Biochimica et Biophysica Acta 1787 (2009) 421–430

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Key regions of VDAC1 functioning in apoptosis induction and regulation by hexokinase

Varda Shoshan-Barmatz *, Miri Zakar [1], Keshet Rosenthal [1], Salah Abu-Hamad

Department of Life Sciences, Ben-Gurion University of the Negev, Beer-Sheva, 84105, Israel
The National Institute for Biotechnology in the Negev, Ben-Gurion University of the Negev, Beer-Sheva, 84105, Israel

ARTICLE INFO

Article history:
Received 10 October 2008
Received in revised form 14 November 2008
Accepted 17 November 2008
Available online 27 November 2008

Keywords:
Apoptosis
Clotrimazol
Hexokinase
Mitochondria
Peptide
VDAC

ABSTRACT

The voltage-dependent anion channel (VDAC), located in the mitochondrial outer membrane, functions as gatekeeper for the entry and exit of mitochondrial metabolites, and thus controls cross-talk between mitochondria and the cytosol. VDAC also serves as a site for the docking of cytosolic proteins, such as hexokinase, and is recognized as a key protein in mitochondria-mediated apoptosis. The role of VDAC in apoptosis has emerged from various studies showing its involvement in cytochrome *c* release and apoptotic cell death as well as its interaction with proteins regulating apoptosis, including the mitochondria-bound isoforms of hexokinase (HK-I, HK-II). Recently, the functional HK–VDAC association has shifted from being considered in a predominantly metabolic light to the recognition of its major impact on the regulation of apoptotic responsiveness of the cell. Here, we demonstrate that the HK–VDAC1 interaction can be disrupted by mutating VDAC1 and by VDAC1-based peptides, consequently leading to diminished HK anti-apoptotic activity, suggesting that disruption of HK binding to VDAC1 can decrease tumor cell survival. Indeed, understanding structure–function relationships of VDAC is critical for deciphering how this channel can perform such a variety of differing functions, all important for cell life and death. By expressing VDAC1 mutants and VDAC1-based peptides, we have identified VDAC1 amino acid residues and domains important for interaction with HK and protection against apoptosis. These include negatively- and positively-charged residues, some of which are located within β-strands of the protein. The N-terminal region of VDAC1 binds HK-I and prevents HK-mediated protection against apoptosis induced by STS, while expression of a VDAC N-terminal peptide detaches HK-I-GFP from mitochondria. These findings indicate that the interaction of HK with VDAC1 involves charged residues in several β-strands and in the N-terminal domain. Displacing HK, serving as the 'guardian of the mitochondrion', from its binding site on VDAC1 may thus be exploited as an approach to cancer therapy.

1. Introduction

It is now well established that mitochondria play a critical role in the regulation of apoptosis in mammalian cells by acting as reservoirs for apoptotic proteins. In response to various apoptotic stimuli, several apoptogenic factors, such as cytochrome *c* and Smac/DIABLO, are released from the intermembrane space of mitochondria into the cytoplasm, where they initiate the activation of downstream destructive programs, including the caspase cascade (reviewed in [1,2]). It remains unclear, however, how these apoptotic initiators cross the outer mitochondrial membrane (OMM) and are released into the cytosol. While some models predict that such release is facilitated by a swelling of the mitochondrial matrix and subsequent rupture of the OMM, other models suggest the formation of protein-conducting channels that are large enough to allow the passage of cytochrome *c* and other proteins into the cytosol, without compromising the integrity of the OMM [3]. The finding that cytochrome *c* can leak from intact mitochondria [4] supports these latter models. Accordingly, accumulating evidence indicates that the voltage-dependent anion channel (VDAC) is involved in the release of apoptogenic proteins via the OMM [5–12]. It should be noted, however, that multiple routes to apoptosis, including VDAC-independent pathways, exist [13].

VDAC lies at a crucial position in the cell, forming the main interface between the mitochondrial and cellular metabolisms. VDAC constitutes a major pathway by which metabolites, such as NAD^+/NADH, ADP/ATP, succinate, citrate, and ions, such as Ca^{2+}, are exchanged between the cytosol and mitochondria [9,14,15]. Although VDAC plays a major role in the physiological processes of solute and metabolite transport, it is also recognized as a key protein in

Abbreviations: HK, hexokinase; hVDAC, human VDAC; mVDAC, murine VDAC; RuR, ruthenium red; STS, staurosporine; VDAC, voltage-dependent anion channel

* Corresponding author. Department of Life Sciences, Ben-Gurion University of the Negev, Beer-Sheva, 84105, Israel.
E-mail address: vardasb@bgu.ac.il (V. Shoshan-Barmatz).

[1] These authors contributed equally to this work.

0005-2728/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2008.11.009

mitochondria-mediated apoptosis. Indeed, several recent reviews have focused on VDAC as an essential player in apoptosis as well as on the contribution of VDAC to the function of mitochondria in cell life and death [5–12]. VDAC, believed to participate in the release of cytochrome *c* and to interact with anti- and pro-apoptotic proteins, is considered a likely candidate for the OMM pore-forming protein [7,10,11,16,17]. In fact, various stimuli were shown to trigger apoptosis by modulation of VDAC, implicating VDAC1 as a component of the apoptosis machinery [10,12,16,18–20].

The role of VDAC in apoptosis also emerged from demonstrations of interactions between VDAC and proteins regulating apoptosis. In fact, the first such demonstration was documented 21 years ago in a study by Nakashima et al. [21] in which a form of hexokinase (HK), now known to be HK-II was shown to bind to VDAC in the OMM of a rapidly growing cancer cell line. Later many other studies demonstrated interactions of VDAC with the mitochondrial-bound isoforms of hexokinase, HK-I and HK-II and with proteins from the Bcl2 family [22–34]. In particular, mammalian HK-I and HK-II were shown to bind to the OMM via interacting with VDAC [10,11,22,23,26–28,34,35]. By binding to VDAC, HK gains direct access to the mitochondrial ATP pool for phosphorylation of glucose [36]. Indeed, one of the signature phenotypes of highly malignant, poorly differentiated tumors is their remarkable propensity to utilize glucose at a much higher rate than normal cells, a property frequently dependent on the marked over-expression of HK [37–39]. HK thus lies at the apex of the glycolytic pathway that provides those metabolic intermediates required by the biosynthetic pathways on which a transformed cell places such heavy demand [40].

Recently, however, the functional HK–VDAC association has shifted from being considered in a predominantly metabolic light to recognition of its major impact on the regulation of apoptotic responsiveness of the cell. Recent studies from our own and other groups have demonstrated that HK can inhibit apoptosis by binding to VDAC and preventing release of cytochrome *c* [11,22,23,26,28,34]. Hence, controlling both bioenergetics and cell death, the HK-mitochondria interaction has been considered as a target for anti-cancer drugs in recent years [11,40–43]. Two main classes of agents affect the HK-mitochondria association. The first class includes competitive and/or allosteric compounds, i.e. inhibitors of HK activity [11,43–46]. The second class includes compounds that compete with VDAC for HK binding, such as VDAC1-based peptides [41], or with HK for VDAC binding sites, such as the peptide representing the N-terminal hydrophobic domain of HK, shown to effectively displace HK from VDAC [24,28].

In this study, we provide insight into the function of VDAC1 in apoptosis and into the regulation of mitochondria-mediated apoptosis, while characterizing the interaction of HK with VDAC and characterizing the protection against apoptosis HK affords.

2. Materials and methods

2.1. Materials

Poly-D-lysine (PDL), propidium iodide and staurosporine (STS) came from Sigma (St. Louis, MO). Mito-Tracker red dye CMXPos was from Molecular Probes, Invitrogen (Eugene, OR). Monoclonal anti-VDAC antibodies raised to the N-terminal region of VDAC1 came from Calbiochem-Novobiochem (Nottingham, UK), while rabbit polyclonal anti-VDAC antibodies, prepared against amino acids 150–250 of hVDAC1, were from Abcam. Monoclonal anti-cytochrome *c* antibodies were obtained from BD Biosciences Pharmingen (San Jose, CA). Monoclonal anti-GFP antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-mouse antibodies were obtained from Promega (Madison, WI). Metafectene was purchased from Biotex (Munich, Germany). RPMI 1640 and DMEM growth media and the supplements, fetal calf serum (FCS), L-glutamine and penicillin-streptomycin, were purchased from Biological Industries (Beit Haemek, Israel). Blasticidin and Zeocin were purchased from InvivoGen (San Diego, CA). Puromycin was purchased from ICN Biomedicals (Eschwege, Germany).

2.2. Plasmids and site-directed mutagenesis

DNA encoding mVDAC1, the LP1, LP2, LP3 and LP4 peptides was generated and cloned into the tetracycline-inducible pcDNA4/TO vector (Invitrogen), as described previously [41]. The four VDAC1 loop-based peptides, LP1 to LP4, were flanked by a tryptophan zipper motif, i.e. the SWTWE amino acid sequence, at the N-terminus of the peptide and the KWTWK sequence at the C-terminus. The pcDNA3-HK-II and pcDNA3.1-HK-I plasmids were kindly provided by J.E. Wilson (Michigan State University). pEGFP-HK-I and pEGFP-HK-II, encoding GFP connected to the HK-I C-terminal, were generated as described [23].

2.3. Cell culture

MCF7 represents a human breast carcinoma cell line, HEK-293 cells correspond to a transformed primary human embryonal kidney cell line, T-REx-293 cells are tetracycline repressor-expressing HEK-293 cells and HeLa cells are derived from cervix cancer. hVDAC1-shRNA T-REx-293 were grown in Dulbecco's modified Eagle's medium (Biological Industries), supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, (all from Biological Industries) and maintained in a humidified atmosphere, at 37 °C with 5% CO_2. hVDAC1-shRNA T-REx-293 cells stably expressing the pSUPERretro vector encoding shRNA-targeting hVDAC1 were transfected with plasmids pcDNA4/TO-mVDAC1, grown as described [18] for 76 to 96 h prior to a 5 h exposure to STS (1.25 μM). Over-expression of mVDAC1- or Δ(26)mVDAC1-pcDNA4/TO was induced by tetracycline (2.5 μg/ml) for 96–112 h and cell viability was analyzed by acridine orange (AcOr) and ethidium bromide (EtBr) staining, as described previously [34]. HEK-T cells were transiently co-transfected with plasmids pEGFP-HK-I and pcDNA/4TO-mVDAC1, or plasmids encoding LP1, LP3 or LP4 peptides, grown for 48 h with tetracycline (1 μg/ml) and then visualized by confocal microscopy.

2.4. Confocal microscopy

HEK-T cells (3×10^5) were grown on PDL-coated coverslips in a 60 mm dish and transfected with pEGFP-HK-I or other plasmids, as indicated in figure or table legends. After 48 h, cells were stained with MitoTracker red dye (15 nM), washed with PBS, fixed for 20 min with 4% paraformaldehyde and rinsed with PBS prior to imaging by confocal microscopy (Olympus 1X81).

2.5. Apoptosis analysis

Apoptosis was analyzed by acridine orange/ethidium bromide staining [34] or by propidium iodide PI uptake and FACS analysis.

2.6. Protein purification and single-channel recording and analysis

mVDAC1 and E72Q-mVDAC1 were expressed in the *Saccharomyces cerevisiae* por1-mutant strain M22-2 [34] or purified from mitochondria [9]. HK-I was purified from rat brain mitochondria [22]. Reconstitution of purified native and E72Q-mVDAC1 into a planar lipid bilayer (PLB), channel recording, and analysis were carried out as described [47].

3. Results

It has been shown that over-expression of human, murine, yeast or rice VDAC1 induces apoptotic cell death, regardless of cell type [18,23,34,48]. On the other hand, the interaction of HK-I with VDAC and protection against cell death as induced by VDAC1 over-

Table 1
Over-expression of recombinant HK-I protects against apoptotic cell death as induced by over-expression of native but not of certain VDAC1 mutants

Experiment	Cells transfected to express:	Apoptotic cells (%)		Protection (%)
		−HK-I	+HK-I	
I	U-937 cells			
	Control	2.9 ± 0.7	3.1 ± 0.4	
	mVDAC1-GFP	73.1 ± 3.0	9.3 ± 3.1	91
	E65Q-mVDAC1-GFP	65.3 ± 4.2	71.1 ± 3.6	0
	E72Q-mVDAC1-GFP	80.0 ± 3.9	78.9 ± 6.3	0
	K73L-mVDAC1-GFP	69.4 ± 2.3	73.6 ± 1.3	0
	D77N-mVDAC1-GFP	72.5 ± 2.1	68.7 ± 2.4	0
II	T-REx-293 cells			
	Control	3.4 ± 0.6	4.1 ± 0.3	
	mVDAC1	74.5 ± 2.8	8.2 ± 2.6	93.4
	G67A-mVDAC1	71.8 ± 10.8	9.0 ± 1.2	91.9
	N75A-mVDAC1	74.6 ± 2.6	8.7 ± 2.9	92.7
	E202Q-mVDAC1	79.2 ± 1.6	66.0 ± 3.7	17.4
III	T-REx-293 cells			
	Control	9.8 ± 0.8	13.0 ± 0	
	mVDAC1	44.7 ± 1.7	10.5 ± 2.2	97
	K20S-mVDAC1	41.9 ± 2.2	37.0 ± 6.7	15.5
	G21A-mVDAC1	44.9 ± 0.9	45.6 ± 1.3	0

In experiment (I), U-937 cells were transfected with plasmid pcDNA3.1-HK-I and grown in the presence of 400 μg/ml neomycin, as described previously [23,34]. The cells were then transfected with pEGFP-N1, encoding native or the indicated mutated VDAC1 and grown in the presence of neomycin (400 μg/ml) for 76 h before analysis of apoptotic cells. In experiment (II), four samples of TREx-293 cell were transfected with pcDNA3.1-HK-I. 48 h later, the expression level of HK-I was analyzed by Western blot. The cell sample with the highest HK-I expression level was selected for a second transfection with pcDNA4/TO encoding native or the indicated mutated VDAC1. Transfected cells were grown for 96 h in the presence of tetracycline (2.5 μg/ml) to induce VDAC1 over-expression. Apoptotic cells were then analyzed. In experiment (III), T-REx-293 cells were co-transfected with pcDNA3.1-HK-I and pcDNA4/TO, encoding mVDAC1, K20S-mVDAC1, G21A-mVDAC1 or E72Q-mVDAC1. Co-transfected cells were grown for 112 h with tetracycline (2.5 μg/ml) and apoptotic cells were then analyzed. Apoptotic cell death was visualized by acridine orange/ethidium bromide staining. Quantitative analysis of apoptosis in the different cells was performed by ANOVA and *t* tests versus control cells. Data shown are the mean ± S.E.M ($n = 3$).

expression has been demonstrated in our previous studies [22,23,34,41].

Table 1 summarizes the effects of HK-I over-expression on apoptotic cell death induced by native and various mVDAC1 mutants. HK-I-mediated protection against apoptosis was analyzed by expression of native or mutated mVDAC1 in U-937 cell, T-REx-293 or hVDAC1-shRNA T-REx-293 cells, the latter resenting cells suppressed for endogenous VDAC1 expression [18], as well as in the same cell lines over-expressing HK-I. HK-I elicited protection against apoptosis induced by over-expression of native and some, but not other VDAC1 mutants. The latter group includes VDAC1 containing mutations at glutamates 65 and 72, aspartate 77, lysine 73, as well as glutamates 188 and 202. The locations of the various mutations in the VDAC1 sequences proposed to be involved in HK interaction are depicted in the previously proposed [15,49] and recently presented [50–52] VDAC1 structural models shown in Fig. 1.

The inability of HK-I to interact with E72Q-VDAC1 was also demonstrated by analyzing the effects of HK-I on the conductance of E72Q-mVDAC1 reconstituted into a planar lipid bilayer (PLB) (Fig. 2). Purified recombinant native and E72Q-mVDAC1 were PLB-reconstituted and channel conductance, before and after addition of purified HK-I were recorded, as a function of the voltage applied (Fig. 2). Upon addition of HK-I to native mVDAC1, channel conductance was reduced at all voltages tested. On the other hand, HK-I had no effect on the conductance of E72Q-mVDAC1. These results suggest that E72 is essential for HK-I interaction with VDAC1 and/or modifying its conductance.

To demonstrate that HK-I does not interact with other VDAC1 mutants, we co-expressed E72Q- or E188Q-mVDAC1 and a HK-I-GFP fusion protein in HEK-T cells. Confocal fluorescence microscopy showed that HK-I-GFP fluorescence is punctuated in cells transiently expressing mVDAC1(Fig. 3). On the other hand, HK-I-GFP fluorescence in cells expressing either E72Q- or E188Q-mVDAC1 was diffused throughout the cytosol. These results indicate that HK-I does not bind to the mutated VDAC1, in agreement with HK inability to confer protection against cell death (Table 1).

Based on these and previously published results [23,34,41], we defined the domains and amino acid residues involved in stabilizing VDAC1 conformation interacting with HK-I (Fig. 1). These VDAC1 regions were expressed in HEK-T cells in the form of the loop-shaped peptides, LP1W, LP2W and LP4W (Fig. 4A), and their effects on the HK-I-GFP interaction with mitochondria were analyzed (Fig. 4B). Peptide LP3W served as a control peptide since it did not interact with HK-I and had no effect on HK-I-mediated protection against apoptosis [41]. Fig. 4B demonstrates that the peptides can modify HK-I-GFP cellular distribution by detaching mitochondria-bound HK-I. Confocal fluorescence microscopy showed that in control cells expressing HK-I-GFP, the fluorescence is punctuated, as expected for a mitochondrial distribution. On the other hand, HK-I-GFP fluorescence in cells expressing the LP1, to lesser extent the LP2 or LP4 peptides (but not peptide LP3) was diffused throughout the cytosol (Fig. 4B). These results indicate that the expressed VDAC1-based peptide detach or prevent HK-I binding to the mitochondria.

The function of the N-terminal region of VDAC1 in the interaction with HK-I and cell death was demonstrated using an N-terminal truncated version of VDAC1 [Δ(26)VDAC1]. As shown in Fig. 5, upon transfection of HEK-293, MCF7 or HeLa cells with mVDAC1, about 10–15% of the cells underwent apoptosis by the third day. This level was increased to 50% by day five (Fig. 5A), due to VDAC1 overexpression (Fig. 5C) [8,10,18,23,34]. Apoptotic cell death was reflected as enhanced nuclear fragmentation, visualized by acridine orange/ethidium bromide staining (Fig. 5B). By contrast, cells expressing Δ(26)mVDAC1 showed 8–15% apoptosis by day 3, a level that did not change by the fifth day of expression. It should be mentioned that these cells express endogenous hVDAC1, and although this VDAC represents about 40% and the N-terminal-truncated mVDAC1 60% of the VDAC pool (Fig. 5C), the effect of the latter is dominant, suggesting that the mutant displays a dominant negative effect (see also [53]).

Finally, not only VDAC1-based peptides can detach HK-I-GFP from its binding site in VDAC, but also chemicals such as clotrimazole (CTM), an anti-fungal azole derivative that, at high concentrations (e.g. 20 μM) has been shown to dissociate HK-II from mitochondria in several cell types [24,28]. Here, we demonstrate the ability of CTM to detach mitochondria-bound HK-I-GFP. The punctuated HK-I-GFP fluorescence, co-localized with MitoTracker, a dye that specifically labels mitochondria in living cells, was converted to diffuse labeling of the cytosol and did not co-localize with MitoTracker in the presence of CTM (Fig. 6A). CTM also induced cell death (Fig. 6B).

4. Discussion

In this study, we addressed the function of VDAC1 in apoptotic cell death and in the regulation of apoptosis via interaction with anti-apoptotic proteins, in particular HK. We and others have explored the N-terminal of VDAC1 as a key element in apoptosis induction and addressed its involvement in apoptosis regulation by HK and Bcl2 [30,53,54] (and Fig. 6). This discussion will thus focus on the HK–VDAC interaction, an interaction sufficient to allow cells to evade apoptosis. Indeed, the notion that the abundant expression of HK-I and HK-II in most cancer cells occurs only to ensure energy supplies has changed, with HK having been defined as an important regulator of mitochondrial function in both normal and cancer cells [40,43]. Hence, HK has become known as the 'guardian of mitochondria' and as such, displacing HK from its binding site in VDAC1 may be exploited as a cancer therapy approach.

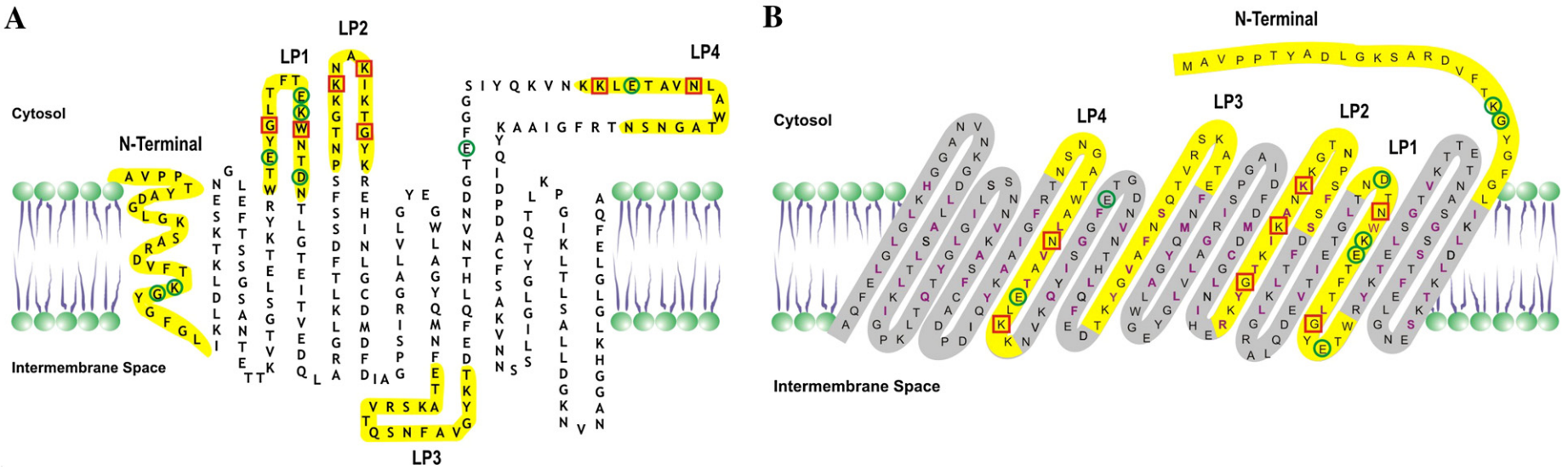

Fig. 1. VDAC1 transmembrane topology models, previously proposed (A) and recently demonstrated (B), highlighting the amino acids mutated and the sequences used for peptides expression. The amino acids mutated in the N-terminus and other domains of VDAC1 are labeled in the previously proposed transmembrane topology (A) [15] and in the newly established structure (B) [50–52]. Green circled amino acids indicate that mutation of these amino acids eliminates HK-I-mediated protection against cell death. Amino acids indicated in red squares, when mutated, had no effect on HK-I-mediated protection against apoptosis. The positions of the sequences of the five peptides (yellow-labeled), expressed as peptides and tested in this work, are illustrated.

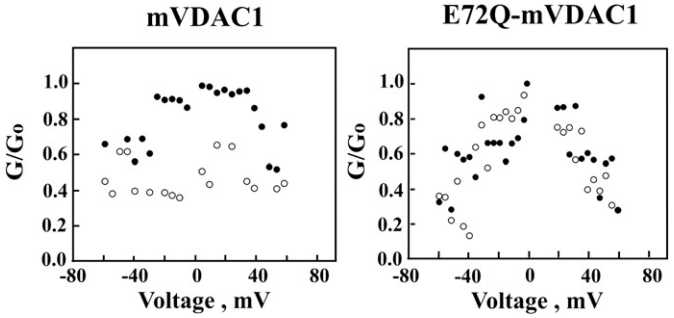

Fig. 2. Hexokinase-based modification of channel conductance of planar lipid bilayer-reconstituted native and mutated VDAC. Multi-channel recordings of the average steady-state conductance of native mVDAC1 or E72Q-mVDAC1 before (closed circles) and 10 min after the addition of HK-I (28.6 mU/ml) (open circles) are shown as a function of voltage. Relative conductance was determined as the ratio of conductance at a given voltage (G) to the maximal conductance (Go). The experiments shown are representative of 4–5 similar experiments.

4.1. VDAC1 domains interacting with HK-I and HK-II

The VDAC1 amino acid residues and domains involved in the interaction with HK-I or HK-II have recently been identified [23,34,41,53]. Certain point mutations in VDAC1 prevented HK-I binding, resulting in HK-I being no longer able to prevent cytochrome *c* release and subsequent apoptosis in cells over-expressing such VDAC1 mutants ([23,34] and Table 1). These mutations include the charged glutamate residues 65 and 72, aspartate 77 and lysine 73, located in the same sequence region, and glutamate 188 and 202 found in another VDAC1 region. According to previously proposed VDAC1 membrane topology models [15,49,55], most of the mutated amino acids listed above were predicated to be exposed to the cytosol. However, according to recently described NMR-based [50,52] and X-ray crystallography-based [51] VDAC1 structure, some but not all of these mutations are located in cytosolic-facing sequences (Fig. 1). The E72Q mutation that interrupts the interaction between VDAC1 and HK was also found to induce increases in mitochondrial swelling and OXPHOS activity, as well as to reduce in mitochondrial membrane cholesterol levels [56].

The N-terminal portion of VDAC1 has been shown to interact with HK-I, as channel conductance of N-terminal truncated VDAC1 was not modified by HK-I [53]. Accordingly, a synthetic peptide corresponding to the N-terminal region of VDAC1 both binds HK-I and prevents HK-I- and HK-II-mediated protection against apoptosis induced by STS [41,53]. Expression of the N-terminal peptide was found to detach HK-I-GFP from mitochondria, supporting the interaction of HK-I with the N-terminal region of VDAC1 [41]. These findings indicate that the interaction of HK with VDAC1 involves charged residues in several β-strands and the N-terminal domain. Recent VDAC1 structural analysis suggests a conformational cross-talk between Glut 72 (Glu-73 when counting the first Met) and the N-terminal part of the VDAC1 [50]. By hydrogen/deuterium exchange coupled to NMR spectroscopy, it has been shown that the N-terminal portion of hVDAC1 (residues in β1–β4) possesses conformational instability and switches between different VDAC1 conformations [50]. Interestingly, the structural instability of the N-terminal part was found to be particularly influenced by Glu-73 [50], which is required for HK-I- and HK-II-mediated modification of channel conductance (Fig. 2 and [23,34]). According to the recently established hVDAC1 3D-structure, Glu-73 points to the membrane to interrupt the amphipathic pattern in strand β4. Mutations that replace the charged Glu-73 with glutamine [23,34] or valine [50] resulted in stabilization of the β1–β4 strands [50]. Such mutations abolished both ruthenium red (RuR)- and HK-I-mediated inhibition of VDAC channel activity and RuR- and HK-I-mediated protection against apoptosis [23,34,57]. Thus, in accord with a recent suggestion [50], the conformational flexibility of the β-strands (β1–β4) is, in addition to the N-terminal region, important for voltage gating and for the interaction of VDAC1 with HK and RuR, both *in vitro* and *in vivo*.

It has been shown that HK interacts with the mitochondria through its N-terminal 12 amino acid hydrophobic sequence (MIAAQLLAYYFT) [28,58,59]. HK might interact thorough its N-terminal region with the amphipatic α-helix N-terminal portion of VDAC1.

On the other hand, the findings presented here indicate that the interaction of HK with VDAC1 involves charged residues. As a highly hydrophobic sequence, the N-terminal of HK cannot directly engage essential charged VDAC1 residues. VDAC1 charged residues may thus be involved in stabilizing the HK-binding VDAC1 conformation. However, the interaction of synthetic VDAC1-based peptides with HK ([41] and Fig. 4), suggests that sequences within these domains are involved in the interaction of VDAC1 with HK. This suggests that HK interacts with several domains of VDAC1, which may explain the detachment of HK by various compounds (see below).

The VDAC1–HK interaction can also apparently be regulated by Glu-6-P and multiple protein kinases (e.g. glycogen synthase kinase (GSK)-3beta [27] and protein kinase C (PKC)), resulting in phosphorylation of VDAC itself or of HK [60]. It was also recently shown that over-expression of HK stimulated VDAC phosphorylation through a PKCε-dependent pathway [61]. It should be noted, however, that VDAC phosphorylation has not been detected by others [62].

4.2. Possible mechanisms by which the detachment of mitochondria-bound HK could lead to cell death

The balance between cytosolic HK-I and mitochondria-bound HK is important in the regulation of glycolysis, depending on physiological conditions and variations in intracellular distribution that could serve as a regulatory mechanism [63,64]. The association between increased HK expression and cell survival is well demonstrated [22,23,28,34,41,64,65]. Several mechanisms have been

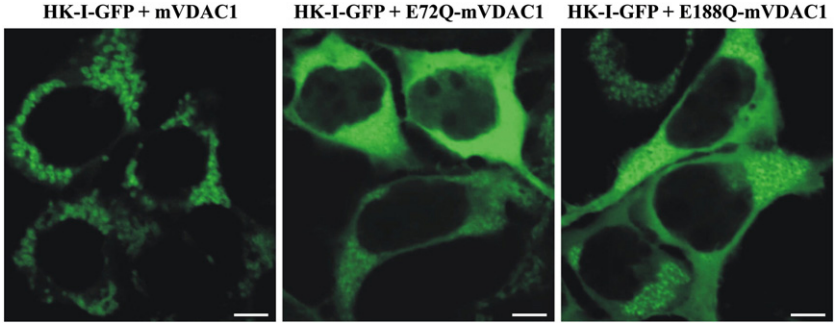

Fig. 3. HK-I-GFP imaging of cells expressing native or mutated VDAC1. HEK-T cells were transiently transfected to express HK-I-GFP alone or co-transfected with plasmid pcDNA/4To, encoding E188Q-VDAC1, E72Q-VDAC1 or mVDAC1 and visualized after 30 h using a confocal microscope (scale bar = 5 μm). Images are representative microscopic fields from one of three similar experiments.

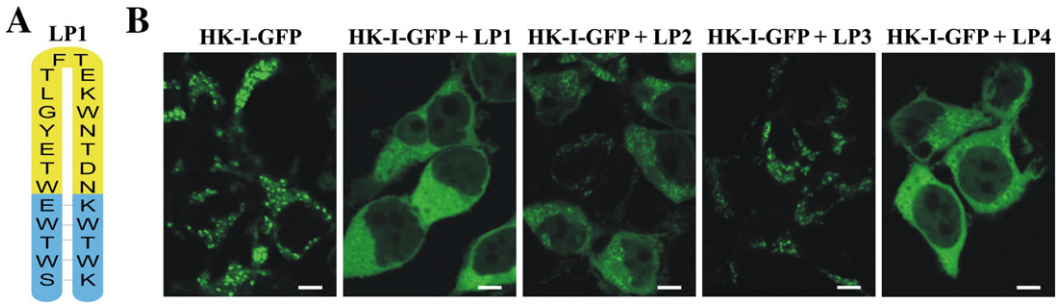

Fig. 4. Detachment of mitochondria-bound HK-I-GFP by VDAC1-based peptides. (A) Schematic presentation of a representative VDAC1-derived peptide (LP1) flanked with a tryptophan zipper, as formed by introducing the tryptophan zipper motif, i.e. the SWTWE amino acid sequence at the N-terminal of the peptide and KWTWK at the C-terminal [41]. These sequences induce the formation of a stable β-hairpin and thus, mimic a VDAC1 loop found in the native protein. (B) HEK-T cells (3×10^5) were transfected using CaPi to express HK-I-GFP alone or with plasmid pcDNA/4To encoding peptide LP1, LP2, LP3 or LP4. After 48 h, cells were visualized using a confocal microscope. Images are representative microscopic fields from one of three similar experiments (scale bar = 5 μm).

proposed for how HK-I and HK-II promote tumor cell survival and anti-apoptotic defense, all involving interaction with VDAC1. HK bound to VDAC on the mitochondrial surface provides both a metabolic benefit and apoptosis-suppressive capacity that gives the cell a growth advantage and increases its resistance to chemotherapy [40]. Mitochondria-bound HK-I and HK-II have direct access to mitochondrial sources of ATP and greater affinity for MgATP supplied by mitochondria [66,67]. In addition, HK bound to the cytosolic face of VDAC acts as a gate, regulating channel ion conductivity (Fig. 2 and [22]). Moreover, mitochondria-bound HK-I is also less sensitive to inhibition by Glu-6-P (CI_{50} of 5 mM, compared with 0.1 mM for the soluble form of the enzyme) [22]. Thus, disruption of the HK–VDAC interaction should interfere with the energy balance of highly demanding cancer cells.

HK-I and HK-II over-expression in cells growing in tissue culture protects against cell death [22,23,27,34,41,68]. The anti-apoptotic effect of HK is mediated through its binding to VDAC, preventing cytochrome *c* release by closing the mitochondrial release pore [22] or/and by preventing Bax translocation to the mitochondria and subsequent apoptosis [28]. Moreover, it has been shown that dissociation of HK from mitochondria induces cytochrome *c* release and apoptosis, even in cells deficient for both Bak and Bax [24]. Thus, if, as suggested, HK–VDAC interaction lies at the heart of apoptosis regulation by HK, interfering with HK binding to VDAC could form the basis for novel cancer treatment.

The mechanism(s) by which HK dissociation from mitochondria leads to apoptosis is not clear. Several possible mechanisms may be considered. As presented above, detachment of mitochondria-bound HK affects mitochondrial and overall cellular bioenergetics. Indeed, agents known to detach mitochondrial HK also induce ATP depletion preceding the decrease in cell viability [69,70]. Another possible mechanism involves the high sensitivity of cytosolic HK to

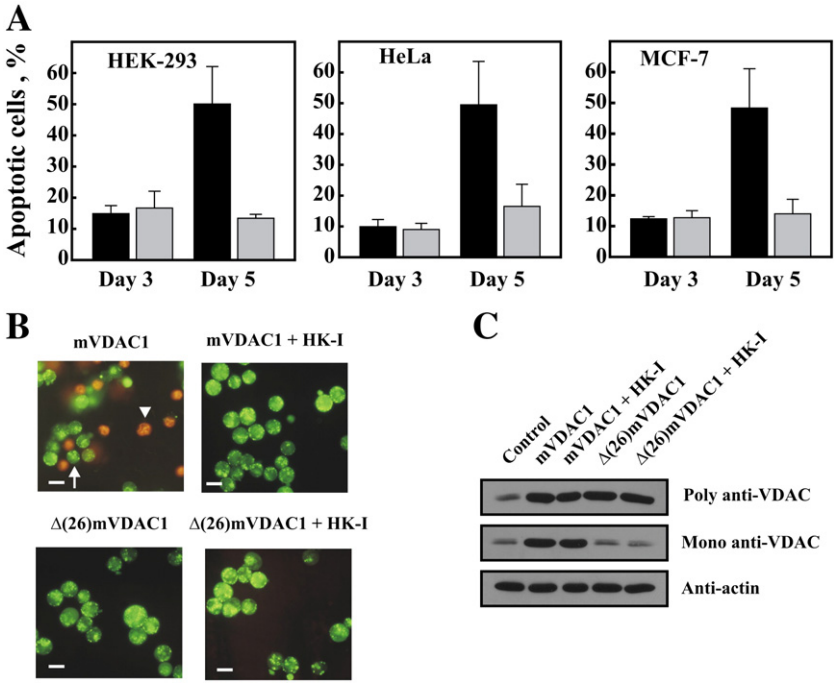

Fig. 5. The N-terminal region of VDAC1 is required for apoptotic cell death and HK-I anti-apoptotic effect. (A) HEK-293, HeLa and MCF-7 cells were transfected to express mVDAC1 (black columns) or Δ(26)mVDAC1 (grey columns), with their over-expression being induced by 2.5 μg/ml tetracycline. Cell viability was determined on day three and on the fifth day, as visualized by acridine orange/ethidium bromide staining. Quantitative analysis of apoptosis in the different treatments was performed by ANOVA and *t* test; $p<0.01$. Data are means ± S.E.M. ($n=4$). (B) Apoptotic cell death visualized by acridine orange/ethidium bromide staining. Arrow indicates cells in an early apoptotic state, reflected by degraded nuclei (stained with acridine orange). Arrow head indicates the late apoptotic state (stained with acridine orange and ethidium bromide) (scale bar = 15 μm). (C) Western blot analysis of VDAC levels in control cells and cells transfected to express mVDAC1 or Δ(26)mVDAC1 (30 μg) using polyclonal and monoclonal anti-VDAC antibodies. For loading controls, actin levels in the samples (15 μg) were compared, using anti-actin antibodies.

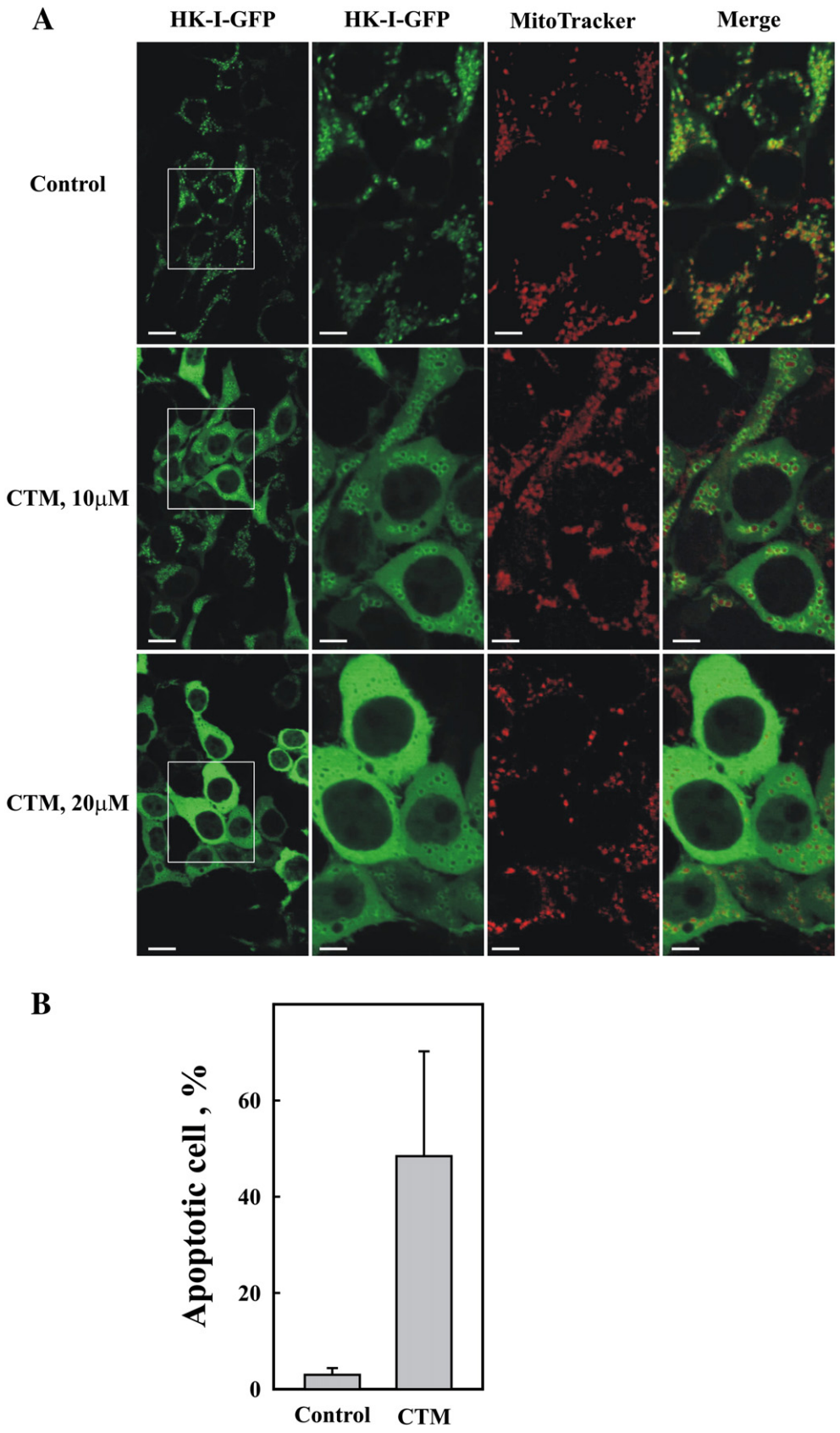

Fig. 6. Clotrimazole (CTM) detaches mitochondria-bound HK-I-GFP. (A) HEK-T cells (3×10^5) were transfected to express HK-I-GFP and after 48 h, were exposed for 2 h to the indicated concentrations of CTM, treated with MitoTracker, as described in Materials and methods, and visualized using a confocal microscope (scale bars = 12.5 μm for the left column and 5 μm for the other columns). The right panel shows the merged images of MitoTracker and HK-I-GFP. Images are representative microscopic fields from one of three similar experiments. (B) HEK-T cells were treated with 20 μM CTM for 11 h or with 0.1% DMSO for control cells in a medium containing 1% FCS. Cell viability was detected by PI uptake and analyzed by FACS.

its product, Glu-6-P. Inhibiting HK activity by Glu-6-P would thus reduce energy production for high energy-demanding cancer cells. In addition, HK-I was shown to inhibit PTP opening [22,71], while Glu-6-P favored PTP opening [22,68], presumably by detaching HK from its binding site [22,46]. Thus, high levels of Glu-6-P not only inhibit HK catalytic activity but also promote PTP opening, and thereby, induction of apoptosis. In addition, as pointed out above, HK-I and HK-II binding to VDAC is decreased by high concentrations of Glu-6-phosphate, leading to their release, thereby allowing for activation of apoptosis [22,72,73].

Another possible mechanism involves the regulation of ROS production by HK. 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 [74]. Expression of HK-II was found to protect against oxidant-induced cell death [75], while HK-I over-expression in an established epithelial cell line leads to protection against oxidation-induced cell death [76]. It was also demonstrated that HK-I and HK-II reduce intracellular levels of ROS [61]. In addition, while glucose increased the rate of oxygen consumption and reduced the rate of H_2O_2 generation, Glu-6-P increased H_2O_2 generation [74]. Thus, over-expression of mitochondria-bound HKI and HKII protects cells against damage from oxidative stress. Accordingly, detachment of HK from its binding site in the mitochondria could lead to increased H_2O_2 generation, thereby activating cell death. These findings suggest that mitochondrial HK activity is pivotal for the physiological functions of mitochondria.

Another possible mechanism for cell death induced by 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 Bcl2-family proteins, such as Bax [28]. One of the proposed models by which Bax would mediate cytochrome *c* release from mitochondria involves the formation of a Bax/VDAC complex [77,78]. It has been shown that VDAC-bound HK renders cells much more resistant to activation of apoptosis by Bax or Bak [28,79]. It has also suggested that Bax and Bid may promote apoptosis by displacing HK from VDAC [28,79]. For this activity, the C-terminal of Bax is required since Bax-ΔC was unable to displace HK or liberate cytochrome *c* from the reconstituted HK–VDAC–ANT (adenine nucleotide translocase) complex [80]. 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, the results presented here strongly suggest that mitochondria-bound HK plays a critical role in mammalian mitochondrial-dependent apoptosis.

4.3. Disruption of the HK–VDAC interaction as an approach to cancer therapy

One of the major hallmarks of tumor cells is their relative resistance against cell death, owing to over-expression of anti-apoptotic proteins of the Bcl2 family and HK [38,39,81–83]. As discussed above, it has been shown that a HK–VDAC1 interaction prevents induction of apoptosis in tumor-derived cells [22,23,26,28,34,41,58]. Thus, promoting detachment of HK from VDAC may be a promising cancer therapy strategy. Several compounds have been shown to induce dissociation of HK from the mitochondria and apoptosis. These include peptides corresponding to the amino terminus of both HK-I [58] and HK-II [28], clotrimazole ([28,84] and Fig. 6), 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) [79], methyl jesmonate [85] and VDAC1-based peptides ([41] and Fig. 4). The VDAC1-based peptides interacted with purified HK-I, detached HK bound to mitochondria isolated from tumor cells and in cells displaced mitochondria-bound HK-I-GFP ([41] and Fig. 4). 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 and enhance the synergistic therapeutic efficacy of conventional chemotherapeutic agents and reducing dose-limiting toxicity.

HK-inhibiting compounds, including glucose metabolites, 2-deoxyglucose, oxamate and 3-bromopyruvate, selectively induce apoptosis in cancer cells and in pre-clinical models of cancer showed selective toxicity against tumor cells metabolizing anaerobically [43,86–88]. These agents act to kill cancer cells by interacting with HK, however, whether these compounds detach HK from its binding sites in the mitochondria is still not clear. It is, nonetheless, proposed that these drugs are likely to enhance the efficacy of the current standard cancer chemotherapeutics and radiation regimens.

Recently [85], we have shown that a plant stress hormone of the jasmonate family, methyl jasmonate (MJ), binds to HK in a specific manner, leading to its detachment from mitochondria isolated from several cancer cell types. The susceptibility of cancer cells to jasmonates is dependent on the expression of HK. This finding, thus, provides an explanation for the selective effects of jasmonates on cancer cells. It should be noted that intra-arterial injection of 3-bromopyruvate, an inhibitor of mitochondria-bound HK, into tumors implanted in rabbit liver killed up to 90% of the tumor cells without significant damage to the surrounding healthy tissue [86].

To conclude, this and other studies discussed here strongly indicate that HK and VDAC are key components in the regulation of apoptosis and that the protective effects of HK against cell death are mediated via its direct interaction with VDAC. Furthermore, these results suggest that HK over-expression in cancer cells not only assures an adequate supply of energy, but also corresponds to an anti-apoptotic defense mechanism. Accordingly, interfering with HK binding to VDAC can serve to guide development of a new selective approach for cancer therapy.

Acknowledgements

This research was supported, in part, by grants from the Israel Science Foundation and the Israel Cancer Association. We would like to thank Dr. Adrian Israelson for providing Fig. 2 and Doron Calo for Fig. 5A.

References

[1] D.R. Green, J.C. Reed, Mitochondria and apoptosis, Science 281 (1998) 1309–1312.
[2] Y.L. Ow, D.R. Green, Z. Hao, T.W. Mak, Cytochrome *c*: functions beyond respiration, Nat. Rev. Mol. Cell Biol. 9 (2008) 532–542.
[3] J.C. Martinou, S. Desagher, B. Antonsson, Cytochrome *c* release from mitochondria: all or nothing, Nat. Cell Biol. 2 (2000) E41–E43.
[4] E. Doran, A.P. Halestrap, Cytochrome *c* release from isolated rat liver mitochondria can occur independently of outer-membrane rupture: possible role of contact sites, Biochem. J. 348 (2000) 343–350.
[5] D.J. Granville, R.A. Gottlieb, The mitochondrial voltage-dependent anion channel (VDAC) as a therapeutic target for initiating cell death, Curr. Med. Chem. 10 (2003) 1527–1533.
[6] M. Le Bras, M.V. Clement, S. Pervaiz, C. Brenner, Reactive oxygen species and the mitochondrial signaling pathway of cell death, Histol. Histopathol. 20 (2005) 205–219.
[7] J.J. Lemasters, E. Holmuhamedov, Voltage-dependent anion channel (VDAC) as mitochondrial governator—thinking outside the box, Biochim. Biophys. Acta 1762 (2006) 181–190.
[8] V. Shoshan-Barmatz, N. Arbel, L. Arzoine, VDAC, the voltage-dependent anion channel: function, regulation and mitochondrial signaling in cell life and death, Cell Science Reviews 4 (2008) 74–118.
[9] V. Shoshan-Barmatz, D. Gincel, The voltage-dependent anion channel: characterization, modulation, and role in mitochondrial function in cell life and death, Cell Biochem. Biophys. 39 (2003) 279–292.
[10] V. Shoshan-Barmatz, A. Israelson, D. Brdiczka, S.S. Sheu, The voltage-dependent anion channel (VDAC): function in intracellular signalling, cell life and cell death, Curr. Pharm. Des. 12 (2006) 2249–2270.
[11] V. Shoshan-Barmatz, N. Keinan, H. Zaid, Uncovering the role of VDAC in the regulation of cell life and death, J. Bioenerg. Biomembr. 40 (2008) 183–191.
[12] Y. Tsujimoto, S. Shimizu, The voltage-dependent anion channel: an essential player in apoptosis, Biochimie 84 (2002) 187–193.

[13] C.P. Baines, R.A. Kaiser, T. Sheiko, W.J. Craigen, J.D. Molkentin, Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death, Nat. Cell Biol. 9 (2007) 550–555.
[14] R. Benz, Permeation of hydrophilic solutes through mitochondrial outer membranes: review on mitochondrial porins, Biochim. Biophys. Acta 1197 (1994) 167–196.
[15] M. Colombini, VDAC: the channel at the interface between mitochondria and the cytosol, Mol. Cell Biochem. 256–257 (2004) 107–115.
[16] M. Madesh, G. Hajnoczky, VDAC-dependent permeabilization of the outer mitochondrial membrane by superoxide induces rapid and massive cytochrome *c* release, J. Cell Biol. 155 (2001) 1003–1015.
[17] N. Zamzami, G. Kroemer, Apoptosis: mitochondrial membrane permeabilization—the (w)hole story? Curr. Biol. 13 (2003) R71–73.
[18] S. Abu-Hamad, S. Sivan, V. Shoshan-Barmatz, The expression level of the voltage-dependent anion channel controls life and death of the cell, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 5787–5792.
[19] N. Tajeddine, L. Galluzzi, O. Kepp, E. Hangen, E. Morselli, L. Senovilla, N. Araujo, G. Pinna, N. Larochette, N. Zamzami, N. Modjtahedi, A. Harel-Bellan, G. Kroemer, Hierarchical involvement of Bak, VDAC1 and Bax in cisplatin-induced cell death, Oncogene 27 (2008) 4221–4232.
[20] Y. Zheng, Y. Shi, C. Tian, C. Jiang, H. Jin, J. Chen, A. Almasan, H. Tang, Q. Chen, Essential role of the voltage-dependent anion channel (VDAC) in mitochondrial permeability transition pore opening and cytochrome *c* release induced by arsenic trioxide, Oncogene 23 (2004) 1239–1247.
[21] R.A. Nakashima, P.S. Mangan, M. Colombini, P.L. Pedersen, Hexokinase receptor complex in hepatoma mitochondria: evidence from N,N′-dicyclohexylcarbodiimide-labeling studies for the involvement of the pore-forming protein VDAC, Biochemistry 25 (1986) 1015–1021.
[22] H. Azoulay-Zohar, A. Israelson, S. Abu-Hamad, V. Shoshan-Barmatz, In self-defence: hexokinase promotes voltage-dependent anion channel closure and prevents mitochondria-mediated apoptotic cell death, Biochem. J. 377 (2004) 347–355.
[23] S. Abu-Hamad, H. Zaid, A. Israelson, E. Nahon, V. Shoshan-Barmatz, Hexokinase-I protection against apoptotic cell death is mediated via interaction with the voltage-dependent anion channel-1: mapping the site of binding, J. Biol. Chem. 283 (2008) 13482–13490.
[24] N. Majewski, V. Nogueira, P. Bhaskar, P.E. Coy, J.E. Skeen, K. Gottlob, N.S. Chandel, C. B. Thompson, R.B. Robey, N. Hay, Hexokinase-mitochondria interaction mediated by Akt is required to inhibit apoptosis in the presence or absence of Bax and Bak, Mol. Cell 16 (2004) 819–830.
[25] J.G. Pastorino, J.B. Hoek, Hexokinase II: the integration of energy metabolism and control of apoptosis, Curr. Med. Chem. 10 (2003) 1535–1551.
[26] J.G. Pastorino, J.B. Hoek, Regulation of hexokinase binding to VDAC, J. Bioenerg. Biomembr. 40 (2008) 171–182.
[27] J.G. Pastorino, J.B. Hoek, N. Shulga, Activation of glycogen synthase kinase 3beta disrupts the binding of hexokinase II to mitochondria by phosphorylating voltage-dependent anion channel and potentiates chemotherapy-induced cytotoxicity, Cancer Res. 65 (2005) 10545–10554.
[28] J.G. Pastorino, N. Shulga, J.B. Hoek, Mitochondrial binding of hexokinase II inhibits Bax-induced cytochrome *c* release and apoptosis, J. Biol. Chem. 277 (2002) 7610–7618.
[29] T.K. Rostovtseva, B. Antonsson, M. Suzuki, R.J. Youle, M. Colombini, S.M. Bezrukov, Bid, but not Bax, regulates VDAC channels, J. Biol. Chem. 279 (2004) 13575–13583.
[30] Y. Shi, J. Chen, C. Weng, R. Chen, Y. Zheng, Q. Chen, H. Tang, Identification of the protein–protein contact site and interaction mode of human VDAC1 with Bcl-2 family proteins, Biochem. Biophys. Res. Commun. 305 (2003) 989–996.
[31] S. Shimizu, A. Konishi, T. Kodama, Y. Tsujimoto, BH4 domain of antiapoptotic Bcl-2 family members closes voltage-dependent anion channel and inhibits apoptotic mitochondrial changes and cell death, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 3100–3105.
[32] S. Shimizu, Y. Matsuoka, Y. Shinohara, Y. Yoneda, Y. Tsujimoto, Essential role of voltage-dependent anion channel in various forms of apoptosis in mammalian cells, J. Cell Biol. 152 (2001) 237–250.
[33] T. Sugiyama, S. Shimizu, Y. Matsuoka, Y. Yoneda, Y. Tsujimoto, Activation of mitochondrial voltage-dependent anion channel by apro-apoptotic BH3-only protein Bim, Oncogene 21 (2002) 4944–4956.
[34] H. Zaid, S. Abu-Hamad, A. Israelson, I. Nathan, V. Shoshan-Barmatz, The voltage-dependent anion channel-1 modulates apoptotic cell death, Cell Death Differ. 12 (2005) 751–760.
[35] R.B. Robey, N. Hay, Mitochondrial hexokinases, novel mediators of the anti-apoptotic effects of growth factors and Akt, Oncogene 25 (2006) 4683–4696.
[36] K.K. Arora, P.L. Pedersen, Functional significance of mitochondrial bound hexokinase in tumor cell metabolism. Evidence for preferential phosphorylation of glucose by intramitochondrially generated ATP, J. Biol. Chem. 263 (1988) 17422–17428.
[37] E. Bustamante, P.L. Pedersen, High aerobic glycolysis of rat hepatoma cells in culture: role of mitochondrial hexokinase, Proc. Natl. Acad. Sci. U. S. A. 74 (1977) 3735–3739.
[38] P.L. Pedersen, S. Mathupala, A. Rempel, J.F. Geschwind, Y.H. Ko, Mitochondrial bound type II hexokinase: a key player in the growth and survival of many cancers and an ideal prospect for therapeutic intervention, Biochim. Biophys. Acta 1555 (2002) 14–20.
[39] A. Rempel, P. Bannasch, D. Mayer, Differences in expression and intracellular distribution of hexokinase isoenzymes in rat liver cells of different transformation stages, Biochim. Biophys. Acta 1219 (1994) 660–668.
[40] P.L. Pedersen, Warburg, me and Hexokinase 2: multiple discoveries of key molecular events underlying one of cancers' most common phenotypes, the "Warburg Effect", i.e., elevated glycolysis in the presence of oxygen, J. Bioenerg. Biomembr. 39 (2007) 211–222.
[41] L. Arzoine, N. Zilbergberg, R. Ben-Romano, V. Shoshan-Barmatz, Voltage-dependent anion channel-1-based peptides interact with hexokinase to prevent its anti-apoptotic activity, J. Biol. Chem. (2008) (in press).
[42] L. Galluzzi, O. Kepp, N. Tajeddine, G. Kroemer, Disruption of the hexokinase-VDAC complex for tumor therapy, Oncogene 27 (2008) 4633–4635.
[43] R.B. Robey, N. Hay, Mitochondrial hexokinases: guardians of the mitochondria, Cell Cycle 4 (2005) 654–658.
[44] T.J. Lampidis, M. Kurtoglu, J.C. Maher, H. Liu, A. Krishan, V. Sheft, S. Szymanski, I. Fokt, W.R. Rudnicki, K. Ginalski, B. Lesyng, W. Priebe, Efficacy of 2-halogen substituted D-glucose analogs in blocking glycolysis and killing "hypoxic tumor cells", Cancer Chemother. Pharmacol. 58 (2006) 725–734.
[45] S. Oudard, F. Poirson, L. Miccoli, Y. Bourgeois, A. Vassault, M. Poisson, H. Magdelenat, B. Dutrillaux, M.F. Poupon, Mitochondria-bound hexokinase as target for therapy of malignant gliomas, Int. J. Cancer 62 (1995) 216–222.
[46] J.E. Wilson, V. Chung, Rat brain hexokinase: further studies on the specificity of the hexose and hexose 6-phosphate binding sites, Arch. Biochem. Biophys. 269 (1989) 517–525.
[47] D. Gincel, H. Zaid, V. Shoshan-Barmatz, Calcium binding and translocation by the voltage-dependent anion channel: a possible regulatory mechanism in mitochondrial function, Biochem. J. 358 (2001) 147–155.
[48] A. Godbole, J. Varghese, A. Sarin, M.K. Mathew, VDAC is a conserved element of death pathways in plant and animal systems, Biochim. Biophys. Acta 1642 (2003) 87–96.
[49] R. Casadio, I. Jacoboni, A. Messina, V. De Pinto, A 3D model of the voltage-dependent anion channel (VDAC), FEBS Lett. 520 (2002) 1–7.
[50] M. Bayrhuber, T. Meins, M. Habeck, S. Becker, K. Giller, S. Villinger, C. Vonrhein, C. Griesinger, M. Zweckstetter, K. Zeth, Structure of the human voltage-dependent anion channel, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 15370–15375.
[51] R. Ujwal, D. Cascio, J.P. Colletier, S. Faham, J. Zhang, L. Toro, P. Ping, J. Abramson, The crystal structure of mouse VDAC1 at 2.3 A resolution reveals mechanistic insights into metabolite gating, Proc Natl Acad Sci U S A 105 (2008) 17742–17747.
[52] S. Hiller, R.G. Garces, T.J. Malia, V.Y. Orekhov, M. Colombini, G. Wagner, Solution structure of the integral human membrane protein VDAC-1 in detergent micelles, Science 321 (2008) 1206–1210.
[53] S. Abu-Hamad, N. Arbel, D. Calo, L. Arzoine, A. Israelson, N. Keinan, R. Ben-Romano, O. Friedman, V. Shoshan-Barmatz, The VDAC1 N-terminal is essential for apoptosis and regulation by anti-apoptotic proteins, J. Cell Sci. (under revision).
[54] V. De Pinto, F. Tomasello, A. Messina, F. Guarino, R. Benz, D. La Mendola, A. Magri, D. Milardi, G. Pappalardo, Determination of the conformation of the human VDAC1 N-terminal peptide, a protein moiety essential for the functional properties of the pore, Chembiochem 8 (2007) 744–756.
[55] S. Reymann, H. Florke, M. Heiden, C. Jakob, U. Stadtmuller, P. Steinacker, V.E. Lalk, I. Pardowitz, F.P. Thinnes, Further evidence for multitopological localization of mammalian porin (VDAC) in the plasmalemma forming part of a chloride channel complex affected in cystic fibrosis and encephalomyopathy, Biochem. Mol. Med. 54 (1995) 75–87.
[56] A.M. Campbell, S.H. Chan, The voltage dependent anion channel affects mitochondrial cholesterol distribution and function, Arch. Biochem. Biophys. 466 (2007) 203–210.
[57] A. Israelson, H. Zaid, S. Abu-Hamad, E. Nahon, V. Shoshan-Barmatz, Mapping the ruthenium red-binding site of the voltage-dependent anion channel-1, Cell Calcium 43 (2007) 196–204.
[58] B.D. Gelb, V. Adams, S.N. Jones, L.D. Griffin, G.R. MacGregor, E.R. McCabe, Targeting of hexokinase 1 to liver and hepatoma mitochondria, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 202–206.
[59] P.G. Polakis, J.E. Wilson, An intact hydrophobic N-terminal sequence is critical for binding of rat brain hexokinase to mitochondria, Arch. Biochem. Biophys. 236 (1985) 328–337.
[60] C.P. Baines, C.X. Song, Y.T. Zheng, G.W. Wang, J. Zhang, O.L. Wang, Y. Guo, R. Bolli, E. M. Cardwell, P. Ping, Protein kinase Cepsilon interacts with and inhibits the permeability transition pore in cardiac mitochondria, Circ. Res. 92 (2003) 873–880.
[61] L. Sun, S. Shukair, T.J. Naik, F. Moazed, H. Ardehali, Glucose phosphorylation and mitochondrial binding are required for the protective effects of hexokinases I and II, Mol. Cell Biol. 28 (2008) 1007–1017.
[62] S. Miyamoto, A.N. Murphy, J.H. Brown, Akt mediates mitochondrial protection in cardiomyocytes through phosphorylation of mitochondrial hexokinase-II, Cell Death Differ. 15 (2008) 521–529.
[63] L. Miccoli, S. Oudard, F. Sureau, F. Poirson, B. Dutrillaux, M.F. Poupon, Intracellular pH governs the subcellular distribution of hexokinase in a glioma cell line, Biochem. J. 313 (Pt 3) (1996) 957–962.
[64] J.E. Wilson, Ambiquitous enzymes: variation in intracellular distribution as a regulatory mechanism, Trends Biochem. Sci. 3 (1978) 124–126.
[65] J.C. Rathmell, C.J. Fox, D.R. Plas, P.S. Hammerman, R.M. Cinalli, C.B. Thompson, Akt-directed glucose metabolism can prevent Bax conformation change and promote growth factor-independent survival, Mol. Cell Biol. 23 (2003) 7315–7328.
[66] E. Bustamante, P.L. Pedersen, Mitochondrial hexokinase of rat hepatoma cells in culture: solubilization and kinetic properties, Biochemistry 19 (1980) 4972–4977.
[67] P.L. Pedersen, Voltage dependent anion channels (VDACs): a brief introduction with a focus on the outer mitochondrial compartment's roles together with hexokinase-2 in the "Warburg effect" in cancer, J. Bioenerg. Biomembr. 40 (2008) 123–126.
[68] H. Sade, N.S. Khandre, M.K. Mathew, A. Sarin, The mitochondrial phase of the glucocorticoid-induced apoptotic response in thymocytes comprises sequential

activation of adenine nucleotide transporter (ANT)-independent and ANT-dependent events, Eur. J. Immunol. 34 (2004) 119–125.
[69] K. Machida, Y. Ohta, H. Osada, Suppression of apoptosis by cyclophilin D via stabilization of hexokinase II mitochondrial binding in cancer cells, J. Biol. Chem. 281 (2006) 14314–14320.
[70] L. Miccoli, A. Beurdeley-Thomas, G. De Pinieux, F. Sureau, S. Oudard, B. Dutrillaux, M.F. Poupon, Light-induced photoactivation of hypericin affects the energy metabolism of human glioma cells by inhibiting hexokinase bound to mitochondria, Cancer Res. 58 (1998) 5777–5786.
[71] G. Beutner, A. Ruck, B. Riede, D. Brdiczka, Complexes between porin, hexokinase, mitochondrial creatine kinase and adenylate translocator display properties of the permeability transition pore. Implication for regulation of permeability transition by the kinases, Biochim. Biophys. Acta 1368 (1998) 7–18.
[72] M. Baijal, J.E. Wilson, Residues putatively involved in binding of ATP and glucose 6-phosphate to a mammalian hexokinase: site-directed mutation at analogous positions in the N- and C-terminal halves of the type I isozyme, Arch Biochem. Biophys. 321 (1995) 413–420.
[73] M. Hashimoto, J.E. Wilson, Membrane potential-dependent conformational changes in mitochondrially bound hexokinase of brain, Arch. Biochem. Biophys. 384 (2000) 163–173.
[74] W.S. da-Silva, A. Gomez-Puyou, M.T. de Gomez-Puyou, R. Moreno-Sanchez, F.G. De Felice, L. de Meis, M.F. Oliveira, A. Galina, Mitochondrial bound hexokinase activity as a preventive antioxidant defense: steady-state ADP formation as a regulatory mechanism of membrane potential and reactive oxygen species generation in mitochondria, J. Biol. Chem. 279 (2004) 39846–39855.
[75] A. Ahmad, S. Ahmad, B.K. Schneider, C.B. Allen, L.Y. Chang, C.W. White, Elevated expression of hexokinase II protects human lung epithelial-like A549 cells against oxidative injury, Am. J. Physiol. Lung Cell Mol. Physiol. 283 (2002) L573–L584.
[76] J.M. Bryson, P.E. Coy, K. Gottlob, N. Hay, R.B. Robey, Increased hexokinase activity, of either ectopic or endogenous origin, protects renal epithelial cells against acute oxidant-induced cell death, J. Biol. Chem. 277 (2002) 11392–11400.
[77] S. Shimizu, M. Narita, Y. Tsujimoto, Bcl-2 family proteins regulate the release of apoptogenic cytochrome *c* by the mitochondrial channel VDAC, Nature 399 (1999) 483–487.
[78] S. Shimizu, Y. Shinohara, Y. Tsujimoto, Bax and Bcl-xL independently regulate apoptotic changes of yeast mitochondria that require VDAC but not adenine nucleotide translocator, Oncogene 19 (2000) 4309–4318.
[79] N. Majewski, V. Nogueira, R.B. Robey, N. Hay, Akt inhibits apoptosis downstream of BID cleavage via a glucose-dependent mechanism involving mitochondrial hexokinases, Mol. Cell Biol. 24 (2004) 730–740.
[80] M. Vyssokikh, L. Zorova, D. Zorov, G. Heimlich, J. Jurgensmeier, D. Schreiner, D. Brdiczka, The intra-mitochondrial cytochrome *c* distribution varies correlated to the formation of a complex between VDAC and the adenine nucleotide translocase: this affects Bax-dependent cytochrome *c* release, Biochim. Biophys. Acta 1644 (2004) 27–36.
[81] S.P. Mathupala, Y.H. Ko, P.L. Pedersen, Hexokinase II: cancer's double-edged sword acting as both facilitator and gatekeeper of malignancy when bound to mitochondria, Oncogene 25 (2006) 4777–4786.
[82] Z. Ding, X. Yang, A. Pater, S.C. Tang, Resistance to apoptosis is correlated with the reduced caspase-3 activation and enhanced expression of antiapoptotic proteins in human cervical multidrug-resistant cells, Biochem. Biophys. Res. Commun. 270 (2000) 415–420.
[83] T. Takehara, X. Liu, J. Fujimoto, S.L. Friedman, H. Takahashi, Expression and role of Bcl-xL in human hepatocellular carcinomas, Hepatology 34 (2001) 55–61.
[84] J. Penso, R. Beitner, Clotrimazole and bifonazole detach hexokinase from mitochondria of melanoma cells, Eur. J. Pharmacol. 342 (1998) 113–117.
[85] N. Goldin, L. Arzoine, A. Heyfets, A. Israelson, Z. Zaslavsky, T. Bravman, V. Bronner, A. Notcovich, V. Shoshan-Barmatz, E. Flescher, Methyl jasmonate binds to and detaches mitochondria-bound hexokinase, Oncogene 27 (2008) 4636–4643.
[86] J.F. Geschwind, Y.H. Ko, M.S. Torbenson, C. Magee, P.L. Pedersen, Novel therapy for liver cancer: direct intraarterial injection of a potent inhibitor of ATP production, Cancer Res. 62 (2002) 3909–3913.
[87] Y.H. Ko, B.L. Smith, Y. Wang, M.G. Pomper, D.A. Rini, M.S. Torbenson, J. Hullihen, P.L. Pedersen, Advanced cancers: eradication in all cases using 3-bromopyruvate therapy to deplete ATP, Biochem. Biophys. Res. Commun. 324 (2004) 269–275.
[88] H. Pelicano, D.S. Martin, R.H. Xu, P. Huang, Glycolysis inhibition for anticancer treatment, Oncogene 25 (2006) 4633–4646.