Journal of Bioenergetics and Biomembranes, Vol. 37, No. 3, June 2005 (© 2005)
DOI: 10.1007/s10863-005-6222-3

Mitochondrial Carrier Homolog 2: A Clue to Cracking the BCL-2 Family Riddle?

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BCL-2 family members are pivotal regulators of the apoptotic process. Mitochondria are a major site-of-action for these proteins. Several prominent alterations occur to mitochondria during apoptosis that seem to be part of the "mitochondrial apoptotic program." The BCL-2 family members are believed to be the major regulators of this program, however their exact mechanism of action still remains a mystery. BID, a pro-apoptotic BCL-2 family member plays an essential role in initiating this program. Recently, we have revealed that in apoptotic cells the activated/truncated form of BID, tBID, interacts with a novel, uncharacterized protein named mitochondrial carrier homolog 2 (Mtch2). Mtch2 is a conserved protein that is similar to members of the mitochondrial carrier protein (MCP) family. This review summarizes the current knowledge regarding BCL-2 family members and the mitochondrial apoptotic program and examines the possible involvement of Mtch2 in this program.

KEY WORDS: BID; mitochondrial carrier homolog 2; BH3-only proteins; BCL-2 family members; mitochondrial carrier proteins; mitochondria; apoptosis.

APOPTOSIS: AN OVERVIEW

Programmed cell death, or apoptosis, is critical for both the development and maintenance of tissues. Caspases, a family of cysteine proteases, are the major executioners of the apoptotic process (Shi, 2002), whereas the BCL-2 protein family members are the major regulators of this process (Danial and Korsmeyer, 2004). The mechanisms by which the BCL-2 proteins regulate cell death are unknown, although it is believed that their function depends mostly on their ability to modulate the release of proteins from the inter-membrane space (IMS) of the mitochondria.

Two major apoptotic pathways, intrinsic and extrinsic, have been identified. The cell-intrinsic apoptotic pathway involves activation of pro-apoptotic BCL-2 family members, which induce the permeabilization of the outer mitochondrial membrane (OMM), resulting in the release of cytochrome-*c* (Cyt-*c*) and other IMS proteins (Wang, 2001). In the extrinsic pathway, apoptosis is initiated through activation of members of the tumor necrosis factor/Fas (TNF/Fas) receptor family (Varfolomeev and Ashkenazi, 2004). Once engaged by ligand, these receptors initiate the formation of the death-inducing signaling complex (DISC), which leads to activation of caspase-8. Activated caspase-8 can initiate both a cascade of caspases, and the cleavage of the pro-apoptotic BCL-2 family member BID. Cleavage of cytosolic BID at Asp59 yields a p15 C-terminal truncated fragment (tBID) that translocates to the mitochondria (Gross *et al*., 1999; Li *et al*., 1998; Luo *et al*., 1998). Targeting of tBID to mitochondria induces the activation of two other pro-apoptotic BCL-2 family members BAX and BAK, resulting in the release of Cyt-*c* (Eskes *et al*., 2000; Wei *et al*., 2000). That BID is an essential component of the extrinsic death pathway was demonstrated in Bid-deficient mice, which were resistant to Fas and TNFα-induced hepatocellular apoptosis (Yin *et al*., 1999; Zhao *et al*., 2001).

THE BCL-2 FAMILY

The founder of this family, the BCL-2 proto-oncogene, was discovered at the chromosomal breakpoint of t(14;18) bearing human B-cell lymphomas. The BCL-2 family of proteins has markedly expanded and includes

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both pro-apoptotic (e.g., BAX) and anti-apoptotic (e.g., BCL-2) proteins (Danial and Korsmeyer, 2004). A major characteristic of these proteins is their frequent ability to form homo- as well as heterodimers suggesting neutralizing competition between these proteins. A further characteristic of functional significance is their ability to associate with membranes.

The BCL-2 family members possess up to four conserved BCL-2 homology (BH) domains designated BH1, BH2, BH3 and BH4, which correspond to α helical segments (Cory and Adams, 2002). Most family members display sequence conservation in the first three domains (multidomain molecules). Many BCL-2 family members also carry a C-terminal hydrophobic domain, which is essential for their targeting to membranes such as the outer mitochondrial membrane (OMM). Deletion and mutagenesis studies argue that the amphipathic α helical BH3 domain serves as a critical death domain in the pro-apoptotic members. This concept is supported by an emerging subset of "BH3-only" pro-apoptotic members (e.g., BID) who display sequence homology only within the BH3 domain (Huang and Strasser, 2000).

Interestingly, the three-dimensional structures of two pro-apoptotic molecules BID and BAX demonstrate a very similar overall α helical content to the structures of the anti-apoptotic molecules BCL-X_L and BCL-2 (Petros *et al*., 2004). However, one should keep in mind that these structures were solved in solution and it is quite possible that in membranes these molecules would adopt different conformations that may explain their opposite cellular functions.

ARE BCL-2 PROTEINS FORMING CHANNELS IN THE OMM?

How are pro-apoptotics inducing apoptosis and anti-apoptotics inhibiting this process at the level of the mitochondrial membrane? An attractive model is that each would form channels in the OMM.

This model has originated from the structural similarity between the BCL-X_L molecule (mainly the two hydrophobic α5 and α6 helices) and the pore forming region of bacterial toxins (Muchmore *et al*., 1996). It was previously demonstrated that recombinant BCL-X_L, BCL-2, BAX or BID form ion-channels in artificial membranes with distinct characteristics (Schendel *et al*., 1998). Perhaps, the tendency of BCL-2 family members to form dimers/multimers in membranes may relate to their ability to form channels.

Recently Kim *et al*. used chemical labeling to elegantly demonstrate that α_5 and α_6 helices of BCL-2 move from the cytoplasm into the lipid bilayer of both the endoplasmic reticulum and mitochondria during apoptosis (Kim *et al*., 2004). Thus, an apoptotic signal might induce BCL-2 to become an "active channel," which protects cells. Surprisingly, a pro-apoptotic peptide triggered a similar conformational change in BCL-2, suggesting that the BCL-2 channel could also act to propagate apoptosis.

BAX AND BAK: KEYS TO THE "APOPTOTIC LOCK" OF MITOCHONDRIA

BAX and BAK are the keys to the "apoptotic lock" of mitochondria, since Bax, Bak double knockout mouse embryonic fibroblasts are resistant to multiple apoptotic stimuli, as well as to tBID and several other BH3-only molecules (Wei *et al*., 2001; Zong *et al*., 2001). Activation of BAX and BAK involves their oligomerization and integration into the cell membrane (Desagher *et al*., 1999; Goping *et al*., 1998; Griffiths *et al*., 1999; Gross *et al*., 1998). *In vitro*, recombinant BAX forms channels in artificial membranes, enabling the passage of large macromolecules through the membrane (Kuwana *et al*., 2002; Saito *et al*., 2000; Schendel *et al*., 1998). In addition, it has been hypothesized that BAX forms lipidic pores, based on its ability to produce a "growing" pore in pure lipid bilayers, which results in membrane breakage (Basanez *et al*., 2002). Thus, the tBID-induced formation of BAX/BAK oligomers, which form non-selective channels/lipidic pores serves as an attractive model for the release of IMS proteins such as Cyt-*c*.

Alternatively, BAX and BAK could regulate the activity of pre-existing channels, rather than form channels themselves. In this regard, it has been proposed that altered conductance of existing channels would eventually lead to mitochondrial swelling, and the non-specific rupture of the OMM (Bernardi *et al*., 2001; Vander Heiden and Thompson, 1999). Recently, Cheng *et al*. demonstrated that the VDAC2 channel (a large channel in the OMM) cross-links to monomeric BAK in non-apoptotic cells. Moreover, VDAC2-deficient cells showed enhanced BAK oligomerization and were more susceptible to apoptotic signals. Based on these findings the authors proposed that VDAC2 acts as an anti-apoptotic molecule by keeping BAK in a monomeric, inactive state. It will be interesting to see whether the interaction between BAK and VDAC also serves to regulate the channel activity of VDAC in non-apoptotic cells and in cells triggered to die. In this respect, it was previously proposed that VDAC channel activity is regulated by anti-apoptotic BCL-2 family members (Shimizu *et al*., 2000; Vander Heiden *et al*., 2001).

Mitochondrial apoptosis-induced channel (MAC) is a putative Cyt-*c* releasing channel that is detected in mitochondrial lysates of apoptotic cells reconstituted into proteoliposomes (Pavlov *et al*., 2001). Recently, it was demonstrated that MAC activity is preferentially associated with the oligomeric, active form of BAX, and that MAC activity was absent in Bax, Bak double knockout mouse embryonic fibroblasts (Dejean *et al*., 2005). Thus, BAX and/or BAK are actively involved in MAC activity. In the future it will be important to demonstrate that MAC activity appears in intact apoptotic cells, and to determine whether BAX and/or BAK are forming the channel themselves or are cooperating with other proteins to form this channel.

BH3-ONLY PROTEINS

The BH3-only proteins are sentinels of intracellular damage (Puthalakath and Strasser, 2002). These proteins are held in check by diverse mechanisms, seemingly at cellular locations at which they can sense specific damage. Following a death signal, these proteins undergo post-translational modifications that enable them to target the OMM.

In the presence of a survival factor, the BH3-only molecule BAD is phosphorylated and sequestered by 14–3-3 scaffold proteins, whereas dephosphorylation enables it to interact with and inhibit the activity of BCL-X_L/BCL-2 at the mitochondria (Zha *et al*., 1996). Recently it has been elegantly demonstrated that in some cell types, BAD resides at the mitochondria in a complex with protein kinase A (PKA), protein phosphatase 1 (PP1), and glucokinase (Danial *et al*., 2003). Most interestingly, Bad-deficient hepatocytes exhibit less mitochondrial-based glucokinase activity and less respiration in response to glucose and are resistant to apoptosis induced by glucose-deprivation. Thus, BAD seems to act as a regulator of glycolysis in non-apoptotic cells and in cells triggered to die. Since ATP is important for execution of both life and the early stages of apoptosis, it will be interesting to see how exactly BAD coordinates between these two opposite processes.

Another BH3-only protein, BIM, is sequestered in healthy cells to the microtubule-associated dynein motor complex through association with the LC8 dynein light chain (Puthalakath *et al*., 1999). Following a death signal, LC8 and BIM dissociate from the motor complex and together translocate to the mitochondria. At the mitochondria, BIM is felt to interact with BCL-2 to antagonize its anti-apoptotic activity. Phosphorylation also seems to be involved in regulating the activity of BIM, since it was recently demonstrated that when BIM is phosphorylated by ERK/MAPK, its pro-apoptotic activity is inhibited (Harada *et al*., 2004); whereas its phosphorylation by JNK causes it to be released from microtubules (Lei and Davis, 2003). Thus, it is possible that BIM acts as a sensor for damage to microtubules.

In the case of BID, the full-length protein is believed to be relatively inactive in the cytosol until proteolytically cleaved by caspase-8. Cleavage of cytosolic BID to tBID triggers its translocation to the mitochondria, where it induces the activation of BAX and BAK. As in the case of BAD and BIM, phosphorylation also seems to regulate BID activity, since it was previously demonstrated that the phosphorylation of BID by casein kinase 1 and/or 2 inhibits its cleavage by caspase-8 and caspase-3 (Degli Esposti *et al*., 2003; Desagher *et al*., 2001). Surprisingly, our recent studies have implicated full-length BID as an active player in the intrinsic pathway of apoptosis, since a caspase-8 non-cleavable BID mutant was as effective as wild-type BID in sensitizing Bid-deficient MEFs to DNA damage-induced apoptosis (Sarig *et al*., 2003). Future studies will determine the mechanism of action of full-length BID in the DNA damage pathway.

Recent studies with BH3 peptides have suggested that there are two different subgroups of BH3-only proteins. One group, represented by BID and BIM, were proposed to induce the mitochondrial apoptotic program via direct activation of BAX/BAK. The other group, including BAD and BIK, was proposed to induce this program indirectly by opposing anti-apoptotic members, such as BCL-2, BCL-X_L, and MCL-1. This led to the classification of BH3-only proteins as either death agonists (activators) or survival antagonists (derepressors) (Kuwana *et al*., 2005; Letai *et al*., 2002; Moreau *et al*., 2003).

BID'S MECHANISM OF ACTION

As mentioned above, it is an attractive model that tBID induces BAX/BAK oligomers, which form non-selective channels in the OMM for the release of IMS proteins such as Cyt-*c*. However, this simple model cannot account for the rapid kinetics and complete extent of Cyt-*c* release during apoptosis (Goldstein *et al*., 2000), since the majority of Cyt-*c* is buried in intramitochondrial cristae.

High-voltage electron microscopic (HVEM) tomography of mitochondria has revealed that the IMS is very narrow (Frey and Mannella, 2000) consistent with functional estimates that only 15–20% of total Cyt-*c* is available in the IMS (Bernardi and Azzone, 1981). Activation of the permeability transition pore (PTP),

a high-conductance IMM channel (Zoratti and Szabo, 1995), that ultimately leads to mitochondrial swelling with secondary rapture of the OMM can lead to the release of these compartmentalized stores of Cyt-*c*. However, this is not the case with tBID since it does not induce detectable swelling of mitochondria (Shimizu and Tsujimoto, 2000). Recently it was elegantly demonstrated that tBID causes the release of these compartmentalized stores of Cyt-*c* (∼85%) by inducing reorganization of the IMM (Scorrano *et al*., 2002). Interestingly, this reorganization did not require tBID's BH3 domain and was independent of BAX and BAK, but was dependent on the transient opening of the PTP, which does not lead to swelling of the mitochondria. Therefore, two distinct pathways are involved in tBID-induced Cyt-*c* release: one, which is BH3/BAX/BAK-depedent and mediates the release of Cyt-*c* across the OMM, and another, which is BH3/BAX/BAK-independent and responsible for the redistribution of Cyt-*c* stored in intramitochondrial cristae.

Recent work in mammalian cells shows that during apoptosis, mitochondria undergo fragmentation (fission) into more numerous and smaller units. This change in mitochondrial morphology occurs with many inducers of apoptosis and close in time to Cyt-*c* release. Interestingly, in apoptotic cells, BAX and BAK co-localize with Drp-1, a protein required for mitochondrial fission, at the sites of mitochondrial fission (Karbowski *et al*., 2002). In addition, a recent study shows that mitochondria also fragment during apoptosis in *C. elegans* and that Egl-1, a BH3-only protein in the worm, triggers this process via the worm Drp-1 (Jagasia *et al*., 2005). These results raise the possibility that tBID might initiate the mitochondrial apoptotic program by triggering the mitochondrial fission apparatus.

We have previously reported that in TNFα-activated haematopoietic FL5.12 cells, tBID becomes part of a single, ∼45 kD cross-linkable mitochondrial complex (Grinberg *et al*., 2002). Importantly, a tBID BH3 mutant (G94E), which does not interact with or induce the oligomerization of either BAX or BAK, formed the 45 kD complex. This mutant is the same mutant that was used by Scorrano *et al*. to demonstrate that tBID-induced reorganization of the IMM is BH3-independent (Scorrano *et al*., 2002). Thus, formation of the 45 kD complex may represent the BAX/BAK-independent mechanism/pathway for tBID-induced reorganization of mitochondria.

We originally proposed that the 45 kD complex represents a tBID homotrimer (Grinberg *et al*., 2002). However, recently we demonstrated that in fact the 45 kD complex represents an association between tBID and mitochondrial carrier homolog 2 (Mtch2), a novel and previously uncharacterized 33 kD protein (Grinberg *et al*., 2005; see below). Mtch2 is a conserved protein that is similar to members of the mitochondrial carrier protein family.

THE MITOCHONDRIAL CARRIER PROTEIN FAMILY

The mitochondrial carrier protein (MCP) family comprises a variety of proteins that catalyze the exchange of substrates across the IMM (Walker and Runswick, 1993). All MCP family members are relatively small proteins, with a molecular mass ranging from 28 to 34 kD in size. Comparison of the amino acid sequences of the different carriers has shown that they are made up of three tandem repeats, each about 100 amino acids in length and known as the mitochondrial carrier domain (MCD) (Fig. 1, *top*). Each repetitive element contains two hydrophobic stretches that are of sufficient length to span the membrane as α-helices, separated by an extensive hydrophilic region. Based on this information, it was proposed that the overall structure of this protein family consists of six transmembrane α-helices, in which both the N- and the C-termini of the proteins are facing the IMS, and the three long hydrophilic segments (connecting the two transmembrane regions of each domain) are facing the matrix (Palmieri, 1994). From the functional characterization of several different carriers that were reconstituted in liposomes, it would appear that the mitochondrial carriers are not only similar in structure but also in function, and are characterized by a common kinetic mechanism (Palmieri, 1994).

THE MITOCHONDRIAL CARRIER HOMOLOG (MTCH) FAMILY

Mitochondrial carrier homolog 2 is an uncharacterized protein first identified as a putative ORF obtained from CD34+ hematopoietic stem/progenitor cells (Zhang *et al*., 2000). It was named after the single conserved MCD it contains. Mtch2 is predicted to be composed of three transmembrane (TM) domains (TMI and TMII that form the MCD, and a third C-terminal TM) (Fig. 1, *bottom*). Our biochemical studies indicate that Mtch2 is an integral membrane protein exposed on the surface of mitochondria, and that all three of its TM domains are likely to span the OMM (Fig. 1; Grinberg *et al*., 2005).

Mtch2 has several close relatives, which together form a "sister family" separate from the other mitochondrial carrier proteins (Fig. 2). All Mtch family members contain a single MCD, three transmembrane

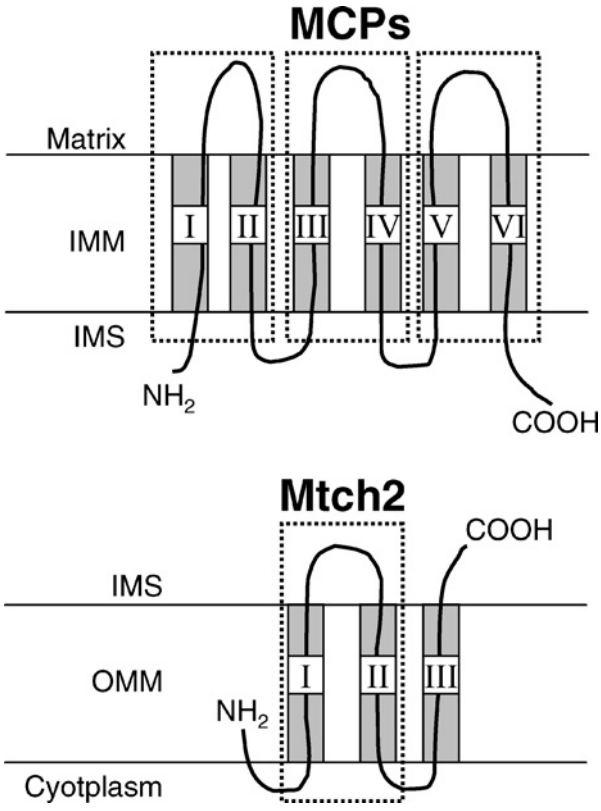

Fig. 1. Membrane topology of mitochondrial carrier proteins (MCP) and of mitochondrial carrier homolog 2 (Mtch2). *Top*: A six-helix model of MCPs. The dotted boxes represent the three tandem structural repeats [mitochondrial carrier domains (MCD)]. Each repeat is proposed to consist of pairs of transmembrane α-helices (I and II, III and IV, V and VI) linked by hydrophilic regions that face the matrix. The N- and C-terminus face the intermembrane space (IMS). *Bottom*: A three-helix model of Mtch2. The dotted box represents the single MCD that consists of transmembrane α-helices I and II, linked by a hydrophilic region that faces the IMS. The hydrophilic region that links transmembrane α-helices II and III, and part of the N-terminus are predicted to face the cytoplasm.

domains, and can be aligned across almost their entire length. These data together with the OMM location of Mtch2, strongly suggest that Mtch proteins are a distinct subgroup of proteins related to MCPs.

The function(s) of Mtch2 and its relatives are unknown. Mtch2 does not seem to be a pro-apoptotic protein, since we found that its overexpression in 293T or in HeLa cells had no effect on cell viability (unpublished data). On the other hand, it was previously reported that human presenilin-1-associated protein (PSAP, also known as human Mtch1, a protein with 48% identity to human Mtch2; see Fig. 2), localizes to mitochondria and induces Cyt-*c* release, caspase activation and apoptosis when overexpressed in 293T cells (Xu *et al.*,

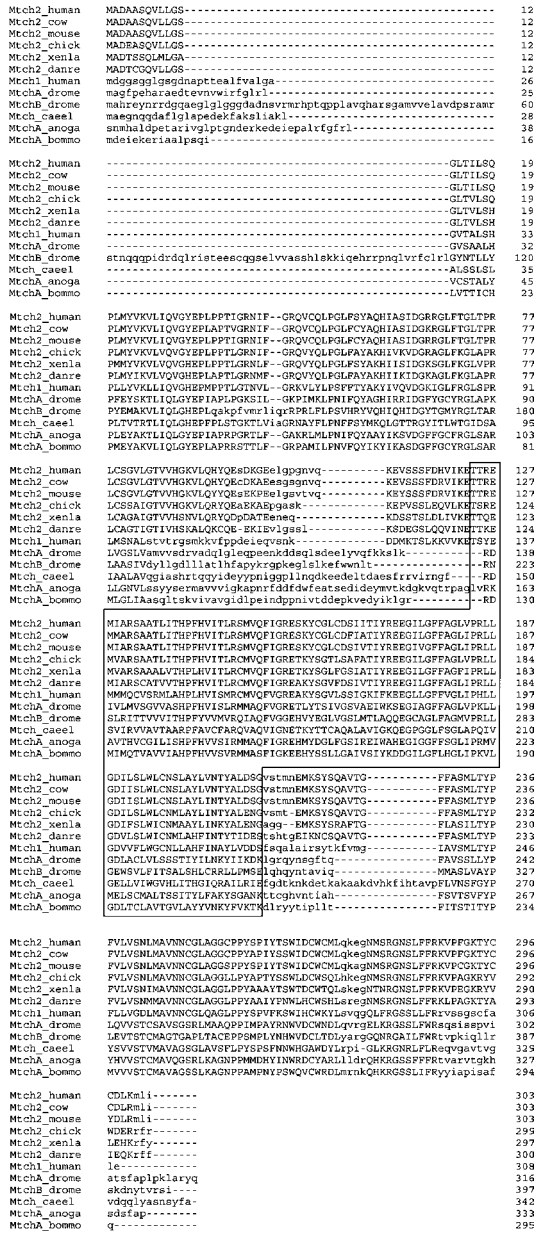

Fig. 2. Multiple sequence alignment of Mtch2 and closely related proteins. Only residues in uppercase letters are confidently aligned. The conserved MCD is marked by a box. NCBI gi codes of the sequences are: Mtch2 human 7657347, Mtch2 cow 7959093 (94% identity to human), Mtch2 mouse 5815347 (93% identity), Mtch2 chick (chicken) 6448447 (79% identity), Mtch2 xenla (xenopus) 16326341 (70% identity), Mtch2 danre (zebra fish) 18859045 (69% identity), Mtch1 human 6995989 (48% identity), MtchA drome (*Drosophila melanogaster* mitochondrial carrier homolog A) 5815351 (38% identity), MtchB drome (*Drosophila melanogaster* mitochondrial carrier homolog B) 21391910 (34% identity), Mtch caeel (*Caenorhabditis elegans*) 17533979 (28% identity), MtchA anoga (*Anopheles gambiae* str. PEST mitochondrial carrier homolog A) 21292540 (36% identity), MtchA bommo (*Bombyx mori* mitochondrial carrier homolog A) 6448449. (37% identity). The conserved MCD is marked by a box.

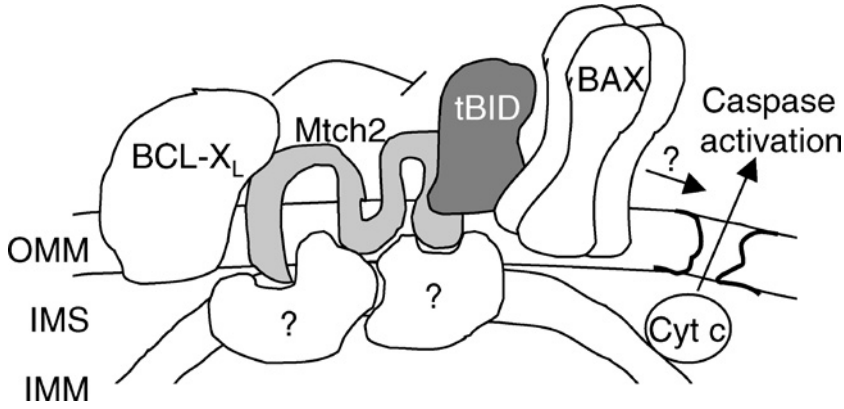

Fig. 3. Interplay between BCL-2 family members and Mtch2 at the mitochondria. Mtch2 resides in the OMM in a relatively large complex that might include IMM proteins. In response to TNFα, tBID and BAX are recruited to the Mtch2-resident complex. At the mitochondria, tBID induces the oligomerization of BAX, which results in the release of Cyt-*c* and caspase activation. In cells overexpressing BCL-X_L, the TNFα-induced recruitment of tBID and BAX to the Mtch2-resident complex is partially inhibited, along with the inhibition of Cyt-*c* release.

2002). Thus, Mtch1 is a pro-apoptotic protein acting at the mitochondria.

MTCH2 IS A TARGET FOR BCL-2 FAMILY MEMBERS IN CELLS SIGNALED TO DIE

To determine the native state of Mtch2 in mitochondria and to determine its relations with BCL-2 family members, we utilized blue-native polyacrylamide gel electrophoresis [BN-PAGE; (Schagger, 2001)]. This method has been proved to be a powerful means for purifying the respiratory chain complexes. Using this method we revealed that Mtch2 resides in an approximately 185 kD resident mitochondrial complex, and activation with TNFα leads to the recruitment of tBID to this complex (Grinberg *et al*., 2005). Moreover, activation by TNFα also recruits BAX to the Mtch2-resident complex. The recruitment of tBID and BAX to the Mtch2-resident complex seems to be important for triggering the mitochondrial apoptotic program since overexpression of BCL-X_L, which inhibits TNFα-induced Cyt-*c* release, inhibits the recruitment of both BAX and tBID to this complex (Grinberg *et al*., 2005). These results (summarized in Fig. 3) raise the possibility that the Mtch2-resident complex is an important player in the mitochondrial apoptotic program.

HOW MIGHT MTCH2 REGULATE THE MITOCHONDRIAL APOPTOTIC PROGRAM?

What might be the function(s) of Mtch2 and its relatives? As described above, the MCP family comprises proteins that reside in the mitochondria and catalyze the transport of metabolites across the IMM (Palmieri, 1994). Thus, one hypothesis would be that Mtch proteins are carrier proteins directly involved in the transport of metabolites. On the other hand, since these proteins contain only one and not three MCDs, it is possible that they are involved in transferring signals (via protein–protein interactions) rather than metabolites (via carrier activity). The fact that Mtch2 is an integral membrane protein, which is likely to be exposed on both sides of the OMM, suggests that it and perhaps other members of the same family play a role in transferring metabolites/signals across the OMM.

The fact that tBID interacts with Mtch2, and that BAX co-resides with both proteins in the native gel, suggests that Mtch2 might also be involved in tBID-induced activation of BAX. It was previously proposed that the interaction between tBID and BAX/BAK is a transient "touch-and-go" interaction (Eskes *et al*., 2000; Wei *et al*., 2000). Since Mtch2 is an integral membrane protein that is exposed on the surface of mitochondria, it is tempting to speculate that it might act as a receptor to stably anchor and/or correctly position tBID in the membrane to enable the transient activation of BAX and/or BAK. This activation might include bringing BAX/BAK into close proximity with a channel/carrier residing in the Mtch2-resident complex.

ACKNOWLEDGMENTS

Research in my laboratory is supported in part by the Israel Science Foundation, Israel Cancer Research

Fund, Minerva Stiftung, the Y. Leon Benoziyo Institute for Molecular Medicine, the Willner Family Center for Vascular Biology, and Mr and Mrs Stanley Chais. A.G. is the incumbent of the Armour Family Career Development Chair of Cancer Research. Due to space limitations, many important studies were not cited in this review.

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