A Novel Isoform of the Mitochondrial Outer Membrane Protein VDAC3 via Alternative Splicing of a 3-Base Exon

FUNCTIONAL CHARACTERISTICS AND SUBCELLULAR LOCALIZATION

Margaret J. Sampson‡, Lyle Ross‡, William K. Decker‡, and William J. Craigen‡§
From the Departments of ‡Molecular and Human Genetics and §Pediatrics, Baylor College of Medicine,
Houston, Texas 77030

Voltage-dependent anion channels (VDACs) are pore-forming proteins found in the outer mitochondrial membrane of all eukaryotes. VDACs are the major pathway for metabolites through the outer mitochondrial membrane and, in mammals, bind several cytosolic carbohydrate kinases. Whereas yeast contain a single VDAC (YV-DAC), to date three isoforms have been described in the mouse that constitute a gene family. We have observed an additional isoform of VDAC3 that appears to be generated via the tissue-specific alternative splicing of a 3-base exon (ATG). The exon is predicted to introduce a methionine 39 amino acids downstream of the amino terminus of the polypeptide. Between exons 3 and 4 is an intrinsic sequence that potentially encodes the exon, with flanking splice enhancer elements. Expression of this alternative form in the mouse is limited to brain, heart, and skeletal muscle. Complementation of YVDAC-deficient yeast by the two isoforms and with other sequence variants of VDAC3 suggests this residue is an important modulator of VDAC3 function. In transfected mammalian cells both isoforms localize to mitochondria. A similar variant is present in humans.

Voltage-dependent anion channels (VDACs) are small, functionally conserved proteins found in the outer mitochondrial membrane of all eukaryotes (reviewed in Ref. 1). VDACs are the binding site for hexokinase, glyceraldehyde-3-phosphate dehydrogenase, and creatine kinase (reviewed in Refs. 2 and 3), and conduct ATP (4), thus providing bound kinases with preferential access to mitochondrial ATP (5). Voltage-sensitive gating of the channel by NADH has been observed, potentially providing a mechanism for coupling glycolytic activity to oxidative phosphorylation (the Crabtree effect; see Refs. 6 and 7). Multiple VDAC isoforms have been identified in a variety of organisms, including yeast (6, 7), mammals (8, 9), plants (e.g. Ref. 10), humans (11), and the mouse (9, 12).

Absence of HVDA1 in skeletal muscle of a child with a severe mitochondrial encephalomyopathy (13) suggests that VDACs have an important regulatory role in energy metabolism.

To date, three mouse VDAC isoforms have been isolated (9, 12). VDAC3 has 65–70% amino acid similarity to the other two murine VDAC isoforms. Unlike VDAC1 and VDAC2, VDAC3 is able to only partially complement a temperature-sensitive YVDAC-deficient yeast strain when grown on glycerol-based media (14), suggesting that this isoform may have distinct physiological functions.

In this report a new VDAC3 isoform with a single amino acid insertion is described. It appears to arise by tissue-specific alternative splicing of a 3-base exon. Using a YVDAC-deficient yeast strain, functional differences between the two VDAC3 isoforms have been investigated. There is no evidence for targeting by either isoform to sites other than mitochondria.

MATERIALS AND METHODS

Isolation of an Alternatively Spliced Mouse VDAC3 cDNA—Mouse brain and testes cDNA libraries were screened with the VDAC3 3'-untranslated region (from nucleotide 1022–1428). A number of VDAC3 cDNAs isolated from each library were sequenced using an ABI model 373A automated DNA sequencer. The DNA sequence was analyzed using the GCG sequence analysis package (15). A genomic fragment containing VDAC3 intron 3 was isolated from a Lambda Fix II phage clone (14) and similarly sequenced.

RT-PCR Analysis of VDAC3 Expression—Total RNA was extracted from various adult mouse tissues using guanidinium isothiocyanate, as described (16). After treatment with RNase-free DNase, 5 μg of RNA from each tissue was used to generate reverse transcribed cDNAs (Superscript RT II first-strand cDNA synthesis kit; Life Technologies, Inc.). A pair of oligonucleotides (sense primer, 5'-GGCATGGTCAAGATAGATCTG-3'; antisense primer, 5'-GTATAAAGCATGACCTGAGTAG-3'; 94 °C for 1 min, 48 °C for 1 min, and 72 °C for 1 min 30 s, 35 cycles) flanking the insertion site was designed to amplify a 74- or 77-bp product, depending on the presence or absence of the ATG codon. The sense oligonucleotide was end-labeled using T4 polynucleotide kinase (Amersham Life Science) and [γ-32P]ATP. A 1:20 dilution of labeled to unlabeled oligonucleotide was used for PCR. The products were separated on a 6% polyacrylamide gel and the gel exposed to x-ray film.

Expression of Mouse VDAC3 Isoforms in Yeast—Oligonucleotide-directed mutagenesis was used to create an AαIII site at the VDAC3 start codon and a NsiI site in the VDAC3 3'-untranslated region. This approach allows the complete open reading frame of the VDAC3 cDNA to precisely replace the YVDAC gene previously cloned into the yeast single-copy shuttle vector pSC58 (provided by M. Forte, Oregon Health Sciences University; Ref. 17). The oligonucleotides were used to amplify a VDAC3 cDNA fragment with the ATG insertion (using a mouse brain cDNA as template), and the product used to replace the YVDAC gene between the NcoI and NsiI sites. From the most 5' ATG codon, the length of the VDAC3 cDNA is 1017 bp. An oligonucleotide was also designed to generate an NcoI site at the ATG insertion sequence 117 bp into the VDAC3 coding sequence, and a truncated VDAC3 PCR fragment was amplified using this oligonucleotide in conjunction with the previous 3'-untranslated oligonucleotide. The truncated cDNA was digested with the restriction enzymes and subcloned as before. This same strategy was also used to design oligonucleotides in...
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250-bp bovine polyadenylation fragment in a pBluescript KSI (−) vector: an A/I113/BgII 92-bp 5′ VDAC3 cDNA fragment, a BgI/BaaiI genomic fragment containing the second part of exon 3, all of intron 3, and the first part of exon 4, and a BsaI/Clai 3′ VDAC3 cDNA coding and 3′-untranslated fragment.

Subcellular Localization of VDAC3 Isoforms—A carboxyl-terminal FLAG epitope (DYKDDDDK) was placed onto the end of each VDAC3 isoform and the truncated version by oligonucleotide-directed mutagenesis using the 5′ primer 5′-GAACACTTGTTCATCGTGTTCCGTTGGTAC-TCGCGTTCCATTCAATCCA-3′. The PCR product was cloned into pBluescript, digested with Apel and SpeI, and subcloned into the mammalian expression plasmid pCDM-SR9. Following transfection of NIH3T3 fibroblasts or the C2C12 mouse myocytic cell line with Superfect Transfection Reagent (Qiagen), cells were incubated with Mitotracker Red CMXRs (Molecular Probes, Inc.), fixed, permethesized, and stained with anti-FLAG IgG and anti-IgG fluorescein isothiocyanate conjugate. Cells were analyzed by fluorescence microscopy for coincidental staining.

RESULTS

An Alternatively Spliced VDAC3 Isoform with a 3-bp Insertion—VDAC3 cDNAs were previously cloned from a mouse liver cDNA library (9). To investigate whether additional VDAC isoforms are present in other mouse tissues, brain and testes cDNA libraries were screened using the 3′-untranslated regions of all three mouse VDAC isoforms. Two VDAC3 cDNAs isolated from a brain cDNA library were completely sequenced and an in-frame ATG insertion was found 117 bp into the coding sequence. Finding the insertion in two distinct cDNAs and subsequently in different mouse and human expressed sequence-tagged cDNAs makes it highly unlikely the insertion is a cloning artifact. The human orthologue of mouse VDAC3, although unreported, can be found in a number of expressed sequence-tagged cDNA sequences. The sequence of human VDAC3 cDNAs without the ATG insertion has been deposited from the Soares pregnant uterus NbHPIU, Stratagene hNT neuron, and fetal heart cDNA libraries (accession numbers AA133349, AA206747, and N86098, respectively). The human VDAC3 cDNAs have greater than 90% sequence homology with the murine VDAC3 isoform. In addition, human VDAC3 cDNAs with the ATG insertion have been deposited from the Soares pregnant uterus NbHPIU, Stratagene hNT neuron, Stratagene muscle, infant brain, and fetal heart cDNA libraries (accession numbers AA114443, AA180978, AA350849, and N89234, respectively). In contrast to the report of Ha et al. (22), who found different isoforms of the human VDAC2 gene, this was the only example of VDAC alternative splicing found in the course of our studies in the mouse. VDAC3 cDNAs were also isolated and sequenced from a mouse testes cDNA library, and these all lacked the ATG insertion.

RT-PCR Analysis of the Variant VDAC3 Expression

1810
tggaaactgtgctacacttatgatattcataaractagtggtggaseatgcactgtac
ccccgggtgggttccctttttctttttttctttttttctttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
Pattern—The VDAC3 isoform with the ATG insertion is expressed in only three of the tissues examined: brain, heart, and skeletal muscle (Fig. 1). Brain and skeletal muscle clearly express both isoforms, whereas the smaller isoform was only detected at very low levels by RT-PCR from heart RNA. A variety of additional tissues were also examined for expression of the larger VDAC3 isoform, including testes, liver, ovary, adrenal, lung, spleen, and kidney; all only expressed the VDAC3 isoform without the ATG insertion (data not shown).

The ATG insertion occurs at the VDAC3 exon 3 and exon 4 splice junction (14). The 3.8-kb intron 3 separating the two exons was isolated from a genomic phage clone and sequenced. An ATG sequence flanked by consensus splice acceptor and donor sites was found 1.9 kb 3' of exon 3 (Fig. 2). Downstream of the putative exon are located guanine-rich splice enhancer elements that have been reported to be necessary for the splicing of very small exons (23). Of course, given the small size, it is possible that the sequence occurs at this location by chance, but the simultaneous presence of the splice enhancer sequences makes this less likely.

To test whether these sequences are sufficient for alternative splicing, a VDAC3 expression plasmid was generated with the coding region of VDAC3 cDNA flanking intron 3. The VDAC2 promoter, which generates high levels of transcription in tissue culture (14), was placed 5' of the VDAC3 sequences to direct mRNA synthesis. A VDAC2-specific oligonucleotide derived from the 5'-untranslated region sequence, when used in conjunction with a VDAC3-specific 3' primer, allows for the amplification of the hybrid transcript. This strategy (outlined in Fig. 3) avoids problems with contamination by the endogenous VDAC3 transcript or from genomic DNA that can give rise to spurious bands due to amplification of processed pseudogenes of VDAC3. The plasmid was transfected into NIH3T3 cells and a mouse skeletal muscle cell line C2C12 and the presence of the ATG determined by DNA sequencing of the RT-PCR product.

Although splicing of the transcript between exons 3 and 4 occurred appropriately, the ATG was not detected in the RT-PCR product (data not shown). Primary cultures of chick myoblasts were then used for transfection and the RT-PCR assay carried out 2 days later. Although this system is capable of

Fig. 3. Strategy for detecting VDAC3 alternative splicing. The endogenous VDAC2 promoter and the first two 5'-untranslated exons of VDAC2 were placed 5' of the VDAC3 cDNA to drive expression in mammalian cells. The complete VDAC3 intron 3 with the proposed trinucleotide ATG exon was inserted in the appropriate region in the VDAC3 cDNA. The plasmid was transfected into the NIH3T3 cells, C2C12 cells, and primary chick myoblasts, and expression of the VDAC3 transgene determined by RT-PCR.

Fig. 4. Complementation of YV- DAC-deficient yeast with VDAC3 and modified forms of VDAC3. Panel a shows the growth of strains containing the indicated amino acids at position 39 at either 30 °C or 37 °C following serial dilution. Panel b shows the results of Western blotting mitochondrial preparations using a HA-specific antibody. Note the smaller size of the truncated cDNA (VDAC3trunc-HA) compared with those cDNAs with (VDAC3(AGT)-HA) or without the additional ATG (VDAC3-HA).
splicing the VDAC3 transcript to the predicted 251-bp fragment, no cDNAs that had incorporated the ATG into the transcript were detected by DNA sequencing. This may be because the plasmid lacks essential regions of the gene necessary for splicing, the chick cells fail to recognize the mouse signals for the small exon, or the insertion of the ATG involves RNA editing and not alternative splicing. This latter explanation seems less plausible given the presence of the predicted sequences within intron 3.

Expression of Mouse VDAC3 Isoforms in Yeast—To determine if any functional differences can be detected between the two VDAC3 isoforms when expressed in YVDAC-deficient yeast, the two isoforms were expressed behind the promoter for two VDAC3 isoforms when expressed in yeast, the two isoforms were expressed behind the promoter for the endogenous YVDAC gene on a single copy plasmid. The two isoforms are indistinguishable in their ability to stimulate the endogenous yeast, the two isoforms were expressed behind the promoter for the endogenous YVDAC gene on a single copy plasmid. The two isoforms are indistinguishable in their ability to stimulate growth, each leading to only partial complementation of growth at 37 °C (Fig. 4A).

Since the insertion encodes a potential start codon and is in-frame, it is possible that the ATG insertion acts as a new translation start site for VDAC3 protein synthesis. To determine whether the downstream ATG is used as a new start site in yeast, an epitope tag was placed on the carboxyl-terminal end of the proteins and protein size examined by Western blotting with an antibody specific to the epitope tag. No difference in the molecular size between the two isoforms was observed (Fig. 4B), indicating that in yeast the inserted ATG is not used for translation initiation. A second construct using the inserted ATG as the obligatory start codon was generated and transfected into the YVDAC-deficient strain. Unlike the full-length VDAC3 constructs, this truncated construct was able to fully rescue the yeast temperature-sensitive growth deficiency (Fig. 4A).

To determine if substitution of the ATG codon with other amino acids affects the ability of VDAC3 to rescue the YVDAC-deficient yeast phenotype, the codon was mutated in the full-length VDAC3 cDNA to that for histidine, leucine, alanine, proline, or glutamic acid. The six mutated constructs exhibited quite variable abilities to complement YVDAC deficiency, with the charged residues histidine and glutamic acid and the small amino acid proline conferring growth comparable to the wild-type yeast strain (Fig. 4A). These results indicate that, in yeast, position 39 plays a significant role in the function of the VDAC3 protein and that levels of protein expression cannot be the sole effect of these amino acid substitutions. In addition, the ability to fully complement the growth deficiency indicates that the amino-terminal region of the protein per se does not inhibit growth, but rather the identity of the 39th amino acid is crucial to the growth phenotype.

Localization of the Isoforms to Mitochondria—Since the mitochondrial location of VDAC3 has not previously been demonstrated and it has been reported that VDACs localize to non-mitochondrial sites (24, 25), we examined its subcellular location using the full-length cDNA with and without the extra ATG. Each isoform was transfected into mouse fibroblasts and C2C12 cells and tested for co-localization with a mitochondria-specific dye (Fig. 5). The VDAC3 protein localizes exclusively to mitochondria and addition of the ATG fails to alter the subcellular location. These results demonstrate that the extra methionine has no effect on membrane targeting. To ensure that location is unaltered if indeed translation initiates at the inserted ATG, the truncated form of the protein was similarly tested for mitochondrial co-localization and the same result obtained. This further implies that the mouse VDAC3 mitochondrial targeting signal is not located at the amino-terminal end of the protein.

DISCUSSION

The discovery of an alternative VDAC3 suggests the existence of an unusual splicing mechanism. Analysis of mouse genomic DNA by PCR demonstrates that the ATG insertion is not encoded by a VDAC3-like intronless gene. Two VDAC3-like intronless genes have been identified in the mouse (14). One is an obvious pseudogene while the other has a complete open reading frame, but neither contains the ATG insertion. Although the use of a 3-bp exon is quite unusual it is not unique. Santoni et al. (26) suggested that the alternatively spliced AAG trinucleotide found in the mouse NCAM gene is also encoded by a 3-bp exon. The AAG trinucleotide (flanked by splice acceptor and donor consensus sequences) is located in the intronic region between the exon splice junction site where the insertion occurs. The sequences of the putative NCAM and VDAC3 3-bp exons are similar in several respects. Both are surrounded by conventional AG... GT dinucleotides, although the acceptor splice site of both exons do not conform well to the consensus sequence (27). The hierarchy of acceptor splice sites is CAG ≈ TAG > AAG ≫ GAG in vivo and in vitro (28). Both the NCAM and VDAC3 proposed small exons have a CAG at the splice acceptor site; however, the classical pyrimidine stretch is not found adjacent to the AG dinucleotide. In contrast, the donor splice sites conform closely with the consensus sequence (26). These similarities, as well as the fact that the alternatively spliced trinucleotides of both genes are found in the brain, suggest that similar mechanisms may be used in the splicing of these very small exons.

The average vertebrate exon is 137 bp and is separated by introns that are typically considerably larger (29). Splice acceptor and donor consensus sequences are relatively short and poorly conserved (30, 31). The splicing machinery is able to overcome this disadvantage by requiring an interaction between 5' and 3' splice sites and the factors that recognize them for the proper regulation of spliceosome assembly (32–34). Because of this it has also been assumed that a minimal separation, and therefore exon size, would be required to prevent
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Steric hindrance among the splicing machinery proteins. Dominski and Kole (35) demonstrated that when a constitutively recognized internal exon is reduced to less than 50 nucleotides it is skipped by the splicing machinery. Exon skipping can be corrected by increasing the strength of the splice sites, suggesting that splice sites are additive factors in exon recognition (36). A number of small exons have been studied and the surrounding intronic sequences analyzed. These studies suggest that small exons require for inclusion special enhancing sequences found in the neighboring introns outside of the strong splice sites (37, 38). It has been hypothesized that these enhancers function as binding sites for splicing factors, thereby artificially extending the exon domain during exon recognition (23). In the region downstream of the VDAC3 exon are multiple splice enhancer sequences previously associated with small exons (39). These sequences may play a role in aiding the recognition of the exon in a tissue-specific manner.

Although the in vivo role of the alternatively spliced VDAC3 isoform remains unknown, its biological importance is presumably reflected in its conservation in human cDNAs. In addition to the cDNAs reported here, there are a number of mouse expressed sequence-tagged cDNAs that contain the ATG, and all have been cloned from cDNA libraries generated from brain, muscle, or heart tissues. Human VDAC3 isoforms that contain the ATG were identified in expressed sequence-tagged cDNAs isolated from a human fetal heart cDNA library, consistent with both the tissue specificity and its occurrence during development. Both mouse VDAC3 isoforms are able to partially rescue the temperature-sensitive phenotype of the yeast, indicating that in yeast the proteins appear to act similarly. In mammalian cells, the VDAC3 ATG insertion may cause a subtle change in the conformation of the protein in the mitochondrial membrane, perhaps altering channel permeability. Alternatively, the methionine insertion may allow the channel to differentially interact with a specific kinase, metabolite, or other protein. Based upon the proposed models of the yeast and human VDAC3 protein, the methionine is located in an extramembrane region facing the intermembrane space of mitochondria (40, 41). This location suggests the amino acid may regulate VDAC3 protein interactions rather than channel selectivity. However, these models may be of limited accuracy and conformational effects of the methionine cannot be excluded.

Another hypothesis is that the ATG insertion acts as an alternative translation initiation site, generating a truncated VDAC3 protein. The VDAC3 start codon (GTTGTAGCTATGT) does not match the Kozak consensus sequence well (GCCGC-CpurCCATGpur; Ref. 42), and the Kozak sequence generated does not match the Kozak consensus sequence well (GCCGC-

In summary, we describe an unusual protein isoform that differs from the more widely expressed protein by only a single amino acid, and this variant appears to be the product of tissue-specific alternative splicing. The functional significance of this splice event remains obscure but does not involve altered translation initiation or subcellular localization.

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