

## Review

# VDAC regulation by the Bcl-2 family of proteins

Y Tsujimoto<sup>\*1</sup> and S Shimizu<sup>1</sup>

<sup>1</sup> Department of Medical Genetics, Biomedical Research Center, Osaka University Graduate School of Medicine, CREST of Japan Science and Technology Corp. (JST), Suita, Osaka, Japan

\* Corresponding author: Y Tsujimoto, Department of Medical Genetics, Biomedical Research Center, Osaka University Graduate School of Medicine, CREST of Japan Science and Technology Corp. (JST), 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel: +81-6-6879-3360; Fax: +81-6-6879-3369; E-mail: tsujimot@gene.med.osaka-u.ac.jp

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## Abstract

The Bcl-2 family of proteins consists of anti-apoptotic and pro-apoptotic members, which determine the life or death of cells by altering mitochondrial membrane permeability. Pro-apoptotic Bcl-2 family members increase mitochondrial membrane permeability, resulting in the release of mitochondrial apoptogenic factors such as cytochrome *c* that activates death proteases called caspases, whereas anti-apoptotic family members prevent this increase of mitochondrial membrane permeability. The release of cytochrome *c* is central to apoptotic signal transduction in mammals, and has been studied extensively, leading to the development of several models for cytochrome *c* release including rupture of the mitochondrial outer membrane and involvement of specific channels. This article describes the important role of a mitochondrial outer membrane channel, the voltage-dependent anion channel (VDAC), in apoptogenic cytochrome *c* release and its regulation by Bcl-2 family members, and also discusses the molecular architecture of the life–death switch in mammalian cells. *Cell Death and Differentiation* (2000) 7, 1174–1181.

**Keywords:** Bcl-2; mitochondria; apoptosis; cytochrome *c*; VDAC

**Abbreviations:** VDAC, voltage-dependent anion channel;  $\Delta\psi_m$ , mitochondrial membrane potential; ANT, adenine nucleotide translocator

## Introduction

In several phylogenetically divergent metazoans including nematodes, fruit flies and mammals, there are structural and functional homologues that play a key role in apoptosis, such as death proteases (caspases), the Bcl-2 family of proteins, and caspase activators, indicating that the basic mechanism of apoptosis has been well conserved throughout evolution. The apoptotic signal

transduction mechanism appears to consist of individual and common pathways. The common pathway is driven by caspases,<sup>1</sup> which cleave a set of cellular proteins including inhibitor of caspase-activated DNase (ICAD),<sup>2,3</sup> Acinus,<sup>4</sup> and many others<sup>1</sup> to cause apoptotic changes in cells, and uniquely constitute an irreversible step in the signal transduction of apoptosis. The activation of caspases is regulated by several sets of genes, among which the best characterized is the bcl-2 family.<sup>5,6</sup> A major role of the Bcl-2 family of proteins in regulating caspase activation is to alter mitochondrial membrane permeability, thus controlling the release of caspase-activating cytochrome *c*. Through different modification of various members, the Bcl-2 family constitutes a convergence point via which a number of apoptotic signals eventually act on the mitochondria.

This article reviews our recent work together with that of others concerning the modulation of mitochondrial membrane permeability by the Bcl-2 family, and discusses how cell viability or death is decided.

## The Bcl-2 family of proteins

Bcl-2<sup>7–9</sup> was initially shown to inhibit cell death induced by deprivation of IL-3<sup>10</sup> and was subsequently found to also inhibit cell death induced by various stimuli, including a chemotherapy agent and heat shock.<sup>11</sup> In recent years, it has been well established that Bcl-2 and its relative Bcl-x<sub>L</sub> can block most forms of apoptotic cell death as well as certain forms of necrotic cell death by preventing mitochondrial changes.<sup>6,12</sup> Based on their structural and functional characteristics, the Bcl-2 family of proteins has been divided into three categories.<sup>5,6</sup> (1) The anti-apoptotic members include Bcl-2, Bcl-x<sub>L</sub>, Bcl-w, Mcl-1, A1 (Bfl-1) and Boo. These proteins mostly share sequence homology within four regions, BH (Bcl-2 homology)1 through BH4, although some members lack an obvious BH4 domain but still carry a conserved  $\alpha$ -helical structure.<sup>13</sup> (2) The pro-apoptotic members include Bax, Bak, Mtd (Bok), and Diva. These proteins share sequence homology of BH1, BH2, and BH3, but not BH4, although a significant BH4 homology has been found in some members. (3) The ‘BH3-only proteins’ are also pro-apoptotic, and they include Bik, Bad, Bid, Bim, Hrk (DP5), Blk, Bnip3, and Bnip3L. These proteins only share sequence homology of BH3 region.

One of the unique features of the Bcl-2 family of proteins is heterodimerization between anti-apoptotic and pro-apoptotic family members, which is considered to inhibit the activity of their partner proteins.<sup>14,15</sup> This heterodimerization process is mediated by the insertion of a BH3 region of a pro-apoptotic protein into a hydrophobic cleft composed of BH1, BH2 and BH3 on an anti-apoptotic protein.<sup>16</sup> In addition to BH1 and BH2, the BH4 domain is required for anti-apoptotic activity.<sup>17–22</sup> In contrast, BH3 is essential and sufficient for pro-apoptotic activity.<sup>23</sup>



One of the known activities of Bcl-2 family members is formation of ion channels in synthetic lipid membranes, with Bcl-2, Bcl-x<sub>L</sub>, Bax, and Bid all having the ability to form such ion channels.<sup>24–28</sup> However, it still remains to be determined whether these proteins actually form ion channels *in vivo* and whether apoptosis is regulated via the creation of ion channels.

## Sites of Bcl-2 family activity: mitochondria versus apoptosome

Mitochondria play an essential role in many forms of apoptosis in mammals<sup>29</sup> by releasing apoptogenic factors such as cytochrome *c*<sup>30</sup> from the intermembrane space into the cytoplasm.<sup>31,32</sup> Mitochondria are also reported to release other apoptogenic factors such as AIF<sup>33</sup> and some of the caspases. Cytochrome *c* release is inhibited by anti-apoptotic Bcl-2 family members, while it is directly enhanced by the pro-apoptotic members.<sup>5,6</sup> Many of the pro-apoptotic family members (such as Bax, Bid, Bim and Bad) are localized in the cytoplasm of living cells.<sup>5,6</sup> Apoptotic stimuli cause various modifications of these proteins, such as proteolytic cleavage and dephosphorylation, probably leading to conformational changes, after which translocation to the mitochondria occurs.<sup>6</sup> In addition to directly preventing an apoptotic increase of mitochondrial membrane permeability, Bcl-2 and Bcl-x<sub>L</sub> inhibit apoptosis by indirectly preventing the translocation of Bax to the mitochondria through a cytoplasmic factor.<sup>34,35</sup>

Bcl-x<sub>L</sub>, but probably not Bcl-2, also has the ability to prevent caspase activation by sequestering Apaf-1.<sup>36,37</sup> Although this is similar to the mechanism through which CED-4 (an Apaf-1 homologue that activates Ced-3 caspase) is regulated by CED-9 (a Bcl-2 homologue) via formation of a protein complex called an apoptosome in *C. elegans*,<sup>38</sup> whether Bcl-2 family proteins actually sequester Apaf-1 to prevent caspase activation in mammalian cells has recently been questioned, based upon the failure to detect a physiologically relevant level of stable interaction between Apaf-1 and Bcl-2/Bcl-x<sub>L</sub>.<sup>39,40</sup> Thus, at least in mammals, modulation of mitochondrial membrane permeability seems to be one of the major mechanisms by which Bcl-2 family proteins regulate apoptosis.

## Bcl-2/Bcl-x<sub>L</sub> and Bax/Bak act independently

In addition to the regulation of apoptosis by heterodimerization of anti-apoptotic and pro-apoptotic members of the Bcl-2 family, some of these proteins have been suggested to regulate apoptosis independently of each other, based on observations in transgenic and knockout mice,<sup>41</sup> although the data have never been conclusive because of the presence of other family members. The independent action of Bcl-2/Bcl-x<sub>L</sub> and Bax/Bak was more clearly shown by recent studies of ours and others on yeast cells, which lack Bcl-2 family proteins.<sup>42–45</sup> It was found that Bax/Bak can induce cytochrome *c* release, which is inhibited by Bcl-2/Bcl-x<sub>L</sub>, in yeast mitochondria<sup>42,44</sup> as well as in yeast cells,<sup>43,45</sup> and that Bcl-x<sub>L</sub> prevents cytochrome *c* release

from isolated mitochondria induced by several chemicals such as ATP and ethanol that are known to induce a ‘permeability transition (PT)-like phenomenon, including  $\Delta\psi$  loss and swelling of isolated mitochondria.<sup>43</sup> Thus, Bcl-2 family members, particularly Bcl-2/Bcl-x<sub>L</sub> and Bax/Bak, seem to regulate apoptotic mitochondrial changes in two ways, i.e., through heterodimerization and through various independent functions.

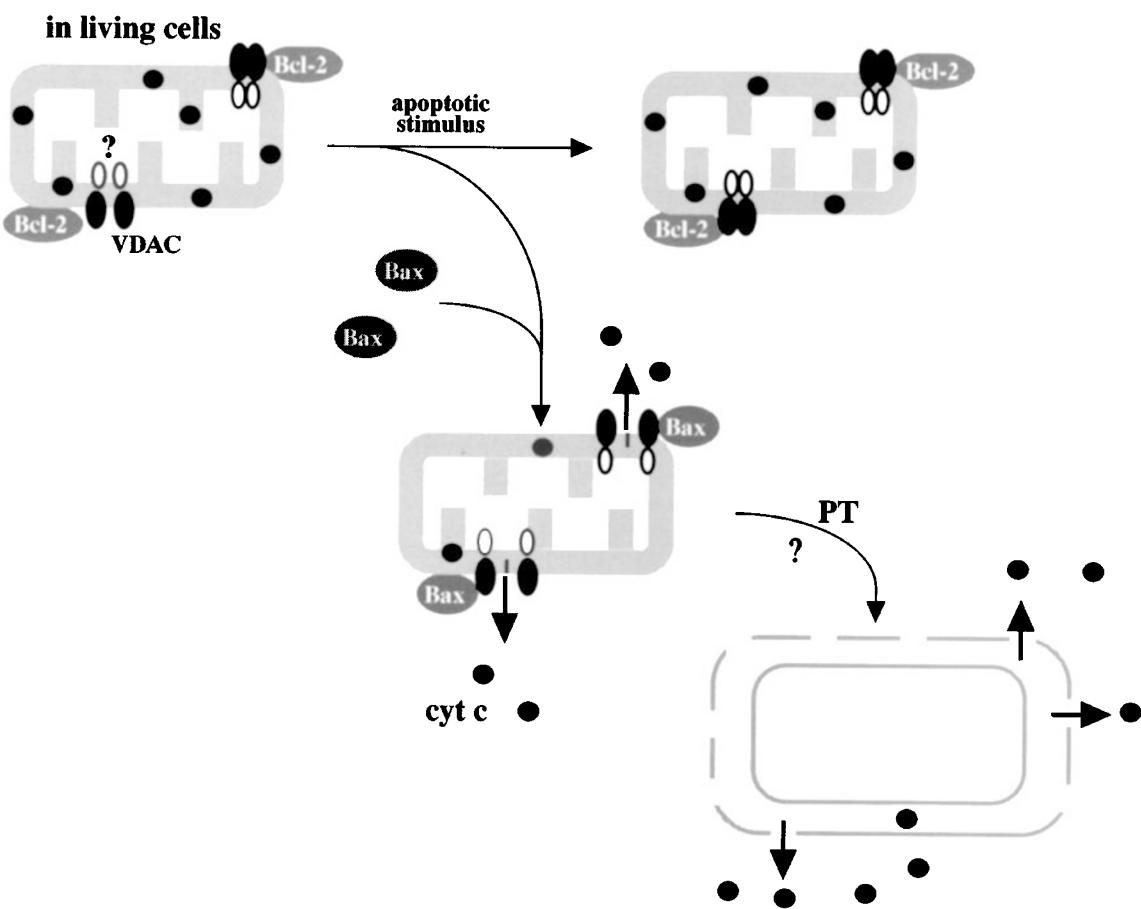
## Regulation of cytochrome *c* release by the Bcl-2 family

Regarding the translocation of mitochondrial cytochrome *c* to the cytoplasm during apoptosis, three models have been proposed.<sup>46</sup> One possibility is that cytochrome *c* release is mediated by physical rupture of the outer mitochondrial membrane, resulting from mitochondrial swelling<sup>47</sup> or membrane instability induced by molecules such as Bax,<sup>48</sup> although previous studies have suggested that mitochondrial swelling and membrane rupture rarely accompany apoptosis. The other models involve release of cytochrome *c* via specific channels such as the VDAC-Bax channel (Figure 1) or a channel constituted by Bax alone (or possibly another pro-apoptotic family member alone).

## Mechanism of cytochrome *c* release through mitochondrial membrane channels

### Permeability transition and Bax/Bak

A system with isolated mitochondria and recombinant Bcl-2 family proteins has proven very useful for studying the mechanisms by which Bcl-2 family proteins regulate apoptotic mitochondrial changes, and has been employed quite extensively. Although there is some controversy about the findings, we and others have shown that addition of recombinant Bax or Bak to isolated mitochondria induces cytochrome *c* release and  $\Delta\psi$  loss (as well as other permeability transition (PT)<sup>49,50</sup>-associated changes such as swelling).<sup>51–55</sup> Note that Bax/Bak-induced mitochondrial swelling is much less severe compared with  $\text{Ca}^{2+}$ -induced swelling<sup>51</sup> (our unpublished observations). We have also shown that, unlike Bax/Bak, some of the BH3-only proteins such as Bid and Bik induce cytochrome *c* release but not the PT ( $\Delta\psi$  loss).<sup>55</sup> Importantly, these observations on isolated mitochondria have been confirmed in cells that were transfected with Bax- or Bid-expressing plasmids. It was found that Bax-induced apoptotic cell death was accompanied by  $\Delta\psi$  loss,<sup>55–57</sup> whereas Bid-induced apoptotic death was not.<sup>55</sup> Expression of BH3-only proteins could also eventually lead to  $\Delta\psi$  loss, which was probably a consequence of cell death. All of the mitochondrial changes induced by pro-apoptotic Bcl-2 members are known to be inhibited by Bcl-2 and Bcl-x<sub>L</sub> in isolated mitochondria as well as in mammalian cells. Consistent with induction of the PT by Bax/Bak, but not by Bid/Bik, Bax/Bak-mediated cytochrome *c* release and  $\Delta\psi$  loss are inhibited by PT inhibitors,



**Figure 1** VDAC-dependent mechanism of cytochrome *c* release. Bax/Bak, once activated interacts with and opens the VDAC through which cytochrome *c* is released. Opening of the VDAC probably affects a channel(s) on the inner membrane and thus induces loss of membrane potential. Anti-apoptotic Bcl-2/Bcl-x<sub>L</sub> has the ability to close the VDAC. It may be possible that Bcl-2/Bcl-x<sub>L</sub> is loosely associated with VDAC in living cells while the channel remains functional, and tightly binds to and closes the VDAC to prevent cytochrome *c* release in response to an apoptotic stimulus. Opening of the VDAC results in cytochrome *c* release and, at least *in vitro*, induction of the PT could eventually occur, leading to further cytochrome *c* release

including cyclosporin A (a cyclophilin D inhibitor),<sup>51,52,54,55</sup> bonkrekic acid (an ANT inhibitor), and calcium depletion,<sup>52,55,58</sup> whereas Bid/Bik-induced cytochrome *c* release is not sensitive to PT inhibitors.<sup>55</sup> More importantly, it should be noted that Bax-induced apoptosis is inhibited by cyclosporine A, but not FK506 (another immunophilin that does not react with mitochondrial cyclophilin D)<sup>57</sup> (our unpublished results). It should also be noted that several modes of apoptosis are inhibited by cyclosporine A and bonkrekic acid.<sup>53</sup> Although PT inhibitors do not block all kinds of apoptotic cell death, this might depend on which pro-apoptotic member of the Bcl-2 family is dominant in different modes of apoptosis, as discussed below. The PT is regulated by the PT pore, which is an oligo-protein channel that consists of the VDAC on the outer membrane, adenine nucleotide translocator (ANT) on the inner membrane, matrix protein cyclophilin D, and probably other proteins,<sup>59</sup> although the molecular nature of this pore is still elusive. Our group and Kroemer's have independently shown that the PT pore is a target for the Bcl-2 family of proteins since Bcl-2/Bcl-x<sub>L</sub> and Bax/Bak interact with the VDAC<sup>42,43</sup> and ANT.<sup>42,60</sup>

#### Bax/Bak induces cytochrome *c* release through the VDAC

We have shown that VDAC activity is directly modulated by the Bcl-2 family of proteins.<sup>42</sup> Bcl-x<sub>L</sub> closes the VDAC on liposomes, whereas Bax/Bak opens this channel so that cytochrome *c* can pass through it (Figure 1). Similarly, although Bax does not affect ANT, it was found to enhance ANT channel activity in the presence of an ANT inhibitor, atractyloside, whereas ANT activity is inhibited by Bcl-2.<sup>61</sup>

An essential role of the VDAC in Bax/Bak-mediated cytochrome *c* was suggested by the observation that Bax/Bak induces cytochrome *c* release from the mitochondria of wild-type yeast cells, but not from the mitochondria of VDAC-1-deficient yeast cells using isolated mitochondria and cells,<sup>42,43</sup> although there was a controversial report that Bax induces cytochrome *c* release, as spectrophotometrically assessed, in yeast cells lacking VDAC-1.<sup>44</sup> Yeast cells carry another VDAC, called VDAC-2, but there is no evidence that VDAC2 forms a channel. Supporting our model involving the VDAC in cytochrome *c* release, we have more recently shown that Bax-induced cytochrome *c*

release and  $\Delta\psi$  loss are prevented in isolated mitochondria by two kinds of anti-VDAC antibodies, both of which inhibit VDAC activity, and also that microinjection of either of these antibodies into cells prevents both Bax-induced cytochrome *c* release and cell death.<sup>62</sup> We also found that Bid-induced cytochrome *c* release and cell death were not inhibited by the same antibodies, providing stronger evidence for the essential role of VDAC in Bax-induced apoptotic mitochondrial changes and cell death.<sup>62</sup> Although it may be argued that Bax kills yeast cells lacking VDAC-1, it should be noted that Bax kills yeast cells by a mechanism which is independent on cytochrome *c* release.<sup>45,63</sup>

How does Bax/Bak make the VDAC permeable to cytochrome *c*? An electrophysiological study of the VDAC-Bax channel has revealed that it is nearly continuously in an open state, unlike the rapidly opening closing VDAC, and its pore is four times as large as that of the VDAC.<sup>64</sup> It was also shown that a single VDAC-Bax channel formed in a planar lipid bilayer is permeable to cytochrome *c*.<sup>64</sup> Although VDAC-Bax channels in liposomes are permeable to cytochrome *c* (14 kDa), but not to GST-GFP with a molecular weight of approximately 50 kDa,<sup>42</sup> the VDAC-Bax channels formed in the mitochondrial membrane in a physiological context might be permeable to larger molecules known to be released from the mitochondria during apoptosis.<sup>65</sup> Bax/Bak might induce conformational changes of the VDAC to form larger pores or might be involved in creating a larger pore together with the VDAC. If Bax/Bak has the same role in inducing cytochrome *c* release and  $\Delta\psi$  loss in yeast mitochondria as in mammalian mitochondria,<sup>42,43,52</sup> since yeast mitochondria lacking the Bcl-2 family of proteins still show inhibition by Bcl-2/Bcl-x<sub>L</sub> of the release of cytochrome *c* in response to chemicals such as ATP and ethanol,<sup>43</sup> the possibility is greater that Bax/Bak induces conformational changes of the VDAC rather than forming a hybrid VADC-Bax channel. However, it is also conceivable that yeast mitochondria might possess a protein that does not belong to the Bcl-2 family but functions similarly to Bax/Bak in forming a hybrid channel.

The stoichiometry of the VDAC-Bax channel, e.g., how many Bax/Bak and VDAC molecules are required to form it, is still to be determined. Mutation studies have indicated that the BH3 domain of Bax/Bak, which is required for inducing cell death, is also indispensable for opening of the VDAC by Bax/Bak, but not for binding to the channel.<sup>42</sup>

## VDAC and anti-apoptotic Bcl-2/Bcl-x<sub>L</sub>

Recombinant Bcl-x<sub>L</sub> inhibits VDAC activity and competes with Bax/Bak on liposomes.<sup>42</sup> A picture of how the VDAC is inhibited by Bcl-x<sub>L</sub> was revealed by an electrophysiological study, which indicated that Bcl-x<sub>L</sub> almost completely closes the channel.<sup>64</sup> Given that the VDAC functions as a gate for various materials moving in and out of the mitochondria, it may be argued that closure of this channel by Bcl-2/Bcl-x<sub>L</sub> in living cells could cause respiratory failure, but this never actually occurs. It might be that only a fraction of the total VDAC, e.g., the VDAC associated with the PT pore, is directly targeted by Bcl-2/Bcl-x<sub>L</sub>. Alternatively, since the interaction of

Bcl-x<sub>L</sub> with the VDAC is considerably enhanced by apoptotic stimuli (our unpublished observations), it might be that Bcl-2/Bcl-x<sub>L</sub> only closes the channel in the presence of apoptotic signals, as seen in an *in vitro* study. Thus, the functional interaction between Bcl-2/Bcl-x<sub>L</sub> and the VDAC might be suppressed in healthy living cells. The BH1 domain of Bcl-x<sub>L</sub> is required for binding to the VDAC,<sup>42</sup> while the BH4 domain of Bcl-2/Bcl-x<sub>L</sub> is necessary and sufficient for Bcl-2/Bcl-x<sub>L</sub> to close the VDAC in liposome and electrophysiological studies.<sup>22</sup> Even after opening the VDAC by Bax, BH4 oligopeptides are able to compete with Bax and close it again.<sup>22</sup> Consistent with the notion that BH4 is essential and sufficient for inhibiting the VDAC, BH4 oligopeptides are able to prevent  $\text{Ca}^{2+}$ -induced apoptotic changes (cytochrome *c* release and  $\Delta\psi$  loss) in isolated mitochondria.<sup>22</sup> Interestingly,  $\text{Ca}^{2+}$  induces cytochrome *c* release and  $\Delta\psi$  loss in a PT inhibitor-sensitive manner as does Bax/Bak.<sup>55</sup> Furthermore, BH4 oligopeptides, when fused with the protein transduction domain of HIV Tat protein (allowing the peptides to cross the plasma membrane), inhibit apoptotic cell death induced by the chemotherapy agent etoposide.<sup>22</sup> Since the BH4 domain is separated by the regions of Bcl-2/Bcl-x<sub>L</sub> ( $\alpha$ -helices 5 and 6) required for formation of an ion channel,<sup>66,67</sup> this finding may imply that ion channel formation by Bcl-2/Bcl-x<sub>L</sub> is not required for anti-apoptotic activity *per se*.<sup>22</sup>

## ANT

Concerning the possible involvement of ANT in Bax/Bak-induced cytochrome *c* release,<sup>60</sup> probably via the PT, our studies on yeast mutants have revealed that none of the three ANTs are essential for Bax/Bak-mediated cytochrome *c* release from isolated mitochondria,<sup>43</sup> apparently ruling out a role of ANTs in Bax/Bak-mediated cytochrome *c* release, although the possibility was not excluded that an ANT-like channel might compensate for the ANT functions.

## $\Delta\psi$ loss and the PT pore

Can the controversy concerning whether or not Bax/Bak induces  $\Delta\psi$  loss (the PT) be resolved? It arises at least partly from differences in the experimental conditions that have been used. Since  $\Delta\psi$  loss (the PT) is  $\text{Ca}^{2+}$ -dependent,  $\text{Ca}^{2+}$  depletion prevents it and this might explain the occurrence of Bax/Bak-induced cytochrome *c* release without  $\Delta\psi$  loss, because  $\text{Ca}^{2+}$  chelator was added to the mitochondrial buffers used in some studies.<sup>68,69</sup> Since the presence of  $\text{Ca}^{2+}$  outside the mitochondria is more physiological and since cells transfected by Bax-expressing DNA show  $\Delta\psi$  loss,<sup>55–57</sup> the  $\Delta\psi$  loss induced in isolated mitochondria by Bax/Bak seems more likely to represent the physiological role of Bax. In the absence of  $\text{Ca}^{2+}$ , Bax/Bak opens the VDAC and allows the efflux of cytochrome *c*, whereas two modes of cytochrome *c* release via the VDAC and through PT-dependent rupture of the outer membrane, can occur in the presence of  $\text{Ca}^{2+}$  (Figure 1). Another possible explanation for the controversy about Bax-induced  $\Delta\psi$  loss came from the study of Pastorino *et al.*,<sup>54</sup> showing that different amounts of Bax can have different effects, even though Bax activates the same PT mechanism, i.e., a small amount of Bax induces a transient

PT with cytochrome *c* release and  $\Delta\psi$  loss that can be reversed subsequently, whereas a larger amount of Bax induces a complete PT with irreversible  $\Delta\psi$  loss. The PT-dependent process, however, need to be assessed carefully because the interaction of Bax/Bak with VDAC in cells might not trigger all of the changes seen during the PT in studies on isolated mitochondria. For example, mitochondrial swelling might be an extreme reaction to the *in vitro* PT that may not occur in actual cells. Furthermore, how extensive the PT actually is during apoptosis might vary between cell types.

How does Bax/Bak induce  $\Delta\psi$  loss? In addition to being essential for cytochrome *c* release, the VDAC has been shown to be required for apoptotic  $\Delta\psi$  loss in yeast mitochondria,<sup>42</sup> whereas ANTs are dispensable.<sup>43</sup> Studies using the neutralizing anti-VDAC antibodies have revealed that the VDAC is essential for Bax/Bak-induced  $\Delta\psi$  loss in isolated mammalian mitochondria.<sup>62</sup> These results suggest that the interaction of Bax/Bak with the VDAC may trigger  $\Delta\psi$  loss. Since the VDAC is a component of the PT pore, it probably communicates with other channels on the inner membrane, and opening of the VDAC sufficiently for cytochrome *c* release might trigger the opening of inner membrane channels, resulting in  $\Delta\psi$  loss. Bax/Bak directly interacts with ANT<sup>42,60</sup> and also possibly with several ANT-like proteins in the mitochondria, such as the Pi/OH<sup>-</sup> antiporter and uncoupling proteins, and these channels might be directly involved in  $\Delta\psi$  loss. It may be that Bax/Bak initially interacts with the VDAC and then translocates to the inner membrane.<sup>60</sup> It also remains possible that Bax might induce  $\Delta\psi$  loss via a totally different mechanism, which might be indirectly regulated by the PT pore.

Since different components of the PT pore complex could interact with each other, involvement of this pore in apoptotic mitochondrial changes could also explain how the ANT inhibitor bonkrekic acid (which is assumed to close the ANT channel) and cyclosporine A (which targets mitochondrial cyclophilin D) can prevent VDAC opening and thereby inhibit cytochrome *c* release. Although Bax induces cytochrome *c* release from isolated mitochondria in both the presence and absence of Ca<sup>2+</sup>, only Bax-mediated cytochrome *c* release occurring in the presence of Ca<sup>2+</sup> is blocked by PT inhibitors. Since the VDAC possesses a Ca<sup>2+</sup>-binding module, Ca<sup>2+</sup> might modify the structure of the channel directly or via other Ca<sup>2+</sup> sensor(s) so that the VDAC comes to interact with other parts of the PT pore complex, and thus, Bax-mediated VDAC opening could be regulated by ANT and cyclophilin D in a Ca<sup>2+</sup>-dependent manner.

### Bax/Bak versus Bid/Bik

Unlike Bax and Bak, BH3-only proteins such as Bid and Bik neither bind to the VDAC nor affect VDAC activity in liposome studies,<sup>55</sup> suggesting that the BH3-only proteins function via a totally different mechanism, via the inhibition of anti-apoptotic Bcl-2 family members or via the activation of pro-apoptotic Bcl-2 family members like Bax and Bak. A third possibility might be supported by the finding that Bid induces conformational changes of Bax and Bak.<sup>70,71</sup> However, support for the first possibility is provided by the finding that

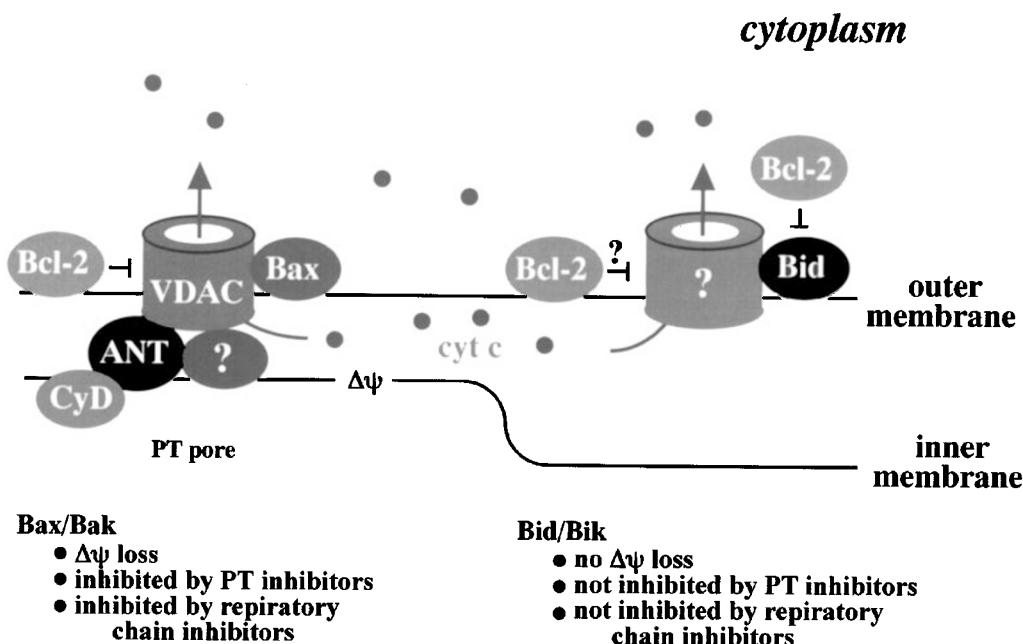
Bax/Bak-induced cytochrome *c* release is substantially different from Bid (or Bik)-mediated cytochrome *c* release under the same experimental conditions<sup>55</sup> (also see Figure 2). One of the notable differences is the concomitant occurrence of  $\Delta\psi$  loss with Bax/Bak-mediated, but not Bid/Bik-mediated, cytochrome *c* release in isolated mitochondria as well as mammalian cells.<sup>55</sup> Consistent with the idea that  $\Delta\psi$  loss is one of the characteristics of the PT, Bax/Bak-mediated (but not Bid/Bik-mediated) cytochrome *c* release from isolated mitochondria can be inhibited by PT inhibitors.<sup>51–53,55</sup> Furthermore, Bax/Bak-mediated cytochrome *c* release is inhibited by respiratory chain inhibitors such as antimycin, protonophore, KCN, and oligomycin, whereas Bid/Bik-induced cytochrome *c* release is insensitive to these drugs,<sup>55</sup> although the detailed mechanism through which Bax/Bak-induced cytochrome *c* release is blocked by respiratory chain inhibitors remains to be determined. Based on these observations, it has been proposed that Bid/Bik probably targets a distinct molecule on the mitochondrial membrane to induce cytochrome *c* release.<sup>55</sup> Although there is considerable variation in the primary structures of BH3-only proteins, their tertiary structure might be sufficiently similar to target the same molecule on the mitochondria, given that the 3D structure of Bid is extremely similar to that of Bcl-x<sub>L</sub> although primary sequence homology only exists within the BH3 domain.<sup>72,73</sup> Since Bid/Bik-induced cytochrome *c* release is prevented by Bcl-2/Bcl-x<sub>L</sub>, these anti-apoptotic Bcl-2 family members might form heterodimers with Bid/Bik to inhibit their activity or directly close the Bid/Bik target channel in a similar manner as occurs with the VDAC (Figure 2).

Given the recent view that different apoptotic signals probably activate different pro-apoptotic Bcl-2 family members,<sup>6</sup> induction of  $\Delta\psi$  loss by Bax/Bak (but not by Bid/Bik) might also explain the previous controversial observations that  $\Delta\psi$  loss is associated with some forms of apoptotic cell death but not others, assuming that different pro-apoptotic Bcl-2 family members may play a major role in certain modes of apoptotic cell death.

### VDAC-Bax channel versus Bax channel: Is one favored over the other?

Given that two models exist for apoptotic cytochrome *c* release involving specific channels,<sup>46</sup> i.e., (1) a channel formed by Bax alone and (2) a channel formed from the VDAC plus Bax, should one model be favored over the other in the light of our current knowledge?

- (1) Independent action of Bcl-2/Bcl-x<sub>L</sub> and Bax/Bak: As discussed above, Bcl-2/Bcl-x<sub>L</sub> and Bax/Bak can function independently from each other. If Bax (or another pro-apoptotic Bcl-2 family member), itself, forms a large pore responsible for cytochrome *c* release, how is Bcl-2/Bcl-x<sub>L</sub> able to prevent this process independently from Bax or without forming heterodimes with Bax? An independent action of anti-apoptotic Bcl-2/Bcl-x<sub>L</sub> and pro-apoptotic Bax/Bak is more favorable for a model of VDAC- or other types of mitochondrial channel-mediated cytochrome *c*



**Figure 2** Functional interaction of Bcl-2/Bcl-x<sub>L</sub> and Bax/Bak, but not Bid/Bik, with the VDAC. Bax/Bak opens the VDAC to induce cytochrome *c* release, while Bcl-2/Bcl-x<sub>L</sub> closes this channel. Bid/Bik does not interact with the VDAC, but probably has the ability to open an unidentified channel(s) that is involved in cytochrome *c* release. The VDAC is a component of the PT pore, which would explain the concomitant induction of  $\Delta\psi$  loss by Bax/Bak and inhibition of Bax/Bak-induced cytochrome *c* and  $\Delta\psi$  loss by PT inhibitors. Bcl-2/Bcl-x<sub>L</sub> inhibits Bid/Bik-induced cytochrome *c* release, probably through heterodimerization with Bid/Bik or by closing an unidentified channel(s)

release. Since yeast mitochondria lack Bcl-2 family proteins, but still have a mechanism for cytochrome *c* release that is Bcl-2/Bcl-x<sub>L</sub>-inhibitable<sup>43</sup> and VDAC-dependent,<sup>42,43</sup> there must be a way to open the VDAC and release cytochrome *c* independently of any of proteins from the Bcl-2 family. A similar mechanism is probably conserved in mammalian mitochondria, underlying the independent action of Bcl-2/Bcl-x<sub>L</sub> and Bax/Bak.

- (2) If Bax (or another pro-apoptotic Bcl-2 family member) forms a large pore responsible for cytochrome *c* release, Bax as well as BH3-only proteins would also be expected to induce cytochrome *c* release from yeast mitochondria. However, only Bax/Bak (and not Bid/Bik) induces cytochrome *c* release from yeast mitochondria<sup>42</sup> (our unpublished observations), making it unlikely that Bid/Bik itself forms a large pore in the mitochondrial membrane that allows cytochrome *c* release.
- (3) Although Bax readily forms ion channels in a synthetic lipid membrane, it does not form its own channel in liposomes incorporating the VDAC,<sup>64</sup> which represent the outer mitochondrial membrane better than does a synthetic lipid membrane.

In addition to our observations obtained with VDAC-deficient yeast cells and anti-VDAC antibodies, these findings also favor the VDAC-Bax channel model over the Bax channel model of cytochrome *c* release. The data also suggest that a target molecule for Bid/Bik probably exists on mammalian mitochondria, which is either missing or less conserved in yeast cells.

## Summary

Apoptotic mitochondrial changes, including release of cytochrome *c* and  $\Delta\psi$  loss, are central to signal transduction in the process of apoptosis. We propose that the Bcl-2 family of proteins, consisting of anti-apoptotic and pro-apoptotic members, functions as a channel regulator to control apoptotic mitochondrial changes rather than providing the actual channel involved, and that the VDAC is one of the major channels directly regulated by the Bcl-2 family. We also suggest that some of the BH3-only proteins may target an unidentified channel on the outer mitochondrial membrane and thus promote cytochrome *c* release.

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