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Genetic strategies for dissecting mammalian and *Drosophila* voltage-dependent anion channel functions

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Abstract Voltage-dependent anion channels (VDACs), also known as mitochondrial porins, are a family of small pore-forming proteins of the mitochondrial outer membrane that are found in all eukaryotes. VDACs are thought to play important roles in the regulated flux of metabolites between the cytosolic and mitochondrial compartments, in overall energy metabolism via interactions with cytosolic kinases, and a debated role in programmed cell death (apoptosis). The mammalian genome contains three VDAC loci termed *Vdac1*, *Vdac2*, and *Vdac3*, raising the question as to what function each isoform may be performing. Based upon expression studies of the mouse VDACs in yeast, biophysical differences can be identified but the physiologic significance of these differences remains unclear. Creation of "knockout" cell lines and mice that lack one or more VDAC isoforms has led to the characterization of distinct phenotypes that provide a different set of insights into function which must be interpreted in the context of complex physiologic systems. Functions in male reproduction, the central nervous system and glucose homeostasis have been identified and require a deeper and more mechanistic examination. Annotation of the genome sequence of *Drosophila melanogaster* has recently revealed three additional genes (*CG17137*, *CG17139*, *CG17140*) with homology to *porin*, the previously described gene that encodes the VDAC of *D. melanogaster*. Molecular analysis of these novel VDACs has revealed a complex pattern of gene organization and expression. Sequence comparisons with other insect VDAC homologs suggest that this gene family evolved through a mechanism of duplication and divergence from an ancestral VDAC gene during the radiation of the genus *Drosophila*. Striking similarities to mouse VDAC mutants can be found that emphasize the conservation of function over a long evolutionary time frame.

Keywords Voltage-dependent Anion Channel · Porin · Mitochondria · Drosophila · Animal models

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Introduction

The voltage-dependent anion channel (VDAC) is an integral membrane protein present in the mitochondrial outer membrane. VDAC is a monomeric, voltage-gated channel that allows passage of molecules up to 5,000 Da and multiple isoforms have been identified in numerous eukaryotic species (Young et al. 2007). VDAC provides the predominant pathway for metabolites such as ATP, ADP, phosphocreatine and small ions across the mitochondrial outer membrane (Colombini 2007). In addition to its role in energy metabolism, VDAC has been tied to basic cellular processes such as cytochrome-c dependent apoptosis (Shimizu et al. 1999; Cheng et al. 2003). While in single cell organisms there appears to be a single VDAC that functions as a channel, the occurrence in multicellular organisms of multiple channel-forming isoforms raises the obvious question of what distinct function exists for each isoform. Genetic approaches to this question (Graham and Craigen 2004) and the, to date, incomplete answers that are

being formulated are the basis of this review, focusing more specifically on studies in the mouse and *Drosophila*. It is hoped that further elucidating known VDAC biological functions and perhaps uncovering potentially new roles in fundamental cellular and organ-specific activities will both enrich our understanding of the biology of VDACs and provide novel therapeutic targets for the treatment of common disorders in which perturbations in mitochondrial function have been implicated.

Mammalian VDACs

Studies in mammalian model systems have begun to detect functional differences in the various VDAC isoforms. In mammals three distinct isoforms are recognized: VDAC1, VDAC2 and VDAC3, each sharing about 65–70% amino acid sequence identity (Sampson et al. 1996a, b; Sampson et al. 1997). The gene structure of each isoform was characterized (Sampson et al. 1997), demonstrating that the intron/exon boundaries are conserved, consistent with a common origin from a single ancestral gene. While each isoform is widely expressed in different tissues they are not ubiquitous and the level of expression in any given tissue varies considerably. There is alternative splicing of the VDAC3 transcript that leads to the inclusion of a three nucleotide exon into the translated portion of the mRNA (Sampson et al. 1998). This alternative splicing event occurs in a tissue-specific fashion and causes the differential incorporation of a single amino acid into the protein in all mammals where it has been sought. While there is ample evidence from EST databases for variable inclusion at this position, the specific function of this single amino acid addition remains obscure.

Expression studies of each isoform in yeast lacking the endogenous VDAC *Por1* has demonstrated that despite the limited sequence similarity (approximately 25% identity) each can completely (VDAC1 and VDAC2) or partially (VDAC3) complement the functional activity of *Por1,* as reflected in the restoration of the capacity to grow on a non-fermentable carbon source. This heterologous expression approach has also been used to examine the channel properties of each isoform following protein purification from VDAC expressing yeast. Each mammalian isoform can confer permeability to liposomes with a similar molecular weight cutoff, based upon permeability to polymers of polyethylene glycol of varying sizes. However, electrophysiological studies of the purified proteins reconstituted into lipid bilayers have demonstrated markedly different channel properties. VDAC1 behaves like a prototypic VDAC, with properties that are highly conserved with those of other species. VDAC2 exhibits normal voltage gating activity but with a second discrete alternate lower conductance and ion selective state. VDAC3 forms channels in phospholipid membranes but does not exhibit clear voltage dependency. Studies of isolated mitochondria expressing the different isoforms demonstrate large differences in their outer membrane permeability to NADH (Xu et al. 1999). To further explore the biophysical properties of mammalian VDACs *in vitro* mutagenesis of the VDAC2 isoform was used to neutralize the voltage sensor domain (Komarov et al. 2005). Mutant channels engineered to lack voltage gating exhibit lower conductance and cationic selectivity yet, surprisingly, remain completely permeable to ATP. Mitochondria containing the mutant isoform similarly remain permeable to NADH and ADP/ATP. These results support the counterintuitive premise that converting a channel from anionic to a cationic selectivity does not substantially influence the flux of negatively charged metabolites, and indicates that ATP permeation is defined by a set of specific interactions between ATP and the channel wall.

To further explore the function of VDACs in mammalian cells loss-of-function mutations were generated for each isoform using homologous recombination and drug selection in embryonic stem (ES) cells, a cell type in which each isoform is highly expressed (Wu et al. 1999). Both alleles for each of the three isoforms were deleted by serial gene targeting and the resulting cell lines characterized for viability, ability to respire and electron transport chain (ETC) enzyme function. Each mutant cell line exhibited reduced oxygen consumption, and partial loss of cytochrome c oxidase activity was observed in cells lacking VDAC1 or VDAC2, indicating that VDACs interact with the respiratory chain in some manner, an observation that had previously been made in yeast lacking *Por1*. Hence, any given VDAC is not required for cell viability and, in fact, all three isoforms can be eliminated or their expression significantly knocked down and the resulting cells remain viable.

VDACs and apoptosis

It has long been postulated that VDACs play some role in the mitochondrial permeability transition and in the execution of a cell death pathway (Tsujimoto and Shimizu 2002). Based upon protein cross-linking studies, VDAC2 was found in interact with Bak, one of two multi-domain proapoptotic proteins of the Bcl-2 family (the other being Bax)(Cheng et al. 2003). Homozygous deficient ES cells were used to create *Vdac2*$^{-/-}$ murine embryonic fibroblasts to further characterize this interaction at a cellular level. VDAC2 is required for the correct import or retention of Bak in the mitochondrial outer membrane, where, in contrast to Bax, it is constitutively located ((Setoguchi et

al. 2006) and unpublished results). Thus, VDAC2 appears to play a role in the regulation of apoptosis via its interaction with Bak. The role of VDACs in cytochrome c release remains controversial; cells lacking each isoform appear to undergo apoptosis in response to a variety of inducers, with a mildly accelerated course in the absence of VDAC2 (Cheng et al. 2003; Baines et al. 2007). Furthermore, induction of the permeability transition appears unperturbed by the absence of each VDAC isoform both *in vivo* and in cell culture. VDAC-deficient mitochondria and cells can carry out cytochrome c release, caspase cleavage and cell death in response to the pro-death Bcl-2 family members Bax and Bid that is indistinguishable from wildtype mitochondria. Thus, it appears that VDACs are dispensable for both the mitochondrial permeability transition and Bcl-2 family member-dependent cell death, however VDAC2 does play a role in the physiologic actions of Bak.

ES cell lines heterozygous for each gene were used to generate chimeric mouse strains in order to begin to study the function of each VDAC gene based upon the phenotypes observed in the absence of each isoform. Following breeding of each chimeric strain, heterozygous and homozygous deficient mice were generated for both *Vdac1* (Anflous et al. 2001) and *Vdac3* (Sampson et al. 2001), however, a null allele of *Vdac2* could not be transmitted through the male germ line, suggesting there is transmission distortion occurring at the *Vdac2* locus, where there is exclusive transmission of the wildtype allele. This is a rare genetic phenomenon (Lyon 2003; Lyon 2005) and may be accounted for by several possible mechanisms that have yet to be explored. A conditional allele of *Vdac2* using LoxP sites and Cre recombinase has subsequently been generated to allow for male germ line transmission and supports this observation (unpublished results).

VDAC mutant mouse phenotypes

Mice deficient for *Vdac1* and *Vdac3* have distinct phenotypes, with $Vdac1^{-/-}$ mice exhibiting partial embryonic lethality (Weeber et al. 2002), depending on the strain background. On a C57Bl6 background there is almost complete lethality, whereas when outcrossed to the more vigorous outbed strain CD-1 survival to weaning is approximately 70% of expected. In addition, VDAC1 deficient mice exhibit abnormal ETC activities and mitochondrial morphology in muscle (Anflous et al. 2001). While $Vdac3^{-/-}$ mice are born in the expected Mendelian proportion, the males demonstrate sperm motility defects leading to obstructive azoospermia and complete infertility, with the most common defect in the epididymal sperm axoneme being loss of a single microtubule doublet at a conserved position within the axoneme. In testicular sperm the defect was only rarely observed, suggesting that instability of a normally formed axoneme occurs with sperm maturation and that it is not due to a developmental assembly defect. Whether this reflects abnormalities in energy production, metabolite transport or a perturbation in structural components that are anchored to mitochondrial via VDAC3 remains unknown. Like $Vdac1^{-/-}$ mice, $Vdac3^{-/-}$ mice exhibit abnormal ETC activities and mitochondrial morphology in muscle (Sampson et al. 2001).

Because the brain has considerable aerobic energy requirements we characterized $Vdac1^{-/-}$, $Vdac3^{-/-}$, and $Vdac1/3^{-/-}$ double mutants with regard to their ability to learn normally and integrate learned behaviors into stable memory, processes that require synaptic plasticity (Graham et al. 2002). All three strains exhibit to differing degrees defects in associative and spatial learning, classic paradigms of learning and memory that probe the functioning of the amygdala and hippocampus. For example, the Morris water maze with a hidden platform test, a well validated test of special learning involving a quantitative assessment of time spent finding a platform just below the water in an opaque tank, was used to demonstrate that $Vdac1^{-/-}$, $Vdac3^{-/-}$, and $Vdac1/3^{-/-}$ double mutant mice have severe deficits in spatial learning (Weeber et al. 2002). At the end of 6 days of training trials, all of the mutant mice exhibited significantly longer escape latencies (the time taken to find the platform hidden below the water) when compared to the wild-type mice, indeed there was virtually no improvement in performance over the course of training. Probe trials, where the hidden platform has been removed following training, revealed that the $Vdac1^{-/-}$, $Vdac3^{-/-}$, and $Vdac1/3^{-/-}$ double mutants did not preferentially spend more time in the trained quadrant of the tank, in contrast to wild-type littermates. Importantly, none of the VDAC mutants showed any deficiencies in the hot-plate test, the open field locomotor activity test, or the rotarod test compared to wild-type controls, thereby excluding sensory or motor deficits as confounding variables. Thus, these mutants demonstrate specific defects in learning and memory, findings that both point to the specific function of VDACs in synaptic plasticity and provide a novel mouse model of mitochondrial encephalopathy.

In conjunction with the behavioral defects, electrophysiological recordings of VDAC-deficient hippocampal slices revealed deficits in long and short term synaptic plasticity. These reflect both pre-synaptic (paired pulse facilitation in VDAC3 deficient mice) and post-synaptic (long term potentiation in VDAC1 deficient mice) deficits in synaptic plasticity. Since neurotransmitter release appeared intact, it would implicate VDACs in other functions such as calcium buffering or calcium-induced calcium release from mitochondria, and

indeed calcium-induced calcium release was subsequently shown to be enhanced in VDAC1 deficient isolated mitochondria (Levy et al. 2003). These findings emphasize the importance of mitochondria for normal synaptic function required for learning and memory, and implicate mitochondrial outer membrane permeability as a potentially important point of control in normal physiologic processes, and not simply the passive sieve view that has long been held.

More recently, attention has been focused on the role of mitochondrial function in normal glucose homeostasis. It has previously been proposed that VDACs are a binding site for metabolically active hexokinase isoforms as a means to couple glucose phosphorylation and glycolytic flux to oxidative phosphorylation. Any disruption of this interaction would be predicted to cause altered metabolic homeostasis and changes in this interaction are thought to underlie altered glycolytic activity and apoptosis in tumors (Gottlob et al. 2001; Mathupala et al. 2006; Pedersen 2007). To address the validity of this model we determined whether mitochondria-bound hexokinase activity is significantly reduced in oxidative muscles (heart and soleus) in *Vdac1*$^{-/-}$ and *Vdac3*$^{-/-}$mice (Anflous-Pharayra et al. 2007). The observed reduction in mitochondria-associated activity was supported by western analysis using HK2-specific antibodies. VDAC deficient mice were also subjected to glucose tolerance testing and exercise-induced stress, each of which involves glucose uptake via different mechanisms. *Vdac1*$^{-/-}$ mice exhibit impaired glucose tolerance whereas *Vdac3*$^{-/-}$ mice have normal glucose tolerance, whereas both strains have normal exercise tolerance. Mice lacking both VDAC1 and VDAC3 have reduced exercise capacity together with impaired glucose tolerance. Hence, a link between VDAC1-mediated mitochondria-bound hexokinase activity and the capacity for glucose clearance was established. We have subsequently observed abnormal lipid metabolism in the absence of VDACs (unpublished results), placing mitochondrial outer membrane permeability in a potentially important role in metabolic regulatory mechanisms.

These studies of VDAC function using loss-of-function mutations in the mouse have begun to shed some light on the important physiologic roles that these proteins play in mammalian physiology. It is anticipated that future work will provide additional mechanistic insights that may be useful in better understanding clinically important areas of disease such as cancer, diabetes, and neuromuscular disorders (Ralph et al. 2006; Shoshan-Barmatz et al. 2006).

Drosophila VDAC

As a powerful model system for genetic analysis *Drosophila melanogaster* offers an opportunity for additional *in vivo* insights into VDAC functions. *D. melanogaster* contains a cluster of four genes (*porin*, *CG17137* [also known as *porin2*], *CG17139*, and *CG17140*) that encode proteins that are homologous to known VDACs (Messina et al. 1996; Ryerse et al. 1997; Oliva et al. 2002). Porin and CG17137 show extensive sequence identity, while CG17139, and CG17140, although far more similar to each other also exhibit significant sequence similarity with other VDACs, especially near the N- and C-termini. Pairwise alignment of these polypeptides reveals 42% identity and 65% similarity between porin and CG17137, while there is 23% identity and 42% similarity between porin and CG17139, and 26% identity and 44% similarity to *CG17140*. The sequences of CG17139 and CG17140 differ from both CG17137 and *D. melanogaster* VDAC in that they contain long, highly charged, N-terminal extensions not found in any other VDAC molecule. The physiologic function of these extensions is unknown.

When expressed in *Por1* deficient yeast, *porin* and *CG17137* can complement the conditional lethal phenotype, while *CG17139* and *CG17140* cannot, demonstrating functional complementation for a subset of these genes (Komarov et al. 2004). When examined in reconstituted lipid bilayers, porin, CG17137, and CG17140 all exhibit biophysical properties such as voltage-dependent anion selectivity typical of other VDACs. In contrast, another study reported that, when expressed in bacteria, CG17137 exhibits voltage-independent cation selectivity *in vitro* (Aiello et al. 2004). The basis for this discrepancy is unclear.

These genes exhibit differential spatiotemporal patterns of expression (Graham and Craigen 2005). *CG17137*, *CG17139*, and *CG17140* are transcriptionally and translationally expressed in a male-specific pattern, while *porin* is equally expressed in both male and female flies. Western blot analysis of dissected adult *Drosophila* tissues has demonstrated a ubiquitous expression pattern for *porin*. In contrast, expression of *CG17137* is predominantly limited to the male reproductive tract (although detectable in other tissues) and *CG17140* exclusively limited to the male reproductive tract In addition, *CG17139* and *CG17140* are expressed as a dicistronic transcript, an unusual arrangement in multicellular eukaryotes (Estes et al. 2003). The intergenic region between *CG17139* and *CG17140* contains a potential hairpin loop within 100 base pairs of the initiator codon for *CG17140* that may act as a type I IRES site for internal translation initiation. Western blot analysis and immunofluorescent microscopy have been used to demonstrate that each of the four proteins localizes to the mitochondrion, and CG17137 and CG17140 are highly abundant in testes. While *porin*, *CG17137*, and *CG17140* are expressed to different degrees during embryogenesis, the expression of all of the *Drosophila* VDACs appears to be down regulated during larvogenesis. This could repre-

sent a progressive dilution of maternally expressed VDACs. However, expression of another mitochondrial protein, cytochrome *c*, is not diminished during larvogenesis and expression of CG17137 and CG17140 is not detected in adult ovary, making this explanation unlikely. This developmental pattern of expression has also been observed in *Drosophila* genes important for mitochondrial biogenesis, including mitochondrial transcription factor A (*TFAM*) (Takata et al. 2001) and mitochondrial single-stranded DNA-binding protein (*mtSSB*) (Ruiz De Mena et al. 2000).

The compact genomic organization of this cluster, the tissue-specific expression patterns for *CG17137*, *CG17139*, and *CG17140*, as well as the dicistronic expression of *CG17139* and *CG17140* all suggest the occurrence of complex regulatory mechanisms functioning at the level of transcription and possibly translation, the significance of which remains to be elucidated.

Database searches of sequenced insect genomes for homologous VDAC sequences and comparison of those sequences with *Drosophila* VDACs reveal that this gene cluster has evolved from a series of gene duplications during radiation of the *Drosophila* species. In all likelihood there was a single ancestral VDAC when insects evolved during the Upper Palaeozoic period 200–250 million years ago (Throckmorton 1968). Based on the emergence of higher Diptera 80–130 million years ago, *Drosophila* began to diverge approximately 80 million years ago and 2 serial duplications of the ancestral VDAC occurred before the separation of the *D. obscura* and *D. melanogaster* lineages approximately 50 million years ago (Yeates and Wiegmann 1999). A final third duplication, leading to *CG17139* and *CG17140*, occurred after the Obscura and Melanogaster divergence. The evolutionary history of this gene cluster is important because it suggests that these *Drosophila*-specific VDACs have either diverged into novel functions or perhaps more partitioned VDAC functions, yet the need to explore this further is tempered by the fact that it appears be limited to *D. melanogaster*.

Oliva, et al. reported a series of *porin* mutants derived from imprecise excision of a P element inserted in the 5' region of *porin* (Oliva et al. 2002). This series consists of mutant alleles containing deletions or insertions upstream of the *porin* coding exons. The reported phenotypes ranged from recessive lethal to viable with male sterility and the phenotypes were attributed to alterations in the level of expression of *porin*, although analysis of expression of the other *Drosophila* VDACs was not reported. We analyzed expression of the downstream three genes in independently derived *porin* deficient strains using isoform-specific antibodies and demonstrated that expression appear unaffected, suggesting the male infertility is inherent to *porin* deficiency, reminiscent of VDAC3 in the mouse. The weight of evidence suggests that *porin* represents a functional ortholog of mammalian VDAC in *Drosophila*. This would predict that mutant *porin* phenotypes should exhibit additional similarities to mammalian mutant VDAC phenotypes, including effects on embryonic survival, synaptic and neuromuscular defects, and mitochondrial morphological abnormalities. Indeed, analyses of mutant *porin* flies demonstrate a striking resemblance to mammalian mutant VDAC phenotypes, including partial embryonic lethality, abnormal retinal and neuromuscular synapses, and "hyperswirl" (Walker and Benzer 2004) mitochondrial morphologic changes (unpublished results). Thus, studies of *porin* provide a powerful approach to the identification of interacting genes and their proteins by using the advantages of unbiased genetic screens for enhancer and suppressor loci that modify the phenotypes associated with *porin* deficiency.

It is anticipated that genetic studies of VDAC function will continue to provide a useful and complementary approach to traditional biophysical experimentation in this research field.

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