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Review

The secrets of the Bcl-2 family

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The Bcl-2 family of proteins is formed by pro- and antiapoptotic members. Together they regulate the permeabilization of the mitochondrial outer membrane, a key step in apoptosis. Their complex network of interactions both in the cytosol and on mitochondria determines the fate of the cell. In the past 2 decades, the members of the family have been identified and classified according to their function. Several competing models have been proposed to explain how the Blc-2 proteins orchestrate apoptosis signaling. However, basic aspects of the action of these proteins remain elusive. This review is focused on the biophysical mechanisms that are relevant for their action in apoptosis and on the challenging gaps in our knowledge that necessitate further exploration to finally understand how the Bcl-2 family regulates apoptosis.
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The proteins of the Bcl-2 family are key regulators of the mitochondrial pathway of apoptosis. They control the permeabilization of the mitochondrial outer membrane (MOM) that releases cytochrome *c* and other apoptotic factors into the cytosol. This results in downstream activation of the caspase cascade and is considered a point of no return in the cell commitment to die. Apoptosis regulation by the Bcl-2 proteins is crucial for tissue homeostasis, for embryo development and for the maturation of blood cells.[1] Importantly, deregulation of the Bcl-2 proteins has a major role in tumor formation and in the cellular responses to anticancer therapy. The Bcl-2 family is also involved in other diseases, such as autoimmune, infectious and neurodegenerative disorders. On the other hand, there is increasing evidence that they also have additional functions in other cellular processes, such as mitochondrial morphology and metabolism, which remain largely unexplored.[2]

Because of their biological relevance and their potential as therapeutic targets, the Bcl-2 proteins have been investigated intensively during the past 25 years. During this time, a number of important questions have been answered. The 20 or so members of the family have been identified and classified according to their function in apoptosis.[3] Classically, three subgroups have been defined: (i) the prosurvival Bcl-2 proteins, such as Bcl-2 itself, Bcl-xL, Bcl-w, Mcl-1 and A1, which inhibit cell death through direct interactions with the proapoptotic members; (ii) the executioners Bax and Bak, which are believed to participate directly in MOM permeabilization; and (iii) the BH3-only proteins, which share a common motif called the BH3 domain and have evolved to sense different stresses in the cell and to initiate apoptosis. The BH3-only proteins have been further classified as 'sensitizer/derepressors', such as Noxa, Bfm or Bik, which can only interact with the prosurvival members and antagonize their function, and 'direct activators', such as Bid and Bim, which in addition have the ability to directly activate Bax and Bak. Under normal conditions, the BH3-only proteins are inactive or exist at low levels in the cell. In the presence of apoptotic stimuli they are activated by post-translational modifications or their expression is increased to induce apoptosis.[4] As a result of BH3-only stimulation, Bax and Bak become activated. It has been observed that upon their activation during apoptosis, Bax translocates from the cytosol to the MOM.[5] Once there, Bax and Bak, which is constitutively bound to the MOM, change conformation, insert into the membrane, oligomerize and induce the release of cytochrome *c*.[6–9] Notably, also some antiapoptotic Bcl-2 members have been shown to translocate and insert into the MOM upon apoptotic stimulation.[5,10] In this scenario, the prosurvival Bcl-2 proteins inhibit MOM permeabilization by direct interactions with the proapoptotic members.

However, there are a number of rather basic questions, still unanswered, that limit our understanding of how the Bcl-2 proteins regulate cell death. For example, we still do not have any clear evidence of how exactly Bax and Bak become activated in the cell and how they mediate MOM permeabilization. It seems that prosurvival Bcl-2 proteins are able to inhibit both BH3-only proteins and Bax and Bak, but the mechanisms involved and their relative importance are only now starting to be uncovered.[9] In addition, we do not understand how the complex interaction network formed by the multiple Bcl-2 members present simultaneously in the cell orchestrates apoptosis signaling. Although the importance of

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Abbreviations: BH domain, Bcl-2 homology domain; MOM, mitochondrial outer membrane; ER, endoplasmic reticulum; FRET, Förster resonance energy transfer; FCS, fluorescence correlation spectroscopy; FRAP, fluorescence recovery after photobleaching; FLIP, fluorescence loss in photobleaching; PALM, photoactivated localization microscopy; STED, stimulated emission depletion; STORM, stochastic optical reconstruction microscopy
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the membrane in the Bcl-2 signaling of apoptosis has recently been acknowledged, the effects of combining signaling events in the cytosol and in the lipid environment of the membrane are not completely clear.

Here, we review the state of our understanding of the function of the Bcl-2 proteins in apoptosis with a focus on the open questions and controversies in the field. We provide a biophysical perspective that may provide a new way of looking at this family of proteins and help find the answers we are looking for. Finally, we comment on new biophysical approaches based on fluorescence microscopy that will have an impact in the progress of the field in the coming years.

The Multiple Activation States of Bcl-2 Homologs

To be grouped as a family member, a protein must contain at least one Bcl-2 homology domain (BH domain). The so-called Bcl-2 homologs or multi-domain members, including Bax and Bak and most prosurvival Bcl-2 members, exhibit a high homology at the level of sequence and structure and contain domains BH1, BH2 and BH3. Such high degree of similarity is striking precisely because some Bcl-2 homologs are proapoptotic, whereas others are antiapoptotic. Yet to this date, the precise features that determine a protein behaving in one way or another remain a major puzzle. Some elusive sequence details in the amphipathic central hairpin seem to have a role, as their exchange in Bcl-xL and Bax inverts their apoptotic activity.[11] Another cause could be the inability of the antiapoptotic members to oligomerize into high molecular weight structures such as Bax and Bak.[12] An interesting observation is that several antiapoptotic Bcl-2 proteins are cleaved during apoptosis and then become proapoptotic.[13,14] The molecular basis for this reversal of function is also unclear. One possibility could be that the truncated versions behave as BH3-only proteins[15] or it could be that they are transformed into Bax/Bak-like proteins. Surely, engineering approaches that manage to control the pro- or antiapoptotic activity of these proteins with designed mutations will shed light on this unresolved issue.[9,11,16]

One fascinating aspect of the Bcl-2 homologs is that they have, at least, two possible stable conformations. On the one hand, in aqueous environments they organize into globular structures.[17,18] This is also believed to be true for mitochondria-associated species that are anchored to the membrane via the C-terminal hydrophobic tail. On the other hand, during apoptosis, they adopt a membrane-inserted conformation with extensive regions of the protein in contact with the hydrophobic part of the lipid bilayer.[9,19–21] This has led to the hypothesis that both pro- and antiapoptotic Bcl-2 can exist in several activation states, involved in interactions with other Bcl-2 members. Yet, the precise molecular mechanisms that regulate such conformational changes, and therefore their activation, remain obscure.

One of the problems arises from the difficulty to experimentally detect interactions of Bax and Bak with the 'direct activators'. So far, there is only one report describing a steady-state, reversible interaction between full-length Bax and tBid in the membrane of liposomes by FRET (förster resonance energy transfer) spectroscopy.[6] These difficulties have led to the proposal of the 'kiss-and-run' hypothesis for Bax and Bak activation.[7,22] In this model, short-lived direct interactions of tBid or Bim with Bax/Bak in the membrane vicinity would induce the conformational change and extensive insertion of the latter in the membrane. In the case of the antiapoptotic Bcl-2 homologs, although direct activations between them with proapoptotic Bcl-2 proteins have been detected, there is still less information about how their different conformational states are regulated. In *in vitro* studies, tight interactions with tBid in the presence of lipid vesicles lead to membrane binding and extensive insertion of soluble Bcl-xL.[23]

How do Bax and Bak Mediate MOM Permeabilization?

Despite intense efforts, there is no clear evidence of how Bax and Bak mediate MOM permeabilization during apoptosis. Upon their activation at the MOM during apoptosis, both Bax and Bak insert extensively into the membrane and oligomerize, which results in the release of cytochrome *c* into the cytosol. Unfortunately, at the moment we only have scattered evidence which of the regions in these proteins are involved in membrane interactions[21,24,25] and oligomerization studies that suggest two contact surfaces, 'nose-to-nose' and 'back-to-back'.[24,26–28] Detailed structural studies that reveal the organization of Bax and Bak in their active form in the membrane would be essential to understand how these proteins mediate MOM permeabilization. However, we have so far been unable to obtain these elusive data and one may speculate that the very nature of the structures adopted by these proteins in the membrane could be the reason behind it.

The MOM is already permeable to molecules smaller than 5 kDa and is a crowded environment, with around 50% protein content.[29] The alterations induced by Bax and Bak allow the simultaneous release of larger molecules: for example cytochrome *c* (12 kDa), SMAC/Diablo (27 kDa) and Omi (48 kDa). One possibility is that Bax and Bak regulate other mitochondrial channels. In such a protein-dense membrane, contacts with other MOM proteins are not difficult to imagine. However, a number of genetic studies question the requirement of such components.[30–32] The most widely accepted model assumes that upon oligomerization, Bax and Bak form a large pore in the MOM that is responsible for the release of the apoptotic factors. This is mainly based on the structural similarities of Bcl-2 homologs with bacterial pore-forming toxins and on the observation that Bax and Bak exhibit pore activity in *in vitro* reconstituted systems with artificial lipid membranes.[8,33,34] However, several antiapoptotic Bcl-2 proteins have also been shown to display similar membrane permeabilizing activity *in vitro*.[23,35–38] This paradox is usually not taken into account.

Nevertheless, if one looks carefully at the data describing the Bax pore in artificial membranes, there are fascinating features to be found. On the one hand, it seems that the presence of cardiolipin, a specific mitochondrial lipid, and tBid are essential for Bax activity. Several groups have reported that the presence of cardiolipin in the membrane is necessary for Bax to oligomerize and to form pores large enough to release cytochrome *c*.[39–41] However, Bax was shown to be able to oligomerize and to induce cytochrome *c* release from cardiolipin-deficient mitochondria.[42] As a result of these contradictory observations, it still remains unclear whether

cardiolipin and tBid form part of the pore organized by Bax, or whether cardiolipin is merely acting as a membrane receptor for tBid, which is merely acting as a receptor to bring Bax to the membrane. In support of the latter, fragments corresponding to helices 5 and 6 in Bax exhibit similar pore activity to the full-length protein, but independently of cardiolipin and tBid.[20,43] This indicates that Bax itself contains the necessary motifs for pore formation and that tBid and cardiolipin are regulators of Bax activity. In summary, how this special lipid and BH3 direct activators contribute to the properties of Bax pore remains to be answered.

On the other hand, there is growing evidence that Bax forms what is known as a toroidal or lipid pore, as schematically shown in Figure 1. Toroidal pores are characterized by having lipids aligned at the rim of the pore. To avoid the high energetic cost of exposing their hydrophobic acyl chains to the aqueous environment, lipids bend and form a highly curved, non-bilayer structure at the pore edge that connects the two monolayers of the membrane with a continuous surface. In this context, lipids can easily exchange monolayers by simple diffusion at the pore. The physical principles governing the energy of toroidal pores have been characterized during the past couple of decades.[44] Pore opening in lipid bilayers is very unlikely, but it happens when the membrane is under stress, known as membrane tension. One example to induce membrane tension is the asymmetric insertion of proteins into one of the membrane leaflets. When a threshold amount of membrane tension is reached, the bilayer structure becomes unstable and a pore will open. Once the pore is open, the creation of a high membrane curvature at the pore rim has an energy cost known as line tension that, together with the membrane tension, contributes to the pore energy. As a result, toroidal pores are in general meta-stable structures with variable size, where the membrane tension tends to enlarge the pore and the line tension is the driving force for pore closure, as it minimizes the energy of the system. Molecules that decrease line tension stabilize the pore edge and increase its lifetime.

Consistent with this model, Bax is able to release high molecular weight molecules from liposomes through a mechanism sensitive to the instrinsic monolayer curvature of the lipids composing the membrane.[45] Moreover, it also induces transbilayer lipid redistribution in liposomes.[39] Helix 5 from Bax exhibits a similar behavior and has been shown to decrease the line tension at the pore edge and to form stable pores.[43,46,47] Interestingly, the structure of toroidal pores was first demonstrated with this Bax peptide.[48] Moreover, cryoelectron microscopy studies on liposomes permeabilized by Bax or Bak have revealed large openings in the lipid bilayer compatible with toroidal pores.[24,34,49] We still need to find out whether Bax localizes to the pore edge, which seems not to be strictly necessary for toroidal pores.[50] Also, it remains unknown whether Bax pores are variable in size, and if so, which parameters determine their size. In any case, the major question of what actually happens in the context of the MOM still needs to be answered.

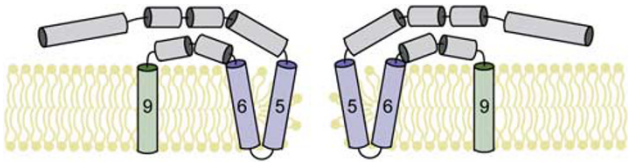

Figure 1 Model for lipid pore induced by Bax and Bak. Once Bax and Bak insert extensively into the MOM and oligomerize, they induce the formation of a toroidal (or lipid) pore. Such pores are thought to be formed by lipids and proteins. In order to avoid the exposure of the hydrophobic acyl tails to the aqueous solvent, the lipids at the pore edge reorganize to form a non-bilayer structure with a very high curvature, which also has an energetic cost. Bax and Bak stabilize the pore by decreasing the energy of the pore rim. According to current models, helices 5 and 6 are inserted into the membrane and are involved in pore formation, and helix 9 is also membrane inserted[25,82]

The ability of Bax to stabilize highly curved membranes in the context of lipid pores may have important consequences for its function in other cellular processes, like for example in the case of mitochondrial morphology.[51] In cells, mitochondria form tubes that continuously fuse and divide. The process of fusion and fission necessarily proceeds via hemifusion/hemifission intermediates characterized by a non-bilayer lipid organization. Interestingly, cardiolipin has a molecular structure that tends to form non-bilayer structures and might have a role in these processes. Indeed, mitochondria undergo extensive fragmentation during apoptosis and Bax has been detected at the fission foci.[52] Recently, it was also reported that fragmented mitochondria are sensitized to Bax activation.[53] In addition, the membrane hemifusion structures induced by Drp1, a dynamin-like protein involved in mitochondrial fission, have been shown to promote Bax oligomerization and activity.[54] The link between mitochondrial fragmentation and MOM permeabilization remains highly controversial.[2] However, given the biophysical similarities between toroidal pores and hemifusion structures, it seems reasonable that Bax may concentrate at those sites to stabilize the non-bilayer structures, thus promoting MOM permeabilization and mitochondrial fission during apoptosis.

What do the Prosurvival Bcl-2 Proteins Really do?

Several mechanisms have been proposed to explain the inhibition of apoptosis by the Bcl-2 proteins (see Figure 2). It seems clear that the prosurvival members of the family can block apoptosis by direct binding both to the executioners Bax and Bak, and to the inducers BH3-only proteins. However, the hierarchy of the interactions that are responsible for apoptosis inhibition remain controversial. Do the prosurvival members inhibit apoptosis mainly by sequestering and blocking the BH3-only proteins, or mostly by binding to Bax and Bak and inhibiting their permeabilisation of the membrane? The dissection of the relative importance of these interactions has only just begun. In a recent study using tBid chimeras with different affinities to prosurvival Bcl-2s, Llambi *et al.*[9] reported that, although both inhibitory mechanisms happen in the cell, inhibition of MOM permeabilization by the prosurvival Bcl-2 members via sequestration of the BH3-only proteins is less efficient than via inhibition of Bax and Bak (Figure 2a).

However, given the multi-step mechanism of Bax and Bak induction of MOM permeabilization, it remains unclear at which level the antiapoptotic Bcl-2 proteins interfere with the process. On the one hand, Bcl-xL has been shown to inhibit Bax association with model membranes,[12] but it is not clear whether this is via direct interactions in the cytosol or by

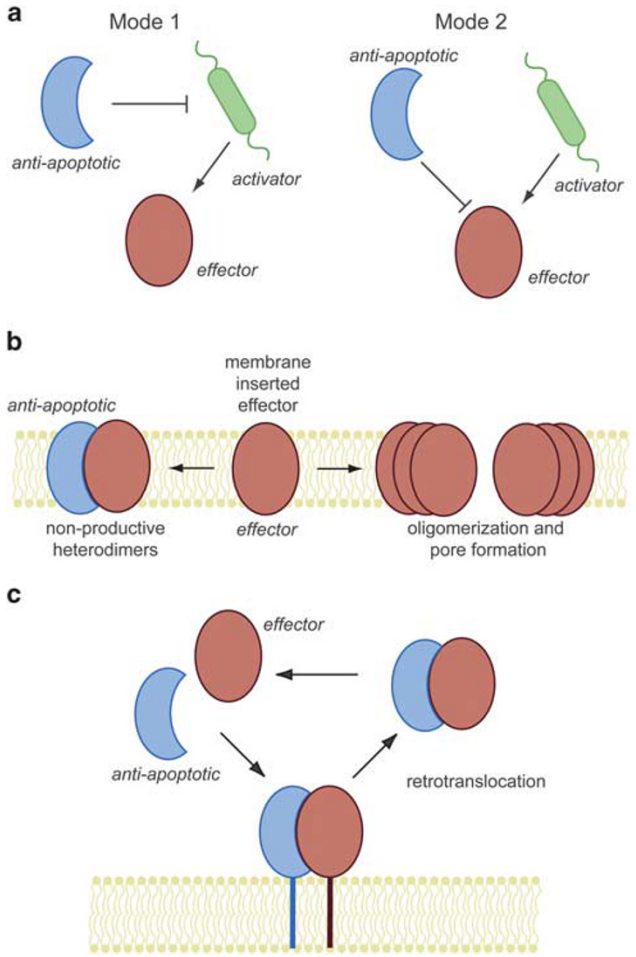

Figure 2 Models for inhibition of MOM permeabilization by the antiapoptotic Bcl-2 proteins. (**a**) Modes 1 and 2 of inhibition proposed by Llambi *et al.*[9] In mode 1, prosurvival Bcl-2 proteins inhibit the BH3-only activators, which cannot further induce the activation of the effectors Bax and Bak. In mode 2, the prosurvival Bcl-2s bind and inhibit directly the effectors. Mode 1 is less efficient than mode 2 to inhibit apoptosis. (**b**) According to the 'embedded together' model, the prosurvival Bcl-2 proteins bind to Bax and Bak in their inserted form at the MOM and form non-productive heterodimers for further oligomerization and MOM permeabilization.[12] (**c**) Bax and Bcl-xL constantly translocate to the mitochondria, where they form a complex and retrotranslocate back to the cytosol.[56] This keeps Bax mainly cytosolic in healthy cells

sequestering cBid, as suggested by the above observations. On the other hand, several lines of evidence point to complexes of the prosurvival and the executioner Bcl-2 proteins being extensively inserted into the MOM.[9,10,23,25,55] In line with this, Bcl-xL was described to inhibit Bax by binding to its membrane-inserted form and preventing its oligomerization and further autoactivation[12] (Figure 2b). Antiapoptotic Bcl-2 homologs would then act as oligomerization-deficient, dominant-negative versions of Bax.

Recently, an additional level of inhibition has been reported. Edlich *et al.*[56] proposed that under normal conditions Bax is constitutively and constantly retro-translocating from mitochondria to the cytosol via a mechanism dependent on transient interactions with Bcl-xL. According to this model, in healthy cells antiapoptotic Bcl-2 proteins would inhibit Bax constitutive binding to the MOM by binding it, detaching it from the membrane and releasing it again in the cytosol (Figure 2c). However, this hypothesis raises many questions. Usually, association/dissociation cycles of proteins with membranes require energy consumption to bring the system out of the energy minimum. In this case, how the relative energy of the protein conformations involved (soluble *versus* inserted) is linked to the retro-translocation process remains to be explained.

Role of the Membrane

Until recently, Bcl-2 models paid no or little attention to the role of the lipid membrane in the regulation of MOM permeabilization. The 'embedded together' model was the first to emphasize that membrane insertion changes the conformation of Bcl-2 proteins, which in turn affects their interactions with other family members.[57] As a consequence, whether the multiple interactions between Bcl-2 family members at the MOM will end in membrane permeabilization depends on the final oligomeric state of Bax and Bak within the membrane environment. But what are the signaling effects of the translocation of Bcl-2 proteins from the polar, 3D environment of the cytosol into the hydrophobic, 2D structure of the MOM?

The lipid environment has been shown to strongly affect interactions between Bcl-2 proteins. A quantitative analysis was essential to uncover this effect. The comparison of the binding affinity between tBid and a form of Bcl-xL lacking the C-terminal domain by fluorescence correlation spectroscopy (FCS) in solution and in model membranes revealed the role of the membrane on promoting the interactions between these proteins.[23] The absolute amounts of proteins required for effective complex formation were much lower when the tBid/Bcl-xL complex was formed within the lipid bilayer than in solution. As these proteins induce each other's binding to the membrane, these results suggested that the inhibitory effect of Bcl-xL over tBid happens mainly at membranes.

But so far, little has been discussed regarding the physical chemical implications of Bcl-2 complex formation in the cytosol *versus* cellular membranes. In most studies in the literature, association between Bcl-2 proteins is promoted in the presence of membranes, most likely due to interactions within the lipid environment. These observations can be explained by a number of mechanisms. First, Bcl-2 proteins are effectively concentrated by their translocation from a 3D into a 2D environment. This concentration effect, together with the molecular crowding existing in cellular membranes, would be the simplest explanation and has been proposed to be the driving mechanism in other cellular processes.[58]

Moreover, the anisotropic environment of the lipid bilayer (in contrast to the cytosol, not equivalent in the *x, y, z* spatial directions) influences the orientation of the proteins as they collide with each other in the membrane plane. Membrane-bound proteins can rotate around the axis vertical to the membrane plane, but their translational movement is restricted. This would then help disposing the two interacting proteins in the right orientation to each other and thus increase the probability of successful collisions for complex formation.

Another possibility is that the energy of the electrostatic bonds in the Bcl-2 complexes is increased in the hydrophobic

milieu of the membrane, where there number of water molecules that compete for these interactions is drastically reduced. In addition, new interaction surfaces formed after the conformational changes associated with membrane insertion may stabilize the Bcl-2 complex. For example, a new interaction interface between α6:α6 helices was recently detected by cross-linking experiments in Bak oligomers at the MOM.[27] Interestingly, a BH3 peptide derived from Bid was able to compete for the interaction between tBid and Bcl-xL in solution, but not within the lipid bilayer, even when it was targeted to the membrane.[23] This suggests that the nature of the interactions in both environments is different, which could be of interest for the development of anticancer drugs targeting prosurvival Bcl-2 proteins bound to membranes.

In principle, these effects could be responsible for a general increase in complex formation between Bcl-2 proteins at cellular membranes. But of course, it would also apply to complexes of Bcl-2 proteins with other cellular components, opening additional possibilities for regulation by competitive binding, and thus situations where complex formation between Bcl-2 members is hindered. Moreover, the slower mobility of proteins in lipid membranes could introduce diffusion-limited effects that counteract complex formation. However, the integration of all these parameters remains a challenge that hinders the *a priori* prediction of membrane effects on Bcl-2 complex formation.

The complexity of the system is increased by the fact that several members of the Bcl-2 family localize not only at the MOM but also at other cellular membranes.[59,60] At the endoplasmic reticulum (ER), they have been implicated in sensing ER stress signals and initiating apoptosis.[61] For example, Bcl-2, Bc-xL and Mcl-1 contribute to apoptosis resistance by modulating the calcium channel activity of the inositol 1,4,5-trisphosphate receptor.[62,63] Moreover, Bax and Bak have been shown to translocate to the ER and increase its permeability during apoptosis.[64] So, it seems that these proteins also function as modulators of membrane permeability at the ER. Interestingly, the permeability of the nuclear membrane is also affected during apoptosis in a Bax/Bak-dependent manner.[65] This suggests that the action of the Bcl-2 members in apoptosis may affect the permeability of several cellular membranes. The physiological importance of these observations and the implication of Bax/Bak pore activity in the process deserve further investigation.

The Complexity of the Bcl-2 Network

As explained above, the members of the family engage in interactions with each other to set the threshold for cell death commitment. A graph with the identified interactions between different Bcl-2 proteins is shown in Figure 3. They form an intricate, fine-tuned interaction network, which combines events in two physically distinct environments, cytosol and membranes. A major controversy in the field is which of these interactions are essential for the regulation of apoptosis. This is illustrated by the proposal of the competing 'direct activation',[40] 'sensitizer/derepressor'[66] and 'embedded together' models,[3] as well as the recent 'unified model',[9] that try to provide an answer to this question. An additional hurdle is that the expression of Bcl-2 proteins differs by cell type,

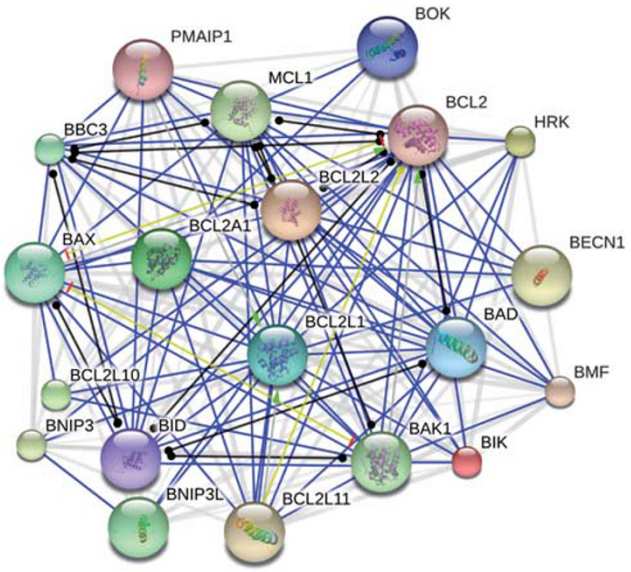

Figure 3 Complexity of the Bcl-2 interaction network. The scheme shows the interactions between different Bcl-2 family members reported in the literature. Modes of action are shown in different colors: blue lines indicate binding, black connectors show reaction and green arrows indicate activation, while red cross lines mean inhibition. Interaction map generated for human Bcl-2 proteins with STRING 9.0 (http://string-db.org). The following nomenclature was used: BBC3 for Puma, Bcl2L11 for Bim, Bcl2L1 for Bcl-xL, Bcl2L2 for Bcl-w, Bcl2L3 for Mcl-2, Bcl2A1 for Bfl-1/A1, PMAIP1 for Noxa, BECN1 for Beclin1 and Bcl2L10 for Bcl-B/Boo/Diva

cellular state and exposure to apoptotic stimuli. Importantly, these patterns are also altered in cancer cells to escape cell death. In this context, the 'primed for death' hypothesis proposes that cancer cells are under continuous death signaling and depend on antiapoptotic Bcl-2 proteins for survival.[67,68] As a result, each cell contains a given set of Bcl-2 proteins that determines the status of the signaling network and its fate. Thus, cells expressing the same subset of Bcl-2 proteins but in different amounts will have a different reaction to the same apoptotic stimuli.

In such a complex scenario, we still fail to understand, and thus to predict, how the interplay between the Bcl-2 proteins in a cell integrates the apoptotic signals and decides whether or not to induce cell death. One has to imagine the Bcl-2 proteins engaged in multiple, competing reactions of complex formation, coexisting in the cytosol and in the membrane of the cellular organelles. In such a web of interactions, the physical and chemical properties of the system are highly integrated and the output of the signaling pathway cannot be explained by the sum of its components. In this sense, MOM permeabilization can be understood as emergent behavior. Emergence in biological (or physical) systems is observed when the individual components of the system integrate to give rise to distinct, collective properties and functions, which are not explained by the full understanding of the individual components in the system.[69] For example, the components of a signaling pathway may interact to form a functional network with novel features, such as self-sustained feedback or alternative outputs depending on the input strength and duration. In the case of the Bcl-2 family, complete knowledge of the structure and activity of individual Bcl-2 proteins does

not suffice to explain the process of MOM permeabilization or its regulation. Moreover, the output of Bcl-2 signaling depends on the intensity of the apoptotic stimuli and their duration.

One of the reasons for emergent behavior is that the intrinsic characteristics of the individual components cannot determine the properties of the whole system. Instead, the system is determined by the organizational dynamics of the individual components, that is, their coordinated interactions in time and space. In this sense, it is the dynamic organization of the interactions between Bcl-2 family members in time and space that determines whether MOM permeabilization takes place or not. Another reason for emergence is the effect of the environment on the dynamic organization of the system. In the case of Bcl-2 signaling, the network status is strongly dependent on the cellular context and the cell death/survival signals arriving from the extracellular environment. A systems biology strategy approaches the emergent behavior of MOM permeabilization as rising from a combination of bottom-up effects (system interactions with the environment) and top-down effects (interactions of the individual components in the system).

Mathematical modeling will contribute to our understanding of how MOM permeabilization is regulated at the systems level. After all, analyzing Bcl-2 signaling one element at a time has so far proved insufficient. The generation of kinetic models of signal transduction pathways is possible for processes that are relatively well understood and for which sufficient biochemical data exist. An example of such a pathway is the caspase cascade. Several models have been proposed to describe the regulation of the extrinsic pathway of apoptosis via death receptors.[70–73] A parallel effort has been undertaken to model the intrinsic, mitochondrial-dependent pathway.[74–77] For example, to deduce a mathematical model of apoptosis regulation by the Bcl-2 family based on ordinary differential equations, one should have estimations of the concentrations of the Blc-2 proteins in individual cells or cell types, their diffusion coefficients and the binding affinities among them, as well as time-resolved data of the different steps of apoptosis progression (for example Bid activation, Bax translocation, cytochrome *c* release) that would allow the experimental validation of the model. A key obstacle in the application of mathematical modeling as a tool to analyze the complexity of the Bcl-2 signaling is the scarce biochemical data on their binding affinities within biological membranes.[23] In addition, quantitative comparisons of the Bcl-2 protein levels in different cells and states are missing. As a consequence, apoptosis regulation by the Bcl-2 proteins has been modeled using simplified approaches that treat the Bcl-2 network as a black box. However, as biochemical and kinetic data on the interactions between the Bcl-2 family members in solution and in membranes becomes available, mathematical modeling will become a promising tool to understand the full complexity of the Bcl-2 network and how it orchestrates apoptosis.[78] The implementation of such models will also open interesting possibilities for the prediction of cellular responses to anticancer therapies.

New Biophysical Approaches to Study the Bcl-2 Family

One may consider that the seemingly current stagnation in the progress of our understanding of the mechanisms of the Bcl-2 proteins is associated with the limitations posed by classical biological methods mostly used so far. Fortunately, the last decade has seen the explosion in the application of a range of powerful biophysical techniques based on advanced fluorescence microscopy to solve relevant biological problems. Their use in the apoptosis field has only recently started, but it is already providing excellent results. For example, the combination of fluorescence recovery after photobleaching with fluorescence loss in photobleaching to investigate the dynamic distribution of Bax in healthy cells was key to uncover its continuous retro-translocation between cytosol and mitochondria controlled by interactions with Bcl-xL.[56] Recently, Andrews and coworkers extended the use of FRET microscopy to investigate complex formation between BH3-only proteins and prosurvival Bcl-2 homologs in living cells.[79] Although this approach cannot provide absolute estimations of the binding affinity between Bcl-2 family members, it could prove useful to study the molecular mechanisms of drugs targeting the Bcl-2 family.

Single-molecule techniques deserve special attention. One powerful possibility is FCS, which analyses the fluctuations in fluorescence intensity due to the diffusion of individual particles in and out of the focal volume of the microscope. In addition to protein concentrations and diffusion coefficients, the dual color version of FCS analyzes the dynamic co-diffusion of proteins labeled with different fluorophores (Figure 4). Importantly, it provides quantitative information

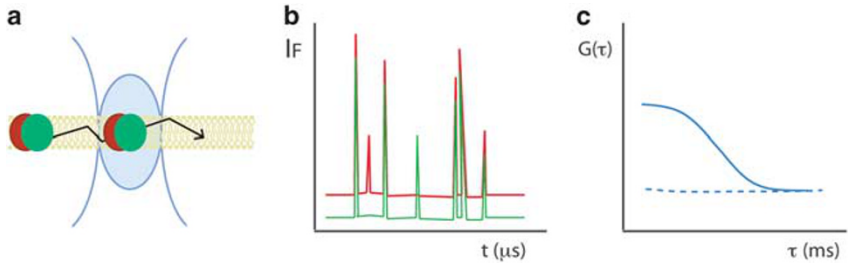

Figure 4 Fluorescence correlation spectroscopy to quantify protein/protein interactions. (**a**) FCS measures the fluorescence fluctuations due to the diffusion of individual molecules through the confocal volume of the microscope. To measure protein/protein interactions in membranes, the confocal volume (blue) is placed on the membrane plane. (**b**) If the proteins of interest (green and red balls in **a**) form a complex, they diffuse simultaneously in and out of the focal volume and the fluorescence spikes they provoke appear simultaneously in the detection channels. (**c**) This co-diffusion is detected and the cross-correlation analysis of the signal allows the quantification of the percentage of proteins that are in complex out of the total pool of proteins of interest. If the two proteins form a complex, the cross-correlation curve has positive amplitude at lag time 0 (solid line), which does not happen if they do not interact (dashed line)

about the extent of complex formation and can be used to calculate binding affinities.[80] Moreover, special FCS approaches can be applied to membranes and were indeed used to quantify for the first time the affinity of the complex between tBid and Bcl-xL within the lipid bilayer,[23] thus revealing the important role of the membrane for Bcl-2 function. Therefore, FCS is a promising tool that may have a key role in clarifying which interactions between Bcl-2 family members are favored, how they are affected by translocation to mitochondria and, finally, how Bcl-2 signaling is integrated.

Other interesting single-molecule strategies to investigate the molecular mechanisms of the Bcl-2 proteins will surely be single molecule imaging and super-resolution microscopy. We still do not have examples in the literature, but one can envision that single-molecule imaging of the dynamics of Bcl-2 proteins in membranes will bring new insight on the molecular mechanisms involved in complex formation between family members. On the other hand, the most popular super-resolution methods are stimulated emission depletion microscopy, which is based on the generation of a fluorescence-emitting spot smaller than the size of a normal diffraction-limited spot via the use of a donut-shaped de-excitation laser that uses the principle of stimulated emission, and photoactivated localization microscopy and stochastic optical resconstruction microscopy, which are based on the precise localization of isolated emitters by fitting their images with the spread point function. For a review on super-resolution microscopy in biology, see Schermelleh *et al.*[81] These techniques achieve an improvement in spatial resolution from around 200 nm to a few tens of nanometers and are very promising to shed light on the nature of the Bcl-2 structures involved in mitochondrial permeabilization during apoptosis.

Concluding Remarks

Despite intense research, there are long-pending questions regarding the mechanism of action of the Bcl-2 proteins that need to be addressed if we want to understand how they regulate apoptosis. Probably, the use of sophisticated techniques that reveal properties will be necessary to bring novel impulse to the field. The structural characterization of complexes between Bcl-2 proteins in membranes, the quantification of their interactions in the lipid environment and the mathematical modeling of the complex Bcl-2 network pose technological challenges that if overcome would yield novel insights into the well-kept secrets of the Bcl-2 family.

Conflict of Interest

The author declares no conflict of interest.

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