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News and Commentary

Bcl-2-related survival proteins

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Bcl-2, the first regulator of core apoptotic pathways to be identified, was discovered as an oncogene in follicular lymphoma by Stan Korsmeyer, Carlo Croce and Mike Cleary. The subsequent discovery in the Korsmeyer lab of Bax, a Bcl-2 homologue with proapoptotic effects, as a binding partner of Bcl-2, led to the rheostat model of opposing Bcl-2 and Bax functions as a setpoint of apoptotic thresholds. The naming of Bax and the general concept of a network of dimer partners evolved in Stan's lab from the recently described Myc–Max transcription factor network:

Zoltan Oltvai: On a Saturday afternoon in the spring of 1993 around 4–5 PM …I went to the lab to develop my first film on the native Bcl-2 versus HA-tagged Bax two way co-IP that I put on film the previous day (Figure 9A in the paper). No one was in the lab other than Stan, sitting in his office and working on some paper. I developed the film, got very excited about it, and decided to show him immediately. We sat down and started to talk about what this means (esp. lane 4 vs. 5 in Fig. 9a). With the initial viability data on the bax overexpressing clones in hand already (Fig. 8) we cooked up this 'balance model' right then (that later became Fig. 10 in the paper): I remember giving him the film and telling him that this looks very similar to the Myc-max story. He leaned back in his chair, turned the film toward the light, and looked at it very carefully for a while. Then he got really excited, and started talking as he was thinking, saying something like: so you are saying that etc. then he asked some more questions etc, and by the end of it, about 5–10 min later, we had the rough model.

The now larger family of Bcl-2-related proteins has revealed functional specialization at the level of binding interactions between anti- and proapoptotic family members. How these proteins regulate apoptotic events and whether there are additional housekeeping functions of these proteins are still areas of active investigation. In this review, we will look at the cellular and molecular functions and structural features of the survival branch of the Bcl-2 protein family.

Cellular Functions of Bcl-2 Antiapoptotic Family Members

The mammalian BCL-2 antiapoptotic protein family includes Bcl-2, Bcl-x_L, Mcl-1, Bcl-w, A1/Bfl-1, NR-13 (human homolog nrh), Bcl-B and Bcl2-L-10. At least, eight viral homologs with survival activity have been identified, including all gamma herpesvirus species examined.[1] A critical step in most examples of apoptosis is the translocation of cytochrome *c* and other proteins normally found in the mitochondrial intermembrane space to the cytoplasm, where they function in caspase activation, inhibition of IAP family proteins, or nuclear DNA degradation.[2–5] The most consistent correlate of Bcl-2 survival function in intact cells is suppression of cytochrome *c* release.[6,7] Furthermore, Bcl-2 protects against apoptotic cell deaths that are dependent on cytochrome *c* release.[8]

David Hockenbery: 'Gabriel Nunez and I started working in Stan's lab at the same time, the summer of 1988. Gabriel decided to work on Bcl-2 as an oncogene, while I chose to work on the normal physiologic functions (these were very early days). Fairly early, we decided to generate an anti-Bcl-2 antibody, and went with hamster monoclonals on the advice of Bob Schreiber one floor down at Washington U. I had never tried this before, but Stan was encouraging and despite numerous mistakes, we came up with a good antibody (6C8) on the first try. When we observed that Bcl-2 had a punctate cytoplasmic distribution by confocal immunofluorescence microscopy, Stan got advice from two classically trained biochemists, Alan Schwartz and Stuart Kornfeld, and we ended up with a pretty rigorous subcellular fractionation to demonstrate a mitochondrial localization. This was a rather controversial result at the time, as it was difficult to imagine how mitochondria could be involved in such a precisely regulated process as apoptosis.'

However, experimental induction of apoptosis by microinjection of cytochrome *c* is also inhibited by Bcl-2/Bcl-x_L,[9] suggesting a second site of action. Bcl-2 and Bcl-x_L can block mitochondrial targeting of cytosolic Bax,[10] as well as inhibiting apoptotic deaths apparently independent of cytochrome *c* release.[11] In this section, we will review the various mechanisms proposed for how Bcl-2 antiapoptotic family members regulate cytochrome *c* release as well as evidence for alternative survival functions.

Regulation of cytochrome *c* release

Several hypotheses have been presented for Bcl-2 suppression of cytochrome *c* release, classified as primary inhibition of specific membrane pores or oppositely, maintenance of physiological membrane permeability. The multidomain proapoptotic Bcl-2 family members Bax and Bak form pores in synthetic membranes and probably *in situ* at mitochondrial membranes.[12] Sizing of Bax pores in synthetic vesicles using dextran indicates pore diameters of 22 Å, sufficient to pass cytochrome *c*.[13] Bcl-2 could inhibit this mechanism directly, by binding to multidomain proteins or sequestering BH3-only factors. This mechanism is an updated Korsmeyer rheostat model of cellular apoptotic threshold as a ratio of

antiapoptotic (Bcl-2) to proapoptotic (Bax) expression/function (Figure 1a1).[14]

Alternatively, cytochrome *c* release has been attributed to an altered conformation of the voltage-dependent anion channel (VDAC), possibility in collaboration with the inner membrane adenine nucleotide transporter (ANT), as part of the permeability transition pore (PTP).[15] PTPs are assembled in response to mitochondrial calcium overload and modulated by reactive oxygen species, thiol oxidants and lysophospholipids, factors associated with apoptosis.[16] Transient depolarization of mitochondria, possibly more relevant to early events in apoptosis, has also been attributed to PTPs.[17] Physical interactions of Bcl-2 and VDAC and ANT can be demonstrated[18] and pores associated with reconstituted VDAC or ANT in lipid vesicles are inhibited by Bcl-2 (Figure 1a2).[19,20] However, against this mechanism of cytochrome *c* release are the results of cyclophilin D knockouts demonstrating preservation of apoptotic responses including cytochrome *c* release despite loss of Ca^{2+}-induced PTP.[21–23]

A third model of cytochrome *c* release is based on osmotic swelling with subsequent stretching and rupture of the OMM. Withdrawal of the growth factor IL-3 from FL5.12 cells leads to accumulation of mitochondrial phosphocreatine in the intermembrane space, suggesting that phosphocreatine transport across the OMM is impaired.[24] Bcl-2 and Bcl-x_L prevent this accumulation, whereas Bcl-x_L maintains the open state of the voltage-dependent anion channel *in situ* (Figure 1a2)[25] and supports adenine nucleotide exchange across the outer mitochondrial membrane. Failure of outer membrane transporters may lead to an osmotic gradient across the mitochondrial membrane, favoring shift of free water and rupture of the limiting outer mitochondrial membrane.

Alternative survival functions

Bcl-2 and/or Bcl-x_L have been reported to inhibit mitochondrial oxidative stress, reduce the density of contact sites between outer and inner membranes, and inhibit glucose oxidation and mitochondrial responses to respiratory substrates.[26–28] These observations suggest that Bcl-2 could have a 'housekeeping' function as a governor or safety valve for mitochondrial electron transport (Figure 1a3). However, the role of mitochondrial oxidative phosphorylation, if any, in apoptosis is poorly understood. Apoptosis proceeds normally in many cases without significant change in ATP levels or mitochondrial morphology.[29,30] Bax-induced cell death in yeast, which

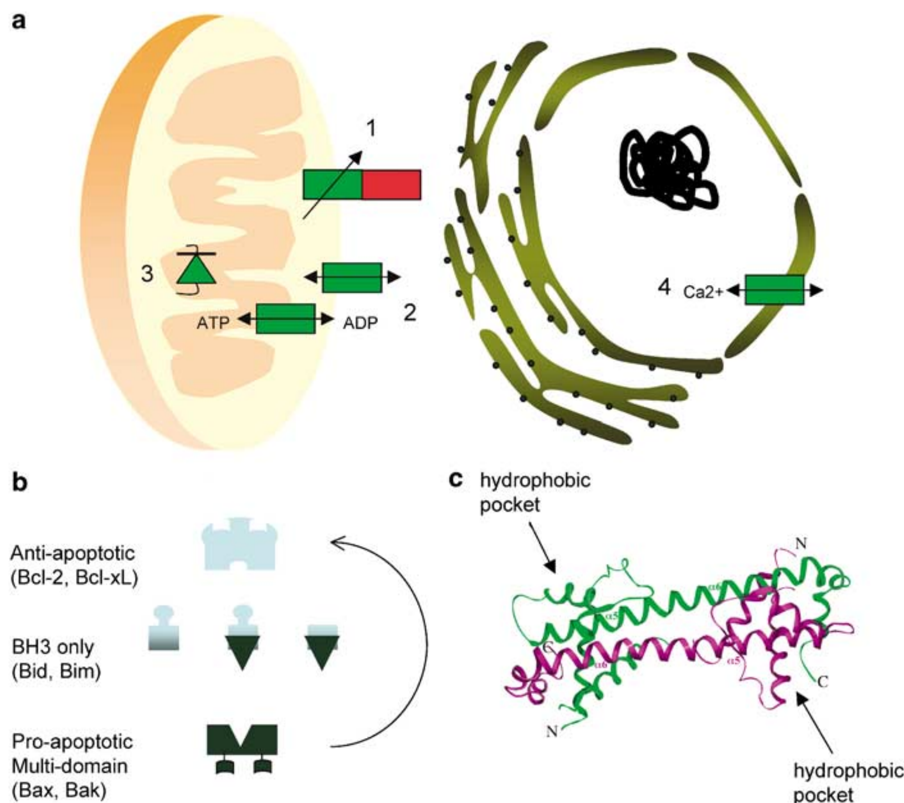

Figure 1 (**a**) Bcl-2 survival functions: proposed models of Bcl-2 survival functions at mitochondria and ER membranes, represented as electrical circuit symbols. (1) Rheostat: interactions with proapoptotic Bcl-2 family members regulate apoptotic threshold. (2) Relay (mitochondrial): Bcl-2 regulates membrane permeability maintaining cytoplasmic–matrix exchange of adenine nucleotides. (3) Relay (ER): Bcl-2 regulates Ca^{2+} release by IP3 receptors. (4) Protection diode: Bcl-2 dampens oxidative stress, perhaps by unloading electron transport chain. (**b**) Bcl-2 family interactions: antiapoptotic proteins engage BH3-only proteins by occupancy of hydrophobic groove, preventing interaction of BH3-only proteins and multidomain proapoptotic effectors. Antiapoptotic proteins may also directly inhibit multidomain Bax and Bak proteins. (**c**) Bcl-x_L homodimer structure: ribbon representation of three-dimensional domain swapped Bcl-x_L homodimer featuring extension of hinge/turn region between α-5 and α-6 helix. Protein half-dimers are represented in different colors

is biochemically similar to mammalian apoptosis, is suppressed in petite strains or in the presence of oligomycin, an inhibitor of the F1F0 ATPase.[31,32]

Endoplasmic reticulum (ER) localization of Bcl-2 and Bcl-x_L has also been plumbed for essential functions related to inhibition. Ca^{2+} homeostasis is altered in cells overexpressing Bcl-2, with evidence both for reduction of stimulated efflux of ER Ca^{2+} as well as continuous leak of ER Ca^{2+} (Figure 1a4).[33,34] Enforced targeting of Bcl-2 to ER membranes inhibits apoptosis in several instances, in some cases better than mitochondrial targeting.[35] As ER-targeted Bcl-2 inhibits mitochondrial cytochrome *c* release, crosstalk between these organelle compartments, perhaps involving Ca^{2+}, is likely involved.[36]

Bcl-2 is also connected to nonapoptotic cell death mechanisms, including necrosis and autophagic death. Autophagy is a regulated process of recycling macromolecules and organelles to assimilate carbon and nitrogen breakdown products for energy production and anabolic cell growth.[37] Although autophagy is a survival response to nutrient deprivation, under certain circumstances, autophagy leads to cell death.[38] Beclin, a Bcl-2-interacting protein, is an essential component in autophagic pathways, and overexpression of Bcl-2 inhibits autophagy.[39,40]

Expression of Bcl-2-related proteins in heterologous systems such as yeast has been undertaken to probe the intrinsic functions of these proteins in a model devoid of endogenous Bcl-2 homologues. Bcl-2 inhibits death triggered by Bax or Bak expression, as well as those associated with prolonged stationary phase, oxidative and NaCl stress and superoxide dismutase-deficient strains.[41–44] Although the relevance of these models to mammalian apoptosis is not proven, they suggest that Bcl-2 has intrinsic functions independent of its mammalian proapoptotic binding partners.

Structure–function Relationships of Bcl-2 Antiapoptotic Family Members

Clues to understanding the detailed function/s of the antiapoptotic Bcl-2 family members may come from understanding their structure. Solution of the tertiary structure of Bcl-x_L by the Fesik group in 1996 brought to light the unanticipated structural homology to bacterial pore-forming proteins, including the membrane translocation domain of diphtheria toxin and bacterial colicins.[45] X-ray crystallographic and NMR structures of Bcl-2, Bcl-w, Bax, Bid and Mcl-1 have shown remarkable conservation of the basic structural features.[46–52] Bcl-2-related proteins have all α-helical folds, with two antiparallel, hydrophobic helices sandwiched by 2–3 amphipathic helices on each side. An unstructured loop of $\sim$60 amino acids bridges the first two helices on one side of the Bcl-2 and Bcl-x_L structure. A narrow hydrophobic cleft is formed on the opposite side between helices 3 and 4, with the floor formed by helices 5 and 6. The C-terminal α-helix in Bcl-w is folded into the hydrophobic groove, whereas available structures for Bcl-2, Bcl-x_L and Mcl-1 were prepared with this domain deleted.[47,48]

Bcl-2-related survival proteins contain four homology domains 1–4 (BH1–4), unlike proapoptotic family members, with the exceptions of Mcl-1 and NR-13, which appear to lack the N-terminal BH4 domain. These relatively short α-helical domains are probably not conserved structural elements, as proapoptotic proteins containing only BH3 domains form highly similar tertiary folds. The most likely selective pressure for conservation of these domains involves binding interactions with proapoptotic proteins, as highly conserved residues in Bcl-2 and Bcl-x_L are found in the hydrophobic groove and mutations in several of these residues diminish binding affinity to proapoptotic partners.[53] Not all of the conserved domains can be explained in this manner; however, implying other molecular functions/interactions are likely important.[54,55]

The observation of a solvent-accessible hydrophobic groove in the tertiary structure of Bcl-x_L fit nicely with the contemporaneous discovery of a conserved domain, BH3, necessary for both the death function and interactions of proapoptotic Bcl-2 family proteins with Bcl-2-related survival proteins.[45,56] The BH3 domain is an amphipathic α-helical segment found within both pro and antiapoptotic family members. Isolated BH3 peptides bind to the hydrophobic groove with the hydrophobic helical surface facing inward, with affinities ranging from nanomolar to micromolar.[57] Part of the variation in affinity correlates with the helical-forming propensity of the peptide in solution, and strategies to stabilize the helical conformation have strong effects on binding affinity.[58,59] Another significant contribution derives from specific hydrophobic interactions between peptide residues and side chains lining the hydrophobic groove. Recent studies by several groups have demonstrated that these interactions form the basis for selective interactions of anti- and proapoptotic family members (Figure 1b).[60–62] In particular, Mcl-1 has low affinity for BH3 peptides derived from Bad, Bid and Bik with high affinity for Noxa, Puma and Bim peptides.

The C-terminal helix is predicted to be membrane-spanning and studies have shown that this segment is both sufficient and necessary for mitochondrial targeting.[63] Bcl-x_L and Bcl-w can be found as soluble cytoplasmic proteins or loosely attached to the outer mitochondrial membrane.[64,65] Apoptotic stimuli, as observed for proapoptotic Bcl-2 family members, trigger mitochondrial translocation and membrane insertion of Bcl-x_L and Bcl-2 antiapoptotic proteins. One set of inducers for relocalization of Bcl-2 proteins are proapoptotic-binding partners.[65,66] The interaction of a proapoptotic BH3 domain with the hydrophobic groove appears to produce a conformational change that promotes membrane binding and insertion. However, opposite results have been described, with BH3 peptides-inhibiting membrane insertion of Bcl-2 and Bcl-x_L, suggesting that binding is not sufficient for insertion but perhaps also requires an additional step of partial protein unfolding.[67]

Both antiapoptotic and proapoptotic Bcl-2 family members exhibit pore-forming activity in synthetic lipid membranes.[68] Electrophysiologic patch-clamping studies indicate both Bcl-2 and Bcl-x_L form high conductance channels with multiple subconductances and mild cation selectivity. Recent *in situ* patch-clamping studies with giant squid axons have demonstrated mitochondrial multiconductance channels following microinjection of Bcl-x_L protein.[69] The relevance of Bcl-2 pore activity to apoptosis has been difficult to assess, as mutations

or small deletions affecting pore activity have not been identified to date.

Homodimers of Bcl-2 and Bcl-x_L have been reported in both soluble cytoplasmic and membrane-associated compartments, and higher order oligomers are also described.[66,70,71] Bax pore activity appears to require dimeric and tetrameric associations, and similar considerations probably apply to antiapoptotic proteins. An X-ray crystallographic structure of Bcl-x_L homodimers was recently published, with an unexpected three-dimensional domain-swapping mechanism of dimerization.[72] For each monomer subunit, the antiparallel arrangement of the central α-5 and 6 helices is reoriented, with the turn as a hinge point for establishment of a continuous helical segment spanning α-5 and -6 (Figure 1c). Protein segments on either side of the hinge region participate in separate 'monomeric' folds, creating an extensive intermolecular interface. Although there is some evidence that BH3 peptides are capable of dissociating Bcl-2/Bcl-x_L homodimers, the hydrophobic groove-binding sites for BH3 peptides are not involved in the three-dimensional dimer interface, and instead are oriented in opposite directions for each half-dimer.[73]

The membrane topology of Bcl-2-related proteins has been studied using a variety of biophysical techniques, including nuclear magnetic resonance, circular dichroism spectroscopy and site-directed spin-labeling electron paramagnetic resonance spectroscopy. In contrast to the initial conception of α-5 and -6 helices spanning the membrane in a perpendicular orientation, several groups have demonstrated a parallel, interfacial orientation.[74] Several possibilities have been put forward as mechanisms for multimerization of these proteins, including alternating interfaces or a daisy-chain domain-swapping mechanism.[71,72]

Molecular Biology of Specific Antiapoptotic Proteins

Gene products

The Bcl-2 gene is juxtaposed with the immunoglobulin heavy-chain locus in t(14,18) follicular lymphomas, leading to its initial discovery. The proximity to the immunoglobulin heavy-chain enhancer deregulates Bcl-2 transcription, an oncogenic event. Bcl-2 has one alternate transcript, Bcl-2 beta, encoding an alternate C-terminal end.

The Bcl-x gene is alternatively spliced into several transcripts, the antiapoptotic Bcl-x_L and Bcl-x_γ and a short proapoptotic version, Bcl-x_S. *Cis*-acting elements controlling alternate splicing have been mapped and several upstream factors promoting Bcl-x_S expression are known.[75] Like Bcl-x_L, Mcl-1 also has a proapoptotic splicing variant, Mcl-1_S. Bcl-w was identified only 5 years ago. High levels of expression are found in the colon, brain and testes, with the latter accounting for a sterility phenotype in knockout mice.[76]

Tissue-specific expression patterns

The expression pattern of the antiapoptotic family members in tissues varies greatly. Bcl-2 is expressed in multiple tissues during embryonic development, including notably proliferating and postmitotic neuronal populations in the brain, double-negative $CD4^-CD8^-$ and single-positive $CD4^+$ and $CD8^+$ thymocytes, pro- and mature B lymphocytes and plasma cells, and sites of mesenchymal–epithelial induction of the developing nephron.[77] Adult expression is more limited, including postgerminal center B lymphocytes, stem cell zones of multiple epithelia and the peripheral nervous system. Homozygous deletion of Bcl-2 leads to several notable phenotypes: premature graying, ablation of lymphoid populations and defective nephrogenesis leading to polycystic disease.[78]

Deb Novack: 'We were very surprised by the graying, and both Stan and I ran over to the mouse room to see for ourselves. As for getting the knockout method going, Stan invited Andy McMahon to visit as we were getting started, and he sent John Shutter and I to Monsanto to see a microinjection setup, but otherwise he was remarkably hands off. We had fun looking at the first ES culture, which differentiated into beating cardiac cells (oops), but quickly mastered the culture part. I did over 50 electroporations and screened >1000 clones with my first vector, constructed from the BALB/c DNA he brought from NIH. When we started we didn't know DNA needed to be from the same strain as the ES cells. Then I made a new targeting vector, using a new library I made from ES DNA. That worked right off. I cringe to think how much LIF I used that year, but Stan never did. He just told me to keep on trying what I thought I needed to do.

Bcl-x_L is also expressed in multiple sites during embryonic development, including early hematopoietic cells in fetal liver and cortical zones, olfactory bulb and Purkinje cells in the brain.[79] However, the expression pattern in thymus is opposite to that of Bcl-2, with expression restricted to $CD4^+CD8^+$ T cells. Adult expression in the central nervous system persists longer than observed for Bcl-2. Homozygous deletion of Bcl-x_L results in embryonic lethality, with excessive apoptosis in the fetal hematopoietic system and developing brain.[80]

Mcl-1 was originally identified owing to its expression in differentiating myeloid cells. Mcl-1 is also highly expressed in hematopoietic stem cells, under the control of stem cell factor.[81] Mcl-$1^{-/-}$ embryos fail to implant or mature beyond early blastocyst stages.[82] Unlike Bcl-2 and Bcl-x_L, expression of Mcl-1 is restricted to apical differentiated cells in complex epithelia (prostate, breast, colon, lung).[83] Both mature lymphocytes and lymphocyte progenitors upregulate Mcl-1 in response to IL-7 signaling, and Mcl-1 is required for IL-7-mediated survival.[84] Within lymphoid follicles, Mcl-1 is highly expressed in germinal centers, opposite to Bcl-2.

Regulation

Bcl-2 transcription is regulated by both positive and negative factors. Several transactivating factors are shared with other prosurvival family members: CREB, STAT-3, NF-κB and Brn-3a, whereas others have only been described for Bcl-2: Myb, Cdx1 and Cdx2, gli-1, PAX8, Oct-2, Ces-2/E2A-HLF, ER, E2F-1, Fli-1, SRF, PPAR-γ, NFAT, C/EBPα and Aiolos.[85] Repressive factors include p53, WT1, Par-4, Egr-1, STAT-1, GADD153 and ICSBP. Post-transcriptional regulation through RNA stability has been reported through AU-rich elements, with rapid degradation following some apoptotic stimuli.

Bcl-2 is a target of phosphorylation with both positive and negative effects on function reported.[86] All of the identified phosphorylation sites are located in the loop domain. Phosphorylation at serine 70 has been ascribed to protein kinase C following growth factor stimulation. Multiple sites (Thr56, Thr74, Ser87 and Ser70) are phosphorylated by cJun NH_2-terminal kinase (JNK) in the G2/M phase of the cell cycle in cells treated with microtubule inhibitors; other putative kinases including cdc2 have been reported. Bcl-2 also undergoes cell cycle-dependent phosphorylation in G2. Phosphorylation has been linked to proteasomal degradation and ER localization.

Transactivating factors for Bcl-x_L include several STATs (STAT-5, 6 and 3), Ets2, PU.1, GATA-1, NF-κB, Brn-3a, PAX3 and CREB, with repression observed with STAT-1, TEL and p53.[87] Like Bcl-2, JNK-dependent phosphorylation has been reported for Bcl-x_L.

A unique post-translational modification of asparagine deamidation occurs in Bcl-x_L.[88] Two asparagines in the loop domain, Asn52 and Asn66, are converted to aspartates or isoaspartates in a cell-type-specific manner following treatment with DNA-damaging agents. Deamidation is associated with loss of Bcl-x_L activity.

Multiple growth factors and cytokines induce Mcl-1 transcription. STAT-3, CREB and PU.1 are known transcriptional activators, whereas E2F-1 is reported to be a repressor. The best-known aspect of Mcl-1 regulation is proteolytic turnover, associated with ubiquitin-dependent proteasomal turnover, possibly related to PEST sequences in Mcl-1.[89] This feature places Mcl-1 at the top of a hierarchy of antiapoptotic proteins, with rapid loss of Mcl-1 expression following multiple apoptotic stimuli affecting protein synthesis.[90] Mcl-1 protein turnover follows Ser159 phosphorylation by glycogen synthase kinase-3 (GSK-3), negatively regulated by growth factors via Akt-dependent inhibition of GSK-3.[91]

Finally, Bcl-2, Bcl-x_L and Mcl-1, are substrates for caspase-mediated cleavage.[92] The COOH-terminal fragment has proapoptotic activity.

Conclusions

Twenty years have elapsed since the initial discovery of Bcl-2. Genetic analyses have demonstrated that antiapoptotic function of Bcl-2 is linked to neutralization of the related proapoptotic factors, confirming the original rheostat model of opposing functions for Bcl-2 and Bax. Recent efforts have embellished this concept, with selective interactions of BH3-only and survival proteins providing insights into the workings of the large Bcl-2 family at the cellular and organism level. Whether we have a complete picture of Bcl-2 functions is unknown at present, with the relationships of Bcl-2 proteins to membrane permeability, mitochondrial oxidative phosphorylation and ER Ca^{2+} dynamics still to be worked out.

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