

Regulation of hexokinase binding to VDAC

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Abstract Hexokinase isoforms I and II bind to mitochondrial outer membranes in large part by interacting with the outer membrane voltage-dependent anion channel (VDAC). This interaction results in a shift in the susceptibility of mitochondria to pro-apoptotic signals that are mediated through Bcl2-family proteins. The upregulation of hexokinase II expression in tumor cells is thought to provide both a metabolic benefit and an apoptosis suppressive capacity that gives the cell a growth advantage and increases its resistance to chemotherapy. However, the mechanisms responsible for the anti-apoptotic effect of hexokinase binding and its regulation remain poorly understood. We hypothesize that hexokinase competes with Bcl2 family proteins for binding to VDAC to influence the balance of pro- and anti-apoptotic proteins that control outer membrane permeabilization. Hexokinase binding to VDAC is regulated by protein kinases, notably glycogen synthase kinase (GSK)-3 β and protein kinase C (PKC)- ϵ . In addition, there is evidence that the cholesterol content of the mitochondrial membranes may contribute to the regulation of hexokinase binding. At the same time, VDAC associated proteins are critically involved in the regulation of cholesterol uptake. A better characterization of these regulatory processes is required to elucidate

the role of hexokinases in normal tissue function and to apply these insights for optimizing cancer treatment.

Introduction

The voltage dependent anion channel (VDAC), also known as mitochondrial porin, is the most abundant protein of the outer mitochondrial membrane (OMM) and the major channel for the exchange of metabolites and ions between the mitochondria and other cellular compartments. As a consequence of its location at the boundary between mitochondria and cytosol, VDAC also is a binding partner for proteins that mediate and regulate the integration of mitochondrial functions with other cellular activities. Notably, VDAC has been reported to bind pro- and anti-apoptotic proteins of the Bcl-2 family that regulate the permeability of the outer membrane (Azoulay-Zohar et al. 2004); it may interact with mitochondrial proteins that form contact sites between the inner and outer membranes (Brdiczka et al. 1998) and that may generate the mitochondrial permeability transition pore complex (Crompton et al. 2002); and it binds to cytosolic proteins, such as hexokinase I and hexokinase II (Wilson 1995; Pedersen et al. 2002), which influences not only its metabolic function, but also its capacity to respond to Bcl-2 family proteins and its role in apoptosis (Pastorino and Hoek 2003). How these different functions of VDAC are regulated remains a matter of much debate, but there is evidence that multiple protein kinases are involved in its control, resulting from the phosphorylation of VDAC itself or its binding partners. In addition, the functional state of VDAC and its capacity to interact with proteins such as hexokinase isoforms may depend on the constituents of the membrane in which it is embedded. In this brief review, we will discuss the regulation of VDAC function with an

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emphasis on the regulation of its interaction with hexokinase and Bcl2-family proteins.

Interactions between hexokinase and VDAC

Early studies on VDAC characterized it as the outer membrane hexokinase-binding protein (Linden et al. 1982; Fiek et al. 1982). Hexokinase isoforms I and II both bind to the outer mitochondrial membrane (OMM) and there is an abundance of evidence that this binding is greatly enhanced by their interaction with VDAC, more specifically the most abundant isoform, VDAC-1. Hexokinase I is highly expressed in brain and hexokinase II is prevalent in cardiac muscle (Wilson 1995). In addition, hexokinase II is highly overexpressed in many tumor cells where it contributes to the tumor's enhanced capacity for oxidative glycolysis, commonly referred to as the Warburg effect (Pedersen et al. 2002; Pastorino and Hoek 2003). However, hexokinase binds equally well to heterologous mitochondria from cells or tissues such as liver that do not normally express these isoforms. Moreover, hexokinase binds effectively to VDAC-1 reconstituted in lipid membranes and affects its ion permeability characteristics, promoting a closed state of the channel (Azoulay-Zohar et al. 2004). Both hexokinase I and II possess a hydrophobic N-terminal sequence of 15 amino acids that has features compatible with an amphipathic α -helix and may be at least partly inserted into the membrane (Xie and Wilson 1988). The N-terminal domain is necessary and sufficient for mitochondrial binding and hexokinase I or II can be displaced from mitochondria by a peptide corresponding to the N-terminal domain (Pastorino et al. 2002). Moreover, the interaction of hexokinase I with the reconstituted VDAC-1 also requires the presence of the N-terminal domain (Azoulay-Zohar et al. 2004). Hexokinase isoforms, such as hexokinase III and hexokinase IV (glucokinase), which lack the N-terminal domain, do not bind directly to mitochondria, although indirect interaction with mitochondria mediated by other proteins may occur (Danial et al. 2003; Arden et al. 2006). However, despite the recognition that the N-terminal α -helix of hexokinase I and II is required for binding to VDAC, it is not clear how the interaction is enhanced by this domain. There is evidence that the hydrophobic tail of the N-terminal domain is inserted into the membrane bilayer (Xie and Wilson 1988), where it may interact with one or more of the transmembrane domains of VDAC-1. It is likely that other regions of hexokinase come in close apposition to the mitochondria and may help to stabilize the interaction of hexokinase with VDAC or other proteins bound to VDAC. This is supported both by antibody interference studies (Hashimoto and Wilson 2000) and by the observation that effective binding

of hexokinase to mitochondria depends on the conformation of the protein. A high concentration of its metabolite glucose-6-phosphate (G6P), which induces a conformational change in the protein, displaces it from the mitochondria (Azoulay-Zohar et al. 2004; Wilson 1995; Pastorino et al. 2002). However, no other specific domains required for mitochondrial binding have yet been identified. Divalent cations such as Mg^{2+} enhance the binding of hexokinase I and II to mitochondria (Wilson 1995; Aflalo and Azoulay 1998). This may involve surface charge shielding or a more specific bridging effect to facilitate the apposition of negatively charged amino acids residues on the enzyme and anionic phospholipids or charged residues of interacting proteins in the OMM. It should be noted that Mg^{2+} also enhances the binding of hexokinase to yeast mitochondria, which do not express a VDAC isoform capable of binding the protein (Aflalo and Azoulay 1998).

As with hexokinase I and II, several regions of VDAC appear to be required for hexokinase binding. Computer modeling of the structure of membrane-embedded VDAC suggests a β -barrel structure containing 13 to 16 antiparallel transbilayer β -strands connected by peptide loops, in addition to an N-terminal α -helical domain presumably localized to the intermembrane space. N,N-dicyclohexylcarbodiimide (DCCD) abolishes hexokinase binding to mitochondria through modification of glutamate 72, presumed to be located in the middle of the first cytoplasmic loop of VDAC (Nakashima et al. 1986; Al Jamal 2005). Indeed, recent mutational studies by Shoshan-Barmatz and coworkers (Zaid et al. 2005) who assessed both hexokinase binding to intact yeast mitochondria expressing VDAC-1 and its effect on VDAC conductance in a reconstituted lipid membrane, confirmed that glutamate 72 is essential for hexokinase binding to VDAC. The E72Q mutation also prevents protection by hexokinase I of VDAC-mediated apoptotic cell death (Zaid et al. 2005). Several other charged residues in VDAC-1 were identified in subsequent studies by the same group as being required for hexokinase I protection against VDAC-1-mediated apoptosis and for channel closure in the reconstituted membrane system. These include glutamate 65, aspartate 77 and lysine 73, all localized within the putative first cytoplasmic loop (Abu-Hamad et al. 2008). In addition, mutations in glutamate 202 and 188, charged residues in the putative fourth cytoplasmic loop, while not essential for binding, were found to stabilize the interaction of hexokinase I with VDAC and to provide additional protection against VDAC-mediated apoptosis (Abu-Hamad et al. 2008). However, the nature of that stabilizing interaction remains poorly defined.

It is interesting to note that the majority of the VDAC-1 residues that have been identified as being required for hexokinase I binding are charged, and are located in the cytoplasm-exposed loops of VDAC-1. This finding lends

further support to the notion that the hydrophobic portion of the N-terminal domain, while required for mitochondrial binding, is not directly engaging these essential residues on VDAC-1. Further characterization of the hexokinase domains involved in these interactions would provide better insight into the nature of the binding interaction.

Recent cross-linking studies (Zalk et al. 2005; Shoshan-Barmatz et al. 2004) and atomic force microscopy analyses of OMM preparations (Goncalves et al. 2007; Hoogenboom et al. 2007) demonstrated that VDAC exists as dimeric, trimeric and tetrameric structures and may be assembled in higher order aggregates in the OMM (Shoshan-Barmatz et al. 2004). The dynamic nature of these oligomerization reactions was suggested by the finding that cross-linking of VDAC oligomers prevented its role in the permeabilization of the OMM and the release of cytochrome c, although transport of ions was not impeded (Zalk et al. 2005). Similarly, hexokinase I assumes a tetrameric structure when bound to rat brain mitochondria (Xie and Wilson 1990), which may be facilitated by the underlying structure of the VDAC oligomers. This is reminiscent of the higher order oligomerization of creatine kinase when bound to VDAC from the interior of the mitochondrial intermembrane space (Brdiczka et al. 1994; Stachowiak et al. 1998). The mechanisms and signaling pathways regulating the formation of VDAC and hexokinase oligomers and their consequences to mitochondrial function and susceptibility to damage largely remain a matter of speculation.

Although the precise nature of the mechanisms by which hexokinase binding protects against apoptosis still remains unclear, other VDAC interacting proteins may contribute to this effect. VDAC has been reported to interact with multiple other proteins that contribute to its role as the mediator of mitochondria-cytosolic interactions. The classical paradigm of VDAC as an essential partner in the permeability transition involves its ability to interact with inner membrane complexes, such as the adenine nucleotide translocator (ANT; Marzo et al. 1998; Halestrap and Brennerb 2003). In cells that express mitochondrial creatine kinase, an octameric form of this protein has been suggested to mediate these interactions (Brdiczka et al. 1994; Stachowiak et al. 1998). The effects of Bcl2 family proteins on VDAC has also been suggested to involve direct binding interactions (Tsujimoto and Shimizu 2000). A recent NMR study identified residues involved in binding of VDAC-1 to Bcl-X_L (Malia and Wagner 2007). This model predicted that the VDAC binding of Bcl-X_L depends primarily on the helices 5 and 6 of Bcl-X_L that are inserted into the membrane. The BH4 domain of Bcl-X_L, which is essential for its anti-apoptotic function, appeared to have only a more superficial interaction with the cytosolic face of VDAC. However, the BH4 domain is essential for its inhibitory effect on VDAC, i.e., the

functionally effective domain was not primarily responsible for its binding (Malia and Wagner 2007).

In these respects, therefore, Bcl-X_L binding resembles the nature of the hexokinase interaction with VDAC-1, where the hydrophobic N-terminal domain is thought to insert into the membrane and other interactions may mediate functional effects. Also, hexokinase binding, similar to Bcl-X_L, inhibits VDAC conductance (Azoulay-Zohar et al. 2004). In this context, it is of interest to point out that we identified a BH4 homology region in hexokinase II, which we postulated to play a role in the anti-apoptotic effects that depend on its binding to VDAC (Pastorino and Hoek 2003). It is conceivable that hexokinase binding displaces Bcl-X_L from VDAC and thereby facilitates its availability for interaction with pro-apoptotic Bcl2 family proteins, such as Bax/Bak that can contribute to outer membrane permeabilization (Fig. 1A).

Interestingly, Malia and Wagner (2007) found that the VDAC-Bcl-X_L interaction shifts the oligomerization equilibrium from a predominantly oligomeric towards a monomeric state of VDAC. This contrasts with the effect of hexokinase binding, which promotes the formation of oligomeric VDAC structures. This observation further suggests that the binding of hexokinase and Bcl-X_L may be mutually exclusive. To what extent this transition plays a role in the protective effects of hexokinase remains to be established. It also remains unclear what role VDAC oligomerization plays in membrane permeabilization.

Signaling pathways that regulate the interaction of hexokinases I and II with VDAC

There is an abundance of evidence that the interaction of hexokinase with mitochondria is regulated to promote its protective anti-apoptotic effects when needed and to facilitate its removal when apoptosis is required. In addition, the metabolic function of hexokinase binding requires integration with other regulatory devices that control metabolic flux and energy supply to meet the demands of the cell (Pastorino and Hoek 2003; Plas and Thompson 2005). Several mechanisms have been identified that affect the hexokinase-VDAC interaction. As pointed out above, hexokinase I and II binding is decreased by high concentrations of its reaction product glucose-6-phosphate, which induces a conformational change in the protein that causes its release from VDAC (Azoulay-Zohar et al. 2004; Wilson 1995; Hashimoto and Wilson 2000). Also, divalent cations are required for hexokinase binding to VDAC-1 (Wilson 1995; Aflalo and Azoulay 1998), although it is not known whether these contribute to the regulation of the interaction in intact cells. In addition, there is evidence that the binding of hexokinase I or II to VDAC is regulated by protein kinases.

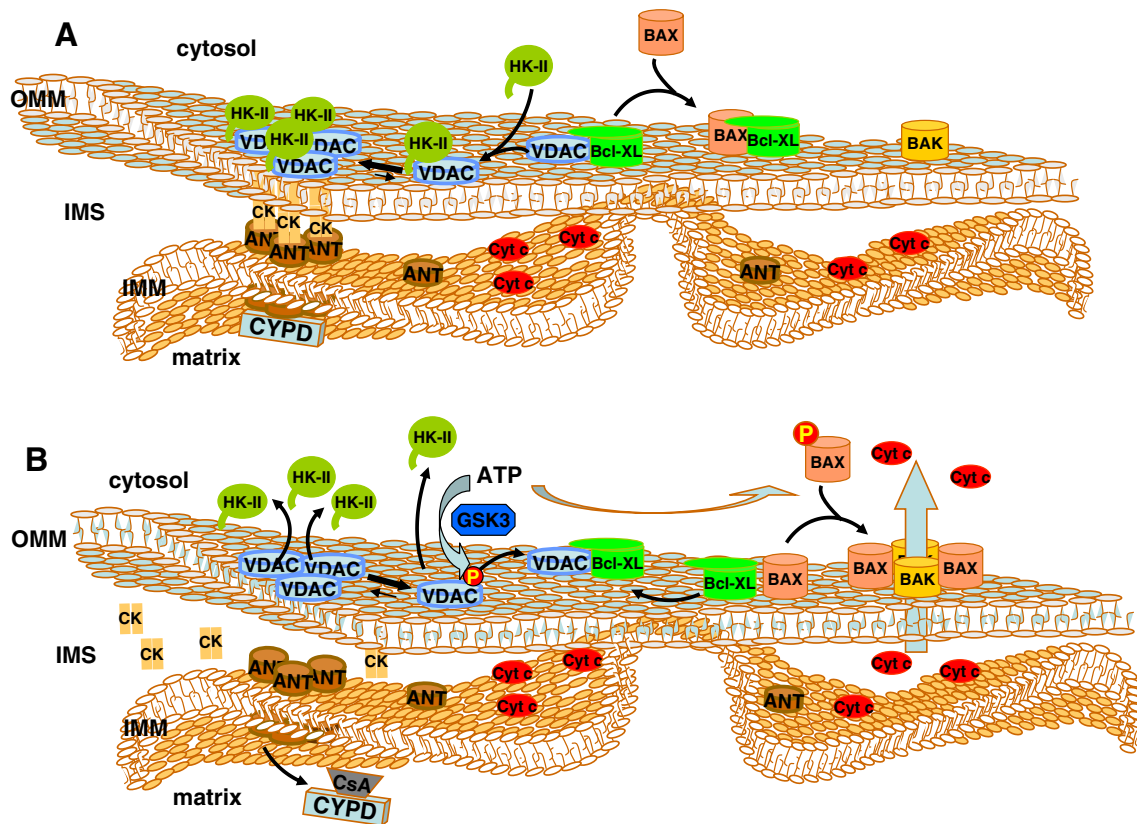


Fig. 1 Proposed mechanism of hexokinase-II-mediated protection against outer mitochondrial membrane (OMM) permeabilization. **A** Hexokinase II (*HKII*) binds to VDAC through both its N-terminal membrane binding domain and its putative BH4 domain (Pastorino and Hoek 2003), which is proposed to compete with VDAC-bound BCL-X_L. Release of BCL-X_L facilitates BAX binding, which prevents BAX-BAK interaction and protects against OMM permeabilization. Hexokinase II binding promotes VDAC oligomerization and the formation of contact sites between the inner and outer membranes in which the adenine nucleotide translocator (*ANT*) and octameric mitochondrial creatine kinase (*CK*) are presumed to play a role.

Akt (also known as protein kinase B) is an oncogene protein kinase that is activated in response to stimulation of phosphatidylinositol 3-kinase (PI3K) and exhibits dysregulated activity in a variety of malignancies (Plas and Thompson 2005; DeBerardinis et al. 2008). Akt hyperactivity in cancer cells is associated with increased glycolysis, at least in part by enhancing the rate of glucose uptake through increased expression of the glucose carriers GLUT1 and GLUT4. Elstrom et al. (2004) demonstrated that Akt activation also enhances binding of hexokinase to the mitochondria. The Akt mediated binding of hexokinase to mitochondria was dependent on the availability of glucose, suggesting that the enzymatic activity of hexokinase is necessary for the interaction with VDAC (Gottlob et al. 2001; Majewski et al. 2004). These authors suggested that Akt would cause an increased binding of hexokinase to the mitochondria by augmenting the uptake and metabolism of glucose. However, despite evidence that enzymatic

Cyclosporin D (*CYPD*) may further enhance ANT binding to VDAC-HKII oligomeric complexes. **B** Phosphorylation of VDAC by GSK3 displaces hexokinase II from VDAC, thereby promoting binding of BCL-X_L to VDAC. Disruption of CYPD interaction with ANT through cyclosporin A or through CYPD knockout would also promote hexokinase II displacement from VDAC, disrupting VDAC oligomers and making monomeric VDAC available for Bcl-XL binding. BAX is now free to interact with BAK to form pore structures that promote the release of cytochrome *c* (*cyt c*) and other intermembrane space (IMS) pro-apoptotic proteins

activity of hexokinase II is required for its effects on apoptosis (Majewski et al. 2004; Sun et al. 2008), the mechanism by which increased glycolytic flux enhanced mitochondrial binding of hexokinase remained unclear in these studies. Other studies discussed below indicate that Akt also has more direct effects on the binding of hexokinase to VDAC by affecting the phosphorylation state of VDAC and/or hexokinase.

The ability of Akt to directly impact mitochondrial function is illustrated by the finding that activated Akt localizes to the mitochondria following activation of the upstream PI3K (Bijur and Jope 2003; Miyamoto et al. 2008). Indeed, Akt can directly phosphorylate hexokinase-II (Bijur and Jope 2003; Miyamoto et al. 2008). Activation of Akt in cardiomyocytes was shown to stimulate association between Akt and hexokinase II, with Akt subsequently phosphorylating hexokinase II at threonine 473, positioned in an Akt phosphorylation consensus sequence.

The phosphorylation of hexokinase II by Akt was accompanied by an increased binding of the enzyme to the mitochondria and also was found to contribute to the anti-apoptotic effects of Akt against ischemia/reperfusion injury. These observations illustrate the ability of Akt to positively regulate the binding of hexokinase II to VDAC.

In addition, work in our laboratory has shown that Akt can also impact the binding of hexokinase II to mitochondria by negatively regulating the activity of glycogen synthase kinase 3 β (GSK3 β) and its phosphorylation of VDAC (Pastorino et al. 2005). Like Akt, GSK3 β can localize to the mitochondria. However unlike Akt, activation of GSK3 β is associated with mitochondrial dysfunction and cell injury (Morrison et al. 2002; Murphy 2004; Jope and Johnson 2004; Juhaszova et al. 2004; Macanas-Pirard et al. 2005; Park et al. 2006; Nishihara et al. 2007). Indeed, it has been demonstrated that many signaling pathways that promote cardioprotection converge at inhibition of GSK3 β activation. Part of the protective effect of GSK3 β inhibition in the heart may be through the preservation of mitochondrial binding of hexokinase II. Similar mechanisms may apply to other cells and tissues that express hexokinase isoforms that can bind to mitochondria, including tumor cells (Pastorino et al. 2005).

GSK3 β can phosphorylate VDAC-1 on threonine 51 resulting in the detachment of hexokinase II from VDAC-1 and a sensitization of cancer cells to chemotherapeutic agents such as paclitaxel and doxorubicin (Pastorino et al. 2005). The location of this threonine residue is not entirely clear. Depending on the structural model of VDAC, threonine 51 would either be located in the putative first cytoplasmic loop or in one of the transmembrane β -sheets (Abu-Hamad et al. 2008, Mannella et al. 1992; Casadio et al. 2002; Colombini 2004) and it is conceivable that phosphorylation of this residue influences the conformation of VDAC-1 or its ability to form oligomeric structures (Fig. 1B). Future mutational studies focusing on the functional consequences of this phosphorylation would be of interest.

The enhanced vulnerability of the mitochondria to pro-apoptotic conditions resulting from this phosphorylation is mediated in part by a heightened sensitivity to the pro-apoptotic protein Bax (Pastorino et al. 2002). How Bax sensitivity is increased has not been well characterized. One model would be that Bax is able to gain direct access to VDAC when hexokinase is not bound. Alternatively, if hexokinase detachment promotes binding of Bcl-X_L to VDAC, this may prevent it from binding Bax, thereby leaving Bax free to interact with Bak and form pore structures in the outer membrane that promote the release of cytochrome c and other apoptosis enhancing proteins (Fig. 1B). This model is also supported by the finding that overexpression of VDAC-1 itself enhances the susceptibility of cells to apoptosis (Abu-Hamad et al. 2006). Additionally,

activation of GSK3 β has been shown to phosphorylate Bax directly, promoting its translocation from the cytosol to the mitochondria (Linseman et al. 2004). This would suggest that GSK3 β activation has the twofold impact of making mitochondria more vulnerable to Bax by detaching hexokinase and tying up the anti-apoptotic Bax binding partner Bcl-X_L to VDAC and then promoting the translocation of Bax to the mitochondrial membrane where it can interact with pro-apoptotic partners, such as Bak (Fig. 1B).

In addition to promoting the lethality of pro-apoptotic proteins such as Bax, activation of GSK3 β decreases the half-life of anti-apoptotic proteins (Maurer et al. 2006; Zhao et al. 2007). GSK3 β phosphorylates the anti-apoptotic protein MCL-1 on serine 159 leading to its increased ubiquitinylation and degradation. A phosphorylation site mutant of MCL-1 demonstrated increased stability and conferred protection against growth factor withdrawal. Stimulation of glucose catabolism brought on by growth factors was shown to prevent MCL-1 degradation by inhibiting GSK3 β activation (Zhao et al. 2007). However, in this instance the signaling pathway identified as mediating the growth factor dependent inhibitory phosphorylation of GSK3 β involved protein kinase C. Whether GSK3 β was a direct or indirect target of protein kinase C was not established in these studies, nor was it determined which isoform of protein kinase C mediated this effect. Nevertheless, this finding exemplifies that different protein kinases can result in phosphorylation and inhibition of GSK3 β activity, thus illustrating the critical node that this enzyme occupies in mediating the vulnerability of the mitochondria to injury.

In contrast to the detachment of hexokinase from VDAC brought about by GSK3 β mediated phosphorylation, phosphorylation of VDAC by PKC- ϵ promotes hexokinase binding (Baines et al. 2003; Korzick et al. 2007). Studies in cardiac mitochondria demonstrated an interaction of PKC- ϵ with VDAC-1. Moreover, *in vitro* studies revealed that PKC- ϵ directly binds to and phosphorylates VDAC-1 (Baines et al. 2003). Intriguingly, over-expression of hexokinase I or II in HEK293 cells caused a 5-10 fold increase in VDAC phosphorylation that was prevented by an inhibitor of PKC- ϵ . Such results suggest that the binding of hexokinase to VDAC may help to trigger VDAC phosphorylation by PKC- ϵ , which then further enhances the affinity of hexokinase for VDAC, thus establishing a positive feedback cycle.

Although these studies did not identify the target site for PKC- ϵ -dependent phosphorylation, a recent mass spectrometric analysis of covalent modifications in OMM proteins detected multiple phosphorylation sites (as well as other covalent modifications) on all three VDAC isoforms in rat liver mitochondria (Distler et al. 2007). Interestingly, VDAC-1 showed evidence of phosphorylation on serine 12 and serine 136, the first being a PKC consensus site located in the N-terminal domain, the second a CAMKII/

GSK3 consensus site, presumably located on the side of the VDAC structure facing the intermembrane space. Recent evidence suggests that a fraction of cellular PKC- ϵ is present in mitochondria, both in the intermembrane space and in the matrix, although the mechanism by which it is translocated to these sites remains to be established (Jaburek et al. 2006).

Other protein kinases have been reported to affect VDAC function. C-Raf kinase, a member of the MAP kinase cascade that can be targeted to mitochondria, forms a complex with VDAC both in vivo and in reconstituted membranes, resulting in its phosphorylation and subsequent closure (Le Mellay et al. 2002). However, it was not determined if C-Raf-mediated phosphorylation of VDAC exhibited any effects on hexokinase binding. Activation of p38 MAP kinase (MAPK) has also been reported to cause phosphorylation of VDAC during myocardial ischemia and reperfusion, with inhibition of p38 activity exerting a cardioprotective effect (Schwertz et al. 2007). However, this study provided evidence that activation of p38 MAPK resulted in tyrosine phosphorylation of the protein, suggesting that this effect is indirect and involves an unknown tyrosine kinase or tyrosine phosphatase that may act downstream of p38 MAPK. The mass spectrometric analysis of Distler et al. (2007) identified a tyrosine phosphorylation site on VDAC-2. However, there is evidence that VDAC-2 does not directly interact with hexokinase I or II (Chiara et al. 2008).

Regulation of hexokinase binding to VDAC by cyclophilin D

Several recent studies have reported that mitochondrial binding of hexokinase II is affected by the activity of cyclophilin D (Chiara et al. 2008; Machida et al. 2006). Cyclophilin D is an immunophilin that exhibits peptidyl-prolyl *cis-trans* isomerase (PPIase) activity and is localized to the mitochondrial matrix. The ability of cyclosporine A to prevent onset of the mitochondrial permeability transition was the first indication that cyclophilin D regulates the permeability transition pore (PTP; Broekemeier et al. 1989). These findings have been confirmed and extended with the utilization of cyclophilin D knockout mice (Nakagawa et al. 2005; Baines et al. 2005; Basso et al. 2005). In the absence of cyclophilin D, the PTP still formed and opened in response to some challenges, but was not responsive to Ca^{2+} or inhibition by cyclosporine A. Additionally, the effects of knockout of cyclophilin D established that opening of the PTP was essential for mitochondrial injury in necrotic cell death but dispensable for mitochondrial mediation of apoptosis. By contrast, several studies have provided evidence that cyclophilin D

suppresses mitochondrial injury and can function as an apoptosis repressor (Lin and Lechleiter 2002; Schubert and Grimm 2004). Moreover, cyclophilin D is up-regulated in a number of human tumors. Overexpression of cyclophilin D in HEK293 cells or rat glioma C6 cells caused desensitization to induction of apoptosis. Importantly, in these studies the PPIase activity of cyclophilin D was found to be necessary for apoptosis suppression.

Recent studies have shown that the anti-apoptotic effects of cyclophilin D may be exerted by stabilization of hexokinase II binding to mitochondria (Chiara et al. 2008; Machida et al. 2006). Inactivation of cyclophilin D with cyclosporine A or knock-down of its expression utilizing siRNA caused a release of mitochondrially bound hexokinase II. Moreover, the anti-apoptotic effects of cyclophilin D were abrogated by the forced detachment of hexokinase II from the mitochondria. Such observations are in agreement with the concept that hexokinase II prevents access of pro-apoptotic proteins such as Bax to the mitochondria or inhibits its pore-forming abilities. However, it is presently unclear how cyclophilin D activity regulates the binding of hexokinase II to the mitochondria. At first sight, it would seem likely that this occurs through modification of the hexokinase II-VDAC interaction, e.g., by inducing a conformational change in VDAC through a mitochondrial constituent that can interact with both proteins. Since cyclophilin D is thought to be localized exclusively to the mitochondrial matrix, the modulation of hexokinase II binding to VDAC by cyclophilin D must occur through an intermediate located in the mitochondrial inner membrane. A promising candidate for this intermediation role is the adenine nucleotide translocator (ANT). Classically, the ANT was thought to be a component of the PTP and to exert its pore-forming function by interacting with VDAC at mitochondrial contact sites. However, this model has come under doubt recently as a result of studies demonstrating that cyclosporin-sensitive PTP formation could occur in the absence of ANT (Bauer et al. 1999). Nevertheless, the ANT-2 isoform has been shown to be expressed in cancer cells that display enhanced glycolysis and in such cells a large proportion of hexokinase II is bound to the mitochondria (Chevrollier et al. 2005). A hypothesis that could account for these effects is illustrated in Fig. 1, where it is proposed that cyclophilin D stabilizes the interaction between ANT and the oligomeric form of VDAC that is enhanced by hexokinase II binding.

However, a recent study by Chiara et al. (2008) suggests that the story may be more complex. These authors showed that detachment of hexokinase II from the mitochondria triggers apoptosis through opening of the PTP and this was accompanied by a disruption of the association of cyclophilin D with ANT. Moreover, inhibition of the ANT or cyclophilin D prevented the onset of the PTP and

cytotoxicity elicited by hexokinase II detachment and, vice versa, inhibition of cyclophilin D or its knockout promoted detachment of hexokinase II from the mitochondria. Remarkably, however, in this study, VDAC was found to be dispensable not only for induction of the PTP and cell death, but also for the hexokinase II detachment induced by inhibition or knockout of cyclophilin D (Chiara et al. 2008). Also, these studies suggested that the PPIase activity of cyclophilin D was not required for its effects on hexokinase II binding. These observations suggest that VDAC and the VDAC-hexokinase interaction are not irreplaceable constituents of the mitochondrial permeabilization process. It may be that other, yet to be identified proteins (or other membrane constituents) can act as surrogate binding partners for hexokinase II at the OMM in the absence of VDAC and also influence opening of the PTP. It will be of interest to establish if such alternate binding partners also interact with anti-apoptotic proteins, such as Bcl-X_L. If so, the essential features of the mechanism by which hexokinase binding protects against Bax-mediated membrane permeabilization may extend to such other binding partners.

Cholesterol enhancement of hexokinase binding to mitochondria

The mitochondrial outer and inner membranes contain lower levels of cholesterol than most other cellular membranes, such as the plasma membrane, the Golgi membrane, or the ER membrane. However, cancer cells have been shown to exhibit a two to tenfold increase in mitochondrial cholesterol content per mg mitochondrial protein compared to liver mitochondria (Baggetto et al. 1992). Most of the increase occurred in the OMM as free (i.e., non-esterified) cholesterol. For instance, the OMM fraction isolated from Ehrlich or AS30-D tumor cell mitochondria was found to contain 3.5 to five times the free cholesterol content found in OMM fractions from rat liver mitochondria, with a much smaller (less than twofold) increase occurring in the inner membrane fraction (Baggetto et al. 1992). Both of these tumor cell lines are highly glycolytic and exhibit an increased expression and mitochondrial binding of hexokinase II. It was suggested that the increased content of cholesterol in the mitochondria of cancer cells could impact the binding of hexokinase to VDAC (Baggetto et al. 1992). Conversely the increased binding of hexokinase to the mitochondria of cancer cells may play a role in mediating an increased synthesis and uptake of cholesterol into the mitochondria of cancer cells. Indeed, an increase in the content of mitochondrial cholesterol has been shown to affect the levels and activity of a number of mitochondrial membrane proteins.

Mitochondria from cancer cells export citrate at up to a fourfold higher rate than liver mitochondria (Parlo and Coleman 1984). This is probably due in part to an increased activity of the tricarboxylate carrier (TCC) responsible for the transport of citrate and isocitrate across the inner mitochondrial membrane. Citrate export is required for the supply of cytosolic acetyl-CoA, the building block of cholesterol, to HMG-CoA reductase, the first committed step of the cholesterol biosynthesis pathway. Intriguingly, this augmented activity of the tricarboxylate carrier in the mitochondria of cancer cells may be associated with their increased content of cholesterol (Fig. 2). Enrichment of liver mitochondria with exogenous cholesterol resulted in a twofold stimulation in the activity of the mitochondrial citrate carrier. Conversely, an increased content of mitochondrial cholesterol can inhibit the activity of other inner membrane proteins. Baggetto et al. (1992) reported that the inner membrane proton permeability (responsible for the basal rate of oxygen uptake in mitochondria at high membrane potential) was decreased as a function of the inner membrane cholesterol content. Elevated levels of mitochondrial cholesterol were found to diminish the ability of the ANT to assist in the onset of the mitochondrial permeability transition (Colell et al. 2003). Additionally, and possibly related to this effect on ANT, the accumulation of cholesterol in mitochondrial membranes inhibited the ability of Bax to permeabilize the mitochondrial membranes and thus prevented stress induced apoptosis (Lucken-Ardjomande et al. 2008). It is not clear whether these effects of cholesterol loading on the activity of mitochondrial constituents are due to the cholesterol content of the inner membrane or whether the OMM, which contains the majority of this cholesterol, contributes to the control of these metabolite transport activities. VDAC is the major gateway for exit of all such metabolites from the mitochondrial intermembrane space to the cytosol and changes in its activity may also contribute to the metabolic consequences of cholesterol loading.

Whereas the increased content of cholesterol in cancer cells may be driven in part by the amplified export rate of citrate from the mitochondria into the cytosol, where cholesterol is synthesized, VDAC has also been implicated in the subsequent import of the resultant cholesterol from the cytosol into the mitochondria (Thomson 2003; Papadopoulos et al. 2007). As mentioned above, glutamate 72 of VDAC is essential for hexokinase binding. Intriguingly, an E72Q mutant that inhibited hexokinase binding to VDAC and mitochondria, also caused a reduction in the mitochondrial cholesterol content of Morris hepatoma cells (Campbell and Chan 2007). These observations suggest that the binding of hexokinase to VDAC by some means helps to stimulate the uptake of cholesterol into the mitochondria. The converse may also arise, in that the elevated cholesterol content of the

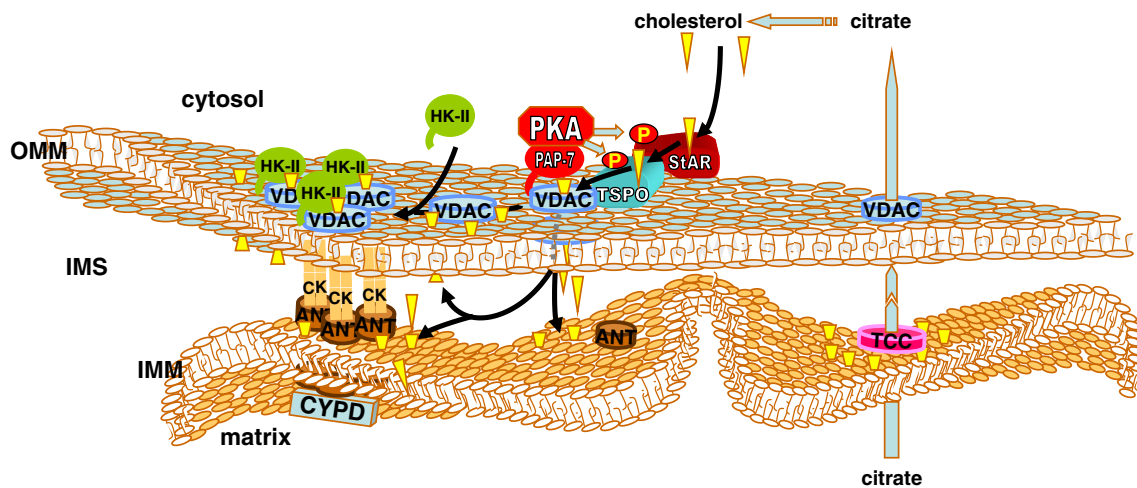


Fig. 2 Mitochondrial cholesterol accumulation promotes hexokinase II binding and protects against OMM permeabilization. The interaction of VDAC with TSPO (also known as the peripheral benzodiazepine receptor) and StAR enhances cholesterol uptake through VDAC. StAR and TSPO are activated by protein kinase A (PKA)-dependent phosphorylation, which is promoted by VDAC binding of the anchoring protein PAP-7. Increased cholesterol content of the mitochondrial membrane promotes binding of HKII to VDAC,

possibly by enhancing the formation of oligomeric complexes involving ANT, resulting in increased protection against OMM permeabilization. Enhanced mitochondrial cholesterol content also promotes citrate export to increase the supply of cholesterol precursor in the cytosol by activation of the tricarboxylate carrier (TCC) located in the inner membrane. The resulting positive feedback loop may cause a switch-like enhancement of mitochondrial cholesterol uptake and hexokinase binding in tumor cells

mitochondria of cancer cells may facilitate the enhanced binding of hexokinase to VDAC. In this way, a positive feedback loop is initiated where hexokinase binding to mitochondria enhances the uptake of cholesterol by VDAC. In turn, the elevated cholesterol content amplifies the binding of hexokinase to VDAC (Fig. 2). These observations, together with the effect of increased mitochondrial cholesterol on the ability of Bax and the ANT to initiate mitochondrial dysfunction, suggest that the loading of cholesterol characteristic of the mitochondria of cancer cells may make them refractory to injury and hence provide the cancer cells with a resistance to induction of apoptosis and a survival advantage.

Importantly, VDAC may be only one component of a complex that mediates the uptake of cholesterol into the mitochondria and is regulated by a number of signaling pathways. The translocator protein 18 kDa (TSPO), formerly known as the peripheral benzodiazepine receptor, is a transmembrane protein located in the OMM (Papadopoulos et al. 1997) that associates with VDAC (McEnery 1992; McEnery et al. 1992, 1993a, b; Kinnally et al. 1993). Importantly, TSPO helps to mediate the uptake of cholesterol into the mitochondria (Li and Papadopoulos 1998). As such, TSPO is found in great abundance in steroidogenic tissues. Additionally, some cancer types such as gliomas with a high glycolytic capacity have been shown to have an amplified expression of TSPO (Hardwick et al. 1999). TSPO contains a cholesterol recognition/interaction amino acid consensus sequence (CRAC domain) at the carboxyl-terminus located on the cytosolic side of the OMM (Li et al.

2001a). Once the cholesterol is bound to the CRAC domain, how it transits through the OMM and IMM is currently unknown. TSPO-associated VDAC may be involved in this process.

Another member of this putative complex is StAR (steroidogenic acute regulatory protein), a 37-kDa protein located in the cytoplasm. Like the TSPO, StAR possesses a cholesterol binding domain (START; Tsujishita and Hurley 2000; Mathieu et al. 2002a, b; Soccio and Breslow 2003). Interestingly, MLN64 (metastatic lymph node 64), a homologue of StAR, is over-expressed in a number of cancers (Watari et al. 1997; Bose et al. 2000a, b). StAR is activated by phosphorylation of serine 195 and is then imported into the mitochondria as a 32-kDa intermediate, which, in turn, is cleaved to a 30-kDa protein once it is imported into the mitochondrial matrix (Bose et al. 2008). StAR has been shown to interact with the cytoplasmically exposed domains of VDAC-1 and to require VDAC expression to exert its ability to mediate cholesterol uptake. However StAR has also been shown to associate with TSPO and TSPO knockdown cells display a loss of StAR activity (Hauet et al. 2005). Interestingly, TSPO binds the anchoring protein, PAP-7 (peripheral benzodiazepine receptor-associated protein-7; Li et al. 2001b; Liu et al. 2003, 2006), a member of the A-kinase anchor protein family, which promotes the interaction of the VDAC-TSPO-StAR complex with the PKA regulatory subunit-1 α (PKAR1 α). PKA is thereby thought to mediate the phosphorylation and consequent activation of StAR. PKA has also been shown to phosphorylate VDAC, however the consequences to

cholesterol uptake and hexokinase binding, if any, are unknown (Bera et al. 1995). PAP-7 possesses a 15 residue targeting element homologous to the N-terminal targeting sequence of hexokinase I and II, suggesting that PAP-7 may interact with VDAC through a mechanism that is similar to that involved in hexokinase II binding (Huang et al. 1999; Ma and Taylor 2002; Affaitati et al. 2003). Whether PAP-7 directly influences hexokinase binding and contributes to the interregulation between cholesterol uptake and synthesis in tumor cells remains to be established. Figure 2 presents a model of the interaction of hexokinase binding to VDAC in the context of the complex of VDAC-interacting proteins that regulate cholesterol transport.

In summary, there are several lines of evidence that suggest an intricate role for the mitochondrial cholesterol content in the binding and anti-apoptotic effects of hexokinase binding. The potential for a positive feedback loop involving hexokinase II binding to VDAC, the mitochondrial cholesterol uptake system, and the mitochondrial tricarboxylate carrier may point to the existence of a bistable system in which enhanced hexokinase binding and cholesterol loading of the mitochondria are mutually reinforcing to promote the special conditions prevailing in tumor cells.

Conclusions and further outlook

The past decade has seen a remarkable shift in our understanding of the nature and implications of the binding interactions of hexokinase I and II to the mitochondrial outer membrane and to VDAC in particular. The functional associations of this interaction have shifted from a predominantly metabolic role to the recognition of its major impact on the regulation of apoptotic responsiveness of the cell. Several recent studies have highlighted the potential application of this understanding for therapeutic purposes (Pastorino et al. 2005; Goldin et al. 2008). As we have pointed out before (Pastorino and Hoek 2003), these findings demonstrate the need for a careful regulation of the binding process so as to integrate the anti-apoptotic protective effects of hexokinase binding with the basic metabolic functions on which the cell depends and which are deregulated in tumor cells. Current evidence suggests that this regulation is at least in part mediated by protein kinase-dependent phosphorylation of VDAC and other proteins involved in the complex formation, most directly by GSK-3 β and PKC- ϵ . However, the regulatory significance of the sites of phosphorylation and the mechanism by which these phosphorylation events affect hexokinase-VDAC interaction remains largely unknown. We hypothesize that VDAC oligomerization and its interaction with ANT and cyclophilin D in the IMM and matrix of the mitochondria may constitute an integral part of these

interactions and may be regulated by VDAC phosphorylation. Also, the recent observation that hexokinase II-mediated protection against apoptotic signals can occur in the absence of VDAC may provide further insight into the regulation of these protective interactions in which hexokinase is involved. It is likely that under such conditions the susceptibility of the cells to protein kinase-mediated regulatory controls would be notably different. In addition, the lipid structure of both the inner and outer mitochondrial membranes may contribute to this regulation as indicated by the consequences of cholesterol loading on the hexokinase binding. How these regulatory processes enable normal cells and tissues to manage the balance between protective effects and the response to pro-apoptotic signals is a topic that deserves much more analysis. Also, hexokinase I and II have a distinct tissue distribution and it is not currently clear what the implications are of the different properties of these isoforms for tissue-specific functions. A better understanding of these functions in normal tissues will greatly contribute to the optimization of therapies for the treatment of tumor cells, where hexokinase II is overexpressed.

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