CHAPTER ELEVEN

Oligomerization of the Mitochondrial Protein VDAC1: From Structure to Function and Cancer Therapy

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Abstract

The voltage-dependent anion channel (VDAC1), lying in the mitochondrial outer membrane (OMM), mediates the transport of ions and metabolites, thus controlling the cross talk between mitochondria and the rest of the cell. VDAC1 has also been recognized as a key protein in mitochondria-mediated apoptosis, is the proposed target

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for the pro- and antiapoptotic Bcl-2-family of proteins and is involved in apoptotic protein release from the mitochondria. Questions, however, remain as to if and how VDAC1 mediates the transfer of apoptotic proteins across the OMM. Our recent studies suggest that upon apoptosis induction, VDAC1 oligomerizes to form a new large pore allowing the passage of a folded protein, like cytochrome *c*. This review provides insight into the central role of VDAC1 in mammalian cell life and death and emphasizes VDAC1 function in apoptosis, focusing on VDAC1 oligomerization as a key step in mitochondria-mediated apoptosis and key structural features of VDAC1 that mediate its apoptotic function.

ABBREVIATIONS

Cyto *c* cytochrome *c*
EGS ethylene glycol bis-(succinimidyl succinate)
IMM inner mitochondrial membrane
OMM outer mitochondrial membrane
PLB planar lipid bilayer
PTP permeability transition pore
RuR ruthenium red
VDAC voltage-dependent anion channel

1. OVERVIEW

Mitochondria, organelles long thought to serve solely as cellular energy generators, are now known to assume functions well beyond this critical bioenergetic role. Mitochondria play a crucial part in the modulation of the cellular redox status, osmotic regulation, pH control, calcium homeostasis, and signaling events. In addition, mitochondria mediate the responses of cells to multiple physiological and genetic stresses, interorganelle communication, and cell proliferation. Mitochondria also serve as a center for the regulation of programmed cell death or apoptosis.

To fulfill the various activities attributed to this organelle, mitochondria contain about 1000 different proteins with tissue-specific features. One of these proteins is the voltage-dependent anion channel, VDAC, located in the outer mitochondrial membrane (OMM), the barrier between the mitochondrial intermembrane space (IMS) and the cytoplasm. VDAC1 plays an important role as a controlled passage for adenine nucleotides, Ca^{2+}, and other metabolites into and out of mitochondria. As such, it has been recognized that VDAC plays a crucial role in regulating the metabolic and energetic functions of mitochondria (Fig. 11.1).

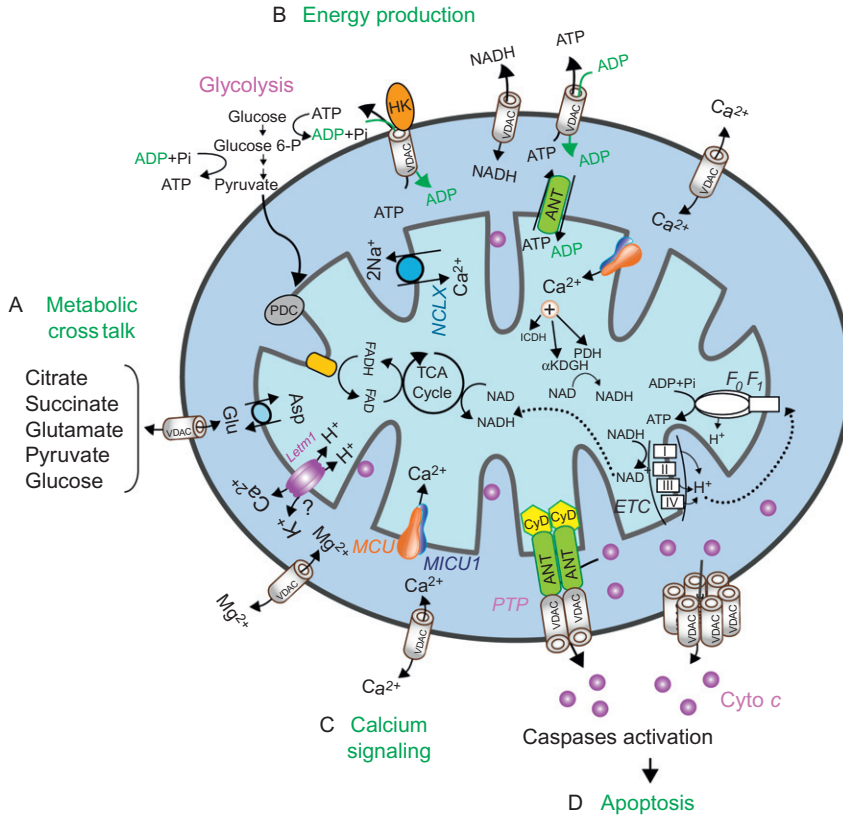

Figure 11.1 Schematic representation of VDAC1 as a multifunctional channel and convergence point for a variety of cell survival and cell death signals. (A) VDAC1 plays a central role controlling cross talk between mitochondria and the rest of the cell. VDAC1 also regulates cellular energy homeostasis (B) by transporting ATP/ADP and NADH between the intermembrane space and the cytosol and by binding HK. (C) VDAC1 is responsible for Ca^{2+} transport across the OMM. Also presented are the Ca^{2+} influx and efflux transport systems in the inner mitochondrial membranes. The uptake of Ca^{2+} into the matrix is mediated by a Ca^{2+}-selective transporter, the mitochondrial Ca^{2+} uniporter (MCU), regulated by a calcium-sensing accessory subunit (MICU1). Ca^{2+} efflux is mediated by NCLX, a Na^{+}/Ca^{2+} exchanger. High levels of matrix Ca^{2+} accumulation trigger the opening of the PTP, a fast Ca^{2+} release channel. Molecular fluxes are indicated by arrows. Ca^{2+}-mediated regulation of the tricarboxylic acid (TCA) cycle is also presented. The activation of pyruvate dehydrogenase (PDH), isocitrate dehydrogenase (ICDH), and α-ketoglutarate dehydrogenase (α KGDH) by intramitochondrial Ca^{2+}, leading to enhanced activity of the TCA cycle, is shown. The electron transport chain (ETC) and the ATP synthase (F_oF_1) are also presented. (D) In the apoptotic process, VDAC1 binds the apoptosis regulatory proteins, that is, Bcl-2 family members and HK, and is active in the release of Cyto *c* into the cytosol. The proposed PTP and VDAC1 oligomer as Cyto *c* release channels are presented.

It is also well accepted that mitochondria play an important role in the regulation of apoptosis via release of proapoptotic agents and/or disruption of cellular energy metabolism.[1–3] Given the importance of mitochondria as integrators and amplifiers of the death program, intense efforts have been directed at exploring the mechanisms of mitochondria-mediated apoptosis. Such efforts include studies on the release of proapoptotic effectors, the participation of Bcl-2 (B-cell leukemia/lymphoma 2) family members in apoptosis, as well as the involvement of reactive oxygen species (ROS) and Ca^{2+} in this event.[4–6] Still, one of the major open questions concerning apoptosis is related to how mitochondrial proapoptoic proteins are released and how this process is regulated.

2. MITOCHONDRIA AND APOPTOSIS

Apoptosis is a cellular process mediated by a large and diverse range of extracellular and/or intracellular signals that allow for the controlled and regulated death of cells. Apoptosis is a genetically regulated process important for tissue homeostasis and development in multicellular metazoans. Defects in the regulation of apoptosis are often associated with disease, such as neuronal degenerative diseases, tumorigenesis, autoimmune disorders, and viral infections.[7–9] Apoptosis can be induced in cells via a number of mechanisms that are either extracellular or intracellular in origin.

In general terms, apoptotic pathways can be subdivided into the extrinsic and the intrinsic pathways. In extrinsic pathways, apoptotic signals are initiated by ligand engagement of cell surface receptors, such as Fas and TNF receptors, as well as by signaling by hormones, growth factors, nitric oxide, or cytokines.[10,11] The intrinsic apoptotic pathway, on the other hand, requires the involvement of mitochondria. During transduction of an apoptotic signal into the cell, an alteration in mitochondrial membrane permeability occurs, causing the release of apoptogenic factors, such as cytochrome *c* (Cyto *c*), apoptosis-inducing factor (AIF), Smac/DIABLO, and endonuclease G from the IMS into the cytosol.[11–14] These proteins participate in complex processes resulting in the activation of proteases and nucleases, leading to protein and DNA degradation, and finally, cell death. Most notable among the released proteins is Cyto *c* that initiates apoptosis by binding to a central apoptotic regulator, Apaf-1, thereby promoting oligomerization of Apaf-1 and activation of caspase 9. Caspase 9 subsequently activates effector caspases, such as caspases 3, 6, and 7, encouraging execution of programmed cell death. How Cyto *c* and other apoptogenic factors are released from mitochondria is not clear, as will be discussed below.

A proapoptotic signal can be generated either inside the mitochondria, for example, by increased Ca^{2+} and/or ROS concentrations, or from outside by proapoptotic proteins, such as tBid and Bax. In the latter case, mitochondrial apoptosis induction is used to amplify the extramitochondrial proapoptotic message coming from death receptors, such as FAS, or increased p53 levels.

The pathways activated by extrinsic and intrinsic signals can overlap to some extent. Receptor binding by an extrinsic signal typically leads to recruitment of adapter proteins that promote caspase oligomerization and autoprocessing. The sensitivity of cells to any of the apoptosis stimuli can vary, depending on a number of factors, such as the expression level of pro- and antiapoptotic proteins (e.g., Bcl-2 proteins or inhibitors of apoptosis proteins), the severity of the stimulus, and the stage of the cell cycle.

3. BAX-, BAK-MEDIATED APOPTOSIS INVOLVES THEIR OLIGOMERIZATION

Oligomerization as a mechanism for the mediation of Cyto *c* release and apoptosis has been previously proposed for two key proteins in the mitochondrial pathway of apoptosis, namely, Bax and Bak (Fig. 11.2). In healthy cells, Bax resides in the cytosol in an inactive state. Upon apoptotic signaling, Bax translocates to the mitochondria. Insertion of Bax into the OMM is accompanied by conformational changes and oligomerization, resulting in mitochondrial OMM permeabilization and release of IMS proteins, such as Cyto *c*[15–17] (Fig. 11.2). It has been shown that TNF-α activation of caspase 8 resulted in the cleavage of Bid to form truncated tBid, which, in turn, interacts with Bax and Bak to generate Bax and Bak oligomers, forming complexes as large as 500 kDa.[18] At the OMM, tBid stimulates the conformational transition of Bax to its active monomeric membrane-integrated form through the insertion of the transmembrane segment and integration of helices 5 and 6 into the bilayer, with activated Bax undergoing autooligomerization to yield an OMM pore.[19,20] It is accepted that Bak follows a similar process,[21] where, following Bak activation, dimers are formed that then multimerize into larger oligomers.[22]

Another suggested mechanism for the activation of Bax/Bak by BH3-only molecules (BH3s) is based on the finding that Bax undergoes stepwise structural reorganization, leading to mitochondrial targeting and homooligomerization. Given this reorganization, it is thought that the α1 helix of Bax keeps the α9 helix engaged in the dimerization pocket,

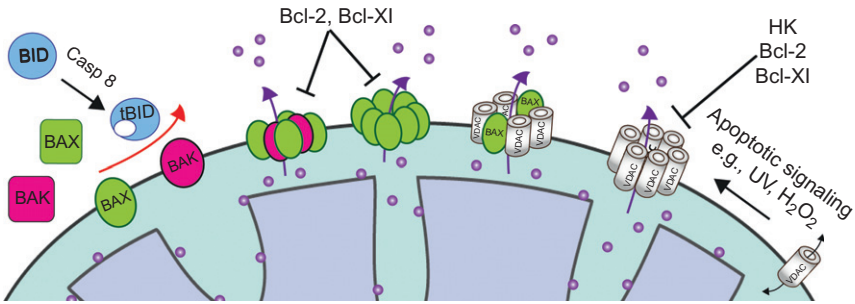

Figure 11.2 Bax- and Bak-mediated Cyto *c* release. The release of Cyto *c* from the mitochondrial intermembrane space is achieved by the activation of pore-forming proteins. Bax is located in the cytosol before translocation to the OMM. Bax and Bak are activated by the product of caspase 8-mediated cleavage of Bid. Upon activation, Bax and Bak begin the pore-formation process as homo- or hetero-oligomers. Bax and Bak translocation to the OMM and subsequent oligomerization are both marked by conformational activation. VDAC1 can be activated by apoptotic signaling to begin the pore-forming process. At least six VDAC1 monomers are required to form the new pore with a calculated diameter of 4 nm, considering an external monomer diameter of 4 nm. In the event that both Bax and VDAC1 are activated, a hetero-oligomer composed of Bax/VDAC1-formed pores appears.

rendering Bax as a monomer in the cytosol. The activator BH3s, tBid/Bim/PUMA, attack and expose the α1 helix of Bax, resulting in secondary disengagement of the α9 helix and mitochondrial insertion. Activator BH3s remain associated with the N-terminally exposed Bax through the BH1 domain to drive homooligomerization. Bak, as an integral mitochondrial membrane protein, can bypass the first activation step, explaining why its killing kinetics are faster than those of Bax.[23] These suggested pathways of Bax/Bak activation gained support with the finding that genetic deletion of *Bid*, *Bim*, and *Puma* prevented homooligomerization of Bax and Bak, and thus Cyto *c*-mediated activation of caspases, in response to diverse death signals.[24]

4. VDAC1 AS A GATEKEEPER OF MITOCHONDRIAL FUNCTION

4.1. VDAC isoforms, structure, and channel activity

Found at the OMM, VDAC1 mediates the complex interactions between mitochondria and other parts of the cell by transporting anions, cations, ATP, Ca^{2+}, and metabolites (Fig. 11.1). VDAC1 thus plays an important

role in coordinating communication between mitochondrion and cytosol. An essential aspect of this management is the transient formation of complexes of VDAC1 with other proteins. Over the past few years, several hypotheses and potential mechanisms have been forwarded for and against a role for VDAC1 in OMM permeability and the subsequent release of apoptosis-promoting proteins (Fig. 11.1). In this section, the proposed structure, channel properties and function, and cellular expression of VDAC will be discussed.

4.1.1 VDAC isoforms and functions

Three different VDAC genes, encoding distinctly expressed isoforms, have been reported in humans, rat, and mice.[25–28] VDAC isoforms appear to serve specialized functions, since the more complex the organism, the more VDAC isoforms it possesses. For instance, *Neurospora crassa* encodes only one form of VDAC, *Saccharomyces cerevisiae* encodes two, while, as noted above, mice and humans each encode three isoforms.[28,29] The three genes that encode the distinct isoforms of mouse VDAC, *VDAC1–3*, have been characterized.[28] Each isoform is 65–70% identical to the others.[30] Phylogenetic analysis indicates that VDAC3 diverged from the primordial VDAC first, with VDAC1 and VDAC2 arising more recently.[31] Mammalian VDAC isoforms are present in a wide variety of tissues and are expressed at high levels in heart, kidney, brain, and skeletal muscle. VDAC2 and VDAC3 were localized to the outer dense fibers of the bovine sperm flagellum,[32] while the localization of VDAC2 to the acrosomal region of bovine spermatozoa was recently demonstrated.[33]

Both VDAC1 and VDAC2 are ubiquitously expressed, with VDAC3 showing a more restricted organ distribution.[28,34,35] In tissues that have been studied, VDAC1 represents the predominant form expressed.[36] However, the tissue-specific distribution and intracellular localization of the different VDAC isoforms are not well established.

The VDAC isoforms also appear to assume different physiological roles.[37,38] In mice, deletion of *VDAC1* and *VDAC2* reduces respiratory capacity,[39] the absence of VDAC3 causes male sterility, while a lack of both VDAC1 and VDAC3 causes growth retardation[40] and is associated with deficits in learning behavior and synaptic plasticity.[41] Embryonic fibroblasts deficient in VDAC2 but not cells lacking the more abundant VDAC1 exhibit enhanced Bak oligomerization and are more susceptible to apoptotic death induced by staurosporine (STS) and etoposide,[37] as supported by results suggesting VDAC2 to be a critical inhibitor of Bak-mediated apoptotic responses.[42]

In this review, we focus on VDAC1, as it is the abundant isoform in most cells and the most studied isoform.[43,44]

4.1.2 VDAC1 membrane topology and molecular structure

4.1.2.1 VDAC1 structure and the N-terminal domain function

In 2008, three independent technical approaches unraveled the three-dimensional structure of VDAC1 at atomic resolution.[45–47] The three structures were found to be almost identical, featuring 19 α-strands connected by flexible loops to form an β-barrel, along with a 25-residue-long N-terminal region[45–47] (see Fig. 11.3). In all three solved VDAC1 structures, a helical conformation is present within the N-terminal region, attached to the channel wall but not part of it.[45–47]

Several functional implications of this architecture were suggested. It was proposed that the N-terminal region lies inside the pore yet could move in the open space.[48] The mobility of the N-terminal region was further supported by studies showing that the N-terminal α-helix exhibits motion during voltage gating,[49–52] and that anti-VDAC1 antibodies raised against the N-terminal region of the protein interact with membranal VDAC1.[53–55]

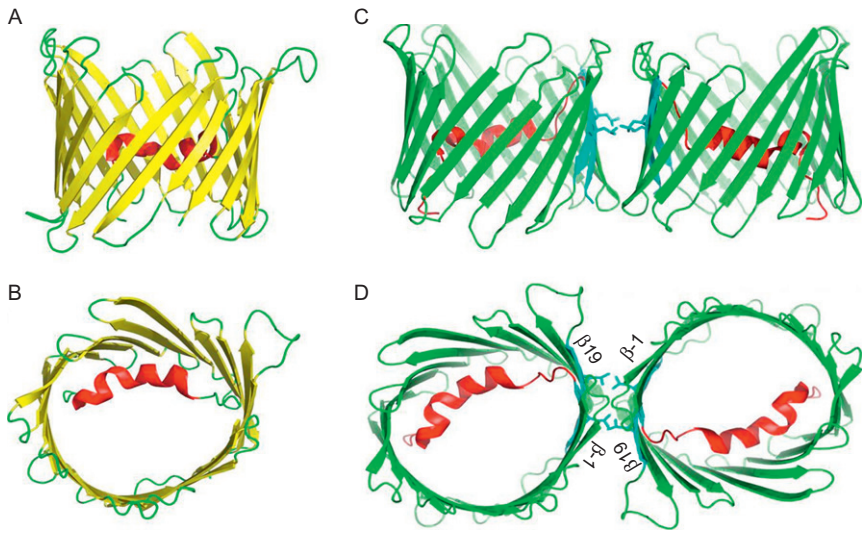

Figure 11.3 VDAC1 monomeric and dimeric structures. Ribbon representation of VDAC1 (PDB ID: 3EMN). Panels (A) and (B) show VDAC1 monomer, while panels (C) and (D) represent VDAC1 dimers. Side views: (A) and (C). Top views: (B) and (D). β-Strands involved in dimerization are marked in blue (β1, β19). The N-terminal domain is colored red.

It is also shown that the N-terminal α-helix interacts with apoptosis-regulating proteins of the Bcl-2 family (i.e., Bax, Bcl-2, and Bcl-xL)[56–59] and hexokinase (HK),[56,60] and that movement of this VDAC1 segment out of the pore modulates the antiapoptotic activities of these binding partners. Moreover, cells expressing N-terminal domain-truncated VDAC1 are resistant to apoptosis.[56] These findings indicate that the N-terminal domain is required for apoptosis induction; interactions with HK-I, Bcl-xL, and Bcl-2; and protection against apoptosis.[56]

Recently,[49] we demonstrated that the VDAC1 N-terminal region lies inside the pore yet can be exposed outside the pore where it interacts with the N-terminal region of a second molecule to form a dimer. Moreover, specific mutations disrupting the α-helix structure or the flexible glycine-rich sequence of this domain affected N-terminal region translocation from the internal pore to the channel surface. The results suggest that N-terminal region mobility is involved in channel gating and interaction with anti-apoptotic proteins.[49]

4.1.2.2 VDAC1 oligomeric structure

Substantial evidence for the formation of higher-order VDAC1-containing complexes has been presented.[61–68] It has been demonstrated that VDAC1 can exist in different oligomeric states, namely, as monomers, dimers, trimers, tetramers, hexamers, and higher-order oligomers.

On the basis of electron microscopy-based analysis of the 2D crystallized OMM, it was noted that VDAC1 pores are organized into ordered arrays, each bearing six monomers with interchannel contacts.[69] Chemical cross-linking experiments of purified VDAC1 and membrane-embedded VDAC1 demonstrated the assembly of dimers, trimers, tetramers, and higher VDAC1 oligomers.[62] The supramolecular organization of VDAC has also been demonstrated using atomic force microscopy to study the native OMM.[64,65] In addition, the application of symmetry operators on the NMR-based structure of recombinant hVDAC1[45] indicated that it may form a parallel dimer, although analysis of the crystal packing of mouse VDAC1 revealed an anti-parallel dimer, which further assembles as hexamers.[70] Recently, it was suggested that the specific lipid composition of the OMM, that changes upon apoptogenic stimuli, significantly enhances VDAC1 oligomerization.[71]

The function of VDAC1 oligomers is not known. It was proposed that an organization of VDAC1 beyond the monomeric or dimeric forms may contribute to stabilizing the protein.[70] On the other hand, it was proposed that the oligomeric assembly of VDAC1 offers a platform for other proteins

to oligomerize, such as HK.[62] HK-I assumes a tetrameric structure that is greatly enhanced when the enzyme is bound to mitochondria[72] or when it interacts with the mitochondria to inhibit permeability transition pore (PTP) opening.[55] Creatine kinase, when bound to VDAC1 at the IMS, forms high-order oligomers,[73,74] interacting with VDAC1 exclusively in the octameric state, with the dimeric state only showing weak affinity for VDAC1.[75]

VDAC1 oligomerization has been proposed to also play important physiological roles in the regulation of VDAC1 function, including apoptosis (see Section 6.1), and in mediating the binding of apoptosis-regulating proteins.[56,62,66]

4.1.3 Channel activity of VDAC1

The functional properties of VDAC1 have been examined following reconstitution into a planar lipid bilayer (PLB). The activity of the channel is reflected in the flow of ions (i.e., current) through a membrane that is otherwise a barrier to ion flow (Fig. 11.4). Bilayer-reconstituted VDAC1 allows voltage-dependent conductance and displays ion selectivity. The single-channel conductance of mitochondrial VDAC1 in 1 M KCl or NaCl ranges between 4 and 5 nS.[77–81] At low voltages (10 mV), the VDAC1 channel is stable in a long-lived open state (up to 2 h). At high positive or negative potentials (>40 mV), VDAC1 presents multiple substates with different

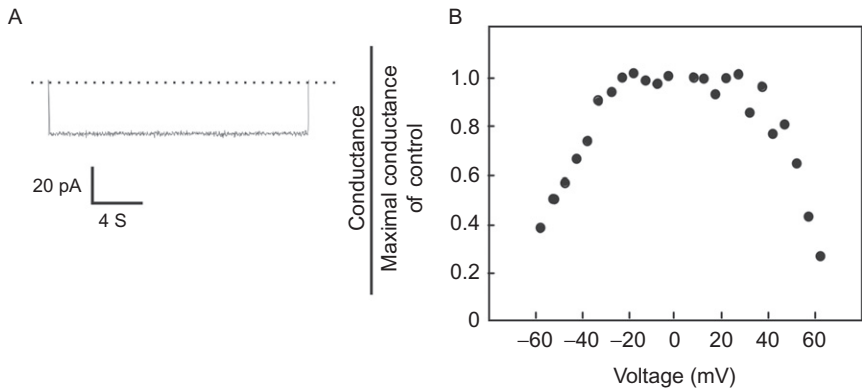

Figure 11.4 VDAC1 channel conductance. Rat liver purified VDAC1 was reconstituted into a PLB and recording of channel conductance was carried out as described previously.[76] (A) Currents through VDAC1 were obtained in response to voltage step from 0 to −10 mV. The broken line indicates the zero current level. (B) VDAC1 conductance was measured as a function of voltage, from 60 to −60 mV. The average steady-state conductance at a given voltage was normalized to the conductance at 10 mV.

ionic selectivities and permeabilities.[79,82] The bell-shaped VDAC1activity profile (Fig. 11.4B) led to characterization of the so-called closed state of the channel. In this low-conducting state, VDAC1 is more permeable to cations. In the fully open state, VDAC1 selectively conducts small ions (e.g., Cl^-, K^+, Na^+), yet also anions, such as glutamate[79] and ATP,[83] and large cations, such as acetylcholine, dopamine,[79] and Tris.[84]

The N-terminal α-helical segment of the channel has been proposed to act as the voltage sensor, gating the pore via its conformational changes or movements.[45,47–50,56]

5. VDAC1 AND APOPTOSIS: STRUCTURE–FUNCTION

5.1. Proposed mechanisms of VDAC1-mediated apoptosis

Several reviews have focused on the role of VDAC1 as an essential player in apoptosis, offering several hypotheses regarding the mechanism of mitochondria-mediated apoptosis implicating VDAC1.[38,85–93] However, none of the current models of mitochondrial membrane permeabilization can account for all of the experimental data. One model proposes that VDAC1 is a component of the PTP that is activated by proapoptotic stimuli.[12,90,91,94–96] Another model proposes that Bax interacts with VDAC1, resulting in Cyto *c* permeation through the OMM.[97,98] A third model proposes that closure of the VDAC1 channel prevents the efficient exchange of ATP and ADP between the cytosol and mitochondrial matrix, followed by swelling and membrane rupture.[99,100] A more recent model proposes VDAC1 oligomerization as mediating the release of proapoptotic proteins.[57,62,66,91] Indeed, the involvement of VDAC1 in the mechanism of Cyto *c* release from the mitochondrial IMS into the cytosol and the regulation of this process has encouraged research at various levels. While numerous studies have shown VDAC1 as a component of the apoptosis machinery,[38,85–93] other studies cast doubt on the contribution of VDAC1 to mitochondrial membrane permeability transition changes leading to cell death.[101,102] In addition, two views as to the relationship between the conducting state of the VDAC1 channel and cell death appear in the literature. While one view suggests that closure, rather than opening of VDAC1, leads to mitochondria outer membrane permeabilization and apoptosis,[99] another model supports a direct relationship between VDAC1-mediated channel opening and release of proapoptotic proteins.[38,85–93,97] These differing views may result from the involvement of two VDAC1-conducting channels, namely, an intrinsic pore (Section 4.1.3) and an intra-VDAC1 subunit

channel (Section 6.1). In the following, findings related to VDAC1 involvement in apoptotic cell death are discussed.

6. VDAC1 AND Cyto *c* RELEASE

There is substantial evidence connecting VDAC1 to apoptosis and suggesting that VDAC1 is a critical player in the release of apoptogenic proteins from mitochondria in mammalian cells.[38,85–93] Cyto *c* release, cell death, and Bax–VDAC1 interaction are all inhibited by anti-VDAC1 antibodies.[103–105] The inhibition of Cyto *c* release and cell death by HK were both shown to specifically rely on interaction with native, but not mutated, VDAC1.[91,106] Similarly, ruthenium red (RuR) interacts with native, but not mutated, VDAC1 to prevent Cyto *c* release and cell death.[106–109] In addition, siRNA-mediated down-expression of VDAC1 prevented cell death induced by cisplatin and strongly reduces cisplatin-induced release of Cyto *c* and AIF and maturation of caspases-3,[110] while depletion of VDAC1 strongly suppresses the cisplatin-induced activation of Bax.[110] Finally, it is also been shown that reducing VDAC1 expression by siRNA attenuates endostatin-induced apoptosis.[111] A proposed mechanism for VDAC1 structure mediating Cyto *c* release is presented here.

6.1. VDAC1 oligomerization and the release of Cyto *c*

When considering models of VDAC1-mediated protein release, one should note the molecular sizes of the released proteins (12–100 kDa). The diameter of the VDAC1 pore at its high conductance state is about 2.6–3.0 nm, sufficient to move nucleotides and small molecules but insufficient to pass a folded protein like Cyto *c*. Yet, release of Cyto *c* via purified VDAC1 reconstituted into liposomes in which Cyto *c* was encapsulated has been demonstrated.[62,103] Therefore, the formation of a VDAC1-composed large channel serving as the Cyto *c* release channel has been proposed.[61,62,66]

Various studies using purified rat liver,[62] brain mitochondria,[55] or recombinant human VDAC1[57] have reported that both soluble purified and membrane-embedded VDAC1 can assemble into dimers, trimers, and tetramers in a dynamic process. In addition, a supramolecular organization of VDAC1 was also demonstrated.[64,65] VDAC1 oligomerization in VDAC1-reconstituted liposomes has been shown by applied FRET technology.[62] Here, purified VDAC1 was labeled with fluorescein isothiocyanate (FITC) or eosin-5-isothiovyanate (EITC) as donor and acceptor fluorophores, respectively, with enhancement in FRET signals reflecting

association between FITC–VDAC1 and EITC–VDAC1 upon the formation of dimers or higher oligomeric combinations. Moreover, FRET intensity was decreased upon addition of an excess of unlabeled VDAC1 during the dissociation/reassociation process, suggesting VDAC1 to exist in a dynamic equilibrium between monomeric and higher-order oligomers. Thus, it was proposed that dynamic VDAC1 oligomerization mediates the formation of a large flexible pore between individual subunits of VDAC1, serving as a channel that allows Cyto *c* to cross the OMM.[56,61,62,91]

This suggestion has gained experimental support by the demonstration that apoptosis induction in cultured cells leads to VDAC1 oligomerization.[66] Apoptosis induction by various inducers was accompanied by an up to 20-fold increase in VDAC1 oligomerization, as revealed by chemical cross-linking, reflecting a shift in VDAC1 organization toward the oligomeric form[66] (see Fig. 11.5A and B). Apoptosis-mediated enhancement of VDAC1 oligomerization was obtained regardless of the cell type or apoptosis inducer used, including STS, curcumin, As_2O_3, etoposide, cisplatin, selenite, TNF-α, H_2O_2, or UV light, all affecting mitochondria yet acting via different mechanisms (Table 11.1). Moreover, correlation between the level of VDAC1 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 VDAC1 oligomerization and apoptosis.[61,66,112]

A VDAC1 oligomeric state was also directly monitored in living cells using BRET2 (bioluminescence resonance energy transfer) technology, where an enhanced BRET signal (three- to five-fold) was observed upon apoptosis induction[66] (Fig. 11.5C and D). In BRET2 technology, VDAC1 proteins are tagged with either *Renilla* luciferase (RLuc) as the donor or a variant of GFP protein (GFP2) as acceptor. Energy transfer occurs when the donor and acceptor are spatially close (<10 nm), making the technique ideal for monitoring protein–protein interactions in biological systems. Enhancement of the BRET2 signal corresponds to the activation of VDAC1 oligomerization, while attenuation of apoptosis-enhanced BRET2 signal indicates inhibition of VDAC1 oligomerization. Indeed, BRET2 technology is applicable for use with the well-defined apoptosis inducers, STS, As_2O_3, and sodium selenite. All these apoptosis inducers enhanced the BRET2 signal (GFP2/Rluc ratio) in rVDAC1–Rluc- and rVDAC1–GFP2-expressing cells, relative to cells not exposed to an apoptosis inducer (Fig. 11.5C).

In addition, it was demonstrated that cells expressing a VDAC1 dimeric fusion protein comprising wild-type and RuR-insensitive E72Q-mutated

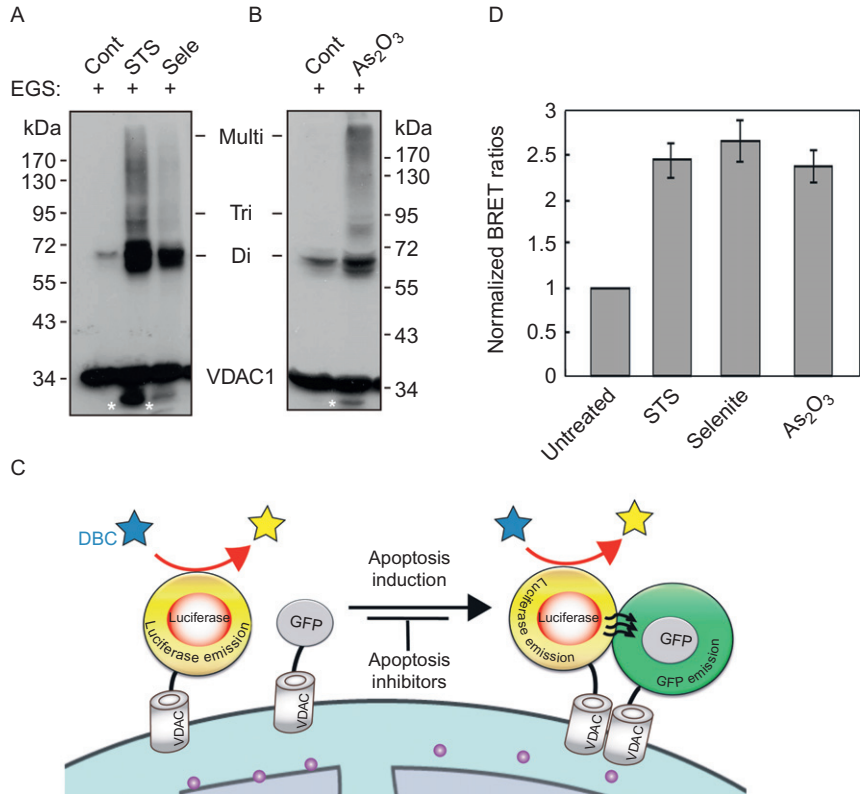

Figure 11.5 Apoptosis induction by STS, selenite, or As_2O_3 is associated with VDAC1 oligomerization. (A) and (B) Apoptosis induction triggers VDAC1 oligomerization. HeLa cells were exposed to the apoptosis-inducing reagent, STS (4 μM, 4 h), selenite (25 μM, 4 h), or As_2O_3 (30 μM, 18 h), washed twice with PBS and incubated (2.5 mg/ml) at 30 °C with 250 μM EGS for 15 min. The cells were then subjected to SDS-PAGE, followed by immunoblotting using anti-VDAC1 antibodies. VDAC1 monomers, dimers, trimers, tetramers, and multimers are indicated. The positions of molecular weight protein standards are provided. (C) Schematic representation of oligomerization detection by BRET2. Energy transfer between VDAC1–luciferase (Luc) (light-producing enzyme) as the donor and VDAC1–GFP2 (fluorophore) as the acceptor that occurs only when the donor and the acceptor are spatially close. The BRET signal is obtained when the two VDAC1 molecules interact physically. (D) The ratios of the BRET2 signals obtained in cells treated with STS (4 μM, 4 h), selenite (25 μM, 4 h), As_2O_3 (30 μM, 18 h), or with the relevant amount of DMSO are given. The shRNA-VDAC1 T-REx-293 cells were cotransfected with VDAC1–Rluc (0.2 μg) and rVDAC1–GFP2-encoding (0.8 μg) plasmids. As control, cells were transfected with the VDAC1–Rluc-encoding plasmid. Luciferase and GFP signals were measured following 48 h of transfection. BRET2 signals were calculated as described.[66] Luciferase activity was measured, following the addition of the substrate, DBC, as luminescence, while GFP fluorescence was measured at 510 nm. All readings were performed with an Infinite 200 ELISA reader.

Table 11.1 Summary of conditions and compounds inducing apoptosis and VDAC1 oligomerization

Agents/compounds inducing apoptosis and VDAC1 oligomerization	Conditions for induction and analysis	Refs.
UV irradiation	HEK-293 cells; UV exposure (15–60 s); EGS cross-linking and immunoblot (after 24 h)	66
TNF-α	HEK-293 cells;10–20 ng/ml TNF-α for 16–24 h; EGS cross-linking and immunoblot	66
Etoposide	HEK-293 cells; 2–5 μM etoposide for 16–24 h; EGS cross-linking and immunoblot	66
Staurosporin (STS)	HEK-293; HeLa, T47D, or A549 cells, 0.5–5 μM STS for 2.5–5 h or 0.1–0.2 μM STS for 16–24 h; EGS, DFDNB, or BMOE cross-linking and immunoblot or BRET	56,66,112–116
Cisplatin	HEK-293 cells; 40–60 μM cisplatin for 16–24 h; EGS cross-linking and immunoblot or BRET	66
Selenite	HEK-293 or HeLa cells; 8–10 μM selenite for 16–24 h; EGS cross-linking and immunoblot or BRET	66,112,115
H_2O_2	HEK-293 or HeLa cells; 0.5–1 mM H_2O_2 for 2–5 h; EGS cross-linking and immunoblot	66,112
Curcumin	HEK-293 cells; 40–60 μM curcumin for 16–24 h; EGS cross-linking and immunoblot	66
As_2O_3	HEK-293, HeLa, A549, or IM-9 cells; 2–30 μM As_2O_3 for 16–24 h; EGS or DSS cross-linking and immunoblot or BRET	66,105,115
VDAC1 overexpression	HEK-293 cells; 24–72 h transfection; EGS or BMOE cross-linking and immunoblot	56,66,116
Lipids, phosphatidyl glycerol	VDAC1 reconstituted into giant unilamellar vesicles, fluorescence cross-correlation spectroscopy	70,71

VDAC1 showed no protection by RuR against STS-induced apoptosis. This dominant-negative VDAC1 mutant reveals oligomeric VDAC1 to be the active unit in mitochondria-mediated apoptosis.[113]

Recently, using structural- and computational-based approaches, in combination with site-directed mutagenesis, cysteine replacement and chemical cross-linking, contact sites between VDAC1 molecules in dimers and higher oligomers were identified.[114] Dimeric VDAC1 with a contact site involving β-strands 1, 2, and 19 was identified. Moreover, the results suggest that VDAC1 exists as a dimer that undergoes conformational changes upon apoptosis induction to assemble into higher oligomeric states with contact sites also involving β-strands 8 and 16[114] (Fig. 11.6A).

The relationship between VDAC1 oligomerization and apoptosis is also reflected in studies attributing the apoptosis-inducing effect of As_2O_3 to an induction of VDAC1 homodimerization.[105] This effect was prevented by overexpression of the antiapoptotic protein, Bcl-xL.[105] Recently, hepatitis E virus ORF3 protein was found to upregulate VDAC1 expression, with ORF3-expressing cells showing higher levels of oligomeric VDAC1, as revealed by chemical cross-linking.[117] It was also demonstrated that VDAC1 oligomerization is encouraged in the presence of Cyto *c* encapsulated in VDAC1-reconstituted liposomes.[62]

6.2. VDAC1 N-terminal and the release of Cyto *c*

Previous studies suggest that the N-terminal region is not required for VDAC1 oligomerization but is important for Cyto *c* release and subsequent apoptosis.[56] The VDAC1 N-terminal region assumes a helical conformation (see Section 4.1.2.1). In the combined NMR/X-ray structure of human VDAC1, the helix comprises residues Tyr7 to Val17 and is folded horizontally inside the barrel wall (Fig. 11.6Ba).[45] Similar positioning is seen in the crystal structure of mouse VDAC1; however, here the helical region is formed by amino acids 6–20 and the hydrogen-bonding pattern is broken at Leu-10 and Gly-11, separating the helix into two segments.[47] On the other hand, residues 11–20 are difficult to observe in solution-state NMR, suggesting the dynamic behavior of this segment[45–47] (Fig. 11.6Bb). Increased mobility of the N-terminal region is provided by a glycine-rich domain,[49] connecting the N-terminal domain to β-strand 1. As proposed above, VDAC1 undergoes oligomerization upon apoptosis induction to form a large pore. The newly formed pore composed of VDAC1 β-barrels would form a hydrophobic channel that would not allow charged proteins, such as Cyto *c*, to pass. However, if the amphipathic N-terminal region of

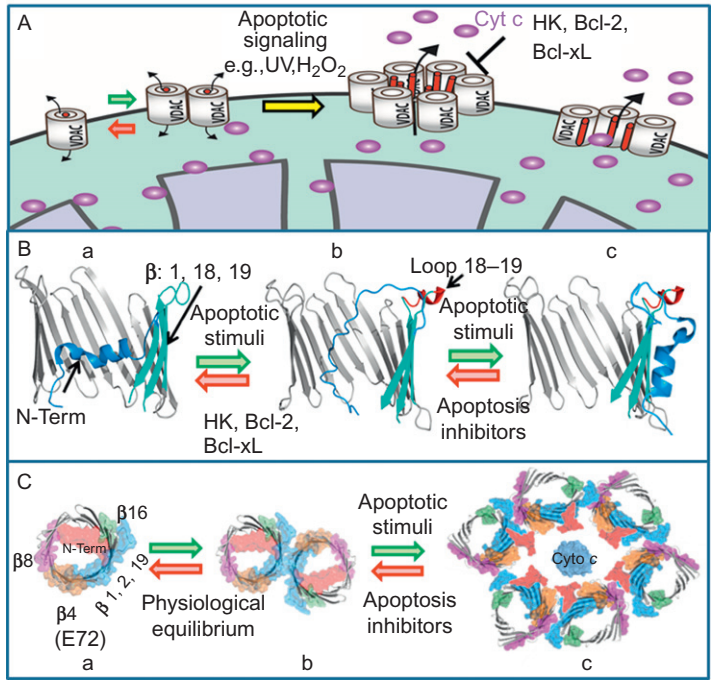

Figure 11.6 Proposed model of VDAC1 oligomerization. (A) A general overview of the VDAC1 oligomerization to form Cyto *c*-conducting channel as induced by apoptotic signaling. VDAC1 exists in equilibrium between the monomeric and dimeric states and is engaged in metabolite and ion exchange functions. Upon apoptosis induction, VDAC1 undergoes oligomerization concomitant with the translocation of the N-terminal domain (red rod) from the pore interior to the inside of a newly formed oligomeric VDAC1 pore (outside the channel). Because of its amphipathic characteristic, the N-terminal domain coats the hydrophobic oligomer pore with a hydrophilic surface, thus allowing Cyto *c* and other apoptogenic proteins to cross the OMM. HK and the anti-apoptotic proteins, Bcl-2 and Bcl-xL, inhibit this process. (B) VDAC1 N-terminal domain structure and location. (a) The crystal structure of VDAC1 (PDB ID: 3EMN) suggests a well-ordered N-terminal domain, interacting through its hydrophobic residues with the interior wall of the channel.[47] (b) The NMR-based structure of human VDAC1 (PDB ID: 2K4T)[46] reveals the unstructured conformation of the N-terminal that may exit the channel to interact with other proteins or to seek stability after apoptotic signaling induces changes in the channel. (c) Here, we propose that the N-terminal region confers stability by interacting with the closest unstable hydrophobic region of VDAC1 (β-strands 1, 2, 19). It should be noted that in the structure of human VDAC1, the loop between β-strands 18 and 19 is more helical, hinting at other changes in this area. Thus, the entire region of dimerization that is directly connected to the N-terminal region may be subjected to conformational activation to reprogram VDAC1 channel activity into a pore-forming unit that releases Cyto *c*. (C) Protein–protein interaction surfaces mapped on VDAC1. (a) Monomeric VDAC1 with the N-terminal domain (red) inside the pore. (b) Dimer formation involves β-sheets 1, 2, 19 (light blue). (c) Upon assembly of higher-order oligomers induced upon

(Continued)

each VDAC1 molecule is moved to line the external surface of the β-barrel, a hydrophilic pore would form (Fig. 11.6C). We propose that upon apoptosis induction, VDAC1 undergoes oligomerization, concomitant with the translocation of the N-terminal domain from the pore interior and into a newly formed oligomeric VDAC1 pore (Fig. 11.6C). This relocated N-terminal domain converts the hydrophobic pore to a pore that presents hydrophilic environment, thus allowing Cyto *c* and other apoptogenic proteins to cross the OMM.[49,56] The newly positioned N-terminal region is in close proximity to Glu72 and is also surrounded by other hydrophilic residues. This hydrophilic surface drives further oligomerization and the creation of a pore large enough to release Cyto *c* into the cytosol. Still, the exact molecular architecture of the Cyto *c*-conducting channel is yet to be determined.

6.3. Signaling mechanisms for induction of VDAC1 oligomerization

The precise mechanisms regulating Cyto *c* release remain unknown. Although substantial evidence support the formation of higher-order VDAC1-containing complexes upon apoptosis induction in cultured cells, the signaling mechanism responsible for induction of VDAC1 oligomerization is not yet known. The dynamic equilibrium that exists between monomers and oligomers can be influenced by intracellular Ca^{2+}, ROS, or ATP levels; by posttranslational modifications; and by associated proteins, such as HK-I or proteins from the Bcl-2 family.

Several studies have shown that Ca^{2+} is a mediator of cell death signaling and that anticancer drugs, such as thapsigargin, STS, selenite, cisplatin, and Ca^{2+} ionophores, induce apoptotic cell death and interfere with Ca^{2+} homeostasis.[118] In addition, mitochondrial Ca^{2+} is involved in apoptosis induction.[119] Ca^{2+} may mediate apoptosis via directly interacting with VDAC1 that mediates Ca^{2+} transport across the OMM and binds this cation.[76] However,

apoptosis induction, the N-terminal domain leaves the pore interior and interacts with the dimer surface (β-strands 1, 2, 19) adjacent to Glu72 and surrounded by other hydrophilic residues leading to dimer interface shifting to involve association of β-sheet 8 and 16 (purple and green, respectively). This newly formed hydrophilic surface drives further oligomerization with the amphipathic properties of the N-terminal domain leading to the formation of a pore large enough to allow release of Cyto *c* (light purple inside the pore) to the cytosolic space. β-Strand 4 (orange), that domain that harbors Glu72 and is associated with HK-, Bcl-xL- and Bcl-2-binding to VDAC1,[58,59,106] is in close proximity to the N-terminal domain.

mitochondrial Ca^{2+} can affect VDAC1 oligomerization via a different mechanism. Most of the Cyto *c* is associated with the anionic cardiolipin (CL), such that Cyto *c* dissociation from CL is a prerequisite step for release of the protein. This could be induced by CL peroxidation,[120] by Ca^{2+} interaction with CL,[121] or by tBid.[122] It was also demonstrated that VDAC1 oligomerization is encouraged in the presence of Cyto *c*.[62] Thus, VDAC1 oligomerization can be regulated through its Ca^{2+} transport activity or via binding of Ca^{2+}.[76]

VDAC1 oligomerization can be induced by H_2O_2,[61,66] implying that the dynamic equilibrium between VDAC1 monomers and oligomers can be influenced by ROS. DIDS, an inhibitor of VDAC1 channel activity, was found to inhibit $O_2^{-\cdot}$-induced apoptosis[103,104,123,124] and VDAC1 oligomerization,[66] suggesting that $O_2^{-\cdot}$ induces Cyto *c* release via VDAC1-dependent permeabilization of the OMM.[103] Moreover, $O_2^{-\cdot}$ was found to evoke Cyto *c* release in VDAC1-reconstituted liposomes.[103] The translocator protein, TSPO, appears to be involved in the generation of ROS, leading to apoptosis induction.[125] In addition, TSPO–VDAC1 interactions are proposed to play a role in apoptosis,[126–128] where the close association of TSPO with VDAC1 is proposed to enhance the concentration of ROS generated by TSPO, leading to apoptosis induction.[129–131] The mechanism by which ROS lead to VDAC1 oligomerization is not clear. ROS may act by inducing upregulation of VDAC1 expression[132] or via modification of amino acid residues affecting VDAC1 conformation, leading to its oligomerization.

7. VDAC1 OVEREXPRESSION LEADS TO OLIGOMERIZATION AND INDUCTION OF CELL DEATH

7.1. Overexpression of VDAC from different sources resulted in apoptotic cell death

It has been demonstrated that apoptosis can be induced by overexpression of human, murine, yeast, and rice VDAC (osVDAC4) regardless of the cells used or VDAC source.[107,133,134] Overexpression of murine or rat VDAC1 or VDAC1–GFP or E72Q–mVDAC1 in U-937 cells resulted in cell death (70–85%) characterized by nuclear fragmentation.[107] Similar results were obtained upon overexpression of rice VDAC in the Jurkat T-cell line.[133] Such cell death was blocked by Bcl-2 or DIDS, also shown to inhibit VDAC1 channel activity.[123,135] Overexpression of mVDAC1 in Neuro-2a cells also induced apoptosis.[136] Moreover, overexpression of *Paralichthys olivaceus* VDAC in fish cells induced apoptosis. These findings suggest that VDAC is a conserved mitochondrial element of the death machinery.

7.2. VDAC1 expression in cancers is enhanced by proapoptotic drugs

Several cancer treatments were found to enhance expression of VDAC1. Upregulation of VDAC1 expression was noted in three different acute lymphoblastic leukemia (ALL) cell lines (697, Sup-B15, and RS4;11) following prednisolone treatment, an observation that can be explored for predicting eventual outcome in childhood ALL.[137] VDAC1 overexpression was observed in a cisplatin-sensitive cervix squamous cell carcinoma cell line (A431) when exposed to cisplatin, while in a cisplatin-resistant cell line (A431/Pt), such treatment resulted in downregulation of VDAC1.[138] Upregulation of VDAC1 has been reported upon UV irradiation of apoptosis-sensitive cells.[139] In A375 human malignant melanoma cells, arbutin (hydroquinone-*O*-β-D-glucopyranoside), a tyrosinase inhibitor and a potential antitumor agent, was found to induce apoptosis by inducing VDAC1 overexpression.[140,141] In addition, ROS were found to induce upregulation of VDAC1 that could be prevented by the ROS chelator, epigallocatechin.[132]

The relationship between VDAC1 expression levels and sensitivity to various treatments was presented in several studies. The PC3 and DU145 prostate cancer cell lines are relatively resistant to apoptosis as induced by G3139 and were found to express less VDAC1 than did G3139-sensitive LNCaP cells.[142] Similarly, reducing VDAC1 expression by siRNA efficiently prevented cisplatin-induced apoptosis and Bax activation in non-small cell lung cancer,[143] attenuated endostatin-induced apoptosis,[111] and inhibited selenite-induced PTP opening.[144] The anticancer activity of furanonaphthoquinones was increased upon VDAC1 overexpression and decreased upon silencing of VDAC1 expression by siRNA.[124,145] It has also been shown that CD45 expression is accompanied by elevated VDAC1 synthesis in myeloma cells sensitized to a diverse set of apoptotic stimuli acting via the mitochondrial pathways.[146]

Hepatitis E virus ORF3 protein was also found to upregulate VDAC1 expression levels.[117] It is known that radiation can also induce the upregulation of VDAC1 in LYas cells[139] and that increased expression of VDAC1 is correlated with uterine epithelial apoptosis after estrogen deprivation.[147] Recently, it was demonstrated that increased expression of VDAC1 sensitizes carcinoma cells to apoptosis induced by cisplatin, mechlorethamine, and its derivative, melphalan.[148] Finally, HEV-infected human hepatoma cells showed enhanced VDAC1 expression, leading to VDAC1 oligomerization and apoptosis.[117] Thus, cell death induction by reagents, such as

arbutin, prednisolone, or cisplatin; by viral proteins; or by UV irradiation, as well as the correlation between drug effectiveness and VDAC1 expression, all suggest that the anticancer activities of these drugs and treatments are associated with VDAC1 levels. This idea is further supported by the finding that cisplatin-induced apoptosis is inhibited in cells silenced for VDAC1 expression.[144,149] In addition, endostatin-induced apoptosis was decreased upon silencing of VDAC1 expression and enhanced by overexpression of VDAC1.[111]

7.3. Proposed mechanism for cell death induction by VDAC1 overexpression

Taken together, the findings presented above suggest that the cellular expression level of VDAC1 is a crucial factor in the process of mitochondria-mediated apoptosis. We propose that the high levels of VDAC1 promote its oligomerization, leading to apoptosis.[61,66]

How can enhanced VDAC1 expression lead to cell death? It is possible that increased VDAC1 levels produce a significant increase in OMM leakage, in turn decreasing cell viability. Since cell death induced by VDAC1 overexpression is prevented by RuR,[107,108] Bcl-2, or DIDS,[133] all shown to interact with VDAC1, it seems that it is not a nonspecific increase in the OMM leakage but rather an increase in VDAC1 functionality that is responsible for apoptotic cell death. Moreover, cells overexpressing HK-I, shown to reduce VDAC1 channel conductance, are resistant to cell death induced by VDAC1 overexpression.[55,106,107] VDAC1 oligomerization was shown to be increased severalfold upon apoptosis induction.[66] Accordingly, an increase in VDAC1 oligomerization in cells overexpressing VDAC1 has been observed.[56] As presented in Section 6.1, dynamic VDAC1 oligomerization is involved in the release of Cyto *c* from mitochondria. As such, VDAC1 overexpression would encourage VDAC1 oligomerization and thus allow for release of proapoptotic proteins from the mitochondrial IMS and, subsequently, apoptosis.

8. VDAC1, BAX, AND BAK HETERO-OLIGOMERS MEDIATE Cyto *c* RELEASE

It has also been hypothesized that a protein-conducting channel may be formed within hetero-oligomers containing VDAC1 and the proapoptotic proteins, Bax or Bak.[91] Bax was demonstrated to increase the pore size of rat brain mitochondrial VDAC1 in the presence of tBid.[150] Similarly, in

PLBs, Bax and VDAC1, when combined, revealed single-channel conductance higher by factors of 4 and 10 over values obtained with VDAC1 and Bax alone, and that could be inhibited by Bcl-xL.[98] It has been shown that As_2O_3-induced Bax and VDAC1 oligomerization and the apoptosis-inducing effects of such rearrangements could be attributed to the induction of homodimerization of VDAC1 molecules.[151] In addition, injection of anti-VDAC1 antibodies into cells prevented Bax-induced Cyto *c* release, the interaction of Bax with VDAC1, and the triggering of cell death.[104] Independent evidence supporting a role for Bax–VDAC1 interaction in apoptosis was provided by the finding that HK-I and II can inhibit apoptosis by binding to VDAC1 and inhibiting VDAC1–Bax interaction.[152] It was further shown that Bax and VDAC1 can form a large pore that is permeable to Cyto *c*.[97,153] It was also proposed that oligomeric VDAC1, as the prime Cyto *c* release channel, is regulated by Bax.[154] In addition, Bax–VDAC1 complexes were identified in digitonin-solubilized cerebellar granule neurons.[155]

Bax–VDAC1 was found to form a protein complex in primary rat hepatocytes and in neonatal rat cerebellum upon acute ethanol exposure that induces oxidative stress and apoptosis.[156,157] Prevention of Bax–VDAC1 interactions by a microinjection of anti-VDAC1 antibodies effectively prevented hepatocyte apoptosis by ethanol.[156] In addition, cerebral ischemia in rat CNS resulted in elevated formation of Bax–VDAC1 heterodimers *in vivo* and in isolated brain mitochondria *in vitro*, with this triggering the release of Cyto *c* from isolated mitochondria.[158]

Interaction between VDAC2 and mitochondrial Bak in living cells was detected using a chemical cross-linking approach. This complex can be disrupted by tBid. VDAC2 thus inhibits BAK activation and mitochondrial apoptosis.[37] However, VDAC2 was reported as being required for tBID-induced mitochondrial apoptosis by recruiting newly synthesized BAK to the mitochondria.[159]

9. PROSPECTIVE

Mitochondrial apoptosis induction is known to effectively kill cells. Thus, one of the mechanisms by which chemotherapeutics destroy cancer cells is via the induction of apoptosis. Indeed, in recent years, it has become clear that a number of experimental chemotherapeutic agents directly affect mitochondria.[160]

The VDAC1 oligomeric assembly formed during apoptosis and possibly associated with the release of Cyto *c* and other apoptogenic factors, thus

inducing cell death, are not yet defined. Future directions should characterize the VDAC1 oligomeric states and their dynamics, as these represent a target for novel therapies that could stabilize VDAC1 homooligomers, leading to apoptosis.

As presented above, it was demonstrated that some anticancer agents select VDAC1–3 isoforms as their targets. Furthermore, some of these drugs increase VDAC1 expression via an unknown mechanism. We propose that this increased level of VDAC1 shifts the equilibrium toward oligomeric VDAC1, thus leading to Cyto *c* release and subsequent apoptosis. Modulating VDAC1 oligomerization can lay down the framework for a novel approach to developing therapeutics for cancers and neurodegenerative diseases. The BRET2 technology developed by our group allows screening for compounds that may stimulate or inhibit apoptosis. Oligomerization inhibitors are expected to inhibit apoptosis induction and can thus be used to combat neurodegenerative diseases, while oligomerization enhancers that induce apoptosis can be used in cancer therapy (Fig. 11.7). Such VDAC1-targeted strategy is superior to approaches that target specific events in apoptosis regulation and should be successful in dealing with the variety of cell phenotypes that arise in a population of transformed cells. In other words, we contend that VDAC1 oligomerization is such a general common

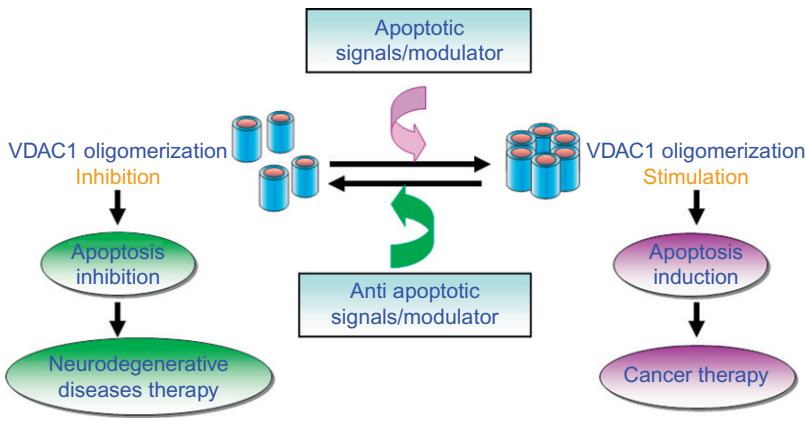

Figure 11.7 Modulation of VDAC1 oligomerization mediates apoptosis to offer novel cancer and neurodegenerative disease therapies. Targeting the VDAC1 oligomeric state provides a new strategy for selectively and directly manipulating apoptosis. This can serve as the basis for developing therapeutic applications for diseases in which apoptosis plays a significant role. Specifically, failure to undergo apoptosis in cancer and the increased apoptosis seen in neurodegenerative diseases would be countered.

denominator of mitochondria-mediated apoptosis that the chances of resistance to its disruption are very remote. As outlined above, the selectivity of this approach toward malignant cells is based on the overexpression of VDAC1 in cancer. Finally, interfering with VDAC1 oligomerization provides the means to overcome tumor chemoresistance and to lay down the foundations for more effective chemotherapy without or with fewer side effects.

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