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REVIEW

The selective BH4-domain biology of Bcl-2-family members: IP_3Rs and beyond

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Abstract Anti-apoptotic Bcl-2-family members not only neutralize pro-apoptotic proteins but also directly regulate intracellular Ca^{2+} signaling from the endoplasmic reticulum (ER), critically controlling cellular health, survival, and death initiation. Furthermore, distinct Bcl-2-family members may selectively regulate inositol 1,4,5-trisphosphate receptor (IP_3R): Bcl-2 likely acts as an endogenous inhibitor of the IP_3R, preventing pro-apoptotic Ca^{2+} transients, while Bcl-X_L likely acts as an endogenous IP_3R-sensitizing protein promoting pro-survival Ca^{2+} oscillations. Furthermore, distinct functional domains in Bcl-2 and Bcl-X_L may underlie the divergence in IP_3R regulation. The Bcl-2 homology (BH) 4 domain, which targets the central modulatory domain of the IP_3R, is likely to be Bcl-2's determining factor. In contrast, the hydrophobic cleft targets the C-terminal Ca^{2+}-channel tail and might be more crucial for Bcl-X_L's function. Furthermore, one amino acid critically different in the sequence of Bcl-2's and Bcl-X_L's BH4 domains underpins their selective effect on Ca^{2+} signaling and distinct biological properties of Bcl-2 versus Bcl-X_L. This difference is evolutionary conserved across five classes of vertebrates and may represent a fundamental divergence in their biological function. Moreover, these insights open novel avenues to selectively suppress malignant Bcl-2 function in cancer cells by targeting its BH4 domain, while maintaining essential Bcl-X_L functions in normal cells. Thus, IP_3R-derived molecules that mimic the BH4 domain's binding site on the IP_3R may function synergistically with BH3-mimetic molecules selectivity suppressing Bcl-2's proto-oncogenic activity. Finally, a more general role for the BH4 domain on IP_3Rs, rather than solely anti-apoptotic, may not be excluded as part of a complex network of molecular interactions.

Keywords Bcl-2 · Bcl-X_L · BH4-domain targets · Inositol 1,4,5-trisphosphate receptors · Ca^{2+} signaling · Apoptosis

Anti-apoptotic Bcl-2-family members counteract pro-apoptotic Bcl-2-family members

Bcl-2-family members play a pivotal role in a cell's decision to initiate apoptosis or to promote cell survival by controlling mitochondrial outer membrane permeabilization (MOMP) [1, 2]. Anti-apoptotic Bcl-2-family members (Bcl-2, Bcl-X_L, Mcl-1, Bcl-W and Bfl-1) have a well-studied and characterized role in scaffolding the Bcl-2 homology (BH) 3 domain of pro-apoptotic Bcl-2-family members, thereby neutralizing their pro-apoptotic activity [3]. A network of interactions has been described in which anti-apoptotic Bcl-2-family members can scaffold the multi-domain pro-apoptotic proteins, Bax and Bak, the pro-apoptotic Bax/Bak-activator BH3-only proteins, Bid and Bim, or the sensitizer BH3-only proteins, Bad, Bik, Noxa, Hrk, Bmf, and Puma [2, 3]. The latter do not directly activate Bax/Bak, but target anti-apoptotic Bcl-2 proteins, thereby alleviating their repressive function on Bax, Bak, Bid, and Bim. Furthermore, these interactions seem to be dynamic and may be important to prevent the mitochondrial accumulation of pro-apoptotic proteins, like Bax [4, 5]. For instance, Bcl-X_L binds Bax at the outer

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mitochondrial membrane, shuttling Bax back in the cytosol, where the Bcl-X_L/Bax complex disassembles resulting in Bax accumulation in the cytosol. On the other hand, Bax activation by BH3-only proteins, like Bim/truncated Bid and Puma, causes a stepwise activation, involving its accumulation at mitochondrial membranes and its oligomerization to a death pore [6, 7]. Besides Bax/Bak, the mitochondrial permeability transition pore can mediate MOMP and cell death in response to apoptotic stimuli that elevate intracellular Ca^{2+} and induce mitochondrial calcium overload [8, 9]. The latter mechanism can be directly targeted and sensitized by Bad in Ca^{2+}-dependent apoptosis through dephosphorylation of Bad by PP2A [10].

As summarized by Letai and coworkers [11], it is clear that while both activator-BH3-only proteins are targeted by all anti-apoptotic Bcl-2-family members, the interaction between anti-apoptotic proteins and the sensitizer BH3-only proteins display a high degree of selectivity [12–16]. For instance, while the BH3 domain of Bad mainly targets Bcl-2, Bcl-X_L, Bcl-W, but not Mcl-1, the BH3 domain of Noxa mainly targets Mcl-1, but not Bcl-2, Bcl-X_L, Bcl-W. The selectivity of BH3-only proteins towards anti-apoptotic Bcl-2-family members has been exploited to derive BH3-domain peptides and to set up a "BH3 profile" of cancer cells, identifying cancer cells as "primed for death" and helping to elucidate to which Bcl-2-family members these cancer cells are addicted [11, 12, 17]. This network also spurred the development of a novel class of anti-cancer drugs, the BH3-mimetic molecules, including the Bad BH3-mimetic ABT-737 (or its orally available variant ABT-263) [18–20].

Bcl-2 family members control Ca^{2+} signaling

The endoplasmic reticulum (ER) and mitochondria are closely connected

The first reports of Bcl-2 affecting ER Ca^{2+} arose in the beginning of the 90s [21, 22]. Since then, it has become increasingly clear that ER, the main intracellular Ca^{2+} store, is tightly controlled by Bcl-2-family members critically regulating Ca^{2+} fluxes from ER to mitochondria [23–27]. In particular, the close connection of the mitochondria and the ER, illustrates the critical role of ER Ca^{2+} homeostasis and ER Ca^{2+} release via inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs) during cell survival and cell death [28–31]. The latter channels are important components of the mitochondria-associated ER membranes (MAMs), which establish physical links between mitochondria and ER through interorganellar multi-protein complexes involving IP_3Rs, glucose regulated protein (GRP) 75, voltage-dependent anion channels (VDACs), mitofusins, chaperones like phosphofurin acidic cluster sorting protein 2, and peptidic tethers [32, 33]. Recently, the ER-stress sensor PKR-like ER-regulated kinase (PERK) has been identified as a novel member of the MAMs [34]. As a consequence, both the steady-state Ca^{2+}-filling level of the ER [35] as well as the IP_3R activity [31] will affect the mitochondria. This is underpinned by recent studies of Foskett's [36] and the Mikoshiba's groups [37]. It was shown that constitutive Ca^{2+} transfer from the ER to the mitochondria through IP_3Rs is essential for mitochondrial bioenergetics and for the production of ATP through oxidative phosphorylation. Suppressing this basal Ca^{2+} firing of IP_3Rs causes the activation of AMP-activated kinase (AMPK) and subsequent induction of macroautophagy, a pro-survival lysosomal delivery pathway [36]. This concept is supported by previous studies showing that inhibition of IP_3R signaling triggered autophagy [38, 39]. In this perspective, lowering the steady-state $[Ca^{2+}]_{ER}$ levels may reduce the amount of Ca^{2+} that is released by spontaneous IP_3R activity and consequently attenuate Ca^{2+}-mediated cross-talk between ER and mitochondria. In addition, lowering the $[Ca^{2+}]_{ER}$ causes the intraluminal ER chaperone, GRP78/BiP, to dissociate from IP_3R1, leading to a decline in the amount of functional IP_3R1 channels, further reducing IP_3R1-mediated Ca^{2+} mobilization and inducing apoptotic cell death, as recently described [37].

Besides the spontaneous IP_3R activity, agonist-triggered IP_3R-mediated Ca^{2+} signals also affect cell survival and cell death. While repetitive and small Ca^{2+} oscillations seem to enhance mitochondrial bioenergetics, thereby promoting survival, large Ca^{2+} transients will inevitably lead to MOMP, thereby promoting cell death [23]. In the latter paradigm, decreasing steady-state $[Ca^{2+}]_{ER}$ will help to avoid mitochondrial Ca^{2+} overloading and will promote survival, while increasing steady-state $[Ca^{2+}]_{ER}$ will enhance apoptosis. Beyond apoptosis, IP_3R activity also seems to be critical for proper autophagy induction during starvation [40, 41] and thus probably for survival responses during adverse conditions. Although the Ca^{2+}-release pathways of the ER have been well established, the mitochondrial Ca^{2+}-uptake pathways remained elusive for a long time. Now, recent work from the Rizzuto group found that VDAC1, but not VDAC2 or VDAC3, is specifically involved in transferring apoptotic Ca^{2+} signals across the outer membrane of the mitochondria [42]. This likely underlies the selective presence of VDAC1 in the MAMs. Furthermore, Ca^{2+} transfer across the inner membrane of the mitochondria is mediated by the recently identified mitochondrial Ca^{2+} uniporter (MCU) [43–45].

Proto-oncogenes and tumor suppressors regulate intracellular Ca^{2+} signals and -release channels

As illustrated above, it is now clear that cell survival and cell death are tightly controlled by Ca^{2+} signaling. Hence, it

is not surprising that proto-oncogenes, like anti-apoptotic Bcl-2-family members, protein kinase B (PKB)/Akt, Bax-Inhibitor-1 (BI-1) and tumor suppressors like promyelocytic leukemia (PML) and fragile histidine triad (FHIT) regulate ER Ca^{2+}-release and mitochondrial Ca^{2+}-uptake mechanisms [46–49]. IP_3Rs are phosphorylated by the pro-survival kinase PKB/Akt, which is activated by phosphatidylinositol-3,4,5-trisphosphate (PIP_3), thereby suppressing IP_3R-channel activity and promoting survival [50, 51]. This is important, since phosphatase and tensin homolog (PTEN), a negative regulator of PKB/Akt signaling through dephosphorylation of PIP_3 to phosphatidylinositol-4,5-bis-phosphate, is one of the most frequent loss functions in human cancers [52]. Another negative regulator of PKB/Akt activity is the tumor suppressor PML, which recruits protein phosphatase 2A to the IP_3R-PKB/Akt-protein complex in the MAMs and suppresses PKB/Akt activity [53–55]. At the mitochondrial level, FHIT seems to target MCU-driven mitochondrial Ca^{2+} uptake, thereby enhancing the transfer of Ca^{2+} into mitochondria during physiological signaling [56]. In addition, anti-apoptotic Bcl-2 proteins can regulate VDAC1-channel activity by directly binding, among other domains, its N-terminal tail, a region important for VDAC-1's pro-apoptotic activity [57]. Importantly, some anti-apoptotic proteins do not target ER Ca^{2+}-release or -uptake mechanisms, but function themselves as Ca^{2+}-leak channels. For instance, ER-stress suppressor BI-1 has been shown to display endogenous Ca^{2+}-leak activity as a Ca^{2+}/H^+ antiporter or Ca^{2+}-release channel, thereby directly controlling the filling state of the ER Ca^{2+} stores [58–60]. In accordance with this, BI-1 overexpression has been proven to lower the Ca^{2+}-filling state of the ER, a mechanism previously shown to act protective against apoptosis [35]. In this regard, we have also recently identified a putative Ca^{2+}-channel pore in the C-terminal part of BI-1 [61]. In addition to this, BI-1 directly binds IP_3Rs through their channel domains, thereby sensitizing these intracellular Ca^{2+}-release channels to IP_3 [62]. This mechanism seems to underlie the autophagy-promoting effect of BI-1, which required the presence of functional IP_3R channels [63].

Bcl-2-family members control Ca^{2+} signaling from the ER

Besides these mechanisms, the best-studied protein family, regulating intracellular Ca^{2+} is the anti- and pro-apoptotic Bcl-2-family. Pinton et al. [64] elucidated a protective role of Bcl-2 at the ER. They found that Bcl-2 overexpression at the ER enhanced the ER Ca^{2+}-leak rate and thus reduced the level of steady-state $[Ca^{2+}]_{ER}$, thereby dampening agonist-induced IP_3R-mediated Ca^{2+} signals originating from the ER and thus reducing the transfer of Ca^{2+} to the mitochondria. This mechanism was underpinned by Scorrano and coworkers [65] who used mouse embryonic fibroblasts lacking Bax/Bak to increase the ratio of anti-apoptotic over pro-apoptotic Bcl-2 family members. Bax/Bak-deficient cells displayed decreased steady-state $[Ca^{2+}]_{ER}$ levels, which protected the cells against apoptotic stimuli. The underlying mechanism involved the hyper-sensitization of the IP_3R1 towards basal IP_3 through a PKA-dependent phosphorylation of the IP_3R, enhancing the basal IP_3R-mediated Ca^{2+} leak from the ER [66]. In contrast, Distelhorst and coworkers initially proposed another mechanism for the protective role of Bcl-2 at the ER, pointing out that Bcl-2 maintained ER Ca^{2+} homeostasis [67]. Successively, Bcl-2 was also reported to recruit calcineurin/PP2B on IP_3Rs [68, 69] or indirectly bind to the IP_3R and suppress IP_3R activity through the phosphatase PP1 [70]. More recent works elucidated direct binding of anti-apoptotic Bcl-2-family members to IP_3Rs, finely regulating their Ca^{2+}-flux properties and consequently cell death outcomes [71–73]. Additionally, Bcl-2-family members are able to indirectly regulate IP_3R signaling by controlling the expression levels of IP_3Rs. For instance, Bcl-X_L overexpression has been shown to decrease the level of IP_3Rs in cells by a decreased binding of the transcription factor nuclear factor of activated T cells (NFAT) cytoplasmic 2 to the IP_3R promoter [74]. Next, anti-apoptotic Bcl-2 was proposed to up-regulate sarco/ER Ca^{2+}-ATPase (SERCA) levels, thereby supporting sustained ER Ca^{2+} filling [75, 76]. This may be due to the direct molecular interactions found between some anti-apoptotic Bcl-2 family members and SERCA [75–77]. However, other studies indicated that the targeting of SERCA1, the skeletal muscle type isoform, by Bcl-2 seemed to destabilize and inactivate the SERCA protein by exposing thiol groups [78], thereby lowering the content of ER Ca^{2+} stores. The mechanisms may involve the translocation of SERCA1 from sarcoplasmic reticulum (SR) lipid-caveolae domains [79]. A recent paper from the same group showed that Bcl-2 also destabilized SERCA2b, the house-keeping isoform of the SERCA-protein family, while heat-shock proteins, chaperones, and other stress-regulated proteins attenuated the negative regulation of SERCA2b by Bcl-2 [80]. These findings are underpinned by recent observations in cystic fibrosis airway epithelium, which displayed decreased SERCA levels, increased Bcl-2 levels and the presence of SERCA/Bcl-2-protein complexes on ER membranes [81]. Finally, Bcl-2 was shown to counteract both the pro-apoptotic and paraptotic effects of p20, a cleaved form of Bap31, via regulation of ER Ca^{2+}. Paraptosis is a form of caspase-independent non-apoptotic programmed cell death that is characterized by cytoplasmic vacuolation initiated by mitochondrial and ER swelling [82–84]. Bap31 is an ER-located protein that plays roles in

protein trafficking [85] as well as ER-associated degradation [86]. In addition, Bap31 was shown to have anti-apoptotic qualities [87]. When Bap31 is cleaved by caspase 8, the resultant ER-located protein, p20, is known to have pro-apoptotic functions. This protein mobilizes ER Ca^{2+}, resulting in MOMP. Via the above-described mechanisms, Bcl-2 is able to counteract these pro-apoptotic signals, allowing cell survival [88]. In addition, a recent paper describes a p20-initiated Bax/Bak-independent paraptotic death pathway [89]. Instead of mobilizing the ER Ca^{2+}, p20 was shown to increase $[Ca^{2+}]_{ER}$ leading to ER remodeling, vacuolization, and both caspase and Bax/Bak-independent paraptotic cell death. Here, the ability of Bcl-2 to lower $[Ca^{2+}]_{ER}$ was shown to protect the cells from these events typically associated with paraptotic cell death.

Finally, in many cells, including pancreatic acinar cells, the ER can come in very close contact with the plasma membrane [90]. Thus, ER-localized Bcl-2 may have plasmalemmal targets and more general cell biological functions in regulating cellular Ca^{2+} homeostasis. A recent report showed that Bcl-2 suppresses cellular Ca^{2+} extrusion through the plasma membrane Ca^{2+} ATPase (PMCA), thereby determining the cell-death pathway that is engaged [91]. In this study, it was shown that Bcl-2-deficient pancreatic acinar cells extrude Ca^{2+} more efficiently, protecting them against excessive necrosis. At the same time, apoptosis was increased in cells exposed to reactive oxygen species (ROS) generated by menadione treatment. However, inhibition of PMCA using a peptide inhibitor promoted necrosis in menadione-treated cells, which may indicate that excessive Bcl-2 accumulation at the ER-plasma membrane junction inhibiting PMCA may be deleterious.

Irrespective of the underlying mechanism, it is clear that Bcl-2 proteins critically regulate ER Ca^{2+} homeostasis and dynamics. This is supported by a recent study, showing that chemical inhibitors of pro-survival Bcl-2-family members like the BH3-mimetic molecules BH3I-2′ and HA14-1 cause a pro-apoptotic depletion of the ER Ca^{2+} stores in part through activation of IP_3R Ca^{2+}-release channels [92].

Bcl-2-family members directly target IP_3Rs

Bcl-2 and Bcl-X_L directly target IP_3Rs, but at different sites

More recent work indicated that Bcl-2 does not primarily act by altering the ER Ca^{2+}-store content. Instead, Bcl-2 directly targets IP_3Rs and functions as an endogenous regulator of IP_3Rs [71, 93–97]. In this paradigm, Bcl-2 suppresses pro-apoptotic IP_3R-mediated Ca^{2+} transients (provoked by strong T-cell-receptor stimulation), while maintaining or even promoting pro-survival Ca^{2+} oscillations (provoked by weak T-cell receptor stimulation). Moreover, Bcl-X_L also directly binds IP_3Rs and sensitizes IP_3R-channel to sub-threshold [agonist] stimulation [72, 98]. IP_3R/Bcl-X_L-complex formation increases the frequency of Ca^{2+} oscillations, mitochondrial bioenergetics, and NFAT-mediated signaling in Bcl-X_L-overexpressing DT40 cells, while not affecting global agonist-induced Ca^{2+} transients. Elegantly, it was shown that Bcl-X_L protection against high [anti-IgM]-induced apoptosis was reduced in the absence of IP_3Rs [98]. Furthermore, the effect of Bcl-X_L on Ca^{2+} signaling depended on the type of IP_3R isoform. Bcl-X_L stimulated IP_3R-mediated Ca^{2+} oscillations for all three isoforms while it lowered $[Ca^{2+}]_{ER}$ in IP_3R3-, but not in IP_3R1- or IP_3R2-, expressing DT40 cells.

At the molecular level, striking differences between Bcl-2 and Bcl-X_L for IP_3R binding were observed. While Bcl-2 binds to the central, modulatory domain of the IP_3R [95, 96], Bcl-X_L binds the C-terminal region close to the Ca^{2+}-channel pore [72, 98] (Fig. 1). This C-terminal tail is also involved in the control of IP_3R-channel gating through the N-terminal suppressor domain of the IP_3-binding domain [99]. Thus, Bcl-X_L may enhance the coupling between the N-terminal IP_3-binding domain and C-terminal channel-pore opening, underlying the observed IP_3R sensitization. The latter region has been proposed to display structural features that mimic the BH3 domain of BH3-only proteins [100]. In this respect, one expects that the hydrophobic cleft formed by BH3, BH1, and BH2 of all anti-apoptotic Bcl-2-family members may participate in the binding the IP_3R. Finally, it has been recently described that not only Bcl-X_L but also Bcl-2 and Mcl-1 target this site on IP_3Rs and cause IP_3R sensitization [73]. In addition to this site, Bcl-2 possesses an additional binding site on the IP_3R with distinct molecular and functional properties. Indeed, Bcl-2 directly binds to a site between amino acids 1389–1408 of IP_3R1. Bcl-2 binding to this central, modulatory domain of the IP_3R causes an inhibition of the Ca^{2+}-flux properties of IP_3R in response to agonist stimulation (Fig. 1). Furthermore, a peptide corresponding to the Bcl-2-binding site on IP_3Rs (a.a. 1389–1408), IP_3R-derived peptide (IDP), completely abolishes the binding of Bcl-2 to the IP_3Rs [95]. A cell-permeable version of IDP enhances IP_3R-mediated Ca^{2+} signaling, thereby potentiating apoptotic signals, similarly to strong TCR stimulation. In this respect, IDP derepresses Bcl-2's inhibitory function on IP_3R1 by specifically targeting its BH4 domain and not the BH3-binding hydrophobic cleft. The only domain of Bcl-2 sufficient for binding, inhibiting, and protecting against IP_3R1-mediated apoptosis [96, 101] is indeed the BH4 domain. This indicates that IDP targets Bcl-2 independently of the compounds that target the hydrophobic cleft, like the BH3-mimetic tools, ABT-737 and HA14-1. Combining IDP with ABT-737 enhanced the potency of

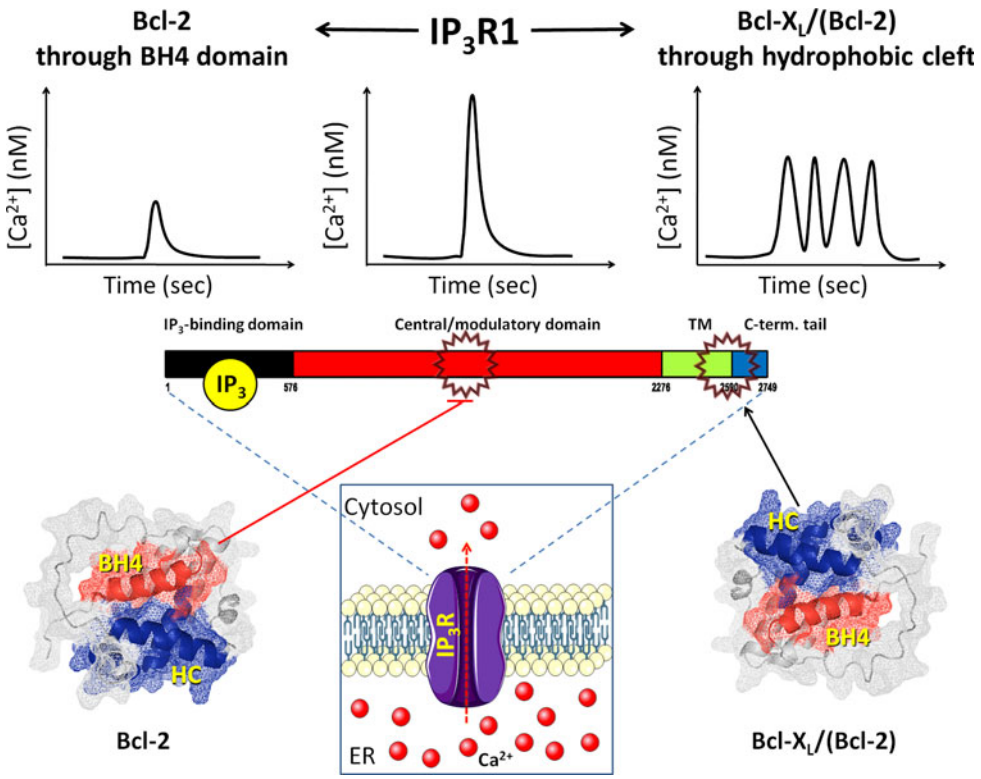

Fig. 1 Differential regulation of IP_3R channels by Bcl-2 versus Bcl-X_L. The Ca^{2+}-flux properties of IP_3R are thought to be critically controlled by Bcl-2-family members to promote cell survival or protect against cell death. We hypothesize that distinct Bcl-2-family members target distinct IP_3R domains. In this paradigm, Bcl-2 through its BH4 domain may primarily target the central, modulatory domain of the IP_3R, thereby reducing large global pro-apoptotic Ca^{2+} transients (*left*), while Bcl-X_L through its hydrophobic cleft (HC) or another domain may primarily target the C-terminal tail of the IP_3R close to the channel pore, thereby increasing IP_3R sensitivity to basal IP_3 levels and promoting pro-survival Ca^{2+} oscillations (*right*). It should be noted that the C-terminal domain of IP_3Rs has been proposed to harbors BH3-like domains and may also recruit Bcl-2. In addition, there is increasing evidence that other Bcl-2-family members may target IP_3Rs, like NrZ, the zebrafish homologue of Bcl-2L10, through its BH4 domain and Mcl-1 through its hydrophobic cleft (HC) or another domain may primarily target the C-terminal tail of the IP_3R close to the channel pore

ABT-737 to induce cell death in lymphocytes obtained from chronic lymphocytic leukemia (CLL) patients [102]. Furthermore, applying a stabilized cell-permeable form of IDP (TAT-$IDP^{DD/AA}$) potently induced cell death through excessive IP_3R-mediated Ca^{2+}-release events in CLL cells, while TAT-$IDP^{DD/AA}$ did not significantly reduce the survival of normal lymphocytes [103].

Bcl-2 and Bcl-X_L regulate various IP_3R-dependent physiological and pathophysiological processes

Bcl-2 and Bcl-X_L-mediated regulation of IP_3Rs is not only relevant for cell death and cancer but also for other physiological processes like embryonic development and pathophysiological conditions, like muscle dystrophy, type-2 diabetes, and bipolar disorders.

A recent paper by Gillet et al. revealed that Nrz (the zebrafish orthologue of human Nrh/Bcl-2L10) through its BH4 domain binds and regulates IP_3R-mediated Ca^{2+} signaling in the developing zebrafish embryo, acting as an inhibitor of IP_3R function [104]. In more detail, Nrz is proposed to suppress Ca^{2+} signaling in yolk syncytial layer (YSL) to facilitate proper blastomere migration from the animal to vegetative pool (known as epiboly, a process that happens before the onset of gastrulation [105]) [104]. Completion of epiboly is characterized by the formation of an acto-myosin contractile ring close to the vegetative pool of the enveloping layer and the deep cell layer. Therefore, it is critically important that during epiboly Ca^{2+} signaling in the YSL is suppressed to prevent premature acto-myosin contractions. This is supported by recent findings showing that *nrz* morphants displayed elevated Ca^{2+} signaling in the YSL causing Ca^{2+}-dependent myosin light chain (MLC) phosphorylation by MLC kinase, thereby affecting cytoskeletal dynamics and cell movements [104]. As a consequence, *nrz* morphants undergo developmental arrest before the onset of gastrulation, resulting in embryonic death without the activation of caspases [106]. This

indicates that Bcl-2 family members as critical Ca^{2+} regulators not only control apoptosis but also developmental processes through Ca^{2+}-dependent processes like actomyosin contraction and/or cell movements [107]. The molecular determinants underpinning this role have not been fully characterized yet but it is intriguing that both Bcl-2's and Nrz's BH4 domains bind and modulate IP_3Rs despite their very divergent primary sequence. Eventually, this may suggest that the concept of Bcl-2-dependent regulation of IP_3Rs is dynamically conserved during evolution.

In Duchenne muscle dystrophy, a lethal disease caused by deficiency in dystrophin, a cytoskeletal protein, the degeneration of muscle is associated with disrupted intracellular Ca^{2+} homeostasis [108]. Overexpression of Bcl-2 in myotubes obtained from dystrophic (*mdx*) mice decreases subsarcolemmal and mitochondrial Ca^{2+} elevations in response to stimulation of the nicotinic acetylcholine receptor [109]. The central role of IP_3Rs in this process was underpinned by experiments performed on saponin-permeabilized myotubes. Myotubes obtained from *mdx* mice displayed more IP_3-induced Ca^{2+} responses than their wild-type counterparts, while Bcl-2 overexpression suppressed these IP_3R-dependent Ca^{2+} signals. These observations correlate with the increased susceptibility of *mdx* myotubes to apoptotic stimuli, which could be counteracted by overexpressing Bcl-2 or an IP_3 sponge.

In the vascular smooth muscle of type 2 diabetes mouse models, the level of Bcl-X_L, but not of Bcl-2, seemed elevated, while IP_3R levels remained constant [110]. Importantly, the rate of IP_3R-mediated Ca^{2+} release from the SR of vascular smooth muscle of type 2 diabetes mouse models was similar to their wild-type counterparts. This enhanced IP_3R activity by Bcl-X_L was counteracted by ABT-737, suggesting IP_3R regulation by Bcl-X_L through its hydrophobic cleft.

Very recently, a single-nucleotide polymorphism (SNP) in the Bcl-2 gene (rs956572) associated with bipolar disorder seemed to affect Ca^{2+} signaling in the lymphoblasts of bipolar disorder patients [111, 112]. This Bcl-2-deficient SNP variant AA is known to be associated with reduced Bcl-2-mRNA and -protein levels and directly affects the brain by significantly decreasing grey matter volume in the ventral striatum of healthy subjects [113]. The striatum's ventral region is important for the neurobiology and pathophysiology of mood disorders [114]. Particularly, Bcl-2-deficient SNP variant AA caused elevated cytosolic $[Ca^{2+}]$ and increased IP_3R-mediated Ca^{2+} release without affecting basal ER and mitochondrial Ca^{2+} levels [111]. These properties were associated with a decline in the Bcl-2-mRNA and -protein levels. In addition, increased IP_3R-mediated Ca^{2+} release could be mimicked by treating lymphoblasts from subjects presenting the normal Bcl-2 SNP variant GG with the Bcl-2 inhibitor BH3-I. Therefore, it is likely that IP_3Rs from lymphoblasts containing the Bcl-2-deficient SNP variant AA are largely depleted from Bcl-2. Nevertheless, this study suggests a critical role for IP_3R/Bcl-2 complexes in regulating intracellular Ca^{2+} dynamics in the brain to control emotional regulation and reward processing.

Collectively, these examples show that Bcl-2 and Bcl-X_L display different functional properties towards IP_3R regulation, underpinning that distinct IP_3R and Bcl-2/Bcl-X_L protein domains are responsible for this phenomenon.

Bcl-2 and Bcl-X_L display different BH4-domain properties at the level of the IP_3R

Despite the fact that Bcl-2 and Bcl-X_L are highly similar in sequence and structure, the BH4-domain biology of Bcl-2 and Bcl-X_L seems totally different [101]. Both their BH4 domains protect against IP_3R-mediated apoptosis, but only the BH4-Bcl-2 domain binds and inhibits IP_3Rs. Indeed, IDP seems to inhibit only Bcl-2 by targeting its BH4 domain without affecting Bcl-X_L's anti-apoptotic function. This is an important therapeutic advantage over the existing BH3-mimetic molecules. For instance, ABT-737 acts as a Bad BH3-mimetic molecule, indicating that it does not discriminate between Bcl-2 and Bcl-X_L, thus inhibiting both proteins. This may not be desirable in cancer patients and cause adverse effects, since Bcl-2 and Bcl-X_L have distinct biological functions. While some types of cancer cells may need the elevated Bcl-2 levels to compensate for the on-going upstream pro-apoptotic signaling and the elevated levels of BH3-only proteins, normal cells may still need Bcl-X_L for their survival. Potent Bcl-2 inhibitors, like the BH3 mimetics ABT-737 and ABT-263, which target the hydrophobic cleft of both Bcl-2 and Bcl-X_L, are already in clinical development and enhance the therapeutic potency of different chemotherapeutical drugs in solid and hematologic malignancies [20, 115–119]. However, in single-use regiments, these compounds lead to in vivo dose-dependent transient thrombocytopenia [120] and thrombocytopathy [121] in a similar range as they kill cancer cells (like CLL). The former is due to the inhibition of Bcl-X_L, which is essential to sustain platelet survival by limiting Bax activity [122]. Since BH3-mimetic molecules do not discriminate between the hydrophobic cleft of Bcl-2 and Bcl-X_L, the treatment of Bcl-2-dependent malignancies by BH3 mimetics like ABT-263 will provoke side effects in patients by limiting the life span of platelets. Hence, specifically targeting the BH4 domain of Bcl-2 with IDP may be a very promising approach to promote cell death via the induction of pro-apoptotic Ca^{2+} signaling in Bcl-2-dependent malignancies [102].

Collectively, these data indicate that Bcl-2 and Bcl-X_L likely have distinct properties at the level of the IP_3R (Fig. 1). We propose that the predominant effect of Bcl-2 is executed via its BH4 domain targeting the central, modulatory domain of the IP_3R, imposing IP_3R inhibition and ultimately preventing large pro-apoptotic Ca^{2+} transients. For Bcl-X_L, we anticipate a dominant role for its hydrophobic cleft targeting the BH3 structure near the C-terminal Ca^{2+}-channel pore of the IP_3R, optimizing IP_3R-channel gating and sensitivity towards IP_3. We do not exclude that Bcl-2 too targets this C-terminal site, but its BH4-domain biology seems to overcome this sensitizing effect.

The conserved Lys17 in the BH4 domain of Bcl-2 determines its selective action on IP_3Rs

Recently, we elucidated one factor in the selective action of Bcl-2 and Bcl-X_L on IP_3Rs [101]. While most residues are conserved among the BH4 domains of Bcl-2 and Bcl-X_L, we identified a critical difference in one single surface-accessible residue in the center of this domain (Fig. 2). We found that Lys17 in BH4-Bcl-2 is not conserved in BH4-Bcl-X_L, in which it corresponds to an Asp residue. We performed a plethora of molecular and functional studies to pinpoint this residue as the underlying factor responsible for the difference in BH4-domain biology between Bcl-2 and Bcl-X_L. Indeed, replacing Asp11 by Lys in BH4-Bcl-X_L led to a variant that is able to bind and inhibit IP_3Rs, while replacing Lys17 by Asp in BH4-Bcl-2 led to a variant that completely lost its IP_3R-binding and inhibitory properties. The importance of this critical difference for the biological properties of these proteins is highlighted by the fact that altering this residue in full-length Bcl-2 impairs its ability to regulate IP_3Rs and to protect against Ca^{2+}-mediated apoptosis. This is further highlighted by the fact that this critical difference in residues is conserved among the five classes of vertebrates in both Bcl-2 and Bcl-X_L (Fig. 3). Indeed, all vertebrate Bcl-2 orthologues contain a positively charged amino acid in the center of their BH4 domain, while all vertebrate Bcl-X_L orthologues contain a negatively charged amino acid. This means that already in the first appearances of Bcl-2 and Bcl-X_L during evolution, this selective function may have been important.

Therefore, while Bcl-2 and Bcl-X_L were considered alike in their respect to regulating Ca^{2+} signaling, we propose selective functions for Bcl-2 and Bcl-X_L at the level of the IP_3R. This idea impinges on the selective environment in which Bcl-2 and Bcl-X_L seems to operate. Bcl-2 seems to operate at different intracellular membranes, including the ER, while Bcl-X_L seems to mainly operate at mitochondrial membranes and in the cytosol. On the one hand, excessive Bcl-2 expression at the mitochondria seems to be toxic for the cells and leads to

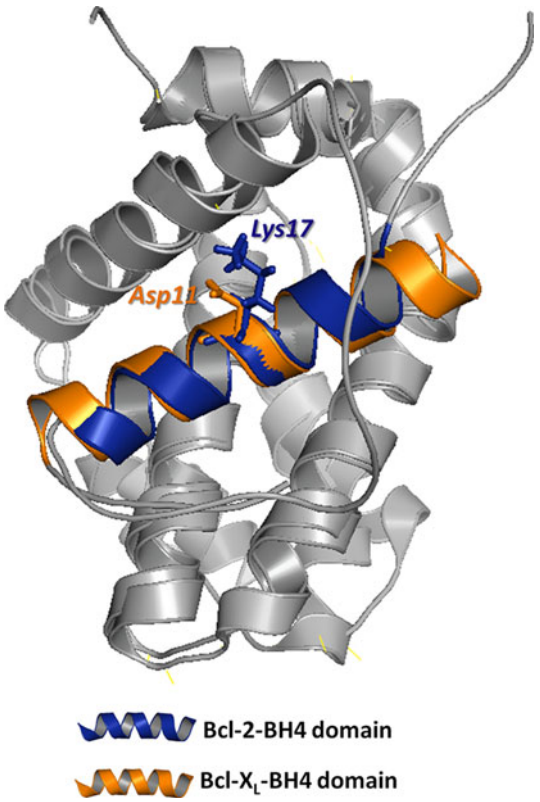

Fig. 2 A representation of the overlapping Bcl-2 and Bcl-X_L structures. Their respective BH4 domains (*blue* for Bcl-2, *orange* for Bcl-X_L) have been indicated together with the critical difference between Bcl-2 (Lys17) and Bcl-X_L (Asp11), which determines the ability of Bcl-2, but not of Bcl-X_L, to interact with the central, modulatory domain of the IP_3R

apoptotic cell death, while Bcl-2 expression at the ER promotes bona fide anti-apoptotic responses [22]. On the other hand, a very recent and elegant study using the recomplementation of Bcl-$X_L^{-/-}$ cells with either ER or mitochondrial-targeted Bcl-X_L showed that the presence of Bcl-X_L is a *conditio sine qua non* for proper protection against apoptotic stimuli [123]. Strikingly, ER-targeted Bcl-X_L expression in Bcl-$X_L^{-/-}$ cells was able to regulate ER Ca^{2+} homeostasis, but this was not sufficient to protect against apoptotic stimuli. The latter required mitochondrial Bcl-X_L, since Bcl-X_L expression in wild-type cells containing endogenous Bcl-X_L provided apoptosis protection. Thus, the ER seems part of the natural environment, in which Bcl-2 would operate in protecting against apoptosis, while the mitochondria may be the natural environment for Bcl-X_L-mediated protection against apoptosis. Finally, alignment of the BH4 domain of the other Bcl-2-family members indicates that the BH4 domain of Bcl-X_L resembles Bcl-2 one's the most. Thus, since BH4-Bcl-X_L is not able to target IP_3Rs, this may suggest a unique role for the BH4 domain of Bcl-2 among the other Bcl-2-family members in repressing pro-apoptotic IP_3R function.

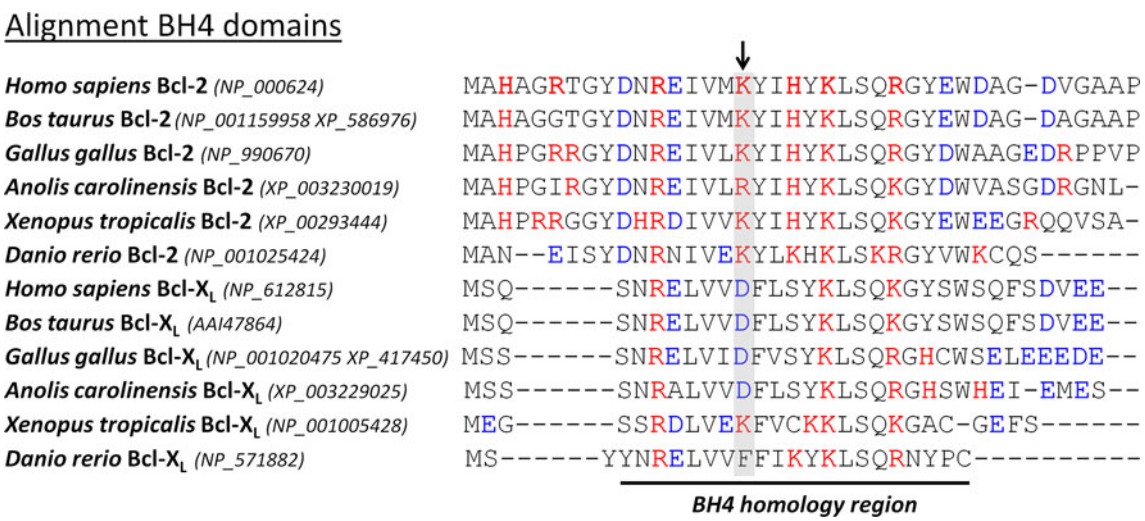

Fig. 3 Sequence alignment of the BH4 domain of different Bcl-2 and Bcl-X_L homologues in the different classes of vertebrates. Bcl-2 and Bcl-X_L homologues were obtained from the DeathBase [152]. The *number between brackets* indicates the accession number of the protein database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/protein). This analysis reveals that the amino acid Lys17 in human Bcl-2 is conserved as a positively charged residue during evolution. The amino acid Asp11 in human Bcl-X_L also seems conserved as a negatively charged residue during evolution, although *Xenopus* Bcl-X_L contains a Lys and zebrafish Bcl-X_L contains a Phe in the corresponding position. The positively charged (*red*) and negatively charged (*blue*) amino acids are depicted in color. The conserved critical Lys residue in the Bcl-2 homologues and its corresponding residue in the Bcl-X_L homologues are displayed on a *gray* background and are indicated by an *arrow*

Other selective BH4-domain targets for Bcl-2 and Bcl-X_L?

Although a number BH4 domain targets of Bcl-2 and/or Bcl-X_L have been identified, a selective role for both proteins has not been investigated. In this respect, most studies either focused on the BH4 domain of Bcl-2 or of Bcl-X_L, providing a growing list of novel targets beyond IP_3Rs, including calcineurin/PP2b, VDACs, Raf-1, Ras, CED-4, paxillin, NF-kB, BI-1 and apoptosis-stimulating of p53 protein 2 (ASPP2) [96]. However, some findings in the literature seem to hint towards a selective regulation of these targets by Bcl-2 versus Bcl-X_L and vice versa [124–126]. Nevertheless, in many cases, firm evidence is lacking, because a side-by-side comparison of the regulation of these targets by Bcl-2 versus Bcl-X_L, or by protein domains derived from them, has never been performed. Since VDAC1 and BI-1 directly control Ca^{2+}-signaling events and apoptosis, we first discuss their BH4-mediated regulation. Finally, we focus on the pro-apoptotic ASPP2 protein, which has been proposed to display selective Bcl-2/Bcl-X_L-binding properties.

VDACs

VDACs are transport proteins located on the outer mitochondrial membranes responsible for exchanging metabolites and ATP between cytosol and mitochondria and for the flux of Ca^{2+} ions from ER into the mitochondria [127, 128]. VDAC proteins seem to be essential for both cell growth and apoptosis [129, 130]. The role in cell growth seems to involve their ability to transport metabolites and energy, but may also be attributed to its Ca^{2+}-flux properties and its localization in MAM's [127]. This flux of Ca^{2+} from the ER into the mitochondria is essential for proper mitochondrial bioenergetics [36]. This correlates with recent observations from White and coworkers showing that Bcl-X_L, at the mitochondrial membranes, enhanced VDAC1-mediated Ca^{2+} flux into the mitochondria, thereby promoting ATP production and increasing mitochondrial bioenergetics (Carl White, pers. comm.). However, Bcl-X_L's regulation of mitochondrial Ca^{2+} uptake might be different during apoptosis, since this anti-apoptotic protein was previously shown to delay Ca^{2+}-mediated MOMP in neuronal cell models [131]. Although the role of VDAC proteins in mitochondria-dependent cell death has always been controversial, recent evidence showed that VDAC1, but neither VDAC2 nor VDAC3, relays IP_3R-mediated pro-apoptotic Ca^{2+} signals into the mitochondria [42]. Additionally, the expression of VDAC1 appears to critically control apoptosis likely by the formation IP_3R/VDAC1 complexes, which are enhanced during apoptotic stress, and by the formation of VDAC1 oligomers [42]. The oligomerization of VDAC1 has been shown to be coupled to its ability to induce apoptosis [132]. Further reports, from Shoshan-Barmatz's laboratory, indicate that anti-apoptotic proteins, like Bcl-2 and hexokinase I and II bind mainly the N-terminal part of VDAC1 and suppress

VDAC's apoptotic function [57, 133–135]. The Bcl-2/Bcl-X_L protein domain regulating VDAC1 activity was proposed to be its BH4 domain [126]. Indeed, the isolated BH4 domains of both Bcl-2 and Bcl-X_L were sufficient to inhibit VDAC1 activity in isolated mitochondria and to prevent apoptosis in intact cells. Likewise, solely a Bcl-X_L not lacking the BH4 domain could display these anti-apoptotic properties. However, in this study [126], a complete, quantitative comparison between the properties of the BH4 domain of Bcl-2 and Bcl-X_L for preventing apoptosis through targeting VDAC1 was not performed. Nevertheless, a sucrose-driven liposomal swelling assay mediated by reconstitution of recombinant VDAC1 into the liposomes showed that both BH4-Bcl-2 and BH4-Bcl-X_L inhibited VDAC1 activity, but BH4-Bcl-X_L seemed more potent than BH4-Bcl-2. In any case, a full side-by-side and quantitative comparison between BH4-Bcl-2 and BH4-Bcl-X_L is needed to unravel their differences in regulating VDAC1 activity and to characterize the importance of Asp11 in BH4-Bcl-X_L and Lys17 in BH4-Bcl-2 for these properties. Furthermore, it will be necessary to determine how the properties of isolated BH4 domains are reflected in the regulation of VDAC1 by full-length Bcl-2 and Bcl-X_L. Indeed, it seems likely that other protein domains of Bcl-2 and Bcl-X_L besides the BH4 domain are involved in the direct interaction with VDAC1, since Bcl-X_L lacking its BH4 domain still interacts with VDAC1 [126, 136]. In addition, the mechanism by which these BH4 domains target VDAC1 is poorly characterized and may involve a complex network of protein interactions.

Bax Inhibitor-1

Seminal work from Reed's laboratory elucidated BI-1 as a highly conserved ER-localized six/seven-transmembrane domain protein that protects cells against apoptosis and counteracts ER stress [137, 138]. Part of BI-1's anti-apoptotic properties have been attributed to its role in controlling ER Ca^{2+} homeostasis through its H^+/Ca^{2+}-antiporter activity [59, 60, 139]. BI-1 overexpression leads to enhanced ER Ca^{2+} leak and decreases the steady-state ER Ca^{2+} levels, while cells deficient for BI-1 display an increase in $[Ca^{2+}]_{ER}$. These BI-1 properties seemed to be highly dependent on its C-terminal domain [59, 140, 141]. These findings are compatible with the recently identified Ca^{2+}-channel pore in the membrane-embedded part of the C-terminal domain of BI-1 [61]. Furthermore, there is now mounting evidence that other BI-1-related proteins like human Golgi anti-apoptotic protein (hGAAP) and TMBIM6/GRINA also control ER Ca^{2+} homeostasis potentially by regulating IP_3Rs [142, 143]. While BI-1's name refers to its discovery as a high-copy suppressor of Bax-induced cell death in yeast, BI-1 is targeted and regulated by anti-apoptotic Bcl-2-family members. Bcl-2 seems to bind BI-1 through its BH4 domain [138]. Furthermore, the BH4 domain of Bcl-2 stimulates BI-1's H^+/Ca^{2+} anti-porter activity by promoting BI-1 oligomerization [139]. In fact, the regulation of the Ca^{2+}-flux properties of BI-1 by anti-apoptotic Bcl-2-family members may underlie the conflicting evidence on whether Bcl-2-family members can lower the ER Ca^{2+}-store content or not. Reed and coworkers showed that Bcl-X_L required the presence of BI-1 to lower $[Ca^{2+}]_{ER}$, since overexpression of Bcl-X_L in BI-1-deficient cells failed to decrease the ER Ca^{2+}-store content, indicating a critical role for BI-1 as downstream targets of Bcl-2 proteins in lowering $[Ca^{2+}]_{ER}$ [60]. In these studies, both Bcl-2 and Bcl-X_L seemed to similarly affect the Ca^{2+}-leak properties of BI-1. While it seems likely that these effects are mediated through their BH4 domains, it is not known whether BH4-Bcl-2 and BH4-Bcl-X_L are equally potent in controlling BI-1 properties.

ASPP2

ASPP2 provokes mitochondrial-dependent cell death by activating tumor suppressors like p53 and by counteracting pro-survival mechanisms like NF-κB and Bcl-2 [144, 145]. Two variants of the pro-apoptotic protein ASPP2 have been discovered: one variant binds to the tumor suppressor p53 and stimulates its pro-apoptotic activity by enhancing the expression of pro-apoptotic proteins at the transcriptional level; the other variant binds to and counteracts the anti-apoptotic Bcl-2 proteins, leading to apoptosis by promoting the release of pro-apoptotic proteins, like BH3-only proteins, from Bcl-2 [146, 147]. Structural studies elucidated four ankyrin repeats and an SH3 domain in the C-terminal part of ASPP2, responsible for interaction with other proteins, including p53, NF-κB, and Bcl-2 [146, 148–151]. An elegant study combining molecular modeling with biophysical analysis revealed the molecular properties of the interaction of C-terminal domain of ASPP2 with anti-apoptotic Bcl-2-family members [125]. Using a peptide array screening, both the BH4 domains as well as the hydrophobic cleft, involved in scaffolding pro-apoptotic BH3 domains, were identified as ASPP2-binding sites. Using quantitative biophysical methods, it was shown that the binding affinity of ASPP2 to BH4-Bcl-2 was about tenfold higher than to BH4-Bcl-X_L or to the Bcl-2-hydrophobic cleft. This indicates a dual selectivity in ASPP2-binding properties of anti-apoptotic Bcl-2-family members. Strikingly, a critical role in the high-affinity binding of BH4-Bcl-2 to ASPP2 was attributed to the surface-exposed Lys17. Lysine's additional positive charge seemed critical, since replacing Lys17 by an alanine or an aspartate (like in BH4-Bcl-X_L) caused a significant reduction in the binding

affinity to ASPP2 or completely abolished ASPP2 binding, respectively. Docking studies revealed that SH3 domain targeted the BH4 domain of Bcl-2/Bcl-X_L, while the ankyrin repeats targeted the hydrophobic cleft of Bcl-2/Bcl-X_L. Hence, ASPP2 may counteract the anti-apoptotic function of both Bcl-2 and Bcl-X_L but with different efficiency. In this way, ASPP2 may discriminate between Bcl-2 and Bcl-X_L targets. Therefore, pro-apoptotic targets of Bcl-2 and Bcl-X_L may be released in a selective manner or time frame upon ASPP2 binding to Bcl-2 and/or Bcl-X_L. In this respect, ASPP2 levels may control the properties of proteins that are targeted by both the BH4 domain and the hydrophobic cleft of Bcl-2 anti-apoptotic proteins.

Conclusions

An essential role of anti-apoptotic Bcl-2 family proteins is due to their regulation of intracellular Ca^{2+} dynamics. Here, we have discussed a selective function of Bcl-2 as endogenous IP_3R inhibitors versus Bcl-X_L as endogenous IP_3R sensitizers. We propose that distinct functional domains of Bcl-2 and Bcl-X_L underlie their divergence in IP_3R-functional regulation. In more detail, Bcl-2 acts on the IP_3Rs primarily via its BH4 domain on the receptor central, modulatory domain while Bcl-X_L via its hydrophobic BH3-domain-binding cleft and on the C-terminal channel-pore domain. We identified a conserved molecular determinant (Lys17) that is critical for the inhibitory action of the BH4 domain of Bcl-2 on IP_3Rs and that is evolutionary conserved among all Bcl-2 orthologues in the five classes of vertebrates. It is one of the most striking differences in surface-accessible residues between BH4-Bcl-2 and BH4-Bcl-X_L underlying the selective action of BH4-Bcl-2 on IP_3Rs. Furthermore, since the sequence of the BH4 domains of other Bcl-2 family members including Mcl-1 deviates a lot from Bcl-2, this suggests a unique role for the BH4 domain of Bcl-2 as an endogenous inhibitor of the IP_3R channel. However, this concept may be too simplistic, considering the recent data showing that the zebrafish's Bcl-2-related protein Nrz is still able to bind and control IP_3Rs activity via its BH4 domain. These data may suggest a broader role for the BH4 domain biology in Ca^{2+} signaling beyond apoptosis modulation, either by a distinct regulation of the Ca^{2+}-flux properties of IP_3R channels or by selective binding and regulation of Ca^{2+}-transport systems in both the ER and the mitochondria. In conclusion, future research is needed to fully characterize BH4-domain biology in the context of Bcl2's proteins physiological and pathophysiological activities, especially considering the growing list of its potential molecular targets besides the IP_3Rs.

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