Apoptosis (2007) 12:869–876
DOI 10.1007/s10495-007-0748-2

Mitochondrial carriers and pores: Key regulators of the mitochondrial apoptotic program?

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Published online: 15 February 2007

Abstract Mitochondria play a pivotal role in the process of apoptosis. Alterations in mitochondrial structure and function during apoptosis are regulated by proteins of the BCL-2 family, however their exact mechanism of action is largely unknown. Mitochondrial carriers and pores play an essential role in maintaining the normal function of mitochondria, and BCL-2 family members were shown to interact with several mitochondrial carriers/pores and to affect their function. This review focuses on the involvement of several of these mitochondrial carriers/pores in the regulation of the mitochondrial death pathway.

Keywords Apoptosis · Mitochondrial carrier proteins · Mitochondrial pores · BCL-2 family members · BID · Mitochondrial carrier homolog 2 (Mtch2) · ADP/ATP translocase (ANT) · Voltage dependent anion channel (VDAC)

1 Apoptosis: an overview

Apoptosis is essential for normal development and maintenance of tissues. Insufficient apoptosis can lead to cancer and autoimmunity while accelerated apoptosis may lead to degenerative diseases. There are two major protein families which are considered as the core of the apoptotic machinery: Caspases, a family of cysteine proteases that are the major executioners of apoptosis [1], and the BCL-2 family of proteins, which are the main regulators of the process [2].

Generally, apoptosis is divided into two pathways, the extrinsic and intrinsic pathways. The extrinsic pathway is initiated by the binding of a ligand to members of the tumor necrosis factor/Fas (TNF/Fas) receptor family on the cell surface [3]. An activated receptor initiates the formation of the death-inducing signaling complex (DISC), which leads to activation of initiator caspase-8, and a downstream cascade of caspases. This ultimately leads to cleavage of cellular targets and death [4]. The intrinsic pathway involves the activation of pro-apoptotic members of the BCL-2 family that exert their function at the mitochondria by inducing the permeabilization of the outer mitochondrial membrane (OMM). This permeabilization results in the release of pro-apoptotic factors from the inter membrane space (IMS), such as cytochrome *c* (Cyt *c*), to activate effector caspases [5]. The two pathways are interconnected via the BH3-only protein BID, which is cleaved by caspase-8. Truncated BID (tBID) translocates to the mitochondria and triggers the activation of the pro-apoptotic BCL-2 family members BAX and BAK, leading to OMM permeabilization. In the extrinsic pathway, this permeabilization serves as an amplification loop for the activation of effector caspases [6–8].

2 The BCL-2 family

The BCL-2 proto-oncogene was the first member of this family to be discovered. This gene was found at the chromosomal

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breakpoint of t(14;18) bearing human B-cell lymphomas. Since then, many other genes homologous to BCL-2 have been found including both pro-apoptotic (e.g., BAX) and anti-apoptotic (e.g., BCL-X_L) proteins [2]. 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 [9]. Most family members display sequence conservation in the first three domains (multidomain molecules). Deletion and mutagenesis studies argue that the amphipathic α helical BH3 domain serves as the 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) that display sequence homology only within the BH3 domain [10, 11]. A major characteristic of the BCL-2 family members 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. Many BCL-2 family members also carry a C-terminal hydrophobic domain, which is essential for their targeting to membranes such as the OMM. The molecular mechanism of OMM permeabilization, which is a key event in the mitochondrial death pathway, is poorly understood. One school of thought proposes that BCL-2 family members themselves regulate OMM permeability by virtue of their ability to form autonomous channels. This idea emerged from the structural similarity between BCL-X_L and the pore forming region of bacterial toxins, and the ability of recombinant BCL-X_L, BCL-2, BAX and BID to form ion-channels in artificial membranes with distinct characteristics [12, 13]. It is yet to be seen whether this ability is of physiological significance. On the other hand there is a growing body of literature suggesting that BCL-2 family members induce OMM permeabilization by regulating the function of resident mitochondrial proteins that in healthy cells function in the maintenance of cell viability, and adopt either a pro- or anti-apoptotic role, in apoptotic cells. In this review we will focus on the involvement of several mitochondrial carriers and pores in this process.

3 The involvement of VDAC in apoptosis

The voltage dependent anion channel (VDAC) is a highly conserved OMM protein that belongs to the porin family. This family forms aqueous channels in membranes that act as pathways for the movement of metabolites by passive diffusion. The mitochondrial channel was first discovered by Colombini [14, 15], who demonstrated its voltage dependency and ion selectivity, with the open state showing preference to anions, and narrowed channel size, caused by alteration in membrane potential, which shows preference to cations.

The involvement of VDAC in the process of apoptosis is unquestionable and extensively reviewed [16–18]. However the molecular mechanism of VDAC regulation in the apoptotic process is highly controversial. One theory promotes the assumption that VDAC serves as the entity that permeabilizes the OMM during apoptosis and therefore is the route through which Cyt *c* and other IMS proteins exit the mitochondria [16]. This theory is based on the observation that several BCL-2 family members interact with VDAC [19–22]. It was reported, using proteoliposomes and electrophysiological measurements, that BCL-X_L promotes the closure of VDAC whereas BAX together with VDAC form a mega channel large enough for the passage of Cyt *c* [21]. Banerjee et al. argued that this increase in the pore size of VDAC by BAX can occur only in the presence of tBID [23]. The regulatory action of BAX on VDAC is further demonstrated by the use of a VDAC-disrupted yeast strain that shows no Cyt *c* release upon BAX expression [20, 24]. Furthermore BAX induced Cyt *c* release can be blocked by specific anti-VDAC antibodies [25], or by a VDAC inhibitor [26]. Interestingly, it was reported that tBID does not have an effect on the function of VDAC [27]. In this study, the authors suggested that the effect of tBID on OMM permeabilization is through another unidentified OMM protein. The importance of VDAC for OMM permeabilization is further supported by recent evidence that overexpression of VDAC induces apoptosis in various cell types, and this effect is inhibited by BCL-2 and VDAC inhibitors [28, 29].

In complete contradiction to the above, there is another theory which claims that in healthy cells VDAC is constitutively open allowing the free exchange of metabolites (e.g., ATP/ADP), whereas in apoptotic cells closure of VDAC blocks the passage of metabolites, thereby inhibiting respiration leading to mitochondrial dysfunction [17]. This theory is supported by data showing no influence of BAX on the properties of VDAC channels under a variety of conditions [30], and an inability to detect a direct interaction between BAX and VDAC in several experimental systems [30, 31]. The holders of this theory also question the results obtained with the expression of BAX in the VDAC-disrupted yeast strain, as it appears that although release of Cyt *c* was blocked, there was no influence on overall cell death, induced by similar BAX levels in both disrupted and wild type cells [32]. Furthermore, it was reported that removal of growth factors leads to adenine nucleotide exchange deficiency, most likely accounted by the closure of VDAC. BCL-X_L restores the adenine nucleotide exchange by promoting VDAC opening [33, 34]. Along that line, there is also a report showing that tBID induces the closure of VDAC [30]. One of the outcomes of VDAC closure is the accumulation of metabolites in the IMS, which exert osmotic pressure leading to matrix swelling and subsequent OMM rupture. However, this mode of OMM permeabilization is rarely

associated with apoptosis but rather with necrosis [35], and was also not observed in the experimental systems described above. Therefore the assumption is that release of apoptogenic factors from the IMS in this setting, must be controlled by another channel(s) [17]. Indeed, previous reports demonstrated the identification of the mitochondrial apoptosis induced channel (MAC) that has functional characteristics which are different from VDAC, and whose activity was detected in the VDAC-disrupted yeast strain [36–39].

4 The involvement of mitochondrial carrier proteins in apoptosis

The mitochondrial carrier (MC) protein family comprises of membrane embedded proteins that catalyze the exchange of solutes across the IMM (reviewed in [40, 41]). These carriers provide a link between mitochondria and cytosol, which is indispensable for the metabolic activities performed by mitochondria. The conserved features of this protein family are a tripartite structure made up of three tandem repeats of about 100 amino acids in length and known as the mitochondrial carrier domain (Mito_Carr domain). Each Mito_Carr domain contains two transmembrane α-helical domains connected by an extensive hydrophilic region (see for an example, the structure of the ADP/ATP translocase in Fig. 1, left panels).

Based on the sequence information together with biochemical analysis of individual MCs it was proposed that the overall structure of each MC monomer is made up of six α-helices transversing the IMM and connected by hydrophilic loops, with both N and C termini facing the IMS. Reconstitution into liposomes and complementation experiments in yeast revealed also common functional characteristics between the different MCs, although the substrates transported differ widely in their structure, charge and size [40].

4.1 ADP/ATP translocase (ANT)

The ADP/ATP translocase (or adenine nucleotide translocator; ANT) is one of the most abundant mitochondrial proteins and was the first MC to be identified, purified and sequenced [42]. Its fundamental role is to transport ATP, which is synthesized inside the mitochondria, to the cytosol, in exchange for ADP. The exchange creates an electrical gradient (ATP^{4-}/ADP^{3-}) that drives the exchange itself [43]. Under normal settings, ANT functions as a highly specific transporter, having two conformational states. This is exhibited by the binding of its two potent inhibitors: carboxyatractyloside, which binds at the cytosolic side of the protein and fixes it in the "c"-conformation, and bongkrekic acid, which binds at the matrix side of the protein and fixes it in the "m"-conformation [44]. In humans, there are three known isoforms of ANT, which differ in their expression level and tissue distribution [45–47]. Under challenging conditions, especially Ca^{2+} overload and oxidative stress, mitochondria undergo massive swelling and depolarization, known as the mitochondrial permeability transition (mPT) [48]. This phenomenon is the result of the opening of a non-specific large pore in the IMM, the permeability transition pore (PTP) [49]. Although the exact composition of this pore is still a debate, it is widely accepted that ANT is a core protein of this pore (also reviewed in [50, 51]). Notably, mitochondria from livers of ANT1, ANT2 double-knockout mice were demonstrated to undergo Ca^{2+}-induced mitochondrial swelling, although at a higher threshold [52, 53]. This phenotype was proposed to be the result of functional compensation by other carrier proteins, based on the structural and functional resemblance between these family proteins. Additional proteins that were proposed to be part of the PTP complex (or to influence its activity) are hexokinase, creatine kinase (CK), the peripheral benzodiazepine receptor (PBR), the mitochondrial matrix cyclophilin D (Cyp D) and VDAC, which was co- purified with ANT on a GST-Cyp D affinity column [54]. However work of others argue that VDAC is not an essential component of the PTP but rather regulates its activity only under certain settings [55].

The involvement of ANT in mPT was demonstrated by the fact that different ANT ligands can affect PTP opening. Reagents that stabilize the "c"-conformation such as carboxyatractyloside, sensitize mPT to Ca^{2+} while reagents that stabilize the "m"-conformation such as ADP and bongkrekic acid, made mPT less sensitive to Ca^{2+} [56–59]. Direct evidence for the presence of ANT in the PTP complex came from work of Woodfield et al. [60] that used a Cyp D affinity column to identify proteins that are part of the PTP. The only protein that retained on their column was the ANT. Furthermore, the binding of ANT to the column was blocked by pretreatment with cyclosporin A, a specific inhibitor of Cyp D and a known effector of PTP opening, and enhanced when the mitochondria used were subjected to oxidative stress. In a similar experiment, Crompton et al. [54] also purified ANT using a GST-Cyp D fusion protein. The interaction between ANT and Cyp D was later demonstrated by reconstitution of recombinant proteins into lipid bilayers that showed the formation of a cyclosporine A-sensitive channel, suggesting that ANT and Cyp D are the only proteins that are essential for the formation of the pore.

4.1.1 ANT, BAX and tBID

BAX was shown to directly interact with ANT both by co-immunoprecipitation and by the yeast two hybrid system [61]. Moreover, this interaction was shown to be important for optimal opening of the PTP in response to atractyloside, both in isolated mitochondria and by *in-vitro* experiments using reconstituted ANT and BAX [62]. This effect of BAX

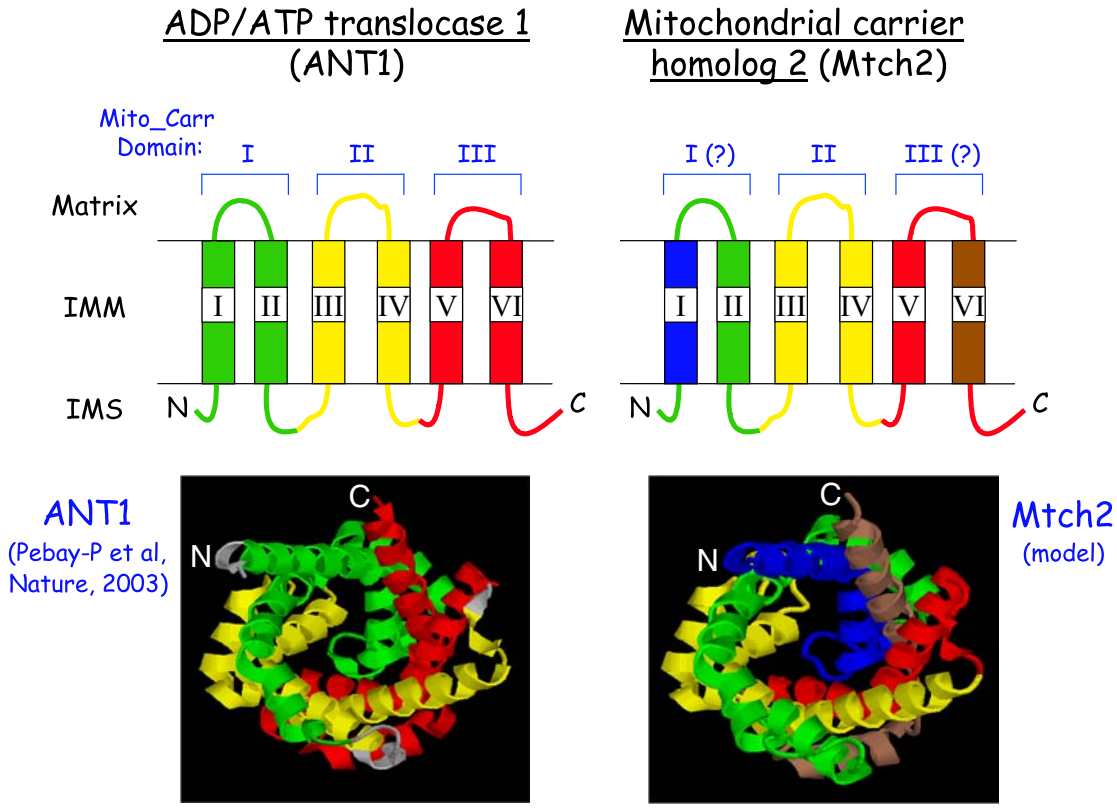

Fig. 1 Comparison of the sequences of ANT1 and hMtch2 and threading of hMtch2 to the ANT1 structure. *Top*: comparison of the sequences of the bovine ADP/ATP translocase 1 [also known as adenine nucleotide translocator 1 (ANT1), or solute carrier family 25 member 4] and hMtch2. ANT1 is a 298 amino acids long sequence composed of three tandem Mito_Carr domains according to Pfam [84] here coloured in green (from amino acids 6 to 101), yellow (from 126 to 206), and red (from 206 to 297), respectively. hMtch2, with a similar length of 303 amino acids, apparently has a single middle Mito_Carr domain from positions 123 to 206 (coloured in yellow). However, sequence comparison indicates that hMtch2 and its homologous sequences such as hMtch1 or their common ancestor in the fly Mtch have extended homology into the neighbouring fragments of the N- and C-terminal Mito_Carr domains of ANT1 including TM helices 2 and 5 (fragments 43-122 and 207-248 in hMtch2, coloured in green and red, respectively). Iterative sequence searches using PSIBLAST [85] with the unmatched N- and C-terminal fragments of hMtch2 (1-42 and 249-303) did not detect any sequences outside the Mtch family. However, we observed that a chimeric sequence constructed using the 249-303 followed by the 1-42 was matched to the complete Pfam Mito_Carr profile with an E-value of 0.081. This suggests that: (1) the actual evolutionary unit in ANT1 would not be the currently defined Mito_Carr domains comprising TM1/TM2, TM3/TM4. and TM5/TM6, but TM2/TM3, TM4/TM5, and TM6/TM1 (the formation of a hybrid N- terminal C-terminal domain has also been described for beta barrels of repeats—like in Kelch—as is known as a "clasp" mechanism that helps the stability of the closing structure); (2) in the Mtch family, the domains TM2/TM3 and TM4/TM5 are present, but the hybrid clasp domain is a very divergent reversed TM1/TM6. *Bottom*: To further support this hypothesis, we threaded the sequence of hMtch2 into the structure of ANT1 (PDB:1ock) [79] using the Phyre web tool [Kelley, unpublished; http://www.imperial.ac.uk/phyre/]. The fold recognition of ANT1 by hMtch2 received an E-value of 2e-15 for a length of 298 amino acids on ANT1 (complete sequence). For illustration of the spatial position of the units the structure of ANT1 (*left*) and the resulting modeled structure for hMtch2 (*right*) are presented. The orientation is such that the axis of symmetry of the barrel runs perpendicular to the page with the TMs running almost perpendicular to the page

was inhibited by BCL-2 which was also shown to directly interact with ANT and compete with BAX [62].

Zamzami et al. [63] demonstrated that tBID-induced permeabilization of the OMM is inhibited by both cyclosporine A and bongkrekic acid, and therefore suggested a functional interaction between tBID and ANT. Since there is no evidence for a direct physical interaction between tBID and ANT, it is tempting to speculate that tBID affects ANT function by interacting with lipids surrounding it. ANT is tightly bound to six molecules of cardiolipin. These interactions are essential for the normal function of ANT [64–66]. In the last several years it has been established that tBID also intimately interacts with cardiolipin [67, 68], and that this interaction is involved in regulating the mitochondrial death pathway [69–73]. Thus, the tBID-cardiolipin interaction might drive a conformational change in ANT, which might trigger the binding of BAX.

4.2 Mitochondrial carrier homolog 2 (Mtch2)

Previously we reported that in TNFα-activated hematopoietic FL5.12 cells, p15 tBID forms a ~45 kD cross-linkable

complex at the mitochondria [74]. More recently, we demonstrated that this complex represents an interaction between tBID and mitochondrial carrier homolog 2 (Mtch2), a novel and previously uncharacterized 33 kD protein [75, 76]. Mtch2 was first identified as a putative ORF obtained from CD34+ hematopoetic stem/progenitor cells [77], named after its single conserved Mito_Carr domain (Mito_Carr domain II; see Fig. 1, right panel). Mtch2 (also known as Mimp) was also shown to be induced by Met-hepatocyte growth factor/scatter factor signaling [78].

Mtch2 has several close relatives, which together form a "sister family" separate from the other mitochondrial carrier proteins [75, 76]. All Mtch family members contain a single conserved Mito_Carr domain, and can be aligned across their entire length. Having only one conserved Mito_Carr domain, it was first predicted to have three trans-membrane (TM) domains (two that form the Mito_Carr domain, and a third C-terminal one) [75, 76]. However, sequence similarity analysis and threading using the 3D structure of ANT1 [79] suggests that the overall structure of Mtch2 resembles the typical structure of MCs (six TM domains connected with hydrophilic loops; Fig. 1, right panels). Of special note, Mtch2 does carry unique features [the N-terminal helix of Mito_Carr domain I (blue) and the C-terminal helix of Mito_Carr domain III (brown)], which are different from the helices in ANT (compare bottom panels in Fig. 1), suggesting that Mtch2 (and probably its close relatives) are related to MCs but distinct.

Biochemical analysis of Mtch2 reveals that it is exposed on the surface of mitochondria, with all its TM domains likely transversing the OMM ([76], and data not shown). This feature seems to be unique to Mtch2, as all other MCs are IMM proteins. This data also fits well with the fact that tBID, which does not seem to reach the IMM [80], directly interacts with Mtch2 [76]. The unique localization of Mtch2 may be explained by its amino acid sequence. As noted earlier, the amino acid sequence of the Mtch2 protein contains a central conserved region (including a region corresponding to Mito_Carr domain II in other MCs), while the N- and C-terminal fragments that are nearby in space and form probably a domain (see Fig. 1, right panels) are less conserved. Studies using deletion mutants of ANT have demonstrated that the insertion mechanism of ANT to the IMM requires cooperate binding of all three Mito_Carr domains to components of the import complexes of both the outer and inner membranes (TOMs and TIMs, respectively; [81]). However the exact residues that are involved in the binding are unknown. Thus, it is possible that the conserved Mito_Carr domain II allows the binding of Mtch2 to the TOM complex, but not its transfer to the TIM complex in the IMM, and therefore it remains stuck in the OMM.

Blue-native gel electrophoresis analysis performed on mitochondria of both healthy cells and cells signaled to die by TNFα revealed that Mtch2 resides in an approximately 185 kD resident complex, and activation with TNFα leads to the recruitment of both tBID and BAX to this complex [76]. The recruitment of pro-apoptotic BCL-2 family members to the complex seems to be important for the induction of the mitochondrial death pathway, since over expression of BCL-X_L, which inhibits TNFα-induced Cyt *c* release, also inhibits the recruitment of both tBID and BAX to the Mtch2-resident complex [76].

4.2.1 What might be the function of Mtch2?

The function of Mtch2 is still unknown. It was previously reported that over expression of human presenilin-1-associated protein (PSAP, also known as human Mtch1, a protein with 48% identity to human Mtch2 [76]), induces Cyt *c* release, caspase activation and apoptosis, implying a pro-apoptotic role for this protein [82]. Over expression of Mtch2 in 293T and HeLa cells, however, did not induce apoptosis [76]. It is important to note that the lack of an effect could be due to the low expression levels that were obtained. Interestingly, it was reported that overexpression of Mtch2/Mimp in mammary adenocarcinoma DA3 cells did not induce apoptosis, but did cause loss of mitochondrial membrane potential [78, 83]. These studies proposed a role for Mtch2 in regulation of cellular growth, motility and tumorigenicity.

Since the members of the Mtch family are close relatives of MC proteins, they might function as carrier proteins, transporting certain metabolites across the OMM rather than across the IMM. This is supported by their apparently similar arrangement of transmembrane domains (Fig. 1). However, the different terminals make them distinct and less symmetrical than their MC partners. The divergent hybrid N-terminal C-terminal domain might hold protein interaction sites specific to the Mtch family.

The fact that both tBID and BAX co-reside with the Mtch2 resident complex in apoptotic cells suggests that Mtch2 might be involved in tBID-induced BAX activation/oligomerization. It is known that tBID activates BAX via its BH3 domain, however the interaction of tBID with Mtch2 is BH3- and BAX/BAK-independent [74]. This may imply that Mtch2 serves as an anchor protein for tBID, that stably anchors and/or correctly positions tBID in the membrane, to allow the activation of BAX via its exposed BH3 domain.

5 Concluding remark

Mitochondria are Jekyll and Hyde entities. In healthy cells they are the power plants providing energy to sustain life activities. At the same time mitochondria also serve as the storage compartment for apoptogenic factors which are

released upon death stimuli. This dual function on the level of the organelle is maintained also on the level of individual mitochondrial proteins. As discussed in this review, mitochondrial carriers and pores, which in healthy cells function to maintain cell viability, might alter their function under certain stress conditions to promote the mitochondrial apoptotic program. We believe that the list of mitochondrial carriers and pores that are involved in this "balancing act" between cell life and death is likely to increase in the future. In a way one can look at this set of proteins as the link between the major regulators of apoptosis (BCL-2 proteins) and the major executioners (caspases), and therefore are likely to represent excellent therapeutic targets.

Acknowledgments Research in my laboratory is supported in part by the Israel Science Foundation, the Israel Cancer Association (through a donation from Teva Pharmaceutical Industries Ltd.), the A-T Children's Project, Minerva Stiftung, MDM ICR Research Award, the USA-Israel Binational Science Foundation (BSF), Joint German-Israeli Research Program in Cancer Research (IMOS-DKFZ), 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.

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