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Review

Bcl-2 and Ca^{2+} homeostasis in the endoplasmic reticulum

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Abstract

Recent data have revealed an unexpected role of Bcl-2 in modulating the steady-state levels and agonist-dependent fluxes of Ca^{2+} ions. Direct monitoring of endoplasmic reticulum (ER) Ca^{2+} concentration with recombinant probes reveals a lower state of filling in Bcl-2-overexpressing cells and a higher leak rate from the organelle. The broader set of indirect data using cytosolic probes reveals a more complex scenario, as in many cases no difference was detected in the Ca^{2+} content of the intracellular pools. At the same time, Ca^{2+} signals have been shown to affect important checkpoints of the apoptotic process, such as mitochondria, thus tuning the sensitivity of cells to various challenges. In this contribution, we will review (i) the data on the effect of Bcl-2 on $[Ca^{2+}]_{er}$, (ii) the functional significance of the Ca^{2+}-signalling alteration and (iii) the current insight into the possible mechanisms of this effect.

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Abbreviations: ER, endoplasmic reticulum; $[Ca^{2+}]_m$, mitochondrial matrix Ca^{2+} concentration; $[Ca^{2+}]_{er}$, endoplasmic reticulum Ca^{2+} concentration; $[Ca^{2+}]_c$, cytosolic Ca^{2+} concentration; GFP, green fluorescent protein; PTP, permeability transition pore; SERCAs, ER Ca^{2+} ATPasi; PMCA, plasma membrane Ca^{2+} ATPase; IP3, inositol trisphosphate; IP3R, inositol trisphosphate receptor

Introduction

In the molecular definition of mammalian apoptosis, experimental oncology highlighted more than two decades ago a key player, the 26 kD protein encoded by the protooncogene bcl-2. The *bcl-2* gene was identified at the t(14;18) translocation of follicular B-cell lymphomas.[1] At the breakpoint, a transcriptional enhancer of an immunoglobulin gene is fused to the *bcl-2* gene. This enhancer/*bcl-2* fusion causes large amounts of Bcl-2 to be expressed in B lymphocytes. These large amounts of Bcl-2 essentially block apoptosis in these mutant B lymphocytes and allows some B cells to survive and accumulate beyond their normal life span accumulating cell proliferation-promoting mutations.

Sequence analysis then demonstrated that Bcl-2 is the homologue of the antiapoptotic protein (CED-9) of the basic machinery of *Caenorhabditis elegans*, and in both normal and neoplastic tissues, its expression proved to protect cells from death by preventing or delaying apoptosis. This role is not restricted to hematopoietic (both lymphoid and myeloid) cells,[2] but proved to be a general concept, holding true, for example also in the survival of central and peripheral neurons.[3]

These data, and the emerging role of Bcl-2 in the control of apoptosis, prompted an intense research on the site and mechanism of its action. As to the first, Korsmeyer and coworkers demonstrated in a seminal study the association of Bcl-2 with cellular membranes and in particular with mitochondrial membranes.[4] This observation was pursued by a number of further studies, and the current consensus is that Bcl-2 shows a heterogeneous distribution to various cells compartments: nuclear envelope, endoplasmic reticulum (ER), outer mitochondrial membrane and, to a lesser degree, inner membrane, with also some retention within the cytosol. Interestingly, mitochondrial staining was patchy, reminiscent of mitochondrial contact zones.[5–7] This complex distribution (and the putative engagement of Bcl-2 in protein complexes) suggests a pleiotropic effect, with specific and likely different functions in different membrane environments.

Major attention was obviously drawn to mitochondria, after increased permeability of the outer mitochondrial membrane emerged as an early step of intracellular death signalling. This mechanism allows apoptogenic factors such as cytochrome *c* to be released into the cytosol where they contribute to caspase activation.[8] As antiapoptotic members of the Bcl-2 family prevent the increase in membrane permeability and protect cells from various death insults, it has been assumed that Bcl-2 family members primarily regulate mitochondrial integrity, and thus the crucial commitment step in many apoptotic routes.[9]

However, in the recent years also the other potential sites of Bcl-2 action regained interest, for at least two reasons. The first is that mitochondrial apoptotic involvement could depend on signals that originate from other cell compartments. Namely, Ca^{2+} rises triggered by release from the ER could induce and/or play a facilitatory role in the apoptotic changes of mitochondria,[10] as discussed later in greater detail. Second, apoptotic signalling can bypass mitochondria and

even occur independently of caspase activation, but still be controlled by Bcl-2. Specifically, the alteration of ER functions could *per se* protect cells from apoptosis. The complexity of the scenario is supported by apparently conflicting results. Serum starvation induced apoptosis in MDCK cells expressing Bcl-2 targeted to the ER but not to the mitochondria, while on the contrary the protein located to the ER was more efficient in protecting from apoptosis Rat-1/myc cells.[11] Lee *et al*.[12] reported that in Rat-1 fibroblasts, Bcl-2 targeted to the ER was able to inhibit Myc- but not etoposide-induced apoptosis. McCullough *et al*.[13] reported that overexpression of gadd153/chop sensitizes cells to ER stress, with ensuing downregulation of Bcl-2 expression, depletion of cellular glutathione, and enhanced production of reactive oxygen species, and restoration of Bcl-2 fully reverts the phenotype.

Thus, Bcl-2 appears capable of managing life-or-death decisions at several intracellular sites, via more than one mechanism and perhaps even in a cell-type-specific manner. In this review, we will analyse the functions of Bcl-2 outside of the mitochondria (extensively described in other reviews presented in this special issue) focusing our attention on the ER and calcium homeostasis.

Bcl-2 and Ca^{2+}: Establishing the Link

Cell death has always been known to be one of the numerous cellular events triggered by increases in intracellular Ca^{2+} concentration evoked by physiological or pathological stimuli. Classically, this has been associated to necrosis, that is the catastrophic derangement of cell integrity and function following different types of cell injury and leading to activation of Ca^{2+}-activated hydrolysing enzymes. A typical example is excitotoxicity, in which glutamate-dependent hyperstimulation leads neurons to death.[14,15] The link to apoptosis was appreciated more recently, and the analysis of Bcl-2 has been a crucial element in attributing a role for Ca^{2+} in apoptosis. When studying the complex mechanism of action of this oncogene, the possibility that it could act, at least in part, by modulating ion fluxes was suggested by its intracellular distribution, that is to organelles playing a key role in Ca^{2+} homeostasis, the ER and mitochondria.[16] Two important contributions corroborated this hypothesis, prompting further research on the topic. First, Bcl-2 and related proteins were shown to act as cation channels. Specifically, the three-dimensional structure of the Bcl-2 homologue, Bcl-X_L, bears a strong resemblance to some pore-forming bacterial toxins.[17] Indeed, Bcl-2 itself was shown to form cation channels of low selectivity in artificial lipid bilayers,[18] although later studies showed that the channel mostly conducts sodium and not calcium.[19]

Second, Bcl-2 expression was shown to modify the changes in Ca^{2+} homeostasis evoked by IL-3 deprivation in haematopoietic cell lines.[20] Specifically, Bcl-2 expression was shown to prevent the reduction in cytoplasmic Ca^{2+} levels and in the size of the TG-releasable pool that was observed upon withdrawal of IL-3. In lymphoma cells, Bcl-2 overexpression was reported to decrease the size of the TG-releasable pool, but not to prevent capacitative Ca^{2+} entry of extracellular calcium. Further work by the same authors showed that Bcl-2 prevents ER depletion at low extracellular Ca^{2+} and maintains Ca^{2+} uptake in TG-treated cells (reviewed in Distelhorst and Shore[21]). Overall, the data, although complex and hard to reconcile into a unifying mechanism, unquestionably associated Bcl-2 and ER Ca^{2+} homeostasis, and prompted a number of researchers to investigate directly, as soon as suitable probes became available, the effect of Bcl-2 on Ca^{2+} levels in the ER lumen and the relevance of this effect for the antiapoptotic activity of the protein.

To further increase the interest in Bcl-2 effects on ER Ca^{2+} homeostasis, in the same years the role of mitochondria in calcium signalling was greatly re-evaluated.[22] Also in this case, the development of novel, protein-based probes allowing the specific measurement of Ca^{2+} concentration within the mitochondrial matrix (mitochondrial matrix Ca^{2+} concentration ($[Ca^{2+}]_m$)) was decisive. With new luminescent[23] or green fluorescent protein (GFP)-based fluorescent[24] probes, in virtually all cell types a large $[Ca^{2+}]_m$ increase was shown to parallel the cytosolic increases occurring upon cell stimulation. In this process, the ER (and its interactions with mitochondria) were shown to play a key role. Indeed, at least in cells in which rapid Ca^{2+} responses depend on the opening of ER Ca^{2+} channels, the strategic location of mitochondria close to the source of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) rise (the ER) allows them to be exposed to microdomains of high $[Ca^{2+}]$, that meet the low affinity of their transporters and allows the rapid and large accumulation of the cation in the matrix.[25,26]

Mitochondrial Ca^{2+} accumulation, in turn, can induce radically different effects, and participate in functionally decoding the Ca^{2+}-mediated signals. On the one hand, by stimulating Ca^{2+}-dependent enzymes[27–29] or metabolite carriers[30] it can stimulate aerobic metabolism[31] and enhance ATP production.[32] On the other hand, Ca^{2+} may synergize with apoptotic mediators and induce large-scale morphological changes of the organelle, possibly by favouring the opening of the large-conductance channel known as permeability transition pore (PTP),[33] and thus induce the release of mitochondrial proapoptotic factors. Not surprisingly, loading of Ca^{2+} in the matrix, and the opening of PTP, has been implicated in the pathogenesis of numerous human disorders, stemming from heart damage upon reperfusion to the pathogenesis of muscular dystrophies.[34,35]

Direct Measurements of Endoplasmic Reticulum Ca^{2+} Concentration ($[Ca^{2+}]_{er}$) in Cells Overxpressing Bcl-2

The development of molecular biology techniques (which enable the modification and expression of exogenous cDNA in heterologous cell types), has been responsible in recent years for the widespread use of protein probes by cell biologists. As far as Ca^{2+} is concerned, the first probe of this type was aequorin, but then the molecular engineering of GFP yielded gene-encoded probes endowed with strong fluorescence and suitable for single-cell imaging studies. In both cases, low-affinity, ER-targeted probes were developed that allowed the direct monitoring of ER Ca^{2+} levels (for methodological

aspects, and the advantages and pitfalls of each class of recombinant probes, we refer to a recent review by Rudolf *et al*.[36]).

Using these different probes, three groups have addressed the issue of Bcl-2 effects on ER Ca^{2+} levels, our own and those of KH Krause and RY Tsien.[37–39] We utilized an ER-targeted aequorin chimera[40] and transiently coexpressed it with Bcl-2 in HeLa cells. No toxicity due to Bcl-2 transient overexpression was observed, in distinction to previous reports on the use of a Bcl-2-GFP chimera.[41] Rather, the cells overexpressing Bcl-2 displayed an enhanced survival upon ceramide treatment,[42] in agreement with previous reports.[43,44] Bcl-2 showed the expected heterogeneous distribution, most of it being located on the ER membrane. As to Ca^{2+} homeostasis (Figure 1), we detected a $\sim$30% reduction in the Ca^{2+} levels within the agonist-sensitive Ca^{2+} stores (i.e. both the ER and the Golgi apparatus). Consequently, stimulus-dependent $[Ca^{2+}]$ increases were reduced both in the cytoplasm and in the mitochondria.[37] The studies also gave some insight into the mechanism of this alteration: no difference in the initial rate of uptake was detected, thus suggesting an increased leak from the organelles, rather than a reduction in the activity of the ER Ca^{2+} ATPase (SERCAs). Direct measurement of the leak rate supported this view.

The other two groups employed GFP-based probes. Krause and co-workers utilized an ER-targeted 'cameleon' and reported a lower steady-state $[Ca^{2+}]_{er}$ in Bcl-2-overexpressing HeLa cells and an increased leak rate upon thapsigargin blockage of SERCA pumps.[38] More recently, Tsien and co-workers developed an optimized cameleon probe, in which the Ca^{2+}-binding site of calmodulin and the calmodulin-binding peptide were coordinately mutated, and the affinity was suitable to the concentration range of the ER lumen. With this probe, that does not perturb calmodulin-dependent signalling (in distinction to the classical probes based on the recombinant expression of wild-type calmodu-

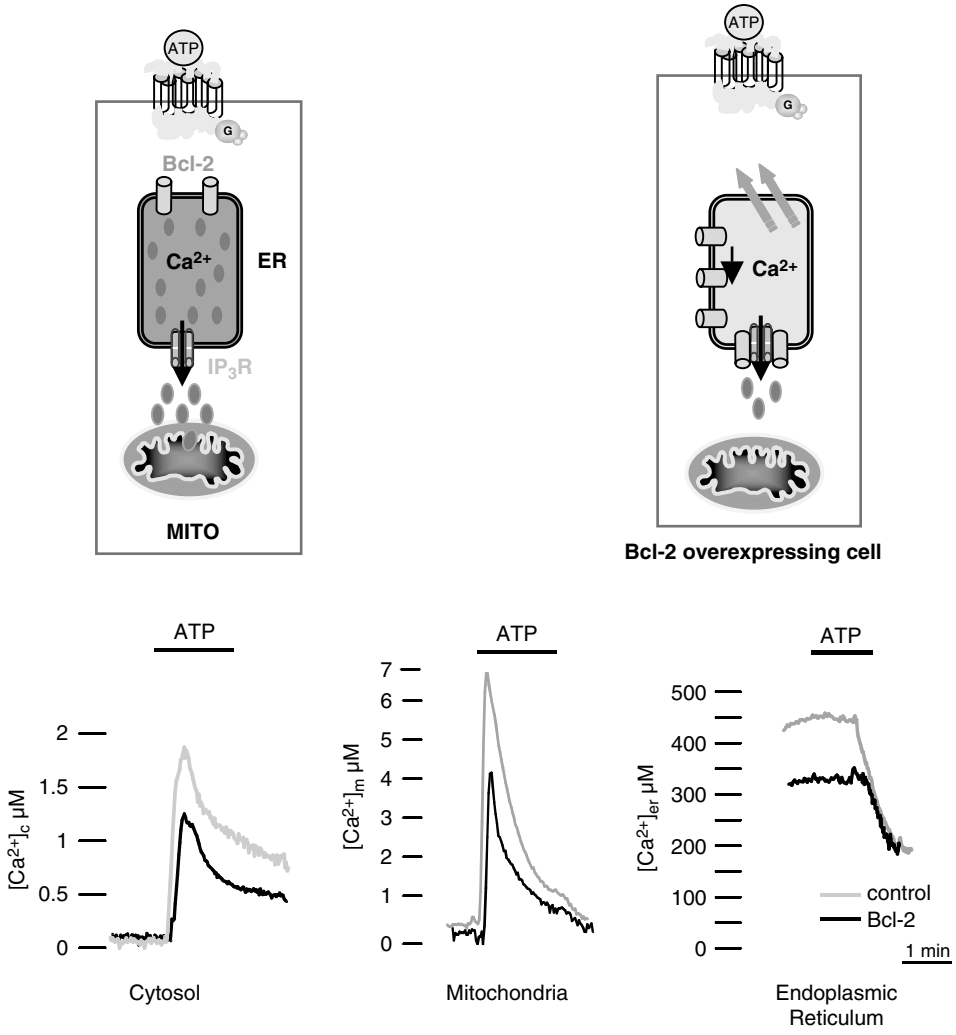

Figure 1 Subcellular analysis of Ca^{2+} homeostasis in control and Bcl-2-overexpressing HeLa cells. The traces show representative $[Ca^{2+}]$ measurements performed in the cell cytoplasm, mitochondria and endoplasmic reticulum. Grey: control cells; black: Bcl-2-overexpressing cells. To monitor $[Ca^{2+}]$ in the different compartments of transiently transfected Bcl-2 or control cells, HeLa cells were cotransfected with specifically targeted aequorin chimeras.[40] Cells were perfused with Krebs–Ringer saline solution and challenged, where indicated, with 100 μM ATP

lin), the notion of a reduced Ca^{2+} level in the ER due to increased Ca^{2+} leak was confirmed. Interestingly, these authors also showed that the green tea compound epigallocatechin gallate, known to bind to Bcl-2 and induce apoptosis in Bcl-2-expressing cells, inhibits Ca^{2+} leakage and restores $[Ca^{2+}]_{er}$ values.[39]

Overall, the three studies provide an amazingly coherent picture: despite the use of radically different probes, with different detection and calibration procedures (which may account for the significant discrepancy in the absolute $[Ca^{2+}]$ values), the direct measurement of $[Ca^{2+}]_{er}$ reveals in all cases a 20–40% decrease of the resting values. Conceptually, similar data were also obtained with other unrelated antiapoptotic proteins. The most striking example was provided by an oncogene recently described in a human hepatocarcinoma. This oncogene is generated by the integration of the hepatitis B (HBx) virus genome in the gene encoding the protein SERCA1. Viral activation was shown to *cis*-activate SERCA1 chimeric transcripts with splicing of exon 4 and/or exon 11. Splicing of exon 11 creates a frameshift and a premature stop codon in exon 12. The encoded protein lacks most of the cytosolic N and P domains and critical Ca^{2+}-binding regions of the transmembrane region. This protein is incapable of active Ca^{2+} pumping,[45] and is causally involved in the neoplastic phenotype. Although the molecular mechanism of this oncogene has not been explained yet, it may be speculated that the mutated SERCA could either interfere with the activity of endogenous pumps and/or it could act as a Ca^{2+} leak pathway from the ER. These data are consistent with the recent observations that overexpression of SERCA in HeLa cells increases the susceptibility of cells to apoptotic agents.[42,46]

The final, unquestionable proof of the relationship between Bcl-2 and Ca^{2+} homestasis came again from the elegant work of Stan and his group. In a seminal paper published in Science, Korsmeyer and co-workers demonstrated that cells from mice in which the genes for Bax and Bak (proapoptotic proteins that associate with mitochondria and initiate organelle dysfunction, but are also localized to the ER) are endowed with partially depleted ER stores, suggesting that these proteins directly counteract the effect of Bcl-2 on calcium signalling (and thus the latter protein, unopposed in the Bax/Bak knockouts (KO), allows lower $[Ca^{2+}]_{er}$ levels to be attained). In support of this possibility, siRNA silencing also of Bcl-2 restored $[Ca^{2+}]_{er}$ values similar to those of controls.[47] Along these lines, we then showed that early after overexpression of Bax in HeLa cells $[Ca^{2+}]_{er}$ levels are higher, whereas at later stages (and progression into the apoptotic phenotype) the difference with control cells becomes virtually undetectable.[48] The common scientific interest was also the opportunity for us to meet Stan Korsmeyer and discuss science with him. The friendly and stimulating discussions, as well as ideas and speculations on 'the bigger picture', turned out even more exciting that our initial enthusiasm that our data and initial hypothesis was supported by the main authority in the field (and his powerful transgenic models). We miss these discussions, as well as the collaborative work that we had planned but did not have time to carry out.

In this context, the observation that Bcl-2 also downregulates Ca^{2+} influx through the plasma membrane is not surprising. In principle, depletion of the ER Ca^{2+} store could bring to activation of capacitative Ca^{2+} influx,[49] and thus induce a prolonged $[Ca^{2+}]_c$ elevation (i.e. a potentiation of Ca^{2+}-mediated effects on apoptosis). Indeed, ER depletion of comparable degree was reported to cause a substantial activation (over 50%) of store-dependent Ca^{2+} influx.[50] Conversely, Bcl-2 expression markedly reduces the $[Ca^{2+}]_c$ increase induced by Ca^{2+} readdition to cells in which the Ca^{2+} store had been fully depleted by SERCA blockers.[37] This effect, that may represent a long-term adaptation to lower $[Ca^{2+}]_{er}$ levels, is most likely due to reduction of the number of functional channels in the plasma membrane.[51] This route appears to be followed also by other antiapoptotic proteins. In analysing the mechanism of neuroendocrine differentiation (that incidentally is a common hallmark of a variety of carcinomas), Vanoverberghe *et al*.[52] showed that in LNCaP cells the same Ca^{2+} signalling alteration (partial ER depletion and reduction of the capacitative Ca^{2+} current) was observed upon Bcl-2 expression and upon induction of neuroendocrine differentiation, although in the latter case different molecular mechanisms may be operative.

The general picture, however, is far from being fully unraveled. Indeed, in the same years a number of reports appeared which did not support the notion that Bcl-2 reduces ER Ca^{2+} levels. The largest body of work was carried out by the group of Distelhorst[19,41,53–55] that in recent papers reported that the size of ionomycin-releasable intracellular Ca^{2+} pools was the same in controls and Bcl-2-expressing cells.[56] Work by other groups support this view. In rat kidney cells[57] and in a murine hypothalamic cell line,[58] after treatment with thapsigargin no difference between control and Bcl-2-expressing cells in the increase of $[Ca^{2+}]_c$ were observed. The obvious implication is that the size of the ER Ca^{2+} pool is not affected. Further complexity is added by the results of Kuo and co-workers who observed a larger $[Ca^{2+}]_c$ increase upon thapsigargin treatment in Bcl-2 overexpressing compared to control cells. The authors suggest that the increase in the amount of Ca^{2+} released in Bcl-2-overexpressing cells is the consequence of the up regulation of SERCA gene expression and a direct activation possibly due to protein–protein interaction of Bcl-2 with SERCA.[59] Finding a reason for these discrepancies is not easy. One could argue that most of these studies monitor $[Ca^{2+}]_{er}$ indirectly (i.e. through the cytosolic peak triggered by physiological or pharmacological emptying of the ER). However, these approaches were employed also in the studies by the other groups, and a difference in the size of the pool was detected.[60] A unifying explanation therefore needs to imply that the situation differs in various cell systems, and thus cell-type-specific regulatory mechanisms may control the amplitude, if not the occurrence, of the Bcl-2 effect. The analysis of the molecular mechanism of the Bcl-2 effect may support such a possibility. Indeed, as we will discuss in detail later, recent data strongly argue for the possibility that isoform-specific interaction with the inositol trisphosphate (IP3) receptor is at the base of the Bcl-2 effect. In this case, it is reasonable to assume that the expression profile of inositol trisphosphate receptors (IP3Rs) or modulatory proteins can tune the responsiveness of cells to the signalling effects of Bcl-2.

The Correlation of the Calcium-Signalling Alteration with Sensitivity to Apoptosis

Although the demonstration of partial ER emptying upon Bcl-2 expression and of the opposite effect of proapoptotic Bcl-2 family members was strongly arguing for a functional role of the signalling alteration, the concept needed experimental verification, that was obtained by different groups. In our own work, we showed that if the Bcl-2 effect on $[Ca^{2+}]_{er}$ was replicated by different pharmacological and molecular approaches (in the absence of the oncoprotein) the cells were protected from ceramide, a Bcl-2-sensitive apoptotic stimulus, while treatments that increased $[Ca^{2+}]_{er}$ had the opposite effect.[42] A similar picture emerged from the study with the Bax/Bak KO. In those cells, when the ER Ca^{2+} levels were restored by recombinantly overexpressing the SERCA, not only mitochondrial Ca^{2+} uptake in response to stimulation was re-established, but the cells regained sensitivity to apoptotic stimuli such as arachidonic acid, C_2-ceramide and oxidative stress.[47] These results are in keeping with previous work by Ma *et al*.[46], demonstrating that SERCA overexpression in Cos cells causes ER Ca^{2+} overload and increases spontaneous apoptosis. Higher $[Ca^{2+}]_{er}$ levels imply a larger amount of Ca^{2+} that can be released into the cytosol (and taken up by mitochondria), but also a different regulation of Ca^{2+}-sensitive luminal processes of the ER (such as protein post-translational modifications and sorting). In order to verify which is the real target of the signalling modulation, we and others have acted on the luminal buffer, the low-affinity Ca^{2+}-binding protein calreticulin and demonstrated that the protective effect depends on the decrease of the releasable Ca^{2+} pool. In calreticulin overexpressing cells, in which the amplitude and duration of Ca^{2+} signals from ER lumen towards cytosol are enhanced[61] without changing $[Ca^{2+}]_{er}$,[62] cell viability is drastically reduced upon ceramide treatment.[63] Conversely, cell lines derived from calreticulin KO, that show a marked decrease in ER Ca^{2+} release upon cell stimulation, are more resistant to apoptosis.[64]

The importance of the size of the releasable pool, that is the concept that the key determinant is the net amplitude of the cytosolic rise (as well as of the mitochondrial loading, as we will discuss later) was further reinforced by the study of two viral proteins. The antiapoptotic Coxsackie viral protein 2B was shown to reduce ER Ca^{2+} levels.[65] On the contrary the proapoptotic protein of HBx augments the cytosolic Ca^{2+} signals evoked by $InsP_3$-linked agonists. However, this is not due to ER Ca^{2+} overload, as the steady-state $[Ca^{2+}]_{er}$ levels are identical to those of mock-transfected cells.[66] The mechanism leading to cell death is similar to what previously reported in staurosporin-treated neurons: the Ca^{2+} extrusion capacity of the cell is impaired by caspase-3-dependent cleavage of the plasma membrane Ca^{2+} ATPase (PMCA).[67] Indeed in HBx-transfected cells there is caspase-3 activation and PMCA cleavage, and recombinant expression of a noncleavable PMCA mutant both restored the Ca^{2+}-signalling patterns and amplitude to those of control cells and reduced the apoptotic efficiency of the viral protein. PMCA represents a very effective target for enhancing Ca^{2+} responses, because it represents the most powerful route allowing the rapid return of $[Ca^{2+}]_c$ to basal level.[66] Along the same lines, the Na^+/Ca^{2+} transporter type 1 is also cleaved by caspase-3 in cerebellar granule neurons undergoing apoptosis.[68]

Two final aspects require attention. Firstly, the idea that a $[Ca^{2+}]_{er}$ decrease protects cells from the effect of apoptotic agents may appear in contrast with the established notion that treatment of cells with SERCA inhibitors (thapsigargin, tBuBHQ, and cyclopiazonic acid) is followed by apoptosis.[69] It should be remembered, however, that the effect on $[Ca^{2+}]_{er}$ is radically different in the two conditions. Upon SERCA blockage the Ca^{2+} depletion is complete and rapid, while in Bcl-2-transfected cells the drop in $[Ca^{2+}]_{er}$ is modest and develops slowly. A drastic reduction in the level of $[Ca^{2+}]_{er}$ might interfere with the basic ER functions (e.g. the regulation of ER protein folding and chaperone interactions), and thus cause a stress response that leads to cell death. The second aspect that needs to be remembered is that calcium is not only associated to cell death, but mediates cell-specific activities and responsiveness to mitogenenic stimuli. Thus, a modification of Ca^{2+}-signalling patterns may have a pleiotropic effect, if both types of signalling are affected. Such a possibility was directly investigated by the Distelhorst group, that analysed in the very same system (T cells) the functional consequences of Bcl-2-dependent reduction of Ca^{2+}-mediated signals. Interestingly, they could show that Ca^{2+} signals elicited by maximal T-cell receptor stimulation (such as those triggered by high concentrations of anti-CD3 antibody) were significantly reduced in Bcl-2-expressing cells, whereas prosurvival Ca^{2+} signals induced by weak anti-CD3 stimulation were unaffected, possibly due to different requirement (or regulation) of the IP3R.[56]

Identifying the Effector: a Primary Role for Mitochondria?

The data so far summarized indicate that, through a reduction in ER Ca^{2+} levels, upon expression of Bcl-2 (and other antiapoptotic proteins) cellular Ca^{2+} signals elicited by physiological and pathological stimuli are reduced, and this exerts a protective effect in some apoptotic routes. The search for the effector systems is complex, and will most likely identify molecular mechanisms operating in different cell compartments. Namely, there is no doubt that a reduction in cytosolic Ca^{2+} signals will affect the activity of enzymes that have been shown to modulate the apoptotic process. To name a few, Ca^{2+}-dependent proteases, such as calpains, have been shown to be active in cell death pathways, and the ablation of their genes associated to muscle-degenerative disorders. As to kinases and phosphatases, a broad literature associates calcineurin and the different isoforms of PKC to the modulation of apoptosis (with specific, even opposite effect of different isoforms).[70,71]

In the past years, however, major attention has been devoted to mitochondria, triggered by two important discoveries. The first was the demonstration of the release during apoptosis of a specific subset of mitochondrial proteins into the cytosol. These proteins, that include cytochrome *c*, Smac/DIABLO and AIF, form with effector caspases a macromolecular machinery (the apoptosome) that precipitates cells into

apoptotic death.[8] The second observation was the functional analysis of a large-conductance mitochondrial channel, the PTP, that is activated by a variety of toxic challenges and cell signals (including increases in $[Ca^{2+}]_m$) and causes mitochondrial swelling and fragmentation. Although the molecular definition of these process is still uncertain, these morphological alterations may represent an efficient route for the release of proapoptotic proteins. Indeed, PTP opening, implicated in many types of cellular dysfunction and death, was a likely candidate for Ca^{2+}-dependent effector of apoptosis.[72,73]

We and others thus verified whether apoptotic challenges lead to mitochondrial alterations, and whether Ca^{2+} was involved in the process. In HeLa cells we observed Ca^{2+} release from the ER upon ceramide treatment and loading into mitochondria (Figure 2). As a consequence, organelle swelling and fragmentation were detected, that were parallel by release of cytochome *c*. These changes were prevented by Bcl-2 expression as well as experimental conditions that preserved $[Ca^{2+}]_{er}$.[42] PTP opening in ceramide-dependent apoptosis was directly demonstrated by Hajnoczky and coworkers who could demonstrate that the lipid mediator facilitates PTP opening. In this case, ceramide acts as a 'mitochondrial sensitizer', that thus transforms physiological IP3-mediated signals into inducers of apoptosis[33](Figure 2). A similar picture emerged with other apoptotic inducers. HBx caused, both in HeLa and hepatic cell lines, a dramatic fragmentation and swelling of the mitochondrial network, that was inhibited (together with the apoptotic efficacy) by the PTP blocker cyclosporin A.[66] As to Bax, its primary effect on Ca^{2+} homeostasis was, early upon expression, an increased ER Ca^{2+} loading, with consequent potentiation of mitochondrial Ca^{2+} responses, then the cells progressed into an apoptotic phenotype (that included drastic reductions of the cytosolic and mitochondrial Ca^{2+} responses upon challenging of cells with stimuli causing ER Ca^{2+} release). In this later phase, no difference became detectable between the steady-state $[Ca^{2+}]_{er}$ levels of controls and Bax-expressing cells. Under those conditions, two features dominated the general picture of Ca^{2+} signalling. The first was a drastic perturbation of mitochondrial three-dimensional structure and of key functional parameters, including mitochondrial membrane potential and the Ca^{2+} uptake capacity of the organelle.[48] This effect also provides a possible explanation for second feature, the gradual decrease in ER Ca^{2+} loading: reduced ATP provision to the SERCA pumps could gradually impair Ca^{2+} (re)accumulation into the ER, thus bringing the $[Ca^{2+}]_{er}$ steady-state levels to those of a control cell. An alternative possibility stems from the recent demonstration that the IP_3R includes a consensus site for caspase-3 cleavage and the cleaved form of the receptor exhibits increased leakiness and reduced sensitivity to IP_3.[74,75] In turn, a smaller Ca^{2+} release results in the reduction of the amplitude of cytosolic Ca^{2+} responses (an effect that cooperates with the direct effect on mitochondria in causing the dramatic, 30–50% reduction of mitochondrial $[Ca^{2+}]$ peaks).

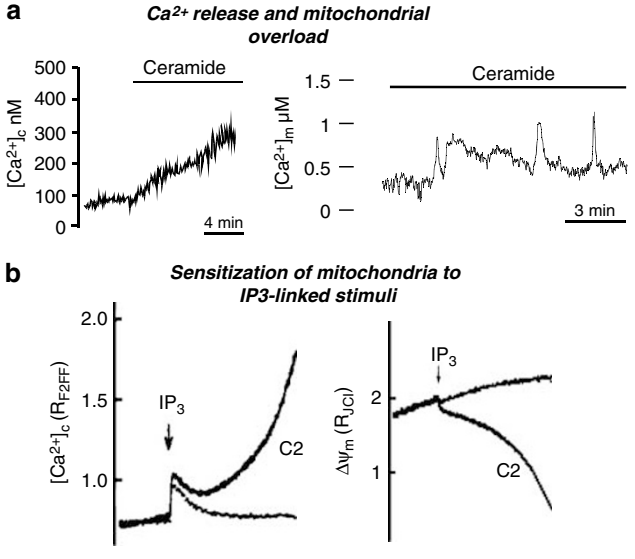

Figure 2 Possible mechanisms of ceramide action on Ca^{2+} homeostasis and mitochondrial function. (**a**) Ceramide can induce leak of stored Ca^{2+} from the ER. Depletion of the ER Ca^{2+} content activates capacitative calcium influx with further $[Ca^{2+}]_c$ increase and mitochondrial Ca^{2+} loading as measured with aequorin-based probes (reproduced with permission from Pinton *et al*.[63]). In turn this causes major morphological alterations of mitochondria, and thus release of proapoptotic factors to the cytoplasm; (**b**) ceramide can induce sensitization of mitochondria to IP3-linked stimuli (reproduced with permission from Szalai *et al*.[33]). Simultaneous measurements of mitochondrial membrane potential ($\Delta\Psi_m$) measured with TMRM and $[Ca^{2+}]_c$ measured using fura2F/FFA during IP3R stimulation in control and ceramide (C2)-treated cells are shown

In summary, Bcl-2 and other antiapoptotic proteins reduce ER Ca^{2+} levels, and consequently they moderate the efficacy of apoptotic mediators that use Ca^{2+} signals (and the involvement of mitochondria as downstream effectors) as a potentiation/commitment factor. Conversely, Bax enhances the loading of the ER Ca^{2+} store, and thus boosts the Ca^{2+} load to which the apoptotic effector systems (including mitochondria) are exposed upon physiological and/or pathological challenges. This effect of Bax coincides with gross perturbation of mitochondrial structure and function, and finally, later in apoptotic progression, to the development of an altered signalling phenotype, that includes impaired ER Ca^{2+} release upon cell stimulation, and thus reduction of cellular Ca^{2+} signals. What is unclear from these studies is whether the ability of Bcl-2 and Bax to regulate ER $[Ca^{2+}]$ is an intrinsic property of these proteins or rather the outcome of a regulatory effect on other proteins.

The Molecular Mechanism

Bcl-2 channel activity

The molecular mechanism by which Bcl-2 alters ER Ca^{2+} leakiness channels is still unresolved. In light of the evidence that Bcl-2 may be an ion channel,[17,18] the simplest explanation for the increased passive leakage observed in Bcl-2-overepressing cells would be a higher density of Bcl-2 channels. In other words, Bcl-2 could facilitate Ca^{2+} leakage from the ER by forming a channel, either by oligomerizing or by interacting with other members of the Bcl-2 family. The conceptual problem with this explanation (strongly supported by the demonstration of ion conductance in lipid bilayers) is that also Bax has structural similarities with pore-forming bacterial toxin and thus can in principle form ion channels in artificial membranes. However, Bax augments, not reduces

$[Ca^{2+}]_{er}$. One could argue that the channel properties of the two pore-forming domains may be different (e.g. for the presence of acidic residues in Bcl-2 where basic ones are present in Bax), thus accounting for different ion selectivity of the channel activity of the two proteins (reviewed in Schendel *et al*.[17]). Specifically, the predicted fifth and sixth α-helices of Bcl-2 and Bax are hypothesized to directly participate in channel formation. These α-helices are positioned in the core of these proteins and are believed to be inserted into the lipid bilayer with the loop connecting α5 and α6 presumably protruding on the other side of the membrane. Indeed, deletion of the α5-α6 regions from Bcl-2 abolishes its ability to form ion channels in synthetic membranes *in vitro*.[18,76] To verify the role of the presumed channel-forming α5–6 helices of Bcl-2 and Bax in Ca^{2+} mobilization were used two chimerical constructs, in which the putative pore domains of the proteins were mutually swapped.[77] The results (Figure 3) showed that when the putative pore-forming domain of Bax replaced that of Bcl-2 in the Bcl-2 polypeptide the chimeric protein was still capable of reducing $[Ca^{2+}]_{er}$. Conversely, the Bax chimera including the putative pore-forming domain of Bcl-2 did not significantly alter $[Ca^{2+}]_{er}$, similarly to the wild-type protein. Accordingly, the two chimeras retained the effects on apoptosis of the original protein: the Bax protein with Bcl-2 (α5–6) helices is proapoptotic, and causes perturbation of mitochondrial structure and function (including the drastic reduction of $[Ca^{2+}]_m$ responses), whereas the Bcl-2 chimera with Bax (α5–6) protects against stimuli, such as ceramide, acting through a calcium- and mitochondria-dependent pathway.[48] The general picture emerging from these results is that Bax and Bcl-2 both have an effect on Ca^{2+} signalling, that does not depend on the region proposed to form an ion channel.

Direct molecular interaction with ER channels

An alternative, intriguing possibility that links Bcl-2 to cellular Ca^{2+} signalling through a mechanism independent of the pore-forming domain, is that Bcl-2 could interact with and alter the function of an endogenous release channel or pore-forming protein in the ER (Figure 4). Recently, strong experimental evidence was obtained indicating that Bcl-2 and related antiapoptotic proteins regulate the most logical target, that is the IP3R, although two conceptually different mechanisms are proposed. The first article was contributed by Stan Korsmeyer's group. Stan and co-workers demonstrated

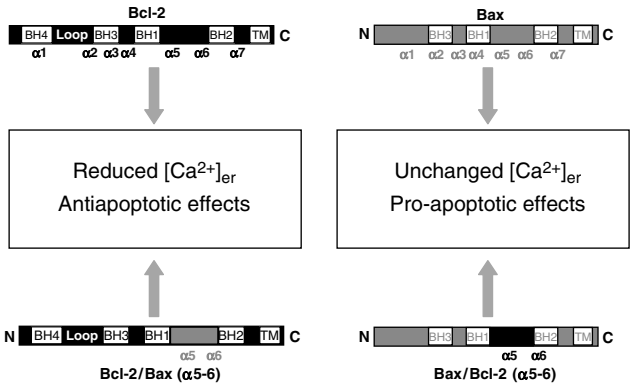

Figure 3 Schematic representation of the Bcl-2 and Bax chimera and of the putative regions of the encoded polypeptide. The predicted positions of the α-helical regions within the human Bcl-2 and Bax proteins are shown. Bcl-2/Bax(α5–6) corresponds to a Bcl-2 construct in which the putative pore-forming domain was replaced with that of Bax. Bax/Bcl-2(α5–6) corresponds to a Bax construct in which the putative pore-forming domain was replaced with that of Bcl-2. BH, Bcl-2 homology domains; TM, transmembrane domaine

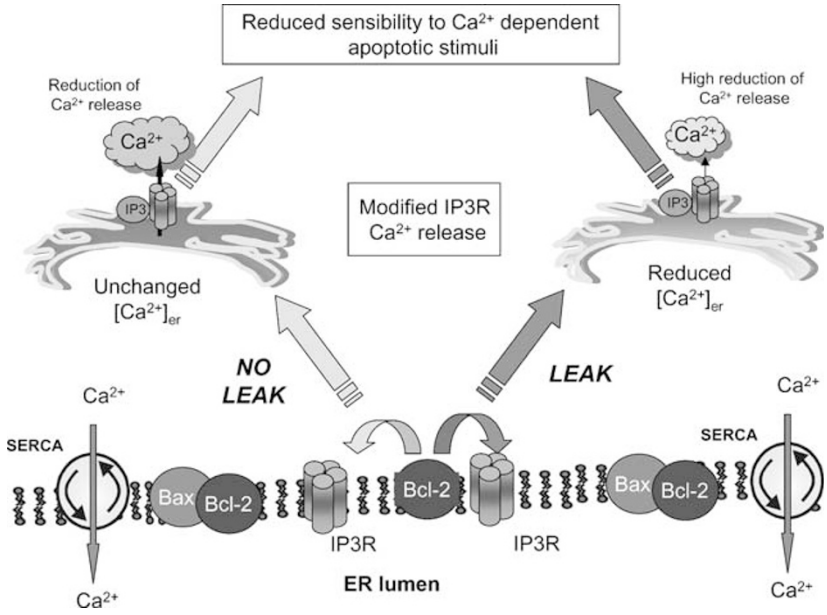

Figure 4 Schematic model of Bcl-2 effects on Ca^{2+} homeostasis. Two nonmutually exclusive mechanisms are presented. In the first (right part) Bcl-2 interacting with the IP3R induces Ca^{2+} leakage from the ER, in turn causing a significant reduction of $[Ca^{2+}]_{er}$ and a consequent impairment of Ca^{2+} release from ER after stimulation. In the second scenario (left part) Bcl-2 interacting with the IP3R (possibly different subtypes) reduces the sensitivity to IP3-linked stimuli. In both cases Bcl-2 and other antiapoptotic proteins, can reduce ER Ca^{2+} release, and consequently moderate the efficacy of apoptotic mediators that use Ca^{2+} signals as a potentiation/commitment factor

that Bcl-2 controls the phosphorylation state of type I IP3R, that in turn regulates the leak rate through the channel. In support of this view, the $[Ca^{2+}]_{er}$ reduction of Bax/Bak KO was reversed by siRNA silencing of IP3R-I.[78] More recently, the groups of Kevin Foskett and Craig Thompson demonstrated that Bcl-X_L directly binds to the IP3R and in single-channel electrophysiology experiments with isolated nuclei both the addition and the recombinant expression of Bcl-X_L sensitized the IP3R to low agonist doses. Recombinant expression (or addition to the patch pipette) of Bax prevented the effect of Bcl-X_L, both in terms of its binding to the IP3R and of capacity of modifying the sensitivity to IP3.[79] The results differ from a previous paper by the Distelhorst group that showed that Bcl-2 reduces the opening probability of IP3Rs inserted into lipid bilayers.[53] As to the mechanism, the Foskett–Thompson paper proposes a direct modulatory role on IP3R (through protein–protein interaction between the two proteins) rather than a phosphorylation event, based on the timing after Bcl-X_L addition and the experimental conditions employed (nonpermissive for phosphorylation). Work needs to be carried out to verify whether the different results of these papers reflect true experimental discrepancies or functional complexity. Indeed, not only the Bcl-2 family members could differ in the signalling mechanism, but the variability between cell lines in IP3R levels, isoform expression (given that IP3RI is proposed to by phosphorylated) and basal levels of IP3 (key regulatory determinant in the Foskett–Thompson paper) could account for the apparently conflicting results. Such a complexity could also be reflected in the previously discussed discrepancies in the Ca^{2+} measurements. Where all papers agree in on the important concept that the IP3R is at the centre of the stage, and Bcl-2 hits the main route for Ca^{2+} release from intracellular stores. This observation well fits with the observation that Bcl-2 alters Ca^{2+} oscillations triggered by agonist acting by IP3R stimulation[39] and with studies reporting a correlation between low expression of IP3R and inhibition of apoptosis.[80] Finally, the Foskett–Thompson paper, by looking at sensitivity to apoptosis of DT40 cells in which all three IP3R isoforms had been deleted, shows that basal activation of the IP3R not only partially depletes the Ca^{2+} store but also provides a 'survival' signal *per se* (possibly by activating mitochondrial metabolism), given that the KO cells are more sensitive to apoptotic challenges than the wild-type cells.[79] Also in this case, given the robust evidence for the role of mitochondrial Ca^{2+} uptake in facilitating PTP opening and proapoptotic mitochondrial changes, it seems fair to conclude that Bcl-2 globally reprogrammes ER Ca^{2+} release and mitochondrial Ca^{2+} loading, as necessary for optimally activating apoptosis (in terms of metabolic requirements as well as prompt release of caspase cofactors into the cytosol).

Conclusions

A broad series of data now allows to state that Bcl-2 expression interferes with cellular Ca^{2+} signals, most likely by acting on the IP3R and thus modifying Ca^{2+} leakage in resting conditions (and thus the steady-state $[Ca^{2+}]_{er}$) and its release kinetics upon stimulation. In turn, this affects the efficacy of apoptotic challenges by influencing the sensitivity of Ca^{2+} effectors, such as mitochondria. Despite the significant advancements of the last years, much remains to be understood, including the final assessment of the molecular mechanism of the Bcl-2 effect and the significance of this process for apoptosis *in vivo*. However, these data already strengthen the view that also apoptotic cell death belongs to the numerous cell functions modulated by Ca^{2+} ions and thus add a well-known pharmaceutical target to the possibilities for modulating the route to cell degeneration.

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