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4 cell life and death**5 Varda Shoshan-Barmatz<sup>a,\*</sup>, Vito De Pinto<sup>b,c</sup>, Markus Zweckstetter<sup>d</sup>,  
6 Ziv Raviv<sup>a</sup>, Nurit Keinan<sup>a</sup>, Nir Arbel<sup>a</sup>7 **Q13** <sup>a</sup>Department of Life Sciences, and the NIBN, Ben-Gurion University, Beer-Sheva, Israel8 <sup>b</sup>Dipartimento Scienze Chimiche, Università di Catania, Catania, Italy9 **Q12** <sup>c</sup>INBB, Rome, Italy10 <sup>d</sup>Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany11 **ARTICLE INFO**12 **Article history:**

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18 **ABSTRACT**

Research over the past decade has extended the prevailing view of the mitochondrion to include functions well beyond the generation of cellular energy. It is now recognized that mitochondria play a crucial role in cell signaling events, inter-organelar communication, aging, cell proliferation, diseases and cell death. Thus, mitochondria play a central role in the regulation of apoptosis (programmed cell death) and serve as the venue for cellular decisions leading to cell life or death. One of the mitochondrial proteins controlling cell life and death is the voltage-dependent anion channel (VDAC), also known as mitochondrial porin. VDAC, located in the mitochondrial outer membrane, functions as gatekeeper for the entry and exit of mitochondrial metabolites, thereby controlling cross-talk between mitochondria and the rest of the cell. VDAC is also a key player in mitochondria-mediated apoptosis. Thus, in addition to regulating the metabolic and energetic functions of mitochondria, VDAC appears to be a convergence point for a variety of cell survival and cell death signals mediated by its association with various ligands and proteins. In this article, we review what is known about the VDAC channel in terms of its structure, relevance to ATP rationing,  $\text{Ca}^{2+}$  homeostasis, protection against oxidative stress, regulation of apoptosis, involvement in several diseases and its role in the action of different drugs. In light of our recent findings and the recently solved NMR- and crystallography-based 3D structures of VDAC1, the focus of this review will be on the central role of VDAC in cell life and death, addressing VDAC function in the regulation of mitochondria-mediated apoptosis with an emphasis on structure-function relations. Understanding structure-function relationships of VDAC is critical for deciphering how this channel can perform such a variety of functions, all important for cell life and death. This review also provides insight into the potential of VDAC1 as a rational target for new therapeutics.

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**Abbreviations:** ANT, adenine nucleotide translocase; CypD, Cyclophilin D; Cyto c, cytochrome c; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; HK, hexokinase; IMM, inner mitochondrial membrane; IMS, inter membrane space; LDAO, lauryl-(dimethyl)-amineoxide; OMM, outer mitochondrial membrane; MMP, mitochondrial membrane permeabilization; MPT, mitochondrial permeability transition; NMR, nuclear magnetic resonance; PLB, planar lipid bilayer; PTP, permeability transition pore; ROS, reactive oxygen species; RuR, ruthenium red; VDAC, voltage-dependent anion channel.

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## 1. Historical overview

In 2008, the structure of VDAC was determined almost simultaneously by three different groups, using different techniques (Bayrhuber et al., 2008; Hiller et al., 2008; Ujwal et al., 2008). The importance of this achievement can be best appreciated if one considers that only one other structure of an integral mitochondrial membrane protein responsible for metabolite transport, i.e. the adenine nucleotide translocase (ANT) carrier (Pebay-Peyroula et al., 2003), has been solved to date. The description of the VDAC structure has filled a void that began in 1976, when Schein et al., in analogy with the recently discovered bacterial porin, detected a pore-forming activity in an extract of *Paramecium tetraurelia* mitochondria (Schein et al., 1976). The existence of the mitochondrial porin, as it was then called (Benz, 1985), or VDAC, as it was subsequently renamed (Colombini, 1979), remained a minor research topic since bioenergetists remained focused on their *primum movens* (center of the world), namely the inner mitochondrial membrane. The outer membrane was considered to be just an envelope without any selectivity role. In the 1980s, most of the work on the protein was devoted to its purification from various tissues and organisms (De Pinto et al., 1987a) and to subsequent characterization of its electrophysiological activity in reconstituted bilayers (Benz, 1994). From these studies, a unifying view of VDAC emerged, namely that VDAC is a protein bearing extremely conserved structural and functional features (despite major difference in sequence), given its obviously important role. The physiological characterization of VDAC, moreover, revealed the voltage-dependence channel activity of the protein, i.e. the ability of the pore to sense the electrical potential imposed onto the membrane and respond with partial closure (Benz and Brdiczka, 1992; Rostovtseva and Colombini, 1997). The implications of this finding for mitochondrial physiology were immediately evident, yet the lack of a clear potential across the poorly-studied outer mitochondrial membrane (OMM) delegated the voltage-dependence of VDAC to the long list of the unexplained, unconventional or simply strange features attributed to the protein. Since those years, there has also the discovery of the hexokinase-binding ability of VDAC (Fiek et al., 1982; Linden et al., 1982a), pointing to VDAC as being deeply involved in bioenergetic metabolism.

The next decade of VDAC studies focused on the quest for structure. After the primary sequence was first determined (Mihara and Sato, 1985), the relatively clear alternation of hydrophilic and hydrophobic residues, surely biased by the recent crystallization of the bacterial porins (Garavito et al., 1983) and by the application of the first simple bioinformatic algorithms, fueled to the prediction of VDAC as assuming a β-barrel structure. Still, no unifying prediction was accepted, since results provided by many experimental techniques, such as site-directed mutagenesis (Blachly-Dyson et al., 1990; Thomas et al., 1993) and modification by proteases and antibodies (De Pinto et al., 1991a; Stanley et al., 1995), led to the proposal of different structures (for a recent review, see (De Pinto et al., 2008)). Although crystallization efforts began in these years, the only positive results offered two-dimensional images at the electron microscope level, with insufficient resolution to describe the arrangement of the pore in detail (Guo et al., 1995). At the same time, the explosion in the number of sequenced genomes revealed the distribution of VDAC genes (Blachly-Dyson et al., 1993; Sampson et al., 1997). It was determined that at least three different VDAC genes exist, as well as various pseudogenes. This was also the decade of discovery of VDAC in other sub-cellular compartments (for review, see Bathori et al., 2000). In this realm, another controversial issue in the field arose when the protein sequence of human VDAC was first determined starting from non-mitochondrial material (Thinnies et al., 1989). Unexpectedly, VDAC was also mainly found in the plasma membrane but also detected in other cellular components, like the sarcoplasmic reticulum (Shoshan-Barmatz et al., 1996). Thus, another poorly understood feature of VDAC, namely its presence in the plasma membrane was noted where it was assigned a redox function (Baker et al., 2004) or channel activity (Dermietzel et al., 1994), was assigned, that required further study.

At the end of the last century, a publication appeared in *Nature* that rocked the field when Tsujimoto et al. proposed that VDAC was a central component of the apoptotic machinery (Shimizu et al., 1999). This report turned the spotlight onto a protein previously considered to be responsible for the almost free permeability of the OMM. As a result, most of the research on VDAC began to address the involvement of the protein in apoptosis and, especially in the permeability transition pore (PTP), that structure responsible of the escape of pro-apoptotic factors from the inter-membrane space (IMS), a role seemingly natural for VDAC. Although the PTP complex has never been isolated, several proposals assigned VDAC, together with the ANT carrier and cyclophilin, as comprising this complex, drawing further attention to VDAC as a pore-forming

protein. Surprisingly, a recent report has cast doubt on the presence of VDAC in the PTP, since deletion of the three VDAC genes did not influence cell death (Baines et al., 2007). This controversial finding will be considered later.

With the structure of VDAC now available, many earlier hypotheses and proposals can be reconsidered. For example, interactions of VDAC with Bcl-2 family members or other proteins found to bind to the VDAC can, moreover, be analyzed. Structural-functional relationships of VDAC can be studied in unprecedented detail, a long-sought goal especially important for the determination of the mechanistic basis of the voltage- (and pore-) gating of the protein. Finally, differences between mitochondrially-targeted VDAC and the same protein targeted to other membranes can be approached.

## 2. The VDAC protein

### 2.1. VDAC purification: a common pattern

Soon after the discovery of a channel-forming component in mitochondria of *Paramecium aurelia* (Schein et al., 1976) and the finding that the outer mitochondrial membrane of a variety of cells contained the newly-defined voltage-dependent anion-selective channel (Colombini, 1979), researchers labored towards isolating the protein, with the aim of characterizing its functional and structural features.

The purification protocols developed to isolate VDAC from various tissues encountered an apparent contradiction. Although the primary structure of the protein predicts a rather polar product (about 50% of residues are hydrophilic), the hydrophobicity of the channel-forming unit is very high. Indeed, it is even higher than that of the total mitochondrial membrane protein pool, including the inner membrane carriers, which present considerably lower polarity than do the VDACs. As a demonstration of this unique feature of VDAC, it was shown that non-ionic detergents with low HLB numbers (corresponding to the hydrophilic/lipophilic balance number) solubilize VDAC better than do the total mitochondrial membrane protein pool (De Pinto et al., 1989b).

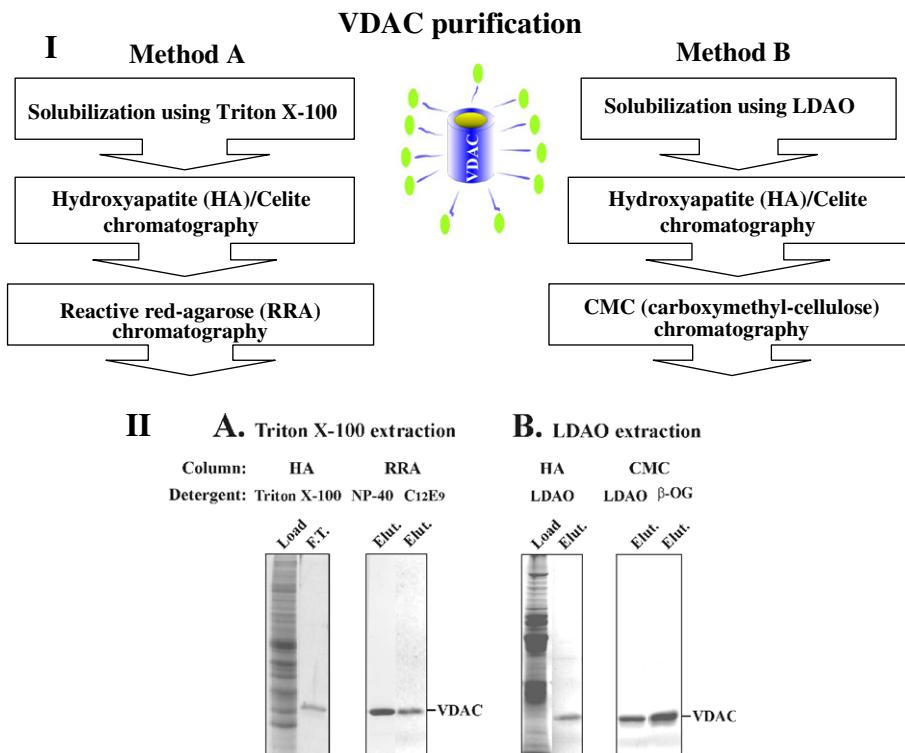
Several different protocols for VDAC purification from tissues, cells or isolated mitochondria were established. VDAC was purified from different membranes, such as rat liver mitochondria (Colombini, 1983; de Pinto et al., 1987b; Gincel et al., 2001; Linden et al., 1982b), human lymphocytes plasma membrane (Thinné et al., 1989), plasma membrane caveolae-related domains (Bathori et al., 1999), skeletal muscle sarcoplasmic reticulum (Shoshan-Barmatz et al., 1996), synaptosomes isolated from sheep or rat brain (Gincel et al., 2000) and from electric organ (Shafir et al., 1998). The purification procedure is clearly dependent on the detergent used. In general, VDAC purification involves protein solubilization with detergent followed by column chromatography. The early protocols started with purified OMM (Linden et al., 1982b), while later, crude or purified mitochondrial membrane preparations were used. Despite the former protocols being conceptually more straightforward, the latter procedures allowed an enormous increase in the yield of purified material and are simple, in comparison with the cumbersome outer membrane purification steps previously required.

The OMM or mitochondria can be dissolved with non-ionic detergents, such as Triton X-100. The extract is then subjected to various chromatographic steps that rely on ionic-exchange materials, like DEAE-Sepharose or CM-Sepharose (Linden et al., 1982b; Roos et al., 1982). The introduction of hydroxyapatite (HTP) was a very important step in VDAC purification. Its power resides on a combination of selectivity, ionic exchange (HTP is a crystal of calcium phosphate) and the properties of adsorption of targets onto the surface of the resin crystals.

In the presence of non-ionic detergents like Triton X-100, Genapol X-80 or other containing polyoxyethylene tails attached to a hydrophobic group, solubilized VDAC presents the peculiar feature of being eluted without interaction with any stationary phase, as mentioned above, HTP included. This trait has allowed for purification of VDAC from a mixture of total mitochondrial proteins in a single chromatographic step (de Pinto et al., 1987b), rendering the protein easily accessible for further study (De Pinto et al., 1987a, 1989a, 1991b). In brief, mitochondria or mitochondrial membrane pellets obtained by osmotic shock are extracted with low salt buffers containing 2.5–3% Triton X-100 or Genapol X-80. The supernatant obtained upon subsequent centrifugation is applied to a dry hydroxyapatite/Celite (ratio 2:1 (w/w)) column in a single chromatographic step. VDAC elutes just after the void volume of the column (de Pinto et al., 1987b). The HTP-eluted VDAC can be further purified using reactive red agarose (Gincel et al., 2000) (RRA), from which it elutes with 0.3 M NaCl. The RRA column enables concentration of the VDAC protein over 10-fold, reduction of the detergent concentration and exchange of detergents, for example, replacing Triton X-100 with NP-40 or with C<sub>12</sub>E<sub>9</sub> but not O-octylglucoside (OG) or lauryl-(dimethyl)amine-oxide (LDAO) (Shoshan-Barmatz and Gincel, 2003) (see Fig. 1). VDAC can also be purified using other columns to which VDAC does not bind but to which most of the other proteins do, like Affi-Gel 501, a resin specific for reduced thiols (De Pinto et al., 1985), or spermine-agarose (Shoshan-Barmatz et al., 1996).

On the other hand, LDAO solubilization of VDAC enables the use of HTP, to which VDAC binds, and from which it is eluted with 20–24 mM NaPi (De Pinto et al., 1989b). LDAO-extracted, HTP-eluted VDAC can be further purified using carboxymethyl (CM)-cellulose. In the presence of low LDAO concentrations (0.25%), VDAC binds to CM-cellulose, from which it can be eluted with buffer containing 0.4 M NaCl and 0.25% LDAO or 0.5% OG (Shoshan-Barmatz and Gincel, 2003), see Fig. 1.

Insight into the mechanism allowing VDAC solubilization was obtained after a systematic survey of the detergents available for membrane solubilization at the beginning of the 1990s. It was found that the factors causing a lack of VDAC interaction with the chromatographic materials used to date was the length of the hydrophilic moiety of the detergent (De Pinto et al., 1989b). Detergents with a long polar arm form micelles embedding most of the VDAC structure. Detergents with a



**Fig. 1.** VDAC purification using different detergents and column chromatography. I. Schematic diagram outlining the different VDAC purification steps used. Membrane-solubilized VDAC with its bound detergent is applied to resins and eluted, as described in B. II. VDAC purification as described in (Shoshan-Barmatz and Gincel, 2003) is shown following SDS-PAGE and staining with Coomassie Blue. Rat liver mitochondria (200 mg of protein) were incubated for 30 min at 0 °C (5 mg/mL) in a solution containing 10 mM Tris, pH 7.4, and 3% Triton X-100 (A) or 2% LDAO (B). After centrifugation at 44,000g for 30 min, the extract was applied to a dry hydroxyapatite/Celite (2:1 w/w) column (0.1 g/mg protein) and eluted with a buffer containing 10 mM Tris-HCl, pH 7.4, and 3% Triton X-100 (A) or 10 mM Tris, pH 7.4, 2% LDAO, 20 mM Na<sub>2</sub>PO<sub>4</sub> and 50 mM NaCl (B). The VDAC-containing fractions were collected, the detergent and diluted to 1% Triton X-100 (A) or 0.5% LDAO (B). The Triton X-100 containing sample was loaded onto either reactive red-agarose column preequilibrated with 10 mM Tris-HCl, pH 7.4, containing 0.4% Nonidet P-40 or 0.5% C<sub>12</sub>E<sub>9</sub> (A). The LDAO containing sample was loaded onto CM-cellulose preequilibrated with 10 mM Tris-HCl, pH 7.4, and either 0.25% LDAO or 0.5% n-octyl-β-D-glucopyranoside (β-OG). The loaded column was washed with the same equilibration buffer, and VDAC was eluted with the same buffer also containing 0.4 M NaCl. Figure was adapted from (Shoshan-Barmatz and Gincel, 2003) with permission.

shorter polar arm, like LDAO, allow the binding of the protein to the resin yet demand elution with solutions of increasing ionic strength (De Pinto et al., 1989b; Palmieri and De Pinto, 1989). These findings provided information on the native structure of VDAC, suggesting that the protein is more embedded in the bilayer than is any other *trans-membrane* carrier, and also allowed for detection of cholesterol molecules permanently attached to the protein (De Pinto et al., 1989b).

Another important point requiring attention is the dilution of the mitochondrial membranes in low ionic strength, detergent-containing buffer. The combination of these two factors indeed regulates the solubilization of membrane phospholipids, together with VDAC. This point was experimentally demonstrated, when, starting from the same mitochondria preparation, HTP chromatography alternatively yielded purified VDAC or various metabolite carriers simply by raising the concentration of the phospholipids present, including added cardiolipin (Bisaccia and Palmieri, 1984; de Pinto et al., 1987b).

The three VDAC isoforms, VDAC1, VDAC2 and VDAC3, were also purified using Triton X-100 and HTP/Celite. Triton X-100 extracts of whole spermatozoa were applied to HTP/Celite, yielding an eluate containing VDAC2 and traces of VDAC3 and VDAC1 (Menzel et al., 2009).

The various purification procedures described above have permitted VDAC purification from numerous tissues and organisms (for review, see (Benz, 1994)). These purified proteins were characterized from the electrophysiological point of view and provided preliminary structural information (Benz, 1994; De Pinto et al., 1987a). The introduction of recombinant DNA technology not only permitted the identification of VDAC genes and transcripts in all eukaryotes but also allowed for the isolation and purification of rare or difficult to purify VDAC isoforms, as well as massive production of recombinant VDAC for crystallization efforts.

Purification of rare or difficult to purify VDAC was pursued in two ways. In one experimental approach, the VDAC coding sequence was introduced into a *Saccharomyces cerevisiae* (*S. cerevisiae*) shuttle vector. The introduced protein is expressed in cells generally lacking VDAC1 and targeted to mitochondria, where the recombinant protein can be isolated following detergent extraction and HTP chromatography. In this manner, the first functional information about alternative isoforms of

mammalian VDACs was obtained (Blachly-Dyson et al., 1989, 1993; Xu et al., 1999). The drawback of this procedure, intended to satisfy the folding and post-translational modification needs of eukaryotic proteins, is low yield. Alternatively, VDAC has been expressed in *Escherichia coli* and isolated through the use of introduced purification tags. This procedure yields enormous amounts of protein and for this reason was adopted by those groups that recently used NMR or crystallization methods to determine the VDAC structure (Bayrhuber et al., 2008; Hiller et al., 2008; Ujwal et al., 2008). The purification tags can be cleaved after the purification of the protein via affinity chromatographies, including Ni-NTA chromatography. The influence of a short added polyhistidine tag was found to be only marginal in terms of recovery of active protein (Aiello et al., 2004; Popp et al., 1996; Ujwal et al., 2008). The *E. coli*-expressed protein is found within inclusion bodies and needs to be dissolved in 1% SDS or high concentrations of urea. Problems associated with the expression of eukaryotic VDAC in a bacterial host have been solved in various ways. For example, adapting unfolding and refolding protocols that consider the presence of lipids that are naturally present in the functional unit (e.g. cholesterol (De Pinto et al., 1989b) or ergosterol for *Neurospora crassa* (Freitag et al., 1982) VDAC) or of non-ionic detergents in the protein solution (Engelhardt et al., 2007; Koppel et al., 1998; Popp et al., 1997; Ujwal et al., 2008) can be considered. Indeed, because of the artificial process of refolding in the presence of detergents, the proposed VDAC1 structure has been questioned (Colombini, 2009).

## 2.2. Sequences of the VDAC proteins

Since the first mitochondrial porin was cloned and sequenced from yeast (Mihara and Sato, 1985), many sequences have been obtained by recombinant DNA technology (Blachly-Dyson et al., 1993; Kleene et al., 1987; Messina et al., 1996; Troll et al., 1992). The porin 31 HL sequence, obtained from human B-lymphocytes, was first determined at the amino acid level and then confirmed by analysis of the corresponding cDNA (Thinnes et al., 1989). The alignment of primary sequences of VDAC1 obtained from sources as distant as yeast, *Dictyostelium discoideum* and man indicates a clear relationship but a very low level of sequence conservation (Benz, 1985). The most remarkable trait seen in all four porins is the GLK triplet near amino acid 90. Otherwise, the degree of identity between porins is rather small over large stretches of the aligned porin sequences. This suggests that the β-barrel structure of membrane channels tolerates extensive amino acid variations without substantial alterations in secondary structure or function.

No identity has been detected between primary sequences of mitochondrial and bacterial porins, especially those of phototrophic bacteria, which are related to the ancestor of mitochondria. On the other hand, the structure and function of bacterial porins as channels are clearly similar to those of the mitochondrial porins, since they both contain a pre-dominant anti-parallel β-barrel forming a hollow cylinder in the OMM and both have similar molecular masses.

## 2.3. Characterization of the VDAC proteins

The mitochondrial porins or VDAC are a quantitatively relevant component of the mitochondrial proteome, calculated to account for up to 50% of the total outer membrane protein in *Neurospora crassa* (Mannella and Bonner, 1975). More recently, VDACs were considered to comprise approximately 0.4% of the total mitochondrial protein population (Yamamoto et al., 2006). The use of purification protocols allowed the isolation of mitochondrial porins from mammalian tissues and from cells of lower eukaryotes with high yield (Colombini, 1983; De Pinto et al., 1987b, 1989b, 1985; Freitag et al., 1982; Linden et al., 1982b; Ludwig et al., 1989, 1988; Roos et al., 1982; Troll et al., 1992; Zalman et al., 1980). When analyzed by SDS-gel electrophoresis, all mammalian porins showed a very similar apparent molecular mass, estimated as 30 kDa (Linden et al., 1982b) or 35 kDa (De Pinto et al., 1985). Comparison of the electrophoretic migration of mammalian VDACs showed them to behave identically, while porins from yeast and *P. aurelia* showed an apparently lower or higher molecular mass. Peptide maps of mammalian VDACs further showed these to be similar, as did the cross-reactivity towards anti-sera raised against bovine heart-purified VDACs, indicating high sequence homology among mammalian VDACs, yet distinctiveness of VDACs in yeast and *Paramecium* (De Pinto et al., 1987a). The functional analysis showed, instead, that VDACs are highly conserved in term of activity, notwithstanding the structural differences revealed at the time (De Pinto et al., 1987a).

It was found that the protein purified and analyzed in several laboratories over the course of two decades was essentially the same orthologous isoform that was later called VDAC1. Most information available is related to this isoform, as it is the most abundant isoform in most cells. Real time PCR experiments demonstrated that in model HeLa cells, VDAC1 is 10 times more abundant than is VDAC2 and 100 times more prevalent than VDAC3 (De Pinto et al., 2010). The predominance of the VDAC1 isoform was also shown by indirect immunological methods (Yamamoto et al., 2006).

The purification of the other VDAC isoforms has been pursued only recently, starting from tissues almost devoid of the VDAC1 isoform, such as spermatozoa (Menzel et al., 2009). Native VDAC2, in particular, has been obtained from this source.

A proteomic survey of the features of mammalian VDAC isoforms showed that VDAC1 displays a lower electrophoretic migration than expected (Yamamoto et al., 2006), as previously noted (De Pinto et al., 1985), whereas VDAC2, despite a higher molecular weight, has the same mobility as VDAC1, while VDAC3 has the highest mobility in SDS-PAGE (Yamamoto et al., 2006). These results were confirmed upon purification of VDAC isoforms from bovine spermatozoa (Menzel et al., 2009). It was demonstrated that when a Triton X-100-solubilized mitochondrial extract was applied to HTP/Celite, the eluate contained three bands that were further separated in 2D electrophoresis, showing that band 2 contained VDAC1 (traces) and VDAC2, while VDAC3 was the faster-migrating band. Interestingly, VDAC2 was also identified in the slower band 1,

**Table 1**

Post-translational modifications of mammalian VDAC proteins.

Modification	Sequence position of the modified amino acid			References
	VDAC1	VDAC2	VDAC3	
N-acetylalanine		2		Kayser et al. (1989), Gauci et al. (2009)
N6-acetyllysine	20, 28, 61, 224, 266	31, 39, 72, 74	20, 28, 61, 63, 90	Distler et al. (2007)
Phosphoserine	13, 137, 101, 104	115	241	Distler et al. (2007), Choudhary et al. (2009), Dephoure et al. (2008), Zahedi et al. (2008), Olsen et al. (2006)
Phosphothreonine	107	236	195	Dephoure et al. (2008), Zahedi et al. (2008), Olsen et al. (2006), Distler et al. (2007), Wang et al. (2008)
Phosphotyrosine	67, 195			Rush et al. (2005), Distler et al. (2007)

Q7 indicating the presence of variants of the same VDAC2 isoform, at least in this cell type (Menzel et al., 2009). Such 2D analysis  
 Q8 always showed that the purified protein is found in more spots (Liberatori et al., 2004; Linden et al., 1982b; Menzel et al.,  
 Q9 2009). This mobility pattern can result from post-translational modifications of VDAC isoforms (see Table 1). Most of these  
 315 modifications have been discovered in large proteomic surveys aimed at the evaluation of specific kinds of modifications  
 316 with functional significance. Analysis of the amino acid sequence of VDAC1 showed that the first methionine is deleted,  
 317 while the second amino acid, an alanine, is acetylated (Gauci et al., 2009; Kayser et al., 1989). Among the other post-trans-  
 318 lation modifications reported in the literature are phosphorylations of serine, threonine and tyrosine (Distler et al., 2007)  
 319 (see Table 1 and Section 12), as well as acetylation of lysines (Choudhary et al., 2009).

320 In yeast, another proteomic survey showed that only active VDAC in this organism is subject to specific carbonylation in  
 321 conditions of oxidative stress (O'Brien et al., 2004), a finding that agrees with a similar result obtained in gerbil synapto-  
 322 somes (Mello et al., 2007). Currently, the influence of these modifications on the activity of VDAC proteins is not clear.

323 The presence of VDACs in spermatozoa is another issue deserving attention. Mammalian spermatozoa are highly differ-  
 324 entiated haploid cells that are unable to synthesize proteins *de novo*. It has been shown that VDAC2 and VDAC3 are present in  
 325 testis (Ficarro et al., 2003; Hinsch et al., 2001) and in spermatozoa (Hinsch et al., 2004; Menzel et al., 2009). In contrast,  
 326 VDAC1 was exclusively localized in Sertoli cells (Hinsch et al., 2001). VDAC2 and VDAC3 have been shown to be present  
 327 in the ODF (outer dense fibre) of the sperm flagellum, an extra-membranous localization (Hinsch et al., 2004). ODF is indeed  
 328 a highly insoluble component of the midpiece and the principal component of the sperm tail. The importance of VDAC in  
 329 spermatozoa has been outlined by Sampson et al. (2001), who showed that VDAC3-lacking mice were male-infertile because  
 330 their mitochondria and the axoneme of their sperm were structurally altered.

#### 334 2.4. Phylogenetic analysis of VDAC sequences

335 Phylogenetic trees were generated for porin sequences available in public databases (Al Bitar et al., 2003; Saccone et al.,  
 336 2003). The most recent phylogenetic analysis of VDAC sequences considered 244 protein sequences and used a combination  
 337 of neighbor-joining and Bayesian methods (Young et al., 2007). These analyses indicated an early separation of VDAC se-  
 338 quences among fungi, plants and animals. The branching patterns of these three monophyletic groups suggest that the ani-  
 339 mal and fungal VDACs are derived from a common ancestor. From phylogenetic analysis, it also appears that the evolution of  
 340 VDACs followed the typical pattern for a highly conserved sequence, since its behavior replicates the expected phyletic posi-  
 341 tion of rRNA (Young et al., 2007).

342 The evolution of metazoan VDAC genes shows the appearance of paralogous genes. In chordates, there are three clades  
 343 corresponding to the VDAC1, 2 and 3 isoforms. There is general agreement among different groups studying VDAC that  
 344 VDAC3 can be considered as the oldest protein. Divergence between VDAC3 and VDAC1/2 was estimated to occur  
 345  $365 \pm 60$  million years (MY) ago, while the divergence between VDAC1 and VDAC2 was estimated to have occurred  
 346  $289 \pm 63$  MY ago (Saccone et al., 2003; Young et al., 2007). VDAC1, apparently the most successful isoform, would be the  
 347 most recent porin. In invertebrates, only one VDAC gene is present, with the exception of the phylum Arthropoda, where  
 348 *Drosophila melanogaster* (*D. melanogaster*) contains four genes for porin (Graham and Craigen, 2005; Oliva et al., 2002). In this  
 349 case, the four genes appear to have evolved through a lineage-specific duplication event, giving rise to a set of orthologs  
 350 (Young et al., 2007). Fungal porins are generally present in a single copy. Paralogs are present in the cluster that includes  
 351 *Saccharomyces*, where they are suspected to be a remnant of earlier genome duplication events (Young et al., 2007).

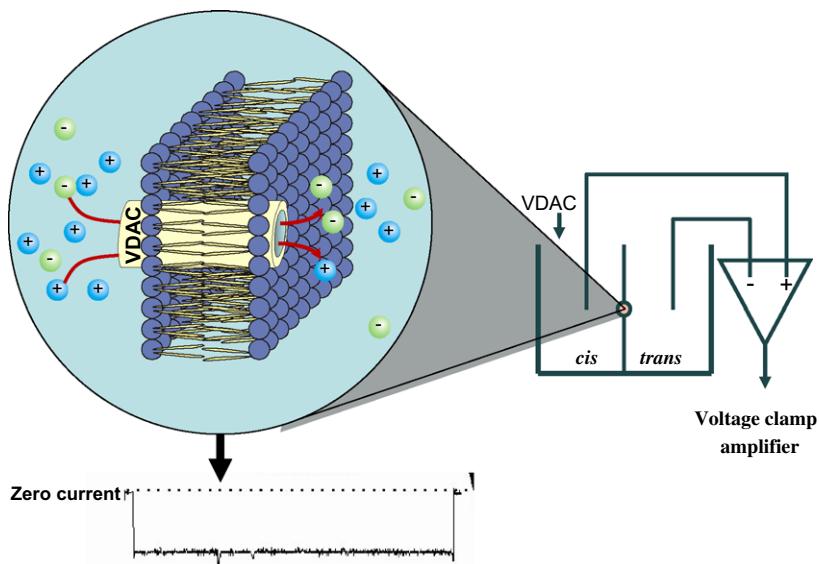
352 Analysis of plant porin sequences has led to the proposal that the duplications that gave rise to the multigene families of  
 353 VDACs probably occurred independently in animals and plants, after the divergence of the two groups (Al Bitar et al., 2003;  
 354 Wandrey et al., 2004). Furthermore, in plants, VDAC duplications probably happened after the divergence of monocotyledons  
 355 and dicotyledons. This means that the number of plant isoforms is unrelated to their animal homologues.

#### 356 3. Channel activity of VDAC

##### 357 3.1. Methods employed for the study of VDAC channel activity

358 As for any membrane protein responsible for an exchange of solutes across a membrane, the functional properties of  
 359 VDAC have been examined in reconstituted systems based on artificially prepared phospholipid bilayers. Two main

## Planar Lipid Bilayer



**Fig. 2.** Schematic presentation showing planar lipid bilayer reconstitution and the channel conductance assay system. f VDAC channel activity is measured following its reconstitution into a PLB and measuring the current passing through the channel when a salt concentration gradient or voltage is applied across the bilayer. The membrane serves as a capacitor, while the ions carry the current. The *cis*-side is defined as the compartment to which VDAC was added. Currents were recorded under voltage-clamp using a Bilayer Clamp BC-535B amplifier (Warner Instruments, Hamden, CT). Currents were measured with respect to the *trans* side of the membrane (ground). The currents were low-pass-filtered at 1 kHz, and digitized on-line using a Digidata 1440-interface board and PCLAMP 10.2 software (Axon Instruments, Union City, CA).

360 membrane systems have been used to study the pore-forming activity of VDACs, i.e. vesicles or planar lipid bilayers (PLB).  
 361 The former, historically used to detect the passage of labeled molecules, is less often employed, given the advantage of the  
 362 smaller amount of active protein required when using the PLB system, all the while producing a refined and large set of infor-  
 363 mation on channel.

364 The reconstitution of VDAC into liposomes loaded with high ( $[^3\text{H}]$ -dextran) and low ( $[^{14}\text{C}]$ -sucrose) molecular weight  
 365 molecules allowed the release of the smaller molecule but not larger ones, indicating specific leak out of the vesicles (Linden  
 366 et al., 1982a; Zalman et al., 1980).

367 A proteoliposome swelling-shrinking assay, as detecting change in the absorbance of the solution, can also be used to fol-  
 368 low VDAC permeability (Colombini, 1980). A solution containing a non-permeable solute leads to liposome shrinking, while  
 369 incubation in solution containing a permeable solute results in liposome swelling. Such experiments, in which carbohydrates  
 370 and polyethylene glycols (PEG) of different molecular masses were used, suggest that the cut off of the pore to be about  
 371 6.8 kDa. From these results, the diameter of the mitochondrial pore was calculated to be around 3–4 nm (Colombini, 1980).

372 The most widespread method for the characterization of the pore-forming activity of mitochondrial VDAC is reconstitu-  
 373 tion of the pore into a PLB that separate two aqueous compartments (see Fig. 2). The activity of the channel is reflected in the  
 374 flow of ions (i.e. current) through a membrane that is otherwise a barrier to ion flow. Thus, the set-up also requires a source  
 375 of continuous current (i.e. a battery), and a more or less sophisticated detector system, able to amplify and record low  
 376 picoamperes currents. This system is so efficient that the activity of even a single channel can be detected, allowing study  
 377 at the molecular level.

378 The channel activity of VDAC in a phospholipid bilayer can be reconstituted in one of two methods. The Montal–Mueller  
 379 method (Benz et al., 1975; Colombini, 1987; Montal and Mueller, 1972) does not require solubilization of phospholipids in  
 380 solvent, since the bilayer is first formed at the air/water interface. The Mueller–Rodin method (Mueller et al., 1962; Benz  
 381 et al., 1979) employs, instead, a solution of phospholipids in organic solvent such as *n*-decane, that is “painted” onto a little  
 382 hole separating the aqueous compartments. It has been debated which method is more physiologically relevant. In the case  
 383 of VDAC, the two PLB formation techniques yield similar results (Colombini, 1979; Roos et al., 1982). For reconstitution, puri-  
 384 fied VDAC (10 ng/ml to 1  $\mu\text{g}/\text{ml}$ ) is added to one of the compartments (termed the *cis*-side) (see Fig. 2). Upon VDAC insertion  
 385 into the membrane, the current is increased by several orders of magnitude within 15–20 min (Colombini, 1979; Roos et al.,  
 386 1982). Such experiments suggest spontaneous insertion of detergent-solubilized VDAC into the phospholipid bilayer, in the  
 387 active conformation. In this artificial system, detergent-solubilized VDAC protein is stabilized in water by having its hydro-  
 388 phobic regions coated with detergent molecules, although loss of some of this detergent coat may result in a metastable state  
 389 (Li and Colombini, 2002). It has been shown that the insertion of the first channel is random, whereas subsequent insertions

are directed by the previously inserted channel(s) (Li and Colombini, 2002; Marques et al., 2004). Indeed, a VDAC channel in the membrane increases the insertion rate of other VDAC channels by 10 orders of magnitude over the rate of insertion into a region of equivalent area of an unmodified phospholipid membrane (Zizi et al., 1995). This process has been termed auto-directed insertion (Xu and Colombini, 1996).

Patch-clamping has also been applied to the study of VDAC channel activity. Either the whole mitochondrial outer membrane of giant mitochondria from mice kept on a cuprizone diet (Moran et al., 1992; Tedeschi and Kinnally, 1987) or vesicles produced by fusing membranes derived from the outer membrane were assayed (Moran et al., 1992; Tedeschi and Kinnally, 1987; Wunder and Colombini, 1991). While typical electrophysiological features of VDAC were detected in some experiments (Tedeschi and Kinnally, 1987; Wunder and Colombini, 1991), others found channels with smaller conductance values ranging from less than 100 pS to about 530 pS in 0.15 M KCl (Moran et al., 1992). Despite much effort, differences in mitochondrial VDAC physiology between lipid bilayer and patch-clamp experiments have been observed, a point that still needs to be clarified.

### 3.2. VDAC conductance and ion selectivity

Upon addition of mitochondrial VDAC to the bilayer set-up described above, the insertion of pore-forming units is spontaneous and appears as single channel incorporation events. The record of this phenomenon is a typical stepwise trace with discrete steps representing conductance of single incorporation events. These steps are caused by the reconstitution of channels, since they are not observed when only detergents are added to the aqueous phase. Most of the conductance steps are directed upwards. Closing steps are only rarely observed at small trans-membrane potentials of about 10–20 mV. As there are excellent works that have reviewed the electrophysiological features of eukaryotic porins or VDAC1 in fungi and metazoa (Benz, 1994; Colombini, 2007; Colombini et al., 1996; Tedeschi et al., 1989), these which will not be discussed here.

The most frequently obtained values for the single-channel conductance of mitochondrial VDAC in 1 M KCl or NaCl range between 4 and 5 nS (Benz, 1994; Colombini, 1980; De Pinto et al., 1985; Gincel et al., 2000; Mannella et al., 1989). A limited number of small steps are interpreted as sub-states of the pore, indicating that VDAC may also exist in different stable conformations. In the main conductance state, VDAC is permeable to small ions (e.g. Cl<sup>-</sup>, K<sup>+</sup>, Na<sup>+</sup>) yet also to large anions, such as glutamate (Gincel et al., 2000) and ATP (Rostovtseva and Colombini, 1997), and to large cations, such as acetylcholine, dopamine (Gincel et al., 2000) and Tris (Benz et al., 1990).

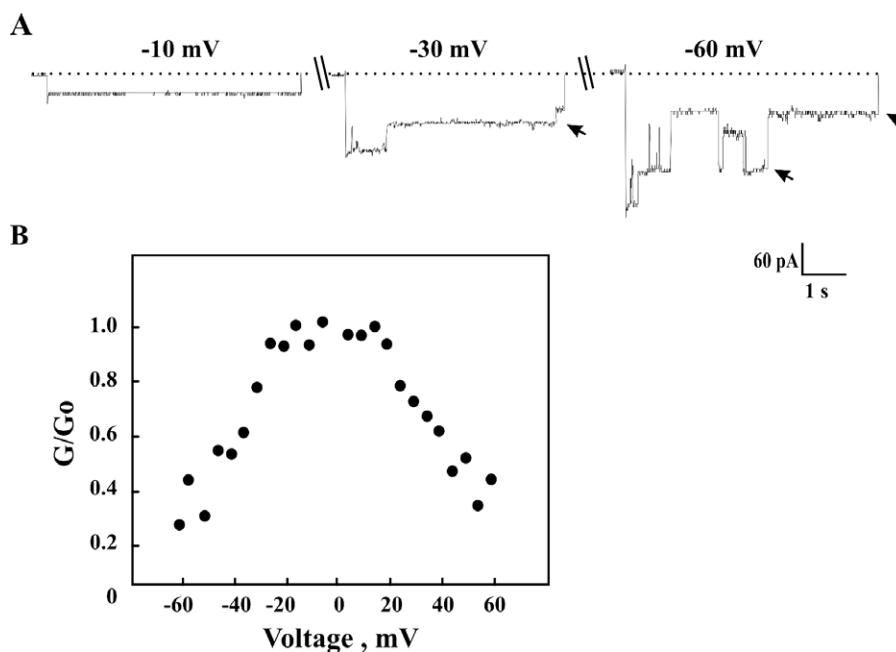
The anion selectivity of the channel, reflected in the protein's name, was originally reported based on a calculated selectivity ratio for the channel of more than 7 for Cl<sup>-</sup> over K<sup>+</sup> (Schein et al., 1976). However, there is now general agreement that the open state of all mitochondrial VDACs characterized thus far are slightly anion-selective for salts composed of equally mobile cations and anions, such as KCl (ratio  $P_{\text{anion}}/P_{\text{cation}} = 2:1$ ) (Gincel et al., 2000). However, the channel also exhibits certain specificity for charged solutes because the single-channel conductance in potassium acetate is somewhat smaller than that in LiCl, despite the same aqueous mobility of lithium ions, as compared to acetate. Recorded measurements of the membrane potential caused by the preferential movement of one sort of ions through the channel allows for evaluation of ionic selectivity in term of ratio  $P_{\text{cation}}/P_{\text{anion}}$  (Goldman-Hodgkin-Katz equation (Benz et al., 1979). The structural data agree that a slight prevalence of positive charged residues line the pore walls, explaining the slight anion selectivity (Ujwal et al., 2008).

One of main aims in measuring single-channel conductance has been to calculate the effective diameter of the pore. This calculation relies upon a simplistic relationship, assuming the pore as being a hollow regular cylinder, leading to an estimated pore diameter of about 1.7 nm. Based on the recently published crystal structure, the pore cavity has been calculated to be 27 × 14 Å at the narrowest point of the channel (Ujwal et al., 2008).

### 3.3. Voltage-dependence of VDAC pores: Open and closed states of the pore

The VDAC channel shows voltage-dependence conductance and ion selectivity. At low voltages (10 mV), the VDAC channel is stable in a long-lived open state (up to 2 h). At high positive or negative potentials (>40 mV), VDAC presents multiple sub-states with different ionic selectivities and permeabilities (Gincel et al., 2000; Hodge and Colombini, 1997). The VDAC channel switches to closed states when the trans-membrane voltage exceeds 20–30 mV (Fig. 3). Closing events become increasingly frequent with rising voltage, with some differences between the various VDAs being noted. For example, application of 35 mV was sufficient to decrease the initial conductance of yeast VDAC to 50% (Ludwig et al., 1988), while 90 mV had to be applied to membranes containing rat brain VDAC to obtain the same effect (Ludwig et al., 1986). It is important to note that voltage-dependence is a phenomenon observed, until now, only in *in vitro* experiments. It is not known whether a membrane potential exists across the OMM. Nevertheless, it has been calculated that the mitochondrial inner membrane (IMM) potential might influence the OMM, depending on the distance between them or in special situations, as at contact sites (Brdiczka, 1991).

This intriguing voltage-dependent channel activity led to the characterization of the so-called closed state of the pore. The average single-channel conductance of the pore is about half the conductance of the open state and shows reduced ion permeability. It has been suggested that the closed state is cation-selective, since for the K-MES combination (a mobile cation combined with a less mobile anion), conductance in the open and in the closed states differs only little. The difference between the open and closed states is more substantial for Tris-HCl (a mobile anion combined with a less mobile cation). This result suggests that the channel indeed is cation-selective in the closed state.



**Fig. 3.** VDAC single and multi-channel activity. Bilayer-reconstituted VDAC single and multi-channel activity was assayed as described previously (Gincel et al., 2000). Purified VDAC (approx 1 ng) was reconstituted into a PLB. In A, a typical recording of the activity of VDAC incorporated into a PLB is presented as current traces obtained in response to voltage steps from 0 mV to either -10 mV, -30 mV or -60 mV. In symmetric solution (1 M NaC), when the voltage was changed from -60 to 10 mV, the channel opens and remains so for up to 2 h. However, when the voltage was changed from 0 to -30 mV or -60 mV, the current first increased, due to a greater driving force, but within less than 1 s, the channel closed to a stable low-conducting state (indicated by arrows). At high positive or negative potentials (>40 mV), VDAC possesses multiple substrates. The dashed line indicates the zero current level, while the sub-state of the channel are indicated by arrowheads. In B, the average steady-state conductance of VDAC is presented as a function of voltage. The conductance ( $G_0$ ) at a given voltage was normalized to the conductance at -10 mV ( $G_{max}$ ). Each point is the average of three experiments. This voltage-dependent behavior is well known for VDAC.

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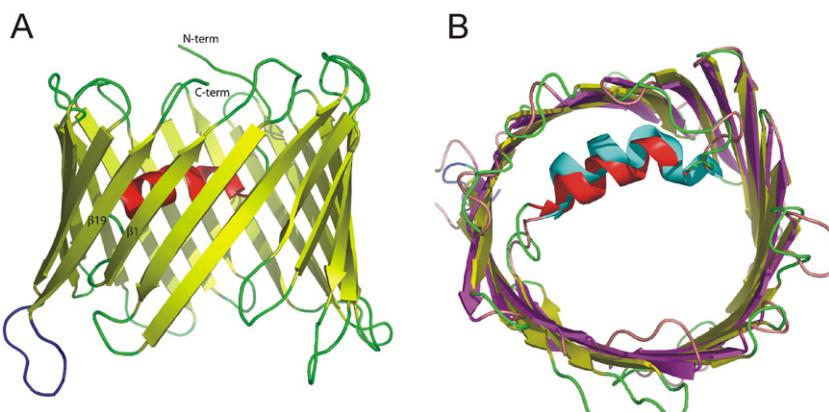
In terms of metabolite flow through the pore, the closed state should hinder the passage of larger molecules, and in particular, of nucleotides. It is assumed that a VDAC-gating process exists and thus, it should be possible to identify charged amino acids and mobile section(s) of the pore that are responsible for the partial pore closure. Electrophysiological recordings, together with simple mathematical interpolation, have estimated that just **two–three** charged residues are involved in the gating process and that the energy needed for channel closure is approximately 7.7 kJ/mol, on the order of one mole hydrogen bond, indicating that channel gating is a low energy process (Benz, 1994).

Numerous studies have focused on the importance of the N-terminal  $\alpha$ -helical segment in channel function. There has been a wide range of predictions as to the functional disposition of this domain, ranging from it forming a segment of the channel wall to acting as the voltage sensor (Colombini et al., 1996; Koppel et al., 1998), to regulating the conductance of ions and metabolites passing through the VDAC pore. It has recently been proposed that the gating of the pore is due to conformational changes or movements of the N-terminal sequence (Abu-Hamad et al., 2009), a 25 residues-long sequence containing  $\alpha$ -helical moieties that lies inside the pore that could move in the open space (Hiller and Wagner, 2009).

#### 4. VDAC structure

##### 4.1. The three-dimensional structure of hVDAC1

In 2008, the three-dimensional structure of isoform 1 of VDAC was determined at atomic resolution by three independent technical approaches (Bayrhuber et al., 2008; Hiller et al., 2008; Ujwal et al., 2008). The structure of human VDAC1 (hVDAC1) was solved in parallel by nuclear magnetic resonance spectroscopy (NMR) (Hiller et al., 2008) and by a novel approach combining nuclear magnetic resonance spectroscopy and X-ray crystallography (Bayrhuber et al., 2008). The three-dimensional structure of VDAC1 from mouse was determined by X-ray crystallography (Ujwal et al., 2008). Mouse VDAC1 differs from human VDAC1 by just four amino acid substitutions, namely threonine 55 to asparagine, methionine 129 to valine, alanine 160 to serine and isoleucine 227 to valine. The three structures are almost identical, featuring a 19-stranded  $\beta$ -barrel and an N-terminal  $\alpha$ -helical region located inside the pore (Fig. 4). As the amino acid sequence of VDAC is highly conserved from yeast to man, it is likely that the overall fold of VDAC and its isoforms is conserved in all eukaryotes (Colombini, 2004).



**Fig. 4.** Three-dimensional structure of VDAC1. A, Side view of the structure of human VDAC1 solved using a combined NMR/X-ray approach. The  $\beta$ -barrel is formed by 19  $\beta$ -strands and the N-terminal helix is folded into the pore interior.  $\beta$ -strands 1–19, the N-terminal  $\alpha$ -helix and the loops are colored yellow, red and green, respectively. The longest loop, which connects  $\beta$ -strands 18 and 19 and is formed by the residue stretch  $^{265}\text{GKNVNAGG}^{272}$ , is highlighted in blue.  $\beta$ -strands 1 and 19 are parallel and close the VDAC1 barrel. N- and C-termini are indicated. B, Top view of a superposition of the NMR/X-ray (PDB code: 2JK4) of human VDAC1 (helix in red) with the crystal structure (PDB code: 3EMN) of mouse VDAC1 (helix in cyan). Figures of this panel were prepared using PyMOL software (DeLano, 2003).

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#### 4.2. The barrel of VDAC1 is formed by 19 $\beta$ -strands

472 The channel pore resembles a slightly elliptical cylinder with dimensions of approximately  $3.1 \times 3.5$  nm in the horizontal  
 473 and approximately 4 nm in the vertical directions (Fig. 4). Similar dimensions for VDAC proteins in the native state have also  
 474 been obtained by high resolution AFM investigations ( $3.8 \times 2.7$  nm diameter) (Goncalves et al., 2007) and electron microscopy studies (diameter of  $\sim 3$  nm) (Guo and Mannella, 1993), both of which relied on *S. cerevisiae* VDAC (scVDAC) in the natural membrane surroundings. hVDAC1 reconstituted into artificial membranes showed identical dimensions (3.7 nm diameter  $\times$  4.3 nm heights) (Dolder et al., 1999). The inner diameter of the pore is approximately  $1.5 \times 1$  nm, therefore leaves space for diffusion of small metabolites.

475 The VDAC1 barrel is formed by an uneven number of 19 strands (Fig. 4). In contrast, bacterial outer membrane proteins  
 476 archetypically show structures with an equal number of strands (Schulz, 2002). The unequal number of strands in the VDAC  
 477 barrel requires one parallel interaction of two adjacent and slightly twisted terminal  $\beta$ -strands ending on the same side ( $\beta$ 1  
 478 and  $\beta$ 19) of the membrane. The average length of these 19 amphipatic  $\beta$ -strands is  $\sim 10$  residues. The average inclination of  
 479 the  $\beta$ -strands, relative to the barrel axis, is  $37^\circ$  and varies between  $27^\circ$  and  $46^\circ$ . At the edges of the barrel, two incomplete  
 480 aromatic girdles separated by a distance of only  $\sim 1.5$  nm on an axis parallel to the membrane normal are present. The 19  $\beta$ -  
 481 strands of VDAC are connected by flexible loops. The longest loop is formed by the residue stretch,  $^{265}\text{GKNVNAGG}^{272}$ , and  
 482 connects  $\beta$ -strands 18 and 19 (Fig. 4).

#### 483 4.3. An N-terminal helical region of VDAC1 is located inside the pore

484 Before the three-dimensional structure of VDAC1 was solved, a wide range of predictions were made towards the structural  
 485 arrangement of the N-terminal 25 residues of VDAC1, ranging from forming a segment of the channel wall (De Pinto  
 486 et al., 1991a; Forte et al., 1987) to being exposed to the cytoplasm (Colombini et al., 1996; Koppel et al., 1998) (see Section  
 487 9.2). All predictions, however, suggested the presence of an  $\alpha$ -helix in the N-terminal region of VDAC. CD and NMR studies  
 488 showed a synthetic peptide corresponding to VDAC1 residues 2–20 exists as an unstructured peptide in aqueous solvent,  
 489 forming a well-ordered  $\alpha$ -helix from residues 5–16 in SDS (De Pinto et al., 2007). In all three solved 3D structures of VDAC1,  
 490 a helical conformation is present within the N-terminal region, which is attached to the channel wall but is not part of it. In  
 491 the combined NMR/X-ray structure of human VDAC1, the helix comprises residues Tyr7 to Val17 and is folded horizontally  
 492 inside the barrel wall approximately at the midpoint of the hydrophobic portion of the membrane (Fig. 4) (Bayrhuber et al.,  
 493 2008). Similar positioning is seen in the crystal structure of mouse VDAC1, however, the helical region is formed by amino  
 494 acids 6–20 and the hydrogen-bonding pattern is broken at Leu-10 and Gly-11, separating the helix into two segments. Residues  
 495 6–9 are attached to the channel wall by hydrophobic interactions involving the methyl groups of leucine 10, valine 143  
 496 and leucine 150. Valine 143 and leucine 150 are the only hydrophobic side chains in the barrel wall pointing to the barrel  
 497 interior. Several hydrogen bond interactions might further stabilize the N-terminus to the barrel wall (Ujwal et al., 2008). On  
 498 the other hand, residues 11–20 are difficult to observe in solution-state NMR, suggesting the dynamic behavior of this seg-  
 499 ment (Bayrhuber et al., 2008; Hiller et al., 2008; Ujwal et al., 2008). Increased mobility in this region could be favored by  
 500 the presence of the helix break at glycine 11 and the presence of multiple glycine residues in the sequence, Gly-21-Tyr-22-Gly-  
 501 23-Phe-24-Gly-25, which connects the  $\alpha$ -helix to strand 1 of the barrel and is highly conserved among mammals. In a lipid  
 502 503 504 505

environment, the N-terminal region is also helical, adopts a well-defined structure and contacts the hydrophobic patch formed by valine 143 and leucine 150 (Schneider et al., *in press*).

#### 508 4.4. Structural mechanism of VDAC gating

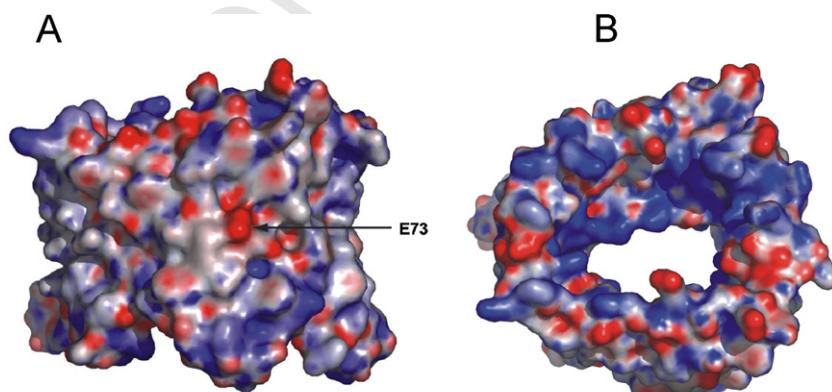
509 In its open conformation, VDAC shows a preference for transporting anions over cations (Colombini et al., 1996). Analysis  
 510 of the 3D structure of VDAC reveals that the exterior of the  $\beta$ -barrel primarily consists of hydrophobic residues that are ex-  
 511 posed to the lipid environment, whereas the interior is extensively hydrophilic (Fig. 5). Fifteen positively-charged residues  
 512 and 11 negatively-charged residues located in the barrel wall point to the interior of the pore. In addition, the pore spanning  
 513 N-terminal  $\alpha$ -helical segment contains three positive (Lys-12, Lys-20, and Arg-15) and two negative (Asp-9 and Asp-16)  
 514 charges. Electrostatic calculations show that the interior of the pore has a higher density of positive versus negative charges,  
 515 potentially favoring the transport of anions in the conformation represented by the 3D structure (Ujwal et al., 2008).

516 In the presence of a slight voltage ( $>30$  mV), VDAC conductance is significantly lowered and the pore becomes weakly  
 517 cationic selective (Schein et al., 1976). Many studies have demonstrated the importance of the N-terminal  $\alpha$ -helical segment  
 518 in voltage gating. For example, several mutations in the  $\alpha$ -helix (conserved Asp-16 and Lys-20) and in  $\beta$ -strands  $\beta$ 1– $\beta$ 5  
 519 (Lys-46, Lys-61, Lys-65 and Lys-84 in scVDAC) affect the voltage-sensing mechanism of scVDAC (Thomas et al., 1993). In  
 520 addition, the presence of the  $\alpha$ -helix inside the VDAC pore (Fig. 4) suggests a strong influence on the exchange of ions  
 521 and overall conductivity.

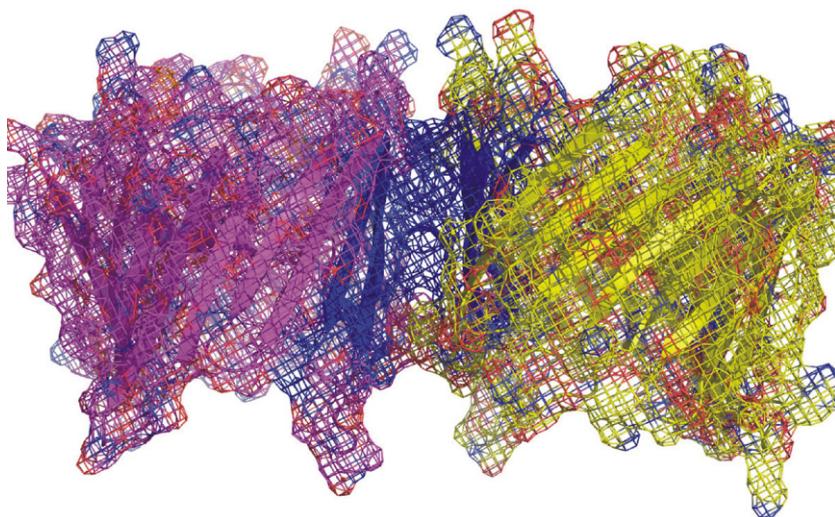
522 Based on the 3D structure of VDAC, different models for voltage-dependent gating in VDAC have been proposed. The first  
 523 class of models emphasizes the blockage of the pore by a movement of the N-terminal helix from the barrel wall towards the  
 524 center of the channel. The movement might involve the complete helix (Ujwal et al., 2008) or only residues 11–20 (Hiller and  
 525 Wagner, 2009), which are difficult to observe in solution-state NMR (Bayrhuber et al., 2008; Hiller et al., 2008). A slight  
 526 movement of the helix is also seen when comparing the structure of hVDAC1 with murine VDAC (Fig. 4). The movement  
 527 of the N-terminal segment might also be coupled to a partial or complete unfolding of the helix. Alternatively, an applied  
 528 electric field may change the favored dipole moment of the helix such that the helix is reoriented and the electrostatic prop-  
 529 erties of the channel are changed. Finally, larger conformational rearrangements upon voltage gating that also involve the  
 530  $\beta$ -barrel have been suggested, based on electron microscopy and electrophysiological data (Peng et al., 1992; Zimmerberg  
 531 and Parsegian, 1986). A conformational instability of the VDAC channel might be favored by the parallel arrangement of  
 532 strands  $\beta$ 1 and  $\beta$ 19 and by the large number of charges in  $\beta$ -strands 1–8 (Fig. 5). Experimental support for conformational  
 533 instability of the N-terminal four to five  $\beta$ -strands of the VDAC1 barrel was provided by hydrogen/deuterium exchange mea-  
 534 surements coupled to NMR spectroscopy, with Glu73 playing an important role in this process (Bayrhuber et al., 2008). In  
 535 addition, solid-state NMR spectroscopy revealed that removal of the N-terminal helix induces a conformational change in  
 536 the barrel for liposome reconstituted human VDAC1 (Schneider et al., *in press*). However, currently no experimental evi-  
 537 dence unambiguously links any of these models to the voltage-gating process.

#### 538 4.5. Orientation of VDAC within the outer mitochondrial membrane

539 Biochemical studies produced mostly conflicting results on the endogenous position of the N and C-termini of VDAC1, i.e.  
 540 whether they are facing the cytosol or the inter-membrane space (De Pinto et al., 1991a; McDonald et al., 2009; Stanley et al.,  
 541 1995). In the 3D structure, the N- and C-termini reside on the same side of the membrane (Fig. 4). In bacterial porins,  
 542 asymmetry in the length of loops connecting the  $\beta$ -strands is characteristic of orientation within the membrane; long loops  
 543 generally point to the extracellular and short turns to the periplasmic side. In the 3D structure of hVDAC1, the asymmetry in



**Fig. 5.** Electrostatic properties of the VDAC1 structure. Side view (A) and top view (B) of the electrostatic potential on the water-accessible surface of murine VDAC (PDB code: 3EMN) at a contour level of  $\pm 15$  kT, as calculated by APBS (Baker et al., 2001). Negative and positive charges are colored red and blue, respectively. In the side view, the position of glutamate 73 is marked.



**Fig. 6.** Dimerization of hVDAC1. Combined surface mesh/cartoon representation of the parallel dimer of hVDAC1 (PDB code: 2JK4). The protomers are shown in yellow and magenta.  $\beta$ -strands of both protomers involved in dimerization are colored blue.

544 the length of the loop regions is less distinct with the longest loop found between  $\beta$ -strands 18 and 19 (Fig. 4). The position of  
 545 the long loop between strands 18 and 19 would thus suggest that the C-terminus of VDAC extends into the inter-membrane  
 546 space. In contrast, antibody binding to a FLAG-epitope (DYKDDDDK) inserted at the C-terminus of scVDAC was equally effi-  
 547 cient in intact and disrupted mitochondria, indicating a cytosolic location of the C-terminus (McDonald et al., 2009). Protease  
 548 treatment suggested that residues 108–119 and 227–229 face the cytosol (Song and Colombini, 1996), which is not possible  
 549 on the basis of the 3D structure of VDAC. Observation of an anti-parallel dimer in crystals of mouse VDAC1 might even  
 550 suggest that VDAC can be present with both orientations in the membrane (Ujwal et al., 2009). Clearly, more studies are  
 551 required to ascertain the orientation of VDAC within the outer mitochondrial membrane.

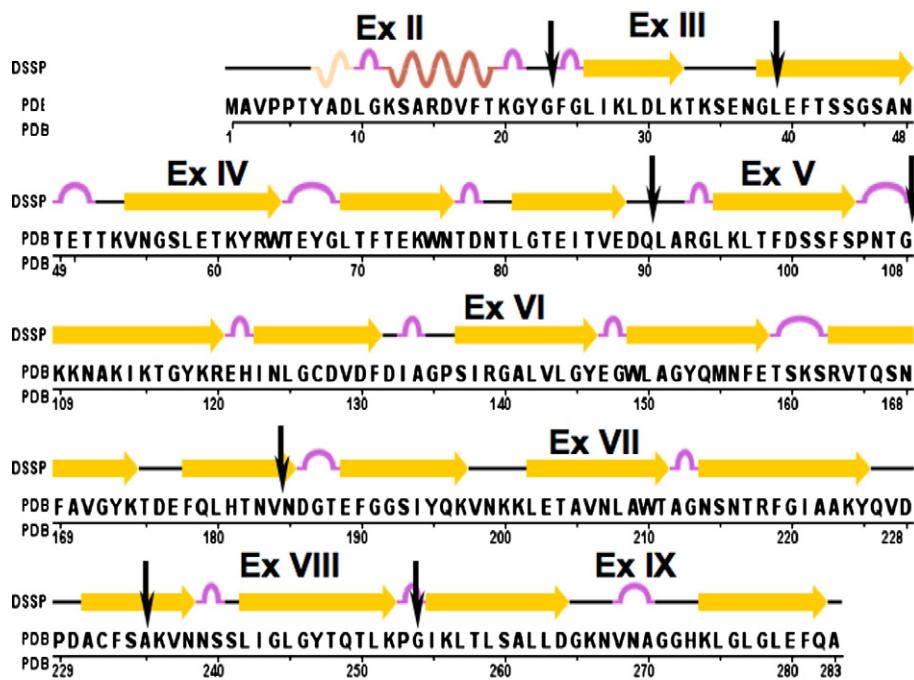
#### 552 4.6. Protein–protein and protein–ligand interactions of VDAC1

553 Various studies have indicated that VDAC can exist in different oligomerization states from monomers to dimers, trimers,  
 554 tetramers, hexamers and higher oligomers (Hoogenboom et al., 2007; Malia and Wagner, 2007; Zalk et al., 2005). Transient  
 555 dimerization is in agreement with chemical cross-linking experiments, where dimers and higher order oligomers were found  
 556 in diluted solutions of human VDAC1 (Zeth et al., 2008). X-ray crystallography of human VDAC1 suggested the formation of a  
 557 parallel dimer (Bayrhuber et al., 2008), while analysis of the crystal packing of mouse VDAC1 revealed an anti-parallel dimer,  
 558 which further assembles as hexamers (Ujwal et al., 2009). Despite the different relative arrangement of the observed dimers  
 559 of human and mouse VDAC, that is parallel versus anti-parallel, in both cases the dimer interface is formed by  $\beta$ -strands  $\beta$ 18,  
 560  $\beta$ 19,  $\beta$ 1 and  $\beta$ 2, extending potentially to  $\beta$ -strands 3 and 4 (Fig. 6) (Bayrhuber et al., 2008; Ujwal et al., 2009). Thus, the dimer  
 561 interface comprises  $\beta$ -strands that are the least stable part of the VDAC pore, according to NMR spectroscopy (Bayrhuber  
 562 et al., 2008). In agreement with the reduced stability of  $\beta$ -strands 1–4 of human VDAC1, computational analysis indicated  
 563 that  $\beta$ -strands located in the interfaces of porin-porin interactions are less stable (Naveed et al., 2009). Interaction of the  
 564 anti-apoptotic protein Bcl-x<sub>L</sub> with VDAC1 occurs close to the dimer interface, thereby potentially influencing the homo-  
 565 oligomerization of VDAC (Hiller et al., 2008; Malia and Wagner, 2007). In addition, the metabolite  $\beta$ -NADH, which is known  
 566 to modulate the voltage-gating process, binds to an interaction surface formed at strands  $\beta$ 17 and  $\beta$ 18 of human VDAC1  
 567 (Hiller et al., 2008). Future studies are expected to reveal the conformational basis of these interactions, as well as those with  
 568 other pro- and anti-apoptotic proteins and with hexokinase, thereby providing insight into the molecular mechanism of  
 569 VDAC-associated apoptosis.

#### 570 5. VDAC genetics

##### 571 5.1. VDAC genes

572 As detailed in Section 2.4, the evolution of VDAC sequences indicates that an ancestor gene lays at the origin of the various  
 573 VDAC genes seen in most groups of organisms. Paralogs have appeared several times in the different lineages as a result of  
 574 clearly different events. In *Drosophila*, for example, segmental duplication has led to the highly divergent forms described in  
 575 (Oliva et al., 2002). In *S. cerevisiae*, the two genes possibly are remnants of a genome duplication process (Kellis et al., 2004).



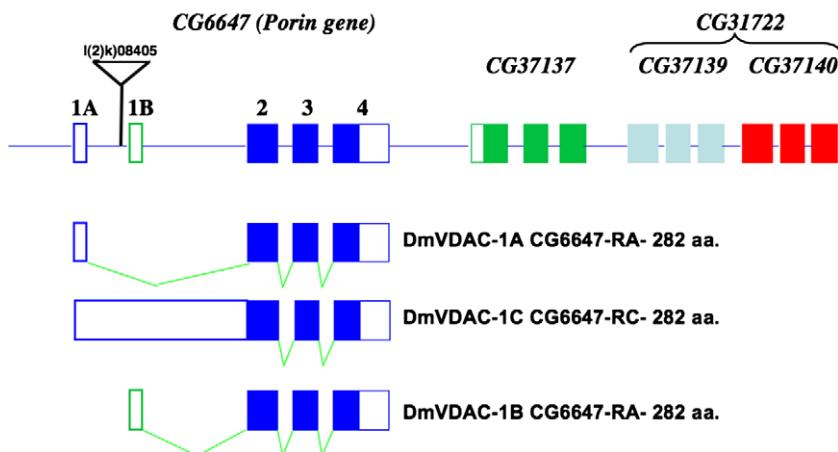
**Fig. 7.** Positions of the exon–intron boundaries (black arrows) in the mouse VDAC1 structure (pdb: 3EMN). Yellow arrows depict the β-strands, purple segments show junction loops, while hatched segments represent the α-helices.

It is possible that evolutionary pressure led to the fixation of similar VDAC genes with slightly different functions, as is apparently the case in plants (Elkeles et al., 1995). In a comparison of VDAC gene structures across a selection of species (Young et al., 2007), it was found that the vertebrate VDAC ORFs usually consist of eight exons, as does the sea urchin gene. *D. melanogaster* contains three exons coding for the protein whereas *C. elegans* includes five exons. The situation in the multi-gene families of chordates supports the notion that genes for VDAC paralogs evolved quite recently, since they share similar structures in their coding regions (Sampson et al., 1997) and even show conserved intron/exon junctions (Messina et al., 2000; Sampson et al., 1997). The observed correspondence between *Drosophila* and chordates exons (Oliva et al., 1998) suggests that structural modules might have been conserved during evolution. The localization of the exons boundaries, with respect to the structure of mouse VDAC1, is reported in Fig. 7. It is evident that the amino acid sequences expressed by the corresponding exons contain distinct structural units. It is thus possible to speculate that porin might have evolved from a simpler structure, also a smaller *trans-membrane* β-barrel.

## 5.2. VDAC in *S. cerevisiae*

VDAC from the yeast *S. cerevisiae* was the first eukaryotic porin to be cloned and sequenced (Mihara and Sato, 1985). In yeast, two genes encoding for porins have been discovered (Blachly-Dyson et al., 1997). *POR1* encodes the primary porin expressed in this organism, a protein of 283 amino acid residues with high sequence homology and undistinguishable pore-forming properties from the other eukaryotic VDAs. Yeast mutants lacking the outer membrane porin were constructed (Dihanich et al., 1987; Michejda et al., 1990). The mutants retained all other major proteins of the mitochondrial outer membrane but were deficient in mitochondrial cytochromes and initially did not grow on the non-fermentable carbon source, glycerol. The strain could, however, slowly adapt to glycerol (Michejda et al., 1990). Another yeast strain lacking the porin gene was constructed by gene displacement and was shown to be able to grow on glycerol at 30 °C but not at 37 °C (Blachly-Dyson et al., 1990). This strain, *Δpor1* M22-2, has been extensively used in many laboratories as a model system to evaluate the complementation pattern of VDAs from other sources.

Upon completion of the yeast genomic sequence, a second porin gene, *POR2*, was identified. The *POR2* gene was discovered by screening for genes which, when overexpressed, can correct the growth defect of *Δpor1* yeast (Blachly-Dyson et al., 1997). The protein encoded by *POR2*, called YVDAC2, shows 49% amino acid sequence identity to the porin protein (also called YVDAC1). Sub-fractionation studies indicate that YVDAC2 is normally present in the outer membrane. However, it was not able to form channels in reconstituted systems. Deletion of the *POR2* gene alone has no detectable phenotype, while *Δpor1* *Δpor2* yeast are viable and show a similar temperature-sensitive growth defect in glycerol at 37 °C as does *Δpor1* (Blachly-Dyson et al., 1997). The physiological function of this second porin isoform in yeast thus remains uncertain.



**Fig. 8.** Genomic organization of the *Drosophila melanogaster* porin genes at locus 32B. The organization and exon/intron structure of the porin gene (*DmVDAC1*) and of the three flanking genes coding *DmVDAC2*, 3, 4 are reported. Three alternative transcripts of *DmVDAC1* are shown. Bars indicate untranslated regions of the exons. The triangle indicates a lethal P-element insertion site.

606

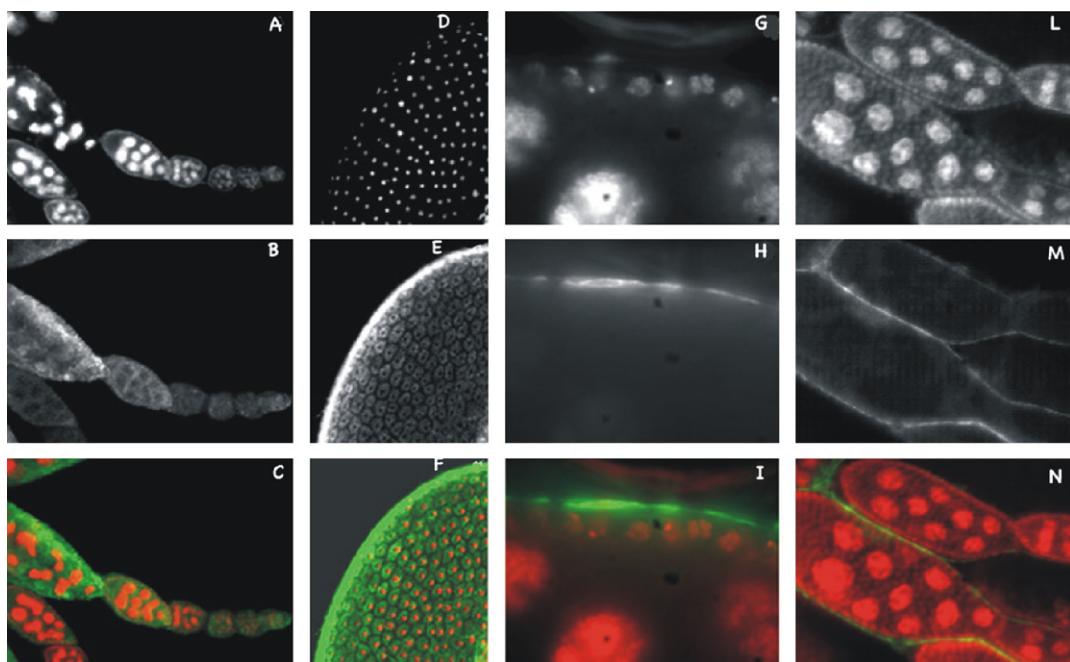
### 5.3. VDAC in *D. melanogaster*

607 Porin (later known as *DmVDAC1* or *DmPorin* isoform 1) was purified from *D. melanogaster* adult mitochondria (De Pinto  
 608 et al., 1989a). It showed very conserved functional features, being about 60% identical to the VDAC isoforms of mammals  
 609 (Messina et al., 1996). The corresponding cDNA was cloned and used to isolate and map (2L32B3-4) the porin gene (Oliva  
 610 et al., 1998); today: CG6647). The gene contained the sequence corresponding to the cDNA divided into four exons. The  
 611 beginning of the first exon was deduced by 5'-RACE-PCR that elongated the cDNA used as a probe by 41 bps. In the same  
 612 experiment, sequence analysis showed the expression of an apparently unrelated 5'-untranslated extension fused to the cod-  
 613 ing sequence. This second sequence surprisingly corresponded to another genomic region, enclosed between exons 1 and 2.  
 614 The structure of the gene was then shown to constitute five exons: Exon 1A indicated the most frequently used 5' exon, while  
 615 exon 1B was named as the exon corresponding to the second 5'-UTR (Fig. 8). Exon 2 (320 bp) and exon 3 (228 bp) exclusively  
 616 contain coding sequences. In particular, the first base in exon 2 precisely corresponds to the first base of the starting ATG  
 617 codon. Exon 4 contains the remaining coding sequence, the stop triplet, TAA, and the 3'-UTR sequence (Oliva et al., 1998).  
 618 Thus, the *DmVDAC1* protein can be expressed through two alternative transcripts. In *D. melanogaster*, alternatively tran-  
 619 scribed genes generating only one protein product are rather common (Misra et al., 2002).

620 Information on these two mRNAs obtained by performing disgenic crosses to remobilize *P* elements inserted into the porin  
 621 transcription unit showed that the absence of the VDAC protein was lethal for the fly. In particular, deletions encompassing  
 622 exon 1A resulted in a lower expression or in a lack of the VDAC protein. On the other hand, removal of the region between  
 623 exon 1A and exon 1B, as well as deletion of exon 1B together with most of the downstream intron, produced an apparently  
 624 normal amount of the VDAC protein in homozygous viable lines (Oliva et al., 2002). The conclusion of these studies was that  
 625 exon 1B is dispensable for fly development and, since 1B-5'-UTR does not add any protein pre-sequence, its functional role  
 626 must be connected to the polynucleotide sequence itself (De Pinto et al., 2003).

627 In this organism, serial duplication of the porin gene gave rise to a cluster of three or four genes with closely related se-  
 628 quences in the 32B3-4 region of chromosome 2L (Graham and Craigen, 2005; Guarino et al., 2006; Oliva et al., 2002). These  
 629 additional transcription units are on the same strand as the porin gene, and they also share a similar exon-intron organiza-  
 630 tion (Fig. 8). The 5'-UTR is present only in the porin and *CG17137* genes. The nucleotide distances separating the four porin-  
 631 like genes are extremely reduced. Alignment of the porin/VDAC sequences deduced from the additional *D. melanogaster*  
 632 genes shows pairwise sequence conservation in pairs. The protein sequence deduced from *CG17137* is 42% identical to *D.*  
 633 *melanogaster* VDAC1 but it is only 26% identical to the deduced amino acid sequences of *CG17140* or *CG31722* (Oliva  
 634 et al., 2002). There is evidence of a different mechanism regulating the expression of the two subgroups of *D. melanogaster*  
 635 porins. *P*-element insertion in regulatory regions of *DmVDAC1* negatively affects the expression of *DmVDAC2* but not of  
 636 *DmVDAC3* and 4 (Oliva et al., 2002).

637 Information about the activity of alternative *D. melanogaster* VDAC genes has been obtained from studies at the transcrip-  
 638 tional and protein levels. The recombinant protein corresponding to *CG17137* was expressed *in vitro* and named *DmVDAC2*.  
 639 *DmVDAC2* is able to form channels upon reconstitution in artificial lipid bilayer membranes, showing conductance similar to  
 640 native and recombinant *DmVDAC1* reconstituted in artificial membranes. This second isoform, nevertheless, shows unique  
 641 traits. *DmVDAC2* is clearly voltage-independent and cation-selective, while its counterpart isoform 1 is voltage-dependent  
 642 and anion-selective (Aiello et al., 2004). A recent report comparing the sequence of 12 genomes of the *Drosophila* genus  
 643 (Clark et al., 2007) showed that the gene encoding *DmVDAC2* is present in all and has a conserved structure (three exons,  
 644 with the only exception being *D. willistoni*, where the gene is split into four exons) (Clark et al., 2007). The protein



**Fig. 9.** Immunolocalization of porin isoforms 1 and 2 in female germ tissues of *Drosophila melanogaster*. Several ovaries from wild-type (*Oregon-R*) females were incubated with anti-porin 1 antibodies (A–F) or anti-porin 2 antibodies (G–N). DNA in the nuclei was stained with DAPI (A, D, G, L). Binding of the primary antibodies were revealed using FITC-labeled secondary antibodies (B, E, H, M). Gray scale images separately obtained by specific filters were computer-colored (DAPI red and FITC green) and merged to give the final image (C, F, I, N). Porin 1 is detected in the follicular cells surrounding the oocyte. In (A–C), an ovariole is shown. (D–F) reports a small region of the oocyte surface. Porin 2 is specifically found in the muscular envelope surrounding the ovariole (H, M). With permission from Specchia et al. (2008).

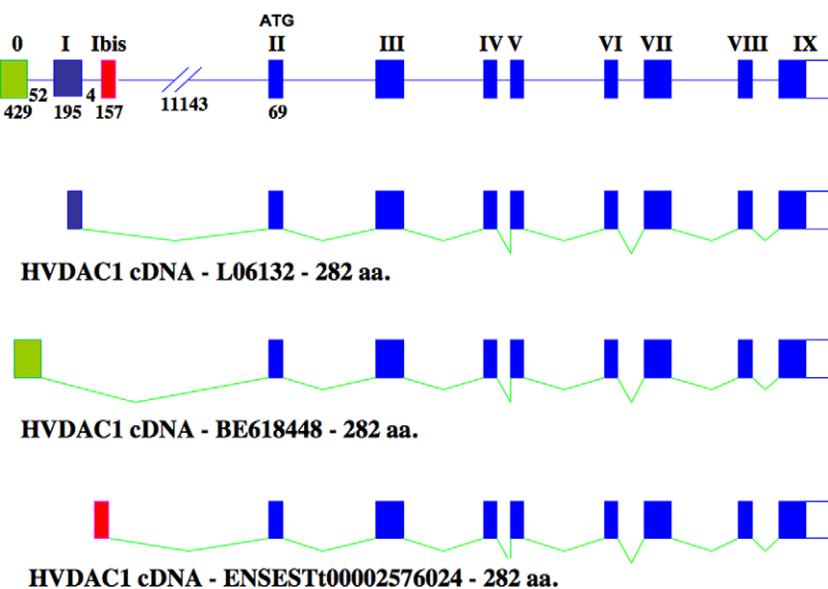
sequence is also well conserved and specific residues, found to be functionally important, like E66 (Aiello et al., 2004), were found to be conserved in 11/12 sequences. This observation indicates that DmVDAC2 is a conserved and useful gene for the fly.

Translated *D. melanogaster* DmVDAC isoforms have been used for several experiments. Reconstitution in planar lipid membranes has, however, shown contradictory results. While DmVDAC2, as was shown for DmVDAC1, forms channels, functional differences have been reported by different investigators (Aiello et al., 2004; Komarov et al., 2004), DmVDAC3 was reported to be inactive in reconstituted membranes, while DmVDAC4 was reported to be active but almost not voltage-dependent (Komarov et al., 2004). DmVDACs appear to be involved in the development of the germinal cell line in the male (Graham and Craigen, 2005; Guarino et al., 2006; Oliva et al., 2002). In male germinal tissue, DmVDAC1 has been found in the Nebenkern bodies, a typical aggregate of mitochondria in developing germinal cells in *Drosophila* (Oliva et al., 2002). In flies carrying the inactivated porin gene, antibodies against DmVDAC1 did not detect any antigen in Nebenkern bodies (Oliva et al., 2002). DmVDAC2 was immunodetected in the spermatozoa tails but not in Nebenkern bodies (Guarino et al., 2006). Antibodies to DmVDAC3–4 were also claimed to detect structures in mature or developing spermatozoa but only a faint and elusive staining was shown (Graham and Craigen, 2005). DmVDAC3 and 4, unlike DmVDAC1 and 2, are not able to rescue the temperature-sensitive conditional-lethal phenotype of VDAC-deficient yeast. This means that DmVDAC3 and 4 support different pathways than do DmVDAC1 and 2.

Immuno-histological stain of ovaries recently showed that DmVDAC1 is selectively targeted to follicular cells, while DmVDAC2 is present in mitochondria of the epithelial sheath cells of the ovariole (Specchia et al., 2008), Fig. 9. The strikingly differentiated expression patterns found in the ovaries of the fly raises questions as to the specific mechanisms controlling gene activity mediating this peculiar distribution, requiring subtle tuning of the two spatially proximal genes. The work on *D. melanogaster* further indicates that the VDACs are connected to the maturation and/or functioning of germ cell lines (Guarino et al., 2007).

#### 5.4. VDAC genes in mouse and man: evidence of alternative splicing

An organic description of the mouse gene family was reported by Sampson et al. (1997). In brief, the mouse VDAC1 gene spans about 28 kbp, is made up of 9 exons and is mapped to chromosome 11. The first exon contains the 5'-UTR, with the start codon being located 4 bp into the second exon. The mouse VDAC2 gene is about 12 kbp long, is divided into 10 exons and is mapped to chromosome 14. The VDAC2 gene contains an additional exon in comparison with VDAC1 and 3 used to express part of the 5'-UTR sequence. The start codon is located in the second exon. While another in frame start codon is



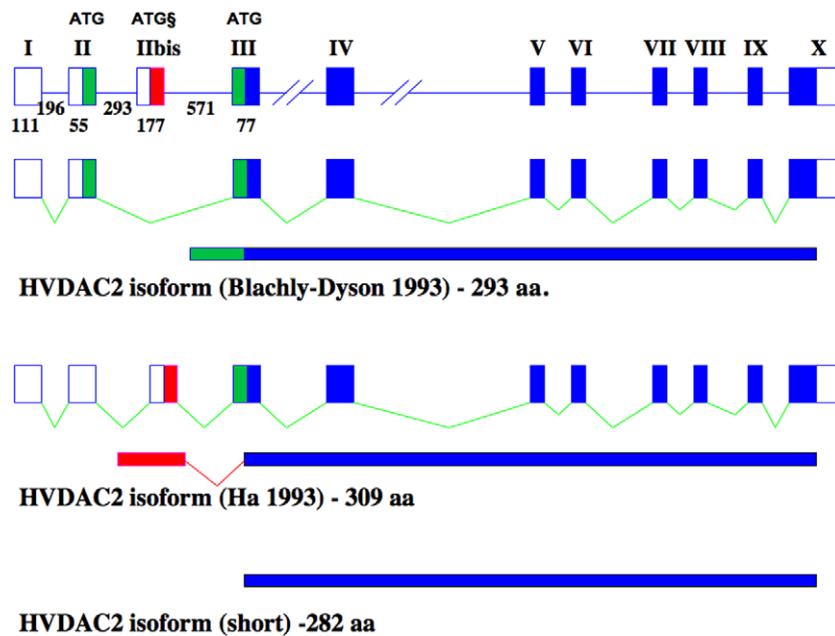
**Fig. 10.** Human VDAC1 gene structure and alternative splicing products. The structure of the gene coding HVDAC1 (5q31–32, UniProtKB/Swiss-Prot P21796) is shown on the top of the figure. Exon I–IX refers to the exon numeration reported by Sampson et al. for mouse VDAC1 (Sampson et al., 1997). The structure of the gene was deduced by a cross-analysis between EST libraries and the human genome. Three 5'-alternative splicing events are shown below in different colors. The solid boxes represent exons coding amino acid sequences. The empty boxes are transcribed but not translated regions. The figure is not drawn to scale.

found in exon 3, the most distal one is used by the ribosome. This gives the VDAC2 polypeptide an extension of 12 amino acids at the N-terminus, relative to the VDAC1 protein. The mouse VDAC3 gene spans about 16 kbp on the chromosome and is, again, made up of 9 exons and is mapped to chromosome 8. The start codon is located in exon 2 (Sampson et al., 1997).

Alternative polyadenylation sites were reported for the mouse VDAC2 gene. Two canonical and one aberrant polyadenylation sequences were found in the various gene sequences. These give rise to different mRNAs with 3'-UTR sequences of different lengths, suggested to be implicated in different usages of the translation product (Sampson et al., 1997). VDAC3 and VDAC1 have single polyadenylation sites (Sampson et al., 1997). Evidence of a VDAC3 internal alternative splicing event was reported (Anflous et al., 1998). A 3-base (ATG) mini-exon was shown to introduce a methionine 39 amino acids downstream of the amino terminus of the mouse VDAC3 protein. This alternative splicing was confirmed by the presence of expressed sequence-tagged cDNAs in the databases for both mouse and for humans. In addition, the alternative ATG-containing VDAC3 isoform is specifically transcribed in brain, heart and skeletal (Sampson et al., 1998). A matter of interest was whether this alternative splicing might cause the production of a shortened form of VDAC3. The alternative isoform of mouse VDAC3, containing the internal ATG, was expressed in *Δpor1* yeast cells. This truncated form of VDAC3 was detectable by antibodies, since a tag sequence was introduced at the protein C-terminus. However, this experiment did not show the presence of a lower molecular weight band in a Western blot, suggesting that the truncated form of the protein is not produced in the cell (Decker and Craigen, 2000). All efforts to observe channel-forming activity by the truncated VDAC3 form were unsuccessful (Xu et al., 1999). It is thus unlikely that the shortened form has any physiological meaning. Moreover, the *in vivo* role of this alternatively spliced VDAC3 isoform remains unknown.

Human porin genes strictly resemble the mouse genes summarized above. They share the same number of exons for each gene isoform and the chromosomal localizations are synthetic (Messina et al., 2000, 1999; Rahmani et al., 1998). The main differences are expanded introns, possibly the result of genome evolution. The porin/VDAC isoform 1 gene was mapped by *in situ* hybridisation to chromosome 5q31–32 (Messina et al., 1999). The human VDAC1 gene spans about 30 kbp and is organized into 9 exons. About half of this region is covered by intron 1 (14.1 kbp) (Fig. 10).

The human VDAC2 gene was mapped to chromosome 10q22. Its locus spans 16.4 kbp and its structure was determined to contain 10 exons (Fig. 11). Also, in humans, as in the mouse, the VDAC2 gene gives rise to three classes of mRNAs, utilizing different polyadenylation sites (Decker and Craigen, 2000). Soon after the discovery of human VDAC2 cDNA (Blachly-Dyson et al., 1993), a new porin-encoding cDNA was fortuitously isolated during cloning of a different human gene (Ha et al., 1993). This new protein sequence (termed Humpor, accession number L08666) showed an N-terminal sequence of 26 amino acids different from the 11 amino acid N-terminal extension discussed above. Synthesis of two proteins of different lengths was shown by translation of this cDNA in a reticulocyte lysate system (Ha et al., 1993). This means that both start sites can be potentially used. Transfection experiments in COS7 cells performed by another group confirmed that both start sites could be apparently used at roughly similar quantitative levels (Yu et al., 1995). ESTs cDNA containing the sequence encoding Humpor are present in the database. Humpor is enriched in heart and B-lymphocytes (Yu et al., 1995).



**Fig. 11.** Human VDAC2 gene structure and alternative splicing products. The structure of the gene coding HVDAC2 (10q22) is shown on the top of the figure. Exon 1–10 refers to the exon numeration reported by Sampson et al. for mouse VDAC2 (1997). According to UniProtKB/Swiss-Prot P45880, three protein products are possible, starting from three ATG codons and considering an alternative splicing event between exon II and IIbis. In blue are the coding regions common to the protein products. In red and green, additional N-terminal sequences characterizing the other products are shown. The empty boxes are transcribed but not translated regions. The figure is not drawn to scale. The green mRNA was first reported by Blachly-Dyson et al. (1993) but contained a false extension of 48 bp at 5' and was missing exon I. The red mRNA originates an ORF starting from ATG\$. The red mRNA (and the corresponding protein) were first reported by Ha et al. (1993) with some corrections. The ATG\$ cannot be used in the green mRNA, since it would alter the coding frame.

707      VDAC3 cDNA was discovered in the process of studying the interaction of the HBX protein, encoded by the hepatitis B  
 708      virus genome, with cellular proteins using the yeast two-hybrid method (Rahmani et al., 1998). The corresponding gene  
 709      was mapped to chromosome 8p11.2, spans 13.3 kbp and is made up of 9 exons (Rahmani et al., 1998). Human VDAC3 mRNA  
 710      is highly enriched in testis but it is also expressed in the many other tissues tested.

711      The protein encoded by this gene is the least characterised and the most intriguing among the various isoforms. From the  
 712      scarce information available for human and mouse VDAC3 (they show only 5 amino acid exchanges over a sequence of 283),  
 713      it seems that this is the only porin unable to complement the incapacity of the yeast *Apor1* M22-2 strain to grow on glycerol  
 714      at 37 °C (Sampson et al., 1997). In reconstituted systems, VDAC3 does not form channels. Moreover, native VDAC3 channels  
 715      are not closed upon application of high voltages, a typical feature of VDAC proteins (Xu and Colombini, 1996).

716      A search with cDNA encoding human VDAC1 and VDAC2 in ESTs collections and cross-analysis between the major human  
 717      sequence databanks (GenBank and Ensembl) revealed the presence of more exons than reported for both genes. The human  
 718      VDAC1 gene showed two additional exons. Exon 1bis (Fig. 10) is 157 bp-long and is located a few bases after exon 1. It ap-  
 719      pears to be a true exon, since it has the canonical splicing consensus sequence at the 3' extremity. Furthermore, ESTs exist  
 720      containing this exon 1 fused to the remaining exons 2–9. This alternative splice variant of VDAC1 does not change the coding  
 721      frame, since exon 1, skipped in this transcript, contains the 5'-UTR, like exon 1bis. Another likely alternative exon is located  
 722      52 bp before exon 1. We call it exon 0 in Fig. 9. It is 429 bp long and has the 3' splicing site. Its use is very similar to exon 1bis  
 723      in that it is present in another splice variant of the transcript containing another alternative 5'-UTR. Several ESTs containing  
 724      this sequence are present in the database and a whole mRNA sequence is also registered in GenBank as AK095989. The pro-  
 725      tein sequence is again not altered by such transcript modification.

726      The human VDAC2 gene shows a similar pattern (Fig. 11). An alternative exon (exon 2bis, 177 bp long) is located between  
 727      exons 2 and 3. Cross-analysis with ESTs and mRNA reveals that this exon codes the N-terminal sequence reported in Humpor  
 728      (Ha et al., 1993). This alternative splicing could thus have potential effects upon proteins, since VDAC2 containing different  
 729      N-end sequence would be expressed. As shown for *D. melanogaster*, the human VDAC gene family seems to use alternative  
 730      splicing at the 5'-extremity of the genes. Alternatively-spliced mRNAs might exert an influence in pre-translational pro-  
 731      cesses, although these are still poorly defined. The functional meaning of such alternative splice variants has to be  
 732      understood.

733      In the human VDAC3 gene, the only alternative splicing event reported in the literature and confirmed by the genome  
 734      sequence corresponds to the single ATG internal exon described above for mouse.

735      Promoters of the VDAC genes have been poorly characterized. A sterol-repressing element (SRE-1) is present in the pro-  
 736      moter region of human and mouse VDAC1 and VDAC2 genes (Messina et al., 2000; Sampson et al., 1997). Sterol-repressing

elements regulate the synthesis of proteins that are involved in cholesterol traffic. VDAC1 binds preferentially to cholesterol (De Pinto et al., 1989a). Furthermore, a NRF-2 (nuclear respiratory factor) element was shown in the human VDAC1 putative promoter. NRFs are transcription factors involved in the regulated expression of proteins belonging to the respiratory complexes (for a review, see (Scarpulla, 2008)). This means that porin may also be regulated together with main components of the respiratory chain. Finally, a SRY element was found. SRY is a protein involved in sex determination and in testis development (Koopman, 1999).

#### 5.5. Human patients lacking VDAC1 have been discovered

The deletion of the VDAC1 gene has been reported in human patients (De Pinto et al., 2000; Huizing et al., 1994, 1996). About 100 suspected patients were investigated for VDAC1-deficiency in the Netherlands and Italy. The patients to be examined were selected on a biochemical basis, having to show (a) diminished substrate oxidation rates not caused by a disturbance in the respiratory chain, citric acid cycle or pyruvate dehydrogenase complex or (b) normal activities of the respiratory chain enzymes and the pyruvate dehydrogenase complex, together with strong suspicion for mitochondrial disorder based upon both clinical and clinical-chemical hallmarks. In these events, a muscle biopsy was obtained and Western blot was performed with anti-VDAC1 antibodies to detect a reduction or absence of the protein. The first case of VDAC1-deficiency was a Dutch five year old male, the third child of healthy, unrelated parents (Huizing et al., 1994). In this subject, biochemical studies of the muscle mitochondria showed obviously impaired rates of substrate oxidation and production of high-energetic compounds. The laboratory results pointed at a functional defect in one of the post-respiratory-chain energy conversion steps, such as ATPase, mitochondrial creatine kinase or the ADP/ATP carrier. Immunological analysis by antibodies against VDAC1 and the ADP/ATP carrier showed the deficiency of VDAC, while ADP/ATP carrier levels were normal. The VDAC deficiency was expressed in a tissue-specific manner in this patient, with muscle tissue showing severe deficiency, and fibroblast VDAC content being nearly normal (Huizing et al., 1994, 1996). In a second case of VDAC1-deficiency, an Italian three year old child showed prominent metabolic acidosis with low plasma electrolytes and ketonuria. Eventually recurrent thrombocytopenia, anemia, neutropenia with untreatable infections led to patient to death one month after admission. Mitochondrial DNA analysis showed the presence of the large common deletion. Western blot of the 600g supernatant from a muscle biopsy showed the lack of VDAC and normal levels of the ADP/ATP carrier (De Pinto et al., 2000). In this case, a small amount of the bioptic material was solubilized with Triton X-100 and subject to a HTP/Celite chromatography and the eluate was subjected to Western blot and electrophysiological characterization. The reconstitution of this material, in comparison with another bioptic control, showed a lack of the typical VDAC1 channel activity, reduced conductance and only slight cation selectivity (De Pinto et al., 2000), possibly indicative of another isoform or pore protein.

Overall, VDAC deficiency in humans cannot be differentiated from other, better known mitochondrial pathologies. For example, the Italian patient was clearly suffering from Pearson's disease (for review, see DiMauro, 2004). Such malfunctioning of mitochondria could also lead to reduced protein synthesis and/or targeting of VDAC and thus, the lack of VDAC could not be the cause but instead was the consequence of another defect. The screening of more than 100 patients thus indicates that a lack of VDAC is a very rare occurrence, posing doubts about a possible lethal phenotype of VDAC1-deficiency in humans.

#### 5.6. Deleting VDAC genes in model animals

Gene deletion is an informative technology that is able to shed light on the physiological role of the gene of interest. Such genetic inactivation, through use of the more recent interference RNA technology that aims at the same result through an easier pathway, does not always give the desired information because compensative mechanisms are triggered to allow the organism to compensate for the defect, even though the gene is considered essential.

Embryonic stem (ES) cell lines lacking each VDAC isoform were generated by homologous recombination (Wu et al., 1999). The strategy used to target the VDAC genes was designed to delete different exons, making the genes incomplete. The cell lines containing the inactivated VDAC1, 2 or 3 gene were examined for loss of respiration and any reduction in respiratory chain activity (Wu et al., 1999). ES cells lacking each isoform were viable but exhibited ~30% reduction in coupled or uncoupled oxygen consumption. VDAC1- and VDAC2-deficient cells showed a partial reduction in cytochrome oxidase (COX) activity, whereas VDAC3-deficient cells had normal COX activity. These results indicate that each mouse VDAC isoform is not essential for cell viability, since the lack of any of them could be compensated and only led to minor metabolite flux reduction (Wu et al., 1999).

The ES cells were used to generate mice deleted of VDAC genes (Anflous et al., 2001; Sampson et al., 2001). Only VDAC1- and VDAC3-deleted mice were produced. The VDAC2-deleted mouse could not be obtained because VDAC2-deficient embryos are non-viable (Cheng et al., 2003). VDAC1-deleted mice were characterized for defects in muscle physiology (Anflous et al., 2001). In VDAC1<sup>-/-</sup> mice, skinned fibers prepared from two oxidative muscles (ventricle and soleus) and a glycolytic muscle (gastrocnemius) showed estimated mitochondrial affinity for ADP and a rate of respiration in the presence the optimal ADP concentrations, indicative of specific modifications for each kind of muscle. These changes were accompanied by striking ultrastructural changes in both subsarcolemmal and intermyofibrillar mitochondria, where the cristae become very compact and the mitochondria were enlarged several fold. Lack of VDAC1 was associated with *in utero* lethality, but interestingly, the surviving VDAC1<sup>-/-</sup> offspring appear normal (Weeber et al., 2002). To determine the role that VDACs may play in

learning and synaptic plasticity, the VDAC-deficient mice were subjected to tests that quantify associative and spatial learning. It was found that VDAC-deficient mice showed differences in spatial learning without the identification of any sensory or motor deficit (Weeber et al., 2002).

Mice lacking VDAC3 in their genome were produced and found to be healthy, but males are infertile (Sampson et al., 2001). Although these mice had a normal sperm number, their spermatozoa exhibited markedly reduced motility. When viewed by electron microscopy, 68% of VDAC3-deficient epididymal sperm axonemes (versus 9% of wild-type axonemes) had some structural aberration, most commonly the loss of one outer doublet from the normal 9+2 microtubule doublet arrangement. Electron microscopy of spermatids in the testes revealed enlarged and abnormally shaped mitochondria along the midpiece. Infertility by sperm immotility may be a consequence of axonemal defects (Sampson et al., 2001).

VDAC2<sup>-/-</sup> mice were never generated, likely due to the protective effect that this VDAC isoform may exert in the context of apoptosis (Cheng et al., 2003). It was found that VDAC2 in the mitochondria of viable cells is present in a complex with the pro-apoptotic protein, Bak. Accordingly, this has been proposed as a mechanism to control the potentially lethal pro-apoptotic Bax and Bak molecules. The protective anti-apoptotic effect of VDAC2 was abolished in ES cells lacking VDAC2, while its re-expression restored protection (Cheng et al., 2003).

A combination of gene deletion and silencing was used to investigate the effect of simultaneous ablation of the three VDAC isoforms, with respect to the apoptotic pathway and, in particular, to the formation of the mitochondrial permeability transition (MPT) (Baines et al., 2007) (see Section 8.2.2). The absence of the three VDAC isoforms should be lethal, unless their function is replaced by other channels in the OMM. Mouse embryonic fibroblasts (MEFs) from wild-type, VDAC1-, VDAC3- and VDAC1–VDAC3-null embryos were used. VDAC1–VDAC3-null embryos further treated with VDAC2 siRNA, followed by treatment with pro-apoptotic chemicals or by overexpressing pro-apoptotic proteins, showed similar levels of cell death. It was thus concluded that the simultaneous loss of all three VDAC isoforms does not disrupt MPT activation and provides no protection from multiple forms of necrotic or apoptotic cell death. However, several observations disagree with this doubt on the contribution of VDAC to changes in MPT (Baines et al., 2007). The function of VDAC in mediating cell death gained support with the demonstration that in cells silenced for VDAC1 expression, cisplatin failed to induce Bax translocation to the mitochondria and apoptosis induction (Tajeddine et al., 2008) (further discussed in Section 7.2) and that the anti-tumor agent, erastin, acts through VDACs (Yagoda et al., 2007). It should be also noted that alternative models for permeability transition pore complex (PTPC)-mediated MPT do exist. Indeed, accumulating evidence suggests that multiple pathways and mechanisms of Cyto c release can co-exist within a single model of cell death, depending on the cell type and the nature of the stimuli.

As to VDAC function in normal cell function, one can ask, however, why as presented above, is it so rare, if not impossible to find living individuals lacking VDAC?

## 825 6. Extra-mitochondrial localization of VDAC

### 826 6.1. VDAC1 in the plasma membrane

827 The extra-mitochondrial localization of porin was shown for the first time by Thinnnes and co-workers (Kayser et al., 1989; 828 Thinnnes et al., 1989; for review, see Bathori et al., 2000), who fortuitously co-purified porin together with human transplantation 829 antigens. Intrigued by this protein, they sequenced it by Edman degradation (Kayser et al., 1989) and called it porin 830 31HL. Later, they produced monoclonal antibodies against the N-terminal end of the protein (Babel et al., 1991). Application 831 of the antibodies to an Epstein Barr virus-transformed lymphocyte line was used to generate secondary immunofluorescence 832 images. The images obtained with this techniques showed fluorescent staining limited to the cell surface (Thinnnes et al., 833 1989).

834 This finding was subsequently confirmed by a number of studies employing several cell lines or tissues (normal and cultured 835 lymphocytes (Cole et al., 1992; Konig et al., 1991), epithelial cells (Puchelle et al., 1993), astrocytes (Dermietzel et al., 836 1994), and the post-synaptic membrane fraction from brain (Moon et al., 1999)), relying on flow cytometry, as well as EM 837 immunogold labeling and immunofluorescence techniques. Immunofluorescence showed a punctuate staining on the cell 838 surface, compatible with a membrane subdomain localization of the protein. In addition, the experiments with monoclonal 839 antibodies indicated that the N-terminal end of the protein is exposed on the cell surface.

840 The major weakness of this line of research is, however, that all immuno-topological evidence of a VDAC plasmalemmatic 841 localization was obtained using the same set of anti-VDAC antibodies and solely by histochemical techniques. The reliability 842 of these findings was thus questioned and the sub-cellular distribution of VDAC isoforms re-examined by investigating the 843 sub-cellular distribution of epitope-tagged VDAC (Yu et al., 1995). In these studies, tags were introduced at the C-terminus of 844 human VDAC1 and VDAC2, which were expressed in cells and subjected to sub-cellular fractionation, immuno-cytochemistry 845 and immuno-EM. The tagged proteins were exclusively detected in fractions or sub-cellular that contained mitochondrial 846 marker proteins. This led to the conclusion that the non-mitochondrial localization of VDAC1 was probably an artefact because of unspecific immunoreactions and/or redistribution of VDAC due to the presence of detergents (Yu et al., 1995).

847 New biochemical evidence assigning VDAC to the plasma membrane came from the finding that VDAC1 is present in 848 caveolae, a specialized domain of the plasma membrane (Lisanti et al., 1994). VDAC1 was later purified from a caveolae 849 preparation (Bathori et al., 1999). To avoid contamination of the caveolar VDAC with mitochondrial VDAC1, intact cells were 850

labeled with the membrane impermeable reagent NH-SS-biotin (Jakob et al., 1995), resulting in biotinylation of proteins exposed to the extracellular environment. The fact that the authors obtained biotinylated VDAC after an HTP/Celite chromatography step, indicated its exclusively originated from the plasma membrane (Bathori et al., 1999). Plasma membrane-derived VDAC1 was subsequently characterized from the biochemical and functional points of view, demonstrating it to be identical to the mitochondrial version of the protein.

The presence of VDAC in the plasma membrane was considered to be a kind of oddity, since a large, unspecific pore in the plasma membrane would be expected to be lethal to the cell (Yu et al., 1995). In spite of this, there are several proposals in the literature claiming functional roles for VDAC in the plasma membrane. One possibility is the presence of channels in the plasma membrane is somehow related to the VDAC protein. A recent review presents the debate considering VDAC1 as the maxi-anion channel of the plasma membrane (Sabirov and Okada, 2009). Furthermore, considering the resting membrane potential across the plasma membrane of about  $\sim 30$  to  $\sim -60$  mV (Moule and McGivan, 1990; Demaurex et al., 1993), VDAC1 in the plasma membrane would be in closed state most of the time (Manella, 1997).

In another recent communication, it has been proposed that plasma membrane VDAC1 can function as a redox enzyme, capable of reducing extracellular ferricyanide in the presence of intra-cellular NADH (Baker et al., 2004). The presence of an NADH:ferricyanide reductase in the plasma membrane is suggested by the finding that oxidation of cytosolic NADH causes concomitant reduction of ferricyanide, an artificial membrane-impermeant electron acceptor (Baker and Lawen, 2000; Navas et al., 1986). This enzymatic activity is referred to as plasma membrane NADH-oxidoreductase (PMOR). The purification of a protein associated with NADH: ferricyanide activity led to its identification as VDAC. Evidence for this result came from MALDI-TOF analysis, immunoprecipitation and purification using different methods (Baker et al., 2004). Although it is unlikely that VDAC1 would function as a reductase in mitochondria, these data showed that in the presence of NADH, VDAC1 can directly catalyze the reduction of ferricyanide. NADH-binding sites in VDAC have been mapped by several investigators (Baker et al., 2004; Yehezkel et al., 2006; Zizi et al., 1994). It should be noted, however, that such activity was not observed with VDAC isolated from mitochondria (shoshan-Barmatz, unpublished results). How might VDAC exert this enzymatic function? Likely explanations are that: (i) the VDAC polypeptide may assume different conformations, depending on its localization and (ii) VDAC1 is controlled by effectors that are able to control the activity of the protein. It was found that a protein modulator located in the IMS was able to influence VDAC activity in mitochondria (Holden and Colombini, 1993). Unfortunately, this modulator has never been isolated. Another possibility is that sub-compartmentalization of the protein, for example, in the case of caveolae, might be strategic to control VDAC function.

Other possible roles for VDAC1 in the plasma membrane have been proposed. VDAC1 was identified in the plasma membrane of human endothelial cells and described as the receptor for plasminogen Kringle 5 (Gonzalez-Gronow et al., 2003). Based upon its specific binding of a neuro-active steroid analogue, VDAC was proposed to be part of the GABA<sub>A</sub> receptor in rat brain membranes (Darbandi-Tonkabon et al., 2003). It has been proposed to play a role in cellular ATP release and volume control (Okada et al., 2004) and it has been suggested to be the plasma membrane maxi-anion channel (Bahamonde et al., 2003; Blatz and Magleby, 1983), a claim that has been rejected by Sabirov et al. (2006), who could not establish any correlation between VDAC expression and maxi-anion channel activity. In neurons, plasma membrane VDAC1 has been implicated in the regulation of  $\beta$ -amyloid-induced neurotoxicity (Marin et al., 2007).

VDAC1 has also been detected in the sarcoplasmic reticulum of skeletal muscle (Jurgens et al., 1995; Lewis et al., 1994; Shafir et al., 1998; Shoshan-Barmatz et al., 1996), however, its function in this compartment is still unknown.

## 6.2. Intra-cellular targeting of VDAC

The biogenesis of  $\beta$ -barrel proteins in mitochondria has been studied with some detail, yet this complex process is still incompletely defined. Several reviews dealing with this process have appeared and the reader is referred to these for a thorough discussion (Paschen et al., 2005; Pfanner et al., 2004). In brief, the VDAC precursor would reach the mitochondria without any chaperone help and be recruited by the TOM (translocase of the outer membrane) complex. Thus, VDAC would not be directly inserted into the outer membrane (as compared with its behavior in reconstituted artificial systems, see Section 4). From the TOM complex, the precursor would be passed to the TOB (topogenesis of outer membrane  $\beta$ -barrel proteins) complex. It remains, however, unresolved how the TOB complex mediates VDAC insertion into the outer membrane and at which point the precursor molecules fold into their native conformation (Paschen et al., 2005). The mechanism proposed here requires initial import of the precursor into the mitochondrial intermembranal space followed by insertion into the outer membrane. This complex process would maintain the topology of the protein and the directionality of insertion.

The targeting of VDAC1 to the plasma membrane was explained by an alternative splicing event that would add a targeting pre-sequence to the VDAC1-coding sequence (Buettnner et al., 2000), as shown for transcription factor A, which is alternatively targeted to the mitochondrion or to the nucleus, depending on the presence of a fused pre-sequence (Larsson et al., 1996). Evidence for the presence of a plasma membrane-targeting pre-sequence on VDAC has also been reported in C1300 mouse neuroblastoma cells (Bahamonde et al., 2003). Reports by the Thinné's group, on the other hand, have raised the possibility that VDAC1 might be targeted to the plasma membrane without a specific sequence. In particular, they have demonstrated that upon transfection of *Xenopus laevis* oocytes with cDNA containing the human VDAC1-coding sequence carrying a FLAG-epitope at the N-terminus but lacking the plasma membrane-targeting sequence, the modified porin could still be detected on the surface of the cells (Steinacker et al., 2000).

910 **7. VDAC silencing, overexpression and cell life and death**

911 In recent years, RNA interference (RNAi) has been proven to be a powerful and specific approach for targeted RNA-depen-  
912 dent gene silencing and is rapidly become a central tool in the study of cell function in a wide range of biomedical applica-  
913 tions (Gunsalus and Piano, 2005; Morita and Yoshida, 2002; Pushparaj et al., 2008). The mechanism of action of RNAi relies  
914 on the endogenous machinery responsible for post-transcriptional gene silencing regulation by micro-RNAs (miRNA) (Chek-  
915 ulaeva and Filipowicz, 2009; Filip, 2007; Ross et al., 2007). Versions of RNAi induced by short hairpin RNA (shRNA) or small  
916 interfering RNA (siRNA) transfection are currently the most time-efficient method for gene knockdown in mammalian cell  
917 lines (Alexander et al., 2007). RNAi has emerged as the basis for innovative nucleic acid-based medicines with potential ther-  
918 apeutic capacities for a broad spectrum of diseases, including cancer (Cejka et al., 2006), because of the possibility of specific  
919 inhibition of mutated oncogenes and other target genes that aid and support the growth of cancer cells (Abdelrahim et al.,  
920 2006; Mocellin et al., 2006). Several studies have employed RNAi for VDAC1 to verify its function in cell life and apoptosis.

921 **7.1. VDAC1 silencing and cell growth**

922 Regulation of mitochondria physiology, indispensable for proper cell activity, requires an efficient exchange of molecules  
923 between mitochondria and cytoplasm at the level of the OMM. The common pathway for metabolite exchange between mito-  
924 chondria and cytoplasm is the VDAC, and it has been recognized that the VDAC channel plays a crucial role in the regulation of  
925 metabolic and energetic functions of mitochondria. Therefore, it is assumed that VDAC knockdown would affect cell metab-  
926 olism and normal function. Indeed, it was demonstrated that silencing hVDAC1 expression in T-Rex-293 cells using shRNA  
927 resulted in reduced ATP production and a decrease in cell growth (Abu-Hamad et al., 2006). Cells in which hVDAC1 expression  
928 was decreased almost all proliferated extremely slowly. Normal growth was, however, restored upon expression of murine  
929 (m)VDAC1. Cells expressing reduced VDAC1 levels showed 4-fold-lower ATP-synthesis capacity, contained low ATP and  
930 ADP levels, and showed strong correlation between ATP levels and cell growth, pointing to limited metabolite exchange be-  
931 tween mitochondria and cytosol (Abu-Hamad et al., 2006). Since cancer cells express high levels of VDAC (Simamura et al.,  
932 2008a), the effect of shRNA directed towards hVDAC1 on cancer cells growth in an animal model was tested, using HeLa cer-  
933 vical cancer cells stably expressing shRNA-VDAC1 (Koren and Shoshan-Barmatz, submitted for publication). In these cells,  
934 VDAC1 expression was decreased almost completely by shRNA-VDAC1, with the cells proliferating much slower than did  
935 parental non-transfected cells, pointing to VDAC1 expression as being essential for normal growth of HeLa cancer cells. Nude  
936 mice subcutaneously inoculated with stably transfected shRNA-hVDAC1 HeLa cells developed about 40-fold smaller tumors  
937 than did mice inoculated with control HeLa cells. Taken together, these results demonstrate the anti-cancer therapeutic po-  
938 tential of VDAC1 down-regulation by means of shRNA (Koren and Shoshan-Barmatz, submitted for publication).

939 **7.2. VDAC silencing and apoptosis**

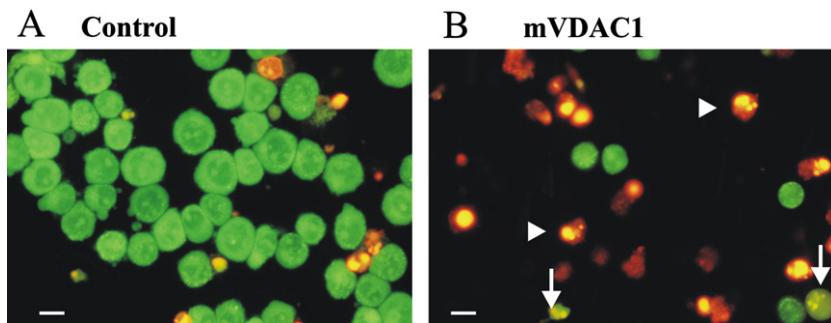
940 Several other studies employing siRNA-based silencing of VDAC1 expression have revealed that VDAC is required for  
941 apoptosis induction by various stimuli. In a delicate research screen for various distinct siRNAs targeting different compo-  
942 nents of the apoptotic machinery, it was found that VDAC1 silencing by siRNA efficiently prevented cisplatin-induced apop-  
943 tosis and Bax activation in non-small cell lung cancer cells (Tajeddine et al., 2008). In the siRNA-VDAC1-expressing cells, the  
944 decrease in cisplatin-induced cell death was correlated with a decrease of apoptosis-associated characteristics, such as mito-  
945 chondrial trans-membrane potential dissipation and plasma membrane permeabilization, inhibition of cytochrome c (Cyto c)  
946 release and AIF translocation, as well as inhibition of caspase-3 activation (Tajeddine et al., 2008). Moreover, VDAC1 knock-  
947 down reduced cisplatin-induced Bax activation but had no apparent effect on Bak activation, implying that VDAC1 acts  
948 downstream of Bak and upstream of Bax in this system. In these VDAC1 knockdown cells, the failure of cisplatin to activate  
949 Bax was reversed by the Bcl-2/Bcl-x<sub>L</sub> antagonist, ABT-737, which concomitantly restored the cytotoxicity of cisplatin (Tajed-  
950 dine et al., 2008).

951 In another study, the involvement of VDAC in endostatin-induced apoptosis was demonstrated. Endostatin is known to  
952 stimulate apoptosis in endothelial cells. Endostatin-induced apoptosis involves Cyto c release and caspase-9 activation in hu-  
953 man microvascular endothelial cells upon endostatin administration. Furthermore, endostatin promoted PTP opening via  
954 VDAC1. Indeed, VDAC1 silencing by siRNA attenuated the endostatin-induced apoptosis effect, while VDAC1 overexpression  
955 enhanced the sensitivity of endothelial cells towards endostatin. Taken together, these findings conclude that VDAC1 plays a  
956 vital role in modulating endostatin-induced endothelial cell apoptosis (Yuan et al., 2008).

957 Recently, it was shown at the single live cell level that overexpression of VDAC1 triggers MPT at IMM, while silencing  
958 VDAC1 expression by siRNA results in the inhibition of MPT induced by selenite (Tomasello et al., 2009). These results, there-  
959 fore, indicate that VDAC1-dependent MPT is an upstream mechanism in oxidative stress-induced apoptosis.

960 **7.3. VDAC overexpression and apoptosis**

961 Several studies have demonstrated that overexpression of VDAC1 from a variety of sources and in different cells types  
962 induces apoptotic cell death. Overexpression of VDAC from different sources ranging from yeast, rice, fish, murine to man



**Fig. 12.** VDAC1 overexpression-induced cell death. U-937 human monocytic were transformed to overexpress recombinant murine VDAC1 (mVDAC1). Control (A) and cells overexpressing VDAC1 (B) were analyzed for cell viability using acridine orange/ethidium bromide staining. The cells were then visualized by fluorescence microscopy (Olympus BX60, equipped with a CCD camera) Arrows indicate cells in an early apoptotic state, reflected by degraded nuclei (stained green with acridine orange). Arrow head indicates late apoptotic state (stained orange with acridine orange and ethidium bromide) (scale bars = 15  $\mu$ m).

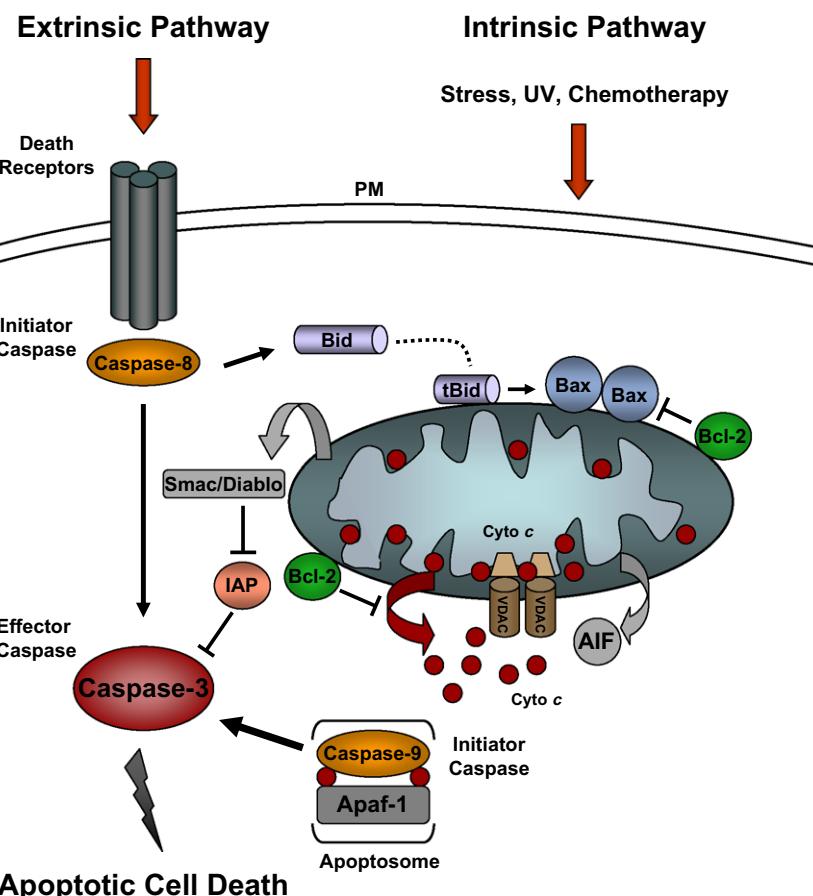
induces apoptotic cell death, suggesting that VDAC is a conserved mitochondrial element of death pathways operating in both animal and vegetal systems (Zaid, 2005; Lu, 2007; Godbole, 2003; Ghosh et al., 2007, see also Fig. 12). Overexpression of murine (m)VDAC or rat (r)VDAC1 in U-937 cells resulted in cell death (70–85%), as characterized by nuclear fragmentation (Zaid, 2005). Moreover, overexpression of rice VDAC induces apoptosis (~70%) (Godbole, 2003), which was blocked by Bcl-2 and the VDAC channel inhibitor, DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid) (Shoshan-Barmatz et al., 1996). Similar results were obtained following transfection of fish cells to express *Paralichthys olivaceus* VDAC (Lu et al., 2007a). It has also been shown that upon human (h)VDAC1 overexpression, the inner mitochondrial membrane becomes depolarized (De Pinto et al., 2007) and triggers MPT at the mitochondrial inner membrane (Tomasello et al., 2009). The effect of VDAC1 overexpression is  $\Delta\psi$  collapse, a phenomenon associated with MPT triggering, a direct consequence of the opening of the PTP complex (Tomasello et al., 2009).

According to these findings, apoptosis induction could be accompanied with VDAC upregulation. Indeed, VDAC was shown to be highly upregulated upon UV irradiation of apoptosis-sensitive cells, as shown using microarray techniques (Voehringer et al., 2000). Similarly, in a proteomic-based analysis of the effect of the anti-cancer drug, arbutin, on the protein expression profile of A375 cells, VDAC was found to be upregulated (Nawaraka et al., 2009). In addition, an increased expression of VDAC is correlated with uterine epithelial apoptosis after estrogen deprivation (Takagi-Morishita et al., 2003). Finally, exposure of a cervix squamous cell carcinoma cell line to cisplatin results in upregulation of VDAC1 (Castagna et al., 2004). All together, these findings indicate that the expression level of VDAC1 may serve as a crucial factor in the process of mitochondria-mediated apoptosis. However, the mechanism underlying cell death induced by VDAC overexpression is not clear.

When seeking such mechanism, several proposals should be considered:

- (1) An increase in VDAC levels might produce an increase in OMM permeability and this, in turn, would decrease cell viability. The results demonstrating that RuR prevents cell death induced by VDAC overexpression and that cells overexpressing HK-I, which reduces VDAC channel conductance, are resistant to cell death induced by VDAC1 overexpression (Zaid et al., 2005), oppose this possibility. It seems that an increase in VDAC1 functionality and not an increase in its total amount is responsible for apoptotic cell death (Zaid et al., 2005).
- (2) Another possibility is that overexpression of VDAC1 leads to an increase in its concentration, shifting the VDAC equilibrium from monomeric to oligomeric species. This VDAC oligomeric state includes a large pore that allows the release of pro-apoptotic proteins, such as Cyto c, from the mitochondrial inter-membrane space ((Zalk et al., 2005), see also Fig. 14). VDAC oligomerization was found to be strongly correlated with apoptosis induction (Keinan, submitted for publication, see Section 8.2.8).
- (3) A third possible mechanism for VDAC1 overexpression inducing cell death may involve its interaction with the ANT at contact sites, thus forming and activating the PTP complex. An increase in expressed VDAC levels would increase VDAC-ANT complexes (see Section 8.2.2, De Pinto et al., 2007). In certain tumor cells, contact sites are absent, suggesting that VDAC-ANT complexes are also missing (Denis-Pouxviel et al., 1987); this may explain suppression of apoptosis in these tumor cells.
- (4) Finally, VDAC has been studied as one of the mediators of oxidative stress-induced apoptosis (Madesh and Hajnoczky, 2001). Therefore it is possible that overexpression of VDAC1 enhances ROS production, thus triggering apoptosis. Indeed, overexpression of VDAC in HeLa cells increased the production of H<sub>2</sub>O<sub>2</sub>. In addition, a correlation between VDAC expression levels, the induction of cell death and H<sub>2</sub>O<sub>2</sub> production by FNQ13 cells has been demonstrated (Simamura et al., 2006).

Hence, while the relationship between VDAC1 overexpression and apoptosis induction is supported by a variety of studies, the mechanism underlying VDAC overexpression-induced cell death is not clear and additional studies are required.



**Fig. 13.** Apoptosis network. A simplified model of the two apoptosis signaling pathways converging to a common execution phase. In the extrinsic, the death receptor-mediated pathway, the death receptor at the plasma membrane (PM) is activated by specific ligands, leading to activation of initiator caspase-8 that activates the executioner caspase-3, which in turn, cleaves cellular substrates and brings about formation of apoptotic bodies. In the intrinsic, mitochondria-mediated pathway, multiple stimuli such as Bax, UV, DNA damage, oxidants, chemotherapies,  $\text{Ca}^{2+}$  overload and ceramide can trigger OMM permeabilization, leading to the release of mitochondrial apoptogenic factors (Cyto c, AIF, Smac/Diablo) from the IMS into the cytosol. The released Cyto c and Apaf-1 form the Apoptosome together with pro-caspase-9, triggering caspase-9 activation which cleaves effector caspases, such as caspase-3. The effector caspases cleave cellular substrates and are responsible for destroying the cell from within, as well as bringing about the formation of apoptotic bodies. The extrinsic apoptotic pathway can activate the mitochondria-mediated intrinsic apoptotic pathway when caspase-8 cleaves Bid. Truncated Bid translocates to the mitochondria and activate the intrinsic apoptotic pathway. The pro-apoptotic molecule, AIF, also released from the mitochondria, is cleaved by calpains and/or cathepsins and translocates to the nucleus, leading to chromatin condensation. Smac/Diablo that is also released from the mitochondria, interacts with IAPs (Inhibitor of Apoptosis Proteins), inhibitors of caspases.

1005

## 8. VDAC as a gatekeeper in mitochondria-mediated apoptosis

1006

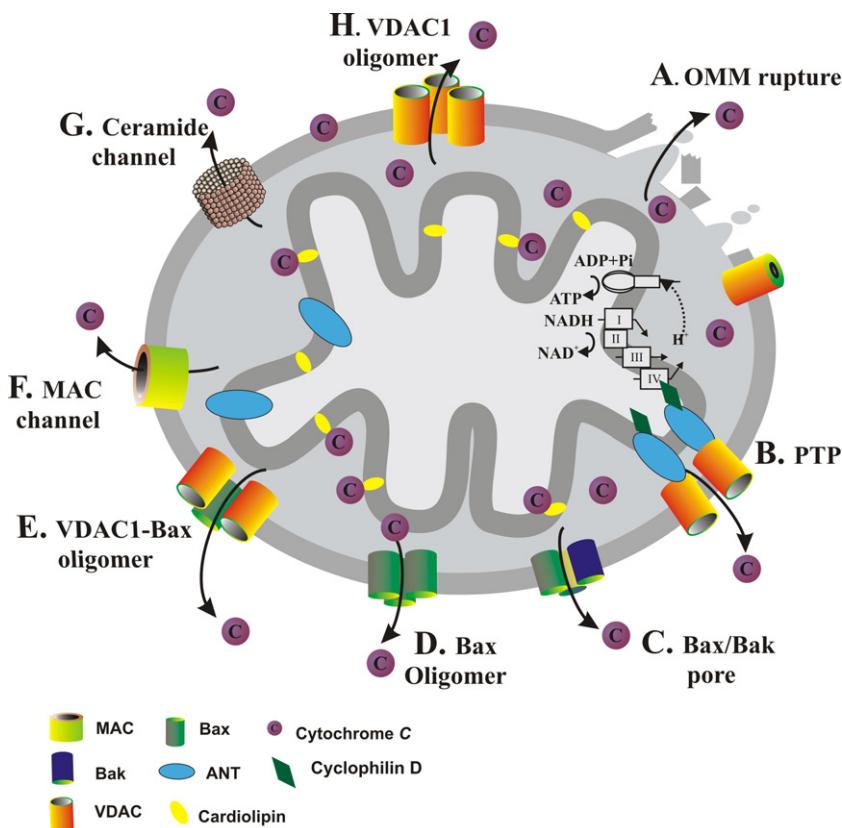
### 8.1. Mitochondria-mediated apoptosis

1007

Cells can undergo death by several modes. One such route involves programmed cell death, or apoptosis. Apoptotic cell death occurs during many physiological conditions, such as during embryonic or immune system development, or in response to infection, DNA damage or disease (Danial and Korsmeyer, 2004; Elmore, 2007; Green, 2003; Hickman, 2002; Johnstone et al., 2002; Olson and Kornbluth, 2001; Tatton and Olanow, 1999). In apoptosis, a cascade of caspases, cysteine protease enzymes capable of cleaving targeted proteins, are activated, subsequently leading to organized cell demise. Defects in the regulation of apoptosis are often associated with disease and drug resistance (Johnstone et al., 2002), with the ability of cells to evade apoptosis being considered a hallmark of cancer (Hanahan and Weinberg, 2000).

1014

Two separate pathways leading to caspase activation have been characterized and are referred to as the intrinsic and the extrinsic pathways (Green, 2000) (Delhalle et al., 2003; Johnstone et al., 2002; Takahashi, 1999) (see Fig. 13). The intrinsic apoptotic pathway involves mitochondria, while the extrinsic apoptotic pathway is activated via death receptors belonging to the tumor necrosis factor alpha ( $\text{TNF-}\alpha$ ) superfamily (Almasan and Ashkenazi, 2003; Baines et al., 2003; Kumar et al., 2005). The intrinsic pathway is initiated in mitochondria in response to different stimuli (Costantini et al., 2000; Green and Reed, 1998; Kroemer, 2003), among which include high levels of cytoplasmic  $\text{Ca}^{2+}$ , reactive oxygen species (ROS), the



**Fig. 14.** Schematic representation of proposed models for the release of apoptogenic proteins from the mitochondrial inter-membrane space mediating the mitochondrial death decision. Different models explaining how the OMM permeability changes during apoptosis induction, allowing the release of apoptogenic factors, such as Cyto c: A, OMM rupture serves as the Cyt c release pathway caused by VDAC closure. B, Permeability transition pore (PTP) – a large conductance pore-forming complex, the PTP, composed of VDAC1 at the OMM, ANT at the IMM and CypD in the matrix, allows apoptogenic proteins release. The PTP is formed in response to radical oxygen species over-production during bioenergetic reactions and  $\text{Ca}^{2+}$  overload. C, Oligomeric Bax forming a Cyto c release channel. D, A pore formed by oligomerized forms of Bax and Bak after their activation by tBid, causing membrane permeabilization. E, Bax- and VDAC1-based hetero-oligomer mediates Cyt c release – the interaction of pro-apoptotic proteins (Bax/Bak) with VDAC1 forms a Cyto c release pathway. F, Mitochondrial apoptosis-inducing channel (MAC), a proteolipid pore formed in response to OMM permeabilization, proposed to serve as a pathway for the release of Cyto c. G, A lipid channel formed by ceramide – a self-assembled ceramide channel is proposed to act as the apoptotic protein release pathway. H, Oligomeric VDAC1 as a channel for the release of apoptotic proteins – a protein-conducting channel is formed within a VDAC1 homo-oligomer. In this manner, VDAC1 oligomerization functions in mitochondria-mediated apoptosis.

activation of pro-apoptotic Bcl-2 family proteins (Keeble and Gilmore, 2007; Kroemer et al., 2007; Le Bras et al., 2005) or UV damage (Denning et al., 2002). The extrinsic apoptotic pathway, however, can also induce activation of the intrinsic pathway, via caspase 8-dependent cleavage of the BH3-only Bcl-2 family protein member, Bid, with its truncated form (tBid) translocating to the mitochondria to activate the intrinsic apoptotic pathway (Berry and Boulton, 2000; Korsmeyer et al., 2000; Yin, 2000). Therefore, tBid links apoptotic signals initiated by death receptors to mitochondria-mediated apoptosis. Thus, mitochondria serve as both initiator and accelerator of apoptotic signals.

The mechanism by which mitochondria are involved in the process of apoptosis has been intensively studied for more than a decade. It is now accepted that the pre-dominant version of apoptosis proceeds through the mitochondrial pathway, with OMM permeabilization leading to the release of apoptotic proteins. Mitochondria contain an arsenal of apoptogenic factors, normally residing in the intermembranal space (IMS), such as Cyto c, AIF (apoptosis-inducing factor), Smac/DIABLO, and endonuclease G, molecules that are critical for the execution stage of apoptosis. In response to an apoptotic signal, these factors are released into the cytosol, as a result of mitochondrial membrane permeabilization (MMP) (Kroemer et al., 2007). Cyto c together with Apaf-1, form the apoptosome, an ATP/Apaf-1/Cyt c complex which is fully functional in recruiting and activating procaspase-9. Activated caspase-9 then cleaves and activates downstream caspases, such as caspase-3, -6, and -7. (Bao and Shi, 2007; Hill et al., 2003; Riedl and Salvesen, 2007; Shi, 2002) (see Fig. 13), which are responsible for destroying the cell from within (Li and Yuan, 2008; Philchenkov, 2003; Salvesen, 2002; Stegh and Peter, 2001). The AIF released from the mitochondria also acts as an effector of apoptotic cell death (Cande et al., 2002). Upon cleavage by calpains and/or cathepsins, AIF is translocated to the nucleus, leading to chromatin condensation (Yuste et al., 2005) and DNA fragmentation (Susin et al., 1999). Thus, release of Cyto c and AIF are key steps in apoptosis induction.

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## 8.2. Proposed models for Cyto c release

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In healthy cells, Cyto c is located in the mitochondrial IMS, where it serves as an electron shuttle between complex III and complex IV in the respiratory chain and most of it bound to cardiolipin (CL). Signals inducing mitochondria-mediated apoptosis resulted in MMP, leading to the release of IMS proteins including Cyto c (see [Section 8.1](#)). The mechanisms by which Cyto c and other pro-apoptotic effector molecules are released have challenged many researchers. Since all of the mitochondrial components known to translocate to the cytoplasm following an apoptotic stimulus reside in the IMS, only the permeability of the OMM needs to be modified. Accordingly, several competing models ([Fig. 14](#)) (Shoshan-Barmatz et al., [2008a](#)) have been proposed to how MMP is induced to allow the release of apoptotic proteins. Some models suggest that release exclusively involves an increase in OMM permeability due to the formation of a channel large enough to allow for the release of proteins, such as Cyto c, while others consider release to be due to disruption of OMM integrity.

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### 8.2.1. Osmotic matrix swelling and OMM rupture, leading to an unspecific release of inter-membrane proteins into the cytosol

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One proposed mechanism suggests that MMP is the results of a sudden increase in IMM permeability to solutes of low molecular weight, leading to MPT. Initial permeabilization of the IMM exerts osmotic forces driving water into the matrix, which undergoes swelling and causes distension of the IMM and eventually, rupture of the OMM, allowing the efflux of IMS proteins, such as Cyto c, to the cytosol (Feldmann et al., [2000](#)). It has been suggested that matrix swelling and OMM rupture results from a defect in mitochondrial ATP/ADP exchange due to VDAC closure (Lemasters and Holmuhamedov, [2006](#); Tan and Colombini, [2007](#); Vander Heiden et al., [2000, 2001](#)) (Model A, in [Fig. 14](#)). This could be obtained, for example, by removal of a normal growth factor that can be restored by the anti-apoptotic Bcl-x<sub>L</sub> protein (Vander Heiden et al., [2000, 2001](#)). Support for the concept of VDAC closure favoring Cyto c release comes with the findings that G3139 reduces OMM permeability to ADP by closing VDAC (Lai et al., [2006](#); Tan and Colombini, [2007](#)) and that ethanol treatment of permeabilized hepatocytes inhibits mitochondrial respiration and decreases permeability of OMM to ATP and respiratory substrates (Lemasters and Holmuhamedov, [2006](#)).

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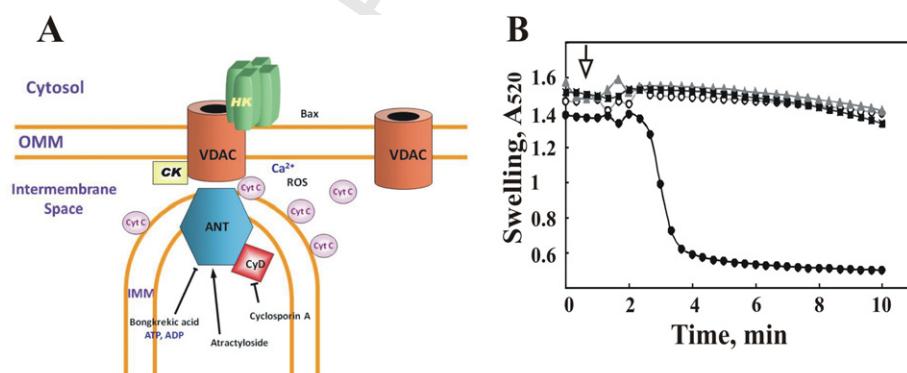
Although, mitochondrial swelling seems to be involved in Cyto c release, many studies offer evidence to the contrary, such as the observation that rat sympathetic neuron mitochondria do not swell during NGF withdrawal-induced apoptosis (Desagher et al., [1999](#)), and that mitochondria from dorsal lateral geniculate nucleus neurons which undergo apoptosis by unilateral occipital cortex ablation in adult rats maintained their morphological integrity until the late, end-stage of apoptosis (Al-Abdulla et al., [1998](#)). Likewise, *Xenopus* oocyte and human cell line HL-60 mitochondria do not swell in response to various apoptotic inductions (von Ahsen et al., [2000](#)). It was also demonstrated that Cyto c release preceded  $\Delta\psi_m$  loss in cerebellar granule neurons undergoing apoptotic death and was not accompanied by mitochondrial swelling and OMM rupture, indicating that mitochondrial swelling might not be involved in Cyto c release in these cells (Wigdal et al., [2002](#)).

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### 8.2.2. The permeability transition pore (PTP) and the release of pro-apoptotic proteins

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A second model (Model B, in [Fig. 14](#)) for MMP suggests the formation of a permeability transition pore (PTP) complex, a large high-conductance multi-protein complex comprising several components and spanning both the IMM and OMM (Bernardi, [1999](#); Halestrap et al., [2000](#); Lemasters et al., [1999](#); Shoshan-Barmatz and Gincel, [2003](#)). The major PTP complex components includes VDAC1 at the OMM, ANT in the IMM, and cyclophilin D (CypD), a mitochondrial peptidyl prolyl-cis,



**Fig. 15.** Proposed molecular architecture of the PTP and its regulation. A. Proposed molecular architecture of the permeability transition pore (PTP) complex and its regulation. PTP involves several trans-membrane proteins: The adenine nucleotide translocator (ANT), VDAC and the peptidyl-prolyl isomerase cyclophilin D (CypD). It also involves members of the Bax/Bcl-2 family, as well as associated proteins, such as HK and mitochondrial creatine kinase (mtCK). Agents or metabolites that facilitate or inhibit PTP opening are indicated. Proteins or peptides carrying the Bcl-2 homology region-3 (BH3) motif may act on either Bax or Bcl-2 (or their homologues) in the OMM, while cyclosporin A acts on CypD. B. Opening of the mitochondrial PTP. When freshly prepared mitochondria generate a membrane potential,  $\text{Ca}^{2+}$  overload can be obtained, subsequently leading to PTP opening. PTP opening can be followed by monitoring mitochondrial swelling, as reflected in the change in absorbance at 520 nm. Mitochondrial swelling was assayed in the absence (○) or the presence of  $\text{Ca}^{2+}$  (●), in the absence of Pi (□) or in the presence of CsA (10  $\mu\text{M}$ ) (△).

trans-isomerase resident in the matrix (Bernardi, 1999; Green and Evan, 2002; Shoshan-Barmatz and Gincel, 2003; Tsujimoto and Shimizu, 2007). In addition, the PTP can interact with the peripheral benzodiazepine receptor (in the OMM), creatine kinase (in the IMS), and hexokinase (HK)-I or HK-II (tethered to VDAC on the cytosolic face of the OMM), as well as with Bax/Bcl-2 proteins (Kroemer et al., 1997; Shoshan-Barmatz and Gincel, 2003; Szabo et al., 1993; Zoratti and Szabo, 1995).

This model is based on the finding that following  $\text{Ca}^{2+}$  accumulation in the matrix, a sudden increase in permeability to solutes (up to 1500 Da) is seen, representing PTP opening (Bernardi, 1999; Halestrap et al., 2000; Lemasters et al., 1999; Shoshan-Barmatz and Gincel, 2003) (Fig. 15). PTP opening could also be caused by other factors, such as changes in the energetic balance of the mitochondria, anoxia and ROS (Crompton, 1999; Kroemer and Reed, 2000; Zoratti and Szabo, 1995). PTP is a regulated pore, corresponding to a large voltage-dependent, non-selective conducting channel with a pore diameter of about 3 nm, features that are compatible with those of VDAC (Zoratti and Szabo, 1995).

Recent studies on the PTP have raised doubts about the importance of PTP in triggering apoptosis (Belizario et al., 2007; Kokoszka et al., 2004; Kroemer, 1997). Using mice lacking CypD, it was revealed that this protein is essential for the occurrence of MPT mediated by  $\text{Ca}^{2+}$  overload, that the CypD-dependent MPT regulates some forms of necrotic cell death but not apoptotic death (Belizario et al., 2007), and that mitochondria isolated from CypD-deficient mice are less susceptible to MPT than are wild-type mitochondria (Kroemer, 1997).

Deletion of both *ant1* and *ant2* genes results in increased resistance of isolated mitochondria to  $\text{Ca}^{2+}$ -induced MPT *in vitro* (Kokoszka et al., 2004). On the other hand, hepatocytes deficient both ANT1 and ANT2 remain responsive to several initiators of apoptosis (Kokoszka et al., 2004), casting doubt on the involvement of ANT in cell death. However, overexpression of the ANT3 isoform in HeLa cells induced apoptosis that was inhibited by bongkrekic acid and cyclosporine A (Zamora et al., 2004). Moreover, a fourth ANT isoform has been identified (Dolce et al., 2005), although its role in MPT has yet to be addressed.

VDAC proteins were reported to be dispensable for  $\text{Ca}^{2+}$ - and oxidative stress-induced PTP opening (Baines et al., 2007). On the other hand, recent studies reported that VDAC1 is an indispensable protein for PTP opening and induction of apoptosis (Tajeddine et al., 2008; Zheng et al., 2004).

Although the mechanism(s) responsible for PTP opening and its physiological function have not yet been resolved, a variety of agents have been found to promote or inhibit PTP opening, including  $\text{Ca}^{2+}$ , inorganic phosphate, various oxidizing agents, glutamate, nucleotides, CypD ligands, gelsolin, ANT, TSPO (PBR), HK, and proteins of the Bcl-2 family. Some of these compounds, such as Bax or Bak, have also been shown to interact with VDAC1 directly and modify its channel activity or become part of the mega-channel that contains VDAC1 (Gincel and Shoshan-Barmatz, 2004; Shimizu et al., 2000b; Shoshan-Barmatz and Gincel, 2003; Shoshan-Barmatz et al., 2006; Sugiyama et al., 2002; Tsujimoto and Shimizu, 2002).

#### 8.2.3. Bax oligomers constitute the Cyto c-conducting channel in the OMM

A third proposed mechanism for MMP and release of Cyto c suggests that Bax, a pro-apoptotic protein, forms selective large channels for Cyto c release (Model C, Fig. 14), with oligomeric Bax serving as the Cyto c-conducting channel in the OMM (Antonsson et al., 2000, 2001; Eskes et al., 2000; Reed, 2006). Indeed, Bax oligomerization has been experimentally implicated in MMP and the release of Cyto c (Kuwana et al., 2002; Zamzami et al., 2000). Cytosolic Bax is weakly associated with mitochondria as a monomer, whereas following apoptosis induction with STS or UV irradiation, Bax became associated with mitochondria as a large oligomer/complex of 96 kDa and 260 kDa. Bcl-2 prevented Bax oligomerization and insertion into the mitochondrial membrane. It has also been demonstrated that tBid directly binds to Bax on the membrane, followed by integration of Bax into the bilayer and subsequent oligomerization of Bax resulting in the formation of pores in membranes, with tBid remaining associated with the pore (Lovell et al., 2008). Although it has been suggested that Bax or Bak homo-oligomerization is sufficient for OMM permeabilization, others suggest that Bax and Bak or VDAC function cooperatively to induce OMM permeabilization (models D and E in Fig. 2). Several studies, however, showed that Bax is not required for apoptosis induction (Lindenboim et al., 2005; Mizuta et al., 2007; Wan et al., 2008).

#### 8.2.4. Bax and Bak oligomers form pores for pro-apoptotic factor efflux during apoptosis

A fourth proposed mechanism to explain MMP and Cyto c efflux (Model D, Fig. 14) suggests that Bax and Bak undergo oligomerization upon apoptotic insult to form pores in the OMM which enable pro-apoptotic effector efflux during apoptosis (Antignani and Youle, 2006; Desagher et al., 1999; Gross et al., 1998; Wei et al., 2000, 2001). Death signaling by TNF- $\alpha$  leads to the activation of caspase-8 which cleaves Bid to tBid, which, in turn, interacts with Bax and Bak to generate Bax and Bak oligomers, forming complexes as large as 500 kDa (Sundararajan et al., 2001). Indeed, Bax and Bak interactions were shown to be augmented by various apoptosis stimuli, resulting in cooperative oligomerization, acting to facilitate formation of mitochondrial membrane pores and cell death in some apoptotic models (Annis et al., 2005; Antonsson et al., 2000; Mikhailov et al., 2003; Sundararajan et al., 2001). At the same time, anti-apoptotic proteins, such as Bcl-2 and Bcl-XL, were shown to protect cells from apoptosis via a blockage of the Bax–Bak interaction, subsequently preventing Cyto c release (Dlugosz et al., 2006; Mikhailov et al., 2003).

#### 8.2.5. Hetro-oligomers composed of VDAC and Bax forms the channel for release of the apoptotic proteins

The possibility that hetro-oligomers composed of VDAC and pro-apoptotic proteins, such as Bax, form the channel for release of apoptotic proteins represents a fifth proposed mechanism for Cyto c efflux (Model E, Fig. 14). It was found that recombinant Bax-induced permeability of liposomes containing intact VDAC but not heat-denatured VDAC, implying that VDAC can induce membrane permeability in the presence of Bax (Shimizu and Tsujimoto, 2000). Furthermore, mitochondria

isolated from VDAC-deficient yeast did not release Cyto c in the presence of Bax, whereas mitochondria from VDAC-deficient yeast expressing the human VDAC1 gene released Cyto c in presence of Bax (Shimizu et al., 1999). In addition, intra-cellular microinjection of neutralizing anti-VDAC antibodies prevented Bax-induced Cyto c release (Shimizu et al., 2001). Electrophysiological studies of Bax and VDAC in planar lipid bilayers revealed that when combined, single-channel conductance rises by factors of 4 and 10 over values attained with VDAC and Bax channels alone, respectively (Banerjee and Ghosh, 2004). Independent evidence supporting the participation of a Bax–VDAC interaction in apoptosis was provided by the fact that HK-I and HK-II can prevent apoptosis by inhibiting interaction of Bax with VDAC, upon binding to VDAC (Pastorino et al., 2002). Another version of this model suggests that oligomeric VDAC is the prime Cyto c release channel and that its pore is regulated by Bax (Debatin et al., 2002). More recently, it was demonstrated that siRNA-mediated down-expression of VDAC1 strongly suppressed cisplatin-induced activation of Bax (Tajeddine et al., 2008).

#### 8.2.6. Mitochondrial apoptosis-induced channel (MAC) as a pathway for Cyto c release

A sixth proposed mechanism (Model F, Fig. 14) involves mitochondrial apoptosis-induced channel (MAC) formation in the mitochondria (Dejean et al., 2005, 2006a,b; Guo et al., 2004; Martinez-Caballero et al., 2004, 2005). The MAC is proposed to be a supramolecular high-conductance channel in the OMM that might be formed during early apoptosis, and through which Cyto c can be released in a manner regulated by Bcl-2 family members (Kinnally and Antonsson, 2007; Klasa et al., 2002). MAC is tightly regulated by Bcl-2 family proteins, with the multi-domain pro-apoptotic proteins, Bax and Bak, being putative components of this channel (Dejean et al., 2005, 2006a,b; Guo et al., 2004; Martinez-Caballero et al., 2004, 2005). The complete molecular identity of MAC is not known (Antonsson et al., 2001).

#### 8.2.7. Ceramides and the release of Cyto c

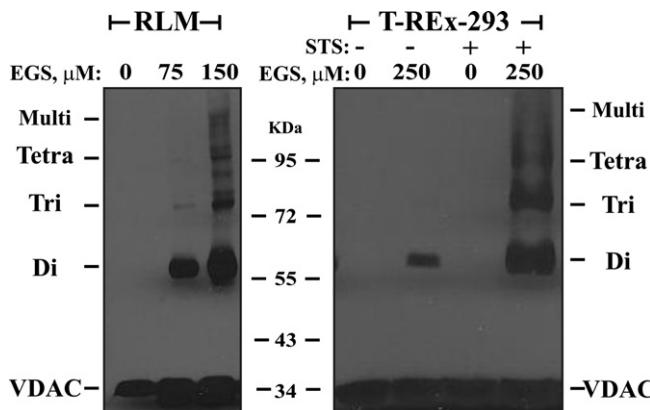
An additional proposed mechanism for the release of Cyto c (Model G, in Fig. 14) suggests the involvement of the lipid, ceramide, acting through the formation of a lipidic channel in the OMM (Siskind et al., 2006; Stiban et al., 2007). Ceramides have been proposed to function as important second messengers in apoptosis signaling pathways and indeed were shown to be produced during apoptosis, where they directly target mitochondria. Ceramides were shown to promote Cyto c release from isolated rat liver mitochondria (Ghafourifar et al., 1999). Three mechanisms have been proposed for the action of ceramides in apoptosis. Colombini's group has shown that ceramides form large stable channels in planar phospholipid membranes (Siskind and Colombini, 2000; Siskind et al., 2006; Stiban et al., 2007), and it is postulated that these may form in the OMM with a diameter large enough to accommodate Cyto c passage. The alternative mechanism proposes that ceramides affect membrane organization (Cremesti et al., 2001), with the primary effect of ceramide being to promote the dissociation of Cyto c by altering lipid microdomains in the IMM, thereby disrupt the association between Cyto c and anionic phospholipids (Yuan et al., 2003). It has suggested that ceramide interacts with Cyto c and affects its ability to interact with the electron transport chain (Ghafourifar et al., 1999; Richter and Ghafourifar, 1999). Another proposal suggest ceramides play a role in Bax activation (Birbes et al., 2005; Oh et al., 2006), with the function of ceramide and cholesterol in the membrane micro-environments being to favor Bax translocation to mitochondria, fostering propagation of the apoptotic cascade (Martinez-Abindis et al., 2009).

#### 8.2.8. VDAC oligomerization and release of Cyto c

A more recent model to describe Cyto c release developed in Shoshan-Barmatz's group suggests that mitochondrial pore formation during apoptosis involves the assembly of homo-oligomers of VDAC1 (Model H, Fig. 14), (Abu-Hamad et al., 2009; Shoshan-Barmatz et al., 2006, 2008a; Zalk et al., 2005). This model is based on a VDAC1 diameter pore (2.5–3.0 nm) sufficient to move nucleotides and small molecules but insufficient to pass a folded protein, like Cyto c. Thus, a model in which Cyto c release takes place through the formation of larger pores by oligomerization of VDAC has been proposed (Abu-Hamad et al., 2009; Shoshan-Barmatz et al., 2006, 2008a; Zalk et al., 2005).

Over the years, much evidence consistent with the oligomerization of VDAC1 has been presented. The existence of dimeric and oligomeric forms of VDAC in rat liver and yeast mitochondria were noted more than 20 years ago (Krause et al., 1986; Linden and Gellerfors, 1983; Pfaller et al., 1985). The hydrodynamic properties of purified rat liver VDAC1 implies that the isolated active protein exists as a dimer after solubilization and purification using Triton X-100 (Linden and Gellerfors, 1983) or oligomers after cross-linking of yeast OMM (Krause et al., 1986). The existence of VDAC1 dimers and oligomers after cross-linking of VDAC1 from *N. crassa* was also demonstrated (Pfaller et al., 1985). A low-resolution (15 Å) surface structure of VDAC1, obtained by metal shadowing and cryoelectron microscopy of human VDAC1 crystals grown in the presence of phospholipids, showed a dimeric organization of VDAC1 (Dolder et al., 1999). Cross-linking experiments of purified VDAC1 and membrane-embedded VDAC1 with five different cross-linking reagents (3–16 Å space) demonstrated the assembly of dimers, trimers, tetramers and higher VDAC oligomers (Zalk et al., 2005) (see Fig. 16). Dynamic VDAC oligomerization was verified by fluorescence resonance energy transfer (FRET) analysis using purified VDAC labeled with FITC or EITC as donor and acceptor fluorophores, respectively, in liposomes (Zalk et al., 2005). The enhancement in FRET signaling upon exposure to oxygen radical-generating conditions indicated that FITC-VDAC and EITC-VDAC joined to form dimers or higher oligomeric combinations. FRET intensity was decreased upon addition of excess unlabeled VDAC during the dissociation–reassociation process. Interestingly, Cyto c-encapsulated liposomes also encouraged VDAC1 oligomerization (Zalk et al., 2005).

The supra-molecular organization of VDAC has also been demonstrated using atomic force microscopy (AFM) of the native outer mitochondrial membrane (Goncalves et al., 2007; Hoogenboom et al., 2007). Purified potato tuber OMM images



**Fig. 16.** Apoptosis induction enhances VDAC oligomerization. A. Rat liver mitochondria (1 mg/ml) were incubated with or without the cell permeable cross-linking reagent, EGS (75 μM, 150 μM) for 10 min at 30 °C in a solution containing 10 mM Tricine, 150 mM NaCl, pH 8.3. B. T-Rex-293 cells were exposed to the apoptosis-inducing reagent, STS (1.25 μM, 2.5 h), washed twice with PBS and incubated (3 mg/ml) at 30 °C with EGS (250 μM) for 15 min. Samples were then subjected to SDS-PAGE, followed by immunoblotting using anti-VDAC antibodies. VDAC monomers, dimers (Di), trimers (Tri), tetramers (Tetra) and multimers (Multi) are indicated. The positions of molecular weight protein standards are provided.

show the native distribution of VDAC membrane-embedded pores in an oligomeric equilibrium ranging from single molecule to hexamers and higher order oligomers (Hoogenboom et al., 2007). AFM images of purified yeast OMM revealed VDAC in the form of monomers, dimers, trimers, hexamers, and arrays of up to 20 molecules (Goncalves et al., 2007). In addition, nuclear magnetic resonance (NMR) studies imply that Bcl-x<sub>L</sub> mediates formation of heterotrimers including two VDAC1 molecules (Bayrhuber et al., 2008; Hiller et al., 2008; Malia and Wagner, 2007). Recently, the application of symmetry operators on the NMR-based structure of recombinant hVDAC1 (Bayrhuber et al., 2008) indicated that it may form a parallel dimer (Fig. 6, further structural details are presented in Section 4).

The most impressive finding of studies addressing VDAC1 oligomerization is related to the direct relationship between this event and apoptosis induction (Abu-Hamad et al., 2009; Alexander et al., 2007; Keinan, submitted for publication; Shoshan-Barmatz et al., 2008a, 2009) (see Fig. 16). Recently, it has been demonstrated that apoptosis induction by various inducers was accompanied by an up to 20-fold increase in VDAC1 oligomerization, as revealed by chemical cross-linking, indicating a shift of VDAC1 organization toward the oligomeric form (Alexander et al., 2007; Keinan, submitted for publication; Shoshan-Barmatz et al., 2008a). A VDAC1 oligomeric state was also directly monitored in living cells using BRET (Bioluminescence Resonance Energy Transfer) technology, showing enhanced BRET signal (3–5-fold) upon apoptosis induction (Keinan, submitted for publication). In this study, apoptosis-mediated enhancement of VDAC oligomerization was obtained regardless of the cell type or apoptosis inducer used, including staurosporine (STS), curcumin, As<sub>2</sub>O<sub>3</sub>, etoposide, cisplatin, selenite, TNF-α, H<sub>2</sub>O<sub>2</sub> or UV, all affecting mitochondria yet acting through different mechanisms. Moreover, correlation between the level of VDAC oligomerization and apoptosis, as a function of the concentration and time of exposure to the apoptosis stimuli, was observed. Conversely, the apoptosis inhibitor, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), prevented STS-induced VDAC oligomerization and apoptosis (Keinan, submitted for publication; Shoshan-Barmatz et al., 2008a).

It was also found that overexpression of VDAC1 shifts its equilibrium status towards the oligomer state (Shoshan-Barmatz et al., 2008a), thus allowing the release of pro-apoptotic proteins, leading to cell death (Abu-Hamad et al., 2008; Azoulay-Zohar et al., 2004; Zaid et al., 2005). These results show that the cellular expression levels and the oligomerization state of VDAC1 are crucial factors in the process of mitochondria-mediated apoptosis. Similarly, the apoptosis-inducing effect of As<sub>2</sub>O<sub>3</sub> was attributed to an induction of VDAC1 homo-dimerization that was prevented by overexpression of the anti-apoptotic protein, Bcl-x<sub>L</sub> (Zheng et al., 2004). Recently, the Hepatitis E virus ORF3 protein was found to upregulate VDAC expression levels, with ORF3-expressing cells showing enhanced levels of oligomeric VDAC, as revealed by chemical cross-linking (Moin et al., 2007).

Hence, the substantial evidence that exists for the formation of higher ordered VDAC1-containing complexes and the enhancement of supra-molecular assembly of VDAC1 in cultured cells upon apoptosis induction supports the involvement of VDAC1 oligomerization in Cyto c release and thus, in apoptosis. Though the signaling mechanism responsible for induction of VDAC oligomerization *in vivo* is not yet known, the dynamic equilibrium between monomers and oligomers can be influenced by Ca<sup>2+</sup>, ROS, low ATP levels and associated proteins, such as HK-I, or proteins from the Bcl-2 family, all known to interact with VDAC (Shoshan-Barmatz et al., 2004, 2008a).

It is, however, likely that alternative models for the release of Cyto c and other apoptotic proteins exist. Indeed, accumulating evidence suggests that multiple pathways and mechanisms of Cyto c release can co-exist within a single model of cell death, depending on the cell type and the nature of the stimulus (Galluzzi and Kroemer, 2007; Gogvadze et al., 2006).

## 1229 8.3. Analysis of VDAC apoptotic activity with anti-VDAC antibodies

1230 Anti-VDAC antibodies have been used for analysis of VDAC function in various processes. Anti-VDAC1 antibodies gener-  
1231 ated by Shimizu et al. (Ab# 25, Shimizu et al., 2001) were found to block VDAC activity. When these antibodies were micro-  
1232 injected into cells, they prevented Bax-induced Cyto c release and subsequent apoptosis but not Bid-induced apoptosis,  
1233 indicating that VDAC is essential for Bax-induced but not Bid-induced apoptotic cell death. In addition, microinjection of  
1234 these antibodies significantly inhibits etoposide-, paclitaxel-, and STS-induced apoptosis. In permeabilized mammalian cells,  
1235 both Cyto c release and loss of  $\Delta\psi$  induced by superoxide radicals were prevented in cells pre-treated with the anti-VDAC1  
1236 antibody Ab#25 (Madesh and Hajnoczky, 2001). Anti-VDAC antibodies also specifically and effectively prevent As<sub>2</sub>O<sub>3</sub>-in-  
1237duced Cyto c release from isolated mitochondria (Zheng et al., 2004) and inhibit the apoptotic process induced by an opening  
1238 of plasma membrane VDAC (Elinder et al., 2005). Microinjection of anti-VDAC antibodies into primary rat hepatocytes effec-  
1239 tively prevents apoptosis and Bax-VDAC interactions, as induced by ethanol (Adachi et al., 2004). The effects of anti-VDAC  
1240 antibodies in inhibition of Cyto c release, cell death and Bax-VDAC interaction by anti-VDAC antibodies emphasize the cen-  
1241 tral role of VDAC in apoptosis.

## 1242 9. VDAC-associated proteins

1243 VDAC1 localization in the OMM makes it not only a major gate for molecules that need to access and/or exit the IMS but  
1244 also makes VDAC a functional anchor point for molecules that interact with the mitochondria. VDAC1, moreover, plays an  
1245 important role in the coordination of communication between the mitochondria and the rest of the cell. A substantial aspect  
1246 of this management involves the transient formation of complexes with other proteins (Vysokikh et al., 2004). It has been  
1247 suggested that VDAC permeability, and hence OMM permeability, is regulated by associated protein(s). Accordingly, VDAC  
1248 displays binding sites for glycerol kinase, hexokinase (HK) and creatine kinase (Beutner et al., 1998). Creatine kinase inter-  
1249 acts with VDAC1 exclusively in the octameric state, with the dimeric state only showing weak affinity for VDAC1 (Schlattner  
1250 et al., 2001). When creatine kinase binds to VDAC1 the affinity of VDAC for HK and Bax decreases (Vysokikh et al., 2004). C-  
1251 Raf kinase has also been reported to negatively regulate VDAC1 and promote cell survival by prohibiting Cyto c release not by  
1252 phosphorylation of VDAC1 but rather by direct interaction (Le Mellay et al., 2002). VDAC also forms complexes with various  
1253 other proteins, such as the ANT, the peripheral benzodiazepine receptor (TSPO) (Kugler et al., 2008; Levin et al., 2005; Veen-  
1254 man et al., 2007), tubulin (Rostovtseva and Bezrukova, 2008; Rostovtseva et al., 2008b), the dynein light chain, mtHSP70, the  
1255 ORDIC channel, glyceraldehyde 3-phosphate dehydrogenase (Shoshan-Barmatz et al., 2008a; Shoshan-Barmatz and Israe-  
1256 lsson, 2005), actin (Xu et al., 2001) and gelsolin (Kusano et al., 2000), as well as and with apoptosis-regulating proteins, namely  
1257 members of the Bcl-2 family (reviewed in Colombini, 2004; Shoshan-Barmatz et al., 2006, 2008a). VDAC1 thus interacts with  
1258 many pro- and anti-apoptotic proteins, making VDAC a key protein in apoptosis (Shoshan-Barmatz et al., 2006, 2008a; Tsu-  
1259 jimoto and Shimizu, 2002).

## 1260 9.1. Hexokinase interaction with VDAC1 and regulating cells bioenergetics and apoptosis

## 1261 9.1.1. Hexokinase expression in cancer cells and apoptosis

1262 HK catalyzes the first step of glycolysis, the phosphorylation of glucose to glucose-6-phosphate (G-6-P). This action of HK,  
1263 the rate-limiting enzyme in glycolysis, sequesters glucose inside the cells. Multiple forms of HK are present in most cell types  
1264 (Wilson, 2003). Of the four mammalian HK isozymes, HK-I and -II are capable of binding to the OMM, more specifically to  
1265 VDAC (Abu-Hamad et al., 2008; Arzoine et al., 2009; Azoulay-Zohar et al., 2004; Pastorino and Hoek, 2008; Pastorino et al.,  
1266 2002, 2005; Shoshan-Barmatz et al., 2006, 2008b, 2009).

1267 Cancer cells are characterized by a high rate of glycolysis which serves as their primary energy-generating pathway  
1268 (Mathupala et al., 2006; Pedersen, 2007; Pedersen et al., 2002). Indeed, one of the signature phenotypes of highly malignant,  
1269 poorly differentiated tumors is their remarkable propensity to utilize glucose at a much higher rate than normal cells, a prop-  
1270 erty frequently dependent on the marked overexpression of HK (Bryson et al., 2002; Gottlob et al., 2001; Pedersen et al.,  
1271 2002; Rose et al., 1974). HK-I and HK-II were found to be overexpressed in many types of cancer, including colon, prostate,  
1272 lymphoma, glioma, gastric adenomas, carcinomas and breast cancers (Bryson et al., 2002; Gottlob et al., 2001). The high  
1273 expression of HK in cancer cells is thought to correlate with the rate of tumor growth. HK thus lies at the apex of the gly-  
1274 colytic pathway that provides those metabolic intermediates required by the biosynthetic pathways on which a transformed  
1275 cell places such heavy demand (Pedersen, 2007). This observation provided a plausible explanation for the observation by  
1276 Warburg over 80 years ago, who, in the 1920s and 1930s, described a consistent phenotype of cancer cells that is now known  
1277 as the Warburg effect. In normal cells, the presence of oxygen suppresses glycolysis, referred to as the Pasteur effect. By con-  
1278 trast, glycolysis is increased in cancer cells at the same time as cells actively consume oxygen for oxidative phosphorylation.  
1279 This aerobic glycolysis suggests that mitochondrial defects may be essential for cancer cell growth.

1280 The elevated levels of mitochondria-bound HK in cancer cells is thus suggested to play a pivotal role in promoting cell  
1281 growth and survival in rapidly growing, highly glycolytic tumors and in protecting against mitochondria-mediated cell death  
1282 (Mathupala et al., 2006). Indeed, mitochondrially-associated HK activity has been shown to protect HeLa and human embry-  
1283 onic kidney cells from entering apoptosis (Bryson et al., 2002). This protection was related to the blockade of the interaction

of the pro-apoptotic protein, Bax, with VDAC (Bryson et al., 2002). Moreover, overexpression of mitochondria-bound HK in the tumor-derived cell lines, U-937 and T-REX-293, or in vascular smooth muscle cells suppressed STS-induced Cyto c release and apoptosis (Arzoine et al., 2009; Azoulay-Zohar et al., 2004; Zaid et al., 2005). A decrease in apoptotic cell death and an increase in cell proliferation have also been reported to be induced by HK-II expression in the NIH-3T3 (Fanciulli et al., 1994) and rat 1a cell lines (Gottlob et al., 2001). In addition, binding of HK-II to mitochondria inhibits Bax-induced Cyto c release and apoptosis (Pastorino et al., 2002). Moreover, it was found that Akt promotes binding of HK-II to VDAC (Gottlob et al., 2001). At the mitochondria, Akt can directly phosphorylate HK-II at threonine 473 (Miyamoto et al., 2008). Such phosphorylation prompts association of HK-II with VDAC. Accordingly, Akt maintains mitochondrial integrity and promotes glycolysis and cell survival. However, other models propose that while Akt regulates HK attachment to mitochondria, this association may prevent Cyto c release, where, depending on the open or closed state of VDAC, HK could be either attached to or detached from VDAC, consequently modulating anti- or pro-apoptotic signals (Robey and Hay, 2006).

#### 9.1.2. VDAC as the mitochondrial target of HK

*In vitro* and *in vivo* studies have shown that HK-I and HK-II play a clear role in protecting against mitochondria-regulated apoptosis through direct docking onto the cytosolic surface of the OMM by binding to VDAC. The interaction of VDAC1 with HK-I and HK-II in cancerous cells promotes cell survival (Abu-Hamad et al., 2008; Arzoine et al., 2009; Azoulay-Zohar et al., 2004; Mathupala et al., 2006; Pastorino and Hoek, 2008; Pastorino et al., 2002; Shoshan-Barmatz et al., 2008a; Zaid et al., 2005). By binding to VDAC, HK gains direct access to the mitochondrial ATP pool for phosphorylation of glucose (Arora and Pedersen, 1988).

The first demonstration showing that a form of HK, now known to be HK-II, bind to VDAC in the OMM of a rapidly growing cancer cell line was documented 23 years ago in a study by Nakashima et al. (1986). Thereafter, several lines of evidence clearly demonstrated the interaction of HK-I and HK-II with VDAC (Arzoine et al., 2009; Galluzzi et al., 2006; Majewski et al., 2004a; Pedersen, 2007; Robey and Hay, 2005). It has been shown that the hydrophobic tail of the HK N-terminal domain is inserted into the membrane where it may interact with one or more of the trans-membrane domains of VDAC1 (Xie and Wilson, 1988). Several other studies indicate that other regions of HK come in close apposition to the membrane and may help stabilize the interaction of HK with VDAC or other proteins bound to VDAC, as supported by antibody interference studies (Hashimoto and Wilson, 2000). HK-I directly interacts with bilayer-reconstituted VDAC to induce closure of the VDAC channel in a manner that is reversed by glucose-6-phosphate (Azoulay-Zohar et al., 2004). In addition, HK-I and VDAC have been co-immunoprecipitated (Shoshan-Barmatz et al., 2008b). Mutagenesis studies have demonstrated that charged residues in VDAC1, i.e. glutamate 65, glutamate 72, aspartate 77 and lysine 73 as being required for HK-I-mediated protection against VDAC1-linked apoptosis and for channel closure in a reconstituted membrane system (Abu-Hamad et al., 2008; Zaid et al., 2005). In addition, mutations in glutamate 202 and 188, while not essential for binding, were found to stabilize the interaction of HK-I with VDAC and to allow for protection against apoptosis (Abu-Hamad et al., 2008). In addition, N-terminally truncated VDAC1 lost its HK binding capacity (Abu-Hamad et al., 2009), while mutation in the N-terminal region of VDAC (i.e. K20S and G21A) was also found to diminish HK-I-mediated protection against apoptosis (Shoshan-Barmatz et al., 2009). Thus, the finding that a single mutation in VDAC1 prevents HK-mediated protection against apoptotic cell death clearly indicates that the anti-apoptotic effect of HK is mediated via its interaction with VDAC (Arzoine et al., 2009) including with its N-terminal region (Abu-Hamad et al., 2009). The regulation of VDAC-HK interaction by phosphorylation involving Akt and glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) was demonstrated (Pastorino et al., 2005). Activation of GSK3 $\beta$  disrupts the binding of HK-II to mitochondria by phosphorylating VDAC, with HK-II being unable to bind to VDAC phosphorylated by GSK3 $\beta$ .

#### 9.1.3. Possible mechanisms by which the detachment of mitochondrial-bound HK could lead to cell death

The molecular mechanisms by which HK promotes cell survival are not yet fully understood. Numerous studies clearly demonstrated that mitochondrial HK activity is pivotal for the physiological functions of mitochondria in cell life and death, particularly in cancer cells. Several models have been proposed to explain how HK-I and HK-II promote tumor cell survival and anti-apoptotic defense, all involving interaction with VDAC1. The direct binding of HK to VDAC and the involvement of VDAC in cell death raise the possibility that a HK-VDAC interaction lies at the base of cell survival and apoptosis regulation by HK, as reflected in the several possible mechanisms that may be considered:

**9.1.3.1. Disrupting the energy balance of highly demanding cancer cells and allowing apoptosis induction.** The balance between cytosolic and mitochondrial-bound HK is important for the regulation of glycolysis. Depending on physiological conditions, variations in intra-cellular distribution could serve as a regulatory mechanism of HK action (Miccoli et al., 1998; Wilson, 1978). HK bound to VDAC on the mitochondrial surface provides both metabolic benefit and apoptosis-suppressive capacity that offers the cell a growth advantage and increases its resistance to chemotherapy (Pedersen, 2007). (i) Mitochondrial-bound HK-I and HK-II have direct access to mitochondrial sources of ATP and greater affinity for MgATP supplied by mitochondria (Bustamante and Pedersen, 1980; Pedersen, 2008). (ii) HK bound to the cytosolic face of VDAC acts as a gate, regulating channel ion conductivity (Azoulay-Zohar et al., 2004). (iii) When bound to the mitochondria, HK is less susceptible to inhibition by its product, Glu-6-P, a metabolic intermediate precursor in most biosynthetic pathways (Rose et al., 1974). Mitochondrial-bound HK-I is less sensitive to inhibition by Glu-6-P, with a  $IC_{50}$  of 5 mM, compared with a  $IC_{50}$  of 0.1 mM for the soluble form of the enzyme (Azoulay-Zohar et al., 2004). An inhibition of HK activity by Glu-6-P would reduce energy production for high energy-demanding cancer cells.

HK-I was shown to inhibit PTP opening, while Glu-6-P favored PTP opening (Azoulay-Zohar et al., 2004), presumably by detaching HK from its binding site (Azoulay-Zohar et al., 2004; Wilson and Chung, 1989). Thus, high levels of Glu-6-P not only inhibit HK catalytic activity but also promote PTP opening, and thereby, induction of apoptosis. (v) HK-I and HK-II binding to VDAC protects against apoptosis, with their release allowing for activation of apoptosis (Azoulay-Zohar et al., 2004; Baijal and Wilson, 1995; Hashimoto and Wilson, 2000; Wilson and Chung, 1989).

Thus, detachment of mitochondrial-bound HK from VDAC affects mitochondrial and overall cellular bioenergetics. Indeed, agents known to detach mitochondrial HK also induce ATP depletion preceding the decrease in cell viability (Machida et al., 2006; Miccoli et al., 1998).

**9.1.3.2. HK-mediated regulation of ROS production.** Another possible mechanism for HK promoting cancer cell survival involves the regulation of ROS production. It had been shown that mitochondrial HK activity fulfilled a key role as a preventive anti-oxidant against oxidative stress, reducing mitochondrial ROS generation through an ADP-recycling mechanism (da-Silva et al., 2004). Expression of HK-II was found to protect against oxidant-induced cell death (Ahmad et al., 2002), while HK-I overexpression in an established epithelial cell line led to protection against oxidation-induced cell death (Bryson et al., 2002). It was also demonstrated that HK-I and HK-II reduce intra-cellular levels of ROS (Sun et al., 2008). In addition, while glucose increased the rate of oxygen consumption and reduced the rate of H<sub>2</sub>O<sub>2</sub> generation, Glu-6-P increased H<sub>2</sub>O<sub>2</sub> generation (da-Silva et al., 2004). Thus, overexpression of mitochondrial-bound HK-I and HK-II protects cells against damage from oxidative stress. Accordingly, detachment of HK from its binding site in the mitochondria could lead to increased H<sub>2</sub>O<sub>2</sub> generation, thereby activating cell death.

**9.1.3.3. Displacing HK from VDAC promotes apoptosis induction by Bax and Bid.** Disruption of HK-VDAC complex may involve an interaction of Bax with the mitochondria. The HK-VDAC interaction changes the susceptibility of mitochondria to pro-apoptotic signals mediated through Bcl-2-family proteins, such as Bax (Pastorino and Hoek, 2008). One of the proposed models by which Bax would mediate Cyto c release from mitochondria involves the formation of a Bax/VDAC complex (see Section 8.2.5). It has been shown that VDAC-bound HK renders cells much more resistant to activation of apoptosis by Bax or Bak (Majewski et al., 2004b; Pastorino et al., 2002). It has also suggested that Bax and Bid may promote apoptosis by displacing HK from VDAC (Majewski et al., 2004b; Pastorino et al., 2002). For this activity, the C-terminal of Bax is required, since Bax-ΔC was unable to displace HK or liberate Cyto c from the reconstituted HK-VDAC-ANT complex (Vyssokikh et al., 2004). Accordingly, displacement of HK from its binding site in VDAC, by either means, would permit the actions of Bax/Bak in mediating apoptosis.

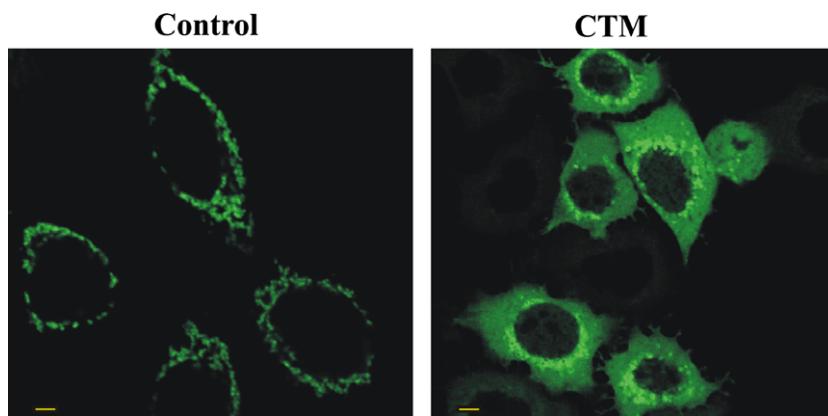
Regardless of the exact mechanism by which dissociation of HK from mitochondria promotes apoptosis, it is well accepted that mitochondrial-bound HK plays a critical role in the regulation of mammalian mitochondria-dependent apoptosis.

#### 9.1.4. Disruption of the HK-VDAC interaction as an approach to cancer therapy

If, as suggested, the HK-VDAC interaction lies at the heart of apoptosis regulation by HK, interfering with HK binding to VDAC would form the basis for novel cancer treatment. One of the hallmarks of tumor cells is their relative resistance to cell death, owing to overexpression of anti-apoptotic proteins of the Bcl-2 family and HK (Ding et al., 2000; Mathupala et al., 2006; Pedersen et al., 2002; Rempel et al., 1994; Takehara et al., 2001). The greatly enhanced expression of HK-II noted in aggressive tumors, such as gliomas (Pastorino and Hoek, 2003), and of HK-II and HK-I in hepatomas (Gelb et al., 1992), make HK-I and HK-II attractive targets for cancer therapy. As discussed above, it has been shown that a HK-VDAC1 interaction prevents induction of apoptosis in tumor-derived cells (Abu-Hamad et al., 2008; Alexander et al., 2007; Arzoine et al., 2009; Gelb et al., 1992; Pastorino and Hoek, 2008; Pastorino et al., 2002). Thus, promoting detachment of HK from its mitochondrial-binding site, i.e. VDAC, offers a promising cancer therapy strategy.

Two main classes of agents affect the HK-VDAC1 association. The first class includes competitive and/or allosteric compounds, i.e. inhibitors of HK activity (Lampidis et al., 2006; Oudard et al., 1995; Robey and Hay, 2005; Shoshan-Barmatz et al., 2008a; Wilson, 1989). The second class includes compounds that compete with VDAC for HK binding. Several compounds of both classes have been shown to induce dissociation of HK from the mitochondria and subsequent apoptosis. These include peptides corresponding to the amino terminus of both HK-I (Gelb et al., 1992) and HK-II (Pastorino et al., 2002), clotrimazole (Pastorino et al., 2002; Penso and Beitner, 1998), and a cell-permeable peptide corresponding to the amino-terminal 23 residues of HK-II fused to six arginines at the carboxyl-terminus (N-HK-II) (Majewski et al., 2004a). Recently, it was shown that the plant stress hormone of the jasmonate family, methyl jasmonate, binds to HK and detaches it from mitochondria isolated from several cancer cell types (Goldin et al., 2008). Apoptosis-inducing reagents, such as clotrimazole (Penso and Beitner, 1998), were found to detach HK from its binding site (see Fig. 17).

Recently, selected VDAC1-based peptides were shown to interact with purified HK-I and detach HK bound to mitochondria isolated from tumor cells (Arzoine et al., 2009) and in cells containing mitochondrial-bound HK-I-GFP (Shoshan-Barmatz et al., 2009). In addition, these VDAC1-based peptides were found to bind to purified HK (Arzoine et al., 2009). These findings suggest that interfering with the binding of HK to mitochondria by VDAC1-based peptides may offer a novel strategy by which to augment apoptosis, thus enhancing the synergistic therapeutic efficacy of conventional chemotherapeutic agents and reducing dose-limiting toxicity.



**Fig. 17.** Detachment of mitochondrial-bound HK-I-GFP by clotrimazole. To demonstrate HK-I binding to mitochondria (i.e. VDAC) as well as detachment, HK-I-GFP fusion protein was expressed in HeLa cells. Confocal fluorescence microscopy showed that in control cells expressing HK-I-GFP, the fluorescence is punctuated, as expected for mitochondrial distribution. On the other hand, clotrimazole (CTM), an anti-fungal azole derivative that has been shown to dissociate HK-II from mitochondria in several cell types (Majewski et al., 2004a; Pastorino et al., 2002), detaches mitochondrial-bound HK-I-GFP. The punctuated HK-I-GFP fluorescence, originally co-localized with mitochondria, was converted to diffuse labeling of the cytosol after exposure to CTM (40 µM), for 3 h. Images are representative microscopic fields from one of three similar experiments. (Scale bar = 5 µm).

#### 9.2. Members of the Bcl-2 family of proteins interact with VDAC

The Bcl-2 family of proteins functions as central regulators of apoptosis in mammals and includes proteins that both up- and down-regulate apoptosis. Members of the Bcl-2 family control the integrity of the OMM and are thus critical in determining the susceptibility of cells to apoptosis induced by the mitochondria-mediated pathway. The Bcl-2 family comprises anti-apoptotic members, such as Bcl-2, Mcl-1, Bcl-x<sub>L</sub> and Bcl-2A1, multi-domain pro-apoptotic members, such as Bax and Bak, and pro-apoptotic BH3-only proteins, including Bad, Bim, Puma, Bid, Bik, Noxa and Bmf (Adams and Cory, 2007; Cory and Adams, 2002; Danial and Korsmeyer, 2004; Huang and Strasser, 2000; Youle and Strasser, 2008).

While anti-apoptotic proteins, such as Bcl-2, are localized to several intra-cellular membranes, including those of the mitochondria, endoplasmic reticulum, and the nuclear envelope (Gross et al., 1999; Krajewski et al., 1993), pro-apoptotic members of the Bcl-2 family, such as Bax and Bak, translocate to mitochondria upon encountering a death signal where they form oligomers leading to permeabilization of the OMM, release of Cyto c and caspase activation (Gross et al., 1998). The anti-apoptotic Bcl-2 family members prevent such release. The rules of engagement between the pro- and anti-apoptotic family members are still contested. Anti-apoptotic members of the Bcl-2-family contribute to tumor initiation, disease progression and drug resistance. In fact, high levels of anti-apoptotic Bcl-2 family members have been associated with resistance of many tumors to chemotherapy (Adams and Cory, 2007; Miyashita and Reed, 1993; Sentman et al., 1991). Despite extensive study, the manner by which Bcl-2 family proteins regulate apoptosis remains speculative (Chipuk and Green, 2008). Nonetheless, it is well-established that their activity is mediated via interactions with the mitochondria and by controlling OMM permeability (Scorrano and Korsmeyer, 2003; Wei et al., 2001; Youle and Strasser, 2008).

Accumulated evidence indicates that both anti- and pro-apoptotic proteins, such as Bax, Bax, Bim and Bcl-xL, interact with VDAC, to regulate mitochondria-mediated apoptosis (Abu-Hamad et al., 2009; Alexander et al., 2007; Arzoine et al., 2009; Malia and Wagner, 2007; Shimizu et al., 2000a; Sugiyama et al., 2002; Tajeddine et al., 2008; Tsujimoto, 2003; Arbel and Shoshan-Barmatz, 2009). It was previously demonstrated that Bax accelerated the efflux of [<sup>14</sup>C] sucrose from VDAC1-containing proteoliposomes, whereas Bcl-x<sub>L</sub> inhibited such efflux (Shimizu et al., 1999). Bax and Bim interact with VDAC, leading to the release of Cyto c, whereas Bcl-x<sub>L</sub> blocks such release (Shimizu et al., 1999; Sugiyama et al., 2002), as do anti-VDAC antibodies (Shimizu et al., 2001). Bax was also found to increase VDAC pore size (Banerjee and Ghosh, 2004). On the other hand, Bid but not Bax was shown to modify the conductance of VDAC channels. Furthermore, oligomeric Bax has no effect on the conductance, selectivity, or voltage-gating properties of VDAC channels (Rostovtseva et al., 2004). The involvement of VDAC1 in Bax-mediated apoptosis has been proposed based on findings that in VDAC1-depleted cells, cisplatin-induced conformational activation of Bax was inhibited, suggesting that VDAC1 is required at an intermediate step in the pathways leading to Bak and Bax activation (Tajeddine et al., 2008). Recently, it has been shown that mitochondrial m-calpain truncated VDAC in Ca<sup>2+</sup>-dependent manner and that such cleavage promotes the mitochondrial accumulation of Bax and the release of tAIF from mitochondria through VDAC-Bax pores (Ozaki et al., 2008, 2009). The requirement of VDAC2 for the pro-apoptotic activity of Bax has also been demonstrated (Yamagata et al., 2009). A possible role for VDAC as the receptor for Bax was, nonetheless, challenged by the observation that ablation of VDAC isoforms 1, 2, and 3 did not affect apoptosis signaling in fibroblasts isolated from VDAC1–3 lacking mice (Baines et al., 2007).

The interaction of Bcl-x<sub>L</sub> with VDAC1 was also demonstrated by NMR spectroscopy (Hiller et al., 2008; Malia and Wagner, 2007). Bcl-x<sub>L</sub> was further shown to modify the oligomerization state of VDAC1, as revealed by chemical cross-linking of microsome-bound VDAC1, shifting the equilibrium from a predominantly oligomeric form towards the monomeric state of VDAC

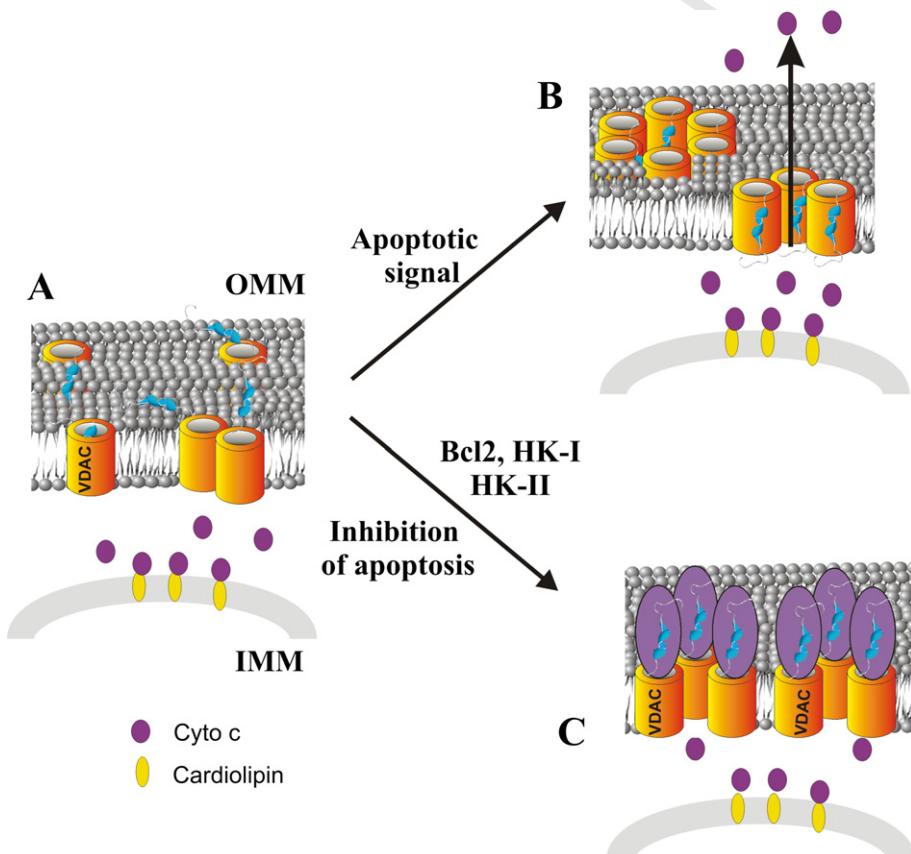
(Hiller et al., 2008; Malia and Wagner, 2007), suggesting that anti-apoptotic proteins may act by preventing VDAC oligomerization. In addition, Bcl-2 and Bcl-x<sub>L</sub> were proposed to interact with VDAC to block As<sub>2</sub>O<sub>3</sub> induced VDAC dimerization (Zheng et al., 2004). These results indicate that Bcl-2 family proteins regulate VDAC-mediated apoptosis and hence, the release of apoptogenic proteins from mitochondria.

Contact sites between hVDAC1 and Bcl-2 family proteins were proposed (Shi et al., 2003b). It has been suggested that Bcl-x<sub>L</sub> interacts with VDAC via a putative loop region of VDAC1 and that VDAC1 interacts with both Bax and Bcl-x<sub>L</sub> to form a tertiary complex (Shi et al., 2003b). NMR-based studies showed the interaction of VDAC1 with Bcl-x<sub>L</sub> and identified residues involved in this interaction (Hiller et al., 2008; Malia and Wagner, 2007).

Recently, an interaction of Bcl-2 with VDAC and the subsequent modulation of apoptosis were demonstrated. Purified Bcl-2(ΔC23) was found to reduce the channel conductance of native but not mutated VDAC1 reconstituted into a planar lipid bilayer (Arbel and Shoshan-Barmatz, 2009). Using site-directed mutagenesis, the VDAC1 domains involved in the interaction of the protein with Bcl-2, conferring protection against apoptosis by the latter, were identified (Arbel and Shoshan-Barmatz, 2009). These VDAC1 domains, including the N-terminal domain of the protein (Abu-Hamad et al., 2009), were used as templates for VDAC1-based recombinant and synthetic peptides. Their interactions with Bcl-2 was demonstrated using surface plasmon resonance or upon peptide expression, preventing Bcl-2-mediated protection against cell death (Arbel and Shoshan-Barmatz, 2009).

All these studies strongly support the involvement of VDAC in the activity of pro- and anti-apoptotic proteins. Such involvement is, however, most likely dependent on cell type and apoptosis stimulus.

The N-terminal region of VDAC1 is proposed to be the target of the apoptosis-regulating proteins, HK-I and Bcl-2 (Abu-Hamad et al., 2009; Arbel and Shoshan-Barmatz, 2009; Arzoine et al., 2009; Shoshan-Barmatz et al., 2009). HK-I was found to decrease channel conductance of bilayer-reconstituted native VDAC but not of N-terminally truncated VDAC1 (Abu-Hamad et al., 2009). In addition, the **VDAC1N-terminal** region was found to bind the anti-apoptotic proteins of the Bcl-2 family



**Fig. 18.** Model for VDAC1 oligomerization and its N-terminal region-mediated cytochrome c release. A, Before apoptosis induction: Side-view across the membrane with VDAC1 in the monomeric state and with the amphipathic  $\alpha$ -helix N-terminal region cytoplasmically exposed (De Pinto et al., 2003), membrane-spanning (Colombini, 2004), lying on the membrane surface (Reymann et al., 1995), or positioned in the pore (Bayrhuber et al., 2008; Hiller et al., 2008; Ujwal et al., 2008). B, Upon an apoptotic signal: VDAC1 oligomerization is enhanced and the amphipathic  $\alpha$ -helix N-terminal region of each VDAC1 molecule flips inside the hydrophobic pore formed by the  $\beta$ -barrels, forming a hydrophilic pore capable of conducting Cyto c release. C, Inhibition of apoptosis by anti-apoptotic proteins: HK or Bcl-2 interact with the N-terminal region of VDAC1 to prevent its translocation and thus, the formation of the hydrophilic pore, thereby inhibiting Cyto c release.

(i.e. Bcl-2 and Bcl-xL) and HK-I (Abu-Hamad et al., 2009; Arbel and Shoshan-Barmatz, 2009; Arzoine et al., 2009; Shi et al., 2003a; Shoshan-Barmatz et al., 2009), and when expressed in cells expressing HK-I, HK-II (Arzoine et al., 2009) or Bcl-2 (Arbel and Shoshan-Barmatz, 2009), prevented their anti-apoptotic activity. Since cells expressing N-terminal truncated-VDAC1 are resistance to apoptosis, it is proposed that by binding the N-terminal part of VDAC, Bcl-2 and HK protect against apoptosis (Fig. 18).

### 9.3. VDAC and the Translocator Protein

The Translocator Protein (TSPO), formerly known as the peripheral-type benzodiazepine receptor (PBR) or isoquinoline-binding protein is an evolutionarily conserved protein that binds benzodiazepines, such as RO5-4864, and isoquinoline carboxamide derivatives, such as PK11195 (Zisterer and Williams, 1997). A 18 kDa protein, TSPO is predominantly located at the surface of the mitochondria, where it is proposed to physically associate with VDAC-ANT in rat kidney (McEnergy et al., 1992). For many years, interactions between TSPO and VDAC were considered to play a role in the activation of the mitochondrial apoptosis pathway (Kugler et al., 2008; Levin et al., 2005; Veenman et al., 2007). In particular, it has been suggested that TSPO may activate PTP opening, causing  $\Delta\Psi$  transition, and leading to apoptosis (Levin et al., 2005; Veenman et al., 2007). It was suggested, however, that interactions between TSPO and VDAC is potentially important for TSPO function (Gavish et al., 1999), but may also affect TSPO binding characteristics (Joseph-Liauzun et al., 1997; Veenman et al., 2002) and have been considered as playing a role in apoptotic cell death (Levin et al., 2005; Veenman et al., 2004).

Due to its proposed function in apoptosis, potentially via its interactions with VDAC, the TSPO has become a target for the development of drugs directed at neurodegenerative diseases and cancer (Veenman and Gavish, 2006; Veenman et al., 2007). In this context, TSPO appears to be involved in the generation of ROS, proposed to be the link between the activation of TSPO and VDAC, leading to the induction of the mitochondria-mediated apoptotic pathway (Veenman et al., 2008). ROS are known to encourage release Cyto c from cardiolipins located at the IMM (Asumendi et al., 2002; Petrosillo et al., 2001). In addition, ROS appear to activate VDAC, facilitating VDAC-mediated release of Cyto c into the cytosol (Madesh and Hajnoczky, 2001; Petrosillo et al., 2003) (see Section 11.2). It has been hypothesized that the close association of TSPO with VDAC (Gavish et al., 1999; McEnergy et al., 1992) may help to ensure that the ROS generated via TSPO can actually affect VDAC. The reported grouping of TSPO molecules around VDAC, potentially reflecting TSPO polymerization (Golani et al., 2001; Veenman et al., 2002, 2007), may help enhance the concentration of ROS generated by TSPO in the proximity of the VDAC, leading to apoptosis induction (see Section 9.3).

### 9.4. VDAC interaction with and regulation by cytoskeletal proteins

Mitochondria have long been known to localize within and to move along the cellular tubulin–microtubule network (Bernier-Valentin and Rousset, 1982; Carre et al., 2002). Accordingly, tubulin binds to isolated mitochondria with high-affinity (Bernier-Valentin and Rousset, 1982). Moreover, tubulin is present in mitochondria isolated from different human cancerous and non-cancerous cell lines. It, thus, was concluded that tubulin is an inherent component of mitochondrial membranes. Mitochondrial tubulin is enriched in acetylated and tyrosinated  $\alpha$ -tubulin, as well as in the class III tubulin isotype, but contains very little of the class IV  $\beta$ -tubulin isotype. Mitochondrial tubulin is likely to be organized in  $\alpha\beta$ -dimers and represents 2.2  $\pm$  0.5% of the total cellular tubulin. The specific association of VDAC with tubulin was shown by co-immunoprecipitation experiments (Carre et al., 2002), while anti-tubulin antibodies induced the release of Cyto c from isolated mitochondria.

Recently, nanomolar concentrations of mammalian dimeric tubulin were found to induce reversible closure of bilayer-reconstituted VDAC (Rostovtseva et al., 2008a). Each tubulin subunit possesses a negatively charged, extended C-terminal tail (Priel et al., 2005; Sackett et al., 1985) and these tails are required for interaction with VDAC. Accordingly, a model of tubulin–VDAC interaction, in which the tubulin C-terminus penetrates into the VDAC channel lumen, interacting with VDAC with high specificity and blocking channel conductance, has been proposed (Rostovtseva and Bezrukova, 2008; Rostovtseva et al., 2008b).

Paclitaxel, a well-known anti-tumor drug which inhibits microtubule dynamics and subsequently induces apoptosis, was found to induce Cyto c release from mitochondria in intact human neuroblastoma cells and isolated mitochondria (Andre et al., 2002). It is likely that paclitaxel and other microtubule-active anti-tumor drugs modify interactions of microtubules and/or tubulin with VDAC and thus deliver a signal for Cyto c release and apoptosis induction (Esteve et al., 2007).

Tubulin, as a potential regulator of VDAC, adds another level of complexity to the VDAC-mediated regulation of mitochondrial signals. It has been proposed that tubulin is a component of a supercomplex referred to as the ATP synthasome, containing MtCK, VDAC and tubulin, and found at contact sites that regulate respiration and energy fluxes in heart (Brdiczka, 2007; Timohhina et al., 2009).

VDAC1 was furthermore reported to interact with other cytoskeletal proteins. By affinity chromatography, MAP2 (microtubule-associated protein 2) was shown to bind VDAC (Linden and Karlsson, 1996). Human recombinant gelsolin, a  $\text{Ca}^{2+}$ -dependent protein that modulates actin assembly and disassembly, was demonstrated to directly bind to VDAC reconstituted into liposomes and to block its activity (Kusano et al., 2000). In a two-hybrid screening, using human VDAC1 the bait protein, it was shown that VDAC1 interacts with the dynein light chain, Tctex-1, and the heat-shock protein peptide-binding protein 74 (Schwarzer et al., 2002). These proteins were expressed as recombinant binding partners and were able to alter the electrophysiological properties of human VDAC1, thus reflecting a functional role in the cell (Schwarzer et al., 2002).

Actin in the monomeric form (G-actin) but not in the fibrous form was able to reduce VDAC-mediated membrane conductance by as much as 85% in reconstituted systems at elevated membrane potentials (Xu et al., 2001). These reports raise the possibility that VDAC1 might act as a mediator of the interactions between mitochondria and cytoskeletal structures. Thus, some domain of VDAC might play a role as a docking structure, in support of the notion that the distribution of mitochondria to high-energy consuming districts in specialized cells may be physiologically relevant (Leterrier et al., 1994; Wagner et al., 2003). Interestingly, most of the interactions with cytoskeleton protein reported in these works affected the pore-forming activity of VDAC1.

Finally, in a recent proteomic survey (Roman et al., 2006) the interactome of VDAC1 was investigated, showing that not all the reported interactions were noted, whereas other interactions were. The systematic study of such interactions is a topic that will need further refinements in the future.

## 9.5. Viruses and VDAC

Many viruses code for proteins that act on mitochondria, in general, and on VDAC, in particular, thus controlling apoptosis of infected cells (Boya et al., 2001, 2003; Everett and McFadden, 2001; Irusta et al., 2003; Verrier et al., 2003). Infections with HIV-1 provide an excellent example of neuronal and immunological apoptosis involving mitochondria (Perfettini et al., 2005; Sui et al., 2004). The HIV-1 envelope protein can induce mitochondrial apoptosis via at least three independent mechanisms (Castedo et al., 2003).

Another virus affecting mitochondria is the Influenza A virus that codes for protein PB1-F2, which targets mitochondria. PB1-F2 contains a C-terminal mitochondria localization signal, which is conserved across the influenza family. PB1-F2 localization to mitochondria resulted in alteration of mitochondrial morphology, dissipation of the mitochondrial membrane potential, and cell death. The PB1-F2 protein interacts directly with mitochondrial VDAC (Zamarin et al., 2005). Recently, molecular interactions between mitochondrial membrane proteins and the C-terminal domain of PB1-F2 have been addressed using an *in silico* approach, which showed the involvement of 12 amino acids of the PB1-F2 protein which forms hydrophobic contacts with 22 VDAC1 amino acids (Danishuddin et al., 2009).

Hepatitis B virus (HBV) is another mitochondria-interacting virus (Boya et al., 2004, 2003; Shirakata and Koike, 2003). The proposed mechanism of HBV action is based on the homology and physical interaction of the HBx protein with mitochondrial VDAC, encouraging the creation of a hetero-hexamer comprising VDAC and HBx proteins. The formation of this hexamer is believed to allow release of Cyto c from mitochondria to the cytosol, where Cyto c initiates the caspase cascade leading to apoptosis (Goncalves et al., 2007; Hoogenboom et al., 2007; Shoshan-Barmatz et al., 2006; Zalk et al., 2005). Such functional roles of HBx in affecting mitochondrial physiology have implications for HBV-induced liver injury and, at the same time, for the development of hepatocellular carcinomas (Rahmani et al., 2000). Furthermore, it was shown that another hepatic virus, HEV, not only induced upregulation of VDAC expression in infected human hepatoma cell lines but also encouraged higher levels of oligomeric VDAC, leading to apoptosis (Moin et al., 2007).

Other strategies used by viruses to influence apoptosis involve mimicking Bcl-2 family proteins, changing the levels of or directly interacting with Bcl-2 family proteins (Boya et al., 2004; Halestrap et al., 2002).

## 10. VDAC regulation by non-protein modulators

In addition to  $\text{Ca}^{2+}$  being proposed to modulate VDAC activity, various other reagents were shown to interact with VDAC and modify its channel activity by increasing the probability of VDAC closure. Moreover, it was demonstrated that VDAC closure by various reagents resulted in the inhibition of PTP opening, Cyto c release and apoptotic cell death. As most of these reagents have been previously discussed (for review, see Shoshan-Barmatz et al., 2006, 2008a; Shoshan-Barmatz and Gincel, 2003), we focus here on a selected few, i.e. DIDS, ruthenium red (RuR), Ru-based reagents and Koenig's polyanion.

### 10.1. VDAC transports $\text{Ca}^{2+}$ and possesses $\text{Ca}^{2+}$ -binding sites

Apart from their role in metabolism, mitochondria act as a major hub of cellular  $\text{Ca}^{2+}$  homeostasis (Szabadkai et al., 2006). Intramitochondrial  $\text{Ca}^{2+}$  modulates critical enzymes of the TCA cycle, fatty acid oxidation, amino acid catabolism, the F1-ATPase and the ANT (Nichols and Denton, 1995). Mitochondria also modulate cytosolic  $\text{Ca}^{2+}$  transients or pulses (Nichols and Denton, 1995) and play a role in apoptosis through the induction of the  $\text{Ca}^{2+}$ -dependent PTP (Hajnoczky et al., 2006; Zoratti and Szabo, 1995). Powered by the mitochondrial membrane potential,  $\text{Ca}^{2+}$  enters this organelle via the uniporter and is extruded by either  $\text{H}^{+}$ - or  $\text{Na}^{+}$ -coupled mitochondrial exchangers (Gunter et al., 1998). Recently, the expression and possible function of mitochondrial  $\text{Na}^{+}/\text{Ca}^{2+}$  exchanger has been reported (Palty et al., 2009). All of the above  $\text{Ca}^{2+}$  transport systems mediate transport of  $\text{Ca}^{2+}$  across the IMM (Gunter et al., 1998) while VDAC mediate transport of  $\text{Ca}^{2+}$  across the OMM (Gincel et al., 2002; Shoshan-Barmatz and Gincel, 2003). VDAC-mediated  $\text{Ca}^{2+}$  transport was shown to be inhibited by L-Glu and DIDS, as demonstrated using VDAC reconstituted into the proteoliposomes or into a tethered biomimetic membrane (Deniaud et al., 2007). In addition, it has been concluded that VDAC possesses higher permeability to  $\text{Ca}^{2+}$  in the closed states than in the open state (Tan and Colombini, 2007). This is in contrast to results demonstrating that VDAC opening promotes calcium flux into mitochondria followed by PTP, mitochondrial swelling and cell death (Gincel et al., 2001;

1571 Shoshan-Barmatz and Gincel, 2003) but are consistent with the view that VDAC closure is a pro-apoptotic signal (Tan and  
1572 Colombini, 2007).

1573 Mitochondrial Ca<sup>2+</sup> overload appears to induce opening of the PTP proposed to mediate Cyto c release. The Ca<sup>2+</sup>-binding  
1574 component regulating PTP opening has not yet been identified. Of the PTP comprising molecules, i.e. VDAC, ANT and CypD  
1575 (see [Section 8.2.2](#)), ANT has been suggested to mediate the Ca<sup>2+</sup> effect (Brustovetsky and Klingenberg, 1996). On the other  
1576 hand, several lines of evidence indicate that VDAC possesses divalent cation-binding sites: (1) La<sup>3+</sup> and Tb<sup>3+</sup> induce VDAC  
1577 channel closure, when assayed in single or multi-channel studies (Gincel et al., 2001; Shoshan-Barmatz and Gincel, 2003).  
1578 (2) Ruthenium red (RuR) (Gincel et al., 2001; Shoshan-Barmatz and Gincel, 2003) and the ruthenium amine binuclear  
1579 complex (Ru360) (Gincel et al., 2002), known to specifically interact with several Ca<sup>2+</sup>-binding proteins (Charuk et al., 1990; Gincel et al., 2001), induce VDAC channel closure in a time-dependent manner and stabilize the channel in a completely closed  
1580 state. (3) The inhibitory effect of RuR is prevented by the presence of CaCl<sub>2</sub> but is re-established upon chelating Ca<sup>2+</sup> with  
1581 EGTA, suggesting that RuR interacts with a Ca<sup>2+</sup>-binding site.

1582 The regulation of VDAC gating by Ca<sup>2+</sup> and the function of Ca<sup>2+</sup> as a regulator of VDAC activity have been demonstrated,  
1583 thereby providing a novel mechanism for the control of OMM permeability to ions and small molecules (Bathori et al., 2006).  
1584 In contrast, it has been argued that VDAC does not require Ca<sup>2+</sup>, functioning normally with or without Ca<sup>2+</sup> (Tan and Colom-  
1585 bini, 2007). This disagreement may be related to the different methods used in preparing planar membranes in the two stud-  
1586 ies (Tan and Colombini, 2007).

1587 The presence and localization of Ca<sup>2+</sup>-binding sites in VDAC were addressed using a novel approach developed for local-  
1588 ization of VDAC Ca<sup>2+</sup>-binding site(s), involving the use of azido ruthenium (AzRu), a photoreactive reagent, that specifically  
1589 interacts with Ca<sup>2+</sup>-binding proteins and can bind irreversibly to Ca<sup>2+</sup>-binding sites upon UV irradiation (Israelson et al.,  
1590 2005). AzRu strongly inhibited the activities of a variety of Ca<sup>2+</sup>-dependent processes, while having no effect on Ca<sup>2+</sup>-inde-  
1591 pendent activities (Israelson et al., 2005). AzRu decreased the conductance of VDAC reconstituted into a bilayer, while Ca<sup>2+</sup>,  
1592 in the presence of 1 M NaCl but not Mg<sup>2+</sup>, prevented this effect. AzRu had no effect on mutated E72Q- or E202Q-VDAC1 con-  
1593 ductance, and [<sup>103</sup>Ru]AzRu labeled native but not E72Q-VDAC1, suggesting that these residues are required for AzRu inter-  
1594 action with the VDAC Ca<sup>2+</sup>-binding site(s). Since AzRu and Ca<sup>2+</sup> share protein-binding sites, AzRu, as a photoreactive reagent,  
1595 was employed to identify Ca<sup>2+</sup>-binding sites in VDAC1. Chymotryptic and tryptic digestion of AzRu-labeled VDAC followed  
1596 by MALDI-TOF analysis revealed two AzRu-bound peptides, corresponding to E72- and E202-containing sequences (Israelson  
1597 et al., 2007). These results suggest that the VDAC Ca<sup>2+</sup>-binding site includes E72 and E202, located, according to a membrane  
1598 topology model proposed for mammalian VDAC1 (Colombini, 2004), in regions facing the cytosol (Israelson et al., 2007).

#### 1600 10.2. The interaction of DIDS with VDAC inhibits Cyto c release and apoptosis

1601 DIDS is a well-known chloride channel blocker affecting the activity of a number of transporters, including Ca<sup>2+</sup>-activated  
1602 chloride channels (Wohlrab et al., 2000), iodide uptake in thyroid-derived cell lines (Amphoux-Fazekas et al., 1998) and the  
1603 exchange of Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> across the plasma membrane (Cabantchik and Greger, 1992), rat heart mitochondria anion channels  
1604 (Tomaskova et al., 2007), as well as mono-carboxylate transporter 1 (Wilson et al., 2009). DIDS has been shown to decrease  
1605 the conductance of VDAC reconstituted into a planar lipid bilayer (Shoshan-Barmatz et al., 1996; Shoshan-Barmatz and Isra-  
1606 elson, 2005; Thennes et al., 1994). Due to its interactions with various transport systems, DIDS can not be considered as a  
1607 specific chemical blocker of VDAC. Yet, DIDS has been used to investigate the role of VDAC in cellular processes. As such,  
1608 DIDS was shown to inhibit the transport of ATP into synaptosomes and sarcoplasmic reticulum, an activity proposed to  
1609 be VDAC-mediated (Shafir et al., 1998; Shoshan-Barmatz et al., 1996). DIDS also inhibits O<sub>2</sub> production from mitochondria  
1610 in a voltage-dependent manner, interpreted as an indication that O<sub>2</sub> exits mitochondria via VDAC channels (Han et al., 2003).  
1611 DIDS prevents superoxide-induced release of Cyto c and apoptosis in a hepatocyte cell line (Madesh and Hajnoczky, 2001),  
1612 and blocks Cyto c release and processing of caspase-9 and caspase-3, and prevents nuclear damage in dexamethasone-treat-  
1613 ed thymocytes, pointing to a role for VDAC in these functions (Sade et al., 2004). It has also been shown that DIDS can block  
1614 apoptosis triggered by overexpression of rice VDAC in mammalian cells (Godbole et al., 2003).

1615 Recently, it was reported that DIDS prevented cisplatin-induced cell death by inhibiting conformational activation of Bax,  
1616 which was shown to depend on VDAC expression (Tajeddine et al., 2008). In another study, DIDS attenuated STS-induced  
1617 apoptosis in cardiomyocytes partly by preventing the translocation of Bax, although apoptosis was mediated by Cyto c  
1618 and caspase activity and not by AIF (Liu et al., 2008). It is intriguing how DIDS prevents the translocation of Bax from the  
1619 cytoplasm to the mitochondria. DIDS, by binding to VDAC, interferes with the interaction of VDAC with Bax (Liu et al., 2008).

1620 Finally, in a recent study it was demonstrated that DIDS efficiently blocked curcumin-induced AIF nuclear translocation in  
1621 fibroblasts, suggesting a role for VDAC in this process (Scharstuhl et al., 2009). DIDS was also found to inhibit ceramide-in-  
1622duced release of apoptogenic factors from mitochondria, pointing to the concerted actions of Bax, VDAC and ceramide in the  
1623 efflux of AIF from mitochondrion (Scharstuhl et al., 2009).

#### 1624 10.3. Ruthenium red and ruthenium-containing reagents interact with VDAC and affect cell death

1625 Ruthenium red (RuR) (Gincel et al., 2001) and the ruthenium amine binuclear complex, Ru360 (Gincel et al., 2002), known  
1626 to specifically interact with several Ca<sup>2+</sup>-binding proteins, decrease the conductance of bilayer-reconstituted VDAC and sta-  
1627 bilize the channel in a completely closed state. RuR was also found to protect against cell death induced by various stimuli.

For instance, RuR inhibits curcumin-induced apoptosis via the prevention of *intra-cellular*  $\text{Ca}^{2+}$  depletion and Cyto c release in U-937 cells (Bae et al., 2003), significantly blocks the ursolic acid-induced increase in *intra-cellular*  $\text{Ca}^{2+}$  concentration. RuR also blocks the effects of ursolic acid on cell viability and apoptosis in HL-60 cells (Bae et al., 1997), while pretreatment with RuR prevented the early mitochondrial  $\text{Ca}^{2+}$  surge and attenuated the subsequent onset of MPT and cell death in microcystin-LR-treated rat hepatocytes (Ding et al., 2001).

The mechanism by which RuR protects against cell death has been proposed to involve interaction with VDAC (Alexander et al., 2007; Gincel et al., 2001; Israelson et al., 2008; Zaid et al., 2005). Given the RuR-insensitive behavior of E72Q-mVDAC1, as reflected by the inability of RuR to inhibit VDAC channel activity in this mutant and to prevent cell death, it appears that RuR-mediated protection against apoptosis induced by the various stimuli reported above is exerted through its direct interaction with VDAC (Israelson et al., 2008).

#### 10.4. Koenig's *polyanion* interacts with VDAC

Koenig's polyanion can induce VDAC closure (Colombini et al., 1987; Mannella and Guo, 1990). In isolated mitochondria, enzymes in the *inter-membrane* space, such as adenylate kinase, have no access to external adenine nucleotides in the presence of Koenig's polyanion (Konig et al., 1977), which induces the low conductance state of VDAC (Benz et al., 1988). Koenig's polyanion blocks Cyto c transit through the liposomal membrane, suggesting that VDAC forms at least part of the functional pore (Budihardjo et al., 1999). On the other hand, Koenig's polyanion was also shown to enhance swelling and amplify Cyto c release from CNS mitochondria (Brustovetsky et al., 2002, 2003). Blockage of VDAC with Koenig's polyanion was demonstrated to inhibit uncoupled and ADP-stimulated respiration of permeabilized hepatocytes upon digitonin-induced plasma membrane permeabilization of cultured hepatocytes (Holmuhammedov and Lemasters, 2009). Koenig's polyanion was also demonstrated to suppress tBid-induced Cyto c release from non-synaptosomal brain mitochondria mediated by Bax but not by Bak. Thus, tBid can induce MPT-independent Cyto c release from brain mitochondria by interacting with exogenous Bax and/or with endogenous Bak in a manner that may involve VDAC.

### 11. Reactive oxygen species, nitric oxide and apoptosis

#### 11.1. Mitochondria, ROS and *oxidative* stress

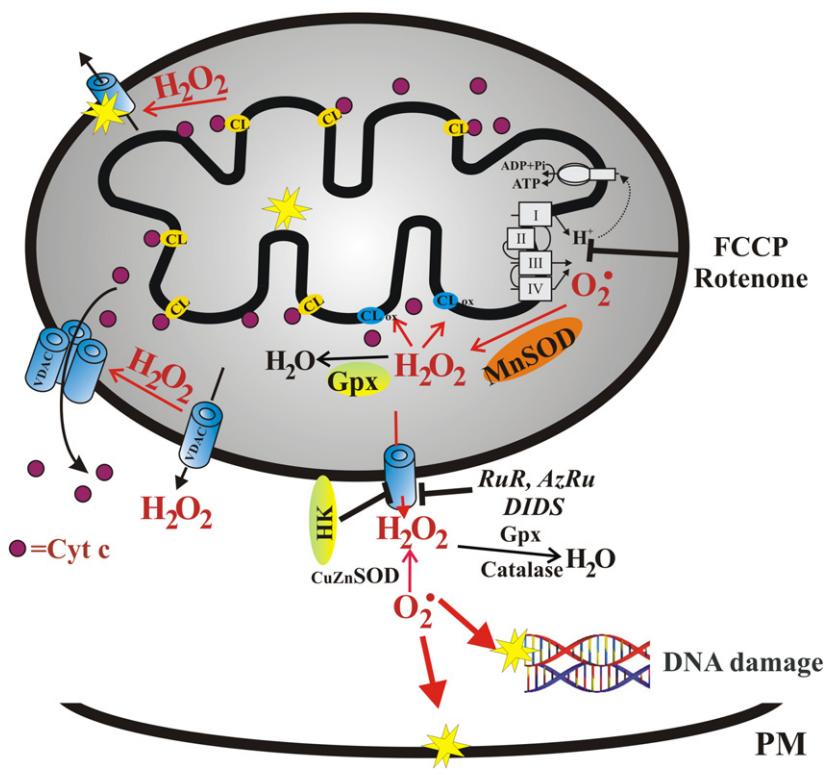
In aerobic organisms, oxygen is essential for efficient energy production but paradoxically, produces chronic toxic stress in cells. Diverse protective systems must, therefore, exist to enable adaptation to oxidative environments. Oxidative stress (OS) results when production of ROS exceeds the capacity of mitochondrial and cellular *anti-oxidant* defenses to remove these toxic species. Unbalanced oxidation/reduction of macromolecular cell components is involved in the pathogenesis of neurodegeneration associated with the disease, trauma, sperm dysfunction, normal ageing and carcinogenesis (Facheris et al., 2004; Moskovitz et al., 2002). At high levels of ROS, apoptosis and necrosis can occur, whereas chronically low levels of ROS promote a wide variety of health problem (Finkel and Holbrook, 2000), such as neurodegeneration (Droge and Schipper, 2007; Swerdlow, 2007) and cancer (Blanchetot and Boonstra, 2008; Lau et al., 2008). Thus, ROS cellular levels must be controlled. Although a number of defense systems have evolved to combat the accumulation of ROS (see Fig. 19), these are not always sufficient to counter the effect of ROS. The ability of the cell to readily detoxify reactive intermediates or easily repair the resulting damage largely determines its ability to reduce OS effects and prevent disease (Kakkar and Singh, 2007; Klaunig and Kamendulis, 2004).

ROS are mainly produced in the mitochondria as by-products of respiratory chain reactions (Orrenius et al., 2007). Approximately 1–5% of the oxygen consumed by mitochondria in human cells is converted to ROS, e.g., superoxide anions ( $\text{O}_2^-$ ),  $\text{H}_2\text{O}_2$ , and hydroxyl radicals (Orrenius et al., 2007).

To combat harmful ROS, cells possess several anti-oxidant defense mechanisms, including catalytic removal of reactive species by enzymes like superoxide dismutase (SOD), catalase, and peroxidase (Barber et al., 2006). Moreover, several enzymes exist within the mitochondria that catalyze the removal of superoxide radicals and which produce oxygen and hydrogen peroxide (Fridovich, 1975) that can be degraded by glutathione peroxidases (Arai et al., 1999; Esworthy et al., 1997) or peroxiredoxin 3 (Rhee et al., 2005; Wood et al., 2003). However, about 1% of the ROS escape elimination and cause oxidative cellular damage. Since ROS are generated in the mitochondria, they can attack the mitochondria directly and, when released to the cytosol, can attack and modify DNA, lipids and proteins (Ott et al., 2007), affecting cell survival (see Fig. 18).

#### 11.2. VDAC function in ROS release and ROS-mediated apoptosis

Upon an imbalance in redox status, depending on the magnitude of ROS encountered, apoptosis may be induced (Raha and Robinson, 2001). ROS play a key role in the promotion of Cyto c release from mitochondria (Petrosillo et al., 2001, 2003). In fact, it has been shown that the apoptosis-inducing agents, such as inorganic arsenic compounds (Ding et al., 2005; Shi et al., 2004) and doxorubicin (Olson et al., 1981; Sokolove, 1994) cause oxidative damage to DNA and protein by inducing ROS generation.



**Fig. 19.** Reactive oxygen species occlusion and degradation in mitochondria can prevent cell damage. A schematic presentation summarizing current knowledge and proposed control mechanisms for ROS production and neutralization. Free radicals generated by the electron transport chain are generally regarded as toxic metabolites and, as such, are decomposed by specialized enzymes, namely catalases, peroxidases, and superoxide dismutases and glutathione peroxidase. Nevertheless, the fraction of ROS that escapes catalytic removal may cause oxidative damage (★) to mitochondrial and cellular proteins, lipids and DNA. In addition, mitochondrial ROS can induce apoptosis. Cyto c (Cyt c) interacts with the mitochondria-specific phospholipid, cardiolipin (CL). ROS-oxidized CL has a markedly lower affinity for Cyt c. ROS can also induce VDAC oligomerization to yield a mega-channel mediating Cyt c release. VDAC inhibitors (RuR, AzRu, DIDS) and hexokinase (HK) can prevent ROS release to the cytosol. CuZnSOD, cytosolic (copper-zinc-containing) superoxide dismutases; MnSOD, mitochondrial (manganese-containing) superoxide dismutases; GPx, glutathione peroxidase.

To be released from the mitochondria to the cytosol, ROS must cross the OMM. This release may be mediated by the VDAC lying in the OMM and serving as major gateway for molecules exit from the IMS. Indeed, VDAC has been proposed to be the mediator for ROS release from the IMS to the cytosol (Han et al., 2003) (see Fig. 18). VDAC involvement in ROS release from the IMS to the cytosol is also supported by the findings that HK-I and HK-II decrease ROS release when overexpressed in HEK cells (Abu-Hamad et al., 2008; Shoshan-Barmatz et al., 2008b; Sun et al., 2008; Zaid et al., 2005), thereby, reducing intra-cellular levels of ROS (Ahmad et al., 2002; da-Silva et al., 2004). Thus, mitochondria-HK association provides not only metabolic advantage but also has a major impact on cell survival, such as decreasing mitochondrial superoxide leakage (Ahmad et al., 2002) and preventing apoptosis (Abu-Hamad et al., 2008; Azoulay-Zohar et al., 2004; Shoshan-Barmatz et al., 2008b; Zaid et al., 2005).

The findings that  $O_2^-$ -induced apoptosis is inhibited by DIDS or anti-VDAC antibodies (Madesh and Hajnoczky, 2001; Simamura et al., 2006), blockers of VDAC channel activity (Shimizu et al., 2001; Shoshan-Barmatz et al., 1996), suggest that  $O_2^-$  induces Cyt c release via VDAC-dependent permeabilization of the OMM (Madesh and Hajnoczky, 2001). Moreover,  $O_2^-$  was found to evoke Cyt c release in VDAC-reconstituted liposomes (Madesh and Hajnoczky, 2001). In other studies, it was found that ROS induced alterations of VDAC and/or ANT can induce MMP selective for Cyt c release, without causing further mitochondrial damage (Le Bras et al., 2005; Madesh and Hajnoczky, 2001). In addition, ROS-induced upregulation of VDAC can be prevented by the ROS chelator, epigallocatechin (Jung et al., 2007). Recently, it has been suggested that ROS-mediated Cyt c release from mitochondria involves VDAC and that SOD1 is released from the mitochondria via VDAC, leading to increased susceptibility of mitochondria to oxidative stress and apoptosis (Li and Yuan, 2008).

The rules governing ROS transport via the OMM and control of this process, as well as the molecular mechanism of ROS activating VDAC to release Cyt c are not clear.

### 11.3. Nitric oxide, mitochondria and cell death

Nitric oxide (NO) is a free radical that functions as a second messenger molecule involved in many physiological and pathological processes within mammalian cells (Hou et al., 1999, 2345). NO plays important and diverse roles in biological

processes such as neurotransmission, inflammatory response, and vascular homeostasis (Kubes et al., 1991; Moncada et al., 1991).

NO can be generated either enzymatically by NO synthases (NOS) or non-enzymatically (Zweier et al., 1995). There are three NOS isoforms, neuronal NOS (nNOS/NOS1), inducible NOS (iNOS/NOS2), and endothelial NOS (eNOS/NOS3). nNOS and eNOS are constitutively expressed and activated in response to calcium-calmodulin signaling. NOS oxidizes the guanine group of  $\text{L-arginine}$  to form NO and  $\text{L-citrulline}$ . The process involves the oxidation of NADPH and the reduction of molecular oxygen (Ignarro, 1990).

NO may prevent or promote cell death, depending on cell type and concentration. NO interacts directly and indirectly with mitochondria (Brown and Borutaite, 2002). Two major NO interaction sites with mitochondria have been identified at complex III and the oxygen binding site of  $\text{Cyto c}$  oxidase (Brookes et al., 2000; Brown, 2001; Cleeter et al., 1994; Poderoso et al., 1999). In addition, NO also stimulates guanylyl cyclase, which leads to an increase in cGMP, an inhibitor of MPT (Nisoli et al., 2003; Takuma et al., 2001). NO possesses both pro- and anti-apoptotic effects, depending on both cell type and NO concentration (Brune et al., 1999; Kim et al., 1999). Specific NO molecular targets include inhibition of Bcl-2 cleavage (Kim et al., 1998) and inactivation of caspases by S-nitrosation (Li et al., 1999). At high NO concentrations (1 mM), inhibition of mitochondrial ATP-synthesis and necrotic cell death were obtained (Leist et al., 1999a,b). While some studies have demonstrated an activation of PTP opening in mitochondria exposed to S-nitrosothiols or high NO concentrations (Balakirev et al., 1997; Borutaite et al., 2000), other reports suggest that NO inhibits PTP opening in a sub-population of mitochondria (Balakirev et al., 1997). The effect of NO on PTP opening has been related to the effect of NO on mitochondrial  $\text{Ca}^{2+}$  accumulation (Schweizer and Richter, 1994) (Ghafoorifar and Richter, 1999). A low physiological release rates, NO was found to inhibit PTP opening in a reverse manner, while at high physiological release rates, NO accelerated PTP opening (Brookes et al., 2000).

VDAC, as a proposed constituent of the PTP complex, was found to bind eNOS, as demonstrated by co-immunoprecipitation experiments followed by mass spectroscopy (Sun and Liao, 2002). *In vitro* binding studies using glutathione S-transferase (GST)-tagged VDAC1 indicated that VDAC binds directly to eNOS and that this interaction amplified eNOS activity. Furthermore, the calcium ionophores, A23187 and bradykinin, both known to activate eNOS, increased VDAC1-eNOS complex formation, suggesting a potential role for intra-cellular  $\text{Ca}^{2+}$  in mediating this interaction. The above results indicate that the interaction between VDAC and eNOS may be important for regulating eNOS activity. This interaction points to highly regulated modulation of VDAC by NO (Sun and Liao, 2002).

## 1731 12. VDAC phosphorylation, its function in apoptosis and modulation by associated proteins

1732 Protein phosphorylation is a major post-translation modification regulatory system, modulating protein stability, enzymatic activity, sub-cellular localization, the ability to interact with binding partners, and more (Cohen, 2002). A cohort of 1733 protein kinases have been detected in mitochondria, e.g. protein kinase A (PKA) (Schwoch et al., 1990), different isoforms 1734 of protein kinase C (PKC) (Majumder et al., 2000), and components of the MAPK signaling pathway (Yuryev et al., 2000), glycogen synthase kinase 3 beta (GSK $\beta$ ) (Hoshi et al., 1995), Akt (Bijur and Jope, 2003), and casein kinase 2 (CK2) (Sarrouilhe and 1735 Baudry, 1996). Accordingly, several phosphatases counterbalancing these kinases activities have also been identified (Dagda 1736 et al., 2003; Lu et al., 2007b; Rosini et al., 2004; Signorile et al., 2002). These and similar cytosolic kinases and phosphatases 1737 may also modulate VDAC activity.

1738 VDAC possesses several potential phosphorylatable residues and several of them were indeed shown to undergo phosphorylation (Baines et al., 2003; Bera et al., 1995; Liberatori et al., 2004). VDAC is phosphorylated by protein kinase A (PKA) (Bera et al., 1995), by protein kinase C $\epsilon$  (PKC $\epsilon$ ) (Baines et al., 2003), while VDAC1 and VDAC2 in guinea pig brain synaptosomes were found to be phosphorylated at a Tyr residue under hypoxic conditions (Liberatori et al., 2004). Recently, the phosphorylation sites in VDAC were identified by MALDI-TOF mass spectrometry, showing that rat liver VDAC1 is phosphorylated under physiological conditions at Ser-12 and Ser-136, and VDAC2 at Tyr-237 and VDAC3 at Ser-241 and Thr-33 (Distler et al., 2007). The effects of VDAC phosphorylation on its activities have been mostly verified *in vitro* (Baines et al., 2003; Bera et al., 1995; Liberatori et al., 2004). VDAC phosphorylation by PKA reduced the single-channel current and opening probability of the protein at negative clamping potentials. c-RAF was shown to interact with and prevent VDAC reconstitution into PLB, yet VDAC phosphorylation by c-RAF was not detected (Le Mellay et al., 2002). There are no reports on the effects of VDAC phosphorylation in terms of apoptosis induction.

1751 It has been shown that GSK3 $\beta$  phosphorylates VDAC on Thr 51 and that this disrupts the binding of HK-II to VDAC. In 1752 other words, in the presence of GSK3 $\beta$ , VDAC is unable to bind HK-II, whereas upon replacing Thr 51 with alanine, VDAC1 1753 could bind HK-II efficiently, even when GSK3 $\beta$  was present. Thus, Akt, by phosphorylating GSK3 $\beta$  and inhibiting its enzymatic 1754 activity, can also induce HK-II binding to VDAC (Pastorino et al., 2005). Another study reported that expression of 1755 VDAC1 mutated at potential phosphorylation sites, i.e. S12A and S103A, effectively reduced the amount of VDAC1 in the cell. 1756 This was prevented by MG132, a well-known proteasome inhibitor. In addition, the S12A and S103A mutants could attenuate 1757 endostatin-induced upregulation of VDAC1 expression in endothelial cells suggesting that endostatin-induced VDAC1 1758 upregulation might result from its phosphorylation (Yuan et al., 2008). Endothelial cell expression of S12A and S103A VDAC1 1759 mutants both abolished endostatin-induced PTP opening and caspase-3 activation (Yuan et al., 2008). It has also proposed 1760 that VDAC1-dependent MPT involves a critical self-amplification loop involving ROS and p38-MAPK (Tomasello et al., 2009).

**Table 2**

VDAC implication in diseases and reagents toxicity.

		References
<b>Q10</b> <i>VDAC-associated diseases</i>		
Down's syndrome	VDAC1 expression level elevated in cerebellum	Yoo et al. (2001)
Alzheimer's disease	Expression levels of VDAC1 decreased in frontal cortex and thalamus and of VDAC2 elevated in temporal cortex	Yoo et al. (2001)
Autoimmune diseases	Antibodies to VDAC1 were found in sera from mixed connective tissue disease (MCTD) patients	Kikuchi et al. (2008)
Cancer	VDAC1–3 transcript levels were significantly higher in tumor cells line (AH130) and that of VDAC1 was elevated in several human cancer cell lines	Shinohara et al. (2000)
Psychomotor and retardation	Reported in a deficient VDAC patient	Huizing et al. (1996)
Epilepsy animal model	VDAC1 expression level is increased and that of VDAC2 is decreased	Jiang et al. (2007)
Influenza virus PB1	PB1 protein interaction with VDAC	Zamarlin et al. (2005)
Ethanol, oxidative stress	Proposed to involve alterations in VDAC activity by reducing its conductance	Lemasters and Holmuhamedov (2006)
<b>VDAC-dependent cytotoxic agents</b>		
Erastin	An anti-cancer agent that binds directly to VDAC2 to induce cell death	Yagoda et al. (2007)
Cisplatin	Direct interaction with VDAC1 and inhibiting PTP opening	Castagna et al. (2004), Yang et al. (2006)
Acrolein	$\alpha,\beta$ -unsaturated aldehydes, carbonylates VDAC in Alzheimer's disease	Mello et al. (2007)
Avicins	Pro-apoptotic plant stress metabolites, target and close VDAC	Haridas et al. (2007)
Endostatin	Induce PTP opening, accompanied by upregulation of VDAC1 expression	Yuan et al. (2008)

1761 Clearly, the relationship between the phosphorylation state of VDAC, apoptosis induction and VDAC association with  
 1762 apoptotic proteins require further study.

### 1763 13. VDAC and human diseases

1764 There is a substantial amount of evidence relating mitochondrial apoptosis to human disease (Mattson, 2000; Olson and  
 1765 Kornbluth, 2001). Mitochondria-mediated apoptosis plays a crucial role in the pathophysiology of several diseases, such as  
 1766 heart attack, stroke, cancer, mitochondrial encephalomyopathies, and aging, as well as in neurodegenerative disorders, such  
 1767 as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis (ALS, also known as Lou Gehrig's disease) (Alex-  
 1768 ander et al., 2007; McEnery et al., 1992; Olson and Kornbluth, 2001; Tatton and Olanow, 1999). VDAC, as a regulator of mito-  
 1769 chondrial function that also serves as a target of pro- and anti-apoptotic proteins and a key factor in mitochondria-mediated  
 1770 apoptosis, might be involved in these diseases (see Table 2).

#### 1771 13.1. VDAC and cancer

1772 Alterations in cell proliferation, cell death or both can lead to cancer development. Most treatments for cancer involve che-  
 1773 motherapy designed to induce apoptosis. Indeed, one of the mechanisms by which chemotherapeutic treatment destroys can-  
 1774 cer cells involves induction of mitochondria-mediated apoptosis (Debatin et al., 2002; Ghobrial et al., 2005; Hail, 2005; Hu and  
 1775 Kavanagh, 2003; Ralph and Neuzil, 2009). A substantial number of anti-cancer drugs, such as etoposide, doxorubicin (Decau-  
 1776 din et al., 1997), ionidamine (Ravagnan et al., 1999), betulinic acid, arsenite, CD437, and several amphiphilic cationic  $\alpha$ -helical  
 1777 peptides (Costantini et al., 2000; Hu et al., 2005), exert their therapeutic actions by inducing mitochondria-mediated apop-  
 1778 tosis (Preston et al., 2001), mainly through activation of the Cyto c/caspase-9 pathway (Kaufmann and Earnshaw, 2000).

1779 A remarkable difference between tumor cells and normal tissues is related to the transcript levels of VDAC isoforms. Human  
 1780 cancer cell lines were found to express higher VDAC1 levels than did normal fibroblast cells (Simamura et al., 2006, 2008b).  
 1781 Also, the transcript levels of the three VDAC isoforms in AH130 cells were significantly higher than in normal liver cells (Shino-  
 1782 hara et al., 2000). Mitochondria from malignant tumor cell lines are also capable of higher HK binding than are normal liver  
 1783 (Shinohara et al., 2000), and as such, are more protected against apoptosis. These results suggest that the high HK-binding capa-  
 1784 bility of malignant tumor cell mitochondria is due to quantitative differences in VDAC-binding sites. Moreover, it was proposed  
 1785 that the highly glycolytic phenotype of many cancers (see Section 9.1.1) may be associated with such upregulation of VDAC.

1786 Anti-apoptotic Bcl-2 family proteins have been shown to be overexpressed in cancers. This contributes to tumor initia-  
 1787 tion, disease progression and has been associated with resistance to anti-cancer drugs (Adams and Cory, 2007; Alexander  
 1788 et al., 2007; Gross et al., 1999; Miyashita and Reed, 1993; Sentman et al., 1991). Members of the Bcl-2 family of proteins were  
 1789 shown to interact with VDAC to mediate their anti-apoptotic effect (see Section 9.2). Thus, targeting VDAC-Bcl-2 association  
 1790 has implications for drug design to treat diseases, such as neurodegeneration and cancer.

#### 1791 13.2. VDAC and neurodegenerative diseases

1792 Extensive evidence suggests a central role for amyloid- $\beta$  peptide ( $A\beta$ ) (a fragment of the amyloid precursor) over-produc-  
 1793 tion in the pathogenesis of Alzheimer's disease (AD) (Yankner et al., 1989). The effects of amyloid precursor protein on

mitochondrial function in Alzheimer's disease have been recently reviewed (Anandatheerthavarada and Devi, 2007; Vina et al., 2007). Several studies indicate direct action of micromolar  $\text{A}\beta$  concentrations on mitochondrial respiration (Canevari et al., 1999), **ATP-synthesis** (Moreira et al., 2003), and the activity of various enzymes involved in energy production (Gibson et al., 1998; Hensley et al., 1994; Shoffner, 1997). Another proposed mode of action of  $\text{A}\beta$  suggests that its accumulation in the brain and muscle mitochondria alters their function, including activation of Cyto c release (Aleardi et al., 2005).

The role of mitochondrial VDAC in AD is not known, although recent pieces of information support the concept that VDAC is cardinal in the pathogenesis of the disease (Ferrer, 2009). It has been demonstrated that VDAC participates in amyloid beta-induced toxicity (Marin et al., 2007, 2008; Ramirez et al., 2009). Studies on murine cortical and hippocampal neurons has revealed the physical interaction of plasmalemmal VDAC (pl-VDAC) and estrogen receptor alpha (mER $\alpha$ ) in a complex that also includes caveolin-1, suggested to act as an anchoring factor to provide additional stability for the integration and functionality of these proteins (Marin et al., 2007, 2008). It was suggested that VDAC in this complex participates in neuroprotection against  $\text{A}\beta$  (Marin et al., 2007). Recently, it has also shown that in Alzheimer's disease brains, VDAC but not ER $\alpha$  was abundant in caveolae, and was prominent in dystrophic neurites of senile plaques, whereas ER $\alpha$  was expressed in astrocytes surrounding the plaques (Ramirez et al., 2009). Based on these findings, it has been suggested that VDAC may be involved in the membrane dysfunction associated with AD neuropathology (Ramirez et al., 2009).

Other studies have demonstrated a huge accumulation of VDAC in the dystrophic neurites of  $\beta$ -amyloid plaques in AD and related transgenic mice models, where  $\text{A}\beta$  is in association with VDAC (Perez-Gracia et al., 2008). Electron microscopy of  $\text{A}\beta$  plaques correlates VDAC with massive amounts of altered mitochondria and lysosomes filled with dense debris, together with vesicles. It has been shown that the extent of oxidative damage to VDAC is reflected in the increase in nitrated VDAC1 in AD (Sultana et al., 2006). Since VDAC provides the main vehicle for trans-membrane transport of ions, ATP and other metabolites through the OMM, it has been suggested that altered VDAC results in disablement of bidirectional energy fluxes across the mitochondrial membrane (Ferrer, 2009).

Changes in the levels of VDAC1 and VDAC2 expression were observed under various pathological conditions. In post-mortem brain regions of patients with Down's syndrome, the level of VDAC1 is elevated, whereas VDAC2 levels are normal, while in Alzheimer's disease, VDAC1 and/or VDAC2 levels are significantly reduced or elevated in different brain regions (Yoo et al., 2001).

In the pathogenesis of familial ALS, alterations in the post-translational modifications of VDAC2 were reported (Fukada et al., 2004). In the hippocampus of a pharma-coresistant rat, serving as an epileptic model, 3-fold increase in VDAC1 levels and 4-fold decrease in VDAC2 levels were reported (Jiang et al., 2007). Finally, in neuronal NMB cells, dopamine was found to decrease VDAC expression, while transfection of the cells to express VDAC decreased the cytotoxic effect of dopamine. These findings support the notion that VDAC is an important participant in dopamine-induced apoptosis (Premkumar and Simantov, 2002).

In a case report, a patient with deficient VDAC showed mitochondriopathy and clinically presented psychomotor retardation and delayed myelination, polymicrogyria and minor dysmorphic features. Biochemical studies on the patient's muscle mitochondria showed almost complete VDAC deficiency, impaired rates of pyruvate oxidation and ATP production but normal mitochondrial enzyme activity and ultra-structure (Huizing et al., 1996).

It thus seems that VDAC levels and isotype composition are important for regulating sensitivity to apoptotic signals, as changes in VDAC levels are associated with an increased apoptotic rate and with certain diseases.

### 13.3. VDAC and muscular and myocardial diseases

In skeletal muscle of the dystrophin-deficient mdx mouse, which suffers from impaired control of energy metabolism, VDAC3 mRNA but neither VDAC1 nor VDAC2 was markedly down-regulated, suggesting a possible involvement of VDAC3 expression in the early pathogenic events of mdx muscular dystrophy (Massa et al., 2000). In addition, in heart mitochondria from streptozocin-treated diabetes rats, down-regulation of VDAC1 expression level was observed (Turko and Murad, 2003).

It was also demonstrated that VDAC2 expression levels were increased in response to hypoxia in mouse cerebral cortical neurons (Jin et al., 2002). In a rabbit model for myocardial ischemia and reperfusion, it was demonstrated that the **p38-MAPK** inhibitor, PD169316, significantly reduced myocardial neutrophil accumulation, necrosis area and creatine kinase release, as compared to a control group (Schwartz et al., 2007). It was also demonstrated that expression of several proteins was altered following myocardial ischemia and reperfusion treatment alone or upon p38 inhibition, including VDAC1 (Schwartz et al., 2007). Moreover, PD169316 significantly reduced p38-mediated phosphorylation of VDAC1, implicating a role for VDAC1 in myocardial ischemia and reperfusion (Schwartz et al., 2007). Activation of the p38 MAP kinase pathway is a known event in myocardial ischemia and reperfusion, ultimately leading to myocardial necrosis and apoptosis.

These observations suggest that interference with VDAC function, reflected as impaired glucose, energy and intermediary metabolism, as well as apoptotic mechanisms, and are involved in numerous pathological conditions.

## 14. VDAC and reagent toxicity

As presented above, mitochondria play a central role in the execution of apoptosis, with VDAC being a critical component in this pathway. As such, VDAC can be considered as a prime target for therapeutic agents designed to modulate apoptosis

(Granville and Gottlieb, 2003). Indeed, several studies have demonstrated VDACs as the pharmacologic target of novel molecules inducing cancer cell death (Table 2). The cancer-selectivity of VDAC-dependent cytotoxic agents could be related to the higher expression of VDACs in cancer cells (Simamura et al., 2008a). These VDAC-dependent pharmacologic agents can be categorized into several groups.

#### 14.1. VDAC1-dependent chemicals acting by production of ROS

ROS are known to activate apoptosis and are able to release Cyto c from cardiolipins located in the IMM (see Section 11.2). In addition, ROS are apparently able to activate VDAC to allow VDAC-mediated release of Cyto c into the cytosol (see Section 11.2). The following are reagents proposed to act via ROS modulation of VDAC activity or expression.

Furanonaphthoquinones (FNQs)-induced caspase-dependent apoptosis occurs via the production of NADH-dependent ROS by VDAC1. The ROS production and the anti-cancer activity of FNQs are increased upon VDAC1 overexpression (Simamura et al., 2006).

Similarly, erastin is a selective anti-tumor agent causing the appearance of ROS and subsequent cell death by inducing RAS-RAF-MEK-dependent non-apoptotic cell death via VDAC2 (Yagoda et al., 2007). RNAi-mediated knockdown of VDAC2 or VDAC3 caused resistance to erastin. Using purified mitochondria expressing a single VDAC isoform, erastin was found to alter the permeability of the OMM by binding directly to VDAC2.

Another proposal for ROS generation affecting VDAC-mediated apoptosis involves TSPO (see Section 9.3). It is proposed that TSPO is involved in the generation of ROS, with the close association of TSPO with VDAC ensuring that TSPO-generated ROS stimulates the ability of VDAC to mediate apoptosis (Veenman et al., 2008).

It has been shown TSPO molecules are grouped around VDAC, possibly aiding in the enhancement of ROS levels generated by TSPO in the proximity of VDAC (Veenman et al., 2008). This hypothesis carries implications for drugs designed to treat diseases, such as neurodegeneration and cancer (Veenman and Gavish, 2006; Veenman et al., 2007).

#### 14.2. Chemicals that directly interact with and modify VDAC activity

Phosphorothioate oligonucleotides have been proposed as specific VDAC inhibitors (Lai et al., 2006). These molecules are stable in the cytosol and are able to bind and block VDAC channel activity in the mitochondria without affecting the respiration complexes, ANT or ATP synthase (Stein and Colombini, 2008). The ability of variable lengths of thymidine phosphorothioate homopolymers to induce Cyto c release from the isolated mitochondria of melanoma cells was found to correlate with the ability of these compounds to interact with VDAC and induce its closure (Lai et al., 2006). It appears that the phosphorothioate oligonucleotide length rather than base sequence determines the ability to block VDAC channels.

One of the studied phosphorothioate oligonucleotide is G3139 (oblimersen), an 18-mer phosphorothioate anti-sense oligonucleotide targeted to the initiation codon region of Bcl-2 mRNA. It was shown that G3139 can induce caspase-dependent apoptosis via the intrinsic mitochondria-mediated pathway in melanoma (Lai et al., 2006) and other cancer cells via a Bcl-2-independent pathway, with mitochondrial Cyto c release preceding the down-regulation of Bcl-2 expression (Lai et al., 2005). G3139 directly binds to the OMM and reduces its permeability to ADP by closing VDAC. The addition of G3139 to a reconstituted VDAC channel resulted in channel closure (Lai et al., 2006; Tan et al., 2007), pointing to VDAC as the pharmacologic target of G3139 (Lai et al., 2006). In addition, down-regulation of enolase-1 and three other glycolytic enzymes indicated a reversion of the cancer-related Warburg effect. It is assumed that these effects are caused by a phosphorothioate-mediated blockage of mitochondrial VDAC (Stessl et al., 2009). Thus, VDAC was proposed as a pharmacological target for G3139.

Avicins represent a novel class of plant stress metabolites that exhibit cytotoxic activity in tumor cells, as well as anti-inflammatory and anti-oxidant properties capable of perturbing mitochondrial function and initiating apoptosis in tumor cells (Haridas et al., 2001a,b 2004, 2005; Gaikwad et al., 2005; Mujoo et al., 2001). Avicins were found to permeabilize the OMM and induce Cyto c release (Lemeshko et al., 2006). Recently, it was shown that avicins interact lipid bilayer-reconstituted VDAC and reduce its channel conductance at negative potentials (Haridas et al., 2007). Closure of VDAC can lead to an overall lowering of cellular energy metabolism, subsequently pushing the cells towards the apoptosis pathway by OMM permeabilization and Cyto c release.

Fluoxetine, also known as prozac, is a clinically-used potent anti-depressant compound, employed to treat a variety of psychiatric disorders (Wong et al., 1995) and has been suggested to also have potential in the treatment of a number of neurological disorders. Specifically, fluoxetine was reported to improve neurological manifestations in patients suffering from Alzheimer's disease, stroke, Huntington's disease, multiple sclerosis, traumatic brain injury, and epilepsy.

Fluoxetine is a selective serotonin reuptake inhibitor with a high selectivity for the 5-hydroxytryptamine (5-HT) transporter, and thus, modulates synaptic serotonin concentration in the brain (Fuller et al., 1991). Besides its well-known action as serotonin reuptake inhibitor, fluoxetine exerts other effects, such as blockade of muscular and neuronal nicotinic receptors (Garcia-Colunga et al., 1997), inhibition of monoamine oxidase A and B (Leonardi and Azmitia, 1994) as well as blocking the activity of the voltage-dependent Na<sup>+</sup> and K<sup>+</sup> and Ca<sup>2+</sup> channels (Deak et al., 2000; Pancrazio et al., 1998). In addition, fluoxetine inhibits the multi-drug resistance extrusion pump and thus enhances responses to chemotherapy. Indeed, fluoxetine enhances doxorubicin accumulation within tumors (Peer et al., 2004). Several studies have also linked fluoxetine with cell proliferation and an increased risk of developing cancer (Deak et al., 2000; Leonardi and Azmitia, 1994; Pancrazio et al., 1998; Peer et al., 2004).

Fluoxetine has been shown to enhance cell proliferation and to prevent apoptosis in the dentate gyrus (Lee et al., 2001), to stimulate DNA synthesis (Brandes et al., 1992) and to inhibit UV-induced DNA fragmentation in U-937 cells (Wright et al., 1994). Contradicting results showing an enhancement of apoptosis in various cell lines have also been reported (Serafeim et al., 2003). Fluoxetine was found to trigger rapid and extensive apoptosis in Burkitt lymphoma cells that could be prevented by overexpression of the anti-apoptotic Bcl-2 protein (Serafeim et al., 2003).

Fluoxetine has been shown to increase the voltage-dependence of bilayer-reconstituted VDAC channel activity (Thinnes, 2005). In a later study (Nahon et al., 2005), fluoxetine was shown to (a) interact directly with purified VDAC reconstituted into a planar lipid bilayer and decrease its channel conductance, (b) prevent PTP opening, as demonstrated by its inhibition of the release of accumulated Ca<sup>2+</sup> and of swelling of energized mitochondria, (c) inhibited release of Cyto c from mitochondria, and (d) protect against STS-induced apoptotic cell death. These results suggest that fluoxetine may mediate its effects on apoptosis by interacting with VDAC.

The platinum-based drug, cisplatin, is a widely used anti-cancer drug which acts by inducing apoptosis. Cisplatin has been used in the clinical treatment of several types of cancer, such as testicular carcinomas, as well as for squamous cell carcinomas (Martinou and Green, 2001; Giaccone, 2000). The cytotoxicity of cisplatin has generally been attributed to its ability to form inter- and intra-strand nuclear DNA cross-links (Zamarín et al., 2005). However, recent studies suggest a different approach of cytotoxicity, implicating mitochondria as a critical target of cisplatin (Cullen et al., 2007; Yang et al., 2006). Recently, it was argued that VDAC1 serves as a cisplatin receptor along the intrinsic mitochondrial or the extrinsic death receptor apoptotic pathways (Thinnes, 2009). Cisplatin was shown to bind mitochondrial DNA and VDAC. When mitochondria were exposed to cisplatin, the drug preferentially bound to VDAC at the OMM (Yang et al., 2006). In addition, exposure of the cervix squamous carcinoma cell line A431 to cisplatin was shown to modulate VDAC1 activity (Castagna et al., 2004). These findings might assist in understanding part of the mechanism by which cisplatin facilitates apoptosis in cancer cells.

Acrolein (2-propen-1-al), the most reactive of the  $\alpha,\beta$ -unsaturated aldehydes, is a toxic compound found in automobile exhaust gases, overheated cooking oils and is a metabolite of cyclophosphamide and allyl alcohol (Halliwell, 1999). Acrolein can also be endogenously produced as a product of lipid peroxidation (Adams and Klaidman, 1993) and from polyamine metabolism (Takano et al., 2005). Acrolein reacts with proteins and DNA, and its levels have been found to increase in pathological conditions associated with oxidative stress, such as spinal cord injury (Luo et al., 2005) and Alzheimer's disease (Lovell et al., 2001). VDAC was recently identified as a selectively oxidized target in the AD brain, with proteomic analysis showing that VDAC was significantly carbonylated by acrolein (Mello et al., 2007; Sultana et al., 2006).

#### 14.3. VDAC-interacting and -based peptides interfere with protection against apoptosis mediated by anti-apoptotic proteins

One of the major hallmarks of tumor cells is their relative resistance to cell death, owing to overexpression of anti-apoptotic proteins of the Bcl-2 family and HK. These excessive levels of Bcl-2 and HK-I or HK-II pose a major problem in terms of resistance to anti-cancer drugs (see Sections 9.1 and 9.2). As these proteins interact with VDAC (see Sections 9.1 and 9.2), VDAC may be a suitable target for therapeutic agents aimed at interfering with VDAC-HK and/or VDAC-Bcl-2 interactions.

Recently, key regions of VDAC1 interacting with HK-I, HK-II and Bcl-2 and required for HK-I-mediated protection against cell death via inhibiting release of Cyto c were identified (see Sections 9.1 and 9.2). VDAC1-base peptides were found to disrupt the HK-VDAC1 interaction, consequently leading to diminished HK anti-apoptotic activity, suggesting that disruption of HK binding to VDAC1 can decrease tumor cell survival. As summarized above (Section 9.1), several compounds have been shown to induce dissociation of HK from the mitochondria and encourage apoptosis. These include peptides corresponding to the amino terminus of both HK-I (Gelb et al., 1992) and HK-II (Pastorino et al., 2002; Penso and Beitner, 1998), a cell-permeable peptide corresponding to the amino-terminal 23 residues of HK-II fused to six arginines at the carboxyl-terminus (N-HK-II) (Majewski et al., 2004b), methyl jasmonate (Goldin et al., 2008) and VDAC1-based peptides (Arzoine et al., 2009). Identification of the exact sequence of the epitopes, i.e. the portion of that agent actually interacting with anti-apoptotic proteins, is important for drug design and could lead to more specific pharmaceuticals and therapies. VDAC1-based peptides were found to overcome the protective effect of the anti-apoptotic proteins, HK-I and HK-II, as well as Bcl-2, allowing these peptides to present a wide therapeutic index.

These finding support the suggestion that VDACS can serve as pharmacologic targets for anti-cancer drugs.

#### 15. Concluding remarks

We have witnessed a significant accumulation of knowledge regarding the function of VDAC in recent years. Following the identification of VDAC as the OMM channel, much has been learned about the protein's structure-function relationships, the biochemical and molecular basis of its activation and inactivation, and the manner by which VDAC activity is modulated within the cell. Biochemical and molecular approaches have revealed a remarkable diversity of regulatory mechanisms controlling VDAC function, ranging from Ca<sup>2+</sup> and adenine nucleotides to phosphorylation and specific associated proteins. Although a high-resolution structure has been presented for recombinant VDAC1, the structure of the native protein and its complexes with associated proteins or modulating ligands or drugs awaits determination at the atomic resolution. However, many interesting and important questions remain. We look forward to acquiring further information concerning the architecture of the channel pore, the location of modulator-binding sites, identification of the regions of the protein involved

in channel gating and the control mechanism of its oligomerization leading to the release of Cyto c. Clearly, additional information on the structural and functional maintenance of multi-subunit complexes as well as on dynamic interactions between VDAC monomers and/or apoptosis-regulating proteins will be essential for precise understanding of the mechanisms underlying the multitude of VDAC functions associated with cell life and death.

The function of the different isoforms should also be explored, as should their involvement in pathogenic conditions. Finally, the identification of VDAC as playing a role at critical control points in the apoptotic pathway points to VDAC as a rational target for the development of a new generation of therapeutics.

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