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Review

Non-apoptotic roles of Bcl-2 family: The calcium connection ☆

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ABSTRACT

The existence of the *bcl-2* (B-cell lymphoma-2) gene was reported nearly 30 years ago. Yet, Bcl-2 family group of proteins still surprises us with their structural and functional diversity. Since the discovery of the Bcl-2 family of proteins as one of the main apoptosis judges, the precise mechanism of their action remains a hot topic of intensive scientific research and debates. Although extensive work has been performed on the role of mitochondria in apoptosis, more and more studies point out an implication of the endoplasmic reticulum in this process. Interestingly, Bcl-2 family proteins could be localized to both the mitochondria and the endoplasmic reticulum highlighting their crucial role in apoptosis control. In particular, in these organelles Bcl-2 proteins seem to be involved in calcium homeostasis regulation although the mechanisms underlying this function are still misunderstood. We now assume with high degree of certainty that the majority of Bcl-2 family members take part not only in apoptosis regulation but also in other processes important for the cell physiology briefly denominated as "non-apoptotic" functions. Drawing a complete and comprehensive image of Bcl-2 family requires the understanding of their implications in all cellular processes. Here, we review the current knowledge on the control of calcium homeostasis by the Bcl-2 family at the endoplasmic reticulum and at the mitochondria. Then we focus on the non-apoptotic functions of the Bcl-2 proteins in relation with the regulation of this versatile intracellular messenger. This article is part of a Special Issue entitled: 12th European Symposium on Calcium.

1. The Bcl-2 family of proteins and apoptosis

Historically, *bcl-2* (B-cell lymphoma-2) gene was discovered at a chromosomal translocation, t(14;18), associated with B-cell follicular lymphoma. This translocation places *bcl-2* under the control of the immunoglobulin gene enhancer leading to abnormal high-level expression of Bcl-2 [1–3]. However, contrary to other oncogenes characterized so far, Bcl-2 acts by inhibiting cell death rather than promoting cell proliferation [4]. Bcl-2 was thus initially characterized as a mitochondrial resident protein that blocks apoptotic programmed cell death [5]. Since the discovery of Bcl-2, extensive work on various animal models, from worms to mammals, uncovered a family of proteins involved in the control of apoptosis and sharing structural homology with the canonical member Bcl-2 [6]. In vertebrates, members of the so called Bcl-2 family are globular proteins containing α-helixes and characterized by the presence of at least one Bcl-2 homology (BH) domain. According to their function in apoptosis, Bcl-2 family members are designated as anti- or pro-apoptotic. Anti-apoptotic proteins (e.g. Bcl-2, Bcl-xL, and Mcl-1) contain four BH domains and a hydrophobic transmembrane (TM) domain essential for their localization into biological membrane. Pro-apoptotic proteins can be further subdivided into two groups. The effector proteins Bax and Bak contain four BH domains and a TM domain whereas BH3-only proteins (e.g. Bad, Bid, and Bik) possess just one BH domain and no TM domain with the exception of Bik and Hrk [7,8].

The Bcl-2 family proteins play a crucial role in the control and the execution of the intrinsic, or mitochondrial, pathway of apoptosis. In this process, some death stimuli (e.g. growth factor deprivation, and DNA damage) activate BH3-only proteins which in turn lead to the activation of Bax and/or Bak either directly or by inhibiting anti-apoptotic members. Activated Bax/Bak then oligomerize at the mitochondria to induce outer mitochondrial membrane (OMM) permeabilization and release into the cytosol of apoptotic factors (e.g. cytochrome c and AIF) which promote caspase activation and subsequent apoptosis execution. Anti-apoptotic members of Bcl-2 family are able to prevent OMM permeabilization via a direct inhibitory interaction with pro-apoptotic members [8].

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2. Endoplasmic reticulum Ca^{2+} and apoptosis

Although the mitochondrion is a key organelle for promoting apoptosis, a growing body of evidence underlies the involvement of endoplasmic reticulum (ER) in this process. The ER is the major site of folding, modification, and trafficking of proteins as well as the main store for intracellular Ca^{2+}. These functions of the ER are tightly connected as Ca^{2+} is required for protein synthesis in almost every cell type. In the ER, Ca^{2+} supports early protein processing and protein glycosylation. In addition, Ca^{2+} may also be implicated in subunit assembly and protein folding [9]. Thus, the reduction of ER Ca^{2+} level can induce the accumulation and aggregation of unfolded proteins, a condition referred to as ER stress. To face this stress, cells activate a prosurvival response known as the Unfolded Protein Response (UPR) to reduce the accumulation of unfolded protein. However if the UPR's mechanisms are insufficient to decrease unfolded protein load, the UPR activates apoptosis [10]. In this case, components of the UPR induce apoptosis via the inactivation of Bcl-2 and the activation of several BH3-only proteins including Bim, Bad, Bik and Puma which then activate Bax and Bak to promote cytochrome c release from the mitochondria. Interestingly, a cross-talk exists between the UPR and apoptosis as Bax/Bak have been proposed to participate in early events of the UPR [11].

Moreover a number of stresses, including ER stress, can lead to the release of the ER Ca^{2+} store and subsequent increase of cytosolic Ca^{2+} levels [10,12]. Cytosolic Ca^{2+} elevation could in turn induce apoptosis via several pathways independent or not from the mitochondria.

The first one is triggered by the caspase-12 independently of mitochondria. This caspase localized at the ER [13,14] can be cleaved and activated by the Ca^{2+}-dependent protease, Calpain [15]. Activated caspase-12 then processes downstream caspases leading to apoptosis independently of OMM permeabilization [16].

Secondly, [Ca^{2+}] rise into the cytosol may lead to OMM permeabilization through BH3-only protein activation. In particular, Calpain-mediated Bid cleavage into its active form tBid was reported to induce apoptosis in a Bax-dependent manner [17]. In addition, Calpain cleavage of Bcl-2 and Bcl-xL N-terminus reduces their anti-apoptotic activity and even promotes Bcl-2 conversion into a pro-apoptotic protein which promotes cytochrome c release on its own [18]. Bad is another BH3-only member which could be activated in response to Ca^{2+} elevation. Under physiological conditions, Bad is phosphorylated and sequesters in the cytosol by 14–3–3 proteins [19]. However, elevation of cytosolic Ca^{2+} levels activates the Ca^{2+}-dependent phosphatase, Calcineurin (also known as PP2B) which dephosphorylates Bad therefore activating the intrinsic mitochondrial pathway [20]. Interestingly, it has also been shown that a slight increase of Ca^{2+} could, on the contrary, promote Bad phosphorylation and sequestration by 14–3–3 via the activation of the Ca^{2+}/Calmodulin dependent protein kinase kinase (CaMKK) highlighting the versatility of Ca^{2+} signaling in cell fate [21].

Finally, massive Ca^{2+} release from the ER can directly trigger cytochrome c release from the mitochondria. Indeed, the ER and the mitochondria have long been known to be tightly associated [22,23]. More recent research established that ER membranes are in close contact with mitochondria, forming microdomains called MAM for mitochondria-associated membranes [24]. MAM are enriched in transmembrane proteins such as PACS-2 or Mitofusin-2 which directly tether the ER and the mitochondria thus creating a physical interaction between them [25,26]. These contact points are crucial for lipid and Ca^{2+} exchanges between ER and mitochondria. Inositol 1,4,5-trisphosphate receptors (IP_3R) located at the ER membrane play an essential role in ER to mitochondria Ca^{2+} trafficking [27,28]. In MAM, IP_3R seems to be part of a protein complex with Grp75 and the mitochondrial Voltage Dependent Anion Channel (VDAC) that promotes Ca^{2+} exchange between ER and mitochondria [29]. [Ca^{2+}] in MAM can reach a very high level compared to the cytosolic concentration [30] which is consistent with a specialized function in Ca^{2+} transfer between ER and mitochondria. Indeed, the recently identified Mitochondrial Calcium Uniporter (MCU) [31,32] requires a high level of Ca^{2+} to open due to its interaction with MICU1 which acts as a gatekeeper by setting a threshold for Ca^{2+} entry into mitochondria [33]. Thus, Ca^{2+} released through IP_3R in MAM can be rapidly uptaken into the mitochondria by VDAC and MCU to enter mitochondrial matrix [34]. Interestingly, IP_3R has been acknowledged to be required for apoptosis in some cases [35–38]. Therefore, upon a stress inducing massive Ca^{2+} release through IP_3R, mitochondria face very high level of calcium and uptake it to buffer cytosolic Ca^{2+}. When Ca^{2+} accumulates in the mitochondria it binds to Cyclophilin D which induces the opening of the permeability transition pore (PTP) and finally leads to cytochrome c release [39].

3. Regulation of ER Ca^{2+} homeostasis by Bcl-2 proteins

The concept that members of the Bcl-2 family could be localized to the ER [40] and control apoptosis through ER Ca^{2+} homeostasis regulation, emerged almost two decades ago. However, in spite of increasing number of studies, the mechanisms by which the Bcl-2 proteins exert this function are still unclear and subject to controversy (Fig. 1).

3.1. Regulation by anti-apoptotic proteins

The first observation suggesting a role for Bcl-2 on intracellular Ca^{2+} homeostasis was made in 1993 [41]. The authors demonstrated that, in Interleukin-3 (IL-3)-dependent hematopoietic cells, IL-3 withdrawal induces apoptosis by a Ca^{2+} transfer from the ER to the mitochondria. In this model, Bcl-2 overexpression prevents apoptosis and blocks this Ca^{2+} flux. In this study, the thapsigargin-releasable Ca^{2+} pool before IL-3 withdrawal was not affected by Bcl-2 suggesting that Bcl-2 does not alter ER Ca^{2+} content but rather acts on Ca^{2+} release from the ER. The year after, two other studies concluded that Bcl-2 decreases Ca^{2+} release from the ER. The first showed that Bcl-2 prevents cytosolic Ca^{2+} increase induced by serum withdrawal in NIH3T3 cells [42]. The second reported that Bcl-2 was able to inhibit thapsigargin-induced apoptosis [43]. Here the authors showed that Bcl-2 reduces Ca^{2+} efflux from the ER following thapsigargin treatment but that this effect was not due to alteration in Ca^{2+} homeostasis within the ER lumen. The same effect was observed upon hydrogen peroxide treatment suggesting that Bcl-2 can reduce Ca^{2+} release from the ER to prevent apoptosis induced by various stresses [44,45]. However, subsequent studies showed that Bcl-2 is able to increase Ca^{2+} uptake in the ER and then enhanced ER Ca^{2+} load under certain conditions. He and colleagues showed that overexpression of Bcl-2 maintains ER Ca^{2+} homeostasis when the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) is inhibited by thapsigargin or when the extracellular [Ca^{2+}] is decreased [46]. Here, Bcl-2 does not seem to prevent SERCA inhibition so the authors concluded that Bcl-2 could mediate Ca^{2+} uptake in the ER. They proposed that Bcl-2, by preventing oxidative damage to the ER membrane, could decrease Ca^{2+} leak through the ER and thus increase Ca^{2+} loading capacity of the ER. They also hypothesized that this effect of Bcl-2 could be due to the formation of cation-selective channel in lipid bilayer by Bcl-2 or Bcl-xL [47,48] thus allowing Ca^{2+} entry into the ER lumen. However, this hypothesis does not take into account the existing calcium gradient across the ER membrane, rendering unlikely the passive calcium transport through Bcl-2 channels. Other studies also reported an increased Ca^{2+} load of the ER when Bcl-2 is overexpressed but in this case it was attributed to an increase level of SERCA expression [49,50]. Interestingly, the authors also described an interaction between Bcl-2 and SERCA which was proposed to enhance SERCA activity [44]. Such interaction was also suggested in *Xenopus* oocytes where Bcl-2 was shown to desensitize SERCA to thapsigargin thanks to its BH4 domain [51]. In all these studies, it was assumed that the anti-apoptotic effect of Bcl-2 was mediated by a

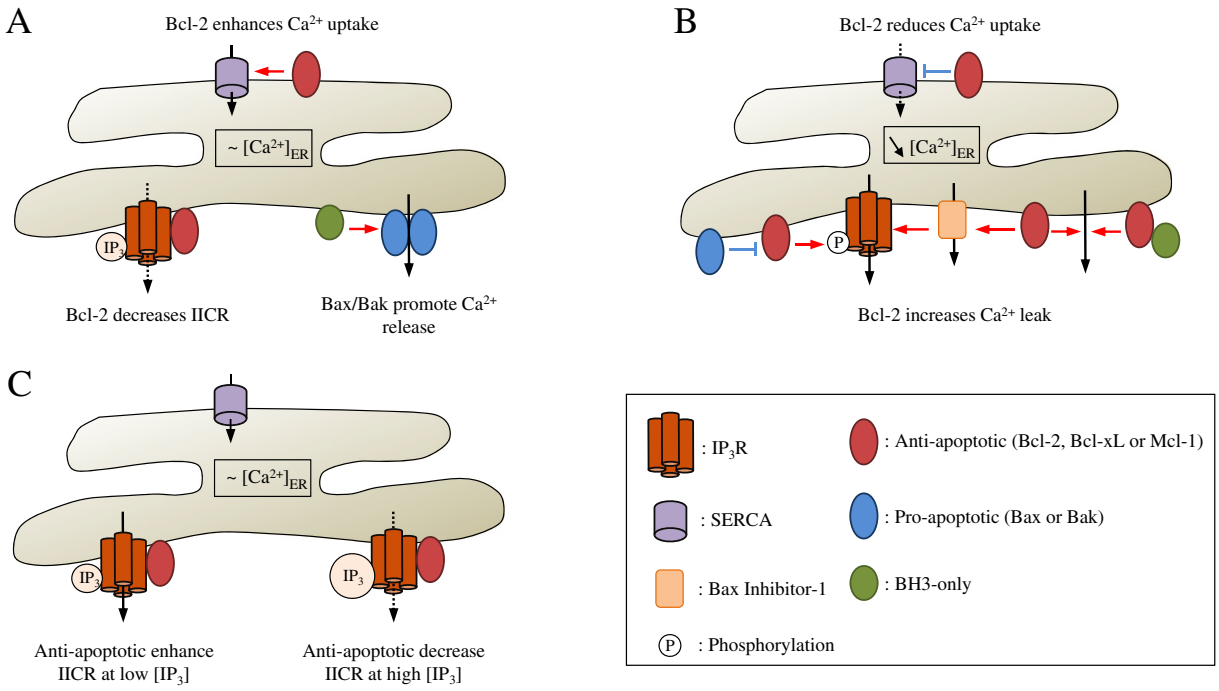

Fig. 1. Schematic representation of the different models for Bcl-2 family activity on ER Ca^{2+} homeostasis. (A) In this model, Bcl-2 reduces cytosolic [Ca^{2+}] to prevent apoptosis induction. Bcl-2 could act either by increasing SERCA activity or by reducing IICR. Bcl-2 may exert its inhibitory activity on IP_3R via a direct interaction with the modulatory and transducing domain of the receptor. Conversely, activated Bax and Bak are proposed to induce Ca^{2+} release from the ER. (B) In this model, Bcl-2 reduces ER Ca^{2+} content to prevent massive Ca^{2+} release which could trigger apoptosis. Bcl-2 is proposed to increase Ca^{2+} leak from the ER by promoting IP_3R phosphorylation or by directly inducing Ca^{2+} leak alone or in combination with a BH3-only protein. Bcl-2 could also reduce SERCA activity by a direct interaction. Bax and Bak may counteract the effect of Bcl-2 by a direct inhibitory interaction. Finally, Bcl-xL could act on BI-1 to increase Ca^{2+} leak from the ER with BI-1 acting as a Ca^{2+} channel or as an IP_3R sensitizer. (C) In this model, anti-apoptotic proteins promote survival by enhancing ER-mitochondria coupling. They are proposed to enhance IICR at low [IP_3] to stimulate bioenergetics whereas at high [IP_3] they could prevent apoptosis by reducing IICR. This effect may be mediated by a direct interaction with the C-terminus of IP_3R. Dashed lines represent a reduction of the calcium flux. Bold lines represent an increase of the calcium flux. Red and blue arrows represent activation and inhibition respectively. IP_3R: inositol 1,4,5-trisphosphate receptor; SERCA: sarco/endoplasmic reticulum Ca^{2+}-ATPase; BI-1: Bax-inhibitor-1; IICR: IP_3-induced calcium release.

reduction of cytosolic Ca^{2+} level either by increasing ER Ca^{2+} load or by reducing Ca^{2+} release from the ER (Fig. 1A).

Nevertheless, in 2000 a new mechanism of action for Bcl-2 at the ER emerged when two groups showed that Bcl-2 was able to decrease ER Ca^{2+} content [47,48]. In their study, Pinton and colleagues demonstrated that Bcl-2 overexpression reduces IP_3-induced Ca^{2+} release (IICR) and attributed this effect to a lower ER Ca^{2+} content due to increased Ca^{2+} leak from the ER [52]. Surprisingly, the authors did not observe any variation in cytosolic [Ca^{2+}] despite the detection of decreased mitochondrial Ca^{2+} uptake. Foyouzi-Youssefi and colleagues also described a reduced ER Ca^{2+} content in Bcl-2 overexpressing cells [53]. As ER [Ca^{2+}] decrease is a signal for capacitative Ca^{2+} entry, authors showed that Bcl-2 enhances unstimulated Ca^{2+} influx and then induced elevated basal cytosolic [Ca^{2+}]. As for the previous study, it was shown that decreased ER [Ca^{2+}] is a consequence of increased Ca^{2+} leak from the ER rather than Ca^{2+} uptake reduction. In both cases authors argued that formation of a cation-selective channel by Bcl-2 may be responsible for the increased Ca^{2+} leak (Fig. 1B). Two subsequent studies also concluded that Bcl-2 could decrease ER Ca^{2+} content. In the first it was demonstrated that Bcl-2 overexpression increases Ca^{2+} leak but also downregulates SERCA and Calreticulin expression to reduce ER Ca^{2+} content [54]. In the second, using a new genetically-encoded Ca^{2+} sensor, Palmer and colleagues measured [Ca^{2+}] within the ER lumen. They found that Bcl-2 decreases ER Ca^{2+} content and, as a consequence, alters IICR [55].

In this model it was proposed that reduction of Ca^{2+} level within the ER mediates the anti-apoptotic activity of Bcl-2 by reducing the amount of releasable Ca^{2+} available to induce apoptosis. Foyouzi-Youssefi and colleagues also speculated that the increased cytosolic [Ca^{2+}] they observed could promote cell survival by the CaMKK-dependent phosphorylation of Bad.

However, the idea according to which this effect is mediated by Bcl-2 pore formation was challenged when Chami and colleagues showed that a Bcl-2 chimera protein containing the pore domain of Bax has the same effect on ER Ca^{2+} homeostasis as the wild-type Bcl-2 [56]. Another possible mechanism emerged from a study which showed that, in contrast to what was proposed earlier, Bcl-2 can interact with and destabilize SERCA [57] (Fig. 1B). Another idea was further put forward to explain the reduction of ER Ca^{2+} content by Bcl-2. In their study, Bassik and colleagues proposed that Bcl-2 phosphorylation within an unstructured loop between its BH4 and BH3 domains can modulate its ability to decrease ER Ca^{2+} content [58]. They showed that in cells expressing a non-phosphorylatable mutant of Bcl-2 (Bcl-2 AAA) the ER Ca^{2+} level is lower than in Bcl-2 expressing cells due to higher Ca^{2+} leak. In this regard Bcl-2 AAA is more efficient at protecting cells from Ca^{2+}-dependent apoptotic stimuli than the wild-type protein. At last, they demonstrated that phosphorylation of Bcl-2 impairs its interaction with Bax and BH3-only proteins and provided evidence suggesting that Bcl-2 interaction with the BH3-only proteins was required to promote the reduction of ER Ca^{2+} content (Fig. 1B). However how a complex between Bcl-2 and a BH3-only can induce Ca^{2+} leak through the ER membrane is not discussed and remains elusive.

Finally, it has been proposed that Bax Inhibitor-1 (BI-1) may be the downstream target of Bcl-xL to lower ER [Ca^{2+}]. Indeed, Xu and colleagues demonstrated that BI-1 is required for ER calcium content reduction induced by Bcl-xL overexpression [59] (Fig. 1B). BI-1 was shown to increase calcium leak from the ER in a pH-dependent

manner [60] and it may act either by forming a Ca^{2+}-permeable channel pore with its C-terminus [61] or by interacting with IP_3R to sensitize the channel to low [IP_3] [62].

Thus, so far, the molecular mechanism by which Bcl-2 can reduce Ca^{2+} level within the ER is still a matter of debate and should be further investigated.

Finally, the group of C.W. Distelhorst proposed that Bcl-2 could regulate calcium homeostasis at the ER by directly interacting with IP_3R. In a work published in 2004 they demonstrated that Bcl-2 is able to decrease IICR without affecting ER calcium level. In a convincing manner they showed that Bcl-2 directly interacts with IP_3R, forming a complex with the receptor. As a consequence, they described that Bcl-2 is able to reduce the channel open probability of IP_3R [63]. These results were confirmed by another study where the authors notably showed that ER-targeted Bcl-2 has the same effect as wild-type Bcl-2 on Ca^{2+} homeostasis [64]. Interaction between Bcl-2 and IP_3R was further characterized and it was demonstrated that Bcl-2 binds to the modulatory and transducing domain of IP_3R between the residues 1347–1426 in the middle of the protein [65] (Fig. 1A). Furthermore, Bcl-2 interacts with IP_3R via its BH4 domain and it has been shown that the BH4 domain alone was sufficient to decrease IICR [66]. At last, a recent study comparing the BH4 domains of Bcl-2 and Bcl-xL has identified a crucial residue within Bcl-2 which seems essential for its regulatory activity on IICR [67]. Contrary to Bcl-2, the BH4 domain of Bcl-xL was able neither to bind to the modulatory and transducing domain nor to reduce Ca^{2+} release through IP_3R. The authors found the Lys17 residue of Bcl-2 to be critical for the activity of Bcl-2 on IP_3R since its mutation in aspartate, as in the BH4 domain of Bcl-xL, abolishes the activity of Bcl-2 BH4 domain on Ca^{2+} signaling.

However, Bcl-xL was also shown to interact with IP_3R but at a different site as it binds the C-terminus of IP_3R. This interaction was shown to sensitize IP_3R to low concentrations of IP_3 thus enhancing Ca^{2+} oscillation in the cytosol. IP_3R sensitization stimulates mitochondrial bioenergetics allowing the authors to propose that Bcl-xL could promote survival by enhancing ER-mitochondria coupling and cellular metabolism [68]. In this case, Bcl-xL was also reported to decrease ER Ca^{2+} content but this effect does not seem to be relevant. Indeed, the same team showed two years later that Bcl-xL interacts with the three IP_3R isoforms but reduces ER Ca^{2+} content only in cells expressing IP_3R3. However, Bcl-xL sensitizes all IP_3R isoforms to IP_3 and enhances cytosolic Ca^{2+} oscillation and apoptosis resistance whatever isoform is expressed [69]. Therefore it is more likely that Bcl-xL action is mediated rather by its interaction with IP_3R than by decreasing ER Ca^{2+} content (Fig. 1C).

These differences between Bcl-xL and Bcl-2 could be attributed, as exposed above, to the differences between the BH4 domains of these proteins. Thus, depending on their BH4 domain, anti-apoptotic members could interact at different sites within IP_3R thus regulating the channel activity in a different manner (for extensive discussion on the topic see [70]).

Nevertheless, Bcl-2 and Mcl-1 seem also able to bind to the C-terminus region of IP_3R and to regulate Ca^{2+} homeostasis in a manner similar to Bcl-xL highlighting the complexity of Bcl-2 family mediated regulation of IP_3R [71,72] (Fig. 1C).

3.2. Regulation by pro-apoptotic members

Pro-apoptotic proteins are also able to localize at the ER and act on ER Ca^{2+} homeostasis. In 2001, Pan and colleagues first proposed a link between Bax and ER Ca^{2+} signaling since apoptosis induced by overexpression of Bax in CHO cells was correlated to a depletion of ER Ca^{2+} store [73]. However they argued that the depletion of the ER Ca^{2+} store rather than cytosolic [Ca^{2+}] increase is responsible for apoptosis induction in their model. The year after Bax and Bak were shown to localize at the ER where they induce ER Ca^{2+} depletion and subsequent cytochrome c release due to Ca^{2+} accumulation in the mitochondria [74]. Bax seems to be required to induce Ca^{2+}-dependent apoptosis as Bax-null cells did not elicit ER Ca^{2+} pool reduction and subsequent apoptosis induced by the staurosporine [75]. Moreover, the group of C. Thompson found that ER stress can induce Bax and Bak oligomerization at the ER leading to caspase-12 activation [76]. Interestingly, they showed that the targeted expression of Bak was sufficient to trigger ER Ca^{2+} depletion and subsequent caspase-12 activation even in the absence of endogenous Bax and Bak. A more recent study is consistent with an activity of Bak at the ER as it was shown that ER-targeted Bak is able to activate BH3-only proteins and subsequent cytochrome c release in part via ER Ca^{2+} release [77]. All these studies therefore assess that once activated Bax and Bak could trigger Ca^{2+} release from the ER to induce apoptosis (Fig. 1A). However, as for the anti-apoptotic proteins contradictory results concerning the role of pro-apoptotic proteins at the ER exist. Indeed, the group of S. Korsmeyer found that in MEF cells deficient for Bax and Bak, ER Ca^{2+} content was significantly reduced suggesting that Bax and Bak act at the ER by increasing [Ca^{2+}] in the lumen of the ER [78]. A few years later, they proposed a model to explain the effect of Bax and Bak on ER Ca^{2+} content. They showed that in cells lacking Bax and Bak IP_3R1 is hyperphosphorylated on Ser 1755. This PKA-dependent phosphorylation renders IP_3R sensitive to basal IP_3 concentration thus increasing Ca^{2+} leak from the ER. They also found that in the absence of Bax and Bak the interaction between Bcl-2 and IP_3R was enhanced and that *bcl-2* knockdown reduces IP_3R phosphorylation in these cells [79]. So this group proposed that at the ER, Bcl-2 might induce IP_3R phosphorylation thus promoting Ca^{2+} leak and ER Ca^{2+} content reduction whereas Bax and Bak, by interacting with Bcl-2, could counteract IP_3R phosphorylation (Fig. 1B).

Finally, besides the multidomain proteins of the Bcl-2 family, the BH3-only protein Bik is also localized at the ER, thanks to its C-terminus TM domain, where it can induce cytochrome c release from the mitochondria [80]. Surprisingly, in this first study the authors argued that Ca^{2+} was not involved in this process. However, in a more recent study, they showed that Bik can induce Ca^{2+} release from the ER by promoting Bax and Bak localization and oligomerization at the ER [81] (Fig. 1A).

At the light of these conflicting results, it is difficult to decipher a common mechanism of action for the Bcl-2 family at the ER. Differences in the cell models as well as the detection methods used by the different groups should be taken into account explaining partially the divergence in the proposed models. Despite these discrepancies, it remains clear that multiple members of Bcl-2 proteins may act as key regulators of calcium homeostasis at the ER. Indeed, all studies at least agree on the fact that anti-apoptotic proteins are able to reduce the amount of calcium released from the ER upon apoptotic stress whereas pro-apoptotic has the opposite effect.

4. Regulation of mitochondrial Ca^{2+} homeostasis by Bcl-2 proteins

Although ER is the main store of Ca^{2+} in the cell, mitochondria also play a critical role in Ca^{2+} homeostasis. As mentioned in Section 2, mitochondria can rapidly uptake Ca^{2+} present in the cytosol acting as a buffer when cytosolic [Ca^{2+}] increases. In mitochondria Ca^{2+} enhances substrate uptake, the rate of NADH production and the activity of the ATP synthase as well as those of the pyruvate, ketoglutarate and isocitrate dehydrogenases of the TCA cycle thus promoting mitochondrial metabolism and ATP production [82]. However, Ca^{2+} overload is detrimental as it impairs mitochondrial respiration and leads to membrane potential drop, mitochondria swelling and finally cytochrome c release. Thus, given the role of the Bcl-2 family on the control of cell fate it can be assumed that this group of proteins can also modulate Ca^{2+} homeostasis directly at the mitochondria. Indeed, several studies have revealed that Bcl-2 proteins could act

on mitochondrial Ca^{2+} homeostasis although, like at the ER, no consensus exists concerning the mechanisms underlying this function (Fig. 2).

The first evidence for a role of Bcl-2 on mitochondrial Ca^{2+} homeostasis was provided in a study on neural cells. In this model, it was shown that overexpression of Bcl-2 allows mitochondria to uptake more Ca^{2+} without any mitochondrial respiratory impairment suggesting that Bcl-2 can protect mitochondria from Ca^{2+} overload [83]. Similar results showing that Bcl-2 enhances the capacity of mitochondria to store Ca^{2+} were obtained by two other groups [45,50,84]. One of these further showed that Bcl-2 in fact induces a reduction of mitochondrial Na^{+}/Ca^{2+} exchanger activity leading to decreased Ca^{2+} efflux from the mitochondria [85] (Fig. 2A). However, other studies found that Bcl-2 had no effect on Ca^{2+} uptake by the mitochondria [58] or even decreases this process although excessive Bcl-2 amount could be detrimental for mitochondria [64].

Concerning the pro-apoptotic members, only few cases depict a role in mitochondrial Ca^{2+} homeostasis. For instance, it has been shown that Bax can enhance cytochrome c release induced by Ca^{2+} on isolated mitochondria suggesting that Bax may contribute to Ca^{2+} entry in this organelle [86]. Another study also concluded that Bax and Bak increase $[Ca^{2+}]$ within mitochondria [74] (Fig. 2B). On the contrary it has also been proposed that Bax can promote Ca^{2+} release from the mitochondria to induce Ca^{2+} waves in the cell and then enhance the apoptotic response [87] (Fig. 2A).

So far, the mechanism by which Bcl-2 family members can act on mitochondrial Ca^{2+} homeostasis is still unknown. As at the ER it can be speculated that the channel properties of the Bcl-2 proteins could be implicated in this function, although Bcl-2 and Bcl-xL channels seem more permeable to monovalent cations and Bax and Bak channel to monovalent anions [88]. One other interesting hypothesis emerged from the fact that Bcl-2 proteins have been shown to interact with VDAC. This channel is the most abundant protein of the OMM and is now acknowledged to be responsible for the import of Ca^{2+} across the OMM [89]. VDAC can be found in two states, an open state where it is mainly permeable to anions and metabolites and a closed state where cations, and in particular Ca^{2+}, could freely permeate the channel [90,91]. The first study reporting such interaction demonstrated that Bcl-xL, Bax and Bak are able to bind to VDAC. While Bcl-xL closes VDAC, Bax and Bak on the contrary promote VDAC opening [92]. The year after, the same group showed that the BH4 domain of Bcl-xL and Bcl-2 was required for exerting their inhibitory effect on VDAC opening [93]. Surprisingly, in this paper authors showed that Bcl-xL could inhibit loss of membrane potential and cytochrome c release from isolated mitochondria submitted to a high $[Ca^{2+}]$. Therefore, if Bcl-xL promotes VDAC closure it can be assumed that it promotes Ca^{2+} entry into mitochondria and thus may not prevent apoptosis in this case. This group also showed that the BH3-only protein Bim could also interact with VDAC and promote its opening [94]. Another recent study also concluded that Bcl-xL could promote VDAC closure via an interaction with the N-terminus domain of the channel [95]. Thus, in this model, anti-apoptotic proteins might enhance Ca^{2+} entry into mitochondria whereas pro-apoptotic proteins might inhibit it (Fig. 2A). However this proposal does not fit the largely accepted idea of Ca^{2+}-sensitive mitochondrial PTP opening. According to this model one could postulate that pro-apoptotic should enhance Ca^{2+} entry into mitochondria to promote cell death and then promote the closure of VDAC. Of note, it has been shown that promoting the closed state of VDAC accelerates the Ca^{2+}-induced PTP opening [96]. More consistent with the Ca^{2+}-sensitive PTP opening, Bcl-xL was shown to promote the open state of VDAC [97] while the BH3-only tBid on the contrary was proposed to close the channel [98]. In addition, Bcl-xL specifically targeted to the mitochondria was shown to be sufficient to protect cells from apoptosis induced by Ca^{2+}-dependent stresses such as H_2O_2 treatment suggesting it may prevent Ca^{2+} entry into mitochondria through VDAC [99]. Moreover, a recent study has implicated VDAC in Ca^{2+}-dependent death induced by Bad [100]. In this paper, Roy and colleagues showed that upon ceramide and staurosporine treatment Bad was dephosphorylated to induce apoptosis independently of Bax and Bak. Apoptosis in this case relies on Ca^{2+} and is triggered by the Ca^{2+}-dependent PTP opening. Interestingly, in these conditions, Bad targets Bcl-xL at the mitochondria and reduces its interaction with VDAC. Thus by breaking Bcl-xL/VDAC interaction Bad may promote VDAC closure and then Ca^{2+} entry into the mitochondria to induce PTP opening (Fig. 2B).

Through all these studies it appears that the exact role of Bcl-2 proteins on VDAC regulation remains largely unclear. Moreover, results presented here provide only indirect clues for an implication of the Bcl-2 family of proteins on mitochondrial Ca^{2+} homeostasis via VDAC regulation. Taking into account that VDAC is also proposed to participate in apoptosis by forming pores in the OMM alone or in combination with Bax to induce cytochrome c release [101], it becomes particularly difficult to decipher the mechanism of action of Bcl-2 proteins on VDAC activity as they can probably act on both VDAC oligomerization and Ca^{2+} fluxes through the channel. Nevertheless, given the importance of Ca^{2+} signaling in mitochondria it can be of particular interest to look directly at the ability of Bcl-2 proteins to control Ca^{2+} homeostasis in this organelle.

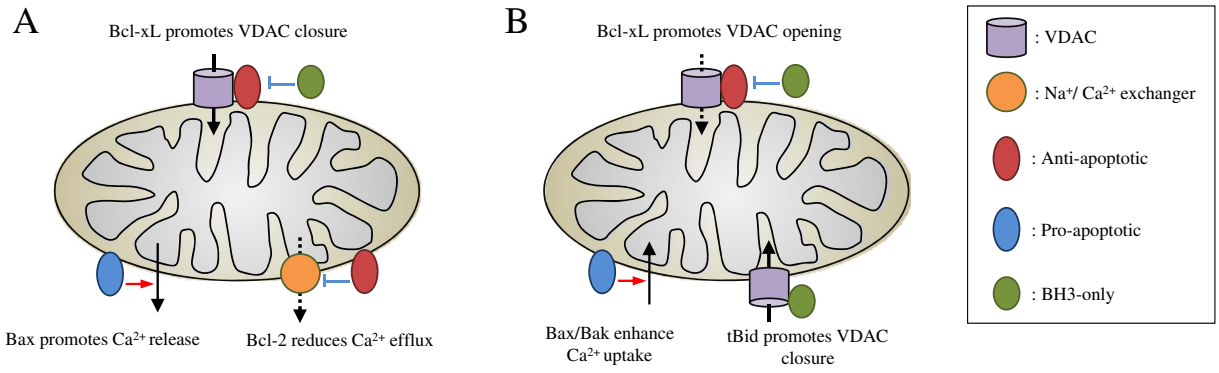

Fig. 2. Schematic representation of the different models for Bcl-2 family activity on mitochondrial Ca^{2+} homeostasis. (A) In this model, anti-apoptotic members increase the mitochondrial capacity to uptake Ca^{2+}. Bcl-2 could reduce the activity of the mitochondrial Na^{+}/Ca^{2+} exchanger and Bcl-xL could promote VDAC closure suggesting it may increase Ca^{2+} entry into the mitochondria. Conversely, Bad may induce VDAC opening whereas Bax may promote Ca^{2+} release from the mitochondria to amplify apoptotic response. (B) In this model, anti-apoptotic members prevent mitochondrial Ca^{2+} overload. Bcl-xL could promote VDAC opening suggesting it may reduce Ca^{2+} entry. Conversely, tBid may induce VDAC closure and Bax/Bak may promote mitochondrial Ca^{2+} uptake. Dashed lines represent a reduction of the calcium flux. Bold lines represent an increase of the calcium flux. Red and blue arrows represent activation and inhibition respectively. VDAC: Voltage Dependent Anion Channel.

5. Calcium in non-apoptotic functions of Bcl-2 family

Beyond their role in apoptosis, there is a growing body of evidence suggesting that Bcl-2 proteins are involved in numerous other cellular processes. However, the mechanisms allowing Bcl-2 proteins to exert their non-apoptotic functions are poorly understood. Here, we will discuss the implication of the regulatory activity of Bcl-2 proteins on Ca^{2+} homeostasis as a possible mechanism for such functions (Table 1).

5.1. Cell cycle

The cell cycle is the process by which cells divide through a series of events leading to DNA replication and cytokinesis. Cell cycle is divided in four phases, the G1 phase, the S phase, the G2 phase (collectively forming the interphase) and the mitosis (M phase). Between the end of mitosis and the G1 phase, cells can enter a quiescent state, known as the G0 phase, were they stop dividing. At the molecular level, the cell cycle is mainly regulated by Cyclin and Cyclin-dependent kinases (CDK) that associate in a specific manner to drive the different phases of the cell cycle.

The implication of Bcl-2 in the cell cycle was quickly suggested after its discovery as in the absence of IL-3 Bcl-2 overexpressing cells not only delayed the onset of apoptosis but are also arrested in G1 phase [4]. Further investigations confirmed this implication of Bcl-2 in cell cycle control. In particular, it has been shown that Bcl-2 deficient cells have an accelerated cell cycle while Bcl-2 overexpression retards the G0/S transition [102]. This was correlated to an increased level of the CDK inhibitor p27 and a downregulation of the nuclear factor of activated T-cells (NFAT) activity. Bcl-xL has the same effect on the cell cycle [103] and it has been confirmed that the effect of anti-apoptotic proteins on the cell cycle is mediated by elevation of p27 level and subsequent inhibition of the CDKs required for G1 phase progression [104]. This effect can be counteracted by Bax and Bad [103,105]; conversely Bax overexpression promotes S phase entry due to lower level of p27 [106]. Thus, anti- and pro-apoptotic exert opposite effects on the cell cycle at least in part by controlling p27 level. However, it has been shown that the anti-apoptotic function of Bcl-2 is independent from its action on the cell cycle. Indeed, mutation of a tyrosine in the BH4 domain of Bcl-2, Bcl-xL and Bcl-W has no effect on their ability to bind pro-apoptotic members and prevent apoptosis whereas it abolishes their effect on the cell cycle [107]. Interestingly, this mutation seems also to perturb the ability of Bcl-2 to decrease IICR [67]. Of note, reduction of ER Ca^{2+} level also induced an arrest in G1 phase correlated with increased p27 level [108]. Consistent with this result, Bax/Bak deficient cells, known to have lower ER Ca^{2+} content, exhibit higher level of p27 and are slower to enter S phase compared to normal cells [109]. Finally, inhibition of the Ca^{2+}-dependent proteins Calmodulin, Calmodulin-dependent kinases (CaMK) and Calcineurin could also lead to cell cycle arrest early in G1 phase indicating that these proteins may be involved in cell cycle progression through the G1 phase [110,111]. In particular, it has been shown that CaMKII can activate the MEK/ERK pathway to promote p27 phosphorylation on the Thr187, a signal for its degradation by the proteasome [112]. Furthermore, Calcineurin is known to trigger NFAT dephosphorylation inducing its translocation to the nuclei and Bcl-2 overexpression decreases NFAT activity. Thus, Bcl-2 and Bcl-xL may reduce the amount of Ca^{2+} released from the ER by either decreasing ER Ca^{2+} content or directly interacting with IP_3R. As a consequence Bcl-2 and Bcl-xL could prevent the activation of Ca^{2+}-dependent proteins resulting in cell cycle arrest whereas pro-apoptotic proteins would exert the opposite effect.

Interestingly, Bcl-2 appears to be phosphorylated in a cell cycle dependent manner. Indeed, Bcl-2 was found to be phosphorylated during mitotic arrest induced by paclitaxel and decreasing Bcl-2 phosphorylation in cells arrested in mitosis promoted interphase re-entry [113]. It has further been shown that such phosphorylation normally occurs in mitosis when cyclinB1/CDK1 activity peaks although this complex is not able to directly phosphorylate Bcl-2 [114]. Furthermore, phosphorylation of Bcl-2 was proposed to occur in G2/M transition via the ASK1/JNK pathway, Bcl-2 being phosphorylated on Ser70, Ser87 and Thr69 by JNK [115]. Of note, as mentioned in Section 3.1, phosphorylation of Bcl-2 on these residues was reported to act on ER Ca^{2+} homeostasis, as illustrated by the fact that a non-phosphorylatable Bcl-2 mutant decreases Ca^{2+} released from the ER [58]. Thus it can be assumed that phosphorylation of Bcl-2 could increase cytosolic Ca^{2+}concentration during the G2/M transition and mitosis. On the other hand, there is a large body of evidence showing that CaMKII activity is essential for progression through the G2 and M phases of the cell cycle [111]. So, a plausible hypothesis might be that phosphorylation of Bcl-2, by increasing Ca^{2+} release from the ER, could participate in CaMKII activation thus promoting cell cycle progression.

Table 1
Table summarizing the non-apoptotic functions of the Bcl-2 family, their effect on Ca^{2+} homeostasis, intermediate effectors involved in these pathways and finally the subsequent effect on cellular processes.

Non-apoptotic function	Bcl-2 family member involved	Effect on $[Ca^{2+}]$	Intermediate effectors	Effect	References
Cell cycle	Bcl-2, Bcl-xL, Bcl-W	↘ $[Ca^{2+}]_c$	↘ CaMK activity ↗ p27 level ↘ Calcineurin activity ↘ NFAT dependent transcription	Arrest in G1	[4,90–92,95]
	Bax, Bak	↗ $[Ca^{2+}]_{ER}$ ↗ $[Ca^{2+}]_c$	↗ CaMK activity ↘ p27 level	Faster G1	[91,93,94]
	Phosphorylated Bcl-2	↗ $[Ca^{2+}]_c$	↗ CaMKII activity	G2/M progression	[101–103]
Neuroplasticity	Bcl-2	↘ $[Ca^{2+}]_{ER}$ ↗ $[Ca^{2+}]_c$	↗ CaMK activity ↗ CREB and Erk dependent transcription ↗ *c-fos* expression	Neurite growth	[109–114,116,117]
Immune response	Bim	↗ $[Ca^{2+}]_c$	↗ Calcineurin activity	Lymphocyte activation	[127]
	Bax, Bak	↗ $[Ca^{2+}]_{ER}$ ↗IICR	↗ NFAT dependent transcription		[129]
Cell migration and invasion	Bcl-2	↗ $[Ca^{2+}]_c$	↗ CaMK activity ↗ AP1 and NF-κB dependent transcription ↗ MMP-2/9 expression	Cell invasion	[131–134,136,137]
Early embryonic development	Mcl-1	↗ $[Ca^{2+}]_c$	↗ Calmodulin activity	Embryo implantation	[140]
	Nrz	↘ $[Ca^{2+}]_c$	↘ MLCK activity ↘ Actin-myosin contraction	Epiboly progression	[151,153]

5.2. Neural plasticity

Neural plasticity, also known as neuroplasticity, refers to the changes in structure, function and organization of the neuronal network. This phenomenon has long been thought to be restricted to the developing nervous system but it is now accepted that it occurs in processes such as learning or memory as well as in recovery after brain injury. Neuroplasticity is driven in part by the formation of new synapses and thus requires the extension of neurites to reach and form these new synapses.

Bcl-2 and Bcl-xL are expressed in the nervous system during embryonic development as well as during the whole lifespan but they harbor different spatial and temporal patterns of expression. Bcl-2 and Bcl-xL are both expressed in the developing central nervous system (CNS). However, postnatally, Bcl-xL expression increases in the CNS while Bcl-2 expression is reduced with aging, being almost restricted to the peripheral nervous system (PNS) [116,117]. While *bcl-x* knockout mice exhibit massive death of developing neurons followed by embryonic death at day 13 [118], *bcl-2* knockout mice do not show any reduction in neuron number following the period of naturally occurring cell death [119], suggesting that Bcl-xL is the main regulator of neuronal apoptosis. On the other hand, it has been shown that Bcl-2 can promote neurite outgrowth and regeneration suggesting that Bcl-2 may play a role in neuroplasticity. Consistently, Bcl-2 is strongly expressed in the developing CNS when axons elongate and its expression declines as neurons lose their ability to generate new axons [120]. In adults its expression is maintained in regions where postnatal neurogenesis takes place, such as the dentate gyrus of the cortex [116]. Thus, a number of studies have demonstrated that Bcl-2 overexpression in CNS neurons promotes axonal growth [121] while axonal elongation is reduced in neurons from Bcl-2-deficient mice [122,123]. Interestingly, Jiao and colleagues demonstrated that the effect of Bcl-2 on axon elongation is dependent on its ER localization and is mediated by the activation of Erk and CREB transcriptional programs [124]. These programs appear to be essential for the regulation of neurite extension and it is well established that they can be activated by cytosolic [Ca^{2+}] increase, in part via CaMKs-dependent phosphorylation [125]. These authors showed that in neurons, Bcl-2 reduces ER Ca^{2+} uptake, probably by acting on SERCA, to enhance cytosolic Ca^{2+} level and then promote axon elongation. Consistent with this result, measurement of intracellular Ca^{2+} store content in PC12 cells by another group also revealed that Bcl-2 decreases ER uptake, suggesting that, in CNS neurons, Bcl-2 may increase cytosolic Ca^{2+} level to promote axon elongation [84]. Furthermore, another publication strengthens the idea that Bcl-2 mediates axon elongation through CREB activation as Bcl-2 could increase *c-fos* gene expression, one of the target genes of CREB required for axonal elongation [126]. Finally, the effect of Bcl-2 on Ca^{2+} homeostasis may have a number of other consequences than CREB activation as many components of the machinery required for axonal elongation are Ca^{2+}-sensitive [127]. In contrast with the results in CNS neurons, Hansen and colleagues found that Bcl-2 overexpression in spiral ganglion neurons inhibits neurites outgrowth [128]. Moreover, in a later study, they showed that this effect could be triggered by Bcl-2 targeted either at the ER or at the mitochondria [129]. The authors argued that this difference could rely on the fact that each type of neuron has a different optimal intracellular [Ca^{2+}] required to promote neurite growth [127]. Thus, in spiral ganglion neuron, Bcl-2, by acting on Ca^{2+} homeostasis at both ER and mitochondria, may alter the intracellular [Ca^{2+}] in a way that inhibits neurite growth.

Of note, neural plasticity seems to be impaired in some pathologies such as anxiety or bipolar disorders and some studies reveal that Bcl-2 may be related to these pathologies [130,131]. In particular, bipolar disorder is associated with perturbations in intracellular Ca^{2+} homeostasis and it was recently showed that a single nucleotide polymorphism (SNP), which decreases Bcl-2 expression, is associated with bipolar disorder [132,133]. Both studies demonstrate that this SNP leads to alterations in intracellular Ca^{2+} homeostasis associated with abnormal Ca^{2+} release through IP_3R.

Thus, Bcl-2 appears to play a crucial role in neuroplasticity although the underlying mechanism is unclear. However, Bcl-2 seems to be implicated in the tight regulation of Ca^{2+} fluxes required for neurite elongation by acting at the ER, on both IP_3R and SERCA, but also possibly at the mitochondria.

5.3. Lymphocyte activation

Ca^{2+} is a crucial second messenger for lymphocyte activation and proliferation following antigen binding to the T-Cell receptor (TCR) or B-Cell receptor (BCR) [134]. Indeed, antigen binding on TCR and BCR, forming the immune synapse, first leads to IP_3 generation and subsequent Ca^{2+} release through IP_3R. This release induces depletion of the ER Ca^{2+} store and then promotes the influx of extracellular Ca^{2+} through the opening of the plasma membrane Ca^{2+}-release activated Ca^{2+} channels (CRAC) by a pathway commonly referred to as store-operated Ca^{2+} entry (SOCE) [134,135]. It has also been shown that mitochondria play a fundamental role in intracellular Ca^{2+} homeostasis after immune synapse formation by moving to the plasma membrane and uptaking some amount of Ca^{2+} to prevent high cytosolic [Ca^{2+}] which leads to CRAC inactivation [136,137]. Finally, this fine intracellular Ca^{2+} regulation leads, among other, to the activation of Calcineurin which dephosphorylates and activates NFAT, a critical step for T-cell activation and proliferation.

Beyond their known function in lymphocyte selection and development by controlling apoptosis [138], Bcl-2 proteins might be involved in Ca^{2+} homeostasis regulation in the immune cells thanks to their ER and/or mitochondria localization. Indeed, recent studies pointed out the role of Bcl-2 proteins in Ca^{2+} release from the ER after T cell receptor activation. Thus the BH3-only protein, Bim, was shown to be crucial for T-cell activation and proliferation [139]. In this study authors demonstrated that T-cells from Bim −/− mice exhibit lower ER Ca^{2+} release and subsequent NFAT dephosphorylation. These severe defects seem to be correlated with an increase of the Bcl-2/IP_3R complex number, leading to partial ER depletion because of the suppression of Bim destabilization effect on Bcl-2/IP_3R interaction. Also, a second team has shown that Bcl-2 could modulate Ca^{2+} release from the ER after strong T-cell activation, but not weak activation, suggesting that Ca^{2+} responses may have different requirements for the IP_3R [140]. Thus, it is possible that Bcl-2 might be implicated in these two different regulation pathways.

Moreover, the group of C. Thompson nicely demonstrated the non-apoptotic role of Bax and Bak in the control of T-cell proliferation by their ability to regulate ER Ca^{2+} release after TCR engagement [141]. Their data indicated that T-cells lacking both Bax and Bak display defects in TCR- and IP_3-inducible Ca^{2+} fluxes from the ER. They argued that decreased IICR is a consequence of ER Ca^{2+} store reduction in Bax −/− Bak −/− T cells. They also showed that Ca^{2+} deregulation leads to a decrease of ROS production which could also be involved in T-cell proliferation.

Finally, another group demonstrated that Bcl-2 could regulate plasma membrane Ca^{2+} ATPases (PMCA) function in pancreatic acinar cells [142]. PMCA has a fundamental role in Ca^{2+} homeostasis control near the CRAC after lymphocyte activation, avoiding CRAC inactivation and thus permitting the SOCE process. We may then hypothesize that during TCR activation, Bcl-2 might also regulate Ca^{2+} extrusion by modulating PMCA function in T-cells.

Thus many studies have provided evidence that Ca^{2+} is crucial for lymphocyte activation with the ER and the mitochondria playing central roles. Given their ability to interact with IP_3R and VDAC, Bcl-2 proteins might be important for IICR and mitochondrial Ca^{2+} buffering during lymphocyte activation.

5.4. Cell migration and invasion

The deregulation of Bcl-2 family protein expression is often associated with cell transformation as it allows tumor cells to escape from apoptosis. However, some studies provide evidence that Bcl-2 proteins can also regulate cellular migration and invasion during tumor progression. It was first reported that the overexpression of Bcl-2 enhances the metastatic potential of breast cancer cell line in vivo and promotes cell migration and invasion in vitro [143]. Bcl-2 was further found to exert the same effect in human glioma malignant cell line; this effect was attributed to an increased level of matrix metalloproteinases (MMP) and in particular MMP-2 and MMP-9 [144]. MMPs are able to cleave components of the extracellular matrix and are associated with tumor progression as they can degrade the basement membrane allowing cells to form metastasis [145]. These results were confirmed in non-small cell lung cancer cells where Bcl-2 also promotes MMP-2 expression and activity [146]. These authors demonstrated that induction of MMP-2 expression is driven here by the AP-1 transcription factor. Interestingly, it has been proposed that elevation of intracellular Ca^{2+} level can promote AP-1 formation via CaMK activation [147]. Thus Bcl-2 either by increasing Ca^{2+} leak at the ER or by reducing Ca^{2+} uptake at the ER and/or at the mitochondria could promote Ca^{2+} increase in the cytosol and subsequent activation of the AP-1 transcriptional complex. Furthermore, two studies showed that Bcl-2 may also promote the activity of the NF-κB transcription factor which can in particular induce MMP-9 expression [148,149]. As for AP-1 it has been reported that intracellular Ca^{2+} increase can activate NF-κB [150]. Together these data suggest that Bcl-2 may promote the activation of transcriptional factors inducing expression of genes involved in migration and invasion of tumor cells by regulating Ca^{2+} homeostasis.

Finally, cell migration is tightly linked to a fine regulation of Ca^{2+} homeostasis within the cell. Indeed, it has been recently demonstrated that migration relies on Ca^{2+} gradients inside the cell which can activate a large number of Ca^{2+}-dependent proteins at the leading edge of the migrating cell [151]. Given, the role of Bcl-2 proteins on Ca^{2+} homeostasis it could be hypothesized that these proteins are differently regulated or expressed within the migrating cell to participate in the formation of Ca^{2+} gradients and thus act on cell migration.

5.5. Early embryonic development

Apoptosis is an essential phenomenon during embryonic development to control cellular homeostasis and shape the future body of the developing embryo. In this regard, Bcl-2 proteins are highly expressed during embryonic development and knockout of their related genes are generally correlated with apoptosis defects during embryogenesis [7]. However, some members of the family seem to exert alternative functions going from oocyte maturation to the control of early embryonic development.

In particular, *mcl-1* is one of the only members of the family which knockout in mice is not correlated with apoptosis defect. Indeed, it has been shown that *mcl-1* null embryos do not harbor any sign of increased apoptosis but these embryos fail to implant in utero and exhibit a delay of maturation [152]. Interestingly, IP_3-dependent Ca^{2+} release has been proposed to participate in mouse pre-implantation development via the activation of Calmodulin [153]. In particular, decreasing the intracellular Ca^{2+} level within the embryo seems to delay the first steps of embryonic development. Moreover, during implantation the fixation of blastocyst with fibronectin induces a rise in intracellular [Ca^{2+}] and reducing the amount of intracellular Ca^{2+} during this process seems to impair implantation [154]. Furthermore, so far the only study on Ca^{2+} regulation by Mcl-1 proposed that it enhanced Ca^{2+} release through IP_3R upon physiological IP_3 stimulation [71]. Thus, in the mouse embryo, absence of Mcl-1 may decrease Ca^{2+} release from the ER then altering development and implantation.

Among the anti-apoptotic members, orthologs of *nr-13* seem to be particularly important for reproduction and embryonic development. Nr-13 was first characterized as an anti-apoptotic protein in quail neuroretina. Interestingly, *nr-13* gene expression was shown to be enhanced by the Rous sarcoma virus thus contributing to virus expansion [155]. In chicken, Nr-13 was shown to be mainly expressed during embryonic development in the bursa of Fabricius and in ovarian follicles [156,157]. Later on, the mouse homolog, Diva/Boo [146] and the human homolog, Nrh (also known as Bcl2l10) [158] were characterized. Of note, these two proteins are only expressed in ovaries and testis [159,160]. In the ovary, Bcl2l10 is implicated in oocyte maturation where it seems to control spindle formation, possibly by interacting with the microtubule-binding protein TCTP [159,161,162]. More recently, we have identified *nrz* as the zebrafish ortholog of *nr-13*. Nrz is highly expressed during the early embryonic development with a specific pattern of expression [163]. Indeed, Nrz is found in an extra-embryonic structure called the yolk syncytial layer (YSL), which results from the merging of marginal cells of the embryo with the yolk sac before the onset of gastrulation. Interestingly, Nrz seems to be involved in the first morphogenetic movement of zebrafish embryogenesis called epiboly. This movement is characterized by the migration of embryonic cells from the animal pole down to the vegetal pole of the embryo. When *nrz* translation is repressed, embryos fail to undergo epiboly and finally die from a detachment of the cells from the yolk sac without any apoptosis activation [163,164]. Further characterization of this phenotype pointed out Ca^{2+} homeostasis alteration within the embryo [165]. Nrz is in fact able to interact with IP_3R and decrease Ca^{2+} release through the channel. Thus, in the absence of Nrz, a large increase in cytosolic Ca^{2+} level occurs during epiboly which activates the CaMK, MLCK. This cascade leads to the hypercontraction of an actin–myosin ring present at the margin of the migrating cells resulting in detachment of the whole embryo from the yolk cell. Interestingly, the normal actin–myosin ring contraction during epiboly is correlated with cyclic Ca^{2+} waves, which cross the embryo in the YSL [166]. Therefore, regarding the function of Nrz at the ER and its impact on actin ring contraction, we could speculate that during epiboly progression Nrz/IP_3R interaction may be modulated to generate these Ca^{2+} waves [167].

6. Conclusion

For the last two decades, Bcl-2 proteins function in apoptosis regulation at the mitochondria has been extensively studied although certain mechanisms still remain unsolved. Moreover, it is now well established that Ca^{2+}, a second messenger crucial for many cellular processes, is also involved in apoptosis and numerous studies emphasize the role of Bcl-2 proteins on Ca^{2+} regulation in this process. However, the mechanisms by which Bcl-2 proteins could regulate Ca^{2+} homeostasis at both the ER and the mitochondria are poorly understood and are still a matter of debate.

Beyond their involvement in apoptosis, a growing set of evidence supports the idea that Bcl-2 proteins also possess non-apoptotic roles as different as cell cycle regulation or neuroplasticity among other. Here, we discussed the hypothesis according to which Ca^{2+} homeostasis regulation may be a common mechanism underlying some of these non-apoptotic functions of Bcl-2 proteins. Therefore, at the light of these evidences, a better understanding of the mechanisms by which Bcl-2 proteins control Ca^{2+} fluxes within the cell could allow us to decipher the large action range of this fascinating family.

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