closely related with each other, at least in multicellular organisms. Increase in $[Ca^{2+}]_{in}$ brings about the activation of several ROS-inducing enzymes, including the stimulation of ROS formation in the respiratory chain of mitochondria [71]. Treatment of nervous cells with 3-nitropropionic acid (3-NP, an irreversible inhibitor of succinate dehydrogenase, increasing the production of $O_2^{\cdot-}$ and, as a consequence, H_2O_2 and $ONOO^-$ by mitochondria) causes rapid increase in $[Ca^{2+}]_{in}$. Chelator of intracellular Ca^{2+} BAPTA AM prevents apoptosis caused by 3-NP. Nifedipine (an inhibitor of L-type Ca^{2+}-channels in cytoplasmic membrane) and dantrolene (an inhibitor of Ca^{2+} release from endoplasmic reticulum) significantly reduced 3-NP-induced apoptosis [72].

ROS and high intramitochondrial Ca^{2+} may act together to trigger the opening of the mitochondrial permeability transition pore (mPTP) [73]. Mitochondrial membrane permeability transition induced by inorganic phosphate, uncouplers, or prooxidants such as *t*-butyl hydroperoxide and diamide is caused by a Ca^{2+}-stimulated production of ROS by the respiratory chain [74]. Studies with submitochondrial particles have demonstrated that the binding of Ca^{2+} to these particles induces lipid lateral phase separation. This condition leads to disorganization of respiratory chain components, favoring ROS production and consequent protein and lipid oxidation. The ROS attack to membrane protein thiols produces cross-linkage reaction, which may open membrane pores upon Ca^{2+} binding [74].

Thus, ROS activate the opening of the mPTP, which causes swelling, rupture of the outer membrane, and the release into the cytoplasm of several intermembrane proteins: cytochrome *c*, procaspase 9, and AIF. Cytochrome *c* forms a complex with cytosolic Apaf-1 and procaspase-9, producing active caspase-9. This in turn activates procaspase 3 and 7. Caspases activation processes are blocked by inhibitors of apoptosis proteins (IAP), which, in turn, are inhibited by Smac/DIABLO. Caspase-12 could activate caspase-9 without participation of cytochrome *c* [39]. Procaspases 3 and 7 are also activated by caspase-8, which, in turn, are activated by so-called "death inducing signal complexes" in plasmalemma. They consist of the "death receptor" and specific adaptor, interacting with procaspase-8. FLIP protein blocks activation of caspase-8 [18].

Caspase-8 releases activated cathepsin B from lysosomes; further one of them (cathepsin B or caspase-8) activates by partial cleaving cytosolic protein Bid. Then activated protein t-Bid activates another pro-apoptotic protein Bax. Interacting with mitochondrial protein porin, it forms a channel for cytochrome *c* and AIF in the outer mitochondrial membrane [41].

AIF directs immediately to the nucleus and causes DNA degradation. Bcl-2 prevents the release of cytochrome *c* from mitochondria. NO blocks apoptosis via selective nitrosylation of effector caspases [11]. Mitochondria are destroyed in the process of apoptotic proteins release. The overall scheme of classical apoptosis is presented in the figure (based on the scheme from [11]).

IS DEATH MACHINERY EVOLUTIONARILY CONSERVED?

Genetic analysis of programmed cell death in *Caenorhabditis elegans* has led to the identification of 13

genes that constitute a developmental pathway of programmed cell death. Two of the three key genes in this pathway, *ced-9*, a cell death suppressor, and *ced-3*, a cell death inducer, were found to encode proteins that share structural and functional similarities with the mammalian proto-oncogene product Bcl-2 and interleukin-1β-converting enzyme (caspase-1), respectively. These results suggest that the genetic pathway of programmed cell death may be evolutionarily conserved from worms to mammals, at least [75]. In relatively primitive Cnidarians, apoptotic cell death plays an important role in morphogenesis and reproduction. A caspase-3-like enzyme has been found in the Cnidarian *Hydra vulgaris* [76]. Furthermore, Bcl-2 related proteins and death receptors have been shown to exist even in sponges [76]. Recent findings indicate that programmed cell death take place also in single-celled eukaryotes [2] and prokaryotes [3]. Let us review the programmed death processes in unicellular organisms from bacteria to ciliates.

APOPTOSIS IN UNICELLULAR ORGANISMS AT DIFFERENT LEVELS OF PHYLOGENY

Prokaryotes. The best understood example of non-animal cell suicide occurs in bacteria. As in metazoa, programmed cell death plays an important role in a number of developmental processes in bacteria, such as lysis of the mother cell in sporulation, lysis of vegetative cells in myxobacterial fruiting body formation, and DNA transformation liberated from cells of streptococci undergoing spontaneous autolysis [2]. In sporulating *Bacillus subtilis*, the mother cell is actively lysed prior to release of the spore. Three autolysins have been identified that participate in mother cell lysis [2]. Fruiting body formation and sporulation in *Myxococcus xanthus* is another example of controlled death aiding development. "Autocides", which include fatty acids as well as glucosamine, induce autolysis in dense cultures of *M. xanthus* and are required for normal fruiting body development and sporulation. The pathway leading from these inducers to autolysis is not known, and neither is the exact role of autolysis in development. Most likely, lysis of vegetative cells is altruistic suicide, and the released nutrients feed the fruiting body and developing spores [2]. In several species, autolysis is part of a well-controlled mechanism of natural transformation. Cells that did not lyse pick up the released DNA. Interestingly, the gene for the main autolysin in *Streptococcus pneumoniae*, *lytA*, is located in the same operon with *recA*, which is responsible for homologous recombination with the incoming DNA. Both RecA and LytA are induced by the quorum-sensing factor, a peptide pheromone that accumulates at high cell density [2]. A considerable body of data suggests that microorganisms also evolved programmed death of defective cells. So, some strains of *Escherichia coli* produce an inactive form of a protease. If a particular phage infects them, one of the phagal polypeptides binds to the protease and activates it. The activated protease cleaves and inactivates a bacterial translation factor (EF-Tu), shutting down synthesis and killing the bacterium, thereby curtailing phagal multiplication and protecting the nearby *E. coli* population from infection. Although this bacterial death program shares a number of features with caspase-dependent apoptosis in animal cells, the proteases involved in the two programmed processes are unrelated [77]. It has been found that *E. coli* has at least two more mechanisms of the same function. One of them includes the identical events in the beginning of the suicide cascade, but the final product is the $tRNA^{Lys}$-specific ribonuclease instead of the EF-Tu-specific protease. The second suicide mechanism was found to produce the RexA and RexB proteins where the former is a phage DNA receptor, the latter being an ion channel activated by the DNA·RexA complex. The channel is assumed to dissipate all the ion gradients of the bacterial membrane and, as a result, kills the cell. Interestingly, all these systems are encoded by "parasitic" elements of the *E. coli* DNA including prophages and plasmids [77] and are likely maintained by natural selection. Several eukaryotic apoptotic proteins have homologs in bacteria. A domain responsible for interaction of some apoptotic proteins shows up in *Streptomyces*, *B. subtilis*, *Synechocystis* sp., and *Rhizobium* spp., and caspase homologs are found in *Streptomyces* spp. In the absence of functional data, it is not possible to decide whether these proteins are involved in programmed cell death in bacteria or have other functions [2].

Autotrophic flagellates. Recent observations concern programmed cell death in the dinoflagellate *Peridinium gatunense*. Its cell death is induced by oxidative stress, confirming the evolutionarily ancient link between ROS and cell suicide. Catalase inhibited cell death in culture. *P. gatunense* exhibits typical markers of apoptosis, DNA fragmentation, and protoplast shrinkage. A cysteine protease inhibitor E-64 prevents cell death, indicating that a protease participates in the suicide program, similar to the situation in higher animals [78]. A unicellular chlorophyte alga *Dunaliella tertiolecta* undergoes apoptosis in the absence of light. Many morphological criteria of apoptotic cell death were observed, including an increase in chromatin margination, degradation of the nucleus, and DNA fragmentation. Caspase-specific inhibitors inhibited the caspase-like activities. Antibodies against mammalian caspases cross-reacted with specific proteins in the alga. The expression of these proteins correlated with the beginning of cell death [79].

Heterotrophic flagellates. A parasitic protozoan *Leishmania major*, a representative of an ancient genus of unicellular eukaryotes, contains only a single giant mitochondria, making it a very convenient object for studies. Staurosporine, a protein kinase C inhibitor [80], causing apoptosis in mammalian cells, was effective in *L. major*,

bringing about the formation of vesicles, phosphatidylserine redistribution, reduction of mitochondrial $\Delta\Psi_m$, release of cytochrome *c*, and at the level of the nucleus, margination and degradation of chromatin and DNA fragmentation, prevented by inhibitors of cysteine proteases. Extract of *L. major* cytoplasm caused characteristic of apoptosis changes in isolated mammalian nuclei, pointing to the presence of AIF-like proteins. *L. major* isolated mitochondria lost cytochrome *c* during incubation with human recombinant Bax even when Bax was deleted of its transmembrane domain required for integration into the outer membrane of mitochondria. Therefore, *L. major* could express proteins that can interact with Bax. Thus, the involvement of cysteine proteases and mitochondria in cell death may have quite ancient origin [81]. Incubation with 4 mM H_2O_2 caused apoptosis-like death of *Leishmania donovani*, involving chromatin margination, nuclear condensation, DNA fragmentation, and reduction of the cell volume. The motility and survival of the parasites reduced with increasing concentration of H_2O_2. After a 30-min exposure, the ability of cellular lysates for cleavage of caspase substrates significantly increased. Specific inhibitor of caspases reduced the number of cells with characteristic traits of apoptosis. Therefore, these protozoa have caspase-like proteins, activated by oxidative stress [82]. Induction of apoptosis by H_2O_2 brought about dose- and time-related loss of mitochondrial $\Delta\Psi$, accompanied by reduction in reduced glutathione and ATP, but not opening of the permeability transition pore. The blocker of the permeability transition pore cyclosporine A did not prevent the $\Delta\Psi_m$ loss, but antioxidant N-acetylcysteine, SH-glutathione precursor [83], facilitated the recovery of the normal $\Delta\Psi_m$ level and prevented the cell death [84]. The death of ookinets accompanied by typical morphological and biochemical traits of apoptosis, including caspase activity in the cytoplasm, was also observed in the malaria plasmodium *Plasmodium berghei* [85]. This death was not simply caused by environmental factors but was spontaneous, occurring prior to the penetration into the primary host mosquito gut as well as *in vitro*. It is likely that the parasite controls its population density with this mechanism [85]. In spite of the external similarity of apoptosis in multicellular organisms and parasitic kinetoplastids, lysosomal cathepsins have not been found in the latter [86].

Yeasts. When the complete genome sequence of *Saccharomyces cerevisiae* became available [87], no relatives of the most central players in apoptosis—e.g., the caspases, members of the Bcl-2/Bax family, or Apaf-1—were found. In 1997, a yeast mutant dying with a typical apoptotic phenotype (exposition of phosphatidylserine, margination of chromatin, and formation of cell fragments) was found. This phenotype was caused by a point mutation of *CDC48* (*cdc48S565G*), coding for a *S. cerevisiae* protein and involved in vesicle fusion [88]. Deletion of *ASF1/CIA1*, a gene coding for a histone chaperone, also results in yeast apoptotic cell death following an arrest at the G2/M transition. Moreover, a reduction in the mitochondrial membrane potential, dysfunction of the mitochondrial proton pump, and release of cytochrome *c* into the cytoplasm was observed [89]. Hydrogen peroxide treatment induces apoptosis together with a caspase-like enzymatic activity in yeast. The inhibitor of cytoplasmic translation cycloheximide, antioxidants, and hypoxia prevented it. This suggests that ROS generation was originally the key event of apoptosis [90]. Ageing mother cells of *S. cerevisiae*, passed more than 20 cleavages, produced ROS [91]. These cells differed from their younger offspring by larger size (10-15 versus 5-7 μm), retarded protein biosynthesis, and uneven surface. Furthermore, they demonstrated typical traits of apoptosis—chromatin fragmentation and phosphatidylserine redistribution [91]. Apoptosis inducing treatment with H_2O_2 caused caspase activity in yeast, which completely disappeared after knockout of the *yor197w* gene and significantly facilitated by superexpression of this gene. A product of the *yor197w* gene, called Yeast Caspase-1 (YCA1) [92], is responsible for the ageing of the culture, emphasizing the adaptive significance of elimination of old cells. The absence of the protein coded by *ygl129c* prevented YCA1-induced apoptosis. This protein is an analog of a mitochondrial protein of animals, DAP-3, involved in cytokine-dependent apoptosis [93]. Functional mitochondria are needed for apoptosis in yeast [94, 95]. Inactivation of telomere-binding protein Cdc13 by mitochondrial protein MTCO3 caused caspase-dependent apoptosis in *S. cerevisiae*, pointing to active participation of yeast mitochondria in apoptosis processes [96]. Bcl-2 family proteins have not been found in yeast. Nonetheless, mammalian pro-apoptotic protein genes expressed in yeast cause their programmed death. For example, expression of pro-apoptotic protein Bax controlled by the GAL10 promoter caused galactose-induced death of *S. cerevisiae* cells [97]. Thus, yeasts represent a unique model, allowing studies of the role of mitochondria in apoptosis at the molecular level [98]. It was yeast models that showed the necessity of C-terminal transmembrane domain of Bax for its cytotoxic effect [97]. They also revealed a new suppressor Bax blocking its integration into the mitochondrial membrane, Ku70 [99].

The expression of either of two mammalian pro-apoptotic Bcl-2 family members, Bax and Bak, in *S. cerevisiae* and *Schizosaccharomyces pombe* results in cytotoxicity, with similar characteristic phenotypes in each case [100]. Bax-expressing *S. cerevisiae* showed margination of chromatin at the nuclear envelope, extensive DNA cleavage, membrane blebbing, and externalization of phosphatidylserine at the plasmalemma. Simultaneous expression of Bcl-2 or Bcl-x_L that inhibit apoptosis in animals prevented Bax-induced cell death [101]. In addition, Bcl-2 expression protected *S. cerevisiae* from death

caused by oxidative stress [102]. A Bax-resistant mutant with reduced quantity of Uth1p protein was obtained. The absence of this protein did not affect the integration of Bax into the outer membrane of mitochondria and the release of cytochrome *c*, but prevented oxidation of mitochondrial membrane lipids and ROS production. Absence of Uth1p brought about resistance to rapamycin, a specific inducer of autolysis [103]. In *S. pombe*, Rad9 protein (SpRad9) containing an amino acid sequence required for SpRad9–Bcl-2 interaction was found. Overexpression of Bcl-2 in *S. pombe* cells increased resistance of Rad9-free mutants toward UV irradiation and ionizing radiation [104]. Recently, one more Rad9 homolog was found which is specific for budding yeast [105]. Genes of apoptosis inhibition proteins (IAP) have been found in genomes of *S. pombe* and *S. cerevisiae* [106]. In rich medium, mutant of *S. cerevisiae* lacking them underwent normal vegetative growth and mitosis. However, on starvation, its diploid cells were poor to develop spores, instead undergoing pseudohyphal differentiation. Most of the developing spores did not sustain more than two divisions after germination. Spores of the same mutant *S. pombe* died at early stages of cleavage after spore germination, terminating at the transition between metaphase/anaphase because of an inability to form normal mitotic spindles [106]. A protein similar to mammalian HtrA-protein has been recently found in *S. cerevisiae* (in mammals, it is an antagonist of XIAP [61]). This protein was named Nma111p (nuclear mediator of apoptosis). Cells lacking this protein better survive at 50°C and show no traits of apoptosis caused by H_2O_2. Overexpression of Nma111p, on the contrary, caused apoptosis [107]. The programmed cell death of yeasts could be induced by a natural substances released by these organisms. Thus, programmed cell death process sharing common features with an apoptotic phenotype can be induced by acetic acid in *S. cerevisiae*. Acetic acid (20-80 mM) caused chromatin condensation along the nuclear envelope suppressed by cycloheximide in *S. cerevisiae*, phosphatidylserine redistribution, and DNA strand breaks [108]. Furthermore, there were ROS generation and the translocation of cytochrome *c* into the cytoplasm and reduction of oxygen consumption and $\Delta\Psi_m$ value [109]. The death of cells caused by 120-200 mM acid was not suppressed by cycloheximide and was accompanied by ultrastructural changes typical of necrosis [108]. The effects of acetic acid on cells of *Candida ablicans* [110] and *Zygosaccharomyces bailii* were similar [111]. Apoptosis in *C. ablicans* was caused by 40-60 mM acid and in *Z. bailii*, by 320-800 mM acid; higher concentrations in each case caused necrosis. α-Factor, a pheromone peptide produced by the α-type haploid cells of *S. cerevisiae*, at concentration below 1 μM forced yeast of the opposite mating type (a) to conjugate with the α-type yeast whereas its higher concentration arrested the a cell cycle and caused their apoptosis [112]. The pheromone-induced death was accompanied by appearance of typical features characteristic of apoptosis in animals. A mutation in the pheromone protein kinase cascade prevented both death and appearance of apoptotic markers [93].

Cytochrome *c* of yeast, however, did not activate caspases in cytosolic extracts of amphibians and mammals. This could be accounted for by almost 45% difference between the amino acid sequences of cytochrome *c* in mammals and yeast; the affinity of mammalian cytochrome *c* to Apaf-1 is very high ($K = 10^{11}$ M^{-1}), which is probably associated with strict specificity of their interaction. The absence of apoptosis activity of yeast cytochrome *c* is caused by its structural characteristics, precluding the interaction with Apaf-1 [113]. The chimerical block consisting of cytochrome *c* and GFP (green fluorescent protein) could function normally in *S. cerevisiae* in the electron transfer chain, but, unlike its natural analog, was not released into the cytoplasm during the expression of Bax. Nonetheless, cells expressing Bax were subjected to apoptosis, indicating that the excretion of cytochrome *c* is not necessary for its induction [114].

Yeasts differ from all other unicellular organisms considered by receptor interacting protein (RIP), interacting with Fas and the mammalian intracellular domain TNFR1. C-Terminal part of RIP contains so-called death domain, which is present in within-cell domains Fas and TNFR1. RIP overexpression caused morphological traits of apoptosis [115].

Slime molds. The multicellular development of the single celled eukaryote, the slime mold *Dictyostelium discoideum*, is induced by starvation and consists of initial aggregation of the isolated amoebae, followed by their differentiation into viable spores and dead stalk cells. These stalk cells retain their structural integrity inside a stalk tube that support the spores in the fruiting body. Terminal differentiation into stalk cells has been shown to share several features with programmed cell death. *Dictyostelium* cell death in its normal development is similar to classical apoptosis in that some cytoplasmic and chromatin condensation occurs but differs from apoptosis because it involves massive vacuolization and, interestingly, lacks DNA fragmentation [116]. Caspase inhibitors did not affect cell death, although some caspase inhibitors (DEVD-fmk) block the development of fruiting bodies [117]. Investigating the genome of amoebae shows that only a limited number of the proteins involved in mammalian apoptosis have homologs in *Dictyostelium*. A *Dictyostelium* homolog of mammalian AIF that is localized in mitochondria and is translocated from the mitochondria to the cytoplasm and the nucleus after the onset of cell death has been recently found [118]. Two homologs, Dd-ALG-2a and -2b, of the mammalian Ca^{2+}-binding protein ALG-2, and homolog of its binding partner, Alix, have been found in the *Dictyostelium*.

Murine Alix formed a heterodimer with -2a but not with -2b, and the interaction was strictly dependent upon Ca^{2+}. The genes encoding both proteins, Dd-ALG-2a and -2b, were expressed in growing cells. The levels of mRNA were at maximum during aggregation and decreases rapidly thereafter. In contrast, the levels of the proteins remained fairly stable. Simple and double knockouts of Dd-ALG-2a/2b produced no obvious developmental phenotype, but an Alix-null mutant had a strong defect in development of fruiting bodies [46]. In the absence of aggregation and differentiation, *D. discoideum* can undergo another form of programmed cell death that closely resembles apoptosis of most mammalian cells, involves loss of mitochondrial transmembrane potential, phosphatidylserine surface exposure, and engulfment of dying cells by neighboring ones [119].

Ciliates are unicellular eukaryotic organisms containing two types of nuclei: macronuclei and micronuclei. After the sexual pathway takes place, a new macronucleus is formed from a zygote nucleus, whereas the old macronucleus is degraded and lysed. The latter is eliminated through a regulated developmental process that can be prevented by inhibitors of gene expression or by gene mutation. In the course of macronuclear differentiation, polytene chromosomes are synthesized but degrade after a few hours. Most of the DNA is eliminated, and the remaining DNA is fragmented into small DNA molecules that are amplified to a high copy number in the new macronucleus. Thus, conjugating ciliates provide a remarkable model system for the study of nuclear death under conditions in which the cell continues to live, selective nuclear apoptosis in a living cell [120]. The programmed nuclear death program involves nuclear condensation (pyknosis) and oligonucleosomal DNA fragmentation in the parental macronucleus. Caspase-8- and -9-like (but not caspase-3-like) activity was detected in the parasitic ciliate *Tetrahymena* [121]. Three species of *Tetrahymena* expressed membrane Fas-receptors. All these species are parasites of teleost fish. Nonspecific cytotoxic cells of fish constitutively express soluble Fas ligand, enabling these cells to initiate programmed cell death of parasitic *Tetrahymena* [122]. The ciliate *Paramecium tetraurelia* synthesizes cathepsins L [123] and S [124]. Cathepsin B was found in the W strain of *Tetrahymena pyriformis* [125]. Staurosporine, an inhibitor of protein kinase C [80], could kill *T. thermophila* cells, blocking the transmission of the survival signals, proliferation, and chemoreception. The death caused by it had several traits characteristic of apoptosis and could be prevented by actinomycin D. So, it is an active process associated with or requiring RNA synthesis *de novo* [126].

Thus, we considered mechanisms of classical apoptosis and compared them with mechanisms of the programmed death in unicellular organisms. It has been shown that apoptosis in the most primitive organisms is triggered by ROS and Ca^{2+}. However, they often have homologs of mammalian apoptosis proteins. Even though the role of special apoptosis proteins significantly increased in phylogeny, the secondary messengers ROS and Ca^{2+} [71] still continue to play important roles in the regulation of programmed death.

On the whole, apoptosis in unicellular organisms has much in common with that in mammals, but its components appeared continuously during evolution. Almost all eukaryotes have inhibitors of apoptosis, probably introduced by viruses at the rise of life. It was probably adaptive for such viruses if their host continued to live, making possible their further reproduction. This virus strategy is characteristic of the parasitic protozoan *Theileria parva*, preventing apoptosis of T-cells of its host by activation of the transcription factor NF-κB [127], and the heterotrophic flagellate *L. major*, increasing production of apoptosis-inhibiting interleukin-8 [128]. Mitochondria and the release by them of proteins cytochrome *c* and AIF are involved in triggering apoptosis in almost all organisms except the protozoan *Trichomonas vaginalis* lacking mitochondria [129]. The patterns of programmed death in eukaryotes and prokaryotes are characteristically different. Whereas inhibitors of apoptosis proteins have viral origin in eukaryotes, the proteins triggering apoptosis are coded by plasmids and prophages in prokaryotes (at least in *E. coli*) [77]. It is possible that bacteria have been infected by the genes of these proteins and this acquisition turned out to be highly adaptive, making it possible to avoid mass death caused by infection of certain individuals by phages. Proteins similar to caspases first appeared in unicellular algae—dinoflagellates [78]. ALG-2 and Alix first appeared in slime molds [46]. Slime molds also have cysteine proteases, but their role in apoptosis has not been determined. The death receptors probably appeared first only in relatively highly organized protozoa: yeasts [115] and ciliates [122]. Proteins which can interact with proteins of the Bcl-2 family probably appeared first in heterotrophic flagellates [81], even though they were identified only in yeast (Rad9 [104]). Cathepsins first appeared in ciliates, but their role in programmed death of these organisms is still unknown [123-125].

Thus, three components are necessary and sufficient for apoptosis: Ca^{2+}, ROS, and at least one cysteine protease. Initiator caspases (-8, -9) probably appear in phylogeny prior to effector caspases (-3). The latter appear only in primitive metazoans, Coelenterata [76]. Mitochondrial factors, cytochrome *c*, and AIF play the primary role in triggering apoptosis in most lower eukaryotes; however, *T. vaginalis* lacking mitochondria [129] in some way manage without these factors. Deletion of ALG-2 homologs in slime molds does not disturb the formation of the fruiting bodies [46], indicating that these proteins are not necessary participants of the apoptosis process. Thus, most proteins involved in the programmed death are used for more detailed regulation of its process-

es, which is hardly possible with a small number of components.

The functions of apoptosis in organisms at different levels of the phylogeny are probably the same. In unicellular organisms, apoptosis represents not only a highly efficient means for the elimination of deficient mutant individuals from the community, but also is involved in the formation of the fruiting bodies in myxobacteria [2] and slime molds [116]. With insufficient feeding, old and damaged yeast cells die so that young cells could survive better. On the other hand, such "altruism" is not adaptive in the complete absence of feeding substrata (such as rainfall water) and the apoptosis does not occur under such conditions [88]. Apoptosis is triggered in bacteria and yeast by factors accumulating with increasing density of the culture. In the *M. xanthus* bacteria, they include fatty acids and glucosamines [2], in yeast, acetic acid [108-111], H_2O_2 [90], and the α-factor [93, 112]. Most probably, this is involved in the maintenance of the optimal population density in unicellular organisms. However, apoptosis in the *T. thermophila* ciliate, on the contrary, occurs at low population density [130]. It is likely that these organisms require relatively high culture density facilitating conjugation. In yeast, apoptosis is triggered by a pheromone, the same substance as for copulation. With such a mechanism, virgin cells (probably damaged) could not take food from healthy cells capable of copulation [93].

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REFERENCES

1. Samuilov, V. D., Oleskin, A. V., and Lagunova, E. M. (2000) *Biochemistry (Moscow)*, **65**, 873-887.
2. Ameisen, J. C. (1996) *Science*, **272**, 1278-1279.
3. Lewis, K. (2000) *Microbiol. Mol. Rev.*, **64**, 503-514.
4. Proskuryakov, S. Y., Gabaj, V. L., and Konoplyannikov, A. G. (2002) *Biochemistry (Moscow)*, **67**, 387-408.
5. Mills, J. C., Stone, N. L., and Pittman, R. N. (1999) *J. Cell. Biol.*, **146**, 703-708.
6. Hanayama, R., Tanaka, M., Miwa, K., Shinohara, A., Iwamatsu, A., and Nagata, S. (2002) *Nature*, **417**, 182-187.
7. Strasser, A., O'Connor, L., and Vishva, M. D. (2002) *Annu. Rev. Biochem.*, **69**, 217-245.
8. Kruman, I. I., and Mattson, M. P. (1999) *J. Neurochem.*, **72**, 529-540.
9. Tonshin, A. A., Saprunova, V. B., Solodovnikova, I. M., Bakeeva, L. E., and Yagughinsky, L. S. (2003) *Biochemistry (Moscow)*, **68**, 875-881.
10. Oleinick, N. L., Morris, R. L., and Belichenko, I. (2002) *Photochem. Photobiol. Sci.*, **1**, 1-21.
11. Kamata, H., and Hirata, H. (1999) *Cell. Signal.*, **11**, 1-14.
12. Earnshaw, W. C., Martins, L. M., and Kaufmann, S. H. (1999) *Annu. Rev. Biochem.*, **68**, 383-424.
13. Liang, H., and Fesik, S. W. (1997) *J. Mol. Biol.*, **274**, 291-302.
14. Köhler, C., Orrenius, S., and Zhivotovsky, B. (2002) *J. Immunol. Meth.*, **265**, 97-110.
15. Grossmann, J., Mohr, S., Lapentina, E. G., Fiocchi, C., and Levine, A. D. (1998) *Am. J. Physiol.*, **274**, G1117-G1124.
16. Salvesen, G. S., and Dixit, V. M. (1999) *Proc. Natl. Acad. Sci. USA*, **96**, 10964-10967.
17. Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. (1998) *Nature*, **391**, 43-50.
18. Philchenkov, A. A. (2003) *Biochemistry (Moscow)*, **68**, 365-376.
19. Hirata, H., Takahashi, A., Kobayashi, S., Yonehara, S., Sawai, H., Okazaki, T., Yamamoto, K., and Sasada, M. (1998) *J. Exp. Med.*, **187**, 587-600.
20. Susin, S. A., Daugas, E., Ravagnan, L., Samejima, K., Zamzami, N., Loeffler, M., Costantini, P., Ferri, K. F., Irinopoulou, T., Prevost, M. C., Brothers, G., Mak, T. W., Penninger, J., Earnshaw, W. C., and Kroemer, G. (2000) *J. Exp. Med.*, **192**, 571-580.
21. Crompton, M. (1999) *Biochem. J.*, **341**, 233-249.
22. Pozzan, T., and Rizzuto, R. (2000) *Eur. J. Biochem.*, **267**, 5269-5273.
23. Bernardi, P. (1999) *Phys. Rev.*, **79**, 1127-1155.
24. Ichas, F., and Mazat, J.-P. (1998) *Biochim. Biophys. Acta*, **1366**, 33-50.
25. Novgorodov, S. A., Gudz, T. I., Brierley, G. P., and Pfeiffer, D. R. (1994) *Arch. Biochem. Biophys.*, **311**, 219-228.
26. Lapidus, R. G., and Sokolove, P. M. (1994) *J. Biol. Chem.*, **269**, 18931-18936.
27. Broekemeier, K. M., Dempsey, M. E., and Pfeiffer, D. R. (1989) *J. Biol. Chem.*, **264**, 7826-7830.
28. Halestrap, A. P., McStay, G. P., and Clarke, S. J. (2002) *Biochimie*, **84**, 153-166.
29. Kim, J. S., He, L., Qian, T., and Lemaster, J. J. (2003) *Curr. Mol. Med.*, **3**, 527-535.
30. Kim, J. S., He, L., and Lemaster, J. J. (2003) *Biochem. Biophys. Res. Commun.*, **304**, 463-470.
31. Budihardjo, I., Oliver, H., Lutter, M., Luo, X., and Wang, X. (1999) *Annu. Rev. Cell. Dev. Biol.*, **15**, 269-290.
32. Robertson, J. D., Orrenius, S., and Zhivotovsky, B. (2000) *J. Struct. Biol.*, **129**, 346-358.
33. Nichols, P. (1974) *Biochim. Biophys. Acta*, **346**, 261-310.
34. Gorbenko, G. P. (1999) *Biochim. Biophys. Acta*, **1420**, 1-13.
35. Rytömaa, M., and Kinnunen, P. K. J. (1995) *J. Biol. Chem.*, **270**, 3197-3202.
36. Ott, M., Robertson, J. D., Gogvadze, V., Zhivotovsky, B., and Orrenius, S. (2002) *Proc. Natl. Acad. Sci. USA*, **99**, 1259-1263.
37. Lorenzo, H. K., Susin, S. A., Penninger, J., and Kroemer, G. (1999) *Cell Death Differ.*, **6**, 516-524.
38. Punj, V., and Chakrabarty, A. M. (2003) *Cell. Microbiol.*, **5**, 225-231.
39. Morishima, N., Nakanishi, K., Takenouchi, H., Shibata, T., and Yasuhiko, Y. (2002) *J. Biol. Chem.*, **277**, 34287-34294.

40. Gogvadze, V., Robertson, J. D., Zhivotovsky, B., and Orrenius, S. (2001) *J. Biol. Chem.*, **276**, 19066-19701.
41. Skulachev, V. P. (2000) *IUBMB Life*, **49**, 365-373.
42. Guicciardi, M. E., Deussing, J., Miyoshi, H., Bronk, S. F., Svingen, P. A., Peters, C., Kaufmann, S. H., and Gores, G. J. (2000) *J. Clin. Invest.*, **106**, 1127-1136.
43. Vousden, K. H., and Vande Woude, G. F. (2000) *Nature Cell. Biol.*, **2**, E178-E180.
44. Nakano, K., and Vousden, K. H. (2001) *Mol. Cell*, **7**, 683-694.
45. Chatellard-Causse, C., Blot, B., Cristina, N., Torch, S., Missotten, M., and Sadoul, R. (2002) *J. Biol. Chem.*, **277**, 29108-29115.
46. Aubry, L., Mattei, S., Blot, B., Sadoul, R., Satre, M., and Klein, G. (2002) *J. Biol. Chem.*, **277**, 21947-21954.
47. Carafoli, E., and Molinari, M. (1998) *Biochem. Biophys. Res. Commun.*, **247**, 193-203.
48. Bordone, L., and Campbell, C. (2002) *J. Biol. Chem.*, **277**, 26673-26680.
49. Kobayashi, S., Yamashita, K., Takeoka, T., Ohtsuki, T., Suzuki, Y., Takahashi, R., Yamamoto, K., Kaufmann, S. H., Uchiyama, T., Sasada, M., and Takahashi, A. (2002) *J. Biol. Chem.*, **277**, 33968-33977.
50. Grook, N. E., Clem, R. J., and Miller, L. K. (1994) *J. Virol.*, **67**, 2168-2174.
51. Hay, B. A., Wassarman, D. A., and Rubin, G. M. (1995) *Cell*, **83**, 1253-1262.
52. Uren, A. G., Pakusch, M., Hawkins, C. J., Puls, K. L., and Vaux, D. L. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 4974-4978.
53. Miller, L. K. (1999) *Trends Cell. Biol.*, **9**, 323-328.
54. Joazeiro, C. A., and Weissman, A. M. (2000) *Cell*, **102**, 549-552.
55. Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. S. (1997) *Nature*, **388**, 300-304.
56. Huang, Y., Park, Y. C., Rich, R. L., Segal, D., Myszka, D. G., and Wu, H. (2001) *Cell*, **104**, 781-790.
57. Yang, Y., and Yu, X. (2003) *FASEB J.*, **17**, 790-799.
58. Suzuki, Y., Kakabayashi, Y., and Takahashi, R. (2001) *Proc. Natl. Acad. Sci. USA*, **98**, 8662-8667.
59. Srinivasula, S. M., Hedge, R., Saleh, A., Datta, P., Shiozaki, E., Chal, J., Lee, R.-A., Robbins, P. D., Fernandes-Alnemri, T., Shi, Y., and Alnemri, E. S. (2001) *Nature*, **410**, 112-116.
60. Nicholson, D. W. (2001) *Nature*, **410**, 33-34.
61. Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K., and Takahashi, R. (2001) *Mol. Cell*, **8**, 613-621.
62. MacFarlane, M., Merrison, W., Bratton, S. B., and Cohen, G. M. (2002) *J. Biol. Chem.*, **277**, 36611-36616.
63. Hu, S., and Yang, X. (2003) *J. Biol. Chem.*, **278**, 10055-10060.
64. Fu, J., Jin, Y., and Arend, L. J. (2003) *J. Biol. Chem.*, **278**, 52660-52672.
65. Deveraux, Q. L., Leo, E., Stennicke, H. R., Welsh, K., Salvesen, G. S., and Reed, J. C. (1999) *EMBO J.*, **18**, 5242-5251.
66. Fleury, C., Mignotte, B., and Vayssiere, J. L. (2002) *Biochimie*, **84**, 131-141.
67. Maulik, N., Yoshida, T., and Das, D. K. (1998) *Free Rad. Biol. Med.*, **24**, 869-875.
68. Tan, S., Sagara, Y., Liu, Y., Maher, P., and Schubert, D. (1998) *J. Cell Biol.*, **141**, 1423-1432.
69. Schulz, J. B., Weller, M., and Klockgether, T. (1996) *J. Neurosci.*, **16**, 4696-4706.
70. Schulz, J. B., Bremen, D., Reed, J. C., Lommatzsch, J., Takayama, S., Wüllner, U., Löschmann, P.-A., Klockgether, T., and Weller, M. (1997) *J. Neurochem.*, **69**, 2075-2086.
71. Gordeeva, A. V., Zvyagilskaja, R. A., and Labas, Y. A. (2003) *Biochemistry (Moscow)*, **68**, 1077-1080.
72. Keller, J. N., Guo, Q., Holtsberg, F. W., Bruce-Keller, A. J., and Mattson, M. P. (1998) *J. Neurosci.*, **18**, 4439-4450.
73. Zoratti, M., and Szabo, I. (1995) *Biochim. Biophys. Acta*, **1241**, 139-176.
74. Vercesi, A. E., Kowaltowski, A. J., Grijalba, M. T., Meinicke, A. R., and Castilho, R. F. (1997) *Biosci. Rep.*, **17**, 43-52.
75. Yuan, J. (1996) *J. Cell. Biochem.*, **60**, 4-11.
76. Seipp, S., Schmich, J., and Leitz, T. (2001) *Development*, **128**, 4891-4898.
77. Skulachev, V. P. (1999) *Biochemistry (Moscow)*, **64**, 1418-1426.
78. Vardi, A., Berman-Frank, I., Rozenberg, T., Hadas, O., Kaplan, A., and Levine, A. (1999) *Curr. Biol.*, **l9**, 1061-1064.
79. Segovia, M., Haramaty, L., Berges, J. A., and Falkowski, P. G. (2003) *Plant Physiol.*, **132**, 99-105.
80. Ha, M. W., Hou, K. Z., Liu, Y. P., and Yuan, Y. (2003) *Ai Zheng*, **22**, 691-694.
81. Arnoult, D., Akarid, K., Grodet, A., Petit, P. X., Estaquier, J., and Ameisen, J. C. (2002) *Cell Death Differ.*, **9**, 65-81.
82. Das, M., Mukherjee, S. B., and Shaha, C. (2001) *J. Cell. Sci.*, **114**, 2461-2469.
83. Liu, J., Shen, H. M., and Ong, C. N. (2001) *Life Sci.*, **69**, 1833-1850.
84. Mukherjee, S. B., Das, M., Sudhandiran, G., and Shaha, C. (2002) *J. Biol. Chem.*, **277**, 24717-24727.
85. Al-Olayan, E. M., Williams, G. T., and Hurd, H. (2002) *Int. J. Parasitol.*, **32**, 1133-1143.
86. Ouaissi, A. (2003) *Kinetoplastid. Biol. Dis.*, **2**, 5.
87. Goffeau, A., Barrell, B. G., Bussey, H., Davis, R. W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J. D., Jacq, C., Johnston, M., Louis, E. J., Mewes, H. W., Murakami, Y., Philippsen, P., Tettelin, H., and Oliver, S. G. (1996) *Science*, **274 (5287)**, 563-567.
88. Madeo, F., Engelhardt, S., Herker, E., Lehmann, N., Maldener, C., Proksch, A., Wissing, S., and Frohlich, K.-U. (2002) *Curr. Genet.*, **41**, 208-216.
89. Yamaki, M., Umehara, T., Chimura, T., and Horikoshi, M. (2001) *Genes Cells*, **6**, 1043-1054.
90. Madeo, F., Fröhlich, E., Ligr, M., Grey, M., Sigrist, S. J., Wolf, D. H., and Fröhlich, K.-U. (1999) *J. Cell. Biol.*, **145**, 757-767.
91. Laun, P., Pichova, A., Madeo, F., Fuchs, J., Ellinger, A., Kohlwein, S., Dawes, I., Fröhlich, K.-U., and Breitenbach, M. (2001) *Mol. Microbiol.*, **39**, 1166-1173.
92. Madeo, F., Herker, E., Maldener, C., Wissing, S., Lachelt, S., Herlan, M., Fehr, M., Lauber, K., Sigrist, S. J., Wesselborg, S., and Frohlich, K. U. (2002) *Mol. Cell*, **9**, 911-917.
93. Skulachev, V. P. (2002) *FEBS Lett.*, **528**, 23-26.
94. Manon, S., Chaudhuri, B., and Guerin, M. (1997) *FEBS Lett.*, **415**, 29-32.
95. Xu, Q., and Reed, J. C. (1998) *Mol. Cell*, **1**, 337-346.

96. Qi, H., Li, T. K., Kuo, D., Nur-E-Kamal, A., and Liu, L. F. (2003) *J. Biol. Chem.*, **278**, 15136-15141.
97. Zha, H., Fisk, H. A., Yaffe, M. P., Mahajan, N., Herman, B., and Reed, J. C. (1996) *Mol Cell Biol.*, **16**, 6494-6508.
98. Priault, M., Camougrand, N., Kinnally, K. W., Vallette, F. M., and Manon, S. (2003) *FEMS Yeast Res.*, **4**, 15-27.
99. Sawada, M., Sun, W., Hayes, P., Leskov, K., Bootman, D. A., and Matsuyama, S. (2003) *Nat. Cell Biol.*, **5**, 320-329.
100. Fraser, A., and James, C. (1998) *Trends Cell. Biol.*, **8**, 219-221.
101. Fröhlich, K.-U., and Madeo, F. (2000) *FEBS Lett.*, **473**, 6-9.
102. Chen, S. R., Dunigan, D. D., and Dickman, M. B. (2003) *Free Rad. Biol. Med.*, **34**, 1315-1325.
103. Camougrand, N., Grelaud-Coq, A., Marza, E., Priault, M., Bessoule, J. J., and Manon, S. (2003) *Mol. Microbiol.*, **47**, 495-506.
104. Komatsu, K., Hopkins, K. M., Lieberman, H. B., and Wang, H.-G. (2000) *FEBS Lett.*, **481**, 122-126.
105. Weinberger, M., Ramachandran, L., and Burchans, W. (2003) *IUBMB Life*, **55**, 467-472.
106. Uren, A. G., Beilharz, T., O'Connell, M. J., Bugg, S. J., van Driel, R., Vaux, D. L., and Lithgow, T. (1999) *Proc. Natl. Acad. Sci. USA*, **96**, 10170-10175.
107. Fahrenkrog, B., Sauder, U., and Aebi, U. (2004) *J. Cell. Sci.*, **117**, 15-26.
108. Ludovico, P., Sousa, M. J., Silva, M. T., Leao, C., and Corte-Real, M. (2001) *Microbiology*, **147**, 2409-2415.
109. Ludovico, P., Rodrigues, F., Almeida, A., Silva, M. T., Barrientos, A., and Corte-Real, M. (2002) *Mol. Biol. Cell*, **13**, 2598-2606.
110. Phillips, A. J., Sudbery, I., and Ramsdale, M. (2003) *Proc. Natl. Acad. Sci. USA*, **100**, 14327-14332.
111. Ludovico, P., Sansonetty, F., Silva, M. T., and Corte-Real, M. (2003) *FEMS Yeast Res.*, **3**, 91-96.
112. Severin, F. F., and Hyman, A. A. (2002) *Curr Biol.*, **12**, R233-235.
113. Abdullaev, Z. Kh., Bodrova, M. E., Chernyak, B. V., Dolgikh, D. A., Kluck, R. M., Pereverzev, M. O., Arseniev, A. S., Efremov, R. G., Kirpichnikov, M. P., Mokhova, E. N., Newmeyer, D. D., Roder, H., and Skulachev, V. P. (2002) *Biochem. J.*, **362**, 749-754.
114. Roucou, X., Prescott, M., Devenish, R. J., and Nagley, P. (2000) *FEBS Lett.*, **471**, 235-239.
115. Stanger, B. Z., Leder, P., Lee, T. H., Kim, E., and Seed, B. (1995) *Cell*, **81**, 513-523.
116. Cornillon, S., Foa, C., Davoust, J., Buonavista, N., Gross, J. D., and Golstein, P. (1994) *J. Cell Sci.*, **107**, 2691-2704.
117. Olie, R. A., Durrieu, F., Cornillon, S., Loughran, G., Gross, J., Earnshaw, W. C., and Golstein, P. (1998) *Curr. Biol.*, **8**, 955-958.
118. Arnoult, D., Tatischeff, I., Estaquier, J., Girard, M., Sureau, F., Tissier, J. P., Grodet, A., Dellinger, M., Traincard, F., Kahn, A., Ameisen, J.-C., and Petit, P. X. (2001) *Mol. Biol. Cell*, **12**, 3016-3030.
119. Tatischeff, I., Petit, P. X., Grodet, A., Tissier, J. P., Duband-Goulet, I., and Ameisen, J. C. (2001) *Eur. J. Cell. Biol.*, **80**, 428-441.
120. Maercker, C., Kortwig, H., Nikiforov, M. A., Allis, C. D., and Lipps, H. J. (1999) *Mol. Biol. Cell*, **10**, 3003-3014.
121. Kobayashi, T., and Endoh, H. (2003) *Cell Death Differ.*, **10**, 634-640.
122. Jaso-Friedmann, L., Leary, J. H., 3rd, and Evans, D. L. (2000) *Exp. Parasitol.*, **96**, 75-88.
123. Volkel, H., Kurz, U., Linder, J., Klumpp, S., Gnau, V., Jung, G., and Schultz, J. E. (1996) *Eur. J. Biochem.*, **238**, 198-206.
124. Maubach, G., Schilling, K., Rommerskirch, W., Wenz, I., Schultz, J. E., Weber, E., and Wiederanders, B. (1997) *Eur. J. Biochem.*, **250**, 745-750.
125. Banno, Y., Yano, K., and Nozawa, Y. (1983) *Eur. J. Biochem.*, **132**, 563-568.
126. Christensen, S. T., Chemnitz, J., Straarup, E. M., Kristiansen, K., Wheatley, D. N., and Rasmussen, L. (1998) *Cell. Biol. Int.*, **22**, 591-598.
127. Heussler, V. T., Machado, J., Jr., Fernandez, P. C., Botteron, C., Chen, C. G., Pearse, M. J., and Dobbelaere, D. A. (1999) *Proc. Natl. Acad. Sci. USA*, **96**, 7312-7317.
128. Aga, E., Katschinski, D. M., van Zandbergen, G., Laufs, H., Hansen, B., Muller, K., Solbach, W., and Laskay, T. (2002) *J. Immunol.*, **169**, 898-905.
129. Chose, O., Noel, C., Gerbod, D., Brenner, C., Viscogliosi, E., and Roseto, A. (2002) *Exp. Cell. Res.*, **276**, 32-39.
130. Christensen, S. T., Sorensen, H., Beyer, N. H., Kristiansen, K., Rasmussen, L., and Rasmussen, M. I. (2001) *Cell. Biol. Int.*, **25**, 509-519.